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TITLE

Isolation and Enrichment of Human Lung Epithelial Progenitor Cells for Organoid Culture

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KEYWORDS:

lung, epithelium, organoid, disease modeling, alveolar Type II cells, FACS

SUMMARY:

This article provides a detailed methodology for tissue dissociation and cellular fractionation approaches allowing enrichment of viable epithelial cells from proximal and distal regions of the human lung. Herein these approaches are applied for the functional analysis of lung epithelial progenitor cells through the use of 3D organoid culture models.

ABSTRACT:

Epithelial organoid models serve as valuable tools to study the basic biology of an organ system and for disease modeling. When grown as organoids, epithelial progenitor cells can self-renew and generate differentiating progeny that exhibit cellular functions similar to those of their in vivo counterparts. Herein we describe a step-by-step protocol to isolate region-specific progenitors from human lung and generate 3D organoid cultures as an experimental and validation tool. We define proximal and distal regions of the lung with the goal of isolating region-specific progenitor cells. We utilized a combination of enzymatic and mechanical dissociation to isolate total cells from the lung and trachea. Specific progenitor cells were then fractionated from the proximal or distal origin cells using fluorescence associated cell sorting (FACS) based on cell type specific surface markers, such as NGFR for sorting basal cells and HTII-280 for sorting alveolar type II cells. Isolated basal or alveolar type II progenitors were used to generate 3D organoid cultures. Both distal and proximal progenitors formed organoids with a colony forming efficiency of 9-13% in distal region and 7-10% in proximal region when plated 5000 cell/well on day 30. Distal organoids maintained HTII-280^{+} alveolar type II cells in culture whereas proximal organoids differentiated into ciliated and secretory cells by day 30. These 3D organoid cultures

can be used as an experimental tool for studying the cell biology of lung epithelium and epithelial mesenchymal interactions, as well as for the development and validation of therapeutic strategies targeting epithelial dysfunction in a disease.

INTRODUCTION:

Airspaces of the human respiratory system can be broadly divided into conducting and respiratory zones that mediate transport of gasses and their subsequent exchange across the epithelial-microvascular barrier, respectively. The conducting airways include trachea, bronchi, bronchioles and terminal bronchioles, whereas respiratory air spaces include respiratory bronchioles, alveolar ducts and alveoli. The epithelial lining of these airspaces changes in composition along the proximo-distal axis to accommodate the unique requirements of each functionally distinct zone. The pseudostratified epithelium of tracheo-bronchial airways is composed of three major cell types, basal, secretory and ciliated, in addition to the less abundant cell types including brush, neuroendocrine and ionocyte[1-3]. Bronchiolar airways harbor morphologically similar epithelial cell types, although there are distinctions in their abundance and functional properties. For example, basal cells are less abundant within bronchiolar airways, and secretory cells include a greater proportion of club cells versus serous and goblet cells that predominate in tracheobronchial airways. Epithelial cells of the respiratory zone include a poorly defined cuboidal cell type in respiratory bronchioles, in addition to alveolar type I (ATI) and type II (ATII) cells of alveolar ducts and alveoli[1,4].

The identity of epithelial stem and progenitor cell types that contribute to the maintenance and renewal of epithelia in each zone are incompletely described and largely inferred from studies in animal models[5-8]. Studies in mice have shown that either basal cells of pseudostratified airways, or club cells of bronchiolar airways or ATII cells of the alveolar epithelium, serve as epithelial stem cells based upon capacity for unlimited self-renewal and multipotent differentiation[7,9-12]. Despite the inability to perform genetic lineage tracing studies to assess stemness of human lung epithelial cell types, the availability of organoid-based culture models to assess the functional potential of epithelial stem and progenitor cells provides a tool for comparative studies between mouse and human[13-17].

Herein we describe methods for the isolation of epithelial cell types from different regions of the human lung and their culture using a 3D organoid system to recapitulate the regional cell types. Similar methods have been developed for the functional analysis and disease modeling of epithelial cells from other organ systems[18-21]. These methods provide a platform for the identification of regional epithelial progenitor cells, to perform mechanistic studies investigating their regulation and microenvironment, and to enable disease modeling and drug discovery. Even though studies of lung epithelial progenitor cells performed in animal models can benefit from the analysis, either in vivo or in vitro, insights into the identity of human lung epithelial progenitor cells have been largely dependent upon extrapolation from model organisms. As such, these methods provide a bridge to relate the identity and behavior of human lung epithelial cell types with their studies investigating regulation of stem/progenitor cells.

PROTOCOL:

Human lung tissue was obtained from deceased tissue donors in compliance with consent procedures developed by International Institute for the Advancement of Medicine (IIAM) and approved by the Cedars-Sinai Medical Center Internal Review Board.

1. Tissue processing for isolation of lung cells from either tracheo-bronchial or small airway/parenchymal (small airways and alveoli) regions

1.1. Prepare and autoclave all dissection instruments, glassware and the appropriate solutions one day prior to cell isolation.

1.2. Upon receiving lung tissue, identify and separate the proximal and distal regions. The trachea and bronchi are considered 'proximal'. For the purposes of this protocol trachea and the first 2-3 generations of bronchi are disected and used for the isolation of "proximal" airway epithelium. Small airways of 2 mm or less in diameter and surrounding parenchymal tissue are, for the purpose of this protocol, considered as 'distal' lung epithelium (**Figure 1A**).

NOTE: All procedures involving processing of human lung tissue should be performed in a biosafety cabinet with use of appropriate personal protective equipment.

2. Enrichment and sub setting of small airway and alveolar epithelial progenitor cells from distal lung tissue

2.1. Distal tissue preparation

2.1.1. Place the distal lung tissue in a sterile Petri dish (150 x 15 mm). Dice tissue into approximately 1 cm^3 pieces and place in a clean 50 mL tube.

2.1.2. Wash the tissue 3x with chilled HBSS, discarding HBSS wash each time to remove blood and epithelial lining fluid.

2.1.3. Place the tissue in a new Petri dish and blot dry with sterile anti-lint wipes. Using forceps and scissors, remove as much visceral pleura (a delicate transparent membrane that covers surface of the lung) as possible.

2.1.4. Use scissors to mince tissue into pieces of approximately 2 mm diameter. Transfer minced tissue into a clean Petri dish and mince further by chopping it to an approximate size of 1 mm with a sterile single sided razor blade.

2.2. Enzyme digestion

NOTE: Liberase stock solution is 5 mg/mL (100x) and DNase stock is 2.5 mg/mL (100x) (**Table of Materials**).

2.2.1. Add 50 µg/mL Liberase and 25 µg/mL DNase into sterile HBSS in a 50 mL conical tube.

2.2.2. Transfer approximately 2-3 g of minced tissue to a new 50 mL conical tube with 25 mL of HBSS, containing Liberase and DNase. Incubate for 40-60 min at 37 ˚C with continuous shaking using a mixer set at 900 rpm. After 30 min of incubation, triturate digested tissue using a 30 mL syringe without a needle to avoid formation of clumps and continue with the incubation.

NOTE: Incubation time with the enzymes can vary depending on the type or condition of the tissue. For example, enzymatic digestion of normal tissue takes approximately 45 min. However, fibrotic tissue from Idiopathic pulmonary fibrosis samples can require a longer incubation time of up to 60 min. Therefore, monitor tissue carefully during this step to prevent damage to the surface markers, which is crucial for FACS.

2.3. Single cell isolation

2.3.1. Triturate the tissue by drawing 5x through a 16 G needle fitted to a 30 mL syringe. Draw the tissue suspension into a wide-bore pipette and pass through a series of cell strainers (500 µm, 300 µm, 100 µm, 70 µm, 40 µm) under vacuum pressure. Wash the strainer with 20 mL of HBSS+ buffer to collect remaining cells. The recipe for HBSS+ buffer can be found in **Table of Materials.**

2.3.2. Add an equal volume of HBSS+ buffer, after 45 min to the filtrate to inhibit Liberase activity and prevent over-digestion.

2.3.3. Centrifuge filtrates at 500 *x g* for 5 min at 4 ˚C. Carefully remove and discard the supernatant. Add 1 mL of Red Blood Cell (RBC) lysis buffer to the pellet, gently rock the tube to dislodge the pellet and incubate on ice for 1 min.

NOTE: The amount and time in the RBC lysis solution depends on the size of the pellet. It is important to maintain the cells on ice and monitor time in RBC lysis solution carefully to prevent lysis of target cells. If RBC lysis is insufficient, repeat the step.

2.3.4. Add 10-20 mL of HBSS+ buffer to neutralize RBC lysis buffer. Centrifuge filtrates at 500 *x g* for 5 min at 4 ˚C.

2.3.5. If lysed red blood cells (ghost cells) forms a cloudy layer above the cell pellet, resuspend pellet in 10 mL of HBSS+ buffer and strain the suspension through 70 µm cell strainer to eliminate the ghost cells. Centrifuge the filtrate at 600 *x g* for 5 min at 4 ˚C and proceed with further steps.

2.4. Depletion of immune cells and endothelial cell (optional step)

2.4.1. Deplete $CD31^+$ endothelial cells and $CD45^+$ immune cells from the pool of total cells using the CD31 & CD45 microbeads conjugated to monoclonal anti-human CD31 and CD45 antibody (isotype mouse IgG1) and LS columns in accordance to the manufacturer's protocol (**Table of Materials**).

2.4.2. Collect the flow through, consisting primarily of epithelial and stromal cells, in a fresh sterile tube and centrifuge it at 600 *x g* for 5 min at 4 ˚C. Perform a cell count to ascertain the total number of cells in the flow through.

2.5. Cell surface staining for fluorescence associated cell sorting (FACS)

2.5.1. Resuspend 1 x 10^7 cells per 1 mL of HBSS+ buffer. Add primary antibodies at the required concentration and incubate the cells for 30 min at 4 ˚C in the dark. In this study, fluorophore conjugated primary antibodies were used unless otherwise stated. Details of antibody sources and titers are described in **Table of Materials**.

NOTE: HTII-280 is currently the best surface reactive antibody that allows subsetting of distal lung cells into predominantly airway (HTII-280^-) and alveolar type 2 (HTII-280^+) cell fractions. A caveat to this strategy is that AT1 cells are not stained using this method and are poorly represented due to their fragility. However, AT1 cells are poorly represented in distal lung preps, presumably due to their fragility and loss during selection of viable cells by FACS and thus only represent a rare contaminant of the airway cell fraction.

2.5.2. Wash cells by adding 3 mL of HBSS+ buffer and centrifuge at 600 *x g* for 5 min at 4 ˚C.

2.5.3. If using unconjugated primary antibodies, add required concentration of an appropriate fluorophore conjugated secondary antibody and incubate for 30 min on ice. Wash off excess secondary antibody by adding 3 mL of HBSS+ buffer and centrifuge at 600 *x g* for 5 min at 4 ˚C.

2.5.4. Discard the supernatant and resuspend cells in HBSS+ buffer per 1 x 10^7 cells/ mL. Filter cells into 5 mL polystyrene tubes through a strainer cap to ensure formation of a single cell suspension. Add DAPI (1 µg/mL) to stain permeable (dead) cells.

NOTE: It is essential to use appropriate single-color and Fluorescence minus one (FMO) controls (i.e., antibody staining cocktail minus one antibody each), to minimize false positives during FACS. In this study positive and negative selection beads were used for empirical compensation for overlap of emission spectra between fluorophores (**Table of Materials**). FACS enrich cell types of interest. Viable epithelial cells are enriched based upon their CD45-negative, CD31-negative, CD236-positive cell surface phenotype and negative staining for DAPI. This epithelial cell fraction can be further subsetted based on staining for cell type-specific surface markers, such as specific staining for HTII-280-positive cells that are enriched for AT2 cells. In contrast, negative selection for HTII-280 allows the enrichment of small airway epithelial cells such as club and ciliated cells (**Figure 2**).

3. Enrichment and subsetting of epithelial progenitor cells from tracheo-bronchial airways

3.1. Tissue preparation

3.1.1. Dissect out proximal airways (trachea/bronchi) from the lungs. Open airways along their length using scissors to expose the lumen and add 50 µg/mL Liberase to fully cover the tissue.

3.1.2. Incubate for 20 min at 37 ˚C with continuous shaking using a mixer set at 900 rpm.

3.1.3. Remove the proximal airway from the centrifuge tube and place it in a sterile Petri dish (150 x 15 mm). Gently scrape the surface of the airway using a scalpel to completely strip luminal epithelial cells from the tissue.

3.1.4. Wash the Petri dish with 5 mL of sterile HBSS+ buffer to collect all dislodged luminal epithelial cells and transfer the dislodged cells to 50 mL conical centrifuge tube. Triturate the suspension by drawing 5x through 16 G needle and 18 G needle fitted to a 10 mL syringe to get single cell suspension.

3.1.5. Centrifuge the suspension at 500 *x g* for 5 min at 4 ˚C. Resuspend the pellet in fresh HBSS+ buffer and store these luminal airway cells on ice, ready to be combined with the single cell suspension generated from the minced proximal airways in the upcoming steps.

3.1.6. Using scissors, cut remaining tracheobronchial tissue along its rings to generate small strips of tissue, and transfer the strips to a fresh Petri dish. Mince the tissue strips using a single sided razor blade to make smaller pieces.

NOTE: Since the proximal airways are cartilaginous, they cannot be minced as finely as the distal lung tissue.

3.1.7. Transfer the minced tissue into the C tubes, add 2 mL of Liberase to the tube ensuring that the tissue is submerged. Load the C tube onto the automated dissociator and run Human Lung Protocol-2 to mechanically dissociate tissue further.

NOTE: The dissociator used in this protocol offers an optimized program called human lung protocol-2 for this specific application (see **Table of Materials**).

3.2. Enzyme digestion and single cell isolation

3.2.1. Transfer approximately 2 g of minced proximal tissue from the C tube into each 50 mL conical centrifuge tube and add 50 µg/mL Liberase and 25 µg/mL DNase solution to each tube.

NOTE: To ensure efficient dissociation, tubes should not be filled beyond the 30 mL mark.

3.2.2. Incubate the minced tissue for 45 min at 37 ˚C with continuous shaking using a mixer set at 900 rpm.

3.2.3. Pass the dissociated tissue suspension through a series of cell strainers (500 µm, 300 µm, 100 µm, 70 µm, 40 µm) under vacuum pressure as mentioned above and collect the flow through. Wash the strainer with 20 mL of HBSS+ buffer to collect remaining cells.

NOTE: Since proximal tissue is cartilaginous and bulky as compared to the distal tissue, there is a higher possibility of clogging of the filters. Using a funnel can help prevent overflowing of the liquid while passing through the strainers.

3.2.4. Add an equal volume of HBSS+ buffer to the filtrate to inhibit Liberase activity and prevent over-digestion. Add the isolated luminal proximal airway cells from 3.1.5 to the cell suspension at this step.

