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Isolation and Characterization of Distal Lung Progenitor Cells

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Abstract

The majority of epithelial cells in the distal lung of rodents and humans are quiescent in vivo, yet certain cell populations retain an intrinsic capacity to proliferate and differentiate in response to lung injury or in appropriate culture settings, thus giving them properties of stem/progenitor cells. Here, we describe the isolation of two such populations from adult mouse lung: alveolar epithelial type 2 cells (AEC2), which can generate alveolar epithelial type 1 cells, and bronchioalveolar stem cells (BASCs), which in culture can reproduce themselves, as well as generate a small number of other distal lung epithelial cell types. These primary epithelial cells are typically isolated using enzyme digestion, mechanical disruption, and serial filtration. AEC2 and BASCs are distinguished from other distal lung cells by expression of specific markers as detected by fluorescence-activated cell sorting, immunohistochemistry, or a combination of both of these techniques.

Keywords

Mouse lung; Alveolus; Terminal airway; Alveolar epithelial type 2 cells; Bronchioalveolar stem cells; Epithelial cell culture; Fluorescence-activated cell sorting; Immunohistochemistry

1. Introduction

With the exception of organs with high rates of turnover, such as gut and skin, adult epithelial cells in normal tissue are quiescent. This is particularly true of adult lung. However, the need to perform repair following injury or tissue replacement via compensatory growth can stimulate normally quiescent lung epithelial cells to divide. Though direct experimental evidence for a distinct hierarchy of progenitors is still lacking for distal lung in vivo, a number of new markers have recently been described that allow prospective characterization of novel lung epithelial subpopulations with stem-cell-like capacity (1). However, traditionally, lung progenitor populations have been characterized by their response to injury. Both newer cell sorting techniques and more classic marker analyses together with fractionation methods have been used to identify and isolate stem/progenitor cells from the small and terminal airways and the alveoli that make up gas exchange structures in the most distal portion of the lung (2–5). A stem/progenitor-like capacity has been ascribed to both the alveolar, surfactant-producing alveolar epithelial type 2 cells (AEC2) and to terminal airway cells of intermediate airway/alveolar phenotype, bronchioalveolar stem cells (BASCs), due to both their response to lung trauma and their ability to regenerate themselves and generate other lung epithelial cell types in vivo and/or in culture (2, 6, 7). AEC2 proliferate during embryonic and fetal life, but in adult lung do not normally divide (8). However, in response to environmental insult, a subpopulation of AEC2 becomes both hypertrophic and hyperplastic. Cells undergo multiple changes, including elevated cyclin and cdk activity, indicating regain of proliferative function (9, 10). BASCs also significantly increase in number following challenge, and under these conditions, their clonogenic and colony-forming properties are much more robust. The ability to remain viable following lung damage by environmental or experimental insult and then increase in number is presumably required so that AEC2, BASCs, and other epithelial progenitors can

drive repopulation and/or regeneration of themselves as well as the downstream target of many commonly used distal lung injury models, the alveolar epithelial type 1 cells (AEC1), which line the alveolus and facilitate gas exchange (11). Recent studies have shown that AEC1, once thought to be terminally differentiated, may have some capacity for self-repair and regeneration (12). However, it is also clear that AEC1 injury initiates extensive activation of surviving progenitors. The ability of healthy lung cell populations to respond to AEC1 damage is hypothesized to be critical for maintaining homeostasis and overall lung health (13, 14). Permanent destruction of distal lung epithelium is also hypothesized to be a first step in the development of a number of lung diseases that affect large numbers of humans worldwide, including pulmonary fibrosis and emphysema/chronic obstructive pulmonary disease (COPD) (15, 16).

In experimental rodent models, AEC2 increase in number during the recovery phase following hyperoxia exposure or the inhalation of a number of caustic and/or particulate substances (13, 17), as well as during the compensatory growth that follows pneumonectomy. Both pneumonectomy and administration of naphthalene, which strips the epithelium from small airway, stimulate increases in BASC numbers (18, 19). The activity of both AEC2 and BASCs is of great interest to those who wish to understand and manipulate the regeneration process in lung. As such, efficient isolation of AEC2 and BASCs from both normal and compromised lung is a useful and powerful technique that allows further experimental analysis of these critical progenitor populations.

