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TITLE:

Generation of 3D Whole Lung Organoids from Induced Pluripotent Stem Cells for Modeling Lung Developmental Biology and Disease

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KEYWORDS:

human pluripotent stem cells; endoderm; lung progenitor cells; 3D whole lung organoids; lung epithelial cells; lung mesenchymal cells; lung development; pulmonary disease modeling

SUMMARY:

The article describes step wise directed differentiation of induced pluripotent stem cells to three-dimensional whole lung organoids containing both proximal and distal epithelial lung cells along with mesenchyme.

ABSTRACT:

Human lung development and disease has been difficult to study due to the lack of biologically relevant *in vitro* model systems. Human induced pluripotent stem cells (hiPSCs) can be differentiated stepwise into 3D multicellular lung organoids, made of both epithelial and mesenchymal cell populations. We recapitulate embryonic developmental cues by temporally introducing a variety of growth factors and small molecules to efficiently generate definitive endoderm, anterior foregut endoderm, and subsequently lung progenitor cells. These cells are then embedded in growth factor reduced (GFR)-basement membrane matrix medium, allowing them to spontaneously develop into 3D lung organoids in response to external growth factors. These whole lung organoids (WLO) undergo early lung developmental stages including branching morphogenesis and maturation after exposure to dexamethasone, cyclic AMP and isobutylxanthine. WLOs possess airway epithelial cells expressing the markers KRT5 (basal), SCGB3A2 (club) and MUC5AC (goblet) as well as alveolar epithelial cells expressing HOPX (alveolar type I) and SP-C (alveolar type II). Mesenchymal cells are also present, including smooth

muscle actin (SMA), and platelet-derived growth factor receptor A (PDGFRα). iPSC derived WLOs can be maintained in 3D culture conditions for many months and can be sorted for surface markers to purify a specific cell population. iPSC derived WLOs can also be utilized to study human lung development, including signaling between the lung epithelium and mesenchyme, to model genetic mutations on human lung cell function and development, and to determine the cytotoxicity of infective agents.

INTRODUCTION:

The lung is a complicated, heterogeneous, dynamic organ that develops in six distinct stages – embryonic, pseudoglandular, canalicular, saccular, alveolar, and microvascular maturation[1,2]. The latter two phases occur pre and postnatally in human development while the first four stages occur exclusively during fetal development unless preterm birth occurs[3]. The embryonic phase begins in the endodermal germ layer and concludes with the budding of the trachea and lung buds. Lung development occurs in part via signaling between the epithelial and mesenchymal cells[4]. These interactions result in lung branching, proliferation, cellular fate determination and cellular differentiation of the developing lung. The lung is divided into conducting zones (trachea to the terminal bronchioles) and respiratory zones (respiratory bronchioles to the alveoli). Each zone contains unique epithelial cell types; including basal, secretory, ciliated, brush, neuroendocrine, and ionocyte cells in the conducting airway[5], followed by alveolar type I and II cells in the respiratory epithelium[6]. Much is still unknown about the development and response to injury of the various cell types. iPSC derived lung organoid models enable the study of mechanisms that drive human lung development, the effects of genetic mutations on pulmonary function, and the response of both the epithelium and mesenchyme to infectious agents without the need for primary human lung tissue.

Markers corresponding to the various stages of embryonic differentiation include CXCR4, cKit, FOXA2, and SOX17 for definitive endoderm (DE)[7], FOXA2, TBX1, and SOX2 for anterior foregut endoderm (AFE)[8], and NKX2-1 for early lung progenitor cells[9]. In embryonic lung development, the foregut divides into the dorsal esophagus and ventral trachea. The buds of the right and left lungs appear as two independent outpouchings around the tracheal bud[10]. During branching morphogenesis, the mesenchyme surrounding the epithelium produces elastic tissue, smooth muscle, cartilage, and vasculature[11]. The interaction between the epithelium and mesenchyme is essential for normal lung development. This includes the secretion of FGF10[12] by the mesenchyme and SHH[13] produced by the epithelium.

Here, we describe a protocol for the directed differentiation of hiPSCs into three-dimensional (3D) whole lung organoids (WLO). While there are similar approaches that incorporate isolation of lung progenitor cells via sorting at the LPC stage to make alveolar-like[14,15] (distal) organoids or airway[16] (proximal) organoids, or generate ventral-anterior foregut spheroids to make human lung organoids expressing alveolar-cell and mesenchymal markers and bud tip progenitor organoids[17], the strength of this method is the inclusion of both lung epithelial and mesenchymal cell types to pattern and orchestrate lung branching morphogenesis, maturation, and expansion *in vitro*.

This protocol uses small molecules and growth factors to direct the differentiation of pluripotent stem cells through definitive endoderm, anterior foregut endoderm, and lung progenitor cells. These cells are then induced into 3D whole lung organoids through important developmental steps, including branching and maturation. The summary of the differentiation protocol is shown in **Figure 1a** with representative brightfield images of endodermal and organoid differentiation shown in **Figure 1b**. **Figure 1c,d** show the gene expression details of endodermal differentiation as well as the gene expression of both the proximal and distal populations of lung epithelial cells after completing the differentiation.

PROTOCOL:

This study protocol was approved by the Institutional Review Board of UCSD's Human Research Protections Program (181180).

1. Definitive endoderm induction from induced pluripotent stem cells (Day 1–3)

1.1. Slowly thaw growth factor reduced (GFR)-basement membrane matrix medium on ice and dilute in equal volume cold DMEM/F12 (1:1). Place P1000 tips in the freezer to chill prior to use.

1.2. Coat each well of a 12-well plate with 500 µL of 50% GFR-basement membrane matrix medium prepared in ice-cold DMEM/F12. Once the desired number of wells are coated, remove any excess medium mixture and/or bubbles from wells and place the plate on ice or refrigerator at 4 °C for 20 min to set. Then, move the plate to the incubator at 37 °C overnight to gel and dry.

1.3. Once hiPSCs reach 70% confluency, add 10 µM of Rock Inhibitor Y-27632 an hour prior to dissociation. Aspirate off the media and wash once with PBS. Dissociate iPSCs by adding cell detachment medium (0.5 mL/12-well) and incubate for 20 min at 37 °C in a 5% CO_2 incubator.

1.4. Remove plates from the incubator and add 0.5 mL/12-well of stem cell passaging medium (**Table 1**) to the wells; gently pipette cells using a P1000 tip to obtain single-cell suspension. Transfer dissociated cells into a 15 mL conical centrifuge tube; centrifuge for 5 min at 300 x *g*.

1.5. Aspirate off the medium and resuspend the cell pellet with 1 mL of mTeSR Plus media supplemented with 10 µM Rock inhibitor (Y-27632). Perform a cell count. Add 2.0 x 10^5 iPSCs in 1 mL of mTeSR per well of a 12-well GFR-medium coated plate. Incubate at 37 °C overnight.

NOTE: Cell seeding number must be optimized per cell line. 24 h after plating, wells should be 50%–70% confluent.

