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Editors

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Adipose-Derived Stem Cells

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Preface

During the past decade, a wide range of scientific disciplines have "found" adipose-derived stem/stromal cells (ASC) as a tool for research and discovery. This is reflected by the exponential growth in the number of publications and citations noted by *ISI Web of Science* (*Thomson-Reuters*) (Fig. 1). However, the cells we now recognize as "ASC" were initially identified as "preadipocytes" in seminal studies initiated by Martin Rodbell and his colleagues during the mid-1960s. His laboratory was among the first to use collagenase digestion to separate mature adipocytes from the heterogeneous stromal vascular fraction (SVF) cells in rat fat pads. Since then, primary cultures of preadipocytes have been utilized by endocrinologists and physiologists to perform in vitro analyses of adipose tissue metabolism. By the late 1990s, investigators at the University of Pittsburgh, University of California – Los Angeles, Zen-Bio, Inc., and other institutions had begun to draw parallels between the ASC and the more thoroughly characterized bone marrow-derived mesenchymal stem/stromal cells (BMSC). Both ASC and BMSC displayed multipotent differentiation potential in vitro, with the ability to form bone, cartilage, hematopoietic supporting cells, and muscle, in addition to adipocytes. Later studies would document that they also share similar immunophenotypic and immunomodulatory characteristics. Unlike bone marrow, subcutaneous adipose tissue is relatively accessible to harvest, abundant, and located in a site that patients are willing, indeed eager, to have biopsied. Consequently, the demand for ASC has grown; they quickly have become the stem cell of choice for many tissue-engineering and regenerative medical projects. Bioengineers, clinicians, entrepreneurs, and research scientists recognized the need to coordinate this emerging field and founded the International Federation of Adipose Therapeutics and Science (IFATS) in 2002. IFATS was envisioned as a society that would promote the free exchange of information and knowledge relating to ASC. In its efforts to provide standardization across laboratories, one of the first steps of IFATS was to establish the acronym "ASC" to describe the collagenase-digested and culture-expanded adipose cell populations. Prior to reaching this consensus in 2004, the nomenclature had been cluttered with multiple terms that led to confusion for newcomers in the field. In this volume of *Methods in Molecular Biology*, we have solicited defined and established protocols from leaders in the field, including many IFATS members. While much of the emphasis is placed on human ASC, additional small and large animal species are included. The chapters are organized around approaches spanning the discovery, preclinical, and clinical processes. While they are designed to be accessible to new students, we hope that they will serve as a reference text for established investigators as well.

The editors have many people to thank for their contributions to this work. First and foremost are the many authors who agreed to take time from their busy schedules and prepare chapters on selected topics (with only a minimum of bribes and arm twisting!). Second is Professor John Walker, Series Editor, whose sage advice and wisdom were greatly appreciated. Third are the members of the Stem Cell Biology Laboratory, Pennington

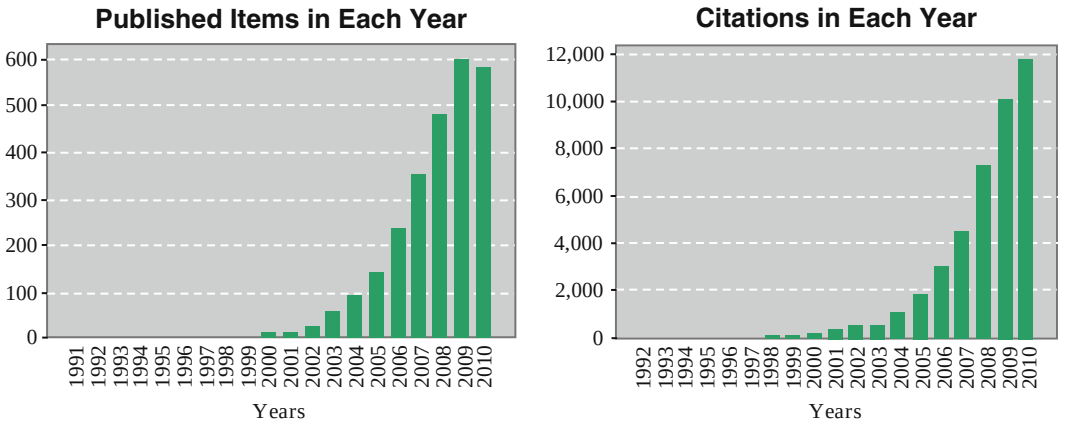

Fig. 1 The ISI Web of Knowledge annual publications and citations for "adipose stem cells"

Biomedical Research Center, and the Center for Stem Cell Research and Regenerative Medicine, Tulane University School of Medicine, for their critiques and insights. Fourth, is Ms. Laura Dallam for her outstanding administrative and editorial assistance. And finally, for sacrificing many evenings and weekends, are our family members: Emma, Jesse, Megan, Paula, and Xiying.

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Part I

Isolation Methods

Chapter 1

Isolation and Culture of Rhesus Adipose-Derived Stem Cells

Christine Gagliardi and Bruce A. Bunnell

Abstract

Adipose tissue is as an abundant and accessible source of stem cells with multipotent properties suitable for tissue engineering and regenerative medical applications. Rhesus monkeys are physiologically and phylogenetically similar to humans, and they and their cells are valuable for biomedical research and evaluation of preclinical therapies. Here, we describe methods for the isolation, culture, and differentiation of rhesus adipose-derived stem cells (rASCs).

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

1. Introduction

Mesenchymal stromal/stem cells (MSCs) 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), there is now independent evidence demonstrating that they are multipotent, with chondrogenic, neuronal-like, and osteogenic differentiation capability (5–8). Consequently, they are now identified as adipose-derived stromal/stem cells or ASCs (9). ASCs have been isolated from adipose tissue of the rhesus macaques and, like human ASCs, are capable of multilineage differentiation along mesodermal and neural lineages (10). Rhesus adipose-derived stem cells (rASCs) have been compared to human ASCs and MSCs and have similar growth kinetics and potential (11). This protocol describes the isolation and culture of ASCs from rhesus adipose tissue (Fig. 1).

Methods to isolate cells from adipose tissue were established by Rodbell and colleagues in the 1960s (12–14), and have subsequently been adapted to isolate cells from human and rhesus adipose

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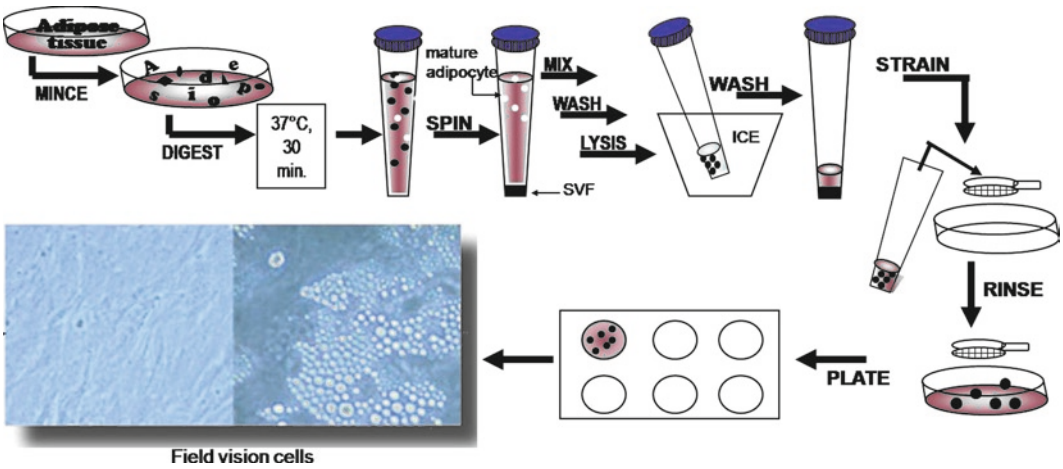

Fig. 1. Overview of the rASC isolation protocol. Reprinted with permission from Elsevier.

tissue (3, 10, 15–17). Subcutaneous adipose tissue samples can be obtained from rhesus monkeys under local anesthesia. The adipose tissue is washed and finely minced by hand. The small fragments are then incubated with a collagenase solution to further digest the tissue. After centrifugation of the sample, mature adipocytes will float and can be removed and discarded with the supernatant. The remaining pellet consists of the stromal vascular fraction (SVF), containing a heterogeneous population of cells, which may include blood cells, fibroblasts, pericytes, endothelial cells, and preadipocytes (12–14). Contaminating red blood cells are lysed and removed by incubation of the SVF in lysis buffer and centrifugation. Multipotent stromal cells can then be isolated based on their ability to adhere to plastic culture dishes.

2. Materials

2.1. Tissue

1. Subcutaneous adipose tissue samples obtained from rhesus macaques under local anesthesia (see Notes 1 and 2).

2.2. Supplies

1. 0.2-μm filter units.
2. 50-mL conical tubes.
3. 15-mL conical tubes.
4. Cryovials.
5. Sterile, disposable scalpels.
6. Personal protective equipment.

7. Multiwell tissue culture dishes.
8. 15-cm round tissue culture dishes.
9. 70-μm cell strainers.
10. Ice bucket.
11. Hemocytometer.
12. Freezing apparatus (alcohol container).
13. Fluorochrome-conjugated monoclonal antibodies against stromal, hematopoietic, endothelial, pericytic, and related cell surface antigens.
14. Phosphate-buffered saline (PBS).
15. Dimethyl sulfoxide (DMSO).
16. Trypsin–EDTA.
17. Ethanol.
18. Isopropanol.
19. Methanol.
20. Sterile H_2O.

2.3. Isolation and Culture Media (see Note 3)

1. Wash buffer: 25 mL of a solution of 10,000 U/mL penicillin and 10,000 μg/mL streptomycin (pen/strep) and 475 mL of PBS.
2. Collagenase solution: 0.75 g type I collagenase and 2 mL of pen/strep solution in 98 mL of sterile PBS. Filter through a 0.2-μm membrane. Use within 1 h.
3. Erythrocyte lysis buffer: 155 mM ammonium chloride (NH_4Cl), 10 mM potassium carbonate (KCO_3), and 0.1 mM EDTA in sterile, distilled water. Filter through a 2-μm membrane. Use within 24 h.
4. Stromal medium: 100 mL fetal bovine serum (FBS, see Note 4), 5 mL of 200 mM L-glutamine solution and 5 mL of pen/strep solution in 394 mL of α-MEM. Filter through a 0.2-μm membrane. Use within 4 weeks.
5. Freezing medium: 8 mL FBS, 1 mL DMEM/Ham's F-12, and 1 mL DMSO. Use within 2 weeks.

2.4. Differentiation Stock Solutions and Media (see Note 5)

1. Dexamethasone, 1 and 10 mM in sterile H_2O (see Note 6).
2. ß-glycerolphosphate, 0.5 M in stromal medium.
3. Ascorbate 2-phosphate, 50 mM and 50 mg/mL in sterile H_2O.
4. Isobutyl-methylxanthine, 5 mM in methanol (this solution may require gentle heating to completely dissolve).
5. Indomethacin, 30 mM in methanol.

6. Transforming growth factor-β3 (TGF-β3), 10 μg/mL.
7. Bone morphogenic protein-6 (BMP-6), 10 μg/mL.
8. Proline, 40 mg/mL.
9. Sodium pyruvate, 100 mg/mL.
10. Epidermal growth factor (EGF), 20 μg/mL.
11. Basic fibroblast growth factor (bFGF), 20 μg/mL.
12. Adipogenic differentiation medium: 5 μM dexamethasone, 0.5 μM isobutylmethylxanthine, and 50 μM indomethacin in stromal medium (Table 1). Filter through a 0.2-μm membrane and store at 4°C. Use within 3 weeks.
13. Oil Red-O staining solution: 0.5 g Oil Red-O in 100 mL of isopropanol, to make a 0.5% stock solution. Filter through a 0.2-μm membrane, and store at room temperature. Immediately before use, combine three parts of 0.5% stock solution and two parts PBS. Wait 10 min before staining. Use within 24 h.
14. Osteogenic differentiation medium: 1 nM dexamethasone, 20 mM β-glycerolphosphate, and 50 μM ascorbate 2-phosphate in stromal medium (Table 1). Filter through a 0.2-μm membrane, and store at 4°C. Use within 3 weeks.
15. Alizarin Red staining solution: 1 g of Alizarin Red in 100 mL of sterile H_2O, adjust the pH to 4.1–4.3 using 0.1% ammonium hydroxide. Filter through a 0.2-μm membrane and store at room temperature.
16. Chondrogenic differentiation medium: 1 μM dexamethasone, 50 μg/mL ascorbate 2-phosphate, 50 μg/mL ITS + premix (Becton Dickinson: 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL bovine serum albumin (BSA), 5.35 mg/mL linoleic acid), 100 μg/mL sodium pyruvate, 40 μg/mL proline, 10 ng/mL TGF-β3, and 500 ng/mL BMP-6 in DMEM with high glucose (Table 1). Use immediately (see Note 7).
17. Toluidine blue staining solution: 1 g of toluidine blue in 1% sodium borate (1 g/100 mL sterile H_2O, dissolve until water is clear). Filter through a 0.2-μm membrane. Store tightly capped in an amber bottle at room temperature. Use within 1 month.
18. Neurosphere medium: Neurobasal medium supplemented with B27, 20 ng/mL bFGF, and 20 ng/mL EGF (Table 1). Use within 3 weeks.
19. Neural differentiation medium: Neurobasal medium with B27 supplement (Table 1). Use within 3 weeks.

Table 1
Differentiation media recipes

	Stock concentration	Final concentration	Volume
Adipogenic differentiation medium			
Stromal medium			200 mL
Dexamethasone	10 mM	5 μM	100 μL
Isobutylmethylxanthine	5 mM	0.5 μM	20 μL
Indomethacin	30 mM	50 μM	333 μL
Osteogenic differentiation medium			
Stromal medium			192 mL
Dexamethasone[a]	1 mM[a]	1 nM[a]	20 μL[a]
β-glycerolphosphate	0.5 M	20 mM	8 mL
Ascorbate 2-phosphate	50 mM	50 μM	200 μL
Chondrogenic differentiation medium			
DMEM, high glucose			50 mL
Ascorbate 2-phosphate	50 mg/mL	50 μg/mL	50 μL
Proline	40 mg/mL	40 μg/mL	50 μL
Sodium pyruvate	100 mg/mL	100 μg/mL	50 μL
ITS Premix			500 μL
Medium without cytokines (above) can be stored at 4°C for 3 weeks Medium with cytokines (below) should be prepared fresh before each use			
Medium w/o cytokines			10 mL
Dexamethasone	1 mM	0.1 μM	1 μL
TGF-β3	10 μg/mL	10 ng/mL	10 μL
BMP-6	10 μg/mL	500 ng/mL	500 μL
Neural differentiation medium			
Neurobasal medium			50 mL[b]
B27 supplement			1 μL[b]
bFGF	20 μg/mL	20 ng/mL	50 μL
EGF	20 μg/mL	20 ng/mL	50 μL

[a]Dilute the 1 mM stock dexamethasone 1:100 in diH_2O, and add 20 μL of the diluted solution
[b]Medium for neural lineage differentiation medium consists of neurobasal medium with B27 supplement only

3. Methods

3.1. Preparation

1. All media, dishes, tubes, and cells should be kept sterile, and therefore all work should be performed under a biosafety hood and sterile techniques should be used.
2. Investigators should be trained in the handling of rhesus tissues and blood borne pathogens prior to initiation of any use of animal tissue.
3. Before beginning the procedure, wash buffer, collagenase solution, erythrocyte lysis buffer, and stromal medium should be warmed in a 37°C water bath.
4. A tissue culture incubator at 37°C, 5% CO_2 should be available for sample incubation.
5. After transportation to the laboratory, the adipose tissue sample can be kept at room temperature for no more than 24 h prior to use.

3.2. rASC Isolation and Culture

1. Wash the tissue sample extensively in wash buffer to remove any contaminating hematopoietic cells. This can be done in a disposable conical tube, or by passing the tissue through several sterile dishes of wash buffer. In order to maintain sterility, all tubes and dishes should only be opened under a biosafety hood.
2. Place the tissue sample in a clean dish with warm collagenase solution to begin tissue digestion. Carefully mince the tissue with two sterile scalpels. Pipette the minced tissue and solution up and down several times with a 25- or 50-mL serological pipette to facilitate digestion. Incubate the sample for 30 min at 37°C in 5% CO_2.
3. After 30 min, neutralize the collagenase enzyme by adding approximately 5 mL of stromal medium. Pipette the solution to disintegrate any aggregates of adipose tissue, and transfer to a sterile 50-mL tube. Any solid pieces of tissue that may be present can be excluded (see Note 8). Centrifuge the sample at 2,000 rpm (~650 × *g*) for 5 min.
4. After centrifugation, there will be a pellet of the SVF. The SVF will usually include a layer of dark red cells. The rASCs are in the SVF, while mature adipocytes will be floating in the supernatant. To complete the separation of the stromal cells from the primary adipocytes, shake the tube vigorously enough to thoroughly disrupt the pellet and mix the cells. Centrifuge again, at 2,000 rpm (~650 × *g*) for 5 min, and then carefully remove the supernatant without disturbing the cell pellet. Leave behind a small volume of collagenase solution above the pellet so that the cells are not disturbed.

