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In Vitro and In Vivo Osteogenic Differentiation of Human Adipose-Derived Stromal Cells

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Abstract

Adipose-derived stromal cells (ASCs) are a promising population of cells that may be useful for the regeneration of human tissue defects. ASCs are capable of forming bone tissue in vitro and in vivo. Further work is required to determine the optimal conditions that will allow human ASCs to regenerate tissue in clinically significant tissue defects. Here we present three experimental protocols that are indispensable for the study of ASC osteogenic activity.

Keywords

Adipose-derived stromal cell; Osteogenesis; Osteogenic differentiation; Bone defect; Calvarial defect

1 Introduction

For decades, bone grafting has been used successfully to treat bone defects caused by infection, congenital conditions, traumatic injuries, and tumors. The advantage of this technique is the use of autologous bone tissue, which eliminates the risk of immune rejection or disease transmission [1]. However, the utility of bone grafting may be limited in patients in whom bone defects are so large or numerous that sufficient autologous bone is not available to fill the defects. For cases such as these, there is interest in the use of synthetic bone substitutes. The advantages of these substitutes are that they are available in unlimited quantities and there is no need for a donor site harvest, which carries its own risks and morbidities [2]. Despite their obvious benefits, synthetic bone substitutes carry a risk of foreign body reaction and infection. Also, their suitability is limited in children, who will rapidly outgrow the implant.

To address the shortcomings of autologous bone grafts and synthetic bone substitutes, there is great interest in the development of regenerative medicine techniques that utilize a patient's own cells or tissue to form new functional tissue. One of the most promising areas of regenerative research involves the use of adipose-derived stromal cells (ASCs). These are a population of multipotent cells that normally reside in the stromal vascular fraction (SVF) of adipose tissue [3]. They have the capacity to differentiate along adipocyte, chondrocyte, and osteoblast lineages by displaying markers associated with these cell types and by exhibiting phenotypes similar to them [4]. However, it remains controversial whether ASCs ever truly transform into adipocytes, chondrocytes, and osteoblasts or simply mimic certain aspects of these cells.

The term ASC is sometimes inaccurately used to refer to all cells of the SVF [4]. In addition to ASCs, the SVF contains many other cell types, including endothelial cells, erythrocytes, lymphocytes, macrophages, and smooth muscle cells [5]. To be precise, ASCs refer specifically to those cells of the SVF that possess multipotent differentiation capacity and that adhere to the plastic culture dish surface [6]. Additionally, ASCs should display the cell surface markers CD34, CD90, CD73, CD105, and CD44 and should be negative for CD45 and CD31 [6].

ASCs hold a number of advantages compared with other methods for filling bone defects. The first is that using a patient's own cells obviates any concern for immune rejection. Also, the risk of a reaction to foreign material is minimized because the ASCs may be delivered using scaffolds that rapidly degrade. ASCs can be easily isolated from fat tissue that is removed from a human patient during a liposuction or abdominoplasty procedure [7]. It is possible to purify ASCs in several hours without exposing the cells to cell culture, which avoids the risk of contaminating the cells with viruses, bacteria, or other human cells [8]. Harvesting of ASCs is invasive but is minimally morbid and could theoretically be performed in multiple sessions over time, if more cell applications were required.

Bone morphogenetic protein (BMP) is a class of proteins that are critical agents of bone formation and maintenance [9]. Not surprisingly, BMPs and BMP analogs as well as other agents that modulate the BMP pathway can increase the osteogenic activity of ASCs [10]. In the future, it may be useful to deliver drugs that affect BMP pathways along with ASCs in order to maximize new bone growth. A typical experiment exploring the effect of BMP-related pathways on ASC function would involve the isolation of ASCs followed by an assay of their osteogenic activity in the presence or absence of a BMP-modulating drug.