1.6. On day 1, aspirate off the mTeSR Plus and add Definitive Endoderm (DE) induction media (**Table 1**) supplemented with 100 ng/mL of human activin A and 5 µM of GSK3β inhibitor/Wnt activator CHIR99021.

NOTE: DE media with GSK3β inhibitor/Wnt activator CHIR99021 should be removed within 20–24 h of day 1 DE induction for successful differentiation.

1.7. On day 2 and day 3, change to DE induction media supplemented with 100 ng/mL of activin A only.

NOTE: DE differentiation should not exceed a total of 72 h, or efficacy will decrease. On day 4, if large cell die-off is observed, decrease total DE media exposure time by 6–12 h.

1.8. To analyze DE efficiency, confirm greater than 90% CXCR4 and/or cKit expression via flow cytometry or immunofluorescence analysis of FOXA2 and/or SOX17 (**Figure 2a**).

2. Anterior foregut endoderm induction (Day 4–6)

2.1. On day 4, change media to serum free basal medium (SFBM) (**Table 1**) supplemented with 10 µM SB431542 and 2 µM Dorsomorphin for AFE induction. Change AFE media daily for 3 days (day 4, day 5, and day 6).

2.2. To analyze AFE efficiency, confirm robust expression of SOX2, TBX1, and FOXA2 via immunofluorescence staining (**Figure 2b**).

3. Lung progenitor cell differentiation (Day 7–16)

3.1. On day 7 thaw growth factor reduced GFR-basement membrane matrix medium on ice. Aspirate off the AFE media and wash well with 1x PBS. Add 1 mL of cell detachment solution and incubate for 10 min at 37 °C.

3.2. Add 1 mL of quenching medium (2% FBS in DMDM/F12) to the wells containing cell detachment solution. Keep cells as aggregates by pipetting up and down gently. Make sure all cells are dislodged and transferred into a 15 mL conical centrifuge tube. Centrifuge for 5 min at 300 x *g*.

3.3. Remove the supernatant and resuspend the cell pellet in LPC induction media (**Table 1**) supplemented with 10 ng/mL of human recombinant BMP4, 0.1 µM of all-trans retinoic acid (RA), 3 µM of GSK3β inhibitor/Wnt activator CHIR99021, and 10 µM of Rock Inhibitor Y-27632.

3.4. Perform a cell count. Add 2.5 x 10^5 cells to 100 µL of cold GFR-basement membrane matrix medium, mix well, and place droplet into a well of a 12-well plate. Incubate the plate at 37 °C for 30–60 min to allow the medium to polymerize. Add 1 mL of LPC media per well ensuring the medium drop is fully submerged and incubate at 37 °C overnight.

3.5. On day 8, 24 h after LPC induction, change LPC medium to remove Rock Inhibitor Y-27632. Change the LPC medium every other day for a total of 9–11 days.

NOTE: If the medium becomes yellow within 24 h, change medium every day.

3.6. To analyze LPC efficiency, confirm robust expression of the intracellular transcription factor NKX2-1 or perform flow cytometry for surface markers $CD47^{hi}/CD36^{low}$[15] or CPM[18] (**Figure 2c**). Grossly, the LPC spheroids should be round and transparent (**Figure 2c**).

NOTE: Do not proceed with lung organoid differentiation if efficiency of NKX2-1 is below 30%.

4. 3D lung organoid induction (Day 16–22)

4.1. On day 17 thaw growth factor reduced GFR-basement membrane matrix medium on ice and place P1000 pipette tips in the freezer to chill. Aspirate the LPC induction medium and wash well 1x with PBS. Add 2 µg/mL of dispase (0.5 mL) into the well and pipette the medium/dispase mixture with a P1000 pipette and place in the incubator for 15 min. Pipette mixture again and incubate for another 15 min.

4.2. Add 1 mL of the quenching media (2% FBS in DMDM/F12) to the dispase containing wells. Transfer cells into a 15 mL conical centrifuge tube and centrifuge for 5 min at 400 x *g*. Remove the supernatant carefully, not to disturb the medium/cell pellet.

4.3. Resuspend the medium/cell pellet with 1 mL of chilled PBS and spin down at 400 x *g* for 5 min. Remove supernatant carefully, not to disturb the medium/cell mixture. Add 1 mL of cell detachment medium to the conical centrifuge tube and pipette up and down to resuspend the medium /cell mixture. Place in incubator at 37 °C for 12 min.

4.4. Add equal volume of quenching media to the conical centrifuge tube and spin down at 400 x *g* for 5 min. Resuspend in quenching medium + 10 µM of Rock Inhibitor Y-27632.

NOTE: Successful lung organoid induction occurs when cells are embedded as aggregates, not single cells, adjust pipetting accordingly.

4.5. Perform a cell count. Calculate the volume needed to obtain 8.0×10^4 cells per well. Aliquot the LPC cell aggregates into 1.5 mL microcentrifuge tubes and centrifuge for 5 min at 300 x *g*. Remove excess supernatant being careful to not agitate the cell pellet. Leave only 10 µL of residual media.

4.6. Re-suspend cell pellet in 200 µL of cold GFR-basement membrane matrix medium and add to cell culture membrane inserts (6.5 mm diameter, 0.4 µm pore, polyester membrane). Incubate the plate at 37 °C for 30–60 min to allow GFR-basement membrane matrix medium to polymerize.

4.7. Add 1 mL of 3D organoid induction medium (**Table 1**) supplemented with FGF7 (10 ng/mL), FGF10 (10 ng/mL), GSK3β inhibitor/Wnt activator CHIR (3 µM), EGF (10 ng/mL), and 10 µM of Rock Inhibitor Y-27632 to the basolateral chamber of the membrane insert. Change the medium every other day for 6 days.

5. 3D Lung organoid branching (Day 23–28)

5.1. On day 23 change to 3D branching medium (**Table 1**) supplemented with FGF7 (10 ng/mL), FGF10 (10 ng/mL), GSK3β inhibitor/Wnt activator CHIR (3 µM), RA (0.1 µM), EGF (10 ng/mL), and VEGF/PIGF (10 ng/mL). Change the medium every other day for 6 days.

NOTE: At day 6 of 3D branching differentiation, there should be multiple branching organoids (**Figure 2**).

6. 3D lung organoid maturation (Day 29–34)

6.1. On day 29 change to 3D maturation medium (**Table 1**), which is the same as 3D branching medium but with the addition of Dexamethasone (50 nM), cAMP (100 µM), and IBMX (100 µM). Change the medium every other day for 6 days.

NOTE: Within 24 h after 3D maturation, the branching organoids should expand and change into transparent spheres.

7. 3D Lung organoid immunocytochemistry

7.1. For 3D whole lung organoid analysis, fix GFR-basement membrane matrix medium in the membrane inserts with 4% PFA for 1 h at 4 ˚C. Embed in paraffin wax and mount onto slides per standard published protocols.

7.2. Perform antigen retrieval prior to staining. Airway markers include KRT5, MUC5AC, and SCGB3A2. Alveolar markers include SP-C, SP-B, HTII-280, HTI-56, and HOPX (**Figure 3**).