5. Resuspend the pellet in 1 mL of erythrocyte lysis buffer, and incubate on ice for 10 min. This will lyse any red blood cells that may be remaining in the cell pellet. After 10 min, add 20 mL of wash buffer, centrifuge at 2,000 rpm (~650 × *g*) for 5 min, and discard the supernatant.
6. Resuspend the cell pellet in 2–3 mL of stromal medium, and filter through a 70-μm cell strainer. Rinse the strainer with an additional 2 mL of stromal medium to obtain any rASCs that may have gotten stuck in the filter. The cell strainer will remove any tissue fragments or large debris that may be left in the suspension. The strainer can be place on top of a sterile 50-mL tube or held over a sterile culture dish to collect the rASCs.
7. Plate the rASC cell suspension onto cell culture-treated multiwell plates, and incubate at 37°C in 5% CO_2. The size and number of plates required will depend on the amount of starting material. In general, one well of a 24-well plate is sufficient for approximately 150–250 mg of starting adipose tissue, or one well of a 12-well plate for 500 mg of tissue (see Note 9).
8. Seventy-two hours after plating, remove the dishes from the incubator and check the cells under a microscope (see Note 10). If the cells appear healthy, aspirate the stromal medium from the well, and wash the cell layer with warm PBS or wash buffer. Pipette the PBS over the cell layer several times in order to remove any tissue fragments and/or blood cells. Aspirate off the PBS and add a volume of fresh, warm stromal medium. The volume will depend on the capacity of the well. Maintain the cells in a humidified tissue culture incubate at 37°C in 5% CO_2. Change the medium every second day until the cells reach 80–90% confluence. At that point, the cells can be harvested for expansion and freezing or directly induced to differentiate.
9. To harvest rASCs, rinse the cell layer by adding a small volume of sterile, warm PBS to the well. Aspirate the PBS, and replace with just enough trypsin–EDTA solution to cover the entire surface of the dish. The volume will depend on the size of the dish. Incubate for at least 5 min at 37°C in 5% CO_2, or until approximately 90% of the cells have detached from the bottom of the dish. Progress can be monitored under a microscope. Add an equal volume of stromal medium to the well. The serum in the medium will neutralize the enzyme and terminate the reaction (see Note 11). Pipette the cell suspension around the well a few times to detach any cells that may be stuck and transfer the suspension to a sterile centrifuge tube. Centrifuge at 1,200 rpm (~240 × *g*) for 5 min. Discard the supernatant and resuspend the cells in a small volume of

stromal medium. Count the cells using the hemocytometer, and proceed with any of the following options.

10. Cryopreservation of rASCs: rASCs should be collected at 80% confluence for freezing. Harvest and count the cells. After counting, suspend the pellet in room-temperature freezing medium at a concentration of $1–2 \times 10^6$ cells/mL. Dispense 1-mL aliquots of the cell suspension to sterile, labeled cryovials. Place cryovials in appropriate freezing apparatus (alcohol container), and place the apparatus in a −80°C freezer. The freezing container will cool the vials slowly, at approximately 1°C every minute, until they reach −80°C. The next day, transfer the cells on dry ice or other frozen material to a liquid nitrogen storage container.
11. Preparation of rASCs for flow cytometry: Harvest approximately 1.5×10^6 rASCs and centrifuge at $300 \times g$ for 5 min at room temperature in a 50-mL tube. Wash the rASCs twice with 10 mL of cold PBS (Ca^{2+} and Mg^{2+} free) and resuspend cells in 500 µl of cold PBS. Aliquot 50 µl of cells into ten 1.5-mL microcentrifuge tubes, add 50 µl of PBS containing a fluorochrome-conjugated monoclonal antibody or isotype control antibody (usually 5–10 µL) 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 the cells with 1 mL of PBS with 1% BSA and centrifuge at $300 \times g$ for 3 min at room temperature to pellet the cells. Repeat the PBS wash three times. After the third wash, resuspend the cells in 500 µL of 1% formaldehyde in PBS to fix the cells. Keep the tubes at 4°C, protected from light exposure, until they can be analyzed on a flow cytometer. Cells should be analyzed within 48 h. See Chapter 10, this volume.
12. Expansion of rASCs: After harvesting and counting cells, replate the cells at approximately 10,000 cells/cm^2. Suspend the pellet in stromal medium following the concentrations listed in Table 2 for dishes of various sizes.

Table 2
rASC plating concentrations

Plate	Area/well (cm^2)	Area/plate (cm^2)	Cells/cm^2	Cells/well	Cells/plate	Media/well (mL)
6 well	10	60	10,000	100,000	600,000	2.5
24 well	2	48	10,000	20,000	480,000	1
96 well	0.33	32	10,000	3,300	316,800	0.2

3.3. rASC Differentiation

1. Adipogenic differentiation of rASCs: Plate 1×10^5 cells per well of a 6-well plate in 2 mL of stromal medium. A lower density can be used if there are not enough cells, as long as each well is seeded at the same density. In a 6-well plate, it is convenient to have two wells for adipogenic differentiation, two wells for osteogenic differentiation, and two wells as controls that will not be cultured in differentiation medium, but will be stained with the others as a negative control. Monitor the cells every day for confluency. Every third day, before the cells reach 100% confluency, remove the stromal medium, rinse the cell layer with wash buffer or PBS, and add fresh stromal medium. After the cells have reached 100% confluency (usually in 2–8 days), remove the stromal medium from the experimental wells, rinse the cell layer with wash buffer or PBS, and add 2 mL of adipogenic differentiation medium (see Note 12). Add stromal medium to the control well. Replace the medium every 3 days for approximately 14–21 days (see Note 13). Mature adipocytes are usually observed within approximately 14 days (see Note 14).

   After 14 days of differentiation, or when lipid vacuoles can be seen, the cells can be fixed with 10% formalin, 4% paraformaldehyde, or 70% ethanol. It should be noted that, when 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 for 1 h, 4% paraformaldehyde for 10 min, or 70% ethanol for 1 h. Remove the fixative, rinse with PBS, and proceed to staining. Fixed cells can be stored in PBS at 4°C for as long as several months, although shorter times are recommended (see Note 15).

   To stain the adipocytes, add 2 mL of Oil Red-O solution to each well, and incubate for 20–60 min at room temperature. Rinse the wells with 2 mL of PBS three times, or until the background is clear. The rinse should become completely clear, with no red coloring. Do not rinse with a larger volume; this may raise the level of the solution in the plastic well and cause staining of the wall of the well. After sufficient washing, add 2 mL of PBS to each well. Examine the cells under an inverted microscope for evidence of fat differentiation.

   Alternatively, stain can be eluted from the cells by adding isopropanol. Elution is immediate. The plate can then be analyzed on a plate reader. Read the OD_{540} and subtract the background staining determined in control wells from the experimental points.

2. Osteogenic differentiation of rASCs: rASCs can be induced to become osteogenic lineage cells in the same manner as adipogenic lineage cells, substituting osteogenic differentiation medium for adipogenic differentiation medium. As for

adipogenesis, the medium is replaced every 3 days for approximately 14–21 days until mineralization is achieved.

After 14 days of differentiation, or when mineralization can be seen, the cells can be fixed in the same manner as the adipocytes. Fixed cells can be stored in sterile H_2O at 4°C for as long as several months, although shorter times are recommended (see Note 15).

Stain for mineralization in the same manner as for adipocytes with Alizarin Red solution and sterile H_2O washes. After sufficient washing, add 2 mL of sterile H_2O to the wells. Examine the cells under an inverted microscope for evidence of bone differentiation (see Note 16).

3. Chondrogenic differentiation of rASCs: For chondrogenic differentiation, the pellet culture system described by Sekiya et al. can be used (18). After harvesting and counting the cells, pellet 2×10^5 cells in a 15-mL conical tube and discard the supernatant. Resuspend the cell pellet in 0.5 mL of complete chondrogenic differentiation medium, and pellet the cells again. Incubate the tube at 37°C in 5% CO_2, and change the medium every 2–3 days for 21 days. It is important to leave the caps loosely on top of the tubes rather than tightening them, in order for the medium to maintain a proper pH. When adding fresh medium, flick the bottom of the tube to dislodge the pellet.

   After 21 days, wash the pellet with PBS and fix in 10% neutral-buffered formalin. The pellets can be embedded in paraffin and sectioned. The sections can then be stained with Toluidine Blue.

4. Neural differentiation: Undifferentiated rASCs cultured at high density will spontaneously form spherical clumps of cells that can be isolated and cultured in Neurobasal medium supplemented with B27 and growth factors on low-attachment dishes (10). The spheres must be maintained at a density of 10–20 cells/cm^2 to prevent self-aggregation. After 4–7 days, the cells can be induced to neural lineage differentiation. Transfer the neurospheres to PDL-laminin coated chamber slides and culture in Neurobasal medium with B27 supplement only. Replace approximately 70% of the medium every 4 days. After 10 days, the cells can be assessed for neural lineage differentiated by immunocytochemistry and RT-PCR for the expression of neuronal- or glial-associated protein and mRNA (see Notes 17 and 18).

4. Notes

1. All animal procedures must conform to the requirements of the Animal Welfare Act and be approved before implementation by the Institutional Animal Care and Use Committee (IACUC) of the institution.

2. Prior to the implementation of this protocol, all personnel involved in the processing of rhesus tissue or primary rASC cultures should complete safety training for the hazards of blood-borne pathogens. Regardless of whether animals have been screened for evidence of infection by any transmissible agents, these precautions are mandatory. In addition, no glass containers or pipettes should be used, and the use of sharp objects (scissors, needles) during the processing steps should be minimized. All procedures involving the rhesus tissue or cells should be conducted in a biological safety cabinet and with appropriate personnel protection equipment.
3. 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 the plate in a humidified, 37°C, 5% CO_2 incubator. After a few days, 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.
4. Before purchase, the fetal bovine serum should be assayed to test for its ability to support cell proliferation and differentiation.
5. The stock solutions can be prepared ahead of time, aliquoted, and stored at −80°C for up to 1 year. Do not filter each stock, filter at the time of media preparation. Some filter materials may be sensitive to specific solvents (e.g., DMSO, methanol) and may disintegrate upon exposure, therefore it is safer to filter stock solutions after they are diluted in the medium. Growth factors should be diluted in recommended solvents, according to manufacturer's instructions. Thawed aliquots should not be refrozen.
6. Water-soluble dexamethasone is supplied encapsulated in 2-hydroxypropyl-β-cyclodextrin. The amount of dexamethasone varies depending on the lot, but it is approximately 60–70 mg dexamethasone/g of material. That means that 100 mg of dexamethasone is actually supplied in approximately 1.5 g of material. This must be taken into account when making up the stock solutions.
7. Medium without cytokines (ascorbate 2-phosphate, proline, and pyruvate stocks plus ITS + premix in high-glucose DMEM) can be prepared and stored for 3–4 weeks at 4°C. TGF-β3, dexamethasone, and BMP-6 stocks can then be added to make complete adipogenic differentiation medium (Table 1). The complete medium should be made fresh as needed.
8. If, after a 1-h digestion, there are some pieces of undigested tissue in the sample, make sure that the collagenase solution is fresh and has not been maintained at room temperature for an extended period of time. 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 can be slowly thawed at room temperature and warmed to 37°C. It is not necessary to have complete digestion before proceeding with the protocol if only a small amount of tissue fragments is observed in the solution.

9. To accelerate cell adhesion, the culture dishes can be precoated with extracellular matrix proteins, such as gelatin or Matrigel.
10. If the cells do not grow very well and do not appear healthy, there are a few options. Supplementation of the culture medium with 20–30% conditioned medium (saved from previous cultures) may facilitate the growth of the cells. If conditioned medium is not available, another alternative is to increase the FBS concentration in the stromal medium; 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. Also, it is important to note that the percent of preadipocytes obtained from the SVF after digestion is donor dependent.
11. It is important to not overexpose the cells to the trypsin–EDTA solution. This could decrease the cell viability.
12. To prevent the adipocytes from detaching, do not dry the well when changing the medium since adipocytes tend to float when new medium is added.
13. According to the protocol, the medium is changed every 3 days. However, as the adipocytes mature, the medium may turn yellow within a day, which may be due to a drop in pH. As the pH falls from 7 to 6.5, cell growth will decline and cell viability decreases at pH between 6.5 and 6. Monitor the pH of the medium, going from red (pH 7) through yellow ($pH \leq 6$), and change the medium more often if needed.
14. Several factors can contribute to poor differentiation of rASCs. The differentiation process is donor 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 animals. To further enhance adipogenesis, the following alternatives can be tried:

    Different PPARγ agonists (troglitazone or pioglitazone, among others) in the medium may enhance differentiation.

    The addition of 5% rabbit serum (RS) can be added to the differentiation medium, as the ethyl acetate contain in the RS has been found to be 35-fold more abundant than in FBS (19).

Another alternative is 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 adipocyte maintenance medium. Repeat this cycle until mature adipocytes are obtained.

15. Cells that have been stained in Oil Red-O and Alizarin Red can be kept after washing for extended periods to allow for microscopic analysis. Add more PBS (for Oil Red-O wells) or water (for Alizarin Red wells) as they evaporate.
16. Confluent culture in bone differentiation plates can tend to lift in sheets or form a ball. The ball can be sliced and made into slides for microscopic analysis for mineral deposition.
17. Contamination – microbial. In order to keep optimal sterile conditions, it is recommended that you open and close the container properly to avoid any potential contaminants. 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 tissue thoroughly.
18. 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 wash thoroughly the cells with PBS. 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 essentially free of EC. Therefore a filtration procedure can be performed by using a nylon mesh filter with a small pore size (25 μm).

References

1. Rangwala, S. M. and Lazar, M. A. (2000) Transcriptional control of adipogenesis. *Annu Rev Nutr* **20**, 535–59.
2. Deslex, S., Negrel, R., Vannier, C., Etienne, J., and Ailhaud, G. (1987) Differentiation of human adipocyte precursors in a chemically defined serum-free medium. *Int J Obes* **11**, 19–27.
3. Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Ailhaud, G., Negrel, R., and Pfeiffer, E. F. (1989) Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. *J Clin Invest* **84**, 1663–70.
4. Halvorsen, Y. D., Bond, A., Sen, A., Franklin, D. M., Lea-Currie, Y. R., Sujkowski, D., Ellis, P. N., Wilkison, W. O., and Gimble, J. M. (2001) Thiazolidinediones and glucocorticoids synergistically induce differentiation of

human adipose tissue stromal cells: biochemical, cellular, and molecular analysis. *Metabolism* **50**, 407–13.

5. Zuk, P. A., Zhu, M., Ashjian, P., De Ugarte, D. A., Huang, J. I., Mizuno, H., Alfonso, Z. C., Fraser, J. K., Benhaim, P., and Hedrick, M. H. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279–95.
6. Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P., an\d Hedrick, M. H. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* **7**, 211–28.
7. Guilak, F., Lott, K. E., Awad, H. A., Cao, Q., Hicok, K. C., Fermor, B., and Gimble, J. M. (2006) Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. *J Cell Physiol* **206**, 229–37.
8. Gimble, J. M., Katz, A. J., and Bunnell, B. A. (2007) Adipose-derived stem cells for regenerative medicine. *Circ Res* **100**, 1249–60.
9. Mitchell, J. B., McIntosh, K., Zvonic, S., Garrett, S., Floyd, Z. E., Kloster, A., Halvorsen, Y. D., Storms, R. W., Goh, B., Kilroy, G. S., Wu, X., and Gimble, J. M. (2006) The immunophenotype of human adipose derived cells: Temporal changes in stromal- and stem cell-associated markers. *Stem Cells* **24**, 376–85.
10. Kang, S. K., Putnam, L. A., Ylostalo, J., Popescu, I. R., Dufour, J., Belousov, A., and Bunnell, B. A. (2004) Neurogenesis of rhesus adipose stromal cells. *J Cell Sci* **117**, 4289–99.
11. Izadpanah, R., Trygg, C., Patel, B., Kriedt, C., Dufour, J., Gimble, J. M., and Bunnell, B. A. (2006) Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. *J Cell Biochem* **99**, 1285–97.
12. Rodbell, M. (1966) Metabolism of isolated fat cells. The similar effects of phospholipase C (*Clostridium perfringens* alpha toxin) and of insulin on glucose and amino acid metabolism. *J Biol Chem* **241**, 130–9.
13. Rodbell, M. (1966) The metabolism of isolated fat cells. IV. Regulation of release of protein by lipolytic hormones and insulin. *J Biol Chem* **241**, 3909–17.
14. Rodbell, M. and Jones, A. B. (1966) Metabolism of isolated fat cells. III. The similar inhibitory action of phospholipase C (*Clostridium perfringens* alpha toxin) and of insulin on lipolysis stimulated by lipolytic hormones and theophylline. *J Biol Chem* **241**, 140–2.
15. Van, R. L., Bayliss, C. E., and Roncari, D. A. (1976) Cytological and enzymological characterization of adult human adipocyte precursors in culture. *J Clin Invest* **58**, 699–704.
16. Bjorntorp, P., Karlsson, M., Pertoft, H., Pettersson, P., Sjostrom, L., and Smith, U. (1978) Isolation and characterization of cells from rat adipose tissue developing into adipocytes. *J Lipid Res* **19**, 316–24.
17. Hauner, H., Wabitsch, M., and Pfeiffer, E. F. (1988) Differentiation of adipocyte precursor cells from obese and nonobese adult women and from different adipose tissue sites. *Horm Metab Res Suppl* **19**, 35–9.
18. Sekiya, I., Colter, D. C., and Prockop, D. J. (2001) BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells. *Biochem Biophys Res Commun* **284**, 411–8.
19. Diascro, Jr., D. D., Vogel, R. L., Johnson, T. E., Witherup, K. M., Pitzenberger, S. M., Rutledge, S. J., Prescott, D. J., Rodan, G. A., and 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**, 96–106.

Chapter 2

Isolation of Human Adipose-Derived Stem Cells from Lipoaspirates

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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.

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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-μm 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. CO_2 Incubator.

2.4. Media Stock Solution (see Note 2)

All the media solutions are filtered through a 0.2-μm 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 ($CaCl_2$). 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 (NH_4Cl), 10 mM potassium carbonate (KCO_3), 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× 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 PPARγ 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 PPARγ2 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 PPARγ 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-cm^2 flask (0.16 ml tissue/cm^2), 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-cm^2 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 × *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×*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×*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×*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/cm^2 of surface area (volume of ~33 ml of tissue for a 175-cm^2 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-cm^2 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 µl of suspended cells to 75 µl 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 ~1.5×10^6 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 µl cold PBS. Aliquot 50 µl of cells into ten 1.5-ml microcentrifuge tubes, add a 50-µl volume of PBS containing a fluorochrome-conjugated monoclonal antibody or isotype control antibody (usually 5–10 µl) 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 µl 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 µl

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 µl Oil Red-O to each well for 15 min at room temperature. Rinse three times or more with 50 µl distilled water. The rinse should become completely clear (no red coloring). Do not rinse with a volume larger than 50 µl. 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 µl isopropanol per well. Elution is immediate. Read the OD_{540} 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-μm 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-μm 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/cm^2 on gelatin-coated 175-cm^2 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 ($pH \leq 6$), 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 PPARγ 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.