3.2.5. Centrifuge the combined cell suspension at 600 *x g* for 10 min. Remove the supernatant and repeat the cell wash in HBSS+ buffer. Perform depletion of $CD45^+$ immune cells and $CD31^+$ endothelial cells as mentioned above in 2.4 (optional step).

3.2.6. Methods for staining is similar to distal lung tissue, follow the steps in 2.5. Enrich viable epithelial cells based upon their CD45-negative, CD31-negative, CD236-positive cell surface phenotype and negative staining for DAPI.

3.2.7. Further subset the epithelial cell fraction based upon staining for cell type-specific surface markers, such as NGFR, allowing enrichment of basal (NGFR positive) and non-basal (NGFR negative; secretory, ciliated, neuroendocrine) cell types (**Figure 3**).

4. Organoid culture

4.1. Add 5,000 (this number can be adjusted to yield the desired density of epithelial organoids) sorted proximal or distal epithelial cells to sterile 1.5 mL tube along with 7.5 x 10^4 MRC-5 cells (human lung fibroblast cell line). Epithelial-mesenchymal interactions are critical for the expansion of progenitor cells.

4.2. Centrifuge at 500 *x g* for 5 min at 4 ˚C.

NOTE: It is important to manually confirm the cell count obtained from the sorter in order to ensure accuracy organoid colony forming efficiency.

4.3. Carefully remove and discard the supernatant and resuspend the cell pellet in 50 µL of ice-cold media supplemented with antibiotics. Keep the cell suspension on ice.

4.4. Add 50 µL of ice cold 1x growth factor depleted basement membrane matrix medium to the vial and gently pipette the suspension on ice to mix.

NOTE: It is important to use ice cold media and maintain cells on ice to avoid premature polymerization of the basement membrane matrix medium.

4.5. Transfer the cell suspension onto 0.4 µM pore-size cell culture insert in a 24 well plate (1.4 x 10^4 cells/cm^2), taking care to avoid introduction of air bubbles.

4.6. Incubate at 37 ˚C for 30-45 min to allow the matrix to solidify.

4.7. Add 600 µL of pre-warmed growth medium to the well.

NOTE: Media was supplemented with antimycotic agents (0.4%) and Pen strep (1%) for the first 24 h after seeding and 10 µM Rho kinase inhibitor for the first 72 h.

4.8. Culture at 37 ˚C in a 5% CO_2 incubator for 30 days, during which time the media should be changed every 48 h.

NOTE: The culture duration can be altered based on the purpose of the experiment. Longer endpoints are used to study differentiation whereas shorter endpoints of 7 days, 14 days etc., can be used if the purpose of the experiment is not to achieve complete differentiation.

4.9. Add 10 µM TGFβ inhibitor to the culture media for 15 days to maintain the cells in the proliferative phase and suppress overgrowth of fibroblasts.

NOTE: Results differ according to the culture medium used for the assay. For e.g., results shown herein were generated using Pneumacult-ALI Medium, which results in the generation of large organoids from distal lung, well differentiated and larger organoids from proximal lung.

5. Organoid staining

5.1. Fixing and embedding of organoids

5.1.1. Aspirate media from both the upper and lower transmembrane insert chambers and rinse once with warm PBS.

5.1.2. Fix the cultures by placing 300 µL of PFA (2% w/v) in the insert and 500 µL in the well for 1hr at 37^{o}C. Remove fixative and rinse with warm PBS taking care not to dislodge the basememt membrane matrix plug.

NOTE: Fixed organoids can be stored submerged in PBS at 4 ˚C for one to two weeks before initiating further steps.

5.1.3. Aspirate PBS, invert the insert and carefully cut the insert membrane around its periphery. Using forceps, remove transwell membrane, taking care not to disturb the matrix plug.

5.1.4. In a Petri dish, tap the insert to recover the matrix plug.

5.1.5. Add a drop of gel necessary for histology and cytology (maintained at 37 ˚C) to the matrix plug and maintain at 4 ˚C until the gel solidifies.

5.1.6. Transfer the plug to an embedding cassette, dehydrate through increasing concentrations of ethanol (70, 90 and 100%), clear in xylene and embed in paraffin wax.

5.1.7. Cut 7 µm sections on a microtome and collect on positively charged slides.

5.2. Immunofluorescence staining of organoids

5.2.1. Place slides at 65 ˚C for 30 min to dewax.

5.2.2. Deparaffinize the sections by immersion in xylene and rehydrate through decreasing concentrations of ethanol.

5.2.3. Perform high temperature antigen retrieval in antigen unmasking solution, citric acid base using a commercially available retriever by dipping slides in the solution for 15 min (**Table of Materials**).

5.2.4. Surround the tissue with a hydrophobic barrier using a pap pen.

5.2.5. Block non-specific staining between the primary antibodies and the tissue, by incubating in Blocking buffer.

5.2.6. Incubate sections in the appropriate concentration of primary antibodies diluted in incubation solution overnight at 4 ˚C in a humidified chamber.

5.2.7. Rinse sections 3x at room temperature with a washing buffer.

5.2.8. Incubate in the appropriate concentration of fluorochrome conjugated secondary antibody for 1 h at room temperature.

5.2.9. Rinse sections 3x at room temperature with 0.1% Tween 20-TBS. Incubate sections for 5 min in DAPI (1 µg/mL). Rinse sections once in TBS (Tris-buffered saline) with 0.1% Tween 20-, dry and mount in a mounting solution (**Figure 4** and **Figure 5**).

NOTE: Source and optimal working dilution of primary and secondary antibodies used for immunofluorescence staining are included in the **Table of Materials**.

REPRESENTATIVE RESULTS:

Source lung tissue

The trachea and extrapulmonary bronchus (**Figure 1A**) were used as the source tissue for isolation of proximal airway epithelial cells and subsequent generation of proximal organoids. Distal lung tissue that includes both parenchyma and small airways of less than 2 mm in diameter

(**Figure 1A**) were used for the isolation of small airway and alveolar epithelial cells (distal lung epithelium) and generation of either small airway or alveolar organoids. Proximal airways lined by a pseudostratified epithelium include abundant basal progenitor cells that are immunoreactive for the membrane protein NGFR (**Figure 1B,C**). In contrast, epithelial cells lining alveoli included a subset showing apical membrane immunoreactivity with the HTII-280 monoclonal antibody, suggestive of their alveolar type 2 cell (AT2) identity (**Figure 1B,D**). These surface markers were used to subset single cell suspensions of epithelial cells isolated from either proximal or distal regions.

Tissue dissociation and cell fractionation

Single cell suspensions of total cells were isolated from either proximal or distal regions of human lung tissue and fractionated using both magnetic bead and FACS to yield enriched epithelial cell populations (**Figure 2** and **Figure 3**). Abundant contaminating cell types including red blood cells, immune cells and endothelial cells were stained using antibodies to CD235a, CD45 and CD31, respectively, followed by magnetic-associated cell sorting for depletion of these cell types from the total pool of lung cells. The resulting "depleted" cell suspensions were significantly enriched for epithelial cell populations in both distal (**Figure 2E**) and proximal (**Figure 3E**) tissue samples, with corresponding increase in FACS efficiency. After depletion of CD235a/ CD45/CD31 positive cells using CD45 & CD31 microbeads the percentage of $CD31^-/CD45^-/CD235a^-$ increased from 14% (**Figure 2A,B**) to 51.7% (**Figure 2E,F**) in distal population. Further FACS depletion of cells staining positively for either CD235a, CD45 or CD31, elimination of cells with positive staining for DAPI and positive selection for the epithelial cell surface marker CD326, led to highly enriched distal cell population that accounted for 33.5% (**Figure 2E,G**) compared to 7% (**Figure 2A,C**) before depletion of negative population. Further subsetting of distal epithelial cell populations was achieved by fractionation based upon surface staining with the HTII-280 monoclonal antibody (**Figure 2D,H**), respectively. Accordingly, distal lung epithelial cells included 4.3% $HTII\text{-}280^+$ and 2.6% $HTII\text{-}280^-$ subsets (**Figure 2D** without depleting of CD31/CD45/CD235a) and 30% $HTII\text{-}280^+$ and 3.6% $HTII\text{-}280^-$ subsets (**Figure 2H** after depleting of CD31/CD45/CD235a).

Total cells isolated from proximal region were depleted for CD235a/ CD45/CD31 positive cells using CD45 & CD31 microbeads and the percentage of $CD31^-/CD45^-/CD235a^-$ increased from 17% (**Figure 3A,B**) to 56.6% (**Figure 3E,F**). Positive selection for the epithelial cell surface marker CD326 in cells isolated from proximal region, led to highly enriched proximal cell population that accounted for 38% (**Figure 3E,G**) of total lung cell fractions compared to 9.3% (**Figure 3A,C**) without depletion of negative population respectively. Further subsetting of proximal epithelial cell populations was achieved by fractionation based upon surface staining with antibodies to NGFR (**Figure 3D,H**), respectively. Accordingly, proximal lung epithelial cells included 2.7% $NGFR^+$ and 6.5% $NGFR^-$ subsets (**Figure 3D** without depleting of CD31/CD45/CD235a) and 13% were $NGFR^+$ and 25% $NGFR^-$ (**Figure 3H** after depleting of CD31/CD45/CD235a).

Lung organoid cultures

Distal lung epithelial organoids were cultured within growth-factor depleted basement membrane matrix in media that were empirically tested to optimize for organoid growth and differentiation. Three different media were evaluated including PneumaCult-ALI medium, small

airway epithelial cell growth medium (SAECG medium) and mouse Basal medium. Optimal organoid growth was obtained using PneumaCult-ALI medium, which was selected for further studies. Cultures of HTII-280^{+} distal lung epithelial cells yielded rapidly expanding organoids with an average colony-forming efficiency of 10% (**Figure 4A,B**). Immunofluorescence staining of day 30 cultures using the HTII-280 and SPC monoclonal antibody revealed lumen-containing organoids composed predominantly of HTII-280^{+} and SPC^{+} distal lung epithelial cells (**Figure 4C,C'** and **Figure 4D,D'**). Cultures of distal lung epithelial HTII-280^{-} cells yielded organoids that were composed of a pseudostratified epithelium resembling that of small airways (not shown).

Proximal lung epithelial organoids were cultured from NGFR^{+} cells seeded into Matrigel and cultured for 30 days in PneumaCult-ALI medium. Large lumen-containing organoids were observed (**Figure 5D,E,F**) with an average colony-forming efficiency of 7.8% (**Figure 5A,B,C**). Organoids were composed of a pseudostratified epithelium composed of self-renewing Krt5+ and NGFR+ basal cells (Fig 5D, 5E and 5F) and differentiated luminal cell types including FoxJ1+ ciliated cells and MUC5AC+ secretory cells (**Figure 5D,E**).

FIGURE AND TABLE LEGENDS:

Figure 1: Sampling of human lung tissue. (**A**) Schematic representation of the human lung showing strategy for sampling proximal and distal regions for cell isolation. (**B**) H&E staining of the proximal and distal regions of the lung. (**C,D**) Immunofluorescent staining of corresponding regions showing NGFR+ basal progenitor cells (red) at the basement membrane of bronchial airways and HTII-280^{+} alveolar type II progenitors (green) in the alveoli. scale bar = 50 μm.

Figure 2: Representative sorting strategy for distal lung cells. (**A,E**) Percentage of various cell populations before and after depletion of CD45^{+}and CD31^{+} population using CD31 and CD45 magnetic beads in distal regions of the lung from one biological sample. (**B,F**) Representative image of FACS plot showing gating strategy of distal CD31^{-}/CD45^{-}/CD235a^{-} population before and after depletion of CD31/CD45/CD235a positive population (**C,G**) Epcam^{+} population before and after depletion of CD31/CD45/CD235a positive population. (**D,H**) HTII-280$^{+/-}$ population before and after depletion of CD31/CD45/CD235a positive cells. Panels A-D are from the same biological sample and panels E-H are from the same biological sample.

Figure 3: Representative sorting strategy for proximal lung cells. (**A,E**) Percentage of various cell populations before and after depletion of CD45+and CD31+ population using CD31 and CD45 magnetic beads in proximal regions of the lung. (**B,F**) Representative image of FACS plot showing gating strategy of proximal CD31^{-}/CD45^{-}/CD235a^{-} population before and after depletion of CD31/CD45/CD235a positive population (**C,G**) Epcam^{+} population before and after depletion of CD31/CD45/CD235a positive population. (**D,H**) NGFR$^{+/-}$ population before and after depletion of CD31/CD45/CD235a positive cells. Panels A-D and E-H were prepared from two different biological samples.

Figure 4: Characterization of distal lung organoids. (**A**) Representative image of the human distal organoids cultured in PneumaCult-ALI medium (2x magnification). (**B**) The Colony forming

efficiency (%CFE) was calculated on triplicate wells of organoids derived from two different biological samples. (**C, C'**) Immunofluorescent staining of corresponding distal organoids cultured in ALI medium showing HTII-280^{+} AT2 cells (green). (**D, D'**) The marker used for isolation of AT2 cells in this study, HTII-280 costains (green) for the another well characterized AT2 cell marker , SPC (red). Scale bar = 50um.

Figure 5: Characterization of proximal organoids from the human proximal lung. (**A,B**) Representative image of the human Proximal organoids cultured in PneumaCult-ALI medium scale bar 50 µm. (**C**) The percentage colony forming efficiency (%CFE) was calculated on triplicate wells of organoids derived from two different biological samples. Immunofluorescent staining of differentiated proximal organoids at day 30 with (**D**) Krt5+ basal cells (green), FoxJ1+ ciliated cells (red) (**E**) Krt5+ basal cells (green) and MUC5AC+ goblet cells (red). (**F**) The marker used isolation of basal cells in this study, NGFR (green) co-stains for the well characterised basal cell marker, Krt5 (red). Scale bar = 50 µm.

DISCUSSION:

We describe a reliable method for the isolation of defined subpopulations of lung cells from human lung tissue for either molecular or functional analysis and disease modeling. Critical elements of methods include the ability to achieve tissue dissociation with preservation of surface epitopes, which allow antibody-mediated enrichment of freshly isolated cells, and the optimization of culture methods for the efficient generation of region-specific epithelial organoids. We focus on the recovery and enrichment of epithelial progenitor cells capable of forming organoids when recombined with stromal support cells in three-dimensional culture. Even though we did not define the clonality of organoids in these cultures, similar studies performed using isolated mouse lung epithelial progenitor cells were shown to be clonal based upon use of mixed cultures of cells harboring distinct fluorescent reporters[22,23].

Methods described herein include adaptations intended to improve cell recovery from digested lung tissue. Digested samples are passed through a 16-gauge needle to further disrupt any remaining undigested clumps and to achieve a homogenous cell suspension. Cell aggregation caused by extruded genomic DNA was mitigated by adding DNase I, which produced a homogeneous cell preparation that provides an uninterrupted fluidics stream during FACS isolation. Together these simple modifications enhance recovery of the target cell populations and avoid delays due to clumping during FACS enrichment.

