Video Article

Isolation & Characterization of Hoechst^{low} CD45^{negative} Mouse Lung Mesenchymal Stem Cells

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Abstract

Tissue resident mesenchymal stem cells (MSC) are important regulators of tissue repair or regeneration, fibrosis, inflammation, angiogenesis and tumor formation. Taken together these studies suggest that resident lung MSC play a role during pulmonary tissue homeostasis, injury and repair during diseases such as pulmonary fibrosis (PF) and arterial hypertension (PAH). Here we describe a technology to define a population of resident lung MSC. The definition of this population *in vivo* pulmonary tissue using a define set of markers facilitates the repeated isolation of a well-characterized stem cell population by flow cytometry and the study of a specific cell type and function.

Video Link

The video component of this article can be found at http://www.jove.com/video/3159/

Protocol

1. Lung Isolation

1. Sacrifice adult mice (8-10 weeks in age; 18-20 g; C57Bl6J) mice with an overdose of isoflurane followed by exsanguination using sterile technique. Profiles will vary slightly between strains.
2. Surgically remove the diaphragm, open up the chest cavity by cutting the ribcage laterally on each side of the mouse. Flush the blood from the lungs by perfusing the right heart ventricle gently using 3-5mls of phosphate buffered saline (PBS).
3. Dissect the lung lobes removing the trachea and large bronchi and place in a petri dish filled with Hanks buffered saline (HBSS). Following collection of all lung lobes forceps are used to place tissue onto the lid of the dish. Tissue is then minced into tiny pieces using disposable scalpels with enough liquid to keep them moist but not so much that they float and move.
4. Dissect a hindlimb of one of the mice and isolate the bone marrow to use as a staining control to set the flow cytometry gates. Stain the bone marrow (BM) as previously described [1].

2. Preparation of a Single Cell Suspension from Lung Tissue

1. Each lung is digested by using an optimized tissue weight to volume ratio of 0.2g: 5mls of pre-warmed 0.2% Worthington type 2 collagenase (Worthington Biochemicals, Lakewood, NJ; cat # LS004202) dissolved in sterile HBSS. The mixture is immersed in a 37°C water bath for 30 min [2,3].
2. Triturate sample well until lung tissue digest flows easily through a 10ml pipette (approximately 10 repetitions). Incubate for additional 15 min at 37°C to complete the tissue digests and ensures a more uniform single cell suspension.
3. Dilute the lung cell suspension with HBSS and triturate with a 5ml pipette to disperse residual tissue fragments.
4. Filter the suspension using a 70μM cell strainer to remove undigested tissue fragments. Sample can be divided into multiple conical tubes at this point to prevent overloading one cell strainer and decrease the time.
5. Pellet the cell suspension for 10 min at 1500 rpm and decant the supernatant.
6. Resuspend cell pellet gently using room temperature RBC lysis buffer (eBiosciences, San Diego, CA; cat # 00-4333-57) and incubate at RT for 5 min. Add an equal volume of HBSS to inactivate the lysis buffer and filter the cell suspension using a 40μM cell strainer to remove debris and cell aggregates.
7. Pipette 10μl of each sample to be stained and count cell numbers for both the lung and BM samples using a hemocytometer. Note: record the total volume of the cells.

8. Pellet the single cell suspension of lung cells by centrifugation at 1500 rpm for 10 min.
9. Resuspend both lung and BM cells at $1x10^6$ cells/ml in prewarmed (37°C) DMEM+ (Dulbecco's modified Eagle's medium, high glucose (Gibco, Carlsbad, CA; cat # 11965-092) containing 2% fetal bovine serum (FBS) and 10mM HEPES.
10. Prepare a 1mg/ml stock solution of Hoechst 33342 dye (Sigma Chemical Company, St.Louis, MO; cat # B2261) in water and filter sterilize [1,4]. The Hoechst dye may be stored as aliquots at -80°C.
11. Add Hoescht 33342 dye to a final concentration of 5µg/ml (a 200x dilution of a 1mg/ml stock) to the single cell suspension.
12. Mix the cells by gently inversion and incubate in 37°C water bath for exactly 90 minutes.

3. Staining and Preparation of the Lung Cell Suspension for Flow Cytometry Analysis

1. After 90 min all steps with Hoechst stained lung cells must be executed at 4°C or on ice to prevent dye efflux. Pellet the cells by centrifugation at 1500 rpm for 10 min at 4°C and resuspend the cells in ice-cold staining buffer (PBS+2%FBS).
2. Resuspend cells at a concentration of no more than $1x10^7$ cells/tube using 300-600µl of PBS containing 2% FBS and 10mM HEPES.
3. Add adequately tittered CD45 antibody (typically 1:200 dilution of CD45-APC (BD Pharmingen, San Diego, CA; cat # 559864) to the lung and BM samples. Incubate on ice for 10-15 min. Including an unstained control during the experimental procedure is useful to set the analysis gates.
4. Pellet cells at 4°C for 5 min at 1500 rpm. Decant the supernatant and resuspend cells in 500 µl of chilled staining buffer (PBS+2%FBS). Transfer to prechilled snap cap polypropylene tubes (Fisherbrand, Schaumburg, IL; cat # 14-956-1D).
5. Add propidium iodide (PI, 200µg/ml stock in PBS) to the samples for a final concentration of 2µg/ml to exclude dead cells. PI must be used in combination with the Hoechst 33342 dye to achieve the correct fluorescence profile upon flow cytometric analysis.
6. Store tubes on ice, protected from light until flow cytometry analysis.

4. Flow Cytometry Analysis to Define and Isolate a Lung Mesenchymal Stem Cell (lung MSC) Population using Hoechst dye Efflux to Detect a Side Population (SP)

1. This assay has the following instrument requirements:
   - 488 nm and UV (350-355 nm) lasers
   - 2 detectors on the UV laser path with blue (450/65) and red (675/50) filters.
   - The UV red detector must have high sensitivity for the red signal. This may be a problem with older cell sorters.
   - Chiller unit to maintain the sample at 5-10°C.

2. Align lasers and set up for cell sorting following the manufacturer's protocol.
3. Collect the red (x-axis) and blue (y-axis) Hoechst signals using a linear scale.
4. Adjust the photomultiplier tube voltages to place the G0/G1 peak in the upper right of center on the plot to allow for adequate display of the trailing side population. A portion of the S-phase and the entire G2 of the cell cycle may be off-scale on the upper right of the plot. The blue signal is relatively bright while the red signal is dim. Typical MoFlo XDP (Beckman Coulter, Miami, FL) voltages are 425 for the blue and 650 for the red.
5. Dead cells are gated out using PI. The standard PI path (excitation 488nm, emission 630nm) can be used. Alternatively, PI is also excited by the UV laser and the dead cell population lies off-scale on the right side of the side population (SP) plot. This alleviates the need to compensate the PI signal from any other fluorochromes such as FITC and PE. The original Goodell method followed the latter procedures [5-7].
6. Maintain a relatively low pressure differential during analysis and sorting to ensure tight resolution of the side population.
7. The SP appears as a side arm apart from the main population of lung cells. This population is termed $Hoechst^{low}$.
8. The $Hoechst^{low}$/SP is analyzed to separate the CD45 positive and negative populations using a histogram [2,3] (Figure 1).
9. Following selection and gating of the $Hoechst^{low}CD45^{neg}$ lung MSC population the cells may be collected by sorting as either a mixed population into a tube or single cells to isolate clones into a 96-well plate [3].
10. For plate sorting the sort stream deflection is adjusted to 25% to create a more vertical sort stream than that usually used for tube sorting.
11. Aim the sort stream by sending a test stream pulse onto the upper left and then the lower right well of a 96-well plate.
12. Set the sorter software plate map to deposit the desired number of cells into each well.
13. To isolate single cell clones a single lung MSC is sorted into the well of a 96-well plate into 150µl of culture media (α-MEM supplemented with 20% FBS). Following the sort an additional 100µl of medium is added.

5. Isolation, Culture and Characterization of lung MSC and Clones

1. Culture and expansion of mixed lung MSC population and single cell clones following sorting is identical using standard culture conditions (37°C, 5% CO_2 and >95% humidity). Cells are allowed to adhere following isolation for 16 hours. Media is replaced every 48 hours (α-MEM supplemented with 20% FBS). When the cells begin to proliferate more rapidly and reach between 75-80% confluence they are passaged (Figure 2).
2. Cells are rinsed with prewarmed HBSS and incubated with 0.5% trypsin/EDTA (Cellgro, Manassas, VA; cat # 25-053-CI) at 37°C for less than 2 min. Cells are monitored using an inverted scope to confirm the appearance of a cell suspension.
3. Lung MSC are then divided at ratios of 1:2 or 1:3 depending on the current proliferation rate of the culture.
4. The ability of lung MSC to differentiate into traditional mesenchymal lineages of osteoblast, adipocyte and chondrocyte and their cell surface expression of accepted mesenchymal markers is evaluated for each cell preparation. Cytogenetic banding analysis is also performed to confirm the absence of gross chromosomal abnormalities [2,3,8-11].

6. Enumeration of lung MSC using a Colony Forming Assay (CFU-F)

1. Dilute cells in Complete MesenCult medium (Stem Cell Technologies, Vancouver, BC) to a final concentration of $1x10^6$ cells/ml.

2. Perform serial dilutions to achieve final concentrations in a 100mm dish of $6x10^4$ cells, $3x10^4$ cells, $1.5 x10^4$ cells and $0.75 x10^4$ cells in 10ml of media. Analyses are performed in duplicate or triplicate for each cell concentration and may be scaled for smaller surface areas.
3. Culture cells for 10 days under standard conditions. On day 10 cells are rinsed with PBS and fixed using 100% methanol for 5 minutes at room temperature.
4. Detect the CFU-F colonies by staining with 0.4% w/v Giemsa staining solution (Sigma Chemical Co, St. Louis, MO) diluted 1:20 with deionized water. Allow samples to air-dry and quantify the number of colonies containing greater than 25 cells per colony (Figure 3).

7. Analysis of the Immunomodulatory Properties of lung MSC on T-cell Proliferation and Apoptosis

1. $6.25x10^4$ lung MSC cells per well are plated in 96-well plates and allowed to stand overnight [2].
2. $CD4^+$ cells from spleens of t-cell receptor (TCR) transgenic mice, OT-II with T cells that recognize the ovalbumin 323-339 peptide, are purified by depleting $CD8^+$, $CD19^+$, MHC-II^+ and $CD25^+$ cells with a Miltenyi AutoMACS cell sorter (Miltenyi, Auburn, CA). Antigen presenting cells (APC) are isolated by positively sorting MHC-II^+ cells [12].
3. APC are fixed in 4% paraformaldehyde, washed 3 times in PBS/5% BSA/2mM EDTA, and loaded with 0.5µg/ml or 5µg/ml OVA323 peptide.
4. Simulated, growth arrested APC are added to the lung MSC in 96-well plates.
5. Greater than 95% pure CD4+ $1x10^5$ cells are labeled with 5µM Carboxyfluorescein succinimidyl ester (CFSE; Sigma, St.Louis MO) in PBS and incubated for 5 min in the dark, washed with PBS-5% BSA, and then added to the APC and lung MSC cells in DMEM 5% FBS media. Cells are then incubated for 96 hours.
6. T-cells are then stained with anti anti-CD40 (Cy5); CD4 (APC-Cy7); CD8 (ViolBlue); Vbeta 5 (PE) and assayed using a Miltenyi MacsQuant 7 channel flow cytometer (Miltenyi, Aurburn, CA) and FloJo analysis software (Treestar, Ashland, OR). Proliferation is measured as the decrease in mean fluorescent intensity of CFSE compared to the background, T cells labeled with CFSE and not exposed to APC (Figure 4). T-cells are counted at this time and viability assessed with trypan blue exclusion.

8. Representative Results:

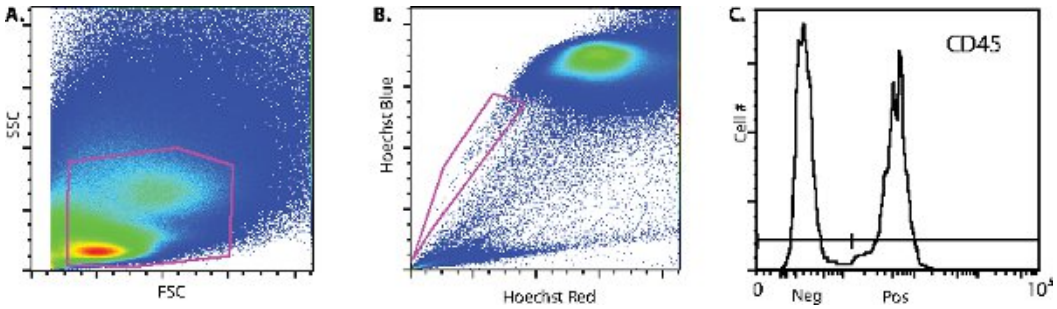

Figure 1. Flow cytometric analysis and Definition of Lung MSC. Single cell suspensions of lung tissue digest are stained with Hoechst 33342 and analyzed by flow cytometry to detect differences in **A**. forward scatter (FSC) and side scatter (SSC) and **B**. Hoechst blue and red fluorescence. The presence of a side population (SP) is visible in the gate. The $Hoechst^{low}$ SP is then analyzed to separate the **C**. $CD45^{negative}$ population of lung MSC. The CD45 positive to negative ratios of lung SP are typically 70:30 / 60:40.

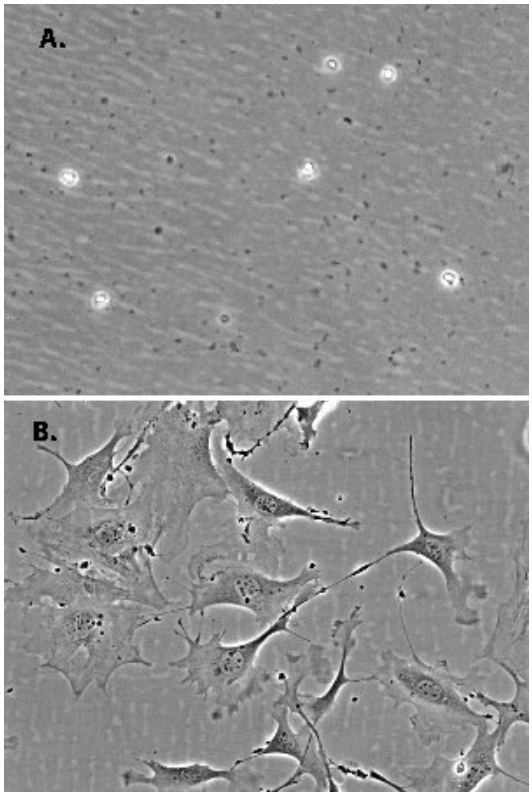

Figure 2. Isolation and Culture of Lung MSC. Following collection of the lung MSC by cell sorting, the cells are plated in 30mm dishes using α-MEM supplemented with 20% FBS. **A**. Freshly sorted cells appear small, round and bright. **B**. After approximately 2-3 weeks colonies with a mesenchymal phenotype become evident and proliferation is more obvious.