8. Removal of whole lung organoids from GFR-basement membrane matrix medium for passage, FACS, or cryopreservation

8.1. To dissociate the organoids from the GFR-basement membrane matrix medium, remove media from the basal chamber and add 2 µg/mL of dispase (1 mL) in the apical chamber.

8.2. Pipette the medium /dispase mixture with a P1000 pipette and place in the incubator for 15 min. Pipette the mixture again and incubate for another 15 min.

8.3. Add 1 mL of chilled PBS (4 °C) and transfer organoids with the matrix medium into a 15 mL conical centrifuge tube. Spin at 400 x *g* for 5 min. Remove the supernatant carefully, not to disturb the cell pellet.

8.4. Wash once more with 1 mL chilled PBS and spin down at 400 x *g* for 5 min. Remove the supernatant carefully, not to disturb the medium/cell mixture.

8.5. Add 1 mL of cell detachment solution to the conical centrifuge tube, pipette up and down to resuspend the GFR-Basement membrane matrix medium/cell mixture. Place in the incubator for 12 min for passaging cells as aggregates or for cryopreservation), or 20 min for single cell suspension (FACS).

8.6. Add equal volume of quenching media and spin down at 400 x *g* for 5 min. Resuspend in quenching medium + 10 µM of Rock Inhibitor Y-27632.

NOTE: At this step, no residual basement membrane medium should be seen in the tube. If residual medium remains, repeat steps 8.5 and 8.6.

8.7. Perform a cell count. Calculate the volume needed for downstream applications.

REPRESENTATIVE RESULTS:

On day 1, iPSC should be 50%–90% confluent. On day 2, DE should be 50%–60% confluent. During DE induction, it is common to observe significant cell death on day 4 but attached cells will retain a compact cobblestone morphology (**Figure 2b**). Discontinue differentiation if majority of adherent cells detach and consider shortening exposure to DE media with activin A by 6–12 h. During AFE induction, cell death is minimal, and cells remain adherent, but will appear smaller and more heterogeneous. Passaging the cells on day 7 must only be done if the yield of double positive SOX2 and FOXA2 is >80%. After passaging into basement membrane matrix for 3D LPC induction, small spheroids will first appear, then grow and some may begin to branch. Gene expression profiles for successful endodermal differentiation include increased SOX17 at DE, increased FOXA2 and SOX2 with decreasing SOX17 and the first appearance of NKX2-1, and increased NKX2-1, along with the presence of SOX2 and FOXA2. Consistent with early embryonic development, the ventralization of AFE occurs for lung bud development (NKX2-1+) and dorsalization of AFE occurs for gastrointestinal development (SOX2+). Cultures at LPC will have a mix of both lung and gastric progenitors.

Lung organoid induction from LPC has been performed using various methods. Some groups sort the cells using NKX2-1 fluorescent reporters or a surface antigen proxy (CPM, $CD26^{low}CD47^{high}$). But those lung organoids contain alveolar type II like cells without alveolar type I cells or mesenchyme. Other groups have collected cell clumps that bud off the AFE/LPC monolayer and embedded them into basement membrane matrix. Those organoids contain a mixed population of lung epithelial and mesenchymal cells but take months to culture[19]. Our protocol includes both the presence of epithelial and mesenchymal cells. The WLOs express proximal epithelial cell markers p63 and KRT5 (basal cells) and SCGB3A2 (club cells) as well as distal epithelial cell markers HOPX (ATI) and proSPC, SPB, and NKX2-1 (ATII). They also express the mesenchymal marker Vimentin at the LPC stage, as well as in the whole lung organoids. PDGFRα is a marker for fibroblasts, which have an important function in the lung during sacculation and alveolarization[20] and is co-expressed with the transcription factor important in distal cell differentiation, SOX9 (**Figure 3**).

Our method efficiently generates NKX2-1-expressing LPC 3D cultures using signaling molecules that occur in fetal lung development to form early lung organoids. When passaging LPCs into GFR-basement membrane matrix medium for lung organoid induction, it is imperative to not over dissociate into single cell suspension, but to instead retain small clumps of cells (10 cells/clump). Cell counting will not be completely accurate, but still necessary to avoid over confluence during the 3-week lung organoid differentiation.

Lung organoid induction should yield small, branching organoids by day 6 of induction (day 23 of differentiation). These should continue to grow during the organoid branching step and maturation step. Twenty-four hours after the introduction of dexamethasone, cAMP, and isobutylxanthine, the branches should expand into transparent spheres. Whole lung organoid analysis can be performed at the end of the differentiation, or the WLOs can be passage into fresh basement membrane matrix with GFR or cryopreserved by freezing down in 10% DMSO.

FIGURES AND TABLES LEGENDS:

Figure 1: Overall schematic of whole lung organoid differentiation from hiPSCs and representative data. (**a**) Schematic of whole lung organoid differentiation from hiPSCs. Circles represent endodermal cell type with identifying markers. Timeline of differentiation is indicated in black bars. Growth factors and/or small molecules for induction of endodermal and lung organoid populations. In summary, stem cells are differentiated into definitive endoderm, anterior foregut endoderm and into lung progenitor cells in approximately 16 days. These cells are then passage into GFR-basement membrane matrix medium containing medium inserts and undergo lung organoid induction, branching, and maturation. The total differentiation takes approximately 35 days. (**b**) Representative phase contrast images of the cells at major endodermal stages and 3D images of whole lung organoids. Scale bar size as indicated in panel. (**c**) qRT-PCR analysis of lung development markers during endoderm and (**d**) whole lung organoid differentiation of proximal and distal cell markers. All data represents an average of 3–5 biological replicates. Error bars represent standard error of the mean and are normalized to actin.

Figure 2: Characterization of endoderm differentiation by flow cytometry and immunocytochemistry. (**a**) Flow cytometry of definitive endoderm marker CXCR4. Left panel shows the gating against the unstained population while the middle panel shows the CXCR4 positive population. The right panel shows immunocytochemistry image of SOX17 (red) overlaid with nuclei (blue). (**b**) Immunocytochemistry image of AFE markers FOXA2 and SOX2 overlaid with nuclei (blue). (**c**) Endogenous expression of NKX2-1-GFP in a reporter cell line in 3D LPC. Images taken from live cell culture in brightfield and GFP. Flow cytometry of lung progenitor intracellular marker NKX2-1 after fixation and permeabilization. Scale bar size = 50 µM.

Figure 3: Characterization of 3D whole lung organoids after 3-week differentiation by immunocytochemistry. (**a**) Proximal lung markers. Left panel shows SOX2 (white) and SOX9 (red) overlaid by nuclei (blue). These markers are important in branching morphogenesis and represent the proximal and distal epithelial populations. Middle panels show P63 (red) and KRT5 (red), both markers of basal cells. The right panel shows SCGB3A2, a marker of club cells. (**b**)

Distal lung markers. Left panel depicts pro-SPC (green) and HOPX (red), markers of alveolar type II ad I cells, respectively, overlaid with nuclei (blue). Middle panel shows proSPC (green) and SPB (red), markers of alveolar type II cells, overlaid with nuclei (blue). The right panel shows NKX2-1 (red) and ZO1 (green) overlaid with nuclei (blue). (**c**) Markers of lung mesenchyme. Left panel shows PDGFRa (red) and SOX9 (white), representing distal mesenchyme. Right panel shows Vimentin (red), which is dispersed throughout the lung. Scale bar size = 50 μM.

