

Chapter 2

Isolation of Human Adipose-Derived Stem Cells from Lipoaspirates

Gang Yu, Z. Elizabeth Floyd, Xiyang Wu, Yuan-Di C. Halvorsen, and Jeffrey M. Gimble

Abstract

Adipose tissue is as an abundant and accessible source of stem cells with multipotent properties suitable for tissue engineering and regenerative medical applications. Here, we describe methods from our own laboratory and the literature for the isolation and expansion of adipose-derived stem cells (ASCs). We present a large-scale procedure suitable for processing >100-ml volumes of lipoaspirate tissue specimens by collagenase digestion and a related procedure suitable for processing adipose tissue aspirates without digestion.

Key words: Adipose-derived stem cells (ASCs), Biopsy, Collagenase, Expansion, Human, Isolation, Lipoaspirate, Mesenchymal stem cells (MSCs), Stromal vascular fraction (SVF)

1. Introduction

Mesenchymal stromal/stem cells (MSC) were initially described in bone marrow and have been found subsequently in multiple tissues, including subcutaneous adipose tissue (1–4). Although adipose-derived stromal cells had been termed “pre-adipocytes” (2, 3), multiple independent investigators have demonstrated that they are multipotent, with chondrogenic, neuronal-like, and osteogenic differentiation capability (5–8). Consequently, they have now been identified as adipose-derived stromal/stem cells or ASCs (9). This protocol, which includes information presented in earlier volumes of this series (10), describes the isolation of primary in vitro cultures of ASCs from human adipose tissue.

2. Materials

2.1. Tissue

Subcutaneous adipose tissue samples obtained from liposuction aspirates (see Note 1).

2.2. Supplies

1. 200-ml plastic centrifugation bottles.
2. 0.2- μ m filter units.
3. 50-ml conical tubes.
4. 2-ml tubes.
5. Scissors.
6. Hemocytometer.
7. Freezing apparatus (alcohol container).
8. Fluorochrome-conjugated monoclonal antibodies against stromal, hematopoietic, endothelial, pericytic, and related cell surface antigens.

2.3. Equipment

1. Inverted microscope – Nikon Eclipse TS100 with Epi-Fluorescence Attachment (Mercury Lamp Illuminator model name: C-SHG) and equipped with a camera photometric cool-snap.
2. MetaMorph imaging software.
3. Shaking water bath.
4. Centrifuge.
5. Biosafety hood.
6. CO₂ Incubator.

2.4. Media Stock Solution (see Note 2)

All the media solutions are filtered through a 0.2- μ m filter unit.

1. *Collagenase solution*: Weigh out 0.1 g of type I collagenase and 1 g of powdered bovine serum albumin (BSA, fraction V). Dissolve these in 100 ml of phosphate-buffered saline (PBS) supplemented with 2 mM calcium chloride (CaCl₂). After sterile filtration, warm the solution to 37°C. This solution should be used with 1 h of its preparation.
2. *Erythrocyte lysis buffer*: Using sterile, distilled water, prepare 155 mM ammonium chloride (NH₄Cl), 10 mM potassium carbonate (KCO₃), and 0.1 mM EDTA. This solution should be used within 24 h of its preparation.
3. *Stromal medium*: To 500 ml of DMEM/Ham's F-12 medium, add 55 ml of fetal bovine serum (10%) and 5.6 ml of antibiotic (penicillin/streptomycin)/antimycotic (amphotericin) 100 \times stock solution. This solution should be used within 4 weeks of its preparation. All fetal bovine serum

should be prescreened prior to purchase for its ability to support both cell proliferation and adipocyte differentiation.

4. *Differentiation medium*: In advance, prepare and aliquot the following stock solutions and store frozen at -20°C until required.
 - (a) A 66-mM stock solution of biotin (2,000-fold concentration) dissolved in 1 N sodium hydroxide.
 - (b) A 34-mM stock solution D-pantothenate (2,000-fold concentration) dissolved in water.
 - (c) A 1-mM dexamethasone solution (1,000-fold concentration) dissolved in water or ethanol, depending on its formulation.
 - (d) A 250-mM stock solution of methylisobutylxanthine (1,000-fold concentration) dissolved in dimethyl sulfoxide.
 - (e) A 200- μM stock solution of human insulin (2,000-fold concentration) dissolved in PBS.
 - (f) A 5-mM stock solution of rosiglitazone or equivalent PPAR γ agonist dissolved in dimethyl sulfoxide. Use this solution within 1 month of its preparation for optimal results.

Prepare the Differentiation medium containing the following final concentrations in DMEM/Ham's F-12: 3% fetal bovine serum, 0.25 mM IBMX, 66 μM biotin, 34 μM D-pantothenate, 5 μM rosiglitazone (or equivalent PPAR γ 2 ligand), 1 μM dexamethasone, 200 nM human insulin. Use this solution within 2 weeks of its preparation.

5. *Adipocyte maintenance medium*: This solution is prepared in an identical manner as Differentiation medium except that it does not contain either the isobutylmethylxanthine or the PPAR γ agonist; these two stock solutions should be omitted. Use this solution within 2 weeks of its preparation.
6. *Oil Red-O staining solution*: Weigh out 0.5 g Oil Red-O. Dissolve in 100 ml isopropanol. Filter through a 0.2- μm filter. Store at room temperature as a stock solution. At the time of use, take 6 ml of Oil Red-O stock solution and add 4 ml of distilled water. Let the solution stand for 1 h at room temperature before use. Use this solution within 24 h of its preparation.
7. *Freezing medium*: The freezing medium consists of 80% fetal bovine serum or 80% calf serum, 10% DMEM/Ham's F-12, and 10% dimethyl sulfoxide. Use this solution within 2 weeks of its preparation.
8. *Medium sterility test procedure*: Prior to use, it is wise to test the sterility of the medium by removing a single milliliter from each bottle, placing it in a single well of a 24-well plate, and incubating it for 48 h in a humidified, 37°C , CO_2 incubator.

After this period, examine the plate using a phase contrast microscope for any evidence of contamination. If contaminated, immediately inactivate all bottles and test plates with 15% bleach solution and discard.

3. Methods

After transportation to the laboratory, the liposuction sample can be kept at room temperature for no longer than 24 h prior to use. Before performing the experiment, warm up the water bath to 37°C.

All of the following procedures are performed in biosafety hoods. Investigators should be trained in the handling of human tissues and human pathogens prior to initiation of any studies (see Note 3).

3.1. Large Volumes of Tissue (Liposuction Aspirates ≥ 100 ml)

1. Warm up buffer (500 ml or more of PBS or KRB). Line the surface of the biosafety hood with a disposable bench protector.
2. Warm up freshly prepared *collagenase solution* in the 37°C water bath.
3. Prepare PBS (or KRB) solution with 1% BSA, filter the solution, and warm it in the 37°C water bath.
4. Prepare stromal medium: cf. *Media stock solution*. This should have been done in advance of the procedure.
5. To maintain optimal sterile conditions, open the surgical container used for the liposuction procedure under the biosafety hood (see Note 4). Dispense a volume of adipose tissue in sterile plastic bottles: for each 175-cm² flask (0.16 ml tissue/cm²), it is recommended that you distribute approximately 33 ml of tissue; each bottle can accommodate ~100 ml of tissue. We routinely process a total of 200 ml of tissue to be plated in six 175-cm² flasks. Add an equal volume of warm PBS. Agitate to wash the tissue and then allow phase separation for 3–5 min. Suction off the infranatant solution (lower liquid phase). The wash is repeated several times until a clear infranatant solution is obtained (usually three to four times).
6. Add an equal volume (60–70 ml) of warm collagenase solution into the 250-ml bottles containing the clean adipose tissue sample. Wrap the bottles with parafilm and place them in a 37°C shaking water bath at ~75 rpm for 60 min until the tissue appears smooth on visual inspection (see Note 5).
7. Isolation of the stromal vascular fraction (SVF): After digestion, spin the samples at $300\times g$ in an appropriate centrifuge for 5 min at room temperature. Take the samples out of the centrifuge and shake them vigorously to thoroughly disrupt

the pellet and to mix the cells. This is to complete the separation of the stromal cells from the primary adipocytes. Repeat the centrifugation step.

8. After spinning, the SVF will form a pellet at the bottom of the bottle or tube (this will usually include a layer of dark red cells). Carefully remove the top layer of oil and fat, the primary adipocytes (a yellow layer of floating cells), and the underlying layer of collagenase solution. Leave behind a small volume of collagenase solution above the pellet so that the cells are not disturbed.

Resuspend the cells in 10 ml of warm PBS (or KRB) solution with or without 1% BSA and transfer the solution containing the cells into a 50-ml conical tube. Centrifuge the cells at $300\times g$ in an appropriate centrifuge for 5 min at room temperature.

Aspirate the remaining collagenase solution. When aspirating, the tip of the pipette should aspirate from the top so that the oil is removed as thoroughly as possible. The cell pellet should be at the bottom of the tubes. At this stage, some protocols suspend the SVF cells in Erythrocyte lysis buffer for 10 min followed by $300\times g$ centrifugation (6); however, we do not find that this step is necessary. Resuspend the cells with 10 ml of stromal medium in each tube. Pool the cells in one 50-ml conical tube and spin the cells at 1,200 rpm ($300\times g$) in an appropriate centrifuge for 5 min at room temperature.

9. After spinning the cells, aspirate off the supernatant and resuspend the cells in 15 ml of stromal medium (see Note 6).

Divide the cells according to the number of flasks. The cells are plated at a density equivalent to approximately 0.18 ml of liposuction tissue aspirate/cm² of surface area (volume of ~33 ml of tissue for a 175-cm² flask).

Divide the cells according to the number of flasks. In this protocol, we use approximately 200 ml of liposuction tissue. Thus, to each 175-cm² flask (times six), we add 2.5 ml of cell suspension and 32.5 ml of stromal medium (see Note 7).

10. Forty-eight hours after plating (this period can vary from 24 to 72 h, depending on the number of cells attached to the plastic surface as observed under a microscope), aspirate the medium from the flask. Wash the cells with prewarmed PBS (see Note 8). Add 35 ml of fresh stromal medium.

The medium is then changed every 2–3 days until the cells achieve 80–90% confluence.

11. Harvesting cells: Remove the medium from the flasks and save the sterile “conditioned media” in a sterile tube for future cell culture application (this media should be sterile-filtered prior to such use). Add 10 ml of sterile warm PBS to the flasks and allow PBS to remain on cells for 2 min while the flasks are in a horizontal position. Replace the PBS with 10 ml

of trypsin–EDTA solution (0.5%) (see Note 9). Incubate in an incubator for 5–10 min. Verify under a microscope that more than 90% of the cells have detached and then add 10 ml of stromal medium to allow the serum contained in the solution to neutralize the trypsin reaction.

Transfer the medium containing the suspended cells from the flask to a sterile 50-ml conical tube. Centrifuge at 1,200 rpm ($300\times g$) for 5 min. Aspirate the supernatant and suspend the cells with a small volume of stromal medium (~2 ml).

Proceed to cell counting by taking an aliquot of cells diluted in Trypan Blue (for a 1:4 dilution: add 25 μ l of suspended cells to 75 μ l of Trypan Blue). Count cells using the hemocytometer.

12. After counting, you have several options:

Cryopreservation

Suspend the cell pellet in room-temperature freezing medium at a concentration of 2×10^6 cells/ml. Dispense 1 ml of aliquots of the cell suspension to sterile cryovials. Place the cryovials in an appropriate freezing apparatus (alcohol container). Freeze the cells to -80°C . The next day, transfer the cells on dry ice or other frozen material to a liquid nitrogen storage container.

Use of cells for flow cytometry

Harvest $\sim 1.5\times 10^6$ ASCs and centrifuge at $300\times g$ for 5 min at room temperature in a 50-ml tube. Wash the ASCs twice with 10 ml cold PBS (Ca and Mg free) and resuspend cells in 500 μ l cold PBS. Aliquot 50 μ l of cells into ten 1.5-ml microcentrifuge tubes, add a 50- μ l volume of PBS containing a fluorochrome-conjugated monoclonal antibody or isotype control antibody (usually 5–10 μ l) to each tube. Mix well. Incubate samples for 20–30 min at room temperature. Critical note: Keep the tubes protected from light exposure to avoid bleaching of the fluorochrome. Wash cells with 1 ml PBS with 1% BSA and pellet cells at $300\times g$ for 3 min at room temperature three times. Resuspend cells in 500 μ l of 1% formaldehyde in PBS to fix cells. Keep the tubes at 4°C protected from light exposure until they can be analyzed on a flow cytometer within a 48-h period.

Replating the cells

After cell counting, suspend the cell pellet in stromal medium following the different concentrations listed in Table 1 to achieve a confluent culture within 24 h of replating.

Adipocyte differentiation

When the cells reach between 80 and 90% confluence (before or after harvesting the cells), the preadipocytes are induced to differentiate. Aspirate the medium, add a small volume (~1.5 ml for a 6-well plate) of prewarmed PBS + 1% antibiotic to wash the cells, and then remove the PBS by aspiration (see Note 10). Next, add the differentiation medium.

Table 1
Table for plating

Plate	Area/plate	Cells/plate	Cells/well	Media/well
6-well plate	60 cm ²	1.8×10^6	30×10^4	2.5 ml
24-well plate	48 cm ²	1.44×10^6	6×10^4	1 ml
96-well plate	31 cm ²	0.93×10^6	10^4	200 μ l

The cells will be maintained in the differentiation medium for 3 days.

Day +3 differentiation

Aspirate the differentiation medium and wash the cells with prewarmed PBS+1% antibiotic (see Note 11). Then add a volume (2.5–3 ml for a 6-well plate) of adipocyte medium.

The adipocyte medium will be changed every 3 days until mature adipocytes are obtained (Day +9 to +12 differentiation) (see Note 12).

Fixation of cells

After 12 days of differentiation, the cells can be fixed using 10% formalin solution, 4% paraformaldehyde, or 70% ethanol (using ethanol, there is a risk that the lipids will be eluted from the cells). After removing the medium and washing the cells with PBS, immerse the cells in the fixative solution: 10% formalin, 4% paraformaldehyde, or 70% ethanol for 30 min, 10 min, or 1 h, respectively. Remove the fixative before staining (fixed cells can be stored at 4°C for as long as several months, although shorter times are recommended).

Cell staining

Add 50 μ l Oil Red-O to each well for 15 min at room temperature. Rinse three times or more with 50 μ l distilled water. The rinse should become completely clear (no red coloring). Do not rinse with a volume larger than 50 μ l. It will raise the level of the solution in the plastic well and cause staining of the wall of the well, resulting in artifactually high background. Elute the stain from the cells by adding 50 μ l isopropanol per well. Elution is immediate. Read the OD₅₄₀ using a plate reader. Subtract the background staining determined in blank wells (no cells) from the experimental points. Determine the relative staining intensity of the differentiated wells compared with the preadipocyte controls.

3.2. Lipoaspirate Fluid (from (11))

1. Aspirate the lipoaspirate fluid fraction using a pipet, separating the fluid fraction from the lipoaspirate tissue fraction.
2. Transfer the lipoaspirate fluid (LAF) to sterile plastic bottles.
3. Centrifuge for 10 min at $400 \times g$ at room temperature.

4. Resuspend the LAF cell pellet in erythrocyte lysis buffer. Let stand at room temperature for 5 min.
5. Filter the LAF cell suspension through a 100- μ m filter.
6. Load cells onto a Ficoll density gradient. Centrifuge for 20 min at $800\times g$ at room temperature.
7. Aspirate cells from gradient interface. Wash with PBS prewarmed to 37°C. Filter LAF cell suspension through 100- μ m filter.
8. Count nucleated cell number using Trypan Blue staining and a hemocytometer.
9. Suspend LAF cells in Stromal medium. Plate at density of 6.4×10^4 cells/cm² on gelatin-coated 175-cm² flasks. Maintain in culture and proceed as described under 3.1.10-3.1.12

3.3. Summary

Recently, there has been increased appreciation for the use of primary cell culture models in the study of human adipocyte differentiation in vitro. This protocol on the cultivation of human adipocyte precursor cells can be used by laboratories with access to human tissues in a scalable manner. The protocol can be adapted to the use of intact human adipose tissue, which can be minced using dissecting scissors and then processed in a manner similar to the lipoaspirates.

4. Notes

1. Prior to the implementation of this protocol, all personnel involved in the processing of human lipoaspirate material or primary ASC cultures should complete safety training for the use of blood-borne pathogens. Regardless of whether tissue donors have been screened for evidence of infection by hepatitis, HIV, or other transmissible agents, these precautions are mandatory. In addition, no glass containers or pipets should be used, and the use of sharp objects (scissors, needles) during the processing steps should be minimized. All procedures involving the human tissue or cells should be conducted in a biological safety cabinet and with appropriate personnel protection gear.
2. Before purchase, the fetal bovine serum should be assayed to test for its ability to support adipogenesis.
3. Contamination – microbial. In order to keep optimal sterile conditions, it is recommended that you open and close the container properly to avoid any potential contaminations. It is vital that the culture be examined regularly to confirm the absence of microbial contamination. To avoid this problem, 5% iodine solution can be added to the initial wash solution. The disinfectant is then washed away in subsequent washes.

In order to avoid this problem in small adipose tissue samples, it is recommended to add in the PBS solution 1% of antibiotic solution and wash the cells thoroughly with this solution.

4. Contamination – cellular. The blood cells could be a source of contamination and may reduce or prevent adhesion of stromal cells; it is then important to thoroughly wash the cells with PBS (1% of antibiotic solution can be added). When removing the PBS from the cells, aspirate the solution up and down until the cells appear clean and free of red cells. An erythrocyte lysis buffer (155 mM NH_4Cl , 5.7 mM K_2HPO_4 , 0.1 mM EDTA at pH 7.3) can serve to remove red blood cells. Endothelial cells (EC) could be another source of contamination. Intraabdominal depots are more subject to this type of contamination when compared with subcutaneous adipose tissue, which is relatively free of EC. Therefore a filtration procedure can be performed by using a nylon mesh filter with a small pore size (25 μm).
5. After 1 h digestion, if pieces of undigested tissue are still observed in the tube, make sure that the collagenase solution is fresh and has not been maintained at room temperature for an extended period. This is necessary to maximize the enzyme efficiency. The collagenase solution can also be stored at -20°C for a few days, with a minor loss of enzyme activity. Prior to use, the frozen solution is slowly thawed at room temperature and prewarmed to 37°C . However, it is all right to not complete the digestion if a small amount of tissue fragments is observed in the solution.
6. A filtration procedure can be performed by using a nylon mesh filter with a small pore size (100 μm). The suspension is then centrifuged at 1,200 rpm ($300\times g$) at room temperature to allow separation of the SVF from the mature adipocytes. The filtration can be performed after removing the floating mature adipocytes cells. This step will remove tissue fragments and is used by some investigators (6). However, the filtration procedure is not recommended for small amounts of adipose tissue.
7. To accelerate cell adhesion, the culture dishes can be precoated with extracellular matrix proteins, such as gelatin or Matrigel.
8. If the cells do not grow very well and do not appear healthy, there are a few options. The percent of preadipocytes obtained from the SVF after digestion is patient dependent. Supplementation of the culture medium with 20–30% conditioned medium (saved from previous cultures) should facilitate the growth of the cells. If you do not have conditioned medium, another alternative is to increase the FBS contained in the stromal medium to 15–20%; however, this

may promote premature adipogenesis. Signs of deterioration, such as granularity around the nucleus, cytoplasmic vacuolations, and/or detachment of the cells from the plastic surface, may indicate inadequate or toxic medium, microbial contamination, or senescence of the primary cells.