2. Materials

All procedures where animals are utilized were established in collaboration with Institute Animal Care Facility veterinarians and were approved by Institutional Animal Care and Use Committees. Animals were cared for in accordance with the US Animal Welfare Act using principles set forth by the National Institutes of Health Office of Laboratory Welfare (OLAW) in the OLAW Guide. The following procedures were developed using C57BL/6J or 129Sv wild-type mice purchased from Jackson Laboratories (Bar Harbor, ME) and maintained under standard conditions in pathogen-free facilities. Both male and female mice can be utilized, and isolations are routinely performed at age 10 ± 2 weeks. All solutions and media should be obtained commercially or prepared using ultrapure (18 MΩ cm) water and then autoclaved or filter sterilized. Reagents used should be analytical grade. Enzymes, antibodies, and media should be stored at 4°C or −20°C as per manufacturer's instructions. All other reagents can be stored at room temperature. Reagent preparation (with the exception of cell culture medium) and initial dissection can be performed on the bench, but once the lung is harvested, all remaining procedures where live cells are handled should be performed in a tissue culture hood.

2.1. Components for Murine AEC2 Isolation and Culture

Standard, sterile tissue culture supplies (pipettes in sizes from 1 to 25 mL, a pipetter, plasticware, a vacuum source) will be required, as will a tissue culture hood.

1. Normal saline: 0.9% NaCl, sterile, room temperature (RT) (see Note 1).
2. Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 1% penicillin/streptomycin and 1% Fungizone (PSF) (Sigma) and 20 mM HEPES (Sigma). This combination is referred to as DMEM throughout the AEC2 isolation procedure.

[1]Phosphate buffered saline (PBS) is not recommended for the AEC2 isolation procedure.

3. Neutral collagenase (dispase): Dissolve powdered enzyme (Worthington) in DMEM at a working concentration of 5 U/ mL. Prepare fresh and keep on ice (see Note 2).
4. 1% low-melting agarose in 0.9% saline. Microwave to dissolve, then keep at 45°C throughout procedure (see Note 3).
5. Sodium pentobarbital (50 mg/mL).
6. 4″ × 4″-sterile drain sponges; 26G needles; 30G needles; 20G plastic angiocatheters; 1-, 3-, and 10-cc syringes; 5-0-silk suture cut in 6″ lengths; 1.5″-sharp surgical scissors; and 6″-curved forceps (blunt and sharp).
7. DNase: 0.01% in PBS.
8. Falcon 50-mL tube cell strainers: 100- and 40-μm (Becton Dickinson) and 25-μm Nytex nylon mesh (Genesee Scientific), pre-sterilized.
9. Mouse IgG plates. 100-mm plates can be prepared 1.5 h—2 weeks before use and stored tightly wrapped with para film at 4°C. To prepare plates, use 10 mL/20 mM Tris pH 9.5 buffer/plate containing the following antibodies: 45 μg/10 mL anti-CD 45 (90 μL of stock obtained from BD/Pharmingen) and 16 μg/10 mL anti-CD 32/16 (32 μL of stock obtained from BD/Pharmingen). Incubate 1 h at room temperature to coat plate. (Plates can be stored containing buffer plus anti-bodies.) Wash 5 × 10 mL PBS immediately before use.
10. Fibronectin-coated tissue culture flasks or dishes and chamber slides with or without removable chambers can be obtained commercially (Becton Dickinson) or can be prepared by adding purified human fibronectin (BD/Pharmingen) at 5 μg/mL in sterilized PBS to tissue culture grade containers of choice and incubating 1 h at room temperature. Wash once with sterile DI water, aspirate thoroughly, and seal coated culture ware with para film. Store at 4°C until use.
11. Fetal bovine serum (FBS).

2.2. Reagents for Murine AEC2 Immunohistochemistry Analysis

1. TBST: 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20.
2. Fixative: 50% acetone, 50% methanol.
3. Blocking buffer: CAS-BLOCK (Zymed).
4. Antibody to human surfactant protein-C (SP-C) (Seven Hills Biotechnology), Cy-3-labeled anti-rabbit secondary antibody (BD/Pharmingen).
5. 4′, 6-diamidino-2-phenyl-indole, dihydrochloride (DAPI) (Invitrogen), Aqua-Mount (Lerner Laboratories).
6. Fibronectin-coated chamber slides (see Subheading 2.1, item 10), 24 × 50-mm cover slips.

2.3. Components for Bronchioalveolar Stem Cell Isolation

1. 1× PBS, sterile, ice cold.

[2]Dispase should ideally be made immediately before use. Solution can be stored at 4°C for 24 h, but note that activity may diminish.
[3]It is preferable to prepare agarose the day before the procedure and equilibrate thoroughly to 45°C before use. Agarose at temperatures higher than 45°C can damage tissue and/or induce stress signaling in isolated epithelial cells. At minimum, agarose should be prepared no later than 1 h before the isolation.