Chapter 3

Isolation of Murine Adipose-Derived Stem Cells

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Abstract

Murine models of obesity or reduced adiposity are a valuable resource for understanding the role of adipocyte dysfunction in metabolic disorders. Primary adipocytes grown in culture and derived from murine adipose tissue are essential for studying the mechanisms underlying adipocyte development and function. Herein, we describe methods for the isolation, expansion, and long-term storage of murine adipose-derived stem cells along with a protocol for inducing adipogenesis in this cell population.

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

1. Introduction

Murine mesenchymal stem cells (MSCs) are commonly used in basic research and have the capacity to undergo adipogenic, osteogenic, chondrogenic, myogenic, and neuronal differentiation in vitro (1–4). MSCs were originally characterized from bone marrow, but are also found in adipose tissue. Harvesting MSCs from adipose tissue (termed adipose-derived stromal/stem cells; muASCs) has several advantages over acquiring bone marrow ASCs, including better accessibility and the greater abundance of the MSCs in adipose tissue. This protocol describes the isolation and expansion of muASCs and the differentiation of the muASCs into adipocytes in vitro based on the method developed by Halvorsen et al. in human ASCs (5).

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2. Materials

2.1. Tissue

Adipose tissue: epididymal/gonadal (surrounding the testes), inguinal, retroperitoneal (surrounding organs in abdominal cavity, one fat pad each side), mesenteric (surrounding intestines, very diffuse).

2.2. Supplies

1. 50-ml plastic centrifugation bottles, autoclave to sterilize.
2. 0.2-μm filter units.
3. 50-ml conical tubes, sterile.
4. 2-ml microtubes, autoclave to sterilize.
5. 75-cm^2 tissue culture (T75) flasks.
6. Scissors and forceps, autoclave to sterilize.
7. Hemocytometer.
8. Freezing apparatus.

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. CO_2 incubator.

2.4. Reagents and Buffers

1. 70% Ethanol.
2. Isopropanol.
3. Dimethyl sulfoxide.
4. Dulbecco's Modified Eagle Medium – high glucose.
5. Fetal bovine serum/calf serum, supplemented with iron.
6. Collagenase type 1.
7. Penicillin/streptomycin/amphotericin antibiotic/antimycotic solution.
8. 0.5% Trypsin/EDTA solution.
9. Biotin.
10. D-Pantothenate.
11. Dexamethasone.
12. Rosiglitazone.
13. Methylisobutylxanthine.
14. Insulin.

15. Oil Red-O.
16. Trypan Blue.
17. 10% Buffered formalin.

All buffers are sterile filtered using a 0.2-μm filter unit.

1. 5% Iodine solution: 5% iodine in Krebs–Ringer bicarbonate buffer (KRB) or phosphate-buffered saline (PBS) (see Note 1).
2. Krebs–Ringer bicarbonate buffer (KRB): 118 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 2 mM KH_2PO_4, 2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5 mM glucose, pH 7.4.
3. Phosphate-buffered saline (PBS): 37 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4.
4. 1% Bovine serum albumin (BSA, fraction V) in PBS: sterile filtered using a 0.2 μm filter.
5. Collagenase solution: Weigh out 0.1 g of type I collagenase and 1 g of powdered BSA (fraction V). Dissolve these in 100 ml of PBS supplemented with 2 mM $CaCl_2$. After sterile filtration, warm the solution to 37°C. This solution should be used within 1 h of its preparation.

2.5. Media Solutions

All solutions are sterile filtered using a 0.2-μm filter unit.

1. *Stromal medium*: DMEM/high glucose with 10% fetal bovine serum, 1× antibiotic/antimycotic solution from 100× stock (penicillin/streptomycin/amphotericin).
2. *Differentiation medium*: In advance, prepare and aliquot the following stock solutions and store frozen at –20°C until required. (1) A 66-mM stock solution of biotin (2,000-fold concentration) dissolved in 1 N sodium hydroxide. (2) A 34-mM stock solution of D-pantothenate (2,000-fold concentration) dissolved in water. (3) A 1-mM dexamethasone solution (1,000-fold concentration) dissolved in water or ethanol, depending on its formulation (see Note 2). (4) A 5-mM stock solution of rosiglitazone or equivalent PPARγ agonist dissolved in dimethyl sulfoxide (1,000-fold concentration).

   Prepare fresh before use: (1) A 500-mM stock solution of methylisobutylxanthine (1,000-fold concentration) dissolved in 50 mM NaOH. (5) A 200-μM stock solution of insulin (2,000-fold concentration) dissolved in 10 mM HCl, made in a plastic tube, *not in glass*.
3. *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 PPARγ agonist; these two stock solutions should be omitted. Use this solution within 2 weeks of its preparation.

4. *Freezing medium*: The freezing medium consists of 80% calf serum, 10% DMEM/high glucose, and 10% dimethyl sulfoxide. Use this solution within 2 weeks of its preparation.
5. *Oil Red-O staining solution*: Weigh out 0.7 g Oil Red-O. Dissolve in 200 ml isopropanol. Filter through a 0.2-μm filter. Store at room temperature as a stock solution. At the time of use, make a 60% Oil Red-O solution in distilled water by adding 30 ml of the Oil Red-O stock to 20 ml distilled water. Mix and let stand at room temperature for 30 min before use and filter again if particulates are present. Use this solution within 24 h of its preparation.

3. Methods

3.1. Obtaining the Adipose Tissue

All procedures are performed in a biosafety hood.

1. Ensure the muASC isolation procedure is initiated within 20 min of tissue extraction, especially if a large number of animals are being completed in 1 day.
2. Warm buffers (at least one to two of 500 ml KRB, PBS, or PBS with 1% BSA) along with the Collagenase solution and Stromal media in the 37°C water bath (see Note 1).
3. Place bench protector down in hood.
4. Sacrifice animal using anesthesia or CO_2 asphyxiation as approved by the AVMA Guidelines on Euthanasia. Saturate the fur with 70% ethanol and place the animal ventral side up.
5. Using sterile tissue forceps, pull up on the skin and cut the skin vertically toward the head.
6. Using sterile scissors and forceps, open the abdominal cavity and remove the desired fat pads.
7. Place the tissue in a preweighed 50-ml tube. Note the tissue weight; it will be used to determine the number of flasks needed for the stromal vascular cells (SVF) in Subheading 3.3, step 6.

3.2. Processing the Adipose Tissue

1. Add enough KRB or 5% iodine solution in KRB (see Note 1) to cover the tissue, and incubate at room temperature for 2–4 min. If using the 5% iodine solution, remove the iodine by rinsing the tissue one to two times with KRB.
2. Mince the adipose tissue into small pieces using sterile scissors.
3. Place approximately 25 ml of the minced tissue into sterile 50-ml centrifuge bottles.

4. Add an equivalent volume of collagenase solution per adipose tissue volume to each centrifuge bottle (i.e., 10 ml collagenase solution per 10 ml adipose tissue).
5. Cap and wipe the bottles with 70% ethanol, dry, then wrap with Parafilm before placing the bottles in a 37°C shaking water bath. Shake at 75 rpm for 45–90 min until the tissue appears smooth on visual inspection (see Note 3). If a shaking water bath is not available, swirl the bottles every 5–10 min in the water bath.

3.3. Isolate the Stromal Vascular Fraction (SVF)

1. After the collagenase digestion, centrifuge the tubes at 300 × *g* at room temperature for 5 min. Then take the tubes out of the centrifuge and shake them vigorously for 5–10 s to thoroughly mix the cells. This will ensure complete separation of the stromal cells from the primary adipocytes. Centrifuge again at 300 × *g* at room temperature for 5 min.
2. Carefully aspirate the oil on top and the primary adipocytes, which will appear as a yellow layer of floating cells (see Note 4). Leave approximately 10 ml of the brown collagenase solution so that the SVF is not disturbed. The SVF will be apparent as the dark red cells pelleted on the bottom.
3. Resuspend the cells in prewarmed PBS with 1% BSA.
4. Centrifuge the resuspended cells at 300 × *g* for 5 min and carefully aspirate the remaining collagenase solution. When aspirating, the tip of the pipette should aspirate from the top so that the oil is removed as much as possible. The stromal cell pellet should be tightly packed at the bottom of the tube.
5. Add 10 ml of stromal media to each centrifuge tube and resuspend the cells. If there are more than one tube, pool the cells into one tube and centrifuge again as in Subheading 3.3.4.
6. Prepare one T75 flask per 40 g tissue harvested. Add 10 ml stromal media to each flask.
7. Divide the cells equally among the appropriate number of flasks.
8. 48 h after plating, aspirate the media from the flask, rinse the cells with prewarmed PBS, and aspirate. Check the cells using the inverted microscope; if excess unattached cells remain after the first PBS rinse, repeat the rinse and aspirate.
9. Add fresh stromal media.
10. The media is changed every 2–3 days until the cells are 80–90% confluent. The cells may begin to detach if the media is removed completely; leave a small amount of media on the cells with each media change. At 80–90% confluence, the cells are either passaged, stored as frozen stocks as passage one (P1), or differentiated.

3.4. Harvesting Cells

1. Remove media from flasks and rinse with 10 ml of prewarmed sterile PBS. Aspirate the PBS and add 2 ml of 0.5% trypsin/EDTA solution. Incubate in a 37°C incubator for 5–10 min. Do not allow the cells to remain in contact with the trypsin/EDTA solution longer than is necessary to detach the cells. When more than 90% of the cells have detached (verify using the inverted microscope), add 10 ml of stromal media to the cells.
2. Transfer the resuspended cells to a sterile 50-ml tube and centrifuge at $300 \times g$ for 5 min at room temperature. Aspirate the media and resuspend the pelleted cells in 1–2 ml of stromal media. Use an aliquot of the cells, diluted with Trypan Blue (a 1:2 or 1:4 dilution is usually convenient) to count the cells using a hemocytometer.

3.5. Replating, Cryopreserving, or Adipcoyte Differentiation of the muASCs

1. *Replating the cells*: Suspend the cells in stromal media and replate at 5×10^3 cells/cm^2 in T75 flasks (see Note 5).
2. *Cryopreservation of the cells*: Centrifuge the cells once more to obtain a cell pellet. Resuspend the pelleted cells in room-temperature freezing media at a concentration of $1–2 \times 10^6$ cells/ml. Dispense 1-ml aliquots of the resuspended cells to sterile cryovials. Place the vials in the freezing apparatus and place at –80°C overnight. Transfer to long-term storage the next day.
3. *Differentiation to adipocytes*: When the cells reach approximately 90% confluency, the muASCs are ready to differentiate. Exchange the stromal media for the differentiation media and leave on the cells for 3 days. On day 3 postinduction, replace the differentiation media with the adipocyte maintenance media. The adipocyte maintenance media will be changed every 2–3 days, and mature adipocytes should be formed by day 9–12 postinduction.

3.6. Cell Fixation and Oil Red-O Detection of Neutral Lipids

1. *Cell fixation*: Once mature adipocytes have formed, the cells can be used for further analysis or experiments. To fix the cells for Oil Red-O staining, the media is removed, the cells are rinsed in PBS, and the PBS is aspirated before adding an amount of 10% buffered formalin sufficient to completely cover the cells. Incubate the cells in the formalin for a minimum of 1 h at room temperature or overnight at 4°C. The cells can remain in the formalin for a longer time, but this is not recommended unless it is necessary to collect multiple plates over several days before staining.
2. *Oil Red-O staining*: Add sufficient Oil Red-O to each plate or well to completely cover the fixed cells. Gently rock at room temperature for 1 h, and then rinse at least three times with distilled water. At this point, the cells can be imaged

using a microscope/camera. If desired, the amount of Oil Red-O staining can be quantified by removing the distilled water and adding 100% isopropanol. Add 0.375 ml of 100% isopropanol/cm^2 (a 6-well plate is 9.6 cm^2/well; a 12-well plate is 4.0 cm^2/well; and a 24-well plate is 2 cm^2/well). As a blank, add an equal amount of isopropanol to an empty well. Elution of the Oil Red-O should be complete within 10–15 min at room temperature. Determine the absorbance of the mixture at 500 nm (or 540 nm if using selected UV filters), and subtract the blank measurement from the other absorbances.

4. Notes

1. Iodine is available at drug or grocery stores. If you are confident of your sterile technique, this step is optional. We recommend that you start without including iodine.
2. Dexamethasone is available in a water-soluble and water-insoluble forms from Sigma. For the water-insoluble form (Sigma D-4902), the 1-mM stock is made by adding 0.39 mg dexamethasome to 1 ml of 100% ethanol. For the water-soluble form (Sigma D-2915), there is 69 mg dexamethasone/100 mg total weight. To obtain a 1-mM stock solution using the water-soluble form, add 6.0 mg of the dexamethasone mixture to 1 ml distilled water.
3. The tissue can be filtered at this step to remove any remaining undigested pieces. Use a 100-μm filter (BD–Falcon) that is available for filtering small volumes. However, if it is desirable to collect the mature adipocytes at a later step, use a nylon filter (sterile) screen with a pore size of 250 μm.
4. As has been reported (6), it is possible to separate small and large mature adipocytes for further analysis by using a 75-μm pore size filter at this step. Rather than aspirating the oil and yellow layer containing the adipocytes, carefully remove the oil and yellow layer and filter (BD-Falcon has filters of various pore sizes that work well for small volumes). The flow-through will contain the smaller adipocytes and the solution remaining on top of the filter will contain adipocytes greater than 75 μm in size.
5. For reasons that are not quite clear, differentiation of the muASCs (and HuASCs) occurs in a higher percentage of the total number of cells when the cells are plated in smaller wells (i.e., the cells will better differentiate in a 12-well plate than a 6-well plate, although both are originally seeded at the same concentration of cells per square centimeter).

References

1. Kelly KA, Tanaka S, Baron R, Gimble JM. (1998) Murine bone marrow stromally derived BMS2 adipocytes support differentiation and function of osteoclast-like cells in vitro. *Endocrinology* 139:2092–2101.
2. Thompson DL, Lum KD, Nygaard SC, Kuestner RE, Kelly KA, Gimble JM, Moore EE. (1998) The derivation and characterization of stromal cell lines from the bone marrow of p53−/− mice: new insights into osteoblast and adipocyte differentiation. *J Bone Miner Res* 13:195–04.
3. Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM, Rice HE. (2002) Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochem Biophys Res Commun* 294:371–379.
4. Zheng B, Cao B, Li G, Huard J. (2006) Mouse adipose-derived stem cells undergo multilineage differentiation in vitro but primarily osteogenic and chondrogenic differentiation in vivo. *Tissue Eng* 12:1891–1901.
5. 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* 50:407–413.
6. Bluher M, Patti ME, Gesta S, Kahn BB, Kahn CR. (2004) Intrinsic heterogeneity in adipose tissue of fat-specific insulin receptor knock-out mice is associated with differences in patterns of gene expression. *J Biol Chem* 279:31891–31901.

Chapter 4

In Vitro Adult Rat Adipose Tissue-Derived Stromal Cell Isolation and Differentiation

Mandi J. Lopez and Nakia D. Spencer

Abstract

Mesenchymal stromal cell research has advanced significantly over the last decade, especially in the area of adult adipose-derived stem cells (ASCs). There are significant species differences in ASC harvest, as well as in vitro doubling time and differentiation. Selection of appropriate animal models for preclinical evaluations is critical for optimization and validation of ASC therapeutic effects. As such, in vitro studies are necessary precursors to in vivo ASC applications. Additionally, to elucidate environmental, disease, or trauma effects on native stromal cell populations, characterization of cells from unexposed individuals is necessary. Rats are an established immunocompetent small animal model for a large number of human disorders and toxin exposures in the majority of organ systems. Isolation and expansion techniques for rat ASCs continue to change with advances in technology and ASC biology. The information presented in this chapter is a summary of rat ASC harvest, isolation, quantification, and in vitro expansion.

Key words: Adult mesenchymal stromal cell, Fat, Adipose, Tissue engineering, Rat

1. Introduction

Stiles documented a morphologically unique phenotype of cells in human adipose tissue in 1975 (1), and, in 2001, Zuk (2) reported the plasticity of the cells, previously termed preadipocytes. Since these original discoveries of a multipotent cell population within adipose tissue, the isolation, expansion, and characterization of adult adipose-derived stromal (stem) cells (ASCs) has advanced significantly. ASCs have been isolated from a variety of species, and significant variations occur among species, although the cells from different species have similar characteristics in part. In order to utilize the best animal model for preclinical studies, detailed knowledge of in vitro ASC behavior is necessary to align the model with the human counterparts.

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The rat is an established immunocompetent small animal model for numerous human injury and disease states as well as environmental toxin exposure and substance abuse. Prior to phase II clinical trials, standard validation of the safety and efficacy of human mesenchymal stem cells is completed in athymic preclinical animal models. Although human cells are successfully applied in the models without complications of host versus graft disease, the models are limited by the lack of a competent immune system (3). Activation of inflammatory cascade components is necessary for normal tissue formation. Additionally, athymic animals are prone to subclinical infections and systemic illness. These limitations can potentially mask the role of mesenchymal stem cells in tissue regeneration and healing processes. Hence, immunecompetent animal models are critical to evaluate relevant biology analogous to clinical application.