Table 1: Table of media.

Table 2: Troubleshooting.

DISCUSSION:

The successful differentiation of 3D whole lung organoids (WLO) relies on a multi-step, 6-week protocol with attention to detail, including time of exposure to growth factors and small molecules, cellular density after passaging, and the quality of iPSCs. For troubleshooting, see **Table 2**. iPSCs should be approximately 70%–80% confluent, with less than 5% spontaneous differentiation prior to dissociation. This protocol calls for mTeSR plus medium; however, mTeSR has also been used with comparable results. For the extracellular matrix, we use growth factor reduced GFR-basement membrane matrix medium. We passage the iPSCs using ReLesR (see **Table of Materials**) to reduce differentiation.

During endoderm differentiation, cells should be visualized daily prior to and after media changes. Specified growth factors/small molecules should be added fresh daily to the base medium to prevent premature degradation. Cell death in definitive endoderm (DE) is common but should be limited during anterior foregut endoderm (AFE) and lung progenitor cell induction. If there is a large die off on the third day of DE (day 4), decrease total time of DE by 6–12 h. New iPSC cell lines may need to be optimized for successful endoderm differentiation. Perform flow cytometry at DE for CXCR4 to confirm successful induction (>85% CXCR4 + cells). The cells should be relatively stable at AFE and will change morphologically with little die off.

Passaging into LPC is another process that must be optimized for cell type. Replating cells at too low a density (<50%) will result in inefficient differentiation. Confirm successful LPC induction with immunocytochemistry for NKX2-1 or flow cytometry for CPM[18] or $CD26^{low}/CD47^{high}$ [15]. Successful LPC induction must have >40% NKX2-1, otherwise the organoids will have greater abundance of dorsal AFE. For LPC induction, growth factors must be added to base media with each media change. If the media becomes yellow later into LPC induction, consider increasing the volume of fresh media, or change the media every day. During 3D whole lung organoid induction, plating number and maintaining cell clusters are key to successful organoid growth. GFR-basement membrane matrix medium is difficult to handle and highly temperature sensitive, so always keep it on ice. If the GFR-basement membrane matrix medium gels too early, then the LPC cells will not integrate within it. We recommend thawing 1 mL aliquots of GFR-basement membrane matrix medium on ice 30 min prior to passaging. Once the cells/clusters have been counted and appropriate aliquots made, place the cell pellet on ice. We suggest preparing plates,

labeling, and addition of cell culture membrane inserts prior to GFR-basement membrane matrix medium handling.

Use pipette tips to add correct volume of liquid GFR-basement membrane matrix medium immediately to cell pellet, keeping on ice. Pipette up and down quickly but gently (nuanced handling) to not introduce bubbles, then place the tube back on ice. Add the cell and GFR-basement membrane matrix medium mixture to the apical portion of the transwell in prepared plates. The mixture should spread and coat the entire transwell; gently tilt plate to ensure coating. After gelling in the incubator for 30–60 min, there should be visible cell clusters in the GFR-basement membrane matrix medium. Add appropriate lung induction media to the basal chamber supplemented with 10 µM Rock Inhibitor Y-27632 and monitor organoid growth every other day.

Future applications of the organoids generated by this protocol include studying the molecular pathways that control early lung lineage commitment and cell fate specification[21–23]. The interaction between the epithelium and mesenchyme can be determined by utilizing gene knock out models[24]. The organoids could also be co-cultured with endothelial cells to determine the importance of tissue specific co-pattern signaling between the lung epithelium, mesenchyme, and the endothelium[25]. Lung development occurs in parallel with vascular development and that relationship may elicit important molecular mechanisms necessary for proper lung development. We have also shown that these whole lung organoids are functional through surfactant secretion assays after GFR-basement membrane matrix medium was removed followed by short-term culture in ultra-low attachment wells[26]. Other strategies include sorting the cells for cell surface markers such as NGFR (basal cells)[27] and HTII-280 (ATII cells)[28] and replacing them as homogenous organoids or a monolayer in air liquid interphase culture conditions.

This protocol is robust and reproducible, but many challenges still exist. Many different iPSC and ESC lines have been tested (>20 lines) but the protocol must be optimized for each cell line. Despite robust DE and AFE induction, LPC induction may be difficult to achieve >40% of NKX2-1 + cells. Other protocols include a sorting step for surface markers of NKX2-1 cells[15,18], but those only yield alveolar type II like organoids without mesenchyme and still contain gastric and hepatic cell populations despite purifying the lung progenitor population[29]. We have also noted a small amount of gastric and hepatic cells in both the LPC and whole lung organoids, possibly due to the presence of dorsal anterior foregut cells contaminating the LPCs. Therefore, the differentiation of pure lung organoids is yet to be achieved, and more research on the development of the lung progenitor cells in human tissue must be completed. Downstream assays must be vigorously benchmarked with gene and protein expression from primary human lung tissue. Prior to transplanting iPSC derived lung cells into patients, quality control must be undertaken, including the identification and removal of contaminating cells, and successful functional assays *in vitro* and *in vivo* in an animal model.

Use of iPSC derived tissue in regenerative medicine to transplant into patients, may be applied in the future as long as the functionality and safety of iPSC-derived cells is confirmed. Undifferentiated cells need to be excluded since they have the capacity to generate teratomas.

One method to determine undifferentiated stem cells in definitive endoderm is to sort the cells out using the pluripotency marker SSEA4. Marker genes for undifferentiated iPSCs were recently detected using single cell RNA sequencing[30]. ESRG, CNMD, and SFRP2 can be used to validate undifferentiated cells at any differentiation step. Once purity is confirmed, the benefit of autologous iPSC derived therapies is the ability for the transplanted cells to avoid rejection since they come from the patient's own cells. The drawbacks include the time it takes to fully differentiate the cells, undergo rigorous clinical grade testing, and transplant the cells into a patient with an acute injury (respiratory distress syndrome, myocardial infarction, or spinal injury). The alternative is to utilize banked allogenic iPSC derived cells[31]. These may be stored and readily available for patients with human leukocyte antigen (HLA) matched donors and they will have undergone thorough testing for contamination. A parallel treatment option is the use of allogenic packed red blood cells for acute hemorrhage. The biggest drawback is the possibility of immune rejection. Immunosuppression may be necessary in allogenic cell transplantation, which is the current reality of allogenic whole tissue transplants. Strategies are being devised to allow the allogenic iPSC derived cells to evade the immune response to safely transplant them into patients[32].

Eventually, iPSC derived whole lung organoids will be utilized to study patient-specific disease models, tailor therapeutics, and enhance regenerative medical research.

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DISCLOSURES:
The authors have nothing to disclose.