2. 1% low-melting agarose (Bio-Rad) in sterile H_2O: microwave to dissolve, then keep at 45°C throughout procedure.
3. Dispase solution (BD), thawed (see Note 4).
4. Collagenase/dispase (Roche) resuspended in sterile H_2O to 100 mg/mL (see Note 5).
5. DNase (Sigma), resuspend in sterile H_2O to make 1% stock. Store at −20°C.
6. Falcon 50-mL tube cell strainers: 100 and 40 μm (Becton Dickinson).
7. Red blood cell (RBC) lysis buffer: 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, in 1-L distilled H_2O; filtered with 0.45-μm filter and stored at RT.
8. Dulbecco's Modification of Eagle's Medium (DMEM).
9. Fetal Bovine Serum (FBS).

2.4. Components for Bronchioalveolar Stem Cell and AEC2 Quantitation and Isolation Using Fluorescence-Activated Cell Sorting

1. Fluorescence-activated cell sorting (FACS) collection tubes: 5-mL polystyrene round-bottom tubes with 40-μm cell-strainer cap (BD/Falcon).
2. 3% bovine serum albumin (BSA) in PBS and 1× PBS for collection tubes (see Note 6).
3. PF10: PBS, 10% FBS.
4. Antibodies: Sca-1-FITC antibody (BD), CD45.2-APC antibody (BD), CD31 (Pecam)-APC antibody (BD) (see Note 7).
5. DAPI, 4′, 6-diamidino-2-phenylindole, (Sigma) 0.1 mg/mL in PBS to detect live cells (see Note 8).

2.5. Components for Bronchioalveolar Stem Cell Culture

1. 0.1% gelatin (Millipore) made up in ultrapure water.
2. Irradiated mouse embryonic fibroblasts (MEFs) (Chemicon).
3. BASC culture medium: High-glucose Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine (Invitrogen), penicillin-streptomycin (Invitrogen), and 10 mM HEPES (Invitrogen) (see Note 9).
4. 0.25% Trypsin-EDTA (Invitrogen).

3. Methods

3.1. Murine AEC2 Isolation and Culture

1. Prepare agarose and dispase.

[4]Dispase solution can be aliquoted and stored at −20°C. Do not refreeze/thaw aliquots.
[5]Collagenase/dispase can be stored at −20°C and thawed just before use.
[6]BSA for collection tubes can be made fresh and filtered or premade and filter sterilized just before use.
[7]The subtype of CD45 antibody used (CD45.1 or CD45.2) depends on the strain of mouse used. A cocktail may be used if unsure of background strain.
[8]As an alternative to DAPI, 7-Aminoactinomycin D (7AAD) resuspended in dimethyl sulfoxide (DMSO) (2 mg/mL solution) may be used for dead cell exclusion.
[9]Do not use Fungizone in BASC media or isolation reagents, as the effect on cultured cell growth is unknown.

2. Sedate mouse using an IP injection of pentobarbital (200 mg/kg; 30 G needle) (see Note 10). Place mouse on its right side on a drain sponge placed on a dissection board and soak midsection using 70% EtOH. Make a small incision in the lower back through the skin and mesentery and excise left kidney. Place mouse in supine position and secure limbs using laboratory tape.

3. Using surgical scissors, make a midline skin incision from sternum to lower jaw. Retract and/or remove skin from chest area, remove ventral rib cage, and gently cut away thymus, if present. Use a 10-cc syringe fitted with a 26G needle to exsanguinate the animal by perfusing ~5 mL DMEM through the right ventricle of the heart (see Note 11). Perfuse till lungs are clear of blood.

4. Retract and/or remove skin from throat area and gently cut and tease away neck strap muscles and salivary glands to expose trachea. Using curved, blunt forceps and scissors where necessary, gently tease away muscles from beneath the trachea and slide 2 6″ lengths of silk suture under the trachea. Knot one suture at the top of the trachea, which will slightly lift the trachea up from the throat cavity. Make a small incision at midpoint of trachea between tracheal rings. Insert a 20G angiocatheter into the incision and secure by knotting the second suture around both trachea and inserted angiocatheter (see Note 12).

5. Deliver 1 mL dispase to airway via angiocatheter using a 3-cc syringe. Being careful to not dislodge angiocatheter, disconnect syringe, fill with 0.5-mL agarose, and deliver to lung via angiocatheter. Leave the syringe in place and immediately cover the chest cavity with crushed ice for 2 min to set agarose (see Note 13).