Reports of cell isolation from rat adipose tissue date as far back as 1964 (4), prior to Friedenstein's (5, 6) description of bone marrow-derived mesenchymal stem cells. A number of tissues have been utilized for rat ASC harvest, including bone marrow (7), as well as epididymal, inguinal (8), omental (3), subcutaneous (9), and perirenal (10) fat depots. Relatively homogenous depots of brown and white fat occur in the rat, unlike many other mammalian species. In addition to distinct differences between white and brown fat (11), in vitro biology differs between depots of white fat (12). Primary ASC culture systems more closely reflect the in vivo characteristics of the native tissue than established cell lines, and are crucial to comparison of in vitro stem cell properties between species (7). A limitation that continues to confound the science of rat ASCs continues to be the lack of reagents to evaluate specific mesenchymal stem cell surface markers. Findings from ongoing research in this arena will further augment the rat as an immunocompetent small animal model.

The majority of adipose tissue harvested from standard elective lipoaspiration is white fat (13), so the focus of translational ASC investigations is primarily on white adipose tissue. Given differences in cellular behavior between white fat sources, consideration of adipose harvest location is warranted when comparing results between investigations. In addition to variations in isolation and culture techniques, differences between harvest locations also likely contribute to variability between investigations (10). As in humans, environment, signalment, and overall health confer properties to rat ASCs elucidated by in vitro investigations (12). The information summarized below is designed to provide methods for rat ASC harvest, isolation, quantification, and in vitro expansion.

2. Materials

2.1. Adipose Tissue Collection

1. Scalpel handles (#3).
2. Scalpel blades (#10).
3. Brown–Adson forceps.
4. 70% Ethanol.
5. 4-L graduated beaker.

2.2. ASC Isolation

1. Scalpel handle (#3).
2. Scalpel blades (#10).
3. Brown–Adson forceps.
4. Centrifuge tubes (15 mL).
5. Microcentrifuge tubes (1,000 µL).
6. Petri dishes.
7. Syringes (10 mL).
8. 0.22-µm sterile syringe filter.
9. Double-distilled water.
10. Phosphate-buffered saline (PBS).
11. Red cell lysis buffer.
12. Dulbecco's Modification of Eagles Medium + Ham's F12 medium (DMEM-Ham's F12).
13. 1% antibiotic/antimycotic solution: 100× penicillin–streptomycin–amphotericin B solution containing 10,000 IU/mL penicillin; 10,000 µG/mL streptomycin; and 25 µG/mL amphotericin B.
14. Characterized fetal bovine serum (FBS).
15. 0.4% Trypan Blue.
16. 0.1% Collagenase: collagenase type-1 and 1% bovine serum albumin (BSA) dissolved in double-distilled water. Filter sterilize with a 0.22-µm syringe filter. Warm to 37°C.
17. 1% BSA: Dissolve BSA in double-distilled water. Filter sterilize with a 0.22-µm filter. Warm to 37°C.
18. Complete stromal medium: DMEM-Ham's F12 supplemented with 1% antibiotic/antimycotic solution and 10% FBS.

2.3. Cell Culture, Maintenance, and Cryopreservation

1. Complete stromal medium.
2. T25 tissue culture flasks.
3. Trypsin–EDTA: store long term at –20°C.
4. Cryopreservation medium: 80% FBS, 10% DMEM-Ham's F-12, 10% dimethylsulfoxide (DMSO).

5. "Mr. Frosty" freezing container (alcohol container).
6. Cryovials.

2.4. Cell Doubling

1. PBS.
2. Trypsin–EDTA.
3. 0.4% Trypan Blue.
4. Hemocytometer.
5. Complete stromal medium.
6. 12-well culture plates.

2.5. Adipogenic Differentiation

1. DMEM-Ham's F-12 medium.
2. Characterized FBS (10%).
3. 1% Antibiotic/antimycotic solution.
4. Biotin (33 μmol/L).
5. Pantothenate (17 μM/L).
6. Insulin (1 μM/L).
7. Isobutylmethylxanthanine (IMBX) (0.5 mM/L).
8. Rosiglitazone (5 μM/L).
9. Dexamethasone (20 nM/L).
10. 1% Paraformaldehyde in PBS.
11. Oil Red-O.
12. Double-distilled water.

2.6. Osteogenic Differentiation

1. β-glycerol phosphate (10 mM/L).
2. Dexamethasone (10 nM/L).
3. Sodium 2-phosphate ascorbate (50 μg/mL).
4. Complete stromal medium.
5. 70% EtOH.
6. 150 mM NaCl in double-distilled water.
7. 2% Alizarin Red: Dissolve in double-distilled water. Adjust pH to 4.1–4.3 with dilute NaOH. Filter solution through a 0.2-μm syringe filter. Store at room temperature covered with aluminum foil to protect from the light.

2.7. Chondrogenic Differentiation

1. Complete Chondrogenic Base Media (R&D Systems, Inc., Minneapolis, MN): Add antibiotic solution to StemXVivo chondrogenic base media at a 1:100 dilution (100 μL antibiotic + 9,900 μL base media). Store at 4°C in the dark.
2. Chondrogenic Differentiation Media (R&D Systems, Inc.): Add StemXVivo Chondrogenic Supplement to the complete chondrogenic base media at a 1:100 dilution (100 μL supplement + 9,900 μL completed base media). Store at 4°C in the dark.

3. Methods

ASCs are morphologically similar to MSCs from other tissues during isolation and culturing. Factors such as donor age, type and location of adipose tissue, harvest procedure, culture conditions, exposure to plastic, plating density, and media formulations influence both proliferation rate and differentiation capacity. Although attachment and proliferation capacity seem to be more pronounced in ASCs from young donors compared with older donors, the differentiation capacity seems to be maintained with aging. In addition to the numerous other factors that can add variability to in vivo ASC studies, the isolation procedure can significantly affect the cells. Not only can viability and differentiation capacity be affected, but also different collagenase batches and centrifugation speeds result in isolation of different cell subsets. Thus, caution is warranted when making direct comparisons between studies with different isolation procedures. Isolated ASCs can be cryopreserved and readily expanded in vitro. Under standard conditions, the cells develop fibroblast-like morphology with increasing passages. The greatest number of ASCs is obtained from cultures plated at low densities. Low plating density and the use of DMEM-Ham's F12 can facilitate ASC differentiation. The media composition also influences gene expression, and use of antioxidants and low calcium concentrations can increase the growth rate and life span of ASCs. Increasing knowledge on the molecular mechanisms regulating ASC proliferation and differentiation will continue to contribute to improved isolation and culture procedures. The methods described below reflect common isolation and expansion procedures.

3.1. Adipose Tissue Collection

1. Immediately following CO_2 asphyxiation or other institutional animal care and use approved humane euthanasia, the abdomen of the rats is clipped and the rats are submerged in a 4-L graduated beaker of 70% ethanol for 1 min. (see Note 1).
2. Place the animal in dorsal recumbency (ventral side up) with the fore- and hind-limbs abducted (see Note 2).
3. Use a #10 scalpel blade to incise the skin along the length of the ventrum and reflect the skin.
4. Carefully incise the abdominal musculature into the peritoneal cavity using a new scalpel blade (see Note 3).
5. The epididymal fat pads are located on either side of the ventral aspect of the bladder. Grasp the fat pad with the forceps and gently excise from the epididymal/testicular tissue with a fresh scalpel blade (see Note 4).

3.2. ASC Isolation

Aseptically perform the steps below in a biosafety cabinet, while wearing appropriate personal protective equipment.

1. Place adipose tissue in a preweighed sterile petri dish and weigh to get a final tissue weight (see Note 5).
2. Mince the fat into small pieces with a sterile scalpel and blade until tissue is no longer fibrous.
3. Place tissue into a 15-mL tube with an equal volume of prewarmed PBS and agitate for 45 s.
4. Allow the mixture to separate into phases for 3–5 min and then remove the infranatant. Continue the process until the infranatant is clear. It takes about four washes (see Note 6).
5. Add an equal volume of collagenase solution to the adipose solution (see Note 7).
6. Clean the outside of the tube with 70% EtOH, cover the top with Parafilm, and place in a 37°C shaking water bath at 75 rpm for 1.5 min or until the tissue becomes homogenous (see Note 8).
7. Vortex for 15 s to thoroughly mix cells and then centrifuge at 1,200 rpm ($300 \times g$) for 5 min.
8. Vortex solution for 10 s to get as many cells out into the media as possible. Then centrifuge again at 1,200 rpm ($300 \times g$) for 5 min.
9. Carefully remove the supernatant consisting of lipids, primary adipocytes, and collagenase solution, leaving the pelleted stromal vascular fraction (see Note 9).
10. Resuspend the pellet in 1% BSA solution. Transfer solution to a new 15-mL centrifuge tube (see Note 10).
11. Centrifuge cells at 1,200 rpm ($300 \times g$) for 5 min and remove the supernatant. When aspirating, keep the tip of the pipette very superficial so the majority of liquid is removed.
12. Resuspend the pellet in 1 mL of stromal media.
13. Centrifuge a 20-µL aliquot in a microcentrifuge tube at 1,200 rpm ($300 \times g$) for 5 min.
14. Remove the supernatant and resuspend the pellet in 20 µL of red cell lysis buffer. Incubate for 5 min at room temperature.
15. Add 20 µL of Trypan Blue and count the cells with a hemocytometer.
16. Plate the cells at the appropriate density in complete stromal media and incubate at 37°C and 5% CO_2.
17. Change the media after 24 h to remove nonadherant cells.
18. Media should be changed about every 3 days.

3.3. Cell Culture (Passage) and Maintenance

1. Aspirate the media from the flasks and rinse the cells with PBS.
2. Add enough trypsin to just cover the surface of the flask and incubate at 37°C for 5 min (see Note 11).
3. Check under the microscope to ensure that the cells are detaching. Once the cells have detached, add complete stromal media. The FBS in the media will stop the enzymatic action of the trypsin.
4. Pipet the cell solution into a prelabeled 15-mL centrifuge tube and repeat for all samples.
5. Centrifuge for 5 min at 1,200 rpm ($300 \times g$) and discard the supernatant.
6. Resuspend the pellet in a known volume of complete stromal medium.
7. Aseptically remove 20 μL of resuspended cells and mix with 20 μL of Trypan Blue.
8. Count the cells under the microscope using the hemocytometer.
9. Seed the cells at the appropriate density.
10. Incubate flasks at 37°C in 5% CO_2.
11. Change medium every 3 days until cells have reached 70–80% confluence and then repeat cell passage.

3.4. Cryopreservation of ASCs

1. Centrifuge tubes at 1,200 rpm ($300 \times g$) for 5 min to pellet cells.
2. Discard supernatant and resuspend cells in freezing media at a concentration of $1–2 \times 10^6$ cells/mL.
3. Aliquot 1 mL of cell suspension to each cryovial.
4. Label cryovials with source, date, cell concentration, and passage information.
5. Store vials in the freezing container at –80°C for 24 h and then transfer the vials to liquid nitrogen.

3.5. Cell Doubling

1. Repeat steps for cell passage.
2. Record cell number and calculate cell doubling time (DT) and doubling number (CD) using the following formulae: (1) $CD = \ln(N_f/N_i)/\ln(2)$ and (2) $DT = CT/CD$. CT = culture time, N_f = final cell number, and N_i = initial seeding density.

3.6. Adipogenic Differentiation

1. Seed cells at the appropriate concentration and incubate at 37°C in 5% CO_2.
2. Change medium every 3 days until cells are 70–80% confluent.

3. Remove the stromal media from each well and add adipogenic induction media.
4. Culture cells in adipogenic induction media for 3 days. Change the media every 3 days.
5. After the 6-day incubation period, remove the media and add adipogenic maintenance media for an additional 6–10 days. Change the media every 3 days.
6. Fix the cells in 1% paraformaldehyde for 20 min at room temperature.
7. Stain the cells with Oil Red-O at room temperature for 20 min.
8. Rinse cells with double-distilled water three times to remove any residual dye.

3.7. Osteogenic Differentiation

1. Seed cells at the appropriate concentration and incubate at 37°C in 5% CO_2.
2. Change medium every 3 days until the cells are 70–80% confluent.
3. When the cells reach 70–80% confluence, replace the stromal media with osteogenic differentiation media.
4. Culture cells in osteogenic differentiation media for 10–15 days with media changes every 3 days.
5. Remove media from each well and wash cells three times with 150 mM NaCl solution.
6. Fix the cells in 70% EtOH for 1 h at 4°C.
7. Stain the cells with 2% Alizarin Red solution at room temperature for 10 min.
8. Rinse cells with double-distilled water three times to remove any residual dye.

3.8. Chondrogenic Differentiation

1. Aliquots of 2.5×10^5 cells are required for chondrogenic induction.
2. Resuspend cells in 500 µL of prewarmed complete chondrogenic base media.
3. Centrifuge cells at 1,200 rpm ($300 \times g$) for 5 min at room temperature.
4. Discard media and resuspend cells in 500 µL of chondrogenic differentiation media.
5. Centrifuge cells at 1,200 rpm ($300 \times g$) for 5 min at room temperature. Do not remove media. Leave the tube cap open to allow gas exchange and incubate at 37°C and 5% CO_2.
6. The cell pellet will form a 1- to 2-mm-diameter sphere in 1–2 days.

7. Change chondrogenic differentiation media every 2 days.
8. The chondrogenic pellet will be ready for harvest in 14–21 days.

4. Notes

1. Adipose harvest should be completed and ASC isolation initiated within 20 min of sacrifice.
2. Clip, shave, or epilate all areas to be incised for adipose tissue harvest to avoid hair contamination.
3. Be careful not to inadvertently incise any organs. This would result in sample contamination.
4. Other abdominal tissue sources often used include the adipose tissue surrounding the kidneys that are separated from the abdominal cavity by the peritoneum (renal, retroperitoneal) and the adipose tissue within the omentum and mesentery surrounding the small and large intestines (mesenteric, omental). The inguinal subcutaneous fat is just beneath the skin on the caudal abdomen. Brown adipose tissue is located beneath the skin between the shoulder blades.
5. If the tissue is covered with blood, it is helpful to rinse it with a few milliliters of PBS in the petri dish and then transfer the rinsed tissue to a new petri dish.
6. The *upper supernatant* consists of the minced and washed adipose tissue. The *infranatant* contains the hemopoietic cells suspended in PBS. It is helpful to aspirate the infranatant with a syringe and needle. Some tissue may settle in the bottom of the tube. It should be removed as well.
7. A volume of 5 mL of collagenase solution is added per gram of harvested adipose tissue.
8. If a shaking water bath is not available, maintain the tissue in a 37°C water bath with vortexing every 10–15 min.
9. Leave approximately 2–3 mL of liquid above the pellet to avoid disrupting the stromal vascular fraction.
10. Resuspend the pellet in the same volume as was used for the collagenase solution.
11. It is important not to exceed 5 min of incubation with the trypsin solution in order to isolate ASCs from monocytes in the adipose tissue. Monocytes will typically not be released with trypsin exposure equal to or less than 5 min.

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References

1. Stiles, J. W., Francendese, A. A., Masoro, E. J. (1975) Influence of age on size and number of fat cells in the epididymal depot. *Am J Physiol.* **229**, 1561–1568.
2. Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P., Hedrick, M. H. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* **7**, 211–228.
3. Tholpady, S. S., Katz, A. J., Ogle, R. C. (2003) Mesenchymal stem cells from rat visceral fat exhibit multipotential differentiation in vitro. *Anat Rec A Discov Mol Cell Evol Biol.* **272**, 398–402.
4. Rodbell, M. (1964) Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J Biol Chem.* **239**, 375–380.
5. Friedenstein, A. J., Gorskaja, J. F., Kulagina, N. N. (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol.* **4**, 267–274.
6. Friedenstein, A. J. (1976) Precursor cells of mechanocytes. *Int Rev Cytol.* **47**, 327–359.
7. Marko, O., Cascieri, M. A., Ayad, N., Strader, C. D., Candelore, M. R. (1995) Isolation of a preadipocyte cell line from rat bone marrow and differentiation to adipocytes. *Endocrinology.* **136**, 4582–4588.
8. Xie, L. W., Fang, H., Chen, A. M., Li, F. (2009) Differentiation of rat adipose tissue-derived mesenchymal stem cells towards a nucleus pulposus-like phenotype in vitro. *Chin J Traumatol.* **12**, 98–103.
9. Yoshimura, H., Muneta, T., Nimura, A., Yokoyama, A., Koga, H., Sekiya, I. (2007) Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell Tissue Res.* **327**, 449–462.
10. Arrigoni, E., Lopa, S., de, G. L., Stanco, D., Brini, A. T. (2009) Isolation, characterization and osteogenic differentiation of adipose-derived stem cells: from small to large animal models. *Cell Tissue Res.* **338**, 401–411.
11. Saggerson, E. D., McAllister, T. W., Baht, H. S. (1988) Lipogenesis in rat brown adipocytes. Effects of insulin and noradrenaline, contributions from glucose and lactate as precursors and comparisons with white adipocytes. *Biochem J.* **251**, 701–709.
12. Caserta, F., Tchkonia, T., Civelek, V. N., Prentki, M., Brown, N. F., McGarry, J. D., Forse, R. A., Corkey, B. E., Hamilton, J. A., Kirkland, J. L. (2001) Fat depot origin affects fatty acid handling in cultured rat and human preadipocytes. *Am J Physiol Endocrinol Metab.* **280**, E238–E247.
13. Tiraby, C., Tavernier, G., Lefort, C., Larrouy, D., Bouillaud, F., Ricquier, D., Langin, D. (2003) Acquirement of brown fat cell features by human white adipocytes. *J Biol Chem.* **278**, 33370–33376.

Chapter 5

In Vitro Adult Canine Adipose Tissue-Derived Stromal Cell Growth Characteristics

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Abstract

Stromal cells are undifferentiated cells found in embryonic and adult tissues. Adult mesenchymal stromal cells (MSCs) possess the properties of self renewal, long-term viability, multipotentiality, and immune privilege, which make them attractive candidates for regenerative medicine applications. In order to develop targeted adult stromal cell therapies for diseased and injured tissues in animals and humans, it is essential to have large-animal models. The dog represents not only a patient population, but is also a valuable experimental model. The dog has contributed significantly to the understanding of various human diseases such as genetic and musculoskeletal disorders. In order to optimize the use of stromal cell therapy in the dog as a patient or disease model, a comprehensive characterization of the cells is required.