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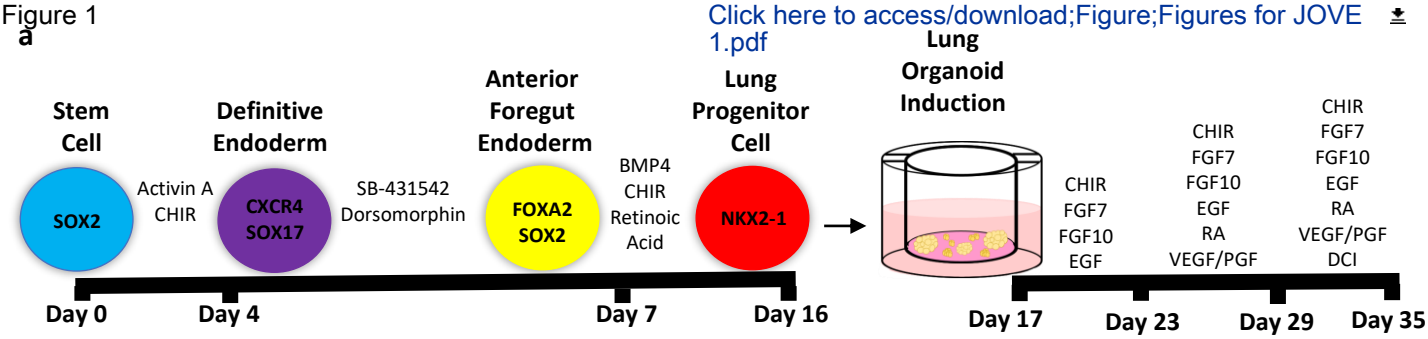
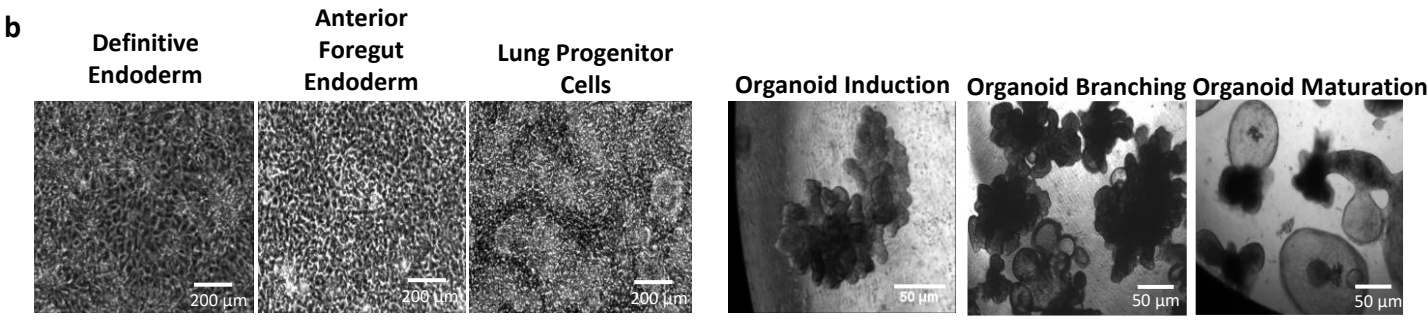
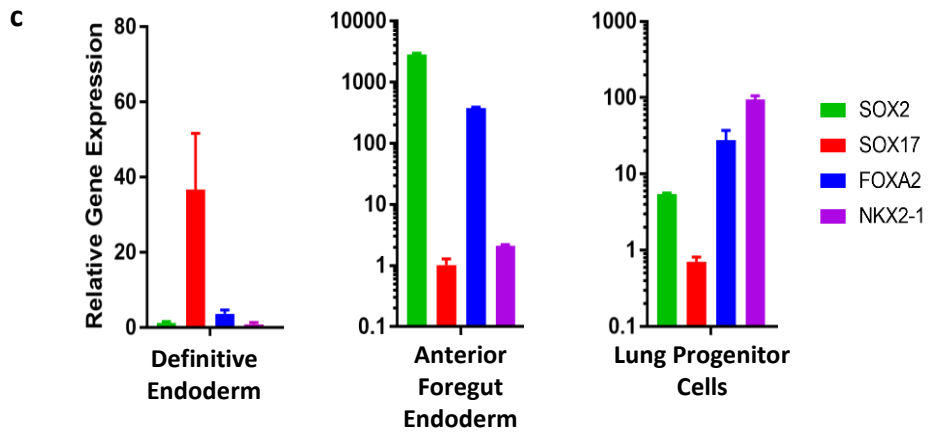
Figure 1

Click here to access/download;Figure;Figures for JOVE 1.pdf







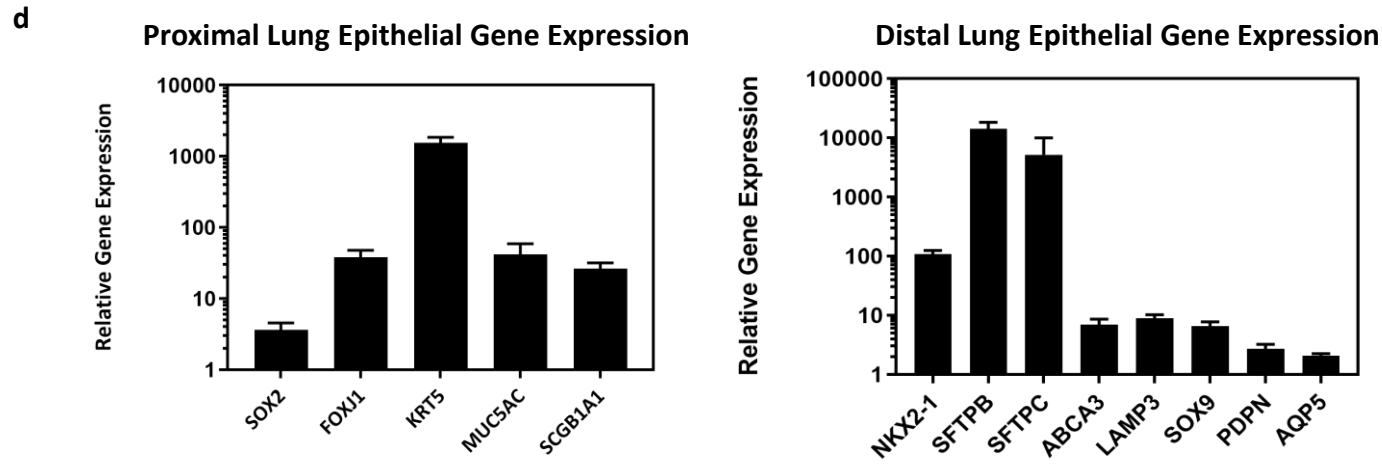
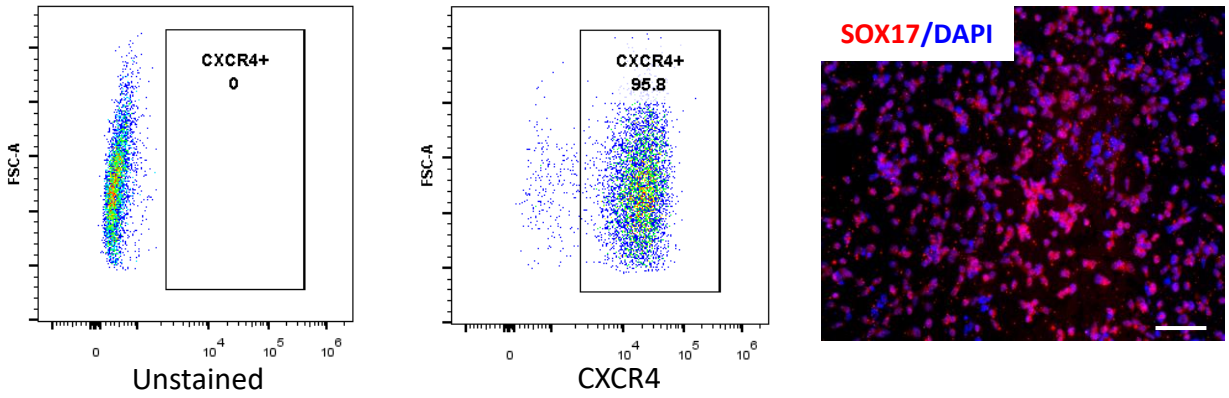


