



## Isolation of Stromal Stem Cells from Human Adipose Tissue

### Abstract

The stromal compartment of mesenchymal tissues is thought to harbor stem cells that display extensive proliferative capacity and multilineage potential. Stromal stem cells offer a potentially large therapeutic potential in the field of regenerative medicine. Adipose tissue contains a large number of stromal stem cells, is relatively easy to obtain in large quantities and thus constitutes a very convenient source of stromal stem cells. Importantly, the number of stem cells obtained is compatible with extensive analyses of the cells in an uncultured, freshly isolated, form. This chapter describes procedures for isolating millions of highly purified stromal stem cells from human adipose tissue and methods of establishing polyclonal and monoclonal cultures of adipose tissue-derived stem cells.

### 1. Introduction

The stromal compartment of mesenchymal tissues is thought to harbor stem cells that display extensive proliferative capacity and multilineage potential. Often called mesenchymal stem cells or stromal stem cells, these cells have been isolated from several mesodermal tissues including bone marrow (1), muscle (2), perichondrium (3) and adipose tissue (4-6). Stromal stem cells isolated from various mesodermal tissues share key characteristics, including ability to adhere to plastic to form fibroblastic-like colonies (called CFU-F), extensive proliferative capacity, ability to differentiate into several mesodermal lineages including bone, muscle, cartilage and fat, and express several common cell surface antigens. Recent evidence suggests that these cells can also form non-mesodermal tissues including neuron-like cells (5,7,8). Considering these characteristics, stromal stem cells may potentially be useful in the field of regenerative medicine.

To date, extensive characterization of stromal stem cells has largely been limited to cultured cells. This is because stromal stem cells are rare and therefore are difficult to isolate in numbers compatible with extensive analyses in an uncultured form. For example, in human bone marrow, only 0.01-0.001% of nucleated cells form CFU-F (1). Until now, their ability to adhere to plastic and proliferate has been used as the conventional isolation method. Therefore, methods to isolate uncultured stromal stem cells in significant numbers are needed to study the biology of these cells.

Adipose tissue contains a large number of stromal stem cells (4). Because it is easy to obtain in large quantities, adipose tissue constitutes an ideal source of uncultured stromal stem cells. We have recently isolated and extensively characterized uncultured human stem cells from the stroma of adipose tissue (9). Cells with a CD45<sup>-</sup>CD31<sup>-</sup>CD34<sup>+</sup>CD105<sup>+</sup> surface phenotype freshly isolated from



adipose tissue form CFU-F, proliferate and can be differentiated towards several lineages including osteogenic, chondrogenic, adipogenic and neurogenic. Here, we describe methods for isolating stromal stem cells from human adipose tissue.