6. Brush away ice, cut away residual thymus and the heart, and place scissors between lungs and liver to make a deep cut across the spine to sever the aorta and esophagus. Turn the dissecting board 180° and remove the lung from the chest cavity en bloc by grasping the trachea and angiocatheter using blunt forceps. Note that some connective tissue under the lung must be cut in order to free the tissue.

7. Place lung into 1 mL dispase in a sterile 50-mL tube. Incubate with gentle rocking for 45 min at room temperature (RT). (If harvesting multiple animals, lungs can be placed on ice at this point until all are ready for the RT incubation.)

8. Transfer lungs to a 100-mm Petri dish and add 100 μL DNase. Tease alveolar tissue from airways using the curved edge of fine tipped curved forceps, then use sharp tips of two pairs of forceps to tear and finely mince parenchymal tissue. Add 7 mL DMEM. Gently shake tissue in medium at room temperature for 10 min.

9. Filter the resulting suspension through 100-μm, then 40-μm cell strainers, then through 25-μm mesh. Centrifuge cells at 130 × *g* for 10 min and resuspend pelleted cells in 10 mL DMEM (see Note 14).

10. Place cell suspension onto a prepared mouse IgG plate and incubate 1 h at 37°C.

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[10] CO_2 asphyxiation is not recommended for sedation, as it can cause pulmonary microhemorrhages and excess red blood cell contamination of the isolate. The preferable level of sedation is one where the animal does not respond to stimuli (toe pinch), but the heart is still beating, which enables efficient exsanguination.

[11] The most efficient exsanguination can be achieved by placing the needle at a 45° angle above horizontal pointing toward the animal's left shoulder. The needle should be placed at a depth no greater than 1 mm just at the apex of the triangle formed by the largest visible coronary artery and the base of ventricle.

[12] The proper placement of the angiocatheter insures even distribution of dispase and agarose. Place the angiocatheter such that the join between the catheter and the luer rests just at the tip of the nose of the animal. This insures it is at the proper depth.

[13] Back pressure from the lung will push dispase back through the angiocatheter after the syringe is removed in order to take up agarose. Excessive loss of dispase can be easily prevented by placing a fingertip over the angiocatheter opening. Note that once agarose has been dispensed, the syringe should be propped up during the icing step in order to prevent it slipping free and/or tearing trachea.

11. Carefully pan epithelial cells off plate by pipetting suspension up and across plate several times. Wash plate with an additional 1 mL DMEM, add to collected cell suspension, and count cells (see Note 15).

12. Following counting, centrifuge as before and suspend cells in DMEM plus 10% FBS such that they can be plated at a density of 2–5 × 10^5 cells/cm^2. Plate cells on fibronectin-coated plates. Cells will attach by 24–36 h. Medium can be changed and cells fixed or harvested up to 48 h (see Note 16).

3.2. Immunohistochemistry Analysis of Murine AEC2 to Determine Purity

1. Remove medium and wash chambers 3 × 5 min using TBST (approximately ~1 mL of TBST per chamber for all washes). Add fixative and incubate 5 min on ice. Wash 3 × 5 min using TBST (see Note 17).

2. Add 500 μL of blocking buffer per chamber and incubate 1 h at RT.

3. Remove blocking buffer. Do not wash. Add 300 μL of anti-SP-C antibody at a dilution of 1:250 in TBST, cover and wrap chamber slide with para film, and incubate 1 h at RT or at 4°C overnight.

4. Remove primary antibody. Wash 3 × 5 min using TBST. Add 300 μL of secondary antibody at a dilution of 1:500 in TBST, cover with aluminum foil, and incubate 30 min at RT. From this point, slides should be kept dark as much as possible.

5. Add 300 μL of DAPI solution (1:500 in TBST) and incubate 5 min at RT.

6. Wash 3 × 5 min using TBST. Remove chamber if using chamber slide with removable chamber. For slides, drop on Aqua-Mount and place coverslip. For live cells (without fixation) in chambers or dishes, add DAPI solution for 30 s then wash 1 × 1 min with water. View cells using a fluorescent microscope (see Note 18). Routinely, adherent cells harvested as described are ~95% SP-C positive.

3.3. Bronchioalveolar Stem Cell Isolation

1. Anesthetize mouse with an IP injection of 400–500 μL Avertin and spray down mouse with 70% ethanol.

2. Quickly cut into ribcage. Using a butter fly needle and 10-mL syringe, perfuse 10 mL of ice-cold PBS through right ventricle until lungs cleared of blood. Cut a slit in left ventricle to allow blood to leave.