Previous protocols call for tissue digestion with Elastase, dispase and tripsin/2 mM EDTA to yield a single cell suspension prior to cell isolation[4,5]. However, this combination of proteases leads to loss of surface proteins and requires that cells are cultured overnight on purcol coated culture dishes for re-expression of surface proteins prior to antibody staining and FACS. By contrast, the combination of Liberase and mechanical agitation to gently disrupt lung tissue provides a more efficient, yet milder, dissociation protocol that can be performed more rapidly while preserving surface epitopes for antibody staining and FACS enrichment. Thus total tissue processing time is condensed and FACS isolation can be performed immediately following tissue dissociation.

These methods allow for the isolation and in vitro culture of epithelial progenitor cells that yield specialized progeny representative of their region of origin. However, these methods can be similarly applied to the identification and enrichment of other cell populations such as immune, vascular and stromal cell types. This could be particularly applicable to the development of regional lung epithelium-on-chip systems which allow for modeling of vascular and epithelial compartments and introduction of other cell types such as immune cells[24-26].

ACKNOWLEDGMENTS :

We appreciate support from Mizuno Takako for IFC and H and E staining, Vanessa Garcia for tissue sectioning and Anika S Chandrasekaran for helping with manuscript preparation. This work is supported by National Institutes of Health (5RO1HL135163-04, PO1HL108793-08) and Celgene IDEAL Consortium.

DISCLOSURES:

Authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Fig 1.jpg

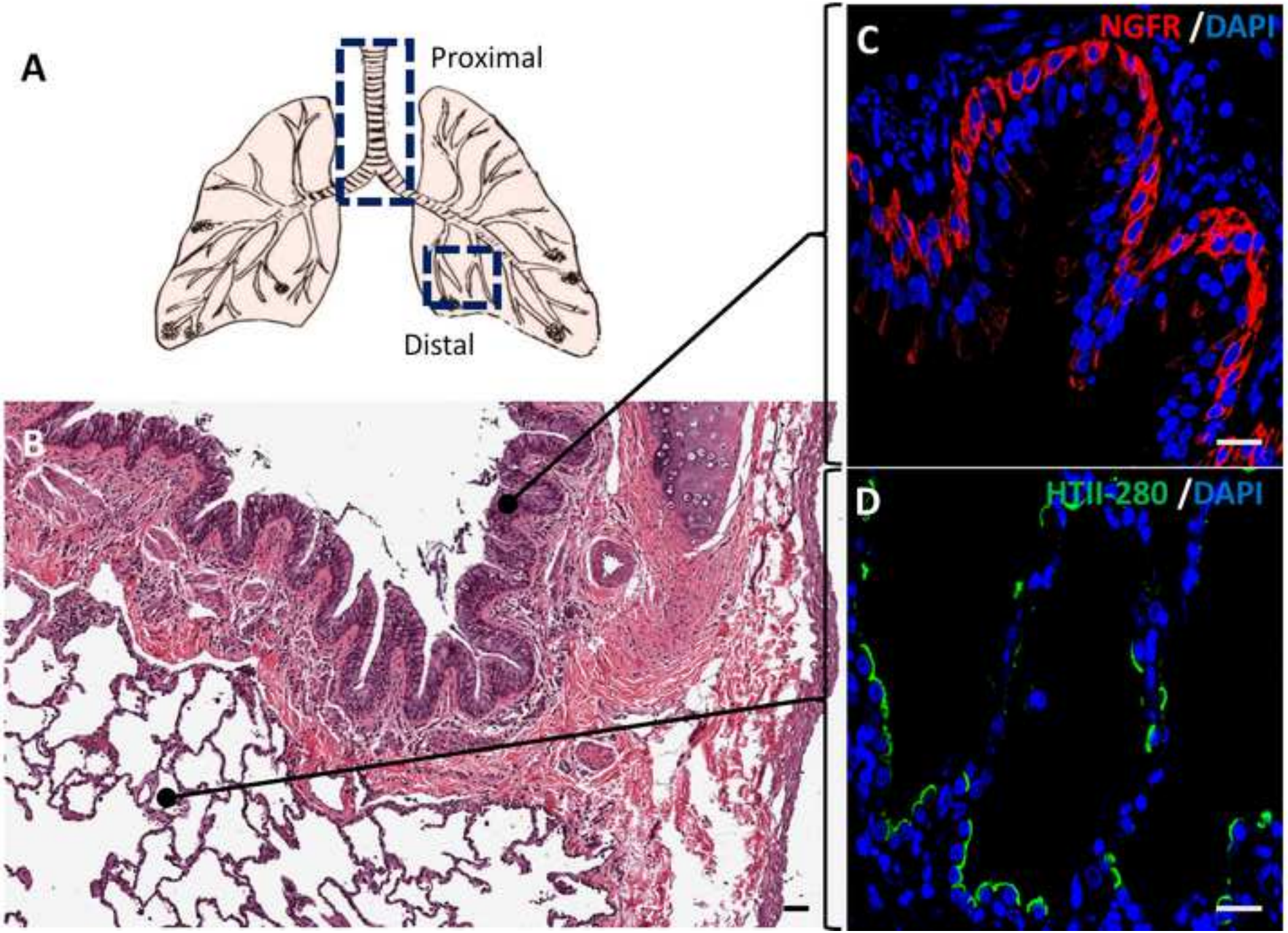
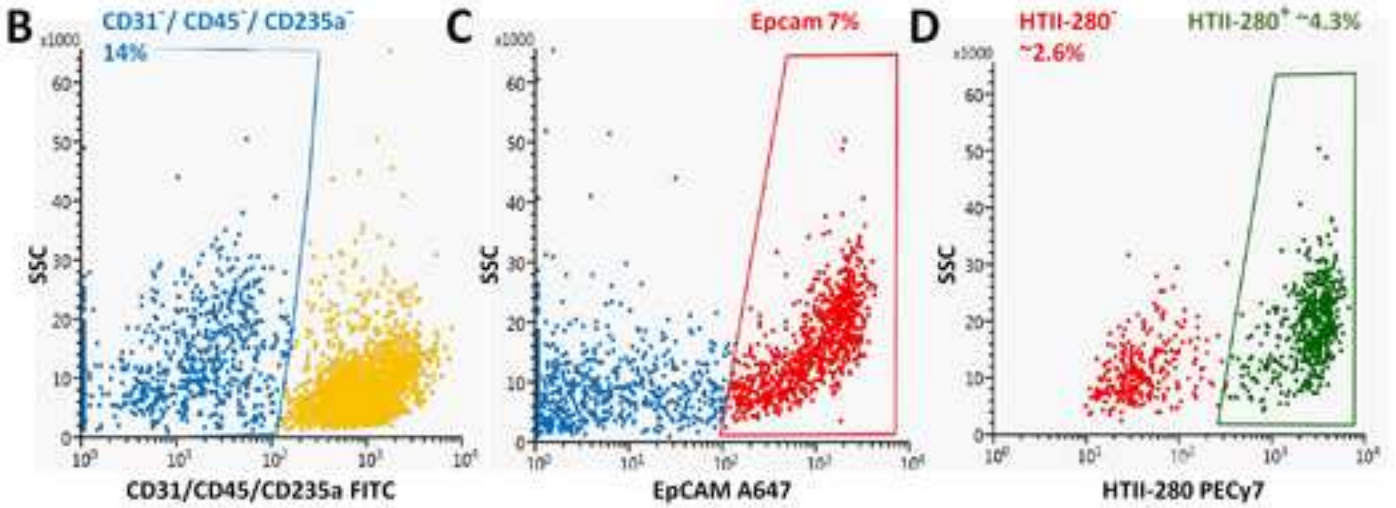


Figure 2

A

	Before depletion of CD45/CD31/CD 235a
Live singlets	61%
Non-CD31/45	14%
Epcam^{+}	7%
HT-II280^{+}	4.3%
HT-II280^{-}	2.6%



E

	After depletion of CD45/ CD31/ CD235a
Live singlets	71%
Non-CD31/45	51.7%
Epcam^{+}	33.5%
HT-II280^{+}	30%
HT-II280^{-}	3.6%

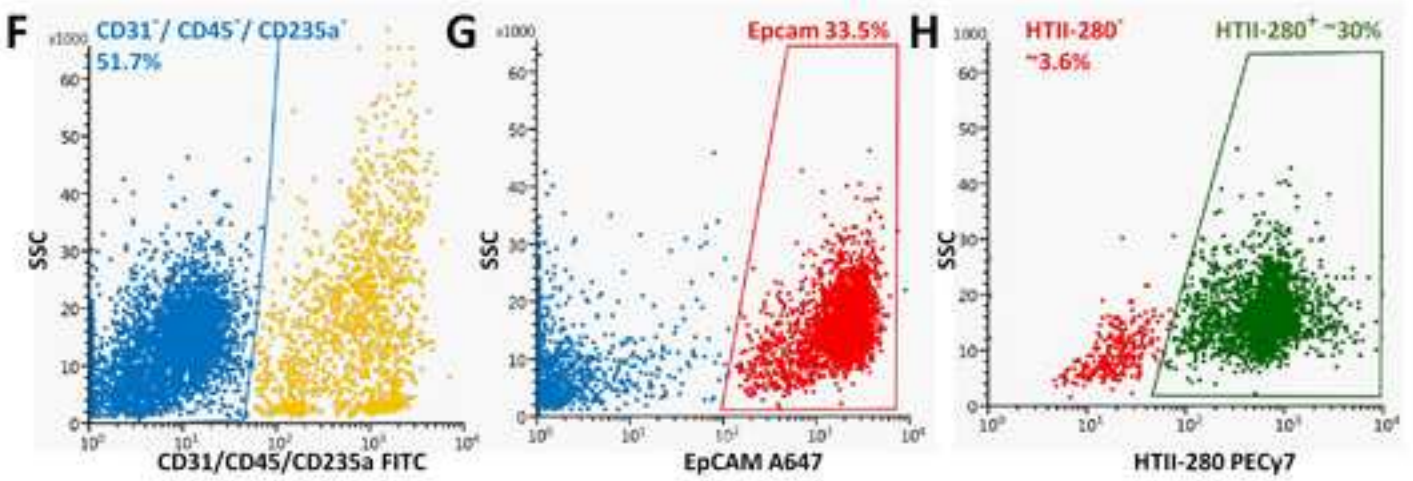
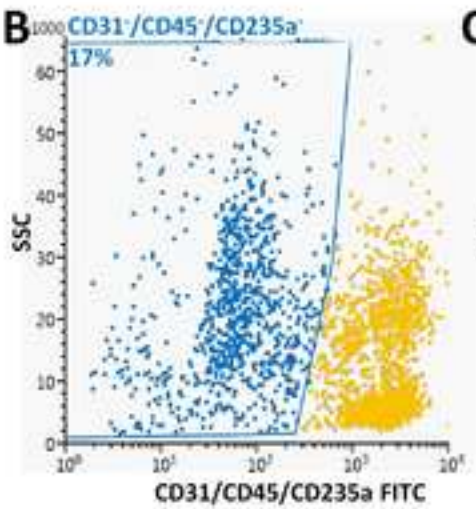
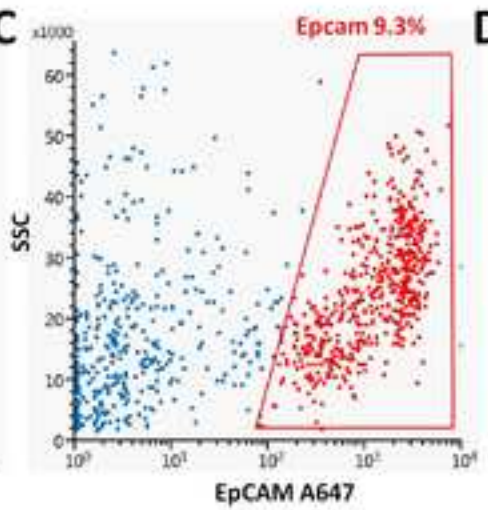


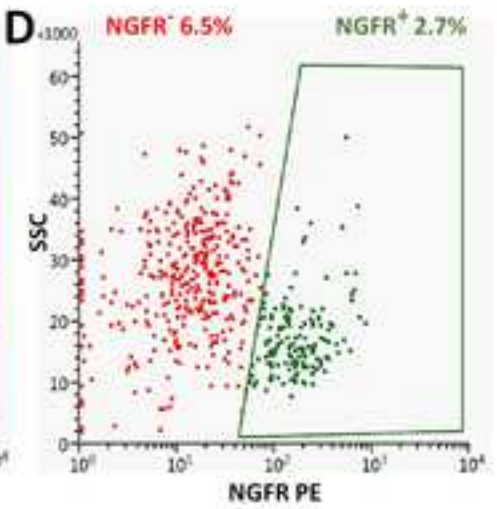
Figure 3

A

	Before depletion of CD45/ CD31/CD235a
Live singlets	52%
Non-CD31/45	17%
Epcam	9.3%
NGFR$^+$	2.7%
NGFR$^-$	6.5%

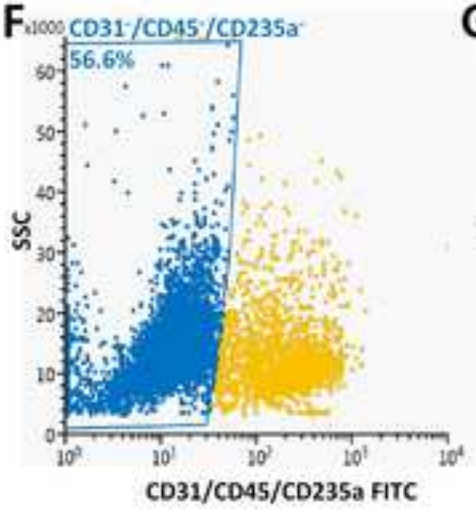


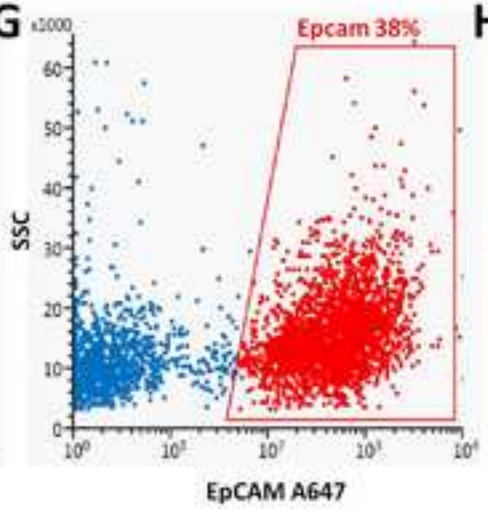




E

	After depletion of CD45/ CD31/CD235a
Live singlets	79%
Non-CD31/45	56.6%
Epcam	38%
NGFR$^+$	13%
NGFR$^-$	25%