## 2. Materials

### 2.1. Materials required for isolation of adipose tissue stromal stem cells

1. Lipoaspirate, commonly obtained from a clinic. The lipoaspirate can be kept at room temperature overnight before stem cell isolation.
2. Hanks Balanced Salt Solution (HBSS, without phenol red; cat. no. 14025-050, Gibco-BRL).
3. Fetal Bovine Serum (FBS; cat. no. 10099-141, Gibco-BRL). Heat-inactivate at 55°C for 30 min. Aliquot and store frozen. Thaw at 4°C.
4. Collagenase A type I (cat. no. C-0130, Sigma-Aldrich).
5. Antibiotics: penicillin-streptomycin mix (100x solution; cat. no. 15140-122, Gibco-BRL).
6. Fungizone (100x solution; cat. no. Gibco-BRL).
7. Histopaque-1077 (cat. no. 1077-1, Sigma-Aldrich).
8. Red blood cell lysis buffer: 2.06 g/L Tris Base, pH 7.2, 7.49 g/L NH<sub>4</sub>Cl. Sterile filter after preparation. Can be kept at room temperature for 4 weeks.
9. Bench media: HBSS with 2% FBS and antibiotics.
10. Falcon 100 µm cell strainers (cat. no. 352360, Becton Dickinson).
11. Falcon 40 µm cell strainers (cat. no. 352360, Becton Dickinson).
12. MACS anti-CD45 FITC-conjugated anti-human monoclonal antibody (cat. no. 130-080-202, Miltenyi Biotec).
13. Anti-CD31 FITC-conjugated mouse anti-human monoclonal antibody (cat. no. MCA 1738F, Serotec).
14. MACS anti-FITC Microbeads (cat. no. 130-048-701, Miltenyi Biotec).
15. Column buffer: phosphate buffered saline (PBS; pH 7.2), 0.5% FBS, 2 mM EDTA.
16. MACS LD columns (cat. no. 130-042-901, Miltenyi Biotec).
17. MidiMACS separation unit (cat. no. 130-048-701, Miltenyi Biotec).
18. 50 ml and 15 ml plastic conical tubes (Corning).
19. 162 cm<sup>2</sup> cell culture flasks (Corning).
20. Empty 500 ml sterile medium bottles (Gibco).
21. 50 ml, 25 ml and 10 ml disposable plastic pipettes (Corning).
22. A swing-out centrifuge with buckets for 50 ml and 15 ml tubes.
23. Fluorescence microscope equipped with a blue light filter for FITC viewing.



## **2.2. Additional materials for the culture of adipose tissue stromal stem cells**

1. Cell incubator set at 100% humidity, 37°C and 5% CO<sub>2</sub> in air.
2. DMEM:F12 (1:1; cat. no. 31331-028, Gibco-BRL).
3. 25 cm<sup>2</sup> cell culture flasks (Corning)

## **3. Methods**

### **3.1. Collection and storage of lipoaspirate**

We have found that stromal stem cells can be isolated from lipoaspirate collected from several regions of the body including hip, thigh and abdominal regions. At least 300 ml of lipoaspirate should be collected into a sterile container to isolate uncultured stem cells in significant numbers (million-range). Using the technique described below, we routinely isolate up to 10<sup>7</sup> adipose stromal stem cells with greater than 95% purity from 300 ml of lipoaspirate. However, yields can vary widely between patients. The actual adipose tissue volume used for digestion after the washing steps is usually about two thirds of the collected lipoaspirate volume. If overnight storage of lipoaspirate is required, we recommend storage at room temperature. We have found overnight storage at 4°C reduces the enzyme digestibility of adipose tissue from the lipoaspirate.

### **3.2. Separation of the stromal vascular fraction**

#### **3.2.1. Lipoaspirate washing**

It is necessary to wash the lipoaspirate extensively to remove the majority of the erythrocytes and leukocytes. The following procedures should be performed under aseptic conditions.

1. Place a maximum of 300 ml of lipoaspirate into a used sterile medium bottle.
2. Allow the adipose tissue to settle above the blood fraction.
3. Remove the blood using a sterile 25 ml pipette.
4. Add an equivalent volume of HBSS with antibiotics and fungizone and firmly tighten the lid.
5. Shake vigorously for 5-10 seconds.
6. Place the bottle on the bench and allow the adipose tissue to float above the HBSS. This will take 1-5 min depending on the sample.
7. Carefully remove the HBSS using a 50 ml pipette.
8. Repeat the above washing procedure (steps 4 to 7) three times.
9. Medium from the final wash should be clear. If it is still red, wash again by repeating steps 4-7.



### 3.2.2. Collagenase digestion

Dispersion of adipose tissue is achieved by collagenase digestion. Collagenase has the advantage over other tissue digestive enzymes that it can efficiently disperse adipose tissue while maintaining high cell viability.