Here we present a protocol for ASC isolation and both in vivo and in vitro testing for osteogenic activity in three parts: (1) isolation of ASCs from human tissue and selection for ASCs bearing specific cell surface markers, (2) use of an in vitro assay to measure ASC osteogenic activity, and (3) use of an in vivo assay to measure new bone formation by ASCs. These techniques can be used together or in combination with other methods to understand the role that BMP-related drugs may have in altering ASC osteogenic activity and bone growth.

2 Materials

1. Dissection and cell culture.
2. 100 µm cell strainers.
3. 15-blade scalpel.
4. 4 mm diamond-coated trephine drill bit.
5. 40 µm cell strainer.
6. Alizarin powder.
7. Antibodies against CD34, CD31, and CD34 that are optimized for flow cytometry.
8. Ascorbic acid.
9. *C. hemolyticum* collagenase type II powder.
10. DAPI (4',6-diamidino-2-phenylindole).
11. Dulbecco's Modified Eagle Medium.
12. Fetal bovine serum (FBS).
13. Fine-toothed forceps.
14. Glass FACS tubes.
15. β-Glycerophosphate.
16. 10,000 U/mL penicillin-streptomycin.
17. Phosphate-buffered saline.
18. PLGA (poly[lactic-co-glycolic acid]) scaffolds coated with hydroxyapatite. Alternately, collagen-based scaffolds that support cell survival may be used.
19. Poloxamer 188.
20. Povidone-iodine solution.
21. Red blood cell lysis buffer.
22. Sodium hydroxide.
23. Sterile surgical drapes.
24. Small sharp scissors.
25. Surgical drill.
26. Veterinary eye ointment.
27. *FACS buffer*: Add 10 mL fetal bovine serum (FBS), 5 mL poloxamer 188, and 5 mL of 10,000 U/mL penicillin- streptomycin solution to 500 mL sterile phosphate-buffered saline (PBS).

28. *Digest mixture*: Add 0.375 g *C. hemolyticum* collagenase type II powder and 5 mL poloxamer 188 to 500 mL sterile Dulbecco's Modified Eagle Medium (DMEM).
29. *Standard medium*: Add 50 mL fetal bovine serum (FBS) and 5 mL of 10,000 U/mL penicillin-streptomycin solution to 500 mL Dulbecco's Modified Eagle Medium.
30. *Alizarin red stain*: Add 1 g alizarin powder to 500 mL distilled water. Use sodium hydroxide to bring the pH to 6.36–6.4. This solution may be stored in the dark for 1 month at 4 °C.
31. *Osteogenic differentiation medium (ODM)*: Prepare standard medium as described above, and add to the mixture 100 mg/mL ascorbic acid and 10 mM β -glycerophosphate.

3 Methods

3.1 Isolation of ASCs from Human Tissue and Selection for Specific Cell Surface Markers

A typical ASC experiment begins with the isolation of ASCs from human tissue. Traditional protocols for isolating ASCs from fresh fat tissue involve culturing the cells for several days in order to keep only those cells that adhere to plastic, which by definition should be ASCs [6]. However, it is clear that the time spent on cell culture plates drastically alters gene expression and cell surface marker profiles in ASCs and other adherent cell types [11]. For this reason, we employ an ASC isolation strategy that takes advantage of fluorescence-activated cell sorting (FACS) for markers known to identify ASCs within the SVF [12]. Specifically, this protocol gates for cells that are positive for CD34 and negative for both CD31 and CD45, which mark hematopoietic and endothelial cells. Erythrocytes are eliminated prior to FACS sorting using erythrocyte-specific lysis buffer. This protocol yields ASCs within several hours of obtaining fat tissue and avoids cell culture. However, cell phenotypes and cell surface marker expression profiles may be altered by the process of sorting them. Indeed, a consensus definition of ASCs based on cell surface marker profile has not been established, and different techniques for isolating ASCs may yield slightly different populations of cells [13].