Key words: Adult mesenchymal stromal cell, Fat, Adipose, Tissue engineering, Canine

1. Introduction

Stromal cells are undifferentiated cells found in embryonic and adult tissues that have self-renewal properties and the capacity to differentiate into various cell types (1–3). Embryonic stem cells (ESC) occur are in the early stage embryo, while adult stromal cells are found in fully differentiated postnatal tissues (2–8). Adult stromal cells are considered to be a viable alternative to ESCs. Although adult stromal cells are less versatile than ESCs, there are no ethical concerns about their tissue source. There are reservoirs of multipotent cells in bone marrow, bone marrow-derived stromal cells (BMSCs), and adipose tissue, adipose-derived stromal cells (ASCs). Given their capacity for self-renewal, long-term viability, multipotentiality, inhibition of apoptosis, suppression

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of inflammation, and immune privilege, these mesenchymal stromal cells (MSCs) are promising candidates for tissue regeneration therapies (9).

Adipose tissue has been established as an alternative to bone marrow as a source of MSCs (10). There are a number of advantages of adipose tissue over bone marrow as an MSC source, including ease of cell isolation, greater stromal cell density, and less harvest site morbidity (11). A higher stromal cell yield from adipose tissue shortens the time to reimplantation following in vitro expansion (12, 13).

Treatment of musculoskeletal disorders in veterinary patients with stromal cell technology has had promising early results (14–17). The dog represents not only a patient population, but is also a valuable experimental model for a number of human pathologies given similarities in physiology, disease presentation, and clinical response between the species (18–23). Close to 200 reported hereditary canine diseases are analogous to those in humans (19). Dogs models are superior to rodent models since the canine life span is more conducive to comprehensive safety studies, and organ sizes can be more closely aligned between dogs and humans (19, 24). Advances in canine adult stromal cell technology will enhance both veterinary and human cell-based therapies.

2. Materials

2.1. Adipose Tissue Collection and ASC Isolation

1. Scalpel handles (#3, 4) and scalpel blades (#10, 20).
2. 50-ml centrifuge tubes.
3. Petri dishes.
4. Phosphate-buffered saline (PBS).
5. 0.1% Collagenase: collagenase type-1 and 1% bovine serum albumin (BSA) dissolved in double-distilled water. Filter sterilize with a 0.22-μm filter. Warm to 37°C.
6. 1% BSA dissolved in double-distilled water. Filter sterilize with a 0.22-μm filter. Warm to 37°C.
7. Red cell lysis buffer.
8. Complete stromal medium: DMEM–Ham's F12 supplemented with 1% antibiotic/antimycotic solution and 20% characterized fetal bovine serum (FBS; see Note 1).
9. Trypan Blue.

2.2. Cryopreservation of ASCs

1. Cryopreservation medium: 80% FBS, 10% DMEM–Ham's F12, 10% dimethylsulfoxide (DMSO).
2. "Mr. Frosty" freezing container (alcohol container, see Note 2).
3. Cryovials.

2.3. Cell Culture and Maintenance

1. Complete stromal medium: DMEM–Ham's F12 supplemented with a 1% antibiotic/antimycotic solution and 20% characterized FBS.
2. T25-cm tissue culture flasks.
3. 12-well culture plates.

2.4. Cell Doubling

1. PBS.
2. 0.05% Trypsin (1:250); 1× liquid; 0.05% porcine trypsin in HBSS with 0.2 g/L ethylenediaminetetraacetic acid (EDTA).
3. Trypan Blue.
4. Hemocytometer.
5. Complete stromal medium: DMEM–Ham's F12 supplemented with 1% antibiotic/antimycotic solution and 20% characterized FBS.

2.5. Adipogenic Differentiation

1. DMEM–Ham's F12 medium.
2. FBS.
3. 100× Penicillin–streptomycin–amphotericin B solution with 10,000 IU/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin B.
4. Biotin (33 μmol/L).
5. Pantothenate (17 μmol/L).
6. Insulin (1 μmol/L).
7. Isobutylmethylxanthanine (IMBX) (0.5 mmol/L).
8. Rosiglitazone (5 μmol/L).
9. Dexamethasone (20 nmol/L).
10. 1% Paraformaldehyde in PBS.
11. Oil Red-O.
12. Double-distilled water.

2.6. Osteogenic Differentiation

1. β-glycerophosphate (10 mM/L).
2. Dexamethasone (10 nM/L).
3. Sodium 2-phosphate ascorbate (50 μg/ml).
4. Complete stromal medium: DMEM–Ham's F12 supplemented with 1% antibiotic/antimycotic solution and 20% characterized FBS.
5. 70% ethyl alcohol (EtOH).
6. 2% Alizarin Red solution dissolved in double-distilled water. Adjust pH to 4.1–4.3 with dilute NaOH. Filter solution through 0.2-μm syringe filter. Solution can be stored at room temperature covered with aluminum foil.
7. 150 mM NaCl dissolved in double-distilled water.

2.7. Chondrogenic Differentiation

1. Complete chondrogenic base medium: Add antibiotic solution to StemXVivo chondrogenic base media at a 1:100 dilution (100 µl antibiotic + 9,900 µl base media). Store at 4°C in the dark.
2. Chondrogenic differentiation medium: Add StemXVivo Chondrogenic Supplement to the complete chondrogenic base media at a 1:100 dilution (100 µl supplement + 9,900 µl completed base media). Store at 4°C in the dark.

2.8. RNA Isolation

1. Trizol.
2. Chloroform.
3. DEPC-treated water.
4. Turbo DNA-free kit.
5. Glyco Blue.
6. Isopropanol.
7. 75% EtOH in DEPC-treated water.

2.9. Complimentary DNA Synthesis with Preprimed 96-Well Plate

1. Preprimed 96-well plate.
2. Nuclease-free water.
3. Flat-cap strips.

2.10. SYBR Green Real-time Polymerase Chain Reaction

1. SYBR Green Master Mix.
2. Nuclease-free water.
3. Forward and reverse primers.
4. Complimentary DNA (cDNA) samples.
5. Low-profile white 96-well plate.
6. Flat-cap strips.

2.11. Papain Digestion

1. Papain from Carica Papaya (25 mg/ml).
2. Double-distilled water.
3. Solution A: 10 mM di-sodium EDTA and 0.4 M sodium acetate dissolved in double-distilled water.
4. Solution B: 200 mM L-cysteine dissolved in double-distilled water.
5. Solution C: Add 1 ml of solution B to 9 ml of solution A before use (1:10 dilution).
6. Solution D: Add 200 µl of papain suspension to 10 ml solution C.

2.12. Hydroxyproline Assay

1. Double-distilled water.
2. Clear flat-bottom 96-well ELISA plate.

3. 12 N Hydrochloric acid (HCl). Dilute 1:2 in double-distilled water for 6 N working solution.
4. 17% Sodium chloride (NaCl) dissolved in double-distilled water.
5. Acetate/citrate buffer: 120 g sodium acetate trihydrate, 12 ml acetic acid, 50 g citric acid monohydrate, 34 g NaOH in 1 L double-distilled water (pH 6.0). Store at 4°C.
6. Oxidant solution: 0.178 g chloramines T in 15 ml of isopropanol and 10 ml of double-distilled water; add 25 ml acetate/citrate buffer. Make fresh (see Note 3).
7. Ehrlich's reagent: 1 g dimethylaminobenzaldehyde in 20 ml isopropanol; combine with 6.6 ml perchloric acid and 15.6 ml double-distilled water. Make fresh (see Note 3).
8. Trans-4-hydroxyproline (1 mg/ml) in double-distilled water. Store at 4°C.

2.13. PicoGreen Double-Stranded DNA Assay

1. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 7.5; dilute 20× TE buffer 1:20 in double-distilled water to a 1× TE working solution.
2. PicoGreen dsDNA dye reagent, dilute 1:200 in 1× TE. Store at 4°C in dark. Make fresh.
3. Black flat-bottom 96-well ELISA plate.
4. Lambda DNA stock (100 μg/ml), dilute 1:50 in 1× TE to 2 μg/ml; dilute standard curve from 1 μg/ml to 1 ng/ml. Store at 4°C in dark. Make fresh.
5. Double-distilled water.

2.14. Lowry Total Protein Assay

1. Biuret's reagent.
2. Folin–Ciocalteu's phenol reagent diluted 1:2 in distilled water.
3. BSA (1 mg/ml) dissolved in distilled water.

2.15. 1,9-Dimethylmethylene Blue Total Sulfated Proteoglycan Assay

1. Double-distilled water.
2. 1,9-Dimethylmethylene blue (DMMB) assay buffer: Dissolve 2 g of sodium formate in 980 ml of distilled water and add 2 ml of formic acid (pH 3.5); in a separate beaker, add 16 mg of DMMB to 5 ml of EtOH and mix until the DMMB is dissolved; add the formate solution to the DMMB solution and bring the volume up to 1 L with double-distilled water. DMMB assay buffer may be stored at 4°C indefinitely.
3. Shark chondroitin-6-sulfate C (1 mg/ml). Prepare glycosaminoglycan (GAG) standards at the following concentrations: 100, 50, 40, 30, 20, 10, and 5 μg/ml in water. Include a blank. The stock and working solutions can be stored at –25°C.

3. Methods

A comprehensive characterization of adult canine stromal cells is necessary for stromal cell therapy in the dog as a patient or animal model. Knowledge of adult canine ASC and BMSC tissue density is required to determine the tissue volume required to provide the necessary multipotent cells for specific tissue regeneration. In turn, the number of required cells is dependent on the amount of tissue to be regenerated. As such, cell doubling rates are necessary to predict the time required between harvest and clinical application. Additionally, differentiation capabilities and efficiency of adult stromal cells are critical to predict appropriate cell numbers and cell lineage induction strategies for tissue regeneration.

3.1. Adipose Tissue Collection and ASC Isolation (25)

1. Using aseptic technique, collect the adipose tissue from the appropriate site (subcutaneous, patellar, etc.).
2. Place in a preweighed sterile petri dish and weigh to get a final tissue weight.
3. Mince tissue using sterile scalpel and blade. Mince/tear until tissue is no longer fibrous.
4. Place tissue in a 50-ml tube with an equal volume of prewarmed PBS and agitate for 15–30 s to wash.
5. Allow to separate into phases for 3 min and then remove the infranatant solution (lower, aqueous phase) (see Note 4).
6. Some tissue may sink to the bottom of the tube. Remove this tissue as well since it is not adipose tissue.
7. Rinse the infranatant tissue with PBS to remove the erythrocytes and white blood cells. Shake for 45 s and allow tissue to float back to the top. Continue rinsing until the infranatant is clear. Add a volume of collagenase solution per volume of adipose volume.
8. Clean the outside of the tube with 70% EtOH, cover with Parafilm, and place in a 37°C shaking water bath at 75 rpm for 1.5 min or until the tissue becomes homogenous. If a shaker is not available, use a lab rotator with water bath and vortex tissue every 10–15 min.
9. Vortex for 15 s to thoroughly mix cells and then centrifuge at $260 \times g$ for 5 min.
10. Vortex the primary adipocyte tissue in the solution to get as many cells out into the media as possible. Then centrifuge again at $260 \times g$ for 5 min.
11. Carefully remove the supernatant consisting of lipids, primary adipocytes, and collagenase solution. Leave behind approximately 2–3 ml of liquid above the pellet so that the stromal vascular fraction is not disturbed.

12. Resuspend cells in 1% BSA solution (see Note 5).
13. Centrifuge cells at 260 × *g* for 5 min and remove the supernatant. When aspirating, keep the tip of the pipette very superficial so the majority of liquid is removed.
14. Resuspend the pellet in a known volume of stromal media.
15. Centrifuge a small aliquot at 260 × *g* for 5 min.
16. Discard supernatant and resuspend pellet in an equal volume of red cell lysis buffer. Incubate for 5 min.
17. Count the cells using Trypan Blue and the hemocytometer.
18. Plate the cells at the appropriate density in complete stromal media.

3.2. Cryopreservation of ASCs

1. Centrifuge tubes at 260 × *g* for 5 min to pellet cells.
2. Freeze cells at a rate of 1–2 million cells/ml of freezing media in a cryovial.
3. Discard supernatant and resuspend cells in freezing media.
4. Store vials in a freezing container at −80°C for 24 h and then transfer to liquid nitrogen.

3.3. Cell Culture (Passage) and Maintenance

1. Aspirate the media from the flasks and rinse the cells with PBS.
2. Add enough trypsin to just cover the surface of the flask and incubate at 37°C for 5 min.
3. Check under the microscope to ensure that the cells are detaching. Once the cells have detached, add complete stromal media to flask. The FBS in the media will stop the enzymatic action of the trypsin.
4. Pipet the cell solution into a prelabeled 15-ml centrifuge tube and repeat for all samples.
5. Centrifuge for 5 min at 260 × *g* and discard the supernatant.
6. Resuspend the pellet in a known volume of complete stromal media (~2 ml) of stromal media per flask harvested.
7. Aseptically remove 20 µl of resuspended cells and mix with 20 µl of Trypan Blue.
8. Count the cells under the microscope using the hemocytometer.
9. Seed the cells at the appropriate density.
10. Incubate flasks at 37°C in 5% CO_2.
11. Change medium every 3 days until cells have reached 70–80% confluence and then repeat cell passage.

3.4. Cell Doubling (26–28)

1. Repeat steps for cell passage.
2. Record cell number and calculate cell doubling time (DT) and doubling number (CD) using the following formulae:

(1) $CD = \ln(N_f/N_i)/\ln(2)$ and (2) $DT = CT/CD$. CT = culture time, N_f = final cell number, and N_i = initial seeding density.

3.5. Adipogenic Differentiation

1. Trypsinize and count cells.
2. Plate cells at the appropriate concentration and incubate at 37°C in 5% CO_2.
3. Change medium every 3 days until cells have reached 70–80% confluence.
4. Once cells are 70–80% confluent, remove the media from each well and add adipogenic induction media.
5. Culture cells in adipogenic induction media for 6 days. Change the media every 3 days.
6. After the 6-day incubation period, remove the media and add adipogenic maintenance media for an additional 6 days. Change the media every 3 days.
7. Fix the cells in 1% paraformaldehyde for 20 min at room temperature.
8. Stain the cells with Oil Red-O at room temperature for 20 min.
9. Rinse cells with double-distilled water three times to remove any residual dye.

3.6. Osteogenic Differentiation

1. Trypsinize and count cells.
2. Plate cells at the appropriate concentration and incubate at 37°C in 5% CO_2.
3. Change medium every 3 days until cells have reached 70–80% confluence.
4. Once cells reach 70–80% confluence, remove the media from each well, and add osteogenic differentiation media.
5. Culture cells in osteogenic differentiation media for 14 days. Change the media every 3 days.
6. Remove media from each well and wash cells three times with 150 mM NaCl solution.
7. Fix the cells in 70% EtOH for 1 h at 4°C.
8. Stain the cells with 2% Alizarin Red solution at room temperature for 10 min.
9. Rinse cells with double-distilled water three times to remove any residual dye.

3.7. Chondrogenic Differentiation

1. If cells are plated, trypsinize and count cells. Aliquots of 2.5×10^5 cells are required for chondrogenic induction.
2. Resuspend cells in 500 µl of prewarmed complete chondrogenic base media.

3. Centrifuge cells at 200 × *g* for 5 min at room temperature.
4. Discard media and resuspend cells in 500 µl of chondrogenic differentiation media.
5. Centrifuge cells at 200 × *g* for 5 min at room temperature. Do not remove media. Open the cap of the tube to allow gas exchange and incubate at 37°C and 5% CO_2.
6. The cell pellet will form a round ball approximately 1–2 mm in diameter after 1–2 days.
7. Change chondrogenic differentiation media every 2 days.
8. Chondrogenic pellet will be ready for harvest after 14–21 days.

3.8. Isolation of RNA (29, 30)

1. Add Trizol to each well at 1 ml Trizol/10^7 cells (see Note 6).
2. Pipet to detach cells from the culture dish surface and place in a 1.5-ml microcentrifuge tube.
3. Add 2 µl of Glyco Blue to the tube (see Note 7). Keep tubes on ice.
4. Vortex each sample for 45 s. Be careful not to let the sample get warm during the vortexing process.
5. Keep the homogenate for 5 min at room temperature.
6. Add 0.2 ml of chloroform per 1 ml of Trizol to the homogenate. Cover the samples tightly and vortex vigorously for 15 s.
7. Keep at room temperature for 15 min at room temperature.
8. Centrifuge at 12,000 × *g* for 15 min at 4°C. The mixture will separate into a lower red–phenol phase, an interphase, and an upper aqueous phase. The RNA is in the upper aqueous phase, while DNA and proteins are in the interphase and organic phase.
9. Carefully transfer the upper aqueous phase to a fresh tube (see Note 8). Precipitate the RNA by adding 0.5 ml of isopropanol per 1 ml of TRI reagent used in the initial homogenization. Mix well by inversion.
10. Store the samples at room temperature for 10 min and centrifuge at 12,000 × *g* for 8 min at 4°C. The RNA pellet is often a translucent white pellet (or blue if using Glyco Blue) adhered to the side or bottom of the tube.
11. Decant the supernatant without losing the pellet. Wash the pellet with 1 ml of 75% ethanol in DEPC water.
12. Centrifuge at 7,500 × *g* for 5 min at 4°C. If the pellet floats, spin tube again at 12,000 × *g*.
13. Decant the ethanol wash and briefly air-dry the pellet for 5–10 min by inverting on a sterile gauze pad or alcohol pad. Do not completely dry the pellet because it will be difficult to resuspend the pellet. Completely evaporate as much ethanol

as possible because ethanol affects Real-time Polymerase Chain Reaction (RT-PCR) (see Note 9).