9. It is important to not overexpose the cells to the trypsin/EDTA solution. This could decrease the cell viability.
10. If the adipocytes are detaching, do not dry the well when changing the medium since adipocytes tend to float when new medium is added.
11. According to the protocol, the medium is changed every 2–3 days. However, as the adipocytes mature, you may observe a yellowing of the culture medium: a drop in pH may account for this. As the pH falls from 7 to 6.5, cell growth will decline and cell viability falls at pH between 6.5 and 6. You can observe this change of pH by looking at the medium color change, going from red (pH 7) through yellow (pH ≤ 6), indicating the need for an immediate change of the medium.
12. If the cells do not differentiate very well, consider that the differentiation process may be patient dependent. The age of the donor can be a factor, since some studies suggest that the differentiation capacity is higher in culture from younger subjects compared with older people. To further enhance adipogenesis, the following alternatives are proposed:
You may try different PPAR γ agonists (troglitazone or pioglitazone, among others).
 - The addition of 5% rabbit serum (RS) can be added to the differentiation medium to enhance differentiation (the ethyl acetate contained in the RS has been found to be 35-fold more abundant than in FBS (12)).
 - Another alternative would be to perform the addition of the differentiation medium multiple times after a 3-day rest period; i.e., 3 days on in the presence of the differentiation medium and 3 days off in the presence of the adipocyte medium. Repeat this cycle until mature adipocytes are obtained.

References

1. Rangwala SM, Lazar MA (2000) Transcriptional control of adipogenesis. *Annu Rev Nutr* **20**:535–59.
2. Deslex S, Negrel R, Vannier C, Etienne J, Ailhaud G (1987) Differentiation of human adipocyte precursors in a chemically defined serum-free medium. *Int J Obes* **11**(1):19–27.
3. Hauner H, Entenmann G, Wabitsch M, Gaillard D, Ailhaud G, Negrel R, Pfeiffer EF (1989) Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. *J Clin Invest* **84**(5):1663–70.
4. Halvorsen YD, Bond A, Sen A, Franklin DM, Lea-Currie YR, Sujkowski D, Ellis PN, Wilkison WO, Gimble JM (2001)

- Thiazolidinediones and glucocorticoids synergistically induce differentiation of human adipose tissue stromal cells: biochemical, cellular, and molecular analysis. *Metabolism* **50**(4):407–13.
5. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**(12):4279–95.
 6. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* **7**(2):211–28.
 7. Guilak F, Lott KE, Awad HA, Cao Q, Hicok KC, Fermor B, Gimble JM (2006) Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. *J Cell Physiol* **206**(1):229–37.
 8. Gimble JM, Katz AJ, Bunnell BA (2007) Adipose-derived stem cells for regenerative medicine. *Circ Res* **100**(9):1249–60.
 9. Mitchell JB MK, Zvonic S, Garrett S, Floyd ZE, Kloster A, Halvorsen YD, Storms RW, Goh B, Kilroy GS, Wu X, Gimble JM (2006) The immunophenotype of human adipose derived cells: Temporal changes in stromal- and stem cell-associated markers. *Stem Cells* **24**:376–85.
 10. Dubois SG, Floyd EZ, Zvonic S, Kilroy G, Wu X, Carling S, Halvorsen YD, Ravussin E, Gimble JM (2008) Isolation of human adipose-derived stem cells from biopsies and liposuction specimens. *Methods Mol Biol* **449**:69–79.
 11. Yoshimura K, Shigeura T, Matsumoto D, Sato T, Takaki Y, Aiba-Kojima E, Sato K, Inoue K, Nagase T, Koshima I, Gonda K (2006) Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. *J Cell Physiol* **208**(1):64–76.
 12. Diascro DD Jr, Vogel RL, Johnson TE, Witherup KM, Pitzenberger SM, Rutledge SJ, Prescott DJ, Rodan GA, Schmidt A (1998) High fatty acid content in rabbit serum is responsible for the differentiation of osteoblasts into adipocyte-like cells. *J Bone Miner Res* **13**(1):96–106.



<http://www.springer.com/978-1-61737-959-8>

Adipose-Derived Stem Cells

Methods and Protocols

Gimble, J.M.; Bunnell, B.A. (Eds.)

2011, XVI, 474 p., Hardcover

ISBN: 978-1-61737-959-8

A product of Humana Press