1. Obtain human abdominal, flank, or thigh subcutaneous fat from a healthy donor undergoing elective liposuction. Fat from other anatomic sources has not been used routinely by our group. Keep the fat in a closed container, such as a plastic suction canister.
2. Add an equal volume of PBS (1:1 ratio) to the fat and mix by gentle agitation for 30 s. After the aqueous and fat layers have separated (aqueous layer to the bottom, fat layer to the top), use a pipette to aspirate and discard the aqueous layer.
3. Decant the fat into a 1 L plastic container, and add an equal volume of the digest mixture. Close the container and clean the outside with 70% ethanol to avoid

contaminating laboratory surfaces with human cells. Seal the cap with paraffin film to prevent spillage.

4. Agitate this mixture using an orbital shaker set to 180 revolutions per minute (rpm) at 37 °C for 30 minutes (min).
5. Neutralize the digestion by adding an equal volume of standard FBS-containing medium.
6. Distribute the mixture equally into plastic conical centrifuge tubes, and centrifuge the mixture at $300 \times g$ for 20 min at 4 °C.
7. Aspirate and discard the supernatant, taking care to leave the pellet intact. Resuspend each pellet in 5 mL standard medium.
8. Combine the cell suspension samples by filtering them through a 100 μ m cell strainer into one 50 mL conical centrifuge tube.
9. Centrifuge at $300 \times g$ for 15 min at 4 °C.
10. Aspirate and discard the supernatant and resuspend the pellet in 5 mL room temperature red blood cell lysis buffer. Allow the mixture to sit for 5 min at room temperature, and then bring the volume to 15 mL with PBS. Centrifuge at $300 \times g$ for 15 min at room temperature.
11. Aspirate the supernatant and resuspend the pellet in 15 mL standard medium. Again, filter through a 100 μ m cell strainer into a single 50 mL conical centrifuge tube.
12. Count the cells using a hemocytometer and determine the total cell number.
13. Centrifuge the cell suspension at $300 \times g$ for 5 min at 4 °C. Resuspend the cells in FACS buffer at a concentration optimized for your FACS antibodies. A typical concentration is 1 million cells per 100 μ L buffer.
14. Transfer 20 μ L of the suspension to a glass FACS tube labeled “Unstained” and bring the volume up to 200 μ L with FACS buffer. Set aside on ice. Optionally, set aside a certain volume of cell suspension into a tube labeled “Unsorted.” These cells will not undergo staining or FACS sorting and will serve as a control in subsequent experiments (*see Note 1*).
15. Label the tube containing the remaining cells as “Stained,” and add FACS antibodies against CD34, CD31, and CD45. The CD31 and CD45 antibodies may be conjugated to the same fluorophore, but the CD34 antibody must be conjugated to a distinct fluorophore. If the CD31 and CD45 antibodies are conjugated to Pacific Blue, eFluor 450, or a similar color, this allows for a “dump channel” when used with DAPI as a viability marker (*see Note 2*).
16. Once the antibodies are added to the “Stained” cells, pipette the suspension up and down several times to mix thoroughly. Place the cells on ice and cover the ice bucket with aluminum foil. Allow the cell/antibody mixture to incubate for

20 min with gentle agitation (or according to the instructions of the antibody manufacturer).

17. When the incubation period is complete, bring the total volume in the “Stained” tube to 2 mL with FACS buffer and centrifuge at $300 \times g$ for 5 min at 4 °C. Aspirate and discard the supernatant. Again, bring the total volume to 2 mL with FACS buffer, and centrifuge at $300 \times g$ for 5 min at 4 °C. Aspirate and discard the supernatant. Resuspend the cells in FACS buffer to a final concentration that is optimized for your FACS machine. We typically use 2 million cells per mL. Filter the cell suspension through a 40 µm cell strainer into a glass FACS tube labeled “Stained.” The use of a FACS tube with a cell strainer built into the cap is helpful.
18. On the FACS machine, use the unstained cells to define a negative gate for the fluorescent markers. Use a viability stain to exclude nonviable cells (*see* Note 2). Then, using a 100 µm nozzle, sort viable CD34⁺CD31⁻CD45⁻ cells into a tube containing standard medium. Ideally the collection tube should be chilled to 4 °C during the sort.

3.2 Alizarin Red Assay for In Vitro ASC Osteogenic Activity

Alizarin red is an in vitro assay performed on cultured cells that quantifies calcification on the surface of culture plates. Cells such as osteoblasts and ASCs with inherent osteogenic behavior will typically produce calcific nodules that adhere to the culture plate. The alizarin red assay is therefore a measurement of the osteogenic activity of cells in culture. Alizarin red staining was first described in the early twentieth century for the histological staining of skeletal specimens [14]. It is currently considered a critical part of characterizing ASCs and other osteogenic cell types. In this protocol, the cultured cells are stained with alizarin red, images are obtained, and the extent of staining is quantified.

1. Following isolation of ASCs, plate the cells in standard medium at a concentration of 100,000 cells per plate onto standard 10 cm polystyrene cell culture plates.
2. When the cells have reached 80% confluency, which typically takes several days, change to osteogenic differentiation medium (ODM). Change the medium three times per week for 14 days. The cells will begin to form bone nodules on the culture plate surface (*see* Note 3).
3. After 14 days in ODM, wash the cell layer three times with PBS.
4. Fix the cells in 100% ethanol for 15 min at room temperature. Then wash three times with distilled water.
5. Stain the cell layer with alizarin red solution for 60 min at room temperature. Use 10 mL for a 10 cm plate.
6. Wash with PBS five times. Make sure to wash away any stain that is adherent to the plastic but not marking bone nodule tissue. Be careful not to dislodge bone nodules during staining or washing. Remove the PBS by aspirating with a

pipette. Place the plates upside down over a paper towel for 2 min to remove the remaining PBS (*see* Note 4).

7. Obtain images of the stained plates using a high-quality computer scanner. Load the images into ImageJ (NIH) or a similar image manipulation program. Convert the images to black and white. Define a threshold that includes all stained bone nodules but excludes background staining. Quantify the surface area of bone nodule in each plate.

3.3 Calvarial Defect Model for Assessing In Vivo Bone Regeneration

While the alizarin red assay measures the osteogenic activity of cells in culture, the calvarial defect model quantifies the ability of cells to assist in closing a bone defect, either by creating new bone tissue themselves or by enhancing endogenous bone growth through paracrine signaling. In vitro assays such as alizarin red can suggest that a cell population is osteogenic, but the use of an in vivo assay such as the calvarial defect model is critical in order to prove that the cells are capable of assisting with bone formation in the complex and hostile environment of a living organism. An added advantage of this model is that human or mouse cells can be used, as long as an appropriately immunosuppressed host mouse is selected. For human ASCs, the athymic CD-1 nude mouse is ideal. If the ASCs are of mouse origin, a syngeneic mouse should be used as the host. We typically use female mice to prevent the risk of fighting between animals.

1. Centrifuge the sorted cells at $300 \times g$ for 5 min at 4 °C. Resuspend the cells in standard medium to a concentration of 1 million cells per 250 μ L.
2. Obtain 4 mm circular PLGA (poly[lactic-co-glycolic acid]) scaffolds coated with hydroxyapatite [15]. Place the scaffolds individually into the wells of a standard 6-well culture plate. Cover each scaffold with 50 μ L of cell suspension containing 200,000 cells. (A different cell number may be chosen based on the needs of your experiment.) Cover the plate with its lid, and incubate in a cell culture incubator at 37 °C for 30 min.
3. Anesthetize an 8–12-week-old female CD-1 nude mouse using inhaled isoflurane gas at 2% in 100% oxygen. Apply eye ointment to prevent desiccation of the cornea. Administer 1 mg/kg buprenorphine SR subcutaneously. Providing analgesia prior to the surgery results in superior pain relief (*see* Note 5).
4. Sterilize the skin of the dorsal aspect of the skull with povidoneiodine solution followed by 70% ethanol three times. Drape with sterile drapes, leaving the surgical site exposed.
5. Using a 15-blade scalpel, make a sagittal midline incision that extends over the majority of the dorsal skull. Using fine-toothed forceps, retract the skin on the right side of the incision to expose the right parietal bone. It may be necessary to use forceps and a spreading motion with small sharp scissors to clear away loose connective tissue and develop a plane between the periosteum of the skull and the skin (*see* Note 6).

6. Using a drill with an autoclaved 4 mm diamond-coated trephine drill bit, gently drill a circular defect through the right-sided parietal bone. It is important to stop drilling once the drill has traversed the bone. Do not extend the defect past bone into the dura mater layer, as this will result in irreversible brain damage. With fine forceps, carefully remove the circle of bone (*see Note 7*).
7. Place a scaffold containing cells into the defect, and then close the skin incision with running nylon suture.
8. Monitor the mouse and provide standard postoperative care according to institutional guidelines (*see Note 8*).
9. Shortly after the procedure, and then at 2, 4, 6, and 8 weeks, use micro CT scanning to measure the rate of calvarial defect closure. At 8 weeks, bone tissue may be excised and formalin fixed, decalcified, and then embedded in paraffin. Sections may be stained with Movat's pentachrome stain and histologically examined to assess the microscopic quality of the new bone tissue.

4 Notes

1. It is easy to forget to set aside an unstained tube prior to initiating antibody staining of the cells. However, not having the unstained cells will make accurate sorting impossible.
2. It is important to use a viability stain during FACS sorting in order to exclude dead or dying cells from the sort. 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) are two common, easy-to-use viability stains. We typically use DAPI, which is added to the cell suspension to a final concentration of 3 μ M just before sorting the cells. Cells that are stained by DAPI are considered to be nonviable and fluoresce strongly in the Pacific Blue channel. If the CD45 and CD31 antibodies are conjugated to Pacific Blue, a "dump channel" can be used to exclude C45⁺, CD31⁺, and nonviable cells simultaneously.
3. Be careful not to allow the cells to become more confluent than 80% before starting osteogenic differentiation. Cells that approach 100% confluency are prone to lifting off of the cell culture plate. When this happens, you will typically find a ball of cell material floating in the medium with no cells adhering to the surface of the culture plate.
4. It is easy to wash too vigorously, which will cause the stained cell layer to lift from the culture plate surface. This makes accurate quantification impossible. The best way to wash away excess alizarin red staining is with multiple gentle washes of PBS.
5. Carefully monitor the respiratory rate of the animal during surgery. Since most of the animal is covered with a drape it can be easy to forget to monitor this parameter. Failure to monitor the respiratory rate can result in the animal expiring during the surgery due to hypoventilation. If the respiratory rate slows, decrease the fraction of inspired isoflurane. Do not reduce the oxygen flow rate.

6. It may be necessary to spend 1–2 min developing the plane between the skin and periosteum by clearing away the loose connective tissue that normally connects these layers. Placement of the scaffold and cells into the defect will be much easier if the periosteal surface is free of connective tissue and the skin lifts freely away from it.
7. Creation of the calvarial defect is perhaps the most critical and challenging step in this protocol. Application of too much force will allow the drill bit to move past the bone and into the dura mater, likely resulting in brain damage. One way to avoid this is to repeatedly apply light pressure to the skull with the drill bit in the same spot in a gentle tapping motion. If you are not sure whether the drill is all the way through the bone, try lifting the bone circle with the forceps. If it does not lift, then more drilling needs to be done.
8. While the mouse is recovering from anesthesia, actively warm it either under a warming lamp or on a warming pad. Mice under anesthesia are very prone to hypothermia. Monitor the mouse until it is able to ambulate independently.