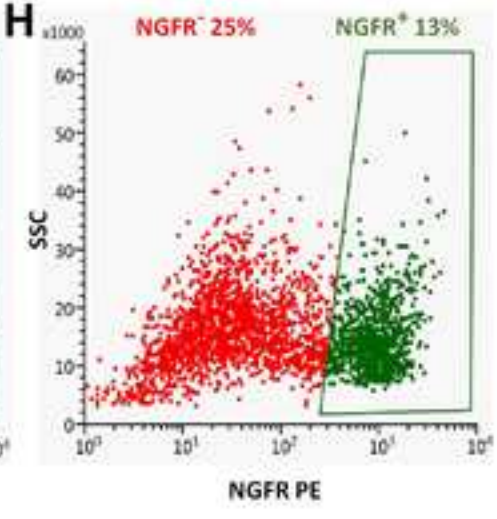


Figure 4

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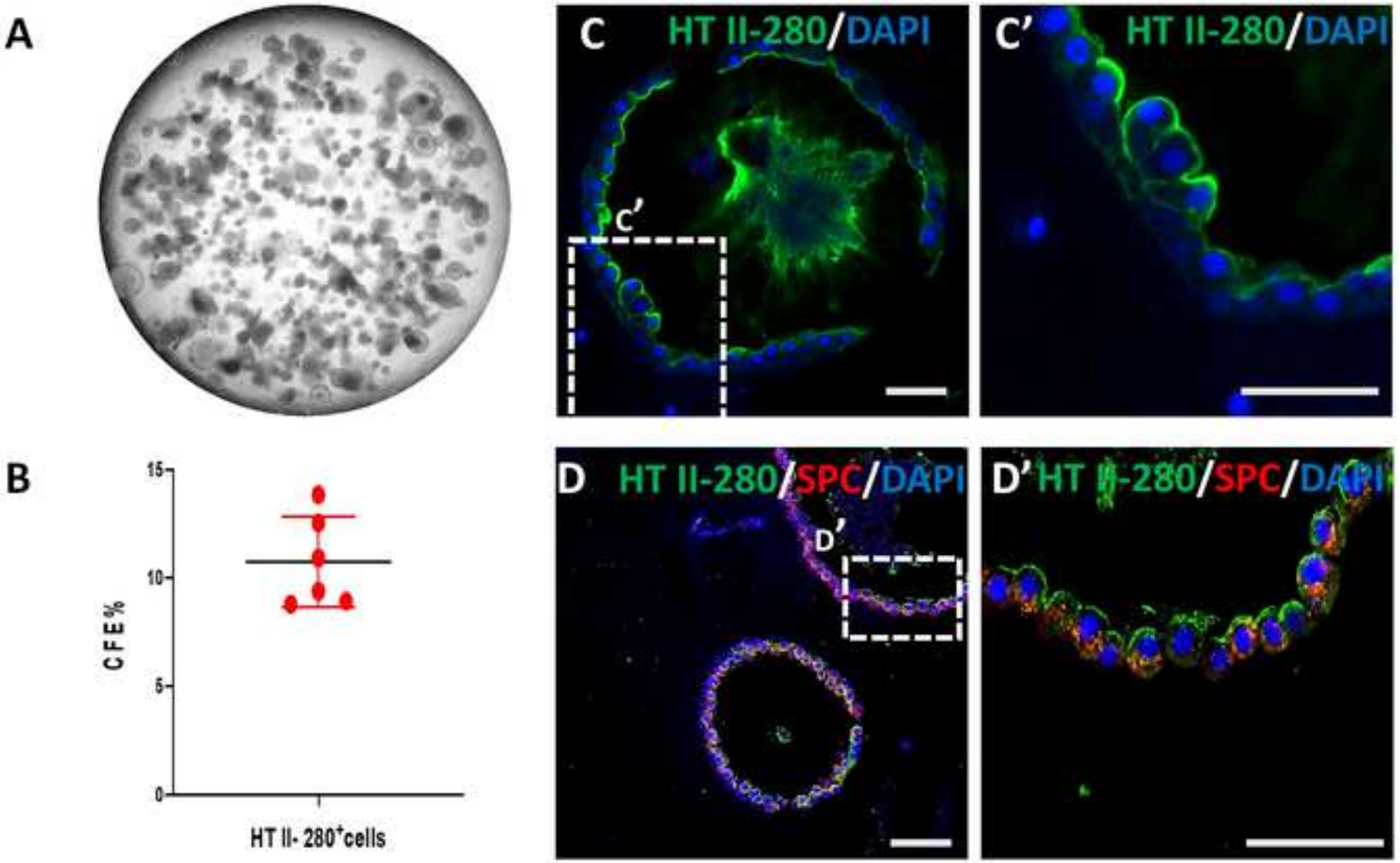

Figure 5

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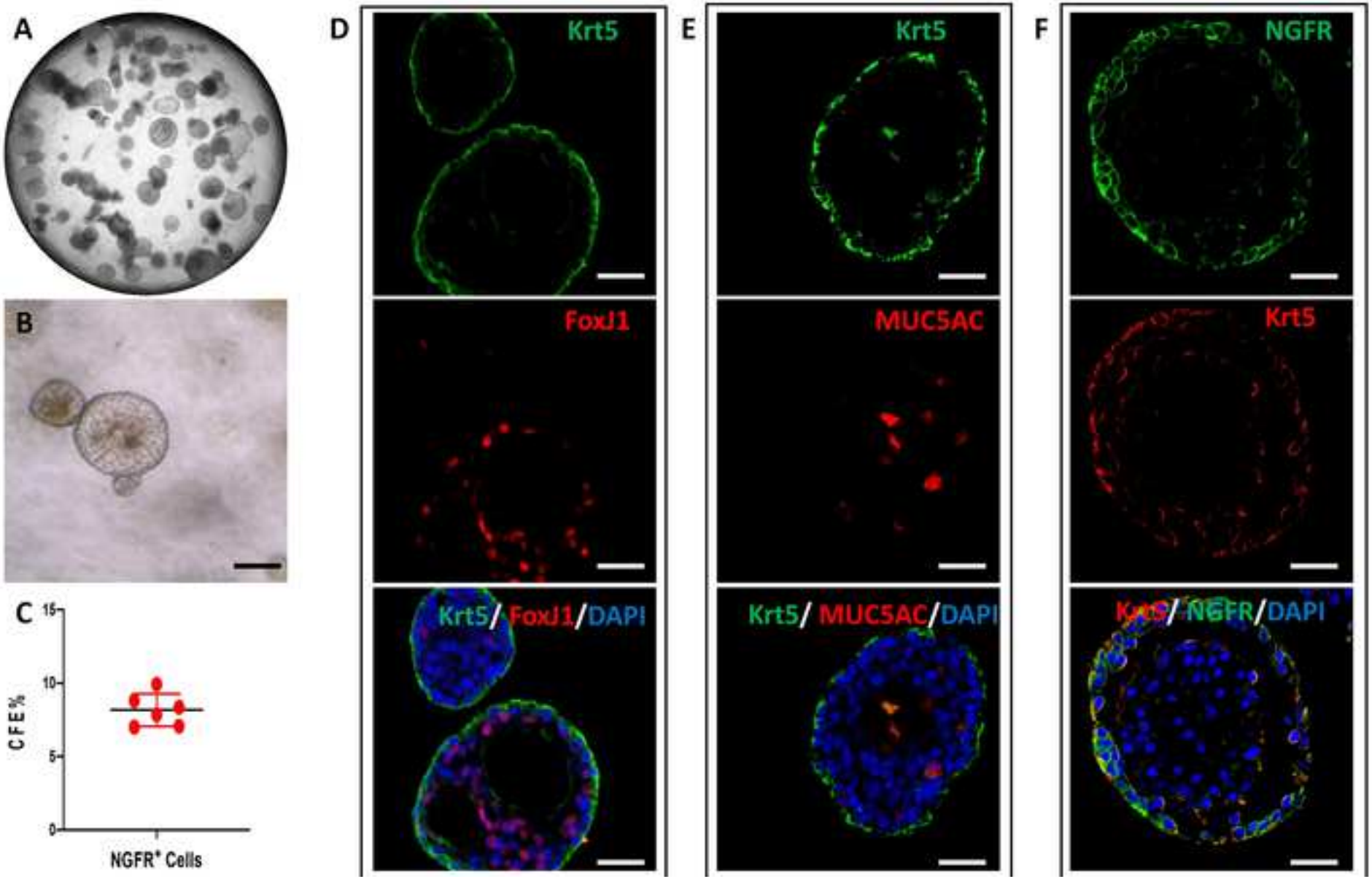


Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials (3) (3) (1).xlsx

Materials	Company	Catalog number	Comments/Description
Cell Isolation			
10 mL Sterile syringes, Luer-Lok Tip	Fisher scientific	BD 309646	
30 mL Sterile syringes, Luer-Lok Tip	VWR	BD302832	
Biohazard bags	VWR	89495-440	
Biohazard bags	VWR	89495-440	
connecting ring	Pluriselect	41-50000-03	
Deoxyribonuclease (lot#SLBF7798V)	sigma Aldrich	DN25-1G	
Disposable Petri dishes	Corning/Falcon	25373-187	
Funnel	Pluriselect	42-50000	
HBSS	Corning	21-023	
Liberase TM Research Grade	sigma Aldrich	5401127001	
needle 16G	VWR	305198	
needle 18G	VWR	305199	
PluriStrainer 100 µm (Cell Strainer)	Pluriselect	43-50100-51	
PluriStrainer 300 µm (Cell Strainer)	Pluriselect	43-50300-03	
PluriStrainer 40 µm (Cell Strainer)	Pluriselect	43-50040-51	
PluriStrainer 500 µm (Cell Strainer)	Pluriselect	43-50500-03	
PluriStrainer 70 µm (Cell Strainer)	Pluriselect	43-50070-51	
Razor blades	VWR	55411-050	
Red Blood Cell lysis buffer	eBioscience	00-4333-57	
Equipment's			
GentleMACS C Tubes	MACS Miltenyi Biotec	130-096-334	
GentleMACS Octo Dissociator	MACS Miltenyi Biotec	130-095-937	
Leica ASP 300s Tissue processor			

LS Columns	MACS Miltenyi Biotec	130-042-401	
MACS MultiStand**	Miltenyi Biotech	130-042-303	
Thermomixer	Eppendorf	05-412-503	
Thermomixer	Eppendorf	05-412-503	
HBSS+ Buffer			
Amphotericin B	Thermo fisher scientific	15290018	2ml
EDTA (0.5 M), pH 8.0, RNase-free	Thermo fisher scientific	AM9260G	500µl
Fetal Bovine Serum	Gemini Bio-Products	100-106	10ml
HBSS Hank's Balanced Salt Solution 1X 500 ml	VWR	45000-456	500ml bottle
HEPES (1 M)	Thermo fisher scientific	15630080	5ml
Penicillin-Streptomycin-Neomycin (PSN) Antibiotic Mixture	Thermo fisher scientific	15640055	5ml
List of antibodies for FACS			
Alexa Fluor 647 anti-human CD326 (EpCAM) Antibody	BioLegend	369820	1:50
BD CompBead Anti-Mouse Ig, K/ Negative control particles set	Fisher Scientific	BDB552843	
CD31 MicroBead Kit, human	Miltenyi Biotec	130-091-935	20µl/ 10^7 total cells
CD45 MicroBeads, human	Miltenyi Biotec	130-045-801	20µl/ 10^7 total cells
DAPI	Sigma Aldrich	D9542-10MG	1:10000
FITC anti-human CD235a	BioLegend	349104	1:100
FITC anti-human CD31	BioLegend	303104	1:100
FITC anti-human CD45	BioLegend	304054	1:100
FITC anti-mouse IgM Antibody	BioLegend	406506	1:500
Mouse IgM anti human HT2-280	Terrace Biotech	TB-27AHT2-280	1:300

PE anti-human CD271(NGFR)	BioLegend	345106	1:50
Composition of Organoid Culture mediums			
MRC-5	ATCC	CCL-171	
PneumaCult -ALI Medium	Stemcell Technologies	5001	
Small Airway Epithelial Cell Growth Medium	PromoCell	C-21170	
ThinCert Tissue Culture Inserts, Sterile	Greiner Bio-One	662641	
Y-27632 (ROCK inhibitor) 100mM stock (1000x)	Stemcell Technologies	72302	
Mouse Basal medium:			
Amphotericin B	Thermo fisher scientific	15290018	50 µl
DMEM/F-12, HEPES	ThermoFisher scientific	11330032	50 ml
Fetal Bovine Serum	Gemini Bio-Products	100-106	5 ml
Insulin-Transferrin-Selenium (ITS - G) (100X)	ThermoFisher scientific	41400045	500 µl
Penicillin-Streptomycin-Neomycin (PSN) Antibiotic Mixture	Thermo fisher scientific	15640055	500 µl
SB431542 TGF-β pathway inhibitor (stock 100 mM)	Stem cell	72234	5 µl
List of antibodies for Immunohistochemistry			
Antigen unmasking solution, citric acid based	Vector	H-3300	937 µl in 100ml water
Histogel	Thermo Scientific	HG-4000-012	
Primary Antibodies			
Anti HT2-280	Terracebiotech	TB-27AHT2-280	1:500
FOXJ1 Monoclonal Antibody (2A5)	Thermo Fisher Scientific	14-9965-82	1:300

Human Uteroglobin/SCGB1A1 Antibody	R and D systems	MAB4218	1:300
Keratin 5 Polyclonal Chicken Antibody, Purified [Poly9059]	Biolegend	905901	1:500
MUC5AC Monoclonal Antibody (45M1)	Thermo Fisher Scientific	MA5-12178	1:300
PDPN / Podoplanin Antibody (clone 8.1.1)	LifeSpan Biosciences	LS-C143022-100	1:300
Purified Mouse Anti-E-Cadherin	BD biosciences	610182	1:1000
Sox-2 Antibody	Santa Cruz biotechnologies	sc-365964	1:300
Secondary Antibodies			
Donkey anti-rabbit IgG, 488	Thermo Fisher Scientific	A-21206	1:500
FITC anti-mouse IgM Antibody	BioLegend	406506	1:500
Goat anti-Hamster IgG (H+L), Alexa Fluor 594	Thermo Fisher Scientific	A-21113	1:500
Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	A-21121	1:500
Goat anti-Mouse IgG2a Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	A-21131	1:500
Goat anti-Mouse IgG2a Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific	A-21134	1:500
Goat anti-Mouse IgG2b Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific	A-21144	1:500
Buffers			

Immunohistochemistry Blocking Solution			3% BSA, o.4% Triton-x100 in TBS (Tris based saline)
Immunohistochemistry Incubation Solution			3% BSA,).1% Triton-X100 in TBS
Immunohistochemistry Washing Solution			TBS with 0.1% Tween 20

Editorial comments:

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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Changed to single line spacing and used Calibri 12 for the font

3. Please provide an email address for each author.

Done

4. Please provide at least 6 keywords or phrases.

Done

5. Please do not make a separate subheading for abbreviations. Please expand all abbreviations during the first-time use.

Removed abbreviations subheading

6. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) without brackets.

Done

7. 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. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Liberase™, Kimwipes, Milteny MACS CD31 & CD45 microbeads, Gentle MACS C tubes, Falcon tube, Thermomixer, MACS Octo Dissociator, PluriSelect cell strainers, TransWell culture insert, MatriGel, etc

Removed all the trademarks

8. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
10. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

Corrected the numbering pattern in the protocol and ensured all the test above was included in the intorduction

11. The Protocol should contain only action items that direct the reader to do something. Please use complete sentences throughout.

Modified
12. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

Modified
13. Please ensure you answer the "how" question, i.e., how is the step performed?
14. The manuscript is a standalone publication. Please do not refer to the video.

Removed video reference from the manuscript.

15. 1: Please make substeps and show how the actions are performed.
16. 2.1.2: How do you visually identify visceral pleura.

Mentioned how to identify visceral pleura: A delicate membrane that covers the surface of the lung.
17. 2.2.2 Amount of tissue used?

Added: 2-3 grams of tissue in 25ml of liberase and DNase. Make sure that the amount of tissue does not exceed the 25ml mark on a 50ml conical tube to ensure efficient enzymatic digestion.

18. 2.3.2: When do you stop the digestion?

Added: 45 minutes

19. Line 151: Is it Antibody or Antigen?

Antibody. Corrected in the manuscript.

20. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less 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.

The isolation of cells from the proximal and distal lungs will be done simultaneously in the video. Even though the highlighted portions exceed 2.75 pages, the video will be within the allocated time. Isolation in both regions will not be showed separately, hence the video will not be long.

21. Please ensure the results are described in the context of the presented technique.

22. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in Figure Legend, i.e. "This figure has been modified from [citation]."

We did not reuse any previously published figures

23. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

24. Please ensure the Acknowledgements section, contains any acknowledgments and all funding sources for this work.

25. Please upload high-resolution figures.

Done

26. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. Please sort the table in alphabetical order. Please combine tables 1-5. They all are essentially a table of materials.

Removed the trademarks and tables are arranged alphabetically.

27. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Tables are uploaded in a separate file and accompanied with appropriate titles and descriptions.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Konda et al, describe a protocol for isolation of epithelial cell types from different regions of the human lungs; in particular HTII280+ type II alveolar cells from the distal region and NGFR+ basal cells from the proximal region.
Both cell types have been shown to have progenitor like features in mouse. Konda et al show that they can form in vitro organoids when combined with human lung fibroblasts.

Minor Concerns:
In the abstract instead of 5000 cells/well please express as cell/surface since it's not clear yet which is the well format.

Added: 1.4 x10^4 cells/cm^2
Table 1: in the text authors mention 60ml and 10ml syringes but only 30ml syringes are listed.

Added: We used 10 ml and 30 ml syringes, and I corrected it and added catalog number for 10ml

I couldn't find the composition of HBSS+, it may be helpful to add a section on buffers used for cell isolation aside from Materials and Equipment.

Added: HBSS+ buffer and FACS buffer are same. I mentioned FACS buffer composition in the table and changed the title.

Please pay attention to refer to the right tables and figures along the protocol, only table 1 and 4 are cited in the first 3 paragraphs. I would suggest this order for the tables:
Table 1: Materials for cell isolation and equipment's
Table 2: Composition of the FACS Buffer
Table 3 (was 4) : List of antibodies for FACS
Table 4 (was 3): Composition of Organoid Culture mediums
Table 5: List of antibodies for immunofluorescence
Table 6: Buffers for immunofluorescence
and cite table 2-3 and figure 2-3 when describing the FACS isolation strategy.

Modified the tables as per the suggestions and mentioned all the tables and figures in the protocol.

The MRC5 cell line seems to be an important part in the organoid preparation, please add the specifics in table 3 and explain what their function is.

Added the details of MRC5 in the table 4 and explained their function.

Line 402, "Large lumen-containing organoids were observed (Fig. 5A and 5B)": I don't think the lumen is really evident from those images.

Added: Large Lumen containing organoids were observed in Fig 5D and 5E

Added new staining's to figure 5.

Figure 2-3: the percentages presented are derived from single experiments and single biological replicates, how much variability would you expect? Is there any advantage of running the optional depletion step? It looks like the percentage of cell of interest is lower after beads depletion, what about the actual number and/or quality of recovered cells?

A. We have repeated this experiment multiple times with different biological samples. We started organoid culture with 1000 cell/well, 2000 cells/well and 5000 cells/well. The colony forming efficiency shown in figures 4 and 5 are from 2 different biological samples with three technical replicates (5000 cells/ well). The variability in %CFE can be up to 2-5% and is dependent on parameters, such as donor age and donor health. %CFE can also be affected by the time required to procure the tissue. Sometimes we receive tissue from East Coast, and it takes 24hr to procure the tissue. Cells from fresher tissue exhibit higher %CFEs.

B. Running the depletion step gets rid of immune and endothelial cells, which comprise of the significant percentage of total isolated cells. This allows for shortened sorting time, thus minimizing sorting expenses. The percentage of cells of interest viz HTII 280+ cells increased from 4.3% to 30% (Figures 2A and E) and that of NGFR+ cells increased from 2.7% to 13% (Figures 3A and E). Initially we didn't perform depletion step for the first couple of samples. We usually need to collect 200k HT II 280^{+}cells for both organoid cultures and run 10x single cell RNA sequencing experiments. Depletion step saves couple of hours of sorting time and sorting goes smoothly.

C. The depletion step does not have any negative impacts on cell quality. After depletion we do sorting, and we collected live $Epcam^{+}$ and HTII-280^{+} cells for organoid culture.

Discussion-line 462: I don't think it's correct to defined these organoids as clonal given the high number of plated cells.

Organoids derived from lung progenitor cells are considered to be clonally derived structures. In our previously published study (Chen et al 2014) we show this using genetic lineage tracing approaches, where GFP negative, GFP low and GFP high lung epithelial progenitor cells self-renew to form clonally distinct organoids. (Ref:18)

Reviewer #2:
The authors present a study regarding the functional analysis of epithelial progenitor cell isolation from the human lung for organoid culture. However, the title does not represent the whole idea. The authors may think going through the title.

- Overall, the idea of the study is interesting and may be useful for further studies. However, the protocol has to be more informative.
- Headline capitalization formats (e.g., Page 2 line 91 and line 108) are not compatible among subheadings.

Fixed all headline and subheadings so that they follow the same format
- Fig2 and Fig3 need higher resolution.
- The text must be strengthened by taking into account the comments.

Page 1, line 35: Please define the abbreviation of HBSS.

Added: Hank's Balanced Salt solution FACS buffer (HBSS+)

Page 2, line 102: This is the only place the authors referring IPF. What does IPF stand for? Is that idiopathic pulmonary fibrosis? Please define.

Changed IPF to Idiopathic pulmonary fibrosis

Page 2, lines 102,103: "However fibrotic tissue from IPF samples can require a longer incubation time of up to 60 minutes"
What is the reference for this information? Does it belong to your previous data? Please provide reference.

In our lab, we isolate cells from normal lung, Idiopathic Pulmonary Fibrosis (IPF) and Chronic Obstructive Pulmonary Disease (COPD) samples. We have isolated more than 20 samples.

Based on those unpublished experiments, IPF needs more time of incubation with Liberase and the COPD samples require additional washes during the processing of lung.

Page 3, line 135: Why is this step optional? The same method is also mentioned in Page 5, line 245. So that it is a bit confusing to consider it as optional. In addition, using "and" between immune cells & endothelial cells instead of comma would be better.

I changed both lines to make the step optional. Performing the immune and endothelial cell depletion step requires access to the magnetic bead-based separation setup and kits. Although this step increases the percentage of target cells in the total pool of cells and reduces time required to sort the cells, it does not affect the quality of cells and organoids formed. Therefore, this step is considered optional.

Page 3, line 144: The capitalization format needs to be fixed. Depending on the headline pattern, it may be edited as "Cell surface staining for fluorescence associated cell sorting" or "Cell Surface Staining for Fluorescence Associated Cell Sorting"

Changed the capitalization format

Page 6, line 258: "Add 2000-5000 sorted proximal or distal epithelial cells and 7.5 x 104 MRC5 cells to a sterile 1.5mL tube"

* How did the authors select the cell ranges for proximal or distal epithelial cells?

While standardizing the organoid culture protocol, we tested various cell densities from 1000 cells/well to 10,000 cells/well. A seeding density of 5000 epithelial cells/trans well insert or 1.4 x10^4 cells/cm2 was found to robustly develop good quality organoids.

* There is no data specified regarding the colony-forming efficiency for the cell numbers used in the range of 2000≤ … <5000.

We chose 5000 cells/well for colony forming efficiency based on consistency on %CFE on different biological sample.

Page 6, line 258: "Add 2000-5000 sorted proximal or distal epithelial cells and 7.5 x 104 MRC5 cells to a sterile 1.5mL tube"

* What is the function of MRC5 fibroblast cells at this point? If the function is acting as a feeder layer, does it have to be MRC5, specifically? Or, do we have a change to use other lung fibroblasts such as CCD-34-Lu cells?

Epithelial- mesenchymal interaction is critical for the successful formation and differentiation of organoids from human lung progenitor cells. The MRC-5 cell line is convenient to use, capable of 42-45 population doublings before the onset on senescence and cells grow rapidly enough to generate sufficient quantities for generation of high-quality organoid cultures. While we have not tried CCD-34-Lu cells in particular, we have cocultured epithelial cells with primary fibroblasts isolated from adult lung tissue, as well as embryonic lung tissue in our lab and observed results consistent with the use of MRC5. However, due to the ease of culturing large quantities of cells, we have developed the current protocol with MRC5. It is possible to use another normal lung fibroblast cell line, such as CCD-34-Lu, however their efficiency to support organoid growth would have to be tested.

Page 6, line 290: "…suppress overgrowth of fibroblasts."
The authors have not indicated MRC5 cells as fibroblasts in line 258. It may be confusing. Please revise.

Mentioned MRC-5 as human fibroblasts in the manuscript.

Page 8, line 353: The authors have already given the abbreviation of alveolar type 2 as ATII, so it must be used. Please revise.

Abbreviation section was removed, and all the abbreviations are mentioned with in the brackets when used for first time.

Page 9, lines 387: The colony-forming efficiency seems quite low. What might be the reason?
I came up with two possible idea. The first one is the method of cell sorting may cause to get lower cell number. Or, the quantity of tissue used for the dissociation is not enough to get sufficient number of progenitor cells. Under either condition, the colony-forming efficiency is possibly related to the maximum cell number that has been used for the organoid culture. Do you think that it is possible to increase the efficiency for this method by increasing the cell numbers?

The colony forming efficiency is in our expected range. Only a small percentage of progenitor cells are stem cells. For example, it has been shown in previous publications that only a small percentage of ATII cells have the stem cell potential (Nabhan et al 8 Science 2018; Zacharias et al Nature 2018). Therefore, we expect only a small percentage of ATII cells to proliferate and clonally expand to form organoids.

While flow sorting can make cells sensitive, we cannot bypass this essential step; our target cells of interest form a limited percentage of the total pool of cells.

In our experience, the seeding density is critical for the formation of good quality organoids as well as for the organoid size. Seeding higher numbers of cells limits the space available for expansion of organoids, and the cells form small clusters, fail to polarize, form a lumen and differentiate into specialized cell types that can secrete surfactant or mucus into the lumen.

Page 11, lines 469: Please prepare an acknowledgement text explaining the main reasons for acknowledging Vanessa Garcia and Anika Sree Chandrasekaran.

Acknowledgments are prepared.

Reviewer #3:

General Comment:

In the manuscript, the authors described the methods of isolating distal and proximal lung epithelial stem cells and culturing the isolated cells in organoids up to 30 days. Among several methods of isolating lung stem cells, they used MACS for depleting the blood cells and endothelial cells and FACS for isolating epithelial cells, in particular, HTII-280+ cells as distal lung stem cells and NGFR+ cells as proximal lung stem cells. They also reported the methods of alveolar and airway organoid culture using the isolated distal and proximal lung stem cells, respectively. Because a lot of researchers face difficulty when they isolate human lung cells for the first time, I think the method of tissue dissociation in this manuscript is suitable for JoVE journal. On the other hand, the part of organoid culture is a little confusing. The authors reported lung organoid culture derived from human lung tissue in the previous paper (Liang J, et al, Nat Med, 2016), but compared to the article, the quality of the organoids, for example, that of immunofluorescence staining shown in this manuscript is a little low. In addition, I wonder why feeder fibroblasts for co-culture with epithelial cells changed from MLg2908 murine lung fibroblasts to MRC5 human fetal lung fibroblasts in this manuscript. It could be a great change for an epithelial cell niche. I think that the human lung organoids shown in this manuscript should be verified more in detail. I recommend the section of lung organoid culture would be investigated in another research. Instead, it might be better to describe how they identify the isolated cells as AT2 cells, basal cells, or other lineages clearly. For the further improvement, I provide comments as follows.

The MRC-5 cell line is convenient to use, capable of 42-45 population doublings before the onset on senescence and cells grow rapidly enough to generate sufficient quantities for generation of high-quality organoid cultures. We have cocultured epithelial cells with

primary fibroblasts isolated from an adult lung tissue, as well as embryonic lung tissue in our lab and observed results consistent with the use of MRC5. However, due to the ease of culturing large quantities of cells, we have developed the current protocol with MRC5. It is possible to use another normal lung fibroblast cell line, such as MLg2908, however their efficiency to support organoid growth would have to be tested.

Major comments:
1. Title. What the "functional analysis" means? It might be better to change the title, because no functional data were shown in the manuscript.

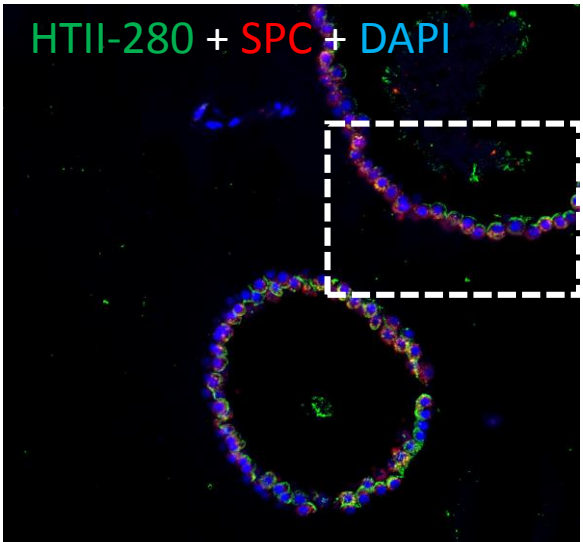
Changed the title.
2. Single cell isolation of distal lung tissue. How many cells can be collected from 1cm3 pieces of human lung tissue? The number of cells in each step is one of the important information for the readers. It is desirable to exhibit the viability of collected cells.