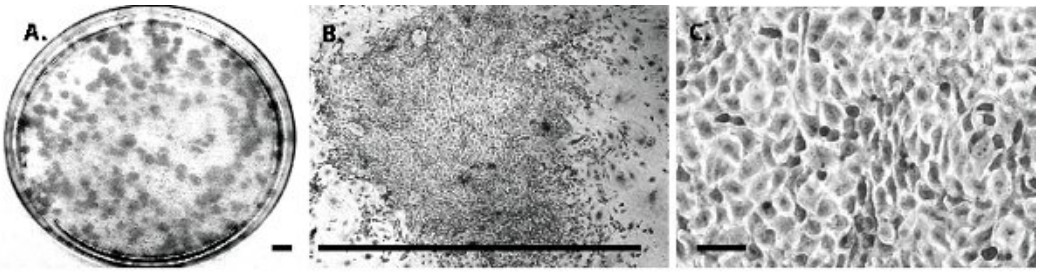

Figure 3. Enumeration of MSC by Colony Forming-Fibroblast Assay. Expanded lung MSC are plated per the described protocol for the CFU-F assay. **A.** After 10 days and Giemsa stain colonies are evident. **B.** Colonies are large and comprised of a few hundred cells. **C.** The cells display a mesenchymal phenotype. A & B, Scale bar =0.5 cm; C Scale bar = 0.14 cm.

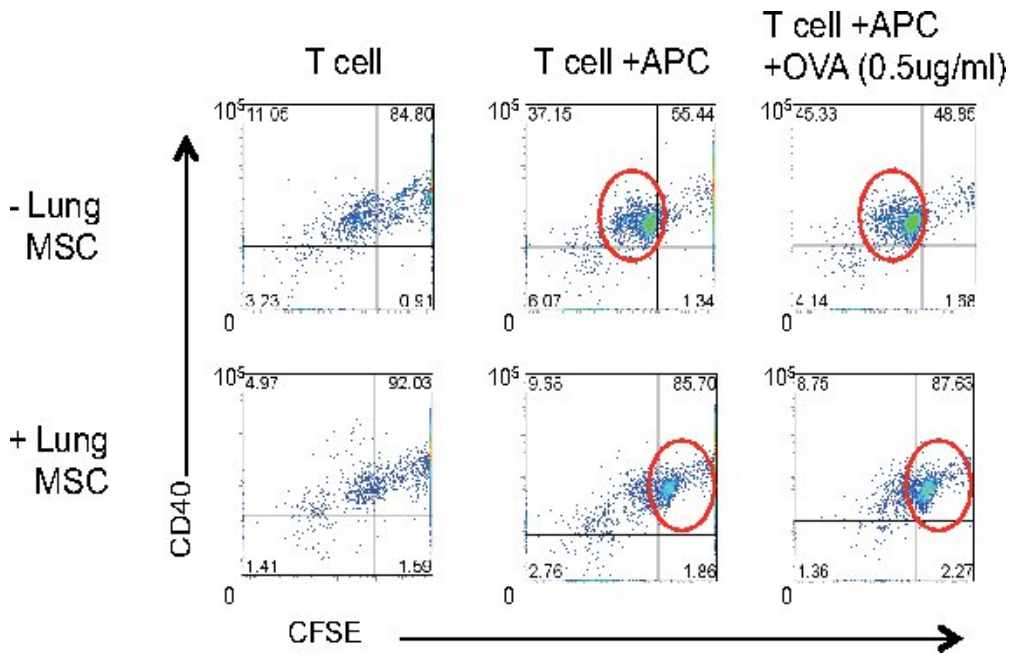

Figure 4. CFSE Based T cell Proliferation Assay. In the absence of lung MSC and presence of antigen presenting cells (APC) +/- ovalbumin (OVA) CD40^{positive} effector T cell demonstrate a decrease in CFSE intensity which indicates proliferation (red circle). CFSE fluorescence intensity of the T-cell membrane decreases stoichiometrically with every cell division ie. with every division. In the presence of lung MSC, APC +/- OVA, CD40^{positive} effector T cell demonstrate no significant change in CFSE intensity which indicates a lack of proliferation (red circle).

Discussion

We have adapted a method initially used to identify BM hematopoietic cells to isolate a specific population of resident lung MSC. Due to the reproducibility of isolation these cells were then well characterized as MSC. Their origin has been defined as resident in the adult mouse lung (as opposed to BM derived) and a phenotypic and molecular profile documented [2]. The ability to repeatedly isolate this characterized population allows the further study of the biological importance and role of the lung MSC during tissue homeostasis and disease. The recent definition of this population *in vivo* in both murine and human pulmonary tissue facilitates the development of a therapeutic strategy directed at the rescue of endogenous cells to facilitate lung repair during injury and disease.

Disclosures

No conflicts of interest declared.

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