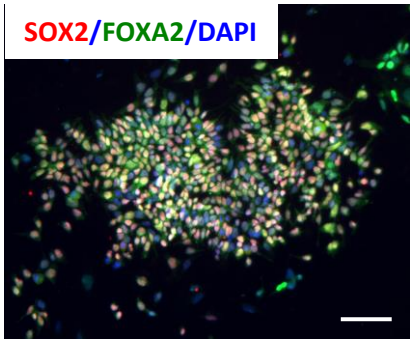
Figure 2

Click here to access/download;Figure;Figures for JOVE 2.pdf

a Definitive Endoderm



b Anterior Foregut Endoderm



c Lung Progenitor Cells

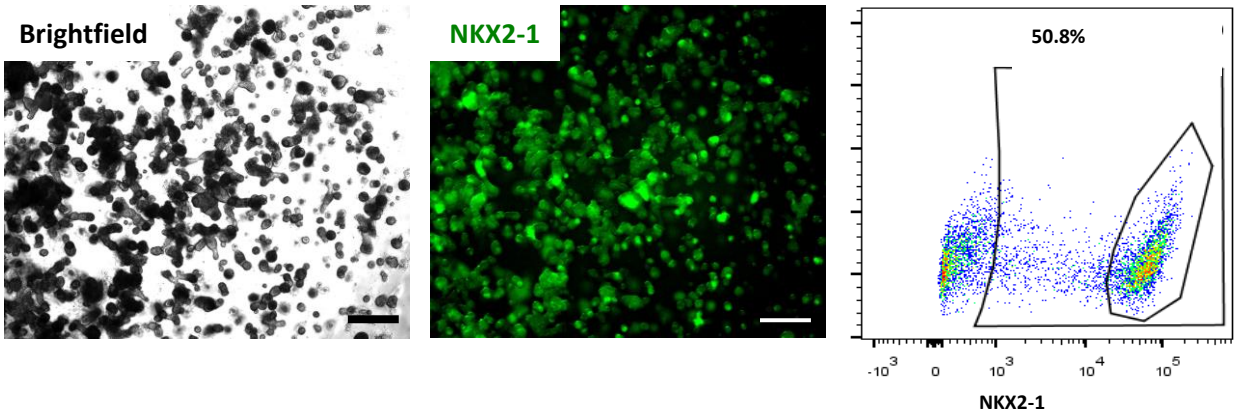


Figure 3

Click here to access/download;Figure;Figures for JOVE 3.pdf

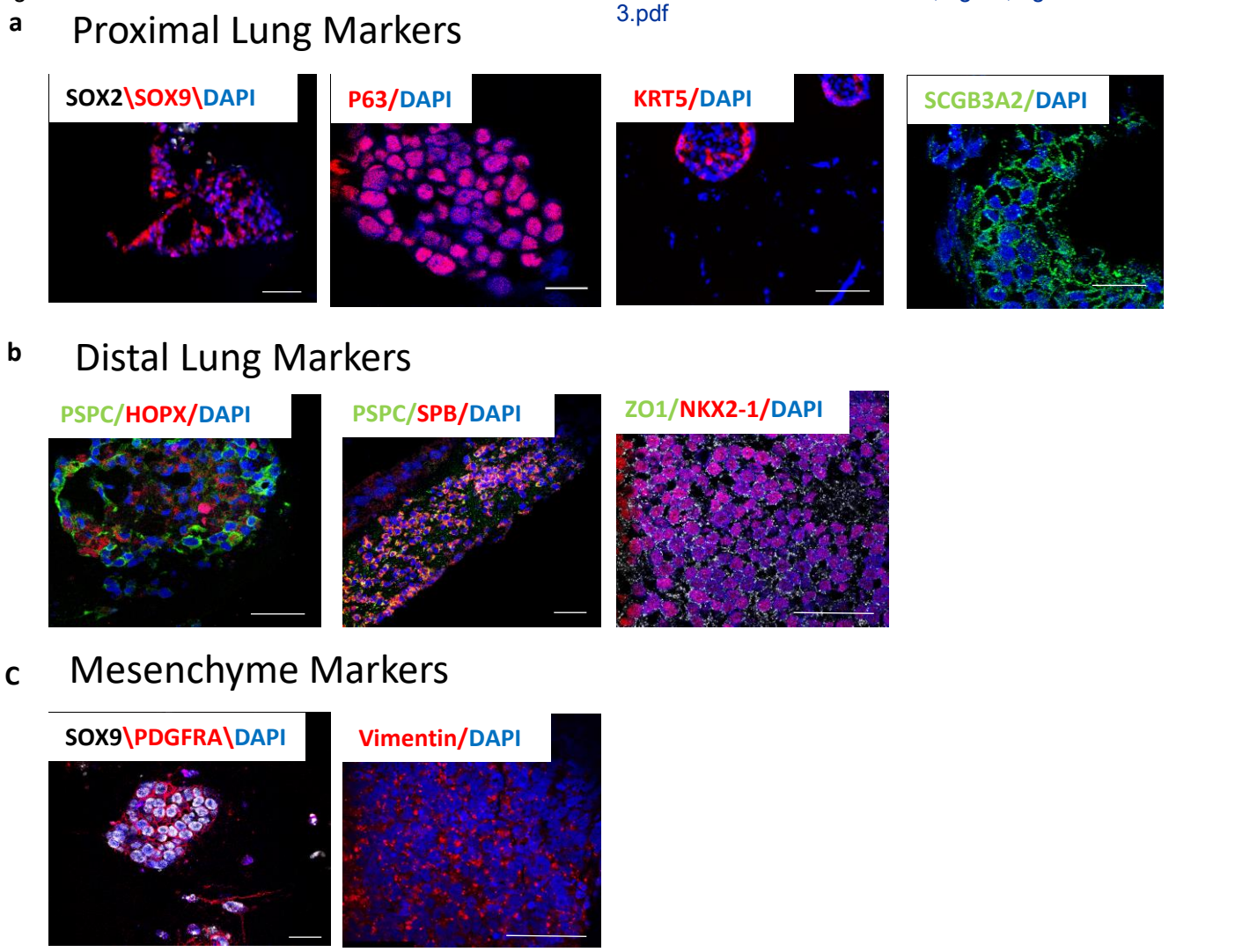


TABLE OF MEDIAS:
REAGENTS AND SOLUTIONS - For company names please see the Table of Materials List