1. Make up collagenase solution just prior to digestion. The final volume required is half that of the washed adipose tissue volume. Add powdered collagenase to HBSS at a final concentration of 0.2%. We dissolve the required amount of collagenase into 40 ml of HBSS, then filter sterilize into the remaining working volume. Add antibiotics and fungizone.
2. Add the washed adipose tissue to large cell culture flasks (100 ml per 162 cm<sup>2</sup> flask).
3. Add collagenase solution.
4. Resuspend the adipose tissue by shaking the flasks vigorously for 5-10 seconds.
5. Incubate at 37°C on a shaker for 1 to 2 h, manually shaking the flasks vigorously for 5-10 seconds every 15 min.
6. During the digestion, prepare Histopaque gradients by dispensing 15 ml of Histopaque-1077 into 50 ml tubes. Two gradients are required for each 100 ml of washed adipose tissue. The gradients must be equilibrated at room temperature before use. Prepare 200 ml of washing medium consisting of HBSS containing 2% FBS, antibiotics and fungizone.
7. On completion of the digestion period, the digested adipose tissue should have a “soup like” consistency.
8. Add FBS to a final concentration of 10% to stop collagenase activity.

### 3.2.3. Separation of the stromal-vascular fraction

After digestion, the ability of lipid-filled adipocytes to float is used to separate them from the stromal vascular fraction (SVF).

1. Dispense the collagenase-digested tissue into 50 ml tubes. Avoid dispensing undigested tissue. Centrifuge at room temperature at 400x g for 10 min.
2. After centrifugation, use a 50 ml pipette to aspirate the floating adipocytes, lipids and the digestion medium. Leave the SVF pellet in the tube.



### **3.3. Separation of stromal stem cells from the SVF**

The SVF predominantly contains erythrocytes, leukocytes, endothelial cells and stromal stem cells. Erythrocytes are removed first, using the red blood cell lysis buffer.

#### *3.3.1. Removal of erythrocytes*

1. Resuspend thoroughly each SVF pellet in 20 ml of cell lysis buffer at room temperature.
2. Incubate at room temperature for 10 min.
3. Centrifuge at 300x *g* for 10 min and aspirate the cell lysis buffer.

#### *3.3.2. Removal of cell clumps and remaining undigested tissue*

It is essential to obtain a cell suspension free from undigested tissue and cell clumps, to effectively separate stromal stem cells from other cell types using antibody-conjugated magnetic beads. The strategies used to achieve this are separation of gross undigested tissue using gravity, straining of cells and gradient separation.

1. Resuspend SVF pellets thoroughly in 2 ml of washing medium using a 1 ml pipette.
2. Pipet the cells up and down several times to reduce clumping.
3. Pool the pellets into two 50 ml tubes.
4. Allow undigested tissue clumps to settle by gravity for ~1 min.
9. Aspirate and pass the suspended cells through 100  $\mu\text{m}$  cell strainers.
10. Pass the filtered cells through 40  $\mu\text{m}$  cell strainers.
11. Add extra washing buffer so that the final volume is equivalent to that of the gradients (i.e., for 4 gradients, the volume of cells in washing buffer should be 60 ml).
12. Hold each tube containing Histopaque at a 45 degree angle and carefully add the cells by running the suspension along the inside wall of the tube at a flow rate of ~1 ml per second. Careful layering of cells onto the gradients is essential for successful cell separation.
13. Centrifuge gradients at exactly 400x *g* for 30 min.
14. Carefully remove the medium (~10 ml) above the white band of cells found at the gradient interface and discard.
15. Carefully remove the white band of cells (~5 ml) by careful aspiration and place into a new 50 ml tube.
16. Add an equivalent volume of washing medium and centrifuge at 300x *g* for 10 min using a low brake setting.
17. Aspirate and resuspend each pellet in 25 ml of washing medium.
18. Centrifuge at 300x *g* for 10 min using a low brake setting.



### 3.3.3. Separation of stromal stem cells from endothelial cells and leukocytes by magnetic cell sorting

Stromal stem cells are separated from remaining cells using magnetic cell sorting. Unwanted endothelial (CD31<sup>+</sup>) and leukocytes (CD45<sup>+</sup>) are magnetically labeled and eliminated from the cell suspension when applied to a column under a magnetic field. Magnetically labeled cells are retained in the column, while unlabeled stem cells with a CD45<sup>-</sup>CD31<sup>-</sup> phenotype pass through the column and are collected. To this end, CD31<sup>+</sup> and CD45<sup>+</sup> cells are labeled with FITC-conjugated anti-CD31 and anti-CD45 antibodies. The stained cells are magnetically labeled by the addition of anti-FITC-conjugated magnetic microbeads. This approach presents the advantage that cell purity after separation can be assessed by flow cytometry or fluorescence microscopy. For the following steps, use cold buffer and work on ice to reduce cell clumping.

1. Resuspend and pool the sedimented pellets in 10 ml of column buffer (PBS containing 2 mM EDTA and 0.5% BSA).
2. Remove all remaining cell clumps by passing the suspension through a 40 µm cell strainer.
3. Perform a cell count.
4. Transfer cells to a 15 ml tube and centrifuge at 300x g for 10 min at 4°C using a low brake setting.
5. Resuspend the cell pellet in column buffer and label with anti-CD31 FITC-conjugated and anti-CD45 FITC-conjugated antibodies according to the manufacturer's recommendations. We resuspend cells in 100 µl of column buffer and add 10 µl of each antibody per 10<sup>7</sup> cells.
6. Mix well and incubate for 15 min in the dark at 4°C (resuspend the cells after 7 min of incubation).
7. Wash the cells to remove unbound antibody by adding 2 ml of column buffer per 10<sup>7</sup> cells. Centrifuge at 300x g for 10 min at 4°C using a low brake setting.
8. Aspirate the supernatant completely and resuspend the cell pellet in 90 µl of column buffer per 10<sup>7</sup> cells. Add 10 µl of MACS anti-FITC magnetic microbeads per 10<sup>7</sup> cells.
9. Mix well and incubate for 15 min at 4°C (resuspend the cells after 7 min of incubation).
10. Wash the cells to remove unbound beads by adding 2 ml of column buffer per 10<sup>7</sup> cells. Centrifuge at 300x g for 10 min at 4°C using a low brake setting.
11. Aspirate the supernatant completely and resuspend the cell pellet in 500 µl of column buffer.
12. For magnetic cell separation, we use the MACS LD column specifically designed for the depletion of unwanted cells. Place a MACS LD column onto the MidiMACS separation unit or onto a compatible unit.
13. Prepare the column by washing with 2 ml of column buffer.



14. Apply the cell suspension to the column and collect the flow-through unlabeled cells in a 15 ml tube.
15. Wash unlabeled cells through the column by twice adding 1 ml of column buffer. Collect the total effluent.
16. Check for stem cell purity as described in **Subheading 3.3.4**.
17. If higher purity is required, centrifuge the collected cells at 300x g for 10 min at 4°C using a low brake setting and repeat steps 11-17.
18. Perform a cell count.
19. Centrifuge at 300x g for 10 min at 4°C using a low brake setting.
20. Use the cells as required or freeze the cells according to standard protocols.

#### *3.3.4. Assessment of stem cell purity*

The success of obtaining pure stromal stem cell samples of high purity varies between donors for unclear reasons. It is therefore important to assess the purity of the sample using a fluorescence-based assay. We find that fluorescence microscopy is sufficient to evaluate purity. A more accurate assessment can be made by flow cytometry; however, this assay requires many more cells.

1. Place 5  $\mu$ l of the collected cell fraction onto a glass slide.
2. View under white light. Stromal stem cells have an evenly round phenotype (See **Figure 1A**), while endothelial cells have an irregular shape (See **Figure 1B**).
3. Observe the samples under epifluorescence.
4. Determine the percentage of fluorescent cells under 5 fields of view (See **Figures 2A** and **2B**).  
The average represents the percentage of contamination of non stem cells in the sample.

#### **3.4. Culture of stromal stem cells**

Culture can also be used to further validate the successful isolation of stromal stem cells from adipose tissue using the above procedure. Stromal stem cells, when cultured, adhere to plastic and acquire a fibroblastic-like morphology. It may take several days before all adherent cells change their morphology. We find that approximately 50% of cells isolated as above will plate under the correct culture conditions. However, plating efficiency can vary substantially between donors. To encourage adherence, we plate isolated stem cells in medium containing 50% FBS in a volume sufficient to smear the medium across the surface of a cell culture flask.



#### 3.4.1. Validation of stem cell isolation by cell culture

1. Resuspend  $10^5$  freshly isolated stromal stem cells in 2 ml of DMEM:F12 medium containing 50% FBS, antibiotics and fungizone.
2. Add the cell suspension to a 25 cm<sup>2</sup> cell culture flask.
3. Smear the cell suspension across the entire surface by rocking the flask.
4. Incubate in a humidified incubator at 37°C, 5% CO<sub>2</sub>.
5. Observe the cells daily using an inverted phase contrast microscope.
6. It usually takes several days before those cells which form a fibroblastic morphology start dividing.
7. Estimate the percentage of cells which adhere to the plastic surface (See **Figure 3A**) and form a fibroblastic-like morphology after 7 days of culture (See **Figure 3B**).

#### 3.4.2. Generation of a stable stromal stem cell line

Generation of stable adipose stem cell lines is required to evaluate their differentiation capacity and proliferative ability. We have kept lines of stem cells generated by the above isolation method for longer than 6 months without loss due to senescence (See **Note 1**).

1. After at least 7 days of culture, replace the medium with DMEM:F12 containing antibiotics, 20% FBS and no fungizone.
2. Sub-culture cells using standard methods of trypsinization after a further week of culture to form a stable cell line (See **Figure 4**).
3. Split the cells weekly at a ratio of 1:3.

#### 4. Notes

1. To evaluate the “stemness” of adipose stem cell lines established, we recommend differentiating the cells towards various mesodermal lineages according to Zuk et al. (**4**). For adipogenic differentiation, incubate cultures in DMEM:F12 medium containing 10% FBS, 0.5 μM 1-methyl-3 isobutylxanthine, 1 μM dexamethasone, 10 μg/ml insulin and 100 μM indomethacin for 3 weeks. Change the medium every 4 days. To visualize lipid droplets, fix the cells with 4% formalin and stain with Oil-Red O (See **Figure 5A**). For osteogenic differentiation, incubate the cells in DMEM:F12 medium containing 10% FBS, 100 nM dexamethasone, 10 mM β-glycerophosphate and 0.05 mM L-ascorbic acid-2-phosphate for 3 weeks. Change the medium every 4 days. Mineralization of the extracellular matrix is visualized by staining with Alizarin Red (See **Figure 5B**).

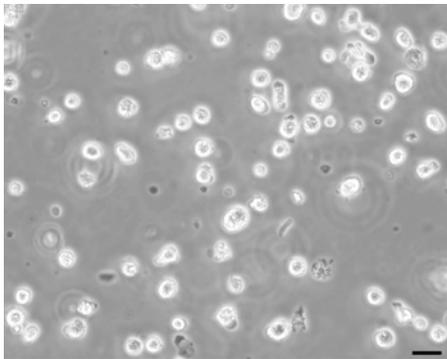


## 5. References

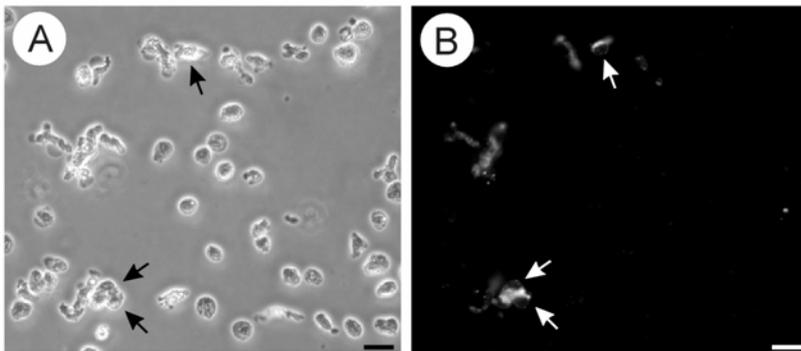
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## Figures