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[14] Isolated AEC2 tend to be sticky and will clump easily, but can also break easily with rough handling. To disperse cells following centrifugation, knock the bottom of two tubes together or tap the tube containing the pelleted cells against some other surface before adding medium to triturate. Trituration alone is not advised to disperse cells. It will inevitably result in clumping, cell breakage, and cell loss. If the cell suspension appears to gelatinize during the isolation process due to freed DNA, an additional aliquot of DNase can be added at any point.

[15] Cells at this point have been mostly depleted of immune cells, which adhere to IgG plate, but some contaminants will remain. When counting, tiny red blood cell and large white blood cell contaminates should not be counted. AEC2 at this point are moderately sized and very bright. Trypan blue can be used as needed to determine viability, but total viability at this point is routinely ~98%.

[16] AEC2 can be plated at lower or higher density depending on the status of the source lung. Control, quiescent cells should be plated at higher density, while cells from lungs stimulated by insult or injury can be plated at lower density. Cell viability is routinely high immediately following isolation but drops significantly in culture. It is normal to lose 60–70% of original cell number after 48 h in culture. Cells harvested or analyzed at time points past 48 h will begin to acquire characteristics of AEC1 and can no longer considered pure AEC2.

[17] Before and during fixation, cells can easily be lost from chambers. Medium, washing buffer, and fixative should be added slowly and gently along one corner of chamber and never pipetted directly onto cells. Liquid should be removed from one corner of chamber using gentle vacuum suction. For this step, vacuum tubing should be fitted with the smallest available pipette tip. Once cells are fixed, more vigorous methods can be used, but some cell loss may still occur.

[18] Immunolabeled cells should be viewed within 3 h if the secondary antibody is labeled with FITC. If the secondary antibody is labeled with Cy3 and cells are kept dark, they can be viewed without loss of signal for up to 2 days.

3. Cut out heart to euthanize mouse.
4. Expose trachea and place forceps under trachea to keep exposed.
5. Inject dispase solution into the trachea just until the lungs inflate (~1–3 mL).
6. Follow with tracheal injection of 0.5–1 mL of 1% low-melting agarose, using a 20G needle.
7. Dissect out lungs en bloc.
8. Place intact lungs on a Petri dish lid on ice. Dissect off each lung lobe.
9. Transfer each harvested lung lobe to the edge of a clean, 50-mL conical tube and add 1 mL PBS. Mince lung tissue inside the tube (tilting tube to allow scissor access) into small pieces using sharp scissors. Lung tissue may be left in PBS on ice while dissecting other mice and before proceeding to the next step.
10. Add 2 mL PBS to tube to wash down minced lung.
11. Add 60 μL collagenase/dispase to minced tissue suspension and rotate at 10 rpm for 45 min at 37°C.
12. Place dish containing digested tissue on ice. Add 7.5 μL of 1% stock of DNase per 3 mL (final concentration 0.025 mg/mL). Mix and leave at RT for no more than 5 min.
13. Filter digested tissue serially through 100- and 40-μm filters into a 50-mL tube. Use an additional 1–2 mL PF10 to wash remaining cells through the 100-μm filter and 1–2 mL PF10 to wash remaining cells through the 40-μm filter. Total final volume is ~5–10 mL.
14. Centrifuge tubes 6 min at 800 rpm at 4°C. Aspirate supernatant.
15. Resuspend each cell pellet in 1 mL of RBC lysis buffer for 90 s at room temperature. After 90 s, immediately neutralize each cell solution with 6 mL DMEM.
16. Add 0.5 mL FBS slowly to bottom of tube by inserting pipette tip all the way through the resuspended cell solution to leave an undisturbed layer of FBS at the bottom of the tube.
17. Centrifuge undisturbed layers for 6 min at 800 rpm. Aspirate the supernatant.
18. Resuspend each pellet in 2–2.5 mL PF10. Cells may be pooled at this point if multiple sets of murine lung were harvested for a single sample.
19. Count cells in each sample.

3.4. Bronchioalveolar Stem Cell/AEC2 Immunostaining and Quantitation Using Fluorescence-Activated Cell Sorting

BASCs can be purified by flow cytometry using the specific surface marker combination Sca-1^{pos}/CD45.2^{neg}/CD31^{neg} and AEC2 with the marker combination Sca-1^{neg}/CD45^{neg}/CD31^{neg}. Live cells can be gated with the addition of DAPI (live cells are DAPI^{neg}) (see Note 19).