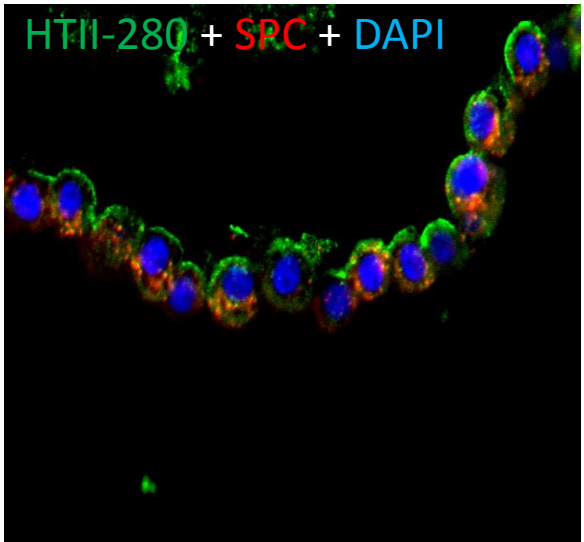
From 1 gram of distal tissue we got around 7 million viable total cells and 130 K HT II-280 Positive cells, and from 1 gram of proximal tissue we get around 1-2 million viable total cells and 10-15 K NGFR positive cells.
3. Page 2, line 113. Did they describe the recipe of HBSS+ buffer in Table 1?

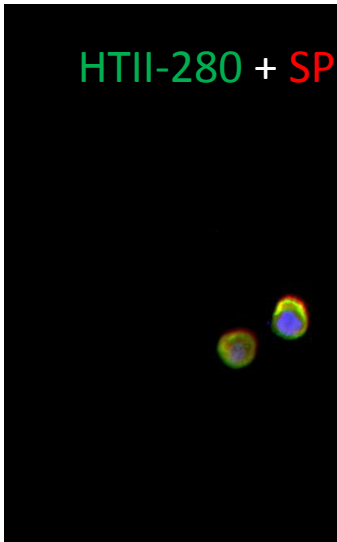
It's mentioned in Table 2
4. Page 4, line 180-185. They should describe how to evaluate the results of HTII-280 positive and negative cell sorting. It is helpful for readers how to know the sorting is successful. Because AT2 cells express various specific markers, such as SP-C and SP-D, I recommend, for example, counterstaining with other anti-AT2 cell markers of cytospinned samples.

We stained HTII-280 positive sorted cells and Organoids generated from HTII-280^{+} with the following antibodies.

Added this figure in Figure 4







5. Page 5, line 216. What is the human lung protocol-2? Is the program specific to the gentleMACS Octo Dissociator or other machines? Is it accessible to the readers of the journal?

This is a preset protocol developed by Gentle MACS Octo Dissociator for ease of isolation of single cells from tissue of interest. Information on pre-set protocol is available on the manufacturers website

6. Page 5, lines 240-241. They mixed the isolated proximal airway cells from 3.1.4 with those from 3.2.4. Do they need to mix the two cell populations? What the approximate ratio of each isolated cell population?

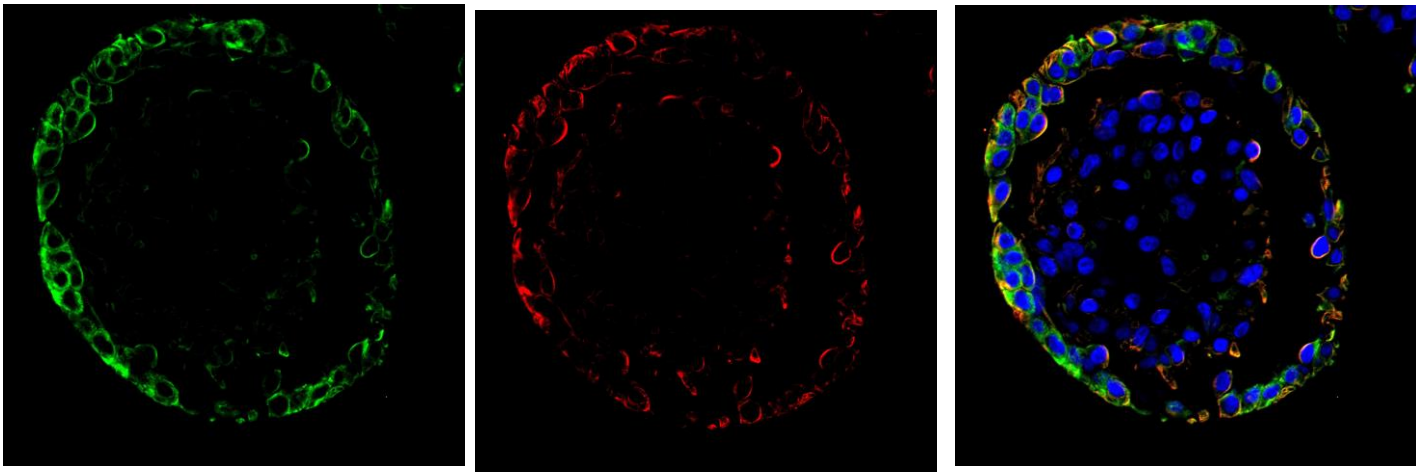
The tracheal epithelium is pseudostratified consisting mainly of ciliated, secretory and goblet cells basal stem cells. Step 3.1.4 yields epithelial cells from tracheal scrapings while step 3.2.4 yields cells that are more tightly associated with the basement membrane. The protocol was optimized to allow maximal recovery of cells and therefore cells from both steps were combined. We have not looked into ratio of cells obtained from scraping vs complete digestion. However, since both cell populations are ultimately sorted based on CD326 positivity, it is practical to sort them as a single sample.

7. Page 6, lines 249-255. They should describe how the readers can evaluate the isolated NGFR positive and negative cells? Are NGFR+ cells positive for p63 and KRT5? Did NGFR negative cells include basal cells?

NGFR (CD273) is a cell surface marker for basal cells (ROCK et al PNAS 2009) and comprises of both p63 and Krt5 positive cells. It is routinely used as a marker for sorting basal cells of the proximal airways, and therefore we relied on the flow profiles to distinguish between NGFR+ and NGFR-ve cells. P63 is nuclear staining and since we are sorting live cells, we preferred NGFR.

Performed NGFR and K5 staining on proximal organoids derived from NGFR+ cells.

Proximal organoids (NGFR + K5 +DAPI) Added in the figure 5



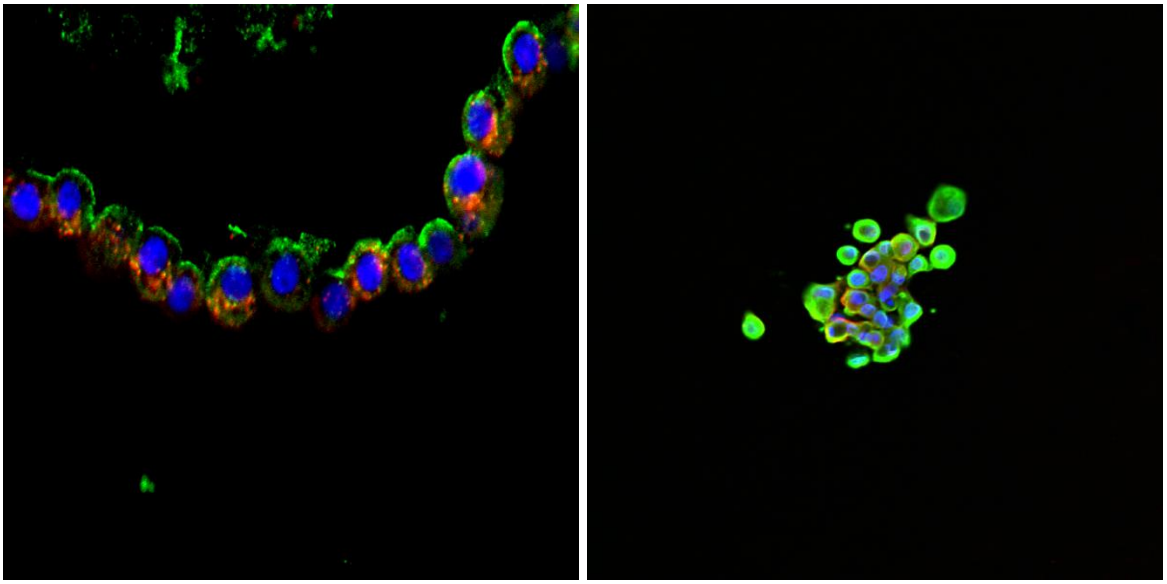
8. Page 6, Section 4, organoid culture. The step 9 (pp 6, lines 289-295) seems preliminary but is of interest. To my knowledge, supplementation of TGFbeta inhibitor to PneumaCult-ALI medium in the step 9 is the novel data for maintenance of alveolar organoids and is still challenging for the community. I think that it might be better for readers to know the advantage of TGFbeta supplementation more clearly. For example, they demonstrated only the expression of HTII-280 in their organoids in Figure 4. If the alveolar organoids are successfully maintained during 30 days, they should demonstrate the immunostaining of other alveolar markers, such as SP-C and SP-D (AT2 cell markers) and AGER and AQP5 (AT1 cell markers) as well as quantitative RT-PCR of related markers. Electron microscopy of lamellar bodies is convincing to demonstrate that AT2 cells are maintained. It's better to clarify the ratio of HTII-280+ cells in the epithelial cells of the organoids.

We performed co-staining of alveolar organoids cultured from HT II-280^{+} cells with HTII-280 antibody and SPC. We ordered AQP5 and tried staining but needed to optimize. We performed 10X single cell RNA sequencing experiment on cultured alveolar organoids, and

the data need to be analyzed. We're planning to publish the 10X data in subsequent publications.

Alveolar organoids (HT II-280 + SPC) and cytospinned cells (HT II-280 +SPC)

Added in the figure 4



9. Page 8, lines 364-374 and 376-385. They described each ratio of cell populations. Were the ratios calculated as a mean value? How many experiments were done for each value? In general, biological replicates should be based on more than three independent experiments.

The figures 2A, 2E, 3A and 3E represent data from the same biological replicate to emphasize the value of incorporating the depletion step. As shown in the figures, depleting immune and endothelial cells raises the percentage of HTII-280+ cells from 4.3% to 30% and the percentage of NGFR+ cells from 2.7% to 13%.

However, using the described protocol with the depletion step, we have routinely and robustly cultured organoids in the same %CFE range

10. Page 9, line 382. Did they compare anti-NGFR with anti-ITGA6 for isolating basal cells? The original paper reported NGFR and ITGA6 as surface markers of basal cells (Rock et al. PNAS, 2009).

We did not use anti-ITGA6 for isolating basal cells. In our hands NGFR is sufficient for enrichment of basal cells.

11. Page 9, line 390-398. As I mentioned above, the alveolar organoid culture is still a challenging issue. They should describe the results more in detail. The immunostaining of

HTII-280 is important but it does not mean that the cultured organoids are really alveolar organoids. What kind of lineage cells are involved in each of HTII-280+ and HTII-280- cell derived organoids, respectively.

We performed 10X single cell RNA sequencing experiments on organoids derived from epithelial progenitor cells. Data needs to be analyzed and will be published in subsequent publications.

12. Figure 1. I recommend the double immunostaining of NGFR and p63 as well as HTII-280 and NKX2.1(and/or SP-C) to show the methods more comprehensively.

We did NGFR and K5 staining and HTII-280 and SPC staining.

13. Page 10. Discussion. Please discuss more. How the methods described in this article outweigh the previous studies? How the authors hope to apply the methods to the future research? What is the limitation and how to improve them? In particular, alveolar organoid culture described here requires coculture with MRC5 cells. Is it possible to culture the isolated AT2 cells without such feeder cells?

We are currently working on using alveolar organoids for drug screening.

We have attempted to culture HT II-280$^+$ cells and NGFR$^+$ cells without stromal support (Data not shown). While NGFR$^+$ can grow, albeit at a lesser %CFE without stromal support, HT II-280$^+$ cells cannot grow without stromal support. To mitigate this issue, it is important to identify epithelial-mesenchymal signaling pathways that are important for maintenance of AT2 cells so that the media can be supplemented with the necessary growth factors. Although this is outside the scope of this study, we are currently working on these studies.

Minor comments:
1. Page 2, Line 95. What is the concentration of DNase in Unit? Or they should add the name of the vendor and batch number of DNase to Table 1, since weight-to-unit ratio varies by batches.

Added to the Table1
2. Page 2, Line 104. What did they specifically pay attention to in this step in order to avoid damaging the cells?

Added: After 30 mins of incubation, pipet up and down with 30ml syringe without needle to make uniform suspension without any clumps and allow uniform access of tissue to the enzyme.

3. Page 3, Line 117. Incubation on ice for 1 minute seems to be too short compared with the manufacturer's protocol (10 minutes' incubation at room temperature). Do they mean that incubation time should be extended or repeated if red blood cells are not lysed enough?

If required, the RBC lysis step can be repeated. Longer incubation is more damaging to the cells. Remaining RBCs can be fractionated using an anti-CD231 antibody during the sorting step.

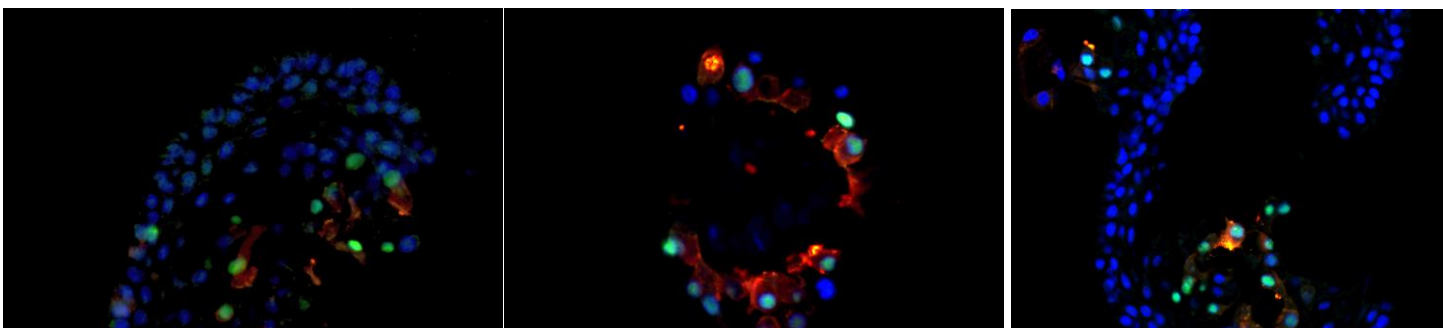
4. Page 3, Line 135. As I mentioned in the general comment, please describe the approximate number and viability of cells before and after MACS per 1cm3 tissue.
5. Page 5, Line 219. How much volume is required for 2 gm of minced tissue?

We repeated cell isolation from recent lung sample, for 5gms of lung tissue. We added 35mL of Liberase and DNase. I later split that into two 50 ml tubes.

We got 45 million viable cells from 5 gm of tissue. After depletion of CD45, CD31 and Red Blood Cells, we got a totals of 18 million viable cells. We sorted 500k Epcam^{+} cells from total viable cells and feezed the rest of the cells for future experiments.

6. Page 9, Line 404. If they describe "AcT+/FOXJ1+ ciliated cells", they should show double-positive cells in Figure 5.

AcT + FOxJ1 + DAPI



7. Figure 2 and 3 legends. (C &F) should be (C & G).

Corrected.

8. Please describe the Acknowledgments more in detail.

Fixed acknowledgments.

9. Table 2. Is the HBSS FACS buffer identical to HBSS+ buffer?

HBSS FACS buffer and HBSS+ are same and its corrected

10. Table 3. Typo. ThinCert should be corrected to Transwell, if applicable.

Corrected to Transwell

11. Table 4. Did they use PE-Cy7 conjugated antibody? It might be better readers to describe how to perform the compensation technique.

We mentioned how to make FMO controls for compensation below 2.5.7.

12. Table 6. "Immunohistochemistry Washing Solution" is not written.

Added

13. Typos.
Line 137: Miltenyi Corrected
Line 209: as finely as Corrected
Line 212, 215: gentleMACS Corrected
Lines 267, 303, 310, 311: Matrigel Corrected
Lines 273, 299, 308, Transwell Corrected
Lines 335: 0.1% Tween-20 in TBS or TBS/0.1% Tween-20 Corrected
Table 6: 3% BSA,).1% Corrected

Figure 1.

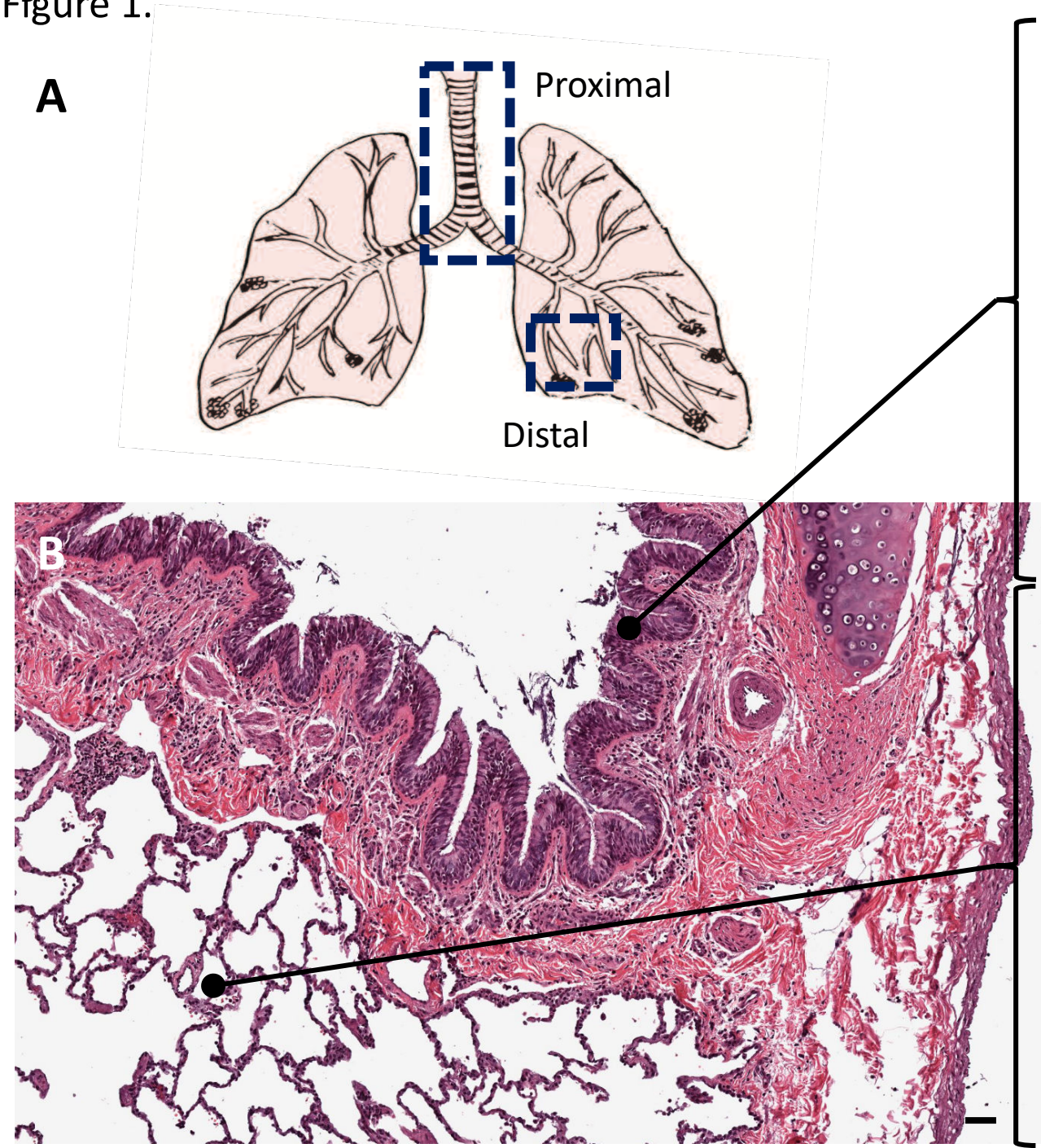
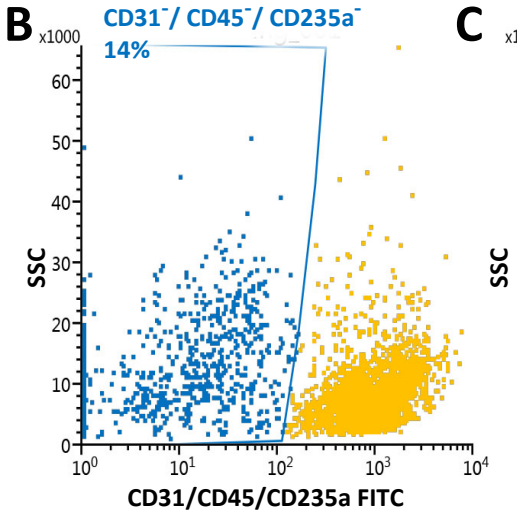
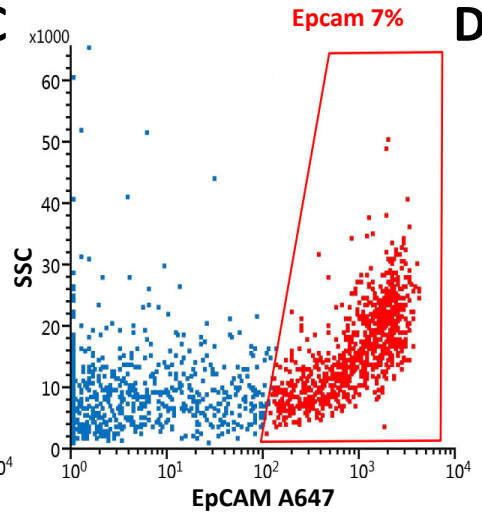


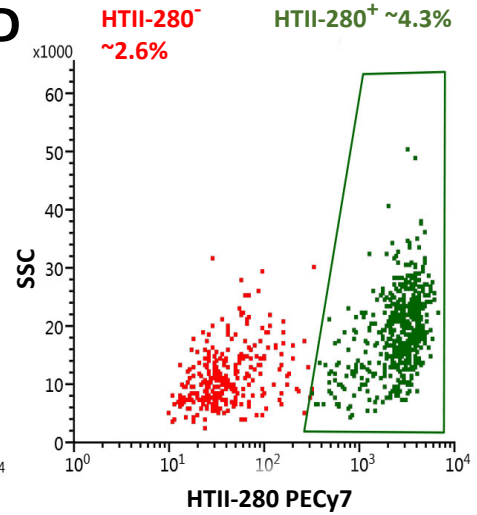
Figure 2.

A

	Before depletion of CD45/CD31/CD 235a
Live singlets	61%
Non-CD31/45	14%
Epcam^{+}	7%
HT-II280^{+}	4.3%
HT-II280^{-}	2.6%

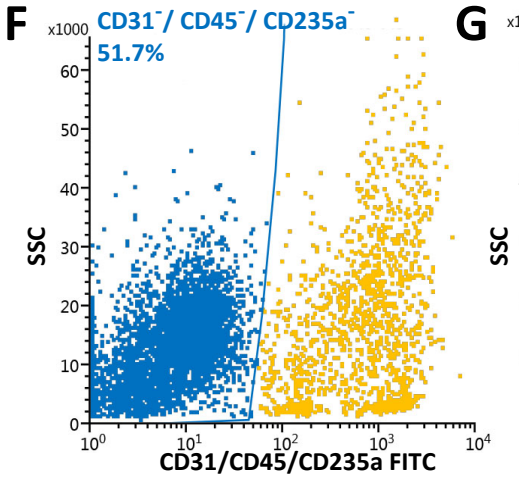


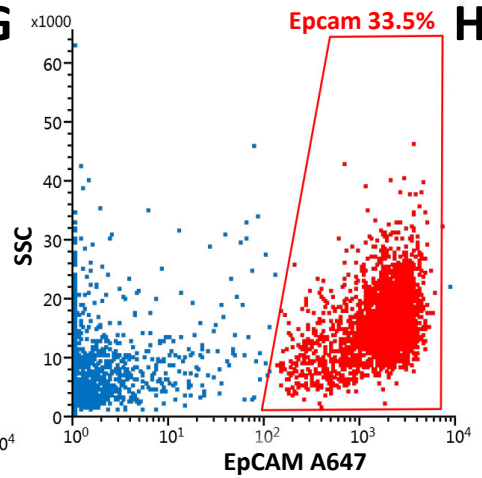




E

	After depletion of CD45/ CD31/ CD235a
Live singlets	71%
Non-CD31/45	51.7%
Epcam^{+}	33.5%
HT-II280^{+}	30%
HT-II280^{-}	3.6%





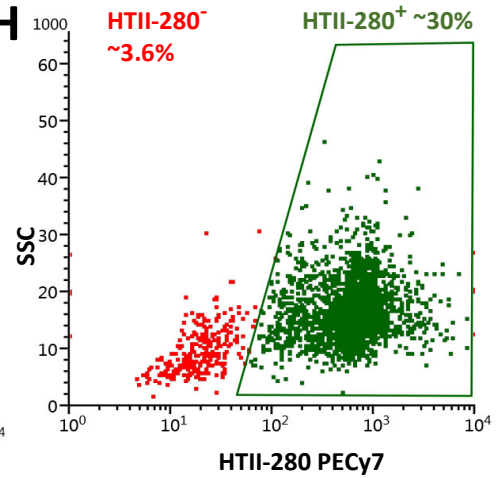
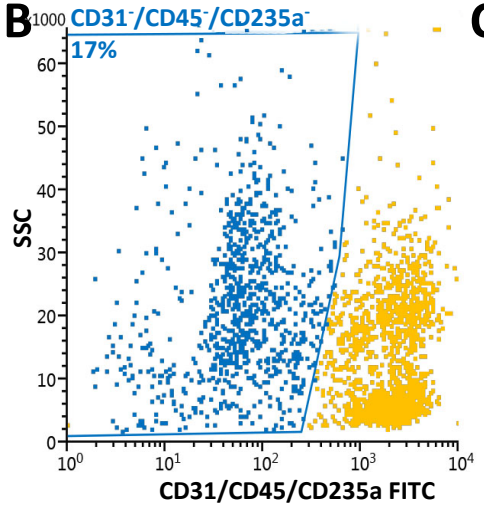
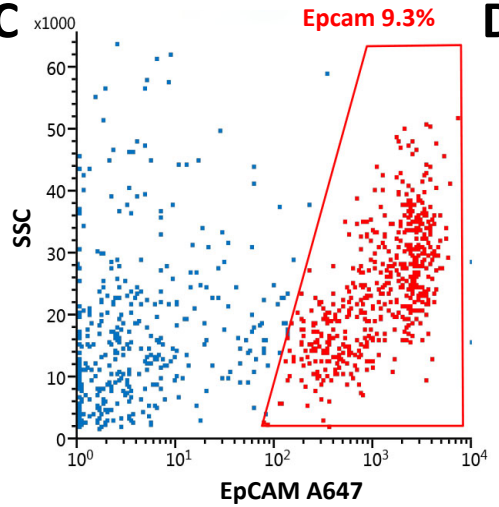


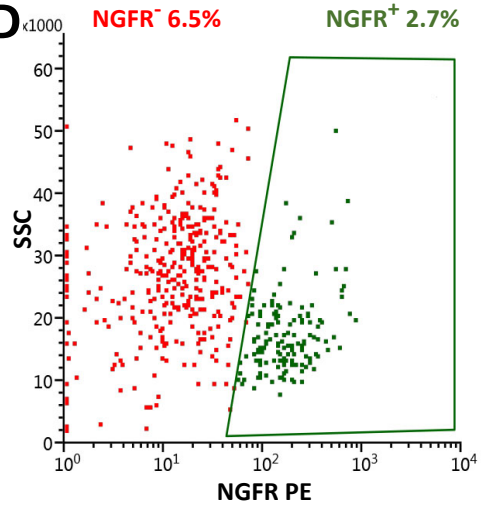
Figure 3.

A

	Before depletion of CD45/ CD31/CD235a
Live singlets	52%
Non-CD31/45	17%
Epcam	9.3%
NGFR$^+$	2.7%
NGFR$^-$	6.5%

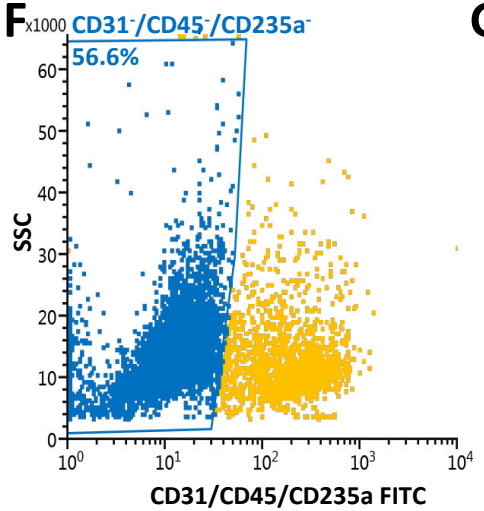


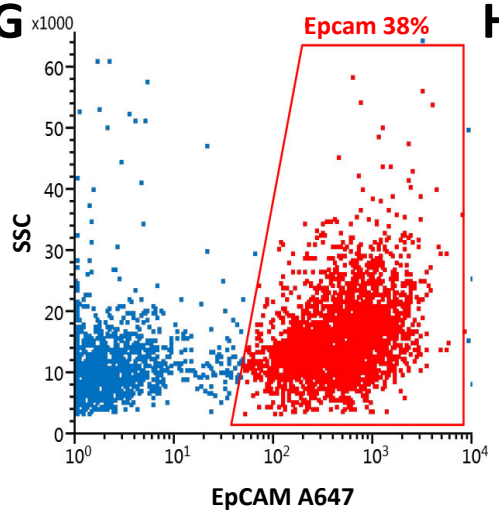




E

	After depletion of CD45/ CD31/CD235a
Live singlets	79%
Non-CD31/45	56.6%
Epcam	38%
NGFR$^+$	13%
NGFR$^-$	25%





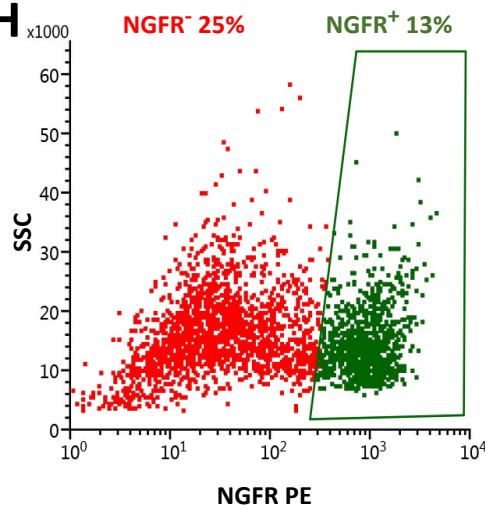


Figure 4.

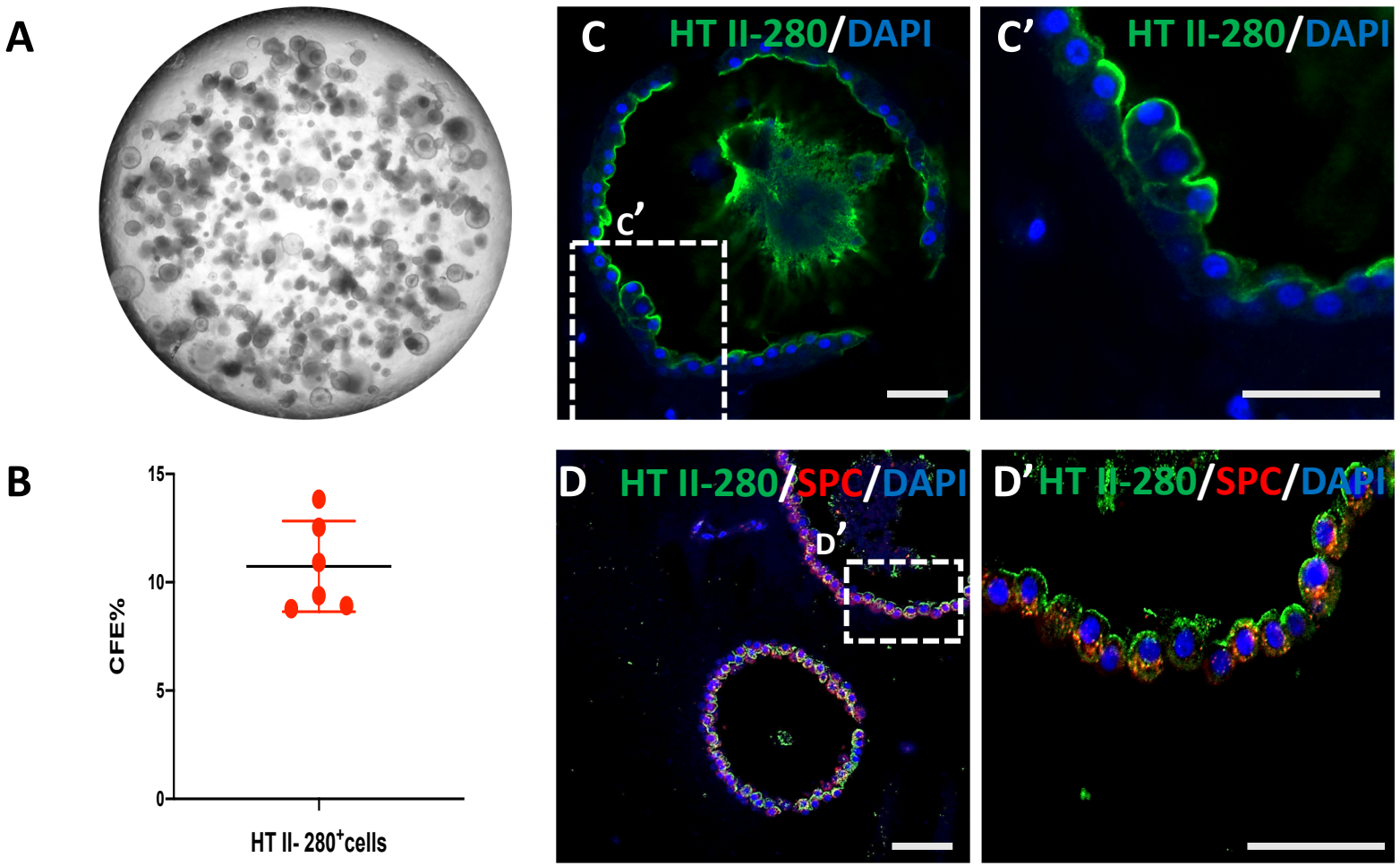
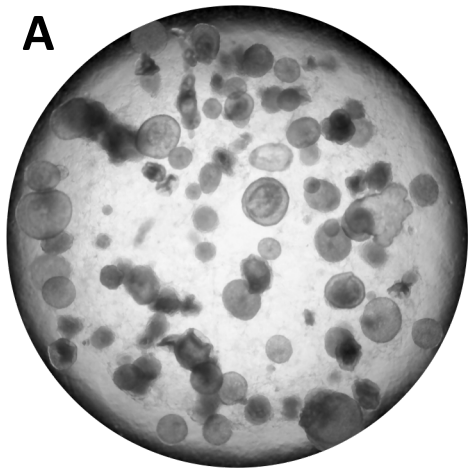
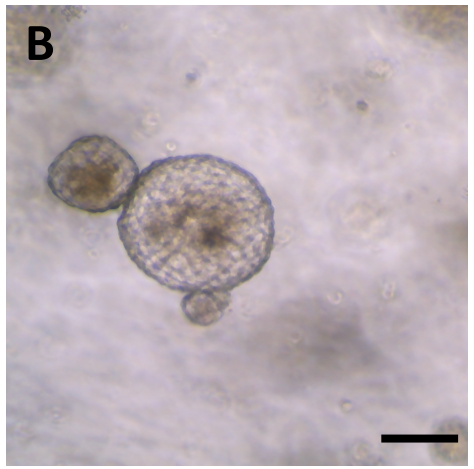
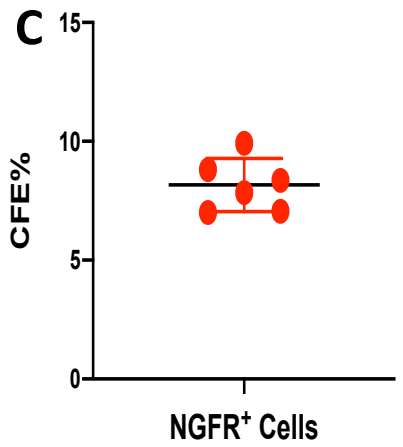
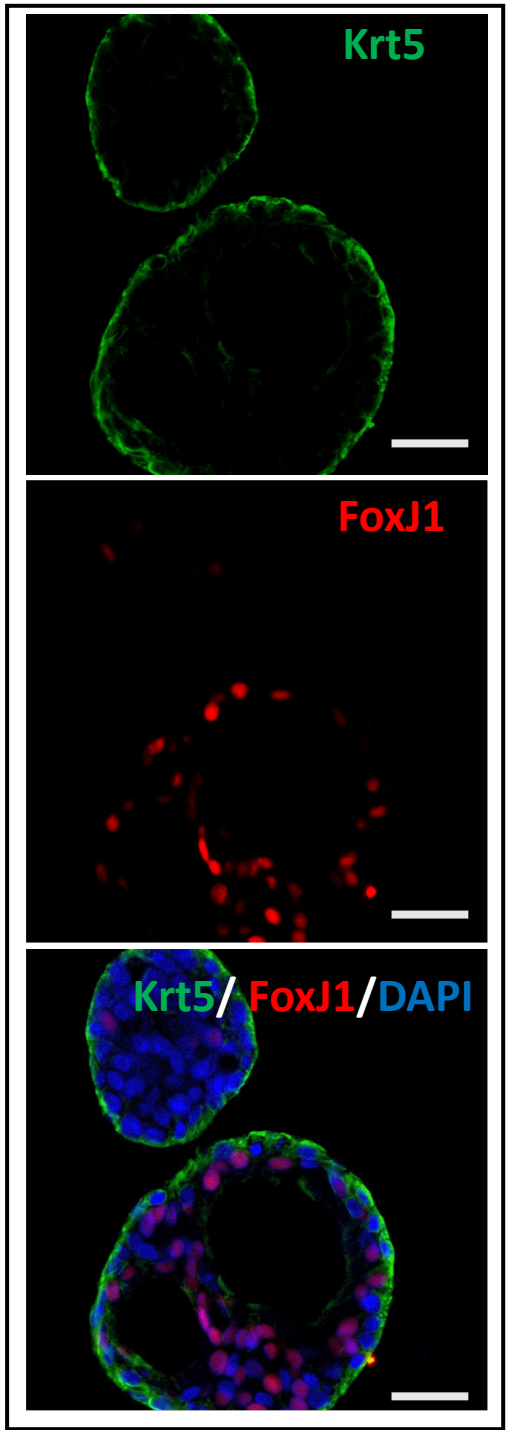

A
B
C
HT II-280/DAPI
C'
C'
HT II-280/DAPI
D
HT II-280/SPC/DAPI
D'
D'
HT II-280/SPC/DAPI
CFE%
15
10
5
0
HT II- 280+cells

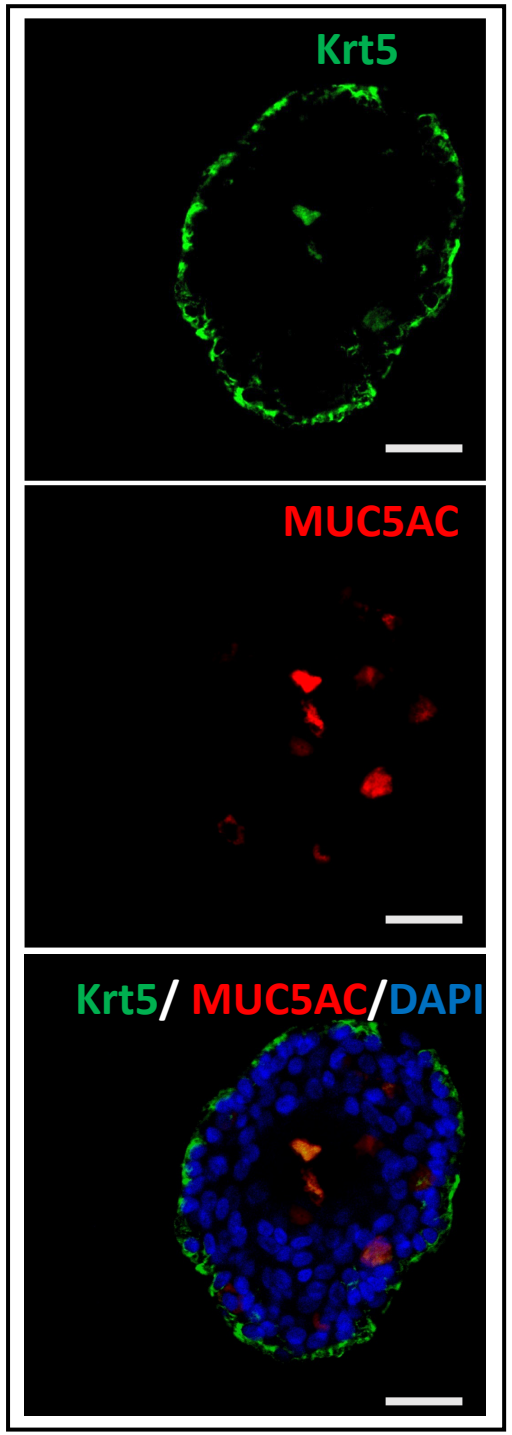
Figure 5.

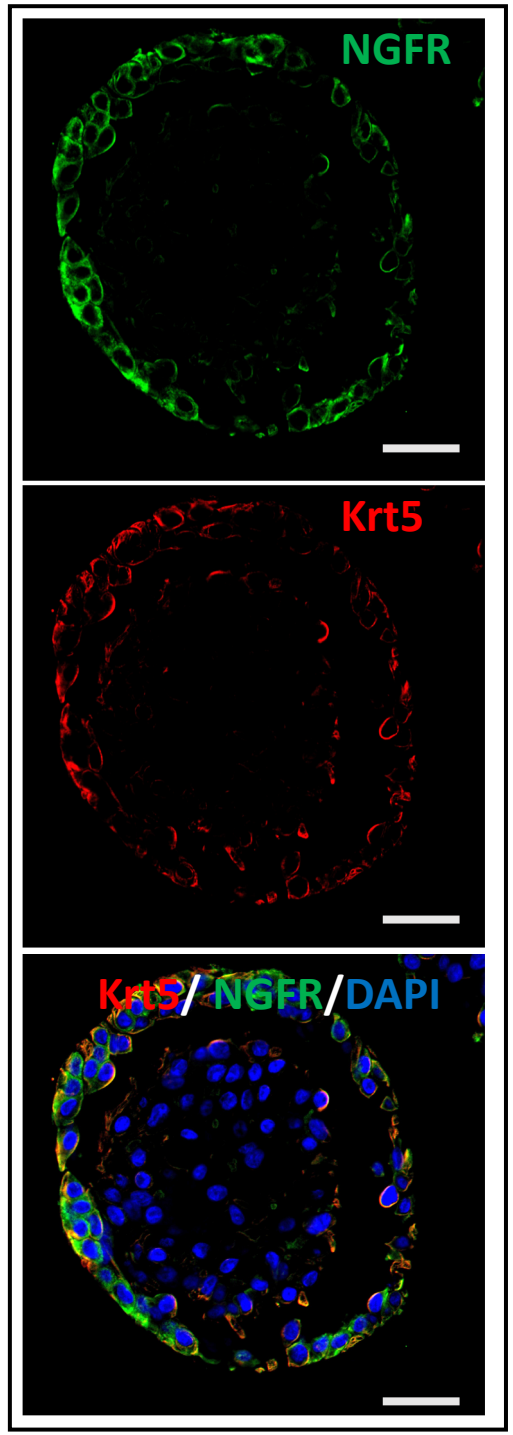

A


B


C
15
10
5
0
CFE%
NGFR+ Cells


D
Krt5
FoxJ1
Krt5/ FoxJ1/DAPI


E
Krt5
MUC5AC
Krt5/ MUC5AC/DAPI


F
NGFR
Krt5
Krt5/ NGFR/DAPI