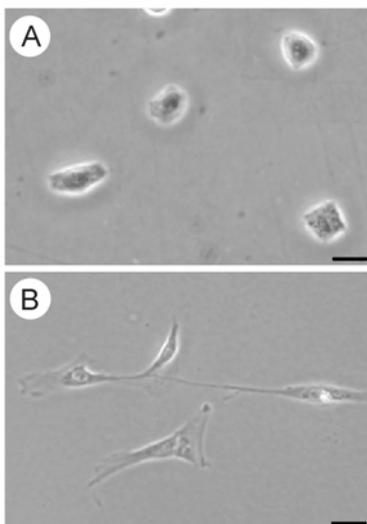


**Fig. 1.** Stromal stem cells purified from collagenase-digested and strained liposuction material. The cells have an even round phenotype. Bar, 20  $\mu\text{m}$ .

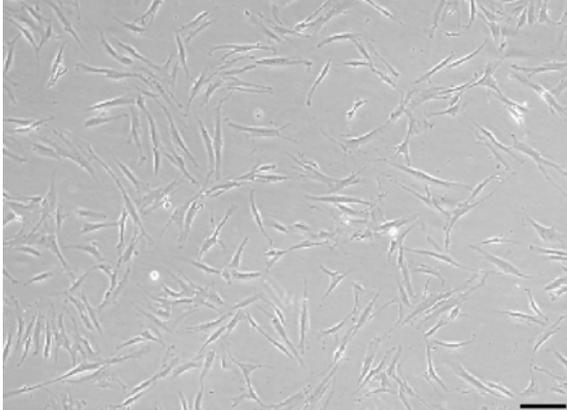


**Fig. 2.** Identification of contaminating  $\text{CD45}^+$  and  $\text{CD31}^+$  cells among stem cells isolated from adipose tissue. Cells recovered from liposuction material were labeled using anti- $\text{CD31}$  FITC- and anti- $\text{CD45}$  FITC-conjugated antibodies prior to purification of  $\text{CD45}^-$  and  $\text{CD31}^-$  cells by negative selection. Unwanted endothelial cells ( $\text{CD31}^+$ ) and leukocytes ( $\text{CD45}^+$ ) were

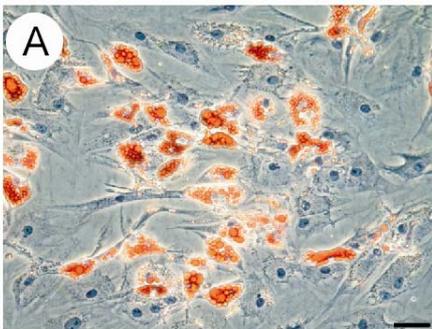
eliminated from the cell suspension. As a quality control assessment, fluorescence microscopy examination of the flow-through cells enables the identification of a low proportion of contaminating  $\text{CD45}^+\text{CD31}^+$  cells (arrows). Bars, 20  $\mu\text{m}$ .



**Fig. 3.** Morphology of isolated and seeded stem cells derived from adipose tissue. (A) Cells adhere to the plastic surface and (B) after 7 days of culture, acquire a fibroblastic-like morphology. Bars, 20  $\mu\text{m}$ .



**Fig. 4.** Monoclonal culture derived from a single adipose tissue-derived stem cell. Bar, 50  $\mu\text{m}$ .



**Fig. 5.** In vitro differentiation of cultured human adipose tissue-derived stem cells towards (A) the adipogenic pathway and (B) the osteogenic pathway. (A) To visualize intracellular lipid droplets, cells were fixed and stained with Oil Red-O. (B) Mineralization of the extracellular matrix was visualized by staining with Alizarin Red. Bars, 50  $\mu\text{m}$ .