[19]It should be noted that other groups have recently isolated distal lung stem/progenitor cells with different techniques, including the development of a three-dimensional Matrigel culture assay (1, 3).

1. For FACS isolation, fill-up collection tubes with sterile 3% BSA and let stand at least 1 h at RT or overnight at 4°C. Just before isolation, aspirate BSA and add 200 μL sterile BASC media or 1× PBS to collection tubes, depending on the purpose of cell isolation.
2. For immunostaining, dilute each sample in PF10 to reach a concentration of 10×10^6 cells/mL.
3. Aliquot $0.5–1.0 \times 10^6$ cells for an unstained negative control as well as an additional $0.5–1.0 \times 10^6$ cells for each single-stain control (see Note 20).
4. Add each primary antibody (Sca-1, CD31, CD45) to each BASC sample as well as associated single-stain control (see Note 21). Recommended concentrations: Sca-1-FITC, CD31-APC, CD45-APC, DAPI at 1:100 (1 in 100 μL).
5. Leave samples on ice 15 min.
6. Quick spin all samples and controls in a standard microfuge (~8 s at 12 rpm for 1.5-mL micro-test tubes, ~10 min at 1,000 rpm for 15-mL or 50-mL test tubes) and aspirate supernatant. Wash cells by adding, at minimum, three times the staining volume of PF10 directly to each sample and control (a larger volume can be used if desired). Quick spin all samples and controls in a standard microfuge. Aspirate supernatant.
7. Resuspend cell pellet in PF10 to a final volume of 500 μL per control/sample. Filter cells through 40-μm filter either directly into a FACS collection tube or into a 50-mL tube and then transfer cell suspension to a FACS tube (see Note 22).
8. For FACS gating, first select single cells by sequentially gating using forward and side scatters as shown in Fig. 1a–c. Next, from the gated single-cell population, select DAPI-negative live cells as shown in Fig. 1d.
9. From this DAPI-negative population, AEC2 are contained in the $CD31^{neg}$, $CD45^{neg}$, and $Sca\text{-}1^{neg}$ population (box on left in Fig. 2, 9.76% in this example) and BASCs, in the $CD31^{neg}$, $CD45^{neg}$, and $Sca\text{-}1^{pos}$ population (gate on right in Fig. 2, 1.02% in this example).
10. Sort cells using low pressure (30 psi for MoFlo, 50 psi for Aria) (see Note 23).

3.5. Bronchioalveolar Stem Cell Culture on Feeder Cells

1. On the day before sorting, prepare feeder cells. MEFs should be irradiated prior to plating using a dose of 26 grays.
2. Prepare the feeder plate by adding 100 μL 0.1% gelatin to all wells of a 96-well plate and incubate at 37°C for 20 min. Aspirate the gelatin and add irradiated MEFs at $2–3 \times 10^6$ cells per 96-well plate. Cells should be suspended in 10 mL BASC medium and distributed by placing 100 μL per well.

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[20] Single-stain controls must be prepared for every antibody used in order to perform FACS compensation.

[21] For optimal fluorescence and cell survival, keep cell aliquots on ice and covered with aluminum foil to minimize light exposure. If cells are being sorted for culture or clonal assays, continue to maintain sterility by performing all immunostaining work in a tissue culture hood.

[22] It is critical to filter final cell suspensions through a 40-μm cell strainer in order to prevent epithelial cells from clumping and clogging the flow cytometer.

[23] Sorting using low pressure is critical for optimal cell growth in culture. Note that a low pressure setting on cell sorters is generally not standard and may require prior set up by the instrument operator. In addition, low pressure sorting usually results in a need for additional machine usage time.

3. BASCs can be sorted directly into the wells of this feeder plate or can be sorted into collection tubes and plated by hand (see Subheading 3.4, step 1 for preparation of collection tubes).
4. After 4 days in culture, supplement culture medium with 100 μL BASC medium/well (see Note 24).
5. After 7 days in culture, BASC colonies can be scored.
6. To passage colonies, aspirate media and wash 1× with PBS, then trypsinize for 10 min using 50 μL of trypsin-EDTA per well. Add 100 μL of media to each well, mix, and replate on a new feeder plate.

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4. Notes

[1]Phosphate buffered saline (PBS) is not recommended for the AEC2 isolation procedure.
[2]Dispase should ideally be made immediately before use. Solution can be stored at 4°C for 24 h, but note that activity may diminish.
[3]It is preferable to prepare agarose the day before the procedure and equilibrate thoroughly to 45°C before use. Agarose at temperatures higher than 45°C can damage tissue and/or induce stress signaling in isolated epithelial cells. At minimum, agarose should be prepared no later than 1 h before the isolation.
[4]Dispase solution can be aliquoted and stored at −20°C. Do not refreeze/thaw aliquots.
[5]Collagenase/dispase can be stored at −20°C and thawed just before use.
[6]BSA for collection tubes can be made fresh and filtered or premade and filter sterilized just before use.
[7]The subtype of CD45 antibody used (CD45.1 or CD45.2) depends on the strain of mouse used. A cocktail may be used if unsure of background strain.
[8]As an alternative to DAPI, 7-Aminoactinomycin D (7AAD) resuspended in dimethyl sulfoxide (DMSO) (2 mg/mL solution) may be used for dead cell exclusion.
[9]Do not use Fungizone in BASC media or isolation reagents, as the effect on cultured cell growth is unknown.
[10]CO_2 asphyxiation is not recommended for sedation, as it can cause pulmonary microhemorrhages and excess red blood cell contamination of the isolate. The preferable level of sedation is one where the animal does not respond to stimuli (toe pinch), but the heart is still beating, which enables efficient exsanguination.
[11]The most efficient exsanguination can be achieved by placing the needle at a 45° angle above horizontal pointing toward the animal's left shoulder. The needle should be placed at a depth no greater than 1 mm just at the apex of the triangle formed by the largest visible coronary artery and the base of ventricle.
[12]The proper placement of the angiocatheter insures even distribution of dispase and agarose. Place the angiocatheter such that the join between the catheter and the luer rests just at the tip of the nose of the animal. This insures it is at the proper depth.
[13]Back pressure from the lung will push dispase back through the angiocatheter after the syringe is removed in order to take up agarose. Excessive loss of dispase can be easily prevented by placing a fingertip over the angiocatheter opening. Note that once agarose has

[24]Never aspirate medium during the first 7 days of BASC culture, as it will disturb the cells.

been dispensed, the syringe should be propped up during the icing step in order to prevent it slipping free and/or tearing trachea.

[14]Isolated AEC2 tend to be sticky and will clump easily, but can also break easily with rough handling. To disperse cells following centrifugation, knock the bottom of two tubes together or tap the tube containing the pelleted cells against some other surface before adding medium to triturate. Trituration alone is not advised to disperse cells. It will inevitably result in clumping, cell breakage, and cell loss. If the cell suspension appears to gelatinize during the isolation process due to freed DNA, an additional aliquot of DNase can be added at any point.

[15]Cells at this point have been mostly depleted of immune cells, which adhere to IgG plate, but some contaminants will remain. When counting, tiny red blood cell and large white blood cell contaminates should not be counted. AEC2 at this point are moderately sized and very bright. Trypan blue can be used as needed to determine viability, but total viability at this point is routinely ~98%.

[16]AEC2 can be plated at lower or higher density depending on the status of the source lung. Control, quiescent cells should be plated at higher density, while cells from lungs stimulated by insult or injury can be plated at lower density. Cell viability is routinely high immediately following isolation but drops significantly in culture. It is normal to lose 60–70% of original cell number after 48 h in culture. Cells harvested or analyzed at time points past 48 h will begin to acquire characteristics of AEC1 and can no longer considered pure AEC2.

[17]Before and during fixation, cells can easily be lost from chambers. Medium, washing buffer, and fixative should be added slowly and gently along one corner of chamber and never pipetted directly onto cells. Liquid should be removed from one corner of chamber using gentle vacuum suction. For this step, vacuum tubing should be fitted with the smallest available pipette tip. Once cells are fixed, more vigorous methods can be used, but some cell loss may still occur.

[18]Immunolabeled cells should be viewed within 3 h if the secondary antibody is labeled with FITC. If the secondary antibody is labeled with Cy3 and cells are kept dark, they can be viewed without loss of signal for up to 2 days.

[19]It should be noted that other groups have recently isolated distal lung stem/progenitor cells with different techniques, including the development of a three-dimensional Matrigel culture assay (1, 3).

[20]Single-stain controls must be prepared for every antibody used in order to perform FACS compensation.

[21]For optimal fluorescence and cell survival, keep cell aliquots on ice and covered with aluminum foil to minimize light exposure. If cells are being sorted for culture or clonal assays, continue to maintain sterility by performing all immunostaining work in a tissue culture hood.

[22]It is critical to filter final cell suspensions through a 40-μm cell strainer in order to prevent epithelial cells from clumping and clogging the flow cytometer.

[23]Sorting using low pressure is critical for optimal cell growth in culture. Note that a low pressure setting on cell sorters is generally not standard and may require prior set up by the instrument operator. In addition, low pressure sorting usually results in a need for additional machine usage time.

[24]Never aspirate medium during the first 7 days of BASC culture, as it will disturb the cells.

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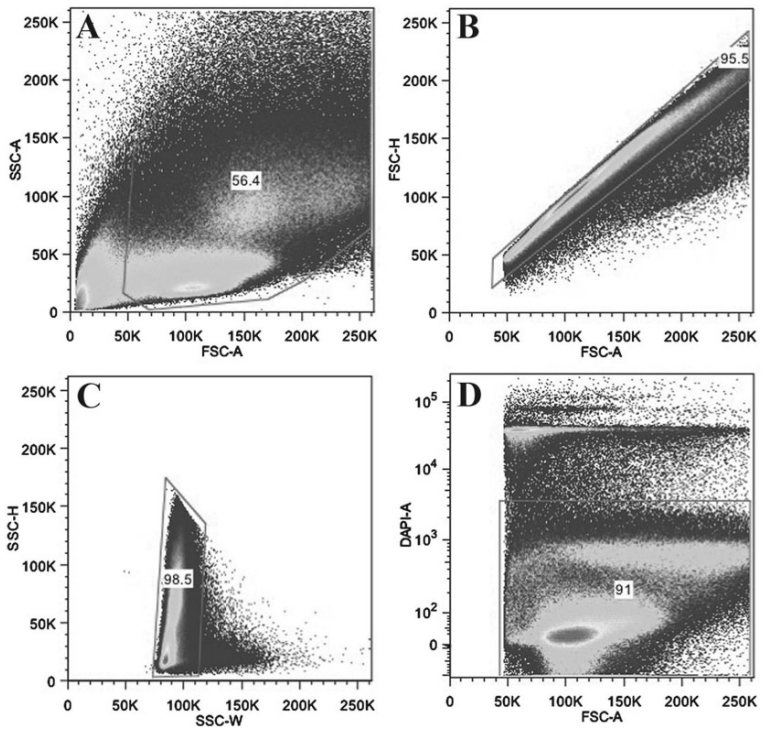

Fig. 1.

Fluorescence-activated cell sorting (FACS) analysis purification of bronchioalveolar stem cells (BASCs) using the specific surface markers. Live cells can be gated with the addition of DAPI.

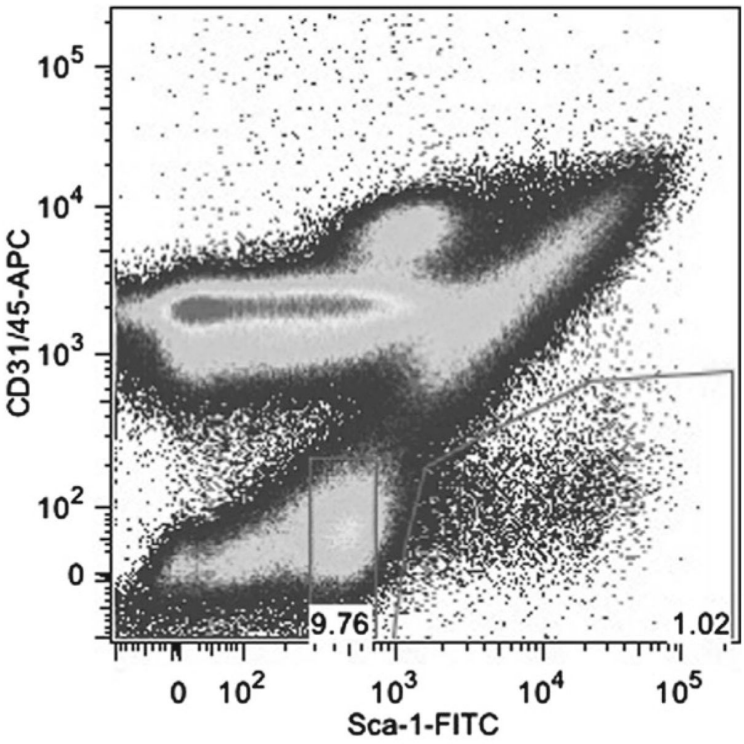

Fig. 2.
FACS analysis purification of BASCs in the $CD31^{neg}$, $CD45^{neg}$, and $Sca\text{-}1^{pos}$ population.