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Isolation and Characterization of Murine Multipotent Lung Stem Cells

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Abstract

Cancer stem cells possess the ability to self-renew and differentiate into specific cells found in tumor types, a characteristic feature of normal multipotent stem cells. These cells harbor within the bulk of tumors and if the tumor suppressor p53 is mutated in these cells, can be more likely to cause relapse and metastasis by giving rise to new tumors. This new paradigm of oncogenesis has been observed in various cancers, including lung cancer. Determining the interaction of critical cellular pathways in the ontogeny of lung tumors is expected to lead to identification of molecular targets for effective therapeutic strategies. To achieve this, it is important to characterize and dissect the differences between the cancer cells with aberrant stem cell like properties and normal multipotent stem cells that contribute to regeneration. This could be accomplished by using cell surface markers unique for certain cell types by employing techniques such as flow cytometry and magnetic bead isolation. This chapter summarizes the isolation process of the resident stem cell Sca1 (+ve), CD-45 (–ve), and CD-31 (–ve) populations for its potential use in assessing correlations between specific p53 gain of function phenotypes in different murine lung cancer models.

Keywords

Lung stem cells; Flow cytometry; MACS

1. Introduction

Stem cells possess distinct characteristic features such as self-renewal and differentiation potential. However, depending on the source of resident stem cells, these characteristics differ; e.g., embryonic stem cells are pluripotent (ability to differentiate into any cell type in a body), while somatic stem cells are multipotent (ability to differentiate into cells of specific lineage). Isolation, characterization, and propagation of these multipotent stem cell lines have been a challenge for researchers. In the context of lung regeneration, the main purpose of the multipotent lung stem cells is to generate new cells and maintain structure and function of the tissue throughout the life of a multicellular organism. Scientists have characterized multipotent stem cells from different tissues and have inferred that different organs use different strategies to renew themselves with results suggesting adoption of a diverse and flexible renewal process by respective tissue and organs (1). The adult lung is a vital and complex organ in multicellular organisms and exhibits a slower regeneration process than rapidly regenerating hematopoietic and epithelial tissues. There is growing evidence of resident multipotent stem cell populations in different anatomical regions of murine lung tissue that possess different differentiating capacities (2, 3).

Recent studies on phenotypical and functional properties of human lung samples have shown the evidence of resident stem cell population that participate in tissue homeostasis and regeneration in animal models (4). The existence of cancer stem cells in different tumor types has set a challenge in identifying and characterizing lung cancer stem cells, which could serve as relevant targets in understanding and treating human lung cancer. The tumor suppressor p53 is mutated in a high number of lung cancers. Certain gain of function phenotypes result from a mutated p53 protein such as increased oncogenicity and tumorigenicity.

Results obtained from the studies of animal models of lung injury and carcinogenesis have led to the idea that several potential stem cell compartments are located along the respiratory tract and in the lung parenchyma (5). Identification of these potential stem cells is primarily based on expression of specific surface markers (2, 3). Based on different studies, it is hypothesized that Sca1 (+ve), CD-45 (–ve), and CD-31 (–ve) cell population harbors one or more endogenous stem cell populations (3). In order to characterize the difference between cancer stem cells and normal stem cells it is important to develop reproducible methods to identify, isolate, and propagate these stem cells. This chapter details cell sorting techniques such as magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS), which take advantage of specific cell surface marker expression in the isolation, sorting, and characterization of resident stem cell populations in the lung. This will enable researchers to study the role mutant p53 may play in cancer stem cells and gain of function effects in those cells throughout the lungs.

2. Materials

2.1. Equipment

1. Sterile Laminar Air flow hood.
2. MACS (Milteny Biotec, Auburn, CA, USA).
3. Fluorescence-activated cell sorter (BD FACS Aria™ II High-Speed Cell Sorter, BD Biosciences).
4. Desktop centrifuge.
5. Flow cytometer (BD Accuri® C6 Flow Cytometer, Accuri, Ann Arbor, MI, USA).

2.2. Anesthesia Preparation

1. *Stock solution*: Add 5 ml T-amyl alcohol to 5 g tribromoethanol (tbe) (Aldrich St. Louis, MO, USA) in a dark environment.
2. *Working solution*: Mix 0.1 ml stock solution with approximately 7.9 ml normal saline, (or PBS), in a glass vessel wrapped in foil or a dark bottle. Dosage is about 0.2 ml/10 g of body weight.

2.3. Dissection Kit

1. Sterile stainless steel narrow blade scalpel, sharp/blunt operating scissors, straight iris scissors, thumb forceps, fine-point forceps, straight teasing needle, surgical thread, syringe, 18, 21, 30 gauge needle, 0.40 μ m mesh filter, 1 ml SubQ Syringe.

2.4. Digestion Media

1. Dispase solution, 0.6–2.4 U/ml working concentration, prepared in sterile Phosphate buffer saline (PBS) (Ca^{2+} and Mg^{2+} free). Alternatively more efficient dissociation of tissue is obtained by mixing the Dispase at 0.3–0.6 U/ml and collagenase (60–100 U/ml) (Invitrogen, Carlsbad, CA, USA) (see Note 1).
2. 0.001% Dnase solution (Invitrogen, Carlsbad, CA, USA), to hydrolyze DNA from damaged cells.

2.5. Sorting Medium

1. Phosphate buffer saline (PBS^{++} with Ca^{2+} and Mg^{2+} ions) with 10% fetal bovine serum (FBS).

2.6. Antibodies, Micro-beads, and Fluorescent Stains

1. Anti-mouse CD-31–PE conjugate (R&D systems, Minneapolis, MN, USA).
2. Anti-mouse CD-45–PE conjugate (R&D systems, Minneapolis, MN, USA).
3. Anti-PE and Anti-APC Micro-beads (Milteny Biotec, Auburn, CA, USA).

2.7. Multipotent Lung Stem Cell Medium

1. DMEM high glucose supplemented with sodium pyruvate and glutamine (Invitrogen, Carlsbad, CA, USA).
2. 1% Penicillin and streptomycin.
3. 1% Nonessential amino acids.
4. 10% FBS (Fig. 1).

3. Methods

3.1. Lung Isolation

The experimental mice used in this study are maintained in our animal facility in pathogen-free conditions according to the guidelines of IACUC and Virginia Commonwealth University animal care facility.

1. Select three 6–8 weeks old mice for dissection.
2. Place the mice on a cage lid and grasp the loose skin behind the ears with your thumb and forefinger. As soon as the mouse's head is restrained pick up the tail and

¹It is important to dissolve lyophilized dispase enzyme in PBS ($\text{Ca}^{2+}/\text{Mg}^{2+}$ free), because these ions reduce the activity of the enzyme. Concentrations higher than 2.4 U/ml are not recommended.

secure it within your ring finger and little finger. Make sure movement of mice is restrained completely.

3. Anesthetize mice by Intra-peritoneal injection method. The injection site should be in the lower left quadrant of the abdomen. Disinfect the site by 70% alcohol and carefully penetrate the tip of the needle into mice abdomen wall at a 45° angle.
4. It will take about 5 min for the mouse to become fully anesthetized (evidenced by lack of response to toe or tail pinch). An additional 0.05–0.1 ml can be given if any sense of pain is observed. Once completely anesthetized, sacrifice the mice by cervical dislocation.
5. Using sterile pointed scissors make a small incision near the posterior end of abdomen. Excise towards the anterior end cutting the rib cage to expose internal organs. Care should be taken to near the throat region to avoid any damage to trachea. Dissect away the platysma and anterior tracheal muscles in order to visualize and access tracheal rings.
6. Slightly make a cut on trachea to insert syringe tip. Inject 1 ml of dispase (2 U/ml) into the trachea using a 1 ml subcutaneous (SubQ) syringe with appropriate needle. The lung inflation is observed during injection. Carefully remove the syringe and tie a surgical thread at cut end of trachea, to prevent draining of dispase.
7. Pulling up trachea with thumb forceps, carefully excise and separate other internal organs to reach lungs. Pull out lungs along with heart, trachea (referred as “pluck”), and place them on a sterile Petri dish. Separate trachea, heart, and other connective tissues to collect lung lobes.
8. Place the lung lobes in cold PBS⁻ (Ca²⁺ and Mg²⁺ free) supplemented with Penicillin and streptomycin, for about 5 min to drain out blood.
9. Discard the mice according to IACUC protocol.

3.2. Lung Digestion

1. In a tissue culture hood remove lung lobes from PBS⁻ and place them on a 100 mm Petri dish. Using a sharp scalpel, mince lung lobes finely into small pieces. Add 10 ml of digestion medium. Incubate for 30 min at 37°C on a rotator. Observe it every 10 min to make sure that all the pieces are immersed in digestion medium.
2. After 30 min, digested lung slurry should be easily pipetted up and down using a 10 ml pipette. This is an indication of good digestion. Repeat this few times (see Note 2).
3. Collect the slurry in a 10 ml syringe and pass through 18 and 21 gauge syringes. Repeat this step few times, until clear slurry is obtained.
4. Filter the slurry using a sterile 0.40 µm mesh filter into a 50 ml conical tube.

²In general, 30 min incubation at 37°C is required for soft tissue digestion. If incomplete digestion is obtained, increase the reaction time accordingly with addition of fresh digestion medium.

5. Centrifuge the filtered solution using desktop centrifuge at $300\times g$ for 5 min and aspirate out the supernatant.
6. Add sterile RBC lysis solution and vortex gently. Incubate it for 2–3 min. Again gently vortex few times and centrifuge at $300\times g$ for 10 min. A white pellet should be observed following centrifugation. Repeat RBC lysis if necessary.
7. Re-suspend the pellet in 1 ml of fresh sorting medium and count the cells using a hemocytometer.

3.3. Depleting CD-31 and CD-45 Positive Cells

1. Re-suspend the pellet in 100 μ l of sorting media (PBS and 10% FBS).
2. Incubate the cell suspension with 10 μ l (for up to 10^7 cells) CD-45–PE conjugate antibody for 15 min at 4°C in dark. After incubation add 1–2 ml of sorting media and wash unbound antibody. Centrifuge at $300 \times g$ for 10 min. Aspirate the supernatant and re-suspend in 100 μ l of sorting media.
3. Incubate the cell suspension with 20 μ l (for up to 10^7 cells) of Anti-PE micro-beads for 15 min at 4°C in dark. After incubation add 1–2 ml of sorting media and wash unbound antibody. Centrifuge at $300 \times g$ for 10 min. Finally re-suspend the pellet in 500 μ l of sorting media.
4. Prepare the MACS column by adding 500 μ l of sorting media. Now add 500 μ l of cell suspension and allow cell suspension to flow through the magnetic columns by gravity. Collect the solution in a 2 ml centrifuge. Wash the column two to three times with 500 μ l sorting media (see Note 3).
5. Repeat the steps 2–4 using CD-31–PE Antibody and Anti-PE micro-beads (see Note 4).

3.4. Collecting Scal Positive Cells

1. After depletion of CD-31 and CD-45 positive cells, re-suspend the pellet in 100 μ l of sorting media (PBS and 10% FBS).
2. Incubate the cell suspension with SCA1-APC conjugate antibody for 15 min at 4°C in dark. After incubation add 1–2 ml of sorting media and wash unbound antibody. Centrifuging at $300\times g$ for 10 min. Aspirate the supernatant and re-suspend in 100 μ l of sorting media.
3. Incubate the cell suspension with 20 μ l (for up to 10^7 cells) Anti-APC micro-beads for 15 min at 4°C in dark. After incubation add 1–2 ml of sorting media and wash unbound antibody. Centrifuge at $300 \times g$ for 10 min. Aspirate the supernatant and re-suspend in 500 μ l of sorting media.

³It is important to allow the flow of cell suspension through the column by gravity. Do not flush the column with force. Please review the use of antibody concentration according to the manufacturer's protocol.

⁴Mouse lung suspension cells have higher number of CD-45 positive cells. Hence it is important to deplete these cells first. Based on the cell numbers obtained from three lungs, depletion of CD-45 and CD-31 could be performed in a single step. If there are more than 10^7 cells obtained from three mice lungs, it is important to perform depletion steps independently, to avoid the clogging of the column. Please review manufacturer's column sizes and cell number recommendations (Milteny Biotec, Auburn, CA, USA).

4. Prepare the MACS column by adding 500 µl of sorting media. Add 500 µl of cell suspension and allow the cell suspension to flow through the magnetic columns by gravity. Remove the MACS column from magnet holder and add 1 ml of sorting media to the column. Using a plunger, force the collected Sca1 positive cells in column into a 1.5 ml centrifuge tube (see Note 5).
5. Centrifuge the cell suspension, re-suspend the pellet in fresh multipotent lung stem cell (MLSC) media, and count the cells using a hemocytometer.

3.5. Plating Sca1 Positive Cells

1. Plate the Sca1 positive lung stem cells on 3-day-old inactivated mouse embryonic fibroblasts (iMEF) dishes. Change the MLSC media daily. Primary colonies should be observed in 7–15 (Fig. 2) (see Note 6).
2. Alternatively, Sca1 positive cells can also be plated on normal tissue culture dishes. Primary colonies may be observed in 20–25 days on tissue culture dishes.
3. Characterization studies could be conducted by flow cytometry (Fig. 3) or immunostaining (see Note 7).

4. Notes

¹It is important to dissolve lyophilized dispase enzyme in PBS ($\text{Ca}^{2+}/\text{Mg}^{2+}$ free), because these ions reduce the activity of the enzyme. Concentrations higher than 2.4 U/ml are not recommended.

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⁵The efficiency of MACS separation is about 70–80%. To further achieve a more homogenous population of Sca1 positive, and CD-45 and CD-31 negative cells, it is important to perform FACS sorting (BD FACSAria™ II High-Speed Cell Sorter). To reduce

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⁶In co-culture with iMEFs, we observe compact spindle-shaped cells (Fig. 2). Once the primary colonies are established these cells can be propagated directly on tissue culture plates without the need of iMEF. Enzymatic passage using 0.25% Trypsin is recommended.

⁷Additional characterization studies are important to establish these isolated cells as multipotent lung stem cells. Immuno-staining, flow cytometry, limiting dilution assays to assess colony forming ability, and differentiation into specific lineage helps us to categorize them as stem cells. Flow cytometry analysis on these isolated lung stem cells is shown in Fig. 3.

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Fig. 1. Schematic detailing the steps involved in isolation of multipotent lung stem cells. *Step 1:* Lung isolation, digestion, sieving tissue debris, Red Blood cell (RBC) lysis, and pelleting by centrifugation. *Step 2:* Separation of Sca1 (+ve), CD-31 (–ve), CD-45 (–ve) cells by magnetic-activated cell sorter (MACS). *Note:* Sca1-APC (*red*), CD-31-PE and CD-45-PE (*yellow*).

Fig. 2. Multipotent lung stem cells propagated on inactivated MEFs. Spindle-shaped cells show up on (a) day 5 and (b) day 8; while they start exhibiting stem cell morphologies of high nuclear–cytoplasmic ratios on (c) day 13 and (d) day 15.

Fig. 3. Flow cytometry analysis of Sca1-APC stained (*red*) and unstained (*blue*) isolated multipotent lung stem cells.