14. Resuspend pellet in 25 µl of DEPC-treated water.
15. DNAsing samples: To each PCR tube, add 25 µl of RNA, 5 µl of buffer, 2 µl of DNAse I, and 18 µl of DEPC water. Keep samples on ice.
16. Spin tubes briefly to mix contents.
17. Incubate for 1 h at 37°C.
18. Add 0.1× volume of DNAse Inactivation reagent and mix.
19. Incubate at room temperature for 2 min.
20. Centrifuge tubes at 9,300×*g* for 1.5 min.
21. Carefully transfer supernatant to a fresh tube without disturbing the pellet.
22. Quantify RNA using a nanodrop or spectrophotometer and then proceed to RT-PCR (cDNA synthesis) or store at –80°C.

3.9. Complimentary DNA Synthesis with BD Sprint Powerscript Preprimed 96-Well Plate

1. Quantify RNA and check the absorbance ratio at 260 and 280 nm. A ratio of the readings 260/280 of 1.6 or higher is acceptable.
2. The maximum quantity of RNA that can be used in each reaction is 1 µg in 20 µl.
3. For the volumes less than 20 µl, add nuclease-free water to a total volume of 20 µl.
4. Vortex gently until everything is in solution. Centrifuge briefly.
5. Thermacycling conditions: 42°C for 1 h, 2.5°C/s to 70°C, 70°C for 10 min, 2.5°C/s to 4°C.
6. Add 80 µl of nuclease-free water to each sample.
7. Samples can be used for RT-PCR or stored at –20°C.

3.10. SYBR Green Real-time Polymerase Chain Reaction

1. Dilute primers and samples as necessary.
2. Prepare master mix for each sample: 12.5 µl SYBR Green + 1.5 µl nuclease-free water + 2 µl forward primer + 2 µl reverse primer.
3. Add 20 µl of master mix to each well.
4. Add 5 µl of sample to each corresponding well.
5. Thermacycling conditions: 50°C, 2 min; 95°C, 3 min; 45 cycles of 95°C, 15 s and 60°C (annealing temperature), 1 min.

3.11. Papain Digestion

1. Add papain solution to each sample to give a final concentration of 25 mg/ml.

2. Incubate papain samples at 60°C overnight. If samples are not digested, vortex and place them back in the incubator. Repeat until samples are completely digested (see Note 10).
3. Papain digested samples can be stored at −80°C until needed.

3.12. Hydroxyproline Total Collagen Assay (31)

1. Dilute samples in an equal volume of 6 N HCl and incubate overnight or 18 h at 110°C (see Notes 11 and 12).
2. Prepare a series of 1:2 serial dilutions (0, 1, 2.5, 5, 10, and 20 µg/well).
3. Add 250 µl each of distilled water and 17% NaCl solution to each sample and mix thoroughly.
4. Add 500 µl of oxidant solution to samples and standards and incubate at room temperature for 5 min.
5. After incubation, add 500 µl of Ehrlich's reagent to each sample and standard and incubate at 60°C for 12 min.
6. Quickly cool samples and standards on ice for 4 min.
7. Pipet 100 µl of each sample and standard onto a microtiter plate.
8. Read absorbance at 550 nm.

3.13. PicoGreen Double-Stranded DNA Assay (32)

1. Prepare standard curve in 1× TE buffer.
2. If necessary, dilute sample in 1× TE buffer so that they fall within the standard curve.
3. Add samples and standards to black microtiter plate.
4. Add 100 µl of 1× PicoGreen Dye solution and incubate for 2–5 min in the dark.
5. Immediately read plate at 485 and 520 nm.

3.14. Lowry Total Protein Assay (33)

1. Prepare a series of serial dilutions (0, 1, 2.5, 5, 10, and 20 µg/well) in duplicate to a final volume of 100 µl.
2. Dilute samples so that they fall within the standard curve range of 0–20 µg/ml in duplicate.
3. Transfer 100 µl of each sample and standard to the flat-bottomed microtiter plate.
4. Add 200 µl of Biuret's reagent to each well and mix thoroughly by pipetting several times.
5. Allow plate to incubate for 10–15 min at room temperature.
6. Add 20 µl/well of 1.0 N Folin–Ciocalteu's reagent. Mix thoroughly with repeated pipetting.
7. Allow color to develop for 30 min at room temperature.
8. Immediately read the plate at 650 nm absorbance.

3.15. 1,9-Dimethylmethylene Blue Total Sulfated Glycosaminoglycan Assay (34, 35)

1. Prepare GAG standards at the following concentrations: 100, 50, 40, 30, 20, 10, and 5 μg/ml in water. Include a water blank.
2. Dilute samples with double-distilled water if necessary so values are within the standard curve.
3. Add 40 μl of each sample and standard to a flat-bottomed microtiter plate.
4. Using a multichannel pipettor, add 250 μl of the GAG assay buffer to each sample and standard.
5. Immediately read the plate at 540 and 600 nm within 5 min (see Note 13).

4. Notes

1. Complete stromal media can be made with 10% FBS instead of 20% FBS. We used 20% FBS in our initial cell culture and continued its use to remain constant throughout the experiments.
2. Mr. Frosty freezing container can be used up to five times before the isopropanol needs to be changed.
3. The oxidant solution and Ehrlich's reagent can be used up to 2 days after initial use if stored at 4°C.
4. We used a 19-gauge, 5-in. needle to remove the infranatant after each wash step. This made it easy to remove the liquid without losing some of the sample.
5. At this point, we found that transferring the solution to a new centrifuge tube helped to reduce the amount of oil contaminating the sample.
6. Lower cell concentrations may be used. See manufacturer's instructions for additional information on cell concentration/Trizol ratio.
7. The RNA pellet is normally a clear or white color. The addition of Glyco Blue in the cell/Trizol solution stains the pellet blue and aids in easier visualization of the pellet. The Glyco Blue does not have an effect on RNA purity.
8. Transfer the aqueous phase starting from the middle of the tube. Place just the tip of the pipette tip into the solution. This helps to minimize phenol and/or DNA contamination.
9. If residual ethanol remains in the tube after the air-drying step, aspirate the ethanol with an insulin syringe and needle.
10. If pieces of tissue still remain after repeated incubation at 60°C, centrifuge the sample and collect the supernatant.

11. We found that the use of screw-cap tubes prevented the tubes from popping open after being exposed to such a high temperature. If snap lid tubes are used, make sure to secure the lids to prevent them from popping open.
12. The sample will have a charred appearance after incubating at 110°C. Mix sample well to dissolve.
13. Read the plate immediately to prevent precipitation of the sample and inaccurate results.

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References

1. Weissman, I.L. (2000) Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* **287**, 1442–1446.
2. Caplan, A.I. (1991) Mesenchymal stem-cells. *J Orthop Res* **9**, 641–650.
3. Caplan, A.I. and Bruder, S.P. (2001) Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med* **7**, 259–264.
4. Hu, Y., Liao, L.M., Wang, Q., Ma, L., Ma, G., Jiang, X., et al. (2003) Isolation and identification of mesenchymal stem cells from human fetal pancreas. *J Lab Clin Med* **141**, 342–349.
5. Jiang, Y., Vaessena, B., Lenvik, T., Blackstad, M., Reyes, M., and Verfaillie, C.M. (2002) Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp Hematol* **30**, 896–904.
6. Meirelles, L.D.S., Chagastelles, P.C., and Nardi, N.B. (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* **119**, 2204–2213.
7. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., et al. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147.
8. Duan, Y., Catana, A., Meng, Y., Yamamato, N., He, S., Gupta, S., et al. (2007) Differentiation and enrichment of hepatocyte-like cells from human embryonic stem cells in vitro and in vivo. *Stem Cells* **25**, 3058–3068.
9. Bocelli-Tyndall, C., Bracci, L., Spagnoli, G., Braccini, L., Bouchenaki, M., Ceredig, R., et al. (2007) Bone marrow mesenchymal stromal cells (BM-MSCs) from healthy donors and auto-immune disease patients reduce the proliferation of autologous- and allogeneic-stimulated lymphocytes in vitro. *Rheumatology (Oxford)* **46**, 403–408.
10. Kim, S.J., Cho, H.H., Kim, Y.J., Seo, S.Y., Kim, H.N., Lee, J.B., et al. (2005) Human adipose stromal cells expanded in human serum promote engraftment of human peripheral blood hematopoietic stem cells in NOD/SCID mice. *Biochemical and Biophysical Research Communications* **329**, 25–31.
11 Neupane, M., Chang, C.C., Kiupel, M., and Yuzbasiyan-Gurkan, V. (2008) Isolation and characterization of canine adipose-derived mesenchymal stem cells. *Tissue Eng Part* A **14**, 1007–1015
12. Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., et al. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279–4295.
13. Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., et al. (2001) Multilineage cells from human adipose tissue:

implications for cell-based therapies. *Tissue Eng* **7**, 211–228.

14. Dahlgren, L. (2006) Use of adipose derived stem cells in tendon and ligament injuries. *Am Coll Vet Surg Symp Equine Small Anim Proc*, 150–151.
15. Harman, R., Cowles, B., Orava, C., et al. (2006) A retrospective review of 62 cases of suspensory ligament injury in sport horses treated with adipose-derived stem and regenerative cell therapy. *Proc Vet Orthop Soc*.
16. Harman, R. (2007) Adipose-derived stem and regenerative cells improves lameness and pain in dogs with osteoarthritis: a summary of results from three clinical trials. *Stem Cells* **25**, 3279.
17. Black, L.L., Gaynor, J., Gahring, D., Adams, C., Aron, D., Harman, S., et al. (2007) Effect of adipose-derived mesenchymal stem and regenerative cells on lameness in dogs with chronic osteoarthritis of the coxofemoral joints: a randomized, double-blinded, multicenter, controlled trial. *Vet Ther* **8**, 272–284.
18. Starkey, M.P., Scase, T.J., Mellersh, C.S., and Murphy, S. (2005) Dogs really are man's best friend – canine genomics has applications in veterinary and human medicine! *Brief Funct Genomic Proteomic* **4**, 112–128.
19. Tsai, K.L., Clark, L.A., and Murphy, K.E. (2007) Understanding hereditary diseases using the dog and human as companion model systems. *Mamm Genome* **18**, 444–451.
20. Cummings, B.J., Head, E., Ruehl, W., Milgram, N.W., and Cotman, C.W. (1996) The canine as an animal model of human aging and dementia. *Neurobiol Aging* **17**, 259–268.
21. Kirchhoff, C. (2002) The dog as a model to study human epididymal function at a molecular level. *Mol Hum Reprod* **8**, 695–701.
22. Sampaolesi, M., Blot, S., D'Antona, G., Granger, N., Tonlorenzi, R., and Innocenzi, A. (2006) Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. *Nature* **444**, 574–579.
23. Ostrander, E.A. and Giniger, E. (1997) What man's best friend can teach us about human biology and disease. *Am J Hum Genet* **61**, 475–480.
24. Ostrander, E.A., Galibert, F., and Patterson, D.F. (2000) Canine genetics comes of age. *Trends Genet* **16**, 205.
25. Aust, L., Devlin, B., Foster, S.J., Halvorsen, Y.D., Hicok, K., du Laney, T., et al. (2004) Yield of human adipose-derived adult stem cells from liposuction aspirates. *Cytotherapy* **6**, 7–14.
26. Rainaldi, G., Pinto, B., Piras, A., Vatteroni, L., Simi, S., and Citti, L. (1991) Reduction of proliferative heterogeneity of Chef18 Chinese-Hamster cell-line during the progression toward tumorigenicity. *In Vitro Cell Dev Biol* **27**, 949–952.
27. Vidal, M.A., Kilroy, G.E., Johnson, J.R., Lopez, M.J., Moore, R.M., and Gimble, J.M. (2006) Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. *Vet Surg* **35**, 601–610.
28. Vidal, M.A., Kilroy, G.E., Lopez, M.J., Johnson, J.R., Moore, R.M., and Gimble, J.M. (2007) Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells. *Vet Surg* **36**, 613–622.
29. Chomczynski, P. and Mackey, K. (1995) Modification of the Tri-Reagent™ procedure for isolation of RNA from polysaccharide-rich and proteoglycan-rich sources. *Biotechniques* **19**, 942–945.
30. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal Biochem* **162**, 156–159.
31. Stegeman, H. and Stalder, K. (1967) Determination of hydroxyproline. *Clin Chim Acta* **18**, 267.
32. Labarca, C. and Paigen, K. (1980) Simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* **102**, 344–352.
33. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.
34. Ratcliffe, A., Billingham, M.E., Saed-Nejad, F., Muir, F., and Hardingham, T.E. (1992) Increased release of matrix components from articular-cartilage in experimental canine osteoarthritis. *J Orthop Res* **10**, 350–358.
35. Ratcliffe, A., Doherty, M., Maini, R.N., et al. (1988) Increased concentrations of proteoglycan components in the synovial-fluids of patients with acute but not chronic joint disease. *Ann Rheum Dis* **47**, 826–832.

Chapter 6

Adipogenic Differentiation of Adult Equine Mesenchymal Stromal Cells

Martin A. Vidal and Mandi J. Lopez

Abstract

Equine adipose tissue-derived mesenchymal stem cells (ASCs) have only recently been investigated for their adipogenic, chondrogenic, and osteogenic differentiation potential. This chapter will briefly outline the molecular mechanisms leading to adipogenesis and the methods of equine adipose tissue harvest, ASC isolation, and adipogenic differentiation. The reader is also directed to other reported methods of adipogenesis for ASCs and mesenchymal stem cells (MSCs) from other tissues.

Key words: Adipokines, Peroxisome proliferator-activated receptor-γ, CCAAT/enhancer binding protein, Adipogenesis, Equine, Horse, Adipose, Stromal cells

1. Introduction

Adipogenesis and associated tissue engineering applications are important considerations in human medicine. Absence or loss of adipose tissue due to injury, disease, or surgical excision often leads to tissue defects and significant dissatisfaction due to poor cosmesis. Design of soft tissue scaffolds that integrate into recipient tissue and provide improved appearance of injured tissues is a current research focus. The ultimate goal is to replace the use of artificial fillers. The focus on cosmetic intervention in equine medicine is not as important as in human medicine, but adipogenesis is important to determination of the multipotential of equine stromal cells, a relatively new area of investigation (1, 2).

Adipogenesis along with osteogenesis and chondrogenesis is part of the classic tripotential of MSCs. Adipogenic methods are reported for MSCs harvested from bone marrow (3, 4), adipose tissue (5), umbilical cord tissue (6), umbilical cord blood (5, 7, 8),

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and peripheral blood (3, 9). Adipogenesis and osteogenesis are intimately related to each other. With the recent discovery of important adipokine endocrine functions, adipose tissue has become recognized for functions beyond energy storage and a physical protection of underlying tissues. Understanding of the intricacies of adipogenic and osteogenic control in marrow fat may provide an understanding for future therapeutic interventions in impaired bone healing, a recognized problem in the horse. This chapter is designed to provide a brief overview of the molecular mechanisms involved in adipogenesis and to summarize techniques for collection, isolation, and adipogenic differentiation of equine bone marrow and adipose tissue.

1.1. Molecular Control of Adipogenesis

Until the 1980s, adipose tissue was largely considered an energy storage depot and it was thought that individuals were born with a set number of adipocytes (10). However, it is now believed that adipogenesis occurs throughout life, and obesity is governed by adipocyte hypertrophy and hyperplasia (11). New light has been shed on the role of adipose tissue in the body with a variety of metabolic regulatory factors such as adipsin (12), tumor necrosis factor (TNF)-alpha (13), and plasminogen activator inhibitor-1 (14), as well as adipokines such as leptin (15) and adiponectin (16). Additionally, adipose tissue is related to insulin sensitivity and obesity, as well as in artherogenesis and inflammation (17).

The very early molecular events determining a primitive mesenchymal precursor cell to differentiate into an adipocytic lineage remain unknown (10). The first stage of fat cell differentiation is characterized by clonal expansion, an initial growth arrest followed by several cell divisions (18). Transcription factors peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding proteins (C/EBP) are expressed during the process, possibly due to their associated antimitotic properties (19, 20) leading to a second growth arrest. Activation of PPARγ appears to correlate with the demise of the DNA-binding activity of E2F/DP, an important transcription factor for many genes associated with cell growth and promoting cell division (21). The transition from a growth-arrested preadipocyte to a fully differentiated fat cell occurs in association with a PPARγ-mediated induction of cyclin-dependent kinase inhibitors such as p18 and p21 (10). The expression of p21 has also been associated with C/EBPα (22).

Attempts to define the beginning and the end of the adipogenesis process in culture are difficult, because the process is asynchronous in nature, occurring progressively in cell clusters, making mRNA level expressions patterns difficult to determine. However, it appears that adipogenesis is subject to a cascade of events. An early biomarker of the differentiation process is lipoprotein lipase (23). The appearance of this protein is closely followed by a number of transcriptional factors, which result in the

expression of the mature adipocyte phenotype and the production of a number of adipogenesis end products. These include glycerophosphate dehydrogenase, fatty acid synthase, the adipocyte-specific fatty acid binding protein (aP2), malic enzyme, the glucose transporter GLUT 4, the insulin receptor, and considerable triglyceride accumulation (24).

1.2. Transcriptional Control of Adipogenesis

Key players involved in the transcriptional cascade of adipogenesis are members of the C/EBP family. The expression of mRNA and protein levels of CEBPβ and C/EBPδ will rise early during adipogenesis followed by C/EBPα, which is induced later in the differentiation process, before other end product genes of fat cells (10). The transcription factor PPARγ governs the function of most adipocyte-specific genes and required for the fat-selective enhancers within the genes encoding aP2, lipoprotein lipase (LPL) (25), and phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme of gluconeogenesis (26). PPARγ ligands, such as the thiazolidinedione (TZD) class of antidiabetic drugs, bind with a range of affinities (K_d = 50–700 nM) (10).