References

1. de Boer HH (1988) The history of bone grafts. *Clin Orthop Relat Res* 226:292–298
2. Kolk A, Handschel J, Drescher W, Rothamel D, Kloss F, Blessmann M, Heiland M, Wolff KD, Smeets R (2012) Current trends and future perspectives of bone substitute materials—from space holders to innovative biomaterials. *J Craniomaxillofac Surg* 40(8):706–718. 10.1016/j.jcms.2012.01.002 [PubMed: 22297272]
3. 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):4279–4295. 10.1091/mbc.E02-02-0105 [PubMed: 12475952]
4. Casteilla L, Planat-Benard V, Laharrague P, Cousin B (2011) Adipose-derived stromal cells: their identity and uses in clinical trials, an update. *World J Stem Cells* 3(4):25–33. 10.4252/wjsc.v3.i4.25 [PubMed: 21607134]
5. Gimble JM, Bunnell BA, Frazier T, Rowan B, Shah F, Thomas-Porch C, Wu X (2013) Adipose-derived stromal/stem cells: a primer. *Organogenesis* 9(1):3–10. 10.4161/org.24279 [PubMed: 23538753]
6. Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM (2013) Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy* 15(6):641–648. 10.1016/j.jcyt.2013.02.006 [PubMed: 23570660]
7. Zielins ER, Luan A, Brett EA, Longaker MT, Wan DC (2015) Therapeutic applications of human adipose-derived stromal cells for soft tissue reconstruction. *Discov Med* 19 (105):245–253 [PubMed: 25977187]
8. Lo DD, Hyun JS, Chung MT, Montoro DT, Zimmermann A, Grova MM, Lee M, Wan DC, Longaker MT (2012) Repair of a critical-sized calvarial defect model using adipose-derived stromal cells harvested from lipoaspirate. *J Vis Exp* (68). 10.3791/4221
9. Sanchez-Duffhues G, Hiepen C, Knaus P, Ten Dijke P (2015) Bone morphogenetic protein signaling in bone homeostasis. *Bone* 80:43–59. 10.1016/j.bone.2015.05.025 [PubMed: 26051467]
10. Fan J, Im CS, Guo M, Cui ZK, Fartash A, Kim S, Patel N, Bezouglaia O, Wu BM, Wang CY, Aghaloo TL, Lee M (2016) Enhanced osteogenesis of adipose-derived stem cells by regulating bone morphogenetic protein signaling antagonists and agonists. *Stem Cells Transl Med* 5(4):539–551. 10.5966/sctm.2015-0249 [PubMed: 26956209]

11. Walmsley GG, Rinkevich Y, Hu MS, Montoro DT, Lo DD, McArdle A, Maan ZN, Morrison SD, Duscher D, Whittam AJ, Wong VW, Weissman IL, Gurtner GC, Longaker MT (2015) Live fibroblast harvest reveals surface marker shift in vitro. *Tissue Eng C Methods* 21(3):314–321. 10.1089/ten.TEC.2014.0118
12. Chung MT, Zimmermann AS, Paik KJ, Morrison SD, Hyun JS, Lo DD, McArdle A, Montoro DT, Walmsley GG, Senarath-Yapa K, Sorkin M, Rennert R, Chen HH, Chung AS, Vistnes D, Gurtner GC, Longaker MT, Wan DC (2013) Isolation of human adipose-derived stromal cells using laser-assisted liposuction and their therapeutic potential in regenerative medicine. *Stem Cells Transl Med* 2(10):808–817. 10.5966/sctm.2012-0183 [PubMed: 24018794]
13. Tevlin R, McArdle A, Brett E, Chung MT, Paik K, Seo EY, Walmsley GG, Duldulao CR, Atashroo D, Zielins E, Vistnes S, Chan CK, Wan DC, Longaker MT (2016) A novel method of human adipose-derived stem cell isolation with resultant increased cell yield. *Plast Reconstr Surg* 138(6):983e–996e. 10.1097/PRS.0000000000002790
14. Dawson AB (1926) A note on the staining of the skeleton of cleared specimens with alizarin red S. *Stain Technol* 1(4):123–123
15. Gentile P, Chiono V, Carmagnola I, Hatton PV (2014) An overview of poly(lactic-co-glycolic) acid (PLGA)-based biomaterials for bone tissue engineering. *Int J Mol Sci* 15(3):3640–3659. 10.3390/ijms15033640 [PubMed: 24590126]