3D organoid induction medium (day 17-22)
Serum-free basal medium (see recipe) supplemented with:
FGF7 (10 ng/mL)
FGF10 (10 ng/mL)
CHIR99021 (3 µM)
EGF (10 ng/mL)

3D organoid branching medium (day 23-28)
Serum-free basal medium (see recipe) supplemented with:
FGF7 (10 ng/mL)
FGF10 (10 ng/mL)
CHIR99021 (3 µM)
All-trans retinoic acid (0.1 µM)
EGF (10 ng/mL)
VEGF/PIGF (10 ng/mL)

3D organoid maturation medium (day 29-34)
3D organoid branching medium (see recipe) supplemented with:
Dexamethasone (50 nM)
Br-cAMP (100 µM)
IBMX (100 µM)

AFE induction medium (day 4-6)
Serum-free basal medium (see recipe) supplemented with:
SB431542 (10 µM)
Dorsomorphin (2 µM)

DE induction medium (day 1-3)
48.5 mL RPMI1640 + Glutamax
1 mL B27 without retinoic acid
500 µl HEPES (1%)
500 µl pen/strep
Human activin A (100 ng/mL)
CHIR99021 (5 µM) - only in the first 24 hours

LPC induction medium (day 7-16)
Serum-free basal medium (see recipe) supplemented with:
BMP4 (10 ng/mL)
All-trans retinoic acid (RA) (0.1 µM)
CHIR99021 (3 µM)

Quenching medium
49 mL DMEM/F12
1 mL FBS

Serum-free basal medium (SFBM)
375 mL Iscove's Modified Dulbecco's Medium (IMDM) + Glutamax
125 mL Ham's F12
5 mL B27 without retinoic acid
2.5 mL N2
500 µl ascorbic acid, 50 mg/mL
19.5 µl monothioglycerol, 500 µg/mL
3.75 mL bovine serum albumin (BSA) Fraction V, 7.5% solution
500 µl pen/strep

Stem cell passaging medium (day 0)
500 mL DMEM/F12
129 mL Knockout serum replacement (KSR)
6.5 mL Glutamax
6.5 mL NEAA
1.3 mL 2-mercaptoethanol
6.5 mL pen/strep

Table 2

Troubleshooting

Problem	Solution
DE differentiation not efficient	24 hours after plating in stem cell medium, cells should be 50-70% confluent
	GSK3β inhibitor/Wnt activator CHIR99021 should be removed within 20-24 hours of day 1 DE induction
	DE differentiation should not exceed a total of 72 hours
AFE differentiation not efficient	Ensure that DE differentiation was successful and the cells express > 80% CXCR4
	Ensure fresh growth factors/small molecules are being added to the media daily
LPC differentiation not efficient	Ensure the AFE differentiation was successful and the cells express > 80% FOXA2/SOX2
	Ensure the AFE cells are passaged as aggregates of 4-10 cells and not single celled
3D lung organoids not growing or differentiating	Ensure the LPC differentation was successful and the cells express > 30% NKX2-1
	Ensure the LPCs were passaged as aggregates of 4-10 cells and not single celled
	Ensure there is no residual matrigel from the LPCs during passaging
	Add Rock Inhibitor Y-27632 with each media change
	Ensure the media is changed on time and fresh growth factors/small molecules are added
	Ensure concentration of growth factors/small molecules is correct

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Cell Culture			
12 well plates	Corning	3512	
12-well inserts, 0.4um, translucent	VWR	10769-208	
2-mercaptoethanol	Sigma-Aldrich	M3148	
Accutase	Innovative Cell Tech	AT104	
ascorbic acid	Sigma	A4544	
B27 without retinoic acid	ThermoFisher	12587010	
Bovine serum albumin (BSA) Fraction V, 7.5% solution	Gibco	15260-037	
Dispase	StemCellTech	7913	
DMEM/F12	Gibco	10565042	
FBS	Gibco	10082139	
Glutamax	Life Technologies	35050061	
Ham's F12	Invitrogen	11765-054	
HEPES	Gibco	15630-080	
Iscove's Modified Dulbecco's Medium (IMDM) + Glutamax	Invitrogen	31980030	
Knockout Serum Replacement (KSR)	Life Technologies	10828028	
Matrigel	Corning	354230	
Monothioglycerol	Sigma	M6145	
mTeSR plus Kit (10/case)	Stem Cell Tech	5825	
N2	ThermoFisher	17502048	
NEAA	Life Technologies	11140050	
Pen/strep	Lonza	17-602F	
ReleSR	Stem Cell Tech	5872	
RPMI1640 + Glutamax	Life Technologies	12633012	
TrypLE	Gibco	12605-028	
Y-27632 (Rock Inhibitor)	R&D Systems	1254/1	
Growth Factors/Small Molecules			
Activin A	R&D Systems	338-AC	
All-trans retinoic acid (RA)	Sigma-Aldrich	R2625	
BMP4	R&D Systems	314-BP/CF	

Br-cAMP	Sigma-Aldrich	B5386	
CHIR99021	Abcam	ab120890	
Dexamethasone	Sigma-Aldrich	D4902	
Dorsomorphin	R&D Systems	3093	
EGF	R&D Systems	236-EG	
FGF10	R&D Systems	345-FG/CF	
FGF7	R&D Systems	251-KG/CF	
IBMX (3-Isobtyl-1-methylxanthine)	Sigma-Aldrich	I5879	
SB431542	R&D Systems	1614	
VEGF/PIGF	R&D Systems	297-VP/CF	

Primary antibodies			**Dilution rate**
CXCR4-PE	R&D Systems	FAB170P	1:200 (F)
HOPX	Santa Cruz Biotech	sc-398703	0.180555556
HTII-280	Terrace Biotech	TB-27AHT2-280	0.145833333
KRT5	Abcam	ab52635	0.180555556
NKX2-1	Abcam	ab76013	0.25
NKX2-1-APC	LS-BIO	LS-C264437	1:1000 (F)
proSPC	Abcam	ab40871	0.215277778
SCGB3A2	Abcam	ab181853	0.25
SOX2	Invitrogen	MA1-014	0.180555556
SOX9	R&D Systems	AF3075	0.180555556
SPB (mature)	7 Hills	48604	1: 1500 (F) 1:500 (W)[a]
SPC (mature)	LS Bio	LS-B9161	1:100 (F); 1:500 (W) [a]

Rebuttal to Editors and Reviewers

Thank you to the editors for your remarks and suggestions which improved the manuscript immensely. We incorporated your suggestions into the manuscript and are re-submitting for your approval. Please see below our responses to the editors and reviewers.

Editorial Changes
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have proofread the manuscript very closely and corrected all errors we uncovered.

2. Please rewrite the following sentences to avoid previously published work: lines 54-56, 68-72.
We have rewritten the sentences to avoid similarities. Please see page 2, lines 55-57 and 71-73. We have also updated our protocol to separate the two publications even further. In our previous publication, we were differentiating LPC in a monolayer, but have changed to a 3D method two years ago. This permits a more robust expression of NKX2-1. Therefore, we have updated the figures, figure legends and text to reflect this change.

3. Please include an ethics statement at the start of the protocol about the conformation of institutional ethics guidelines for this protocol.
We have included an ethical statement. Please see page 2, lines 85-86.

4. Use “mL” instead of “ml” (e.g., lines 132, 141, etc.). Also include a space between the quantity and its unit: “1 mm” instead of “1mm” (e.g., line 135, etc.).
We have adjusted the units and added spaces after the numbers.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. E.g., Matrigel, Eppendorf tube, etc. Please include details about manufacturers of specific reagents/materials only in the table of materials and not in the protocol. E.g., line 108-109, etc.
We have removed commercial language from the manuscript and updated the Table of Materials.

6. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
We have highlighted the most pertinent parts of the protocol.

7. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Journal name. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate the journal title.
We have adjusted the references to follow the layout provided.

8. Do not embed tables in the text file. Instead, upload them separately as .xls files through the editorial manager. (e.g., lines 314- 376 – upload this as a separate table). Sort the Materials Table alphabetically by the name of the material.
We have created a separate “Table of Medias” as an excel file and sorted them alphabetically.

Reviewers' comments:

Reviewer #1:

The JOVE manuscript titled. "Generation of Induced Pluripotent Stem Cell-derived Human Whole Lung Organoids to Model Development and Disease" is a methods video article of importance. However, I have the following suggestions:

1. The title can be modified to depict inclusivity. Maybe the authors can consider revising the title as: "Generation of 3D whole lung organoids from iPSCs for modelling the lung-developmental biology and disease".
Thank you for the suggestion, and we have modified the title of the manuscript to depict inclusivity.

2. Page 3: Line 82, under the heading "Protocol", please mention the entire scheme of differentiation and depict the same with a Figure number a.
We have added a summary paragraph with the full differentiation (lines 88-91 on page 2) and adjusted the figure legend for figure 1a (Page 7, lines 306-309).

3. Page 3: Line 85, please express the concentration of matrigel in percentage, say here it is 50%.
Done.

4. Page 3: Line 86-87, Rephrase the sentence, "Add 5ml ……bottom" and make it crisp, as " Coat each well of a 12 well plate with 500 microlitres of 50% GFR-M prepared in ice-cold DMEM/F12.
Done.

5. Page 4: Line 89, please mention 'or refrigerator at 4 deg C' next to the word 'ice'.
Done.

6. Page 4: Line 93, Rephrase the sentence, 'Add accutase to the stem cells' with 'Dissociate iPSCs by adding Accutase'.
Done.

7. Page 4: Section 1.3, Line 96, this is not clear whether Accutase is added separately, or else, stem cell passaging media is used, along-with Accutase. Please rephrase this point to clarify. Merge the points 1.2 and 1.3.
We have rephrased the paragraph to make it more clear and merged the sections. Please see page 2, section 1.2.

8. Page 4: Section 1.3, Line 96, Rephrase, "Add……passage media" as "Add iPSC passaging media."
Done

9. Page 4: Line 101, write Rock inhibitor prior to the name of the compound.
Done.

10. Page 4: Line 109, please write 'GSK3β inhibitor/Wnt activator before the name of the compound CHIR99021.
Done.

11. Page 4: Line 122, Next to the heading under point 2: Mention the Day number here, say Day 4 to Day 6. Follow the same for all the headings mentioning each step of multistep differentiation.
We have updated each heading with the days that each step of the differentiation is performed.

12. Page 5: Line 132, Rephrase the first sentence as ' On Day XXX, aspirate off.'
Done.

13. Page 5: Line 135, It should be 15 ml conical centrifuge tube.
Done

14. Page 5: Section 3.2, please correct the syntax errors. Also, write the broad categories of the compound such as Rock inhibitor before Y-27632 etc. Please follow the same for the entire manuscript regarding mentioning the general group of the compound before the compound name.
We have corrected the syntax errors and added the general group of the compound before the compound name throughout the manuscript.

15. Page 6: Line 176, please add the missing articles such as 'the.'
Done
Done

16. Page 7: Representative results section, please mention about the appearance of lung mesenchyme as well as the epithelial cells especially line 257, in detail such as the marker PDGFRα. Mention the figure numbers for this sentence.
We have mentioned the appearance of both epithelial and mesenchymal lung populations in the Representative Results section, page 7, lines 281-288 and linked them the figure 3.

17. Page 7: Line 257, 258, "These organoids contain a mixed population of lung epithelial and mesenchymal cells but take months to culture"-Please elaborate this point in the discussion section about their usefulness for cell therapy or drug screening especially if the time required for these organoids is so much.
We mentioned the drawback of using autologous cells in acute diseases due to the long time is takes to fully differentiate them and undergo subsequent quality controls. See page 10, lines 419-422.

18. Page 9: Table of Materials, please mention the company name against each of the reagents mentioned, although the same has been mentioned in the reagent list.
We have created a Table of Medias with the recipes of all the medias and the company names are included in the Table of Materials.

19. Page 10,'Discussion' section-Please add a sentence on how you can estimate the percentage of undifferentiated iPSCs during first step of differentiation. Discuss regarding the feasibility of generation of human lung organoids from allogeneic or autologous iPSCs.
We have added a paragraph in the Discussion section incorporating your suggestions. Please see page 10, lines 409-427.

20. Page 10, Line 385, Mention the company of ReLesR.
We have been directed by the editor that we cannot include company names in the manuscript. We have guided the reader to access the Table of Materials after the discussion of ReLesR. (see Page 9, line 351).

21. Mention one section on 'Troubleshooting' and how to ensure the efficiencies of differentiations in each step in a tabular format.
We have created a separate table called “Troubleshooting” which can help the scientist improve the differentiations.

Reviewer #2:
Manuscript Summary:
The authors describe their method for generation whole lung organoids from iPSCs. Successful differentiation and branching are demonstrated.

Major Concerns:
The authors mention that the purity of these lung organoids is a limitation to this study. Additional information is warranted given concerns about contamination critical for subsequently assays.
Thank you for the suggestion. We have added a couple of sentences addressing the purity of the lung organoids, specifically in their use in regenerative medicine. See page 10, lines 409-415.

Minor Concerns:
No clear why the authors did not reference novel work from the Spence lab and how their approach differs.
We apologise for this oversight. We follow the work of the Spence lab and recognize their important contributions to the lung organoid field. We added a summary of their iPSC lung organoid differentiation and the reference. Please see page 1, lines 78-80.