Transcriptional control of adipogenesis follows along a cascade of events whereby hormonal cues may initially activate CEBPβ and C/EBPδ, which affect expression of the PPARγ–RXRα complex, subsequently causing the activation of C/EBPα. The adipocyte determination and differentiation-dependent factor 1/sterol regulatory element-binding protein (ADD1/SREBP1) is also induced during adipogenesis and is regulated in response to variations in food intake (27, 28). It has also been associated with cholesterol homeostasis (29), adipocyte development, and expression of fatty acid synthase (FAS) and lipoprotein lipase (LPL), two key regulators of fatty acid metabolism (28). The exact role is still not completely understood but its contribution to adipogenesis in regulating triglyceride and fatty acid metabolism has been established. It is considered a weaker stimulus for adipogenesis compared with PPARγ or the C/EBP proteins (10), yet it is believed that ADD1/SREBP1-expressing cells in conditioned medium can activate PPARγ directly or indirectly via promotion of an endogenous PPARγ ligand (27).

1.3. Cellular and Hormonal Control of Adipogenesis

Hormonal induction of adipogenesis requires activation of preadipocyte IGF-1 receptors (IGFR) by IGF-1 or insulin (10) to activate two separate signal transduction pathways, Akt (30) and Ras (31). Elevation of cyclic AMP (cAMP) has been associated with early adipogenic events and is strongly upregulated via the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) (32). Cyclic AMP has been associated with the suppression of Wnt10b (33) and Sp1 (34) and induction of C/EBPβ. Adipogenic pathways are also mediated either through glucocorticoids (35), which bind to the glucocorticoid receptor (GR),

thereby resulting in C/EBPδ activation, and preadipocyte factor (Pref1), a cell surface glycoprotein with antiadipogenic effects (36). A variety of growth factors and cytokines act as inhibitors of adipogenesis (10) affecting mitogen-activated protein kinase (MAPK), which phosphorylates PPARγ and its heterodimerization partner RXR (37).

1.4. Equine Adipose Tissue Collection

Unlike other species such as the human or the dog, horses have relatively few reliable sites for adipose tissue harvest and little amount of adipose tissue in areas that are cosmetically or functionally not detrimental. Therefore the collection of adipose tissue in the horse is mostly limited to the area over the rump and near the tail head. The texture of equine adipose tissue in this location, however, is much firmer and more fibrous than adipose harvests from humans and is therefore surgically removed by sharp dissection via a small incision. Liposuction techniques for better cosmetic results are currently under investigation.

1.5. ASC Isolation from Adipose Tissue

The process of isolating MSCs from adipose tissue is based on collagenase digestion of the tissue followed by a series of centrifugation steps to separate adipocytes from other nucleated cells based on buoyancy differences (2). The nucleated cell fraction contains MSCs and is referred to as the stromal vascular fraction (SVF). The SVF is currently commercially applied to treat equine tendons, ligaments, and joints without further in vitro cell expansion. The quantity of MSCs within the SVF appears to be directly related to the quantity of adipose tissues collected whereby there is considerable variation from one animal to another. Average nucleated cell yields from equine adipose tissue of $3.0 \pm 1.4 \times 10^5$ nucleated cells/mL of tissue have been reported (2). Therefore collecting more fat may be advantageous for clinical cases but may also be limited by body condition. Factors contributing to the variability in equine ASC harvest have so far not received much attention. Certainly age and fat depot location appear to affect functional characteristics of cell proliferation and sensitivity to apoptosis in humans (38). Morbidity may also limit ASC collection. In the attempt to better characterize whether MSCs are associated with perivascular niches, da Silva Meirelles and coworkers have demonstrated a relationship between ASC numbers and blood vessel density in fat samples (39).

Up until recently the lack of available antibodies and cross-reactivity with the equine species has hampered determination of surface proteins to reliably identify mesenchymal stem cells. The limited number of equine-specific or validated antibodies for MSC identification has forced the majority of work on equine MSCs to rely on adherence to plastic surfaces. However, Ratcliff and coworkers recently reported various surface markers for bone marrow-derived mononuclear cells (40). These cells showed variable

temporal expression in surface markers, whereby CD44 and CD29 remained robustly positive in culture over 30 days, CD90 expression increased as MPC cultures became more homogeneous whereas expression of CD11a/CD18 and CD45RB molecules decreased over time. The findings should help to improve MPC isolation methods for BMSCs and may well apply to ASCs as well. Based on work with non-equine specific antibodies, equine ASCs also express CD90 and CD44 (41). The use of antibodies against CD34 and CD45 to determine hematopoietic contaminants in culture has also been recently described for horses (42).

Ideal culture conditions maintain MSC with: (a) native phenotypic and functional characteristics similar, (b) indefinite proliferation, and (c) a capacity to differentiate into multiple lineages (43). The self-renewal potential of MSCs varies greatly according to methodology and species (44), but cells can be expected to expand for at least 40 population doublings (PDs) before their growth rate decreases significantly (45). Isolation of MSCs from various tissue sources is achieved through plastic adherence of primary nucleated cells in culture. Nonadherent cells are removed within 24–72 h with a media change. After 7–10 days with twice-weekly medium changes, a bone marrow stroma culture of heterogeneous MSCs develops (46). A relatively homogeneous culture develops by P2. Trypsin digestion for longer than 5 min tends to result in passage of other myeloid cell populations. Primary MSCs isolated from adipose or cord tissue typically form homogenous cultures within days after seeding the nucleated cell as long as the medium is changed within 24 h to remove nonadherent cells. Culture media may vary, but the most frequently used are Dulbecco's modified Eagle's medium (DMEM) and α-minimum essential medium (47).

Friedenstein et al. (48) showed that cyclic division that occurs in cell culture is not characteristic of in vivo behavior. The artificial environment of serum and exogenous growth factors may induce the cells to enter and remain in the cell cycle. As such, cells can expand up to 50 population or cell doublings (CD) compared with control basal media cultures, which have fewer than 50 CDs (49, 50). Addition of various growth factors at different concentrations probably contributes to heterogeneity observed in MSC types. Fibroblast growth factor (FGF)-2, increases human MSCs to more than 70 PDs (50). Hebert showed that epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) increased proliferation potential of human ASCs threefold, and ASCs treated with bFGF and EGF under adipogenic conditions showed more robust functionality (51). Cell doubling time was also decreased by approximately 50% in equine ASCS and BMSCs when bFGF was added to the stromal culture medium while maintaining differentiation potential of these cells (52).

Cell seeding density is a further factor that affects expansion rate but has so far not yet been investigated in equine MSCs (53, 54). Colter and coworkers showed dramatic differences in expansion rates when human MSCs were seeded at low versus high densities (53). A recent report showed that human bone marrow-derived MSCs seeded at 10 cells/cm^2 expanded approximately 500-fold within 12 days with a maximum cell doubling time of approximately 10 h (55). When the same cells were seeded at 1,000 cells/cm^2, they expanded only 30-fold. Also, effects of seeding density on MSC differentiation was illustrated by Sekiya (55), who showed that, after adipogenic induction of P3 human MSCs, there was significant reduction in Oil Red O staining when cells were seeded at 1,000 cells/cm^2 compared with those seeded at 50 cells/cm^2.

1.6. Adipogenic Induction of Equine ASCs

Methods of equine ASC adipogenic induction have been largely adapted from protocols for human MSCs (56–58). Initial work in our laboratory using a protocol (59) for human ASCs resulted in limited adipogenesis of equine ASCs. Rabbit serum enhanced adipogenesis in vitro for human (60–63), rat (63), and mouse (63) MSCs. Our studies demonstrated that the addition of 5% rabbit serum was necessary to achieve any reliable adipogenesis and fat droplet accumulation in equine ASCs (1, 2). Cell seeding density (64), culture media, and additives such as thiazolidinediones (65) and rabbit serum (60) all influence the rate of equine ASC adipogenic differentiation. There is also individual-dependent variation, as previously reported for human ASCs (66). Protocols for human equine ASC adipogenesis typically contain a mixture of insulin, dexamethasone, IBMX, and indomethacin (56). This protocol has been modified in our laboratory (59), replacing indomethacin with rosiglitazone (5 mM). Rosiglitazone is a member of the hypoglycemic drug family, thiazolidinediones or glitazones. The drugs are used against Type 2 diabetes due to selective PPARγ antagonism that affects insulin sensitivity (65).

2. Materials

2.1. Adipose Tissue Collection

1. Detomidine HCl (0.04 mg/kg intravenously).
2. Butorphanol (0.01 mg/kg intravenously).
3. Lidocaine (2%).

2.2. Isolation of Bone Marrow-Derived MSCs

1. Stromal growth medium: DMEM–Ham's F12 medium, characterized fetal bovine serum (10%), antibiotic/antimycotic solution (1%).

2. PBS solution: 1% bovine serum albumin (BSA Type V; Sigma–Aldrich), collagenase Type I (0.1%).
3. Red cell lysing buffer.

2.3. Method of Adipogenic Induction for Equine ASCs

1. Adipogenic induction medium: DMEM–Ham's F12, characterized fetal bovine serum (3%), 1% antibiotic/antimycotic solution, biotin (33 μmol/L), pantothenate (17 μmol/L), insulin (1 μmol/L), dexamethasone (1 μmol/L), isobutylmethylxanthine (IBMX; 0.5 mmol/L), rosiglitazone (5 μmol/L; Avandia™, Glaxo Smith Kline, Cidra, Puerto Rico), rabbit serum 5% (see Note 1).
2. Adipogenic maintenance medium: same as adipogenic induction medium without the IBMX and rosiglitazone.
3. Oil Red O solution (stock): weigh out 0.5 g Oil Red O, dissolve in 100 mL isopropanol, filter through a 0.2-μm filter, and store at room temperature as stock solution.
4. Formalin (10%): made from 37% formaldehyde commercial stock (vol:vol).

3. Methods

3.1. Adipose Tissue Collection

The emphasis of this chapter is the mesenchymal stromal cell of adipose tissue and hence these methods of tissue collection are described here. The reader is also directed to references for the collection of other commonly harvested tissues such as bone marrow (67–69), equine umbilical cord tissue (6), and umbilical cord blood (7, 8).

1. Horses are sedated with detomidine HCl (0.04 mg/kg intravenously (IV)) and butorphanol (0.01 mg/kg IV).
2. The area over the dorsal gluteal muscles or the head of the tail is aseptically prepared.
3. The skin and subcutaneous tissues are desensitized by local infiltration of 2% lidocaine directly into the surgical site or via an inverted L-block around the planned incision site.
4. A 3- to 5-cm incision is made in a sagittal plane over the head of the tail or abaxial and parallel to the vertebral column.
5. The amount of adipose tissue is harvested will depend on the body condition score of the horse over the superficial gluteal fascia, and the skin incision apposed with nylon suture material (see Note 2).

3.1.1. Isolation of Adipose Tissue-Derived MSCs

1. Fresh adipose tissue is minced with a sterile surgical blade, washed, and briefly agitated with an equal volume of

phosphate-buffered saline (PBS) solution to promote separation into two phases (see Note 3).

2. The upper phase consists of the minced and washed adipose tissue, and the liquid infranatant containing hematopoietic cells suspended in PBS is removed.
3. The tissue is then digested in an equal volume of a filtered (0.2-mm filter) PBS solution containing 1% BSA (Type V; Sigma–Aldrich) and 0.1% collagenase (Type I; Worthington Biochemical, Lakewood, NJ) with continuously shaking at 37°C for ~50 min (see Note 4).
4. The sample is then centrifuged at 260×*g* for 5 min.
5. To complete stromal cell separation from primary adipocytes, the sample is briefly and vigorously agitated again followed by centrifugation at 260×*g* for 5 min, resulting in a SFV pellet.
6. The supernatant composed of oil, primary adipocytes, and collagenase solution is carefully removed after centrifugation to avoid disturbance of the SVF pellet, which is then resuspended in 10 mL stromal medium (see Note 5).
7. The SVF is the remaining nucleated cell fraction of adipose tissue, which contains the ASCs.
8. A small aliquot of the cell suspension is centrifuged, and the pellet resuspended in an equal volume of a red cell lysing buffer (Sigma–Aldrich) for nuclear Trypan Blue staining and hemocytometer assessment of total nucleated cell numbers per unit volume of adipose tissue.

3.2. Method of Adipogenic Induction for Equine ASCs

The following procedure is used in our laboratory for adipogenic induction of bone marrow and adipose tissue-derived MSCs:

1. Before the adipogenic induction, early passage MSCs are grown to confluence in stromal medium (DMEM–Ham's F12, 10% characterized fetal bovine serum, 1% antibiotic/antimycotic solution).
2. MSCs are then exposed to an adipogenic induction medium for 3 days (see Note 6).
3. Thereafter, the same medium without the IBMX and the Avandia™ is used to maintain the adipocyte cell culture (see Note 7).
4. The cells cultures are then gently rinsed three times with PBS and fixed in PBS containing 10% formalin and placed on a cold plate at 4°C for 1 h.
5. Lipid accumulation is stained via Oil Red O (66, 70). The cells are stained for 20 min followed by three rinses with distilled water. Take 6 mL of Oil Red O stock solution. Add

4 mL of distilled water. Let stand for 1 h at room temperature. Remove all formaldehyde from well. The well should be dry after fixation and removal. Take Oil Red O solution that has been sitting for 1 h and filter it through a 0.2-μm filter. Add 50 μL Oil Red O to each well (96-well plate) for 15 min at room temperature. Rinse three times or more with 50 μL distilled water until the rinse becomes completely clear (no red coloring). Do not rinse with a volume larger than 50 μL. 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.

6. Elute the stain from the cells by adding 50 μL isopropanol per well. Elution is immediate.
7. Read the OD_{540} using a plate reader.
8. 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.

3.3. Other Reported Methods

Table 1 is a summary of all equine related reports to date in chronological order, which have used adipogenesis in their experiments. The table includes studies of other MSC types and briefly describes the method used as well as the references for adipogenic protocols quoted in these studies. It is interesting to note that robust adipogenesis in MSCs from bone marrow and adipose tissue was only achieved by those authors (1, 2, 7–9) who used rabbit serum as part of their adipogenic protocol. Koerner and his coworkers had adopted the method by Janderova et al. (60) in which MSCs were exposed to repeated cycles of adipogenic induction and maintenance media because three cycles of the differentiation cocktail the C/EBP protein had completely disappeared, which suggested that by that point the majority of the hMSCs had progressed through the adipocytic "commitment" stage (60). Koerner et al., however, reported much weaker droplet formation in bone marrow and peripheral blood progenitor cells as it is typically seen in human marrow-derived MSCs. This might suggest that existing adipogenic protocols merely render equine MSCs at the stage of commitment but not terminal differentiation. Interestingly, MSCs from equine umbilical cord apparently differentiated into the adipogenic lineage without the addition of rabbit serum (6). These disparate results further support the notion that there are profound differences among mesenchymal stem cells from different tissue sources, which is the focus of much of the current research efforts in order to attribute the suitable MSC for appropriate applications in regenerative medicine.

Table 1
Other methods reported for equine mesenchymal stromal cells

References	Cell type	Adipogenic method
Koerner et al. (32)	PBMSC, BMSC	Cells cultured until confluence. Then adiopogenic induction medium (10% FCS), 10 g/mL insulin (Gibco), 1 M dexamethasone, 100 µM indomethacin, and 500 µM IBMX (Sigma–Aldrich) for 72 h followed by adipogenic maintenance medium for 24 h (one 96-h cycle). This cycle of treatments was repeated four times in total, with an additional week in maintenance medium (3, 25)
Vidal et al. (56)	BMSC	See above description
Vidal et al. (57)	ASC	See above description
Koch et al. (30)	CBMSC	The cells are expanded to 100% confluency in regular expansion media. Authors quote protocols by Pittenger et al. (42) and Janderova et al. (25)
Hoynowski et al. (23)	CTMSC	Authors quote the protocol by Wang et al. (58)
Giovannini et al. (16)	PBMSC	Authors quote protocols by Pittenger et al. (42) and Janderova et al. (25). Cells are grown to confluence and then exposed to 4 cycles of: 72 h in induction medium (DMEM–F12, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1 mM dexamethasone [Sigma, Buchs, Switzerland), 100 mM indomethacine [Sigma), 500 mM 3-IBMX [Sigma), 1,700 nM insulin [Life Technologies)), and rabbit serum. Followed by 24 h in maintenance medium (DMEM–F12, 10% FBS, 1% penicillin/streptomycin supplemented with 1,700 nM insulin)
Mambelli et al. (34)	ASC	Authors quote Zuk et al. (59) and describe their own protocol: cells are cultured to a monolayer for 72 h in basal medium. Adipogenic induction (DMEM–HG culture medium supplemented with 2% KSR, 1 mM dexamethasone, 100 mM indomethacin [Sigma], 0.5 mM IBMX [Sigma], 10 mg/mL insulin [Sigma], and 1% antibiotic solution). Cells are washed and fixed by Day 4 and 7
Koch et al. (31)	CBMSC, BMSC	Cells are cultured for 10 days in adipogenic induction medium (BulletKit, Lonza) consisting of 1 mM dexamethasone, 0.5 mM 3-IBMX, 10 mg/mL recombinant human (rh) insulin, 0.2 mM indomethacin, and 10% fetal calf serum (FCS) in DMEM. The FCS included in the commercial medium kit for adipogenic induction was discarded and substituted with 15% rabbit serum (Sigma)

BMSC bone marrow-derived mesenchymal stem cells, *ASC* adipose tissue-derived mesenchymal stem cells, *PBMSC* peripheral blood-derived progenitors, *CT-MSC* umbilical cord tissue progenitor cells, *CB-MSC* umbilical cord blood progenitor cells

4. Notes

1. The IBMX should be used fresh.
2. The author prefers to limit the surgical incision length to 3–4 cm due to cosmetic appearance and to avoid postoperative serum accumulation, swelling, and scaring.
3. Due to the fibrous nature of equine adipose tissue harvested from the region described above, diligent mincing of the tissues is important and in our hands has resulted in higher yields of mononuclear cells.
4. Incubation periods in collagenase solution of more than 60–90 min appears to result in lower mononuclear cell yields.
5. After removal of the oil, primary adipocytes and part of the collagenase solution, there is often still fibrous material above the SVF pellet. The use of a sterile tea strainer and stromal medium will allow separation and washing of this fibrous material from the SVF. The remnant cell suspension is centrifuged again to obtain the SVF pellet, the remnant collagenase solution is removed, and the pellet is resuspended in a known amount of stromal medium.
6. Adipogenic conversion is typically noted within 24 h of culture in adipogenic induction media. The cell morphology changes from elongated spindle to oval cells. The cells have a distinct ring of dark granules around the cell periphery, which appear as fat droplets after Oil Red O staining (Fig. 1) (2).
7. The ASCs often begin to detach from the plastic culture dishes after 6–8 days in adipogenic culture media. Quantitative DNA and protein assessments should be made before then.

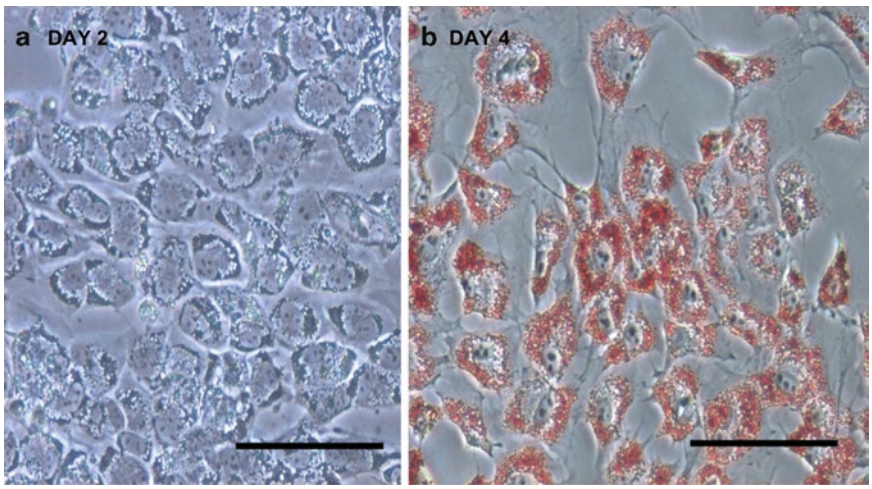

Fig. 1. *Panel a* shows unstained equine ASCs with characteristic cytoplasmic droplets by Day 2 after adipogenic induction. *Panel b* shows Oil Red O-stained equine ASCs 4 days after adipogenic induction. Reprinted from (2).

References

1. Vidal, M. A., Kilroy, G. E., Johnson, J. R., Lopez, M. J., Moore, R. M., and Gimble, J. M. (2006) Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity, *Vet Surg 35*, 601–610.
2. Vidal, M. A., Kilroy, G. E., Lopez, M. J., Johnson, J. R., Moore, R. M., and Gimble, J. M. (2007) Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells, *Vet Surg 36*, 613–622.
3. Koerner, J., Nesic, D., Romero, J. D., Brehm, W., Mainil-Varlet, P., and Grogan, S. P. (2006) Equine peripheral blood-derived progenitors in comparison to bone marrow-derived mesenchymal stem cells, *Stem Cells 24*, 1613–1619.
4. Arnhold, S. J., Goletz, I., Klein, H., Stumpf, G., Beluche, L. A., Rohde, C., Addicks, K., and Litzke, L. F. (2007) Isolation and characterization of bone marrow-derived equine mesenchymal stem cells, *Am J Vet Res 68*, 1095–1105.
5. Reed, S. A. and Johnson, S. E. (2008) Equine umbilical cord blood contains a population of stem cells that express Oct4 and differentiate into mesodermal and endodermal cell types, *J Cell Physiol 215*, 329–336.
6. Hoynowski, S. M., Fry, M. M., Gardner, B. M., Leming, M. T., Tucker, J. R., Black, L., Sand, T., and Mitchell, K. E. (2007) Characterization and differentiation of equine umbilical cord-derived matrix cells, *Biochem Biophys Res Commun 362*, 347–353.
7. Koch, T. G., Heerkens, T., Thomsen, P. D., and Betts, D. H. (2007) Isolation of mesenchymal stem cells from equine umbilical cord blood, *BMC Biotechnol 7*, 26.
8. Koch, T. G., Thomsen, P. D., and Betts, D. H. (2009) Improved isolation protocol for equine cord blood-derived mesenchymal stromal cells, *Cytotherapy 11*, 443–447.
9. Giovannini, S., Brehm, W., Mainil-Varlet, P., and Nesic, D. (2008) Multilineage differentiation potential of equine blood-derived fibroblast-like cells, *Differentiation 76*, 118–129.
10. Rosen, E. D. and Spiegelman, B. M. (2000) Molecular regulation of adipogenesis, *Annu Rev Cell Dev Biol 16*, 145–171.
11. Klyde, B. J. and Hirsch, J. (1979) Increased cellular proliferation in adipose tissue of adult rats fed a high-fat diet, *J Lipid Res 20*, 705–715.
12. Flier, J. S., Cook, K. S., Usher, P., and Spiegelman, B. M. (1987) Severely impaired adipsin express\ion in genetic and acquired obesity, *Science 237*, 405–408.
13. Min, H. Y. and Spiegelman, B. M. (1986) Adipsin, the adipocyte serine protease: gene structure and control of expression by tumor necrosis factor, *Nucleic Acids Res 14*, 8879–8892.
14. Venugopal, J., Hanashiro, K., and Nagamine, Y. (2007) Regulation of PAI-1 gene expression during adipogenesis, *J Cell Biochem 101*, 369–380.
15. Leibel, R. L. (2002) The role of leptin in the control of body weight, *Nutr Rev 60*, S15–S19; discussion S68–84, 85–17.
16. Ouchi, N., Kihara, S., Funahashi, T., Matsuzawa, Y., and Walsh, K. (2003) Obesity, adiponectin and vascular inflammatory disease, *Curr Opin Lipidol 14*, 561–566.
17. Nuttall, M. E. and Gimble, J. M. (2004) Controlling the balance between osteoblastogenesis and adipogenesis and the consequent therapeutic implications, *Curr Opin Pharmacol 4*, 290–294.
18. Cornelius, P., MacDougald, O. A., and Lane, M. D. (1994) Regulation of adipocyte development, *Annu Rev Nutr 14*, 99–129.
19. Tang, Q. Q., Gronborg, M., Huang, H., Kim, J. W., Otto, T. C., Pandey, A., and Lane, M. D. (2005) Sequential phosphorylation of CCAAT enhancer-binding protein beta by MAPK and glycogen synthase kinase 3beta is required for adipogenesis, *Proc Natl Acad Sci USA 102*, 9766–9771.
20. Umek, R. M., Friedman, A. D., and McKnight, S. L. (1991) CCAAT-enhancer binding protein: a component of a differentiation switch, *Science 251*, 288–292.
21. Altiok, S., Xu, M., and Spiegelman, B. M. (1997) PPARgamma induces cell cycle withdrawal: inhibition of E2F/DP DNA-binding activity via down-regulation of PP2A, *Genes Dev 11*, 1987–1998.
22. Timchenko, N. A., Wilde, M., Nakanishi, M., Smith, J. R., and Darlington, G. J. (1996) CCAAT/enhancer-binding protein alpha (C/EBP alpha) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein, *Genes Dev 10*, 804–815.
23. MacDougald, O. A. and Lane, M. D. (1995) Transcriptional regulation of gene expression during adipocyte differentiation, *Annu Rev Biochem 64*, 345–373.

24. Spiegelman, B. M., Choy, L., Hotamisligil, G. S., Graves, R. A., and Tontonoz, P. (1993) Regulation of adipocyte gene expression in differentiation and syndromes of obesity/diabetes, *J Biol Chem 268*, 6823–6826.
25. Robinson, C. E., Wu, X., Morris, D. C., and Gimble, J. M. (1998) DNA bending is induced by binding of the peroxisome proliferator-activated receptor gamma 2 heterodimer to its response element in the murine lipoprotein lipase promoter, *Biochem Biophys Res Commun 244*, 671–677.
26. Tontonoz, P., Hu, E., Devine, J., Beale, E. G., and Spiegelman, B. M. (1995) PPAR gamma 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene, *Mol Cell Biol 15*, 351–357.
27. Kim, J. B., Sarraf, P., Wright, M., Yao, K. M., Mueller, E., Solanes, G., Lowell, B. B., and Spiegelman, B. M. (1998) Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1, *J Clin Invest 101*, 1–9.
28. Kim, J. B. and Spiegelman, B. M. (1996) ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism, *Genes Dev 10*, 1096–1107.
29. Brown, M. S. and Goldstein, J. L. (1997) The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor, *Cell 89*, 331–340.
30. Xu, J. and Liao, K. (2004) Protein kinase B/AKT 1 plays a pivotal role in insulin-like growth factor-1 receptor signaling induced 3T3-L1 adipocyte differentiation, *J Biol Chem 279*, 35914–35922.
31. Benito, M., Porras, A., Nebreda, A. R., and Santos, E. (1991) Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of ras oncogenes, *Science 253*, 565–568.
32. Farmer, S. R. (2006) Transcriptional control of adipocyte formation, *Cell Metab 4*, 263–273.
33. Bennett, C. N., Ross, S. E., Longo, K. A., Bajnok, L., Hemati, N., Johnson, K. W., Harrison, S. D., and MacDougald, O. A. (2002) Regulation of Wnt signaling during adipogenesis, *J Biol Chem 277*, 30998–31004.
34. Tang, Q. Q., Jiang, M. S., and Lane, M. D. (1999) Repressive effect of Sp1 on the C/EBPalpha gene promoter: role in adipocyte differentiation, *Mol Cell Biol 19*, 4855–4865.
35. Tomlinson, J. J., Boudreau, A., Wu, D., Atlas, E., and Hache, R. J. (2006) Modulation of early human preadipocyte differentiation by glucocorticoids, *Endocrinology 147*, 5284–5293.
36. Wang, Y., Kim, K. A., Kim, J. H., and Sul, H. S. (2006) Pref-1, a preadipocyte secreted factor that inhibits adipogenesis, *J Nutr 136*, 2953–2956.
37. Solomon, C., White, J. H., and Kremer, R. (1999) Mitogen-activated protein kinase inhibits 1,25-dihydroxyvitamin D3-dependent signal transduction by phosphorylating human retinoid X receptor alpha, *J Clin Invest 103*, 1729–1735.
38. Schipper, B. M., Marra, K. G., Zhang, W., Donnenberg, A. D., and Rubin, J. P. (2008) Regional anatomic and age effects on cell function of human adipose-derived stem cells, *Ann Plast Surg 60*, 538–544.
39. da Silva Meirelles, L., Sand, T. T., Harman, R. J., Lennon, D. P., and Caplan, A. I. (2009) MSC frequency correlates with blood vessel density in equine adipose tissue, *Tissue Eng Part A 15*, 221–229.
40. Radcliffe, C. H., Flaminio, M. J., and Fortier, L. A. (2010) Temporal analysis of equine bone marrow aspirate during establishment of putative mesenchymal progenitor cell populations, *Stem Cells Dev*.
41. de Mattos Carvalho, A., Alves, A. L., Golim, M. A., Moroz, A., Hussni, C. A., de Oliveira, P. G., and Deffune, E. (2009) Isolation and immunophenotypic characterization of mesenchymal stem cells derived from equine species adipose tissue, *Vet Immunol Immunopathol*
42. Martinello, T., Bronzini, I., Maccatrozzo, L., Iacopetti, I., Sampaolesi, M., Mascarello, F., and Patruno, M. (2010) Cryopreservation does not affect the stem characteristics of multipotent cells isolated from equine peripheral blood, *Tissue Eng Part C Methods* .
43. Meirelles Lda, S. and Nardi, N. B. (2003) Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization, *Br J Haematol 123*, 702–711.
44. Bianco, P., Riminucci, M., Gronthos, S., and Robey, P. G. (2001) Bone marrow stromal stem cells: nature, biology, and potential applications, *Stem Cells 19*, 180–192.
45. Bruder, S. P., Jaiswal, N., and Haynesworth, S. E. (1997) Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation, *J Cell Biochem 64*, 278–294.
46. Islam, M. Q., Meirelles Lda, S., Nardi, N. B., Magnusson, P., and Islam, K. (2006) Polyethylene glycol-mediated fusion between primary mouse mesenchymal stem cells and mouse fibroblasts generates hybrid cells with

increased proliferation and altered differentiation, *Stem Cells Dev 15*, 905–919.

47. Otto, W. R. and Rao, J. (2004) Tomorrow's skeleton staff: mesenchymal stem cells and the repair of bone and cartilage, *Cell Prolif 37*, 97–110.
48. Friedenstein, A. J., Chailakhyan, R. K., Latsinik, N. V., Panasyuk, A. F., and Keiliss-Borok, I. V. (1974) Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo, *Transplantation 17*, 331–340.
49. Castro-Malaspina, H., Gay, R. E., Resnick, G., Kapoor, N., Meyers, P., Chiarieri, D., McKenzie, S., Broxmeyer, H. E., and Moore, M. A. (1980) Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny, *Blood 56*, 289–301.
50. Bianchi, G., Banfi, A., Mastrogiacomo, M., Notaro, R., Luzzatto, L., Cancedda, R., and Quarto, R. (2003) Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2, *Exp Cell Res 287*, 98–105.
51. Hebert, T. L., Wu, X., Yu, G., Goh, B. C., Halvorsen, Y. D., Wang, Z., Moro, C., and Gimble, J. M. (2009) Culture effects of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) on cryopreserved human adipose-derived stromal/stem cell proliferation and adipogenesis, *J Tissue Eng Regen Med 3*, 553–561.
52. Colleoni, S., Bottani, E., Tessaro, I., Mari, G., Merlo, B., Romagnoli, N., Spadari, A., Galli, C., and Lazzari, G. (2009) Isolation, growth and differentiation of equine mesenchymal stem cells: effect of donor, source, amount of tissue and supplementation with basic fibroblast growth factor, *Vet Res Commun* 33:811–821.
53. Colter, D. C., Class, R., DiGirolamo, C. M., and Prockop, D. J. (2000) Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow, *Proc Natl Acad Sci USA 97*, 3213–3218.
54. Colter, D. C., Sekiya, I., and Prockop, D. J. (2001) Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells, *Proc Natl Acad Sci USA 98*, 7841–7845.
55. Sekiya, I., Larson, B. L., Smith, J. R., Pochampally, R., Cui, J. G., and Prockop, D. J. (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality, *Stem Cells 20*, 530–541.
56. Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999) Multilineage potential of adult human mesenchymal stem cells, *Science 284*, 143–147.
57. Wang, H. S., Hung, S. C., Peng, S. T., Huang, C. C., Wei, H. M., Guo, Y. J., Fu, Y. S., Lai, M. C., and Chen, C. C. (2004) Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord, *Stem Cells 22*, 1330–1337.
58. Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P., and Hedrick, M. H. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies, *Tissue Eng 7*, 211–228.
59. Mitchell, J. B., McIntosh, K., Zvonic, S., Garrett, S., Floyd, Z. E., Kloster, A., Di Halvorsen, Y., Storms, R. W., Goh, B., Kilroy, G., Wu, X., and Gimble, J. M. (2006) Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers, *Stem Cells 24*, 376–385.
60. Janderova, L., McNeil, M., Murrell, A. N., Mynatt, R. L., and Smith, S. R. (2003) Human mesenchymal stem cells as an in vitro model for human adipogenesis, *Obes Res 11*, 65–74.
61. Hicok, K. C., Thomas, T., Gori, F., Rickard, D. J., Spelsberg, T. C., and Riggs, B. L. (1998) Development and characterization of conditionally immortalized osteoblast precursor cell lines from human bone marrow stroma, *J Bone Miner Res 13*, 205–217.
62. Houghton, A., Oyajobi, B. O., Foster, G. A., Russell, R. G., and Stringer, B. M. (1998) Immortalization of human marrow stromal cells by retroviral transduction with a temperature sensitive oncogene: identification of bipotential precursor cells capable of directed differentiation to either an osteoblast or adipocyte phenotype, *Bone 22*, 7–16.
63. Diascro, D. D., Jr., Vogel, R. L., Johnson, T. E., Witherup, K. M., Pitzenberger, S. M., Rutledge, S. J., Prescott, D. J., Rodan, G. A., and 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*, 96–106.
64. Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Ailhaud, G., Negrel, R., and Pfeiffer, E. F. (1989) Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium, *J Clin Invest 84*, 1663–1670.

65. Scheen, A. J. (2002) (Medication of the month. Rosiglitazone (Avandia)), *Rev Med Liege 57*, 236–239.
66. Sen, A., Lea-Currie, Y. R., Sujkowska, D., Franklin, D. M., Wilkison, W. O., Halvorsen, Y. D., and Gimble, J. M. (2001) Adipogenic potential of human adipose derived stromal cells from multiple donors is heterogeneous, *J Cell Biochem 81*, 312–319.
67. Orsini, J. A. and Divers, T. J. (1998) *Manual of Equine Emergencies: Treatment and Procedures*, 1 ed., Saunders, Philadelphia, PA.
68. Smith, R. K., Korda, M., Blunn, G. W., and Goodship, A. E. (2003) Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment, *Equine Vet J 35*, 99–102.
69. Fortier, L. A., Nixon, A. J., Williams, J., and Cable, C. S. (1998) Isolation and chondrocytic differentiation of equine bone marrow-derived mesenchymal stem cells, *Am J Vet Res 59*, 1182–1187.
70. Halvorsen, Y. D., Bond, A., Sen, A., Franklin, D. M., Lea-Currie, Y. R., Sujkowski, D., Ellis, P. N., Wilkison, W. O., and Gimble, J. M. (2001) Thiazolidinediones and glucocorticoids synergistically induce differentiation of human adipose tissue stromal cells: biochemical, cellular, and molecular analysis, *Metabolism 50*, 407–413.

Chapter 7

Isolation and Culture of Porcine Adipose Tissue-Derived Somatic Stem Cells

Kellie J. Williams, Robert A. Godke, and Kenneth R. Bondioli

Abstract

Adipose tissue-derived stem cells (ASCs) have been described for a number of laboratory animals and humans. Improved culture conditions and cellular characteristics of ASCs have been identified. ASCs can self-renew and differentiate into multiple tissue lineages. Further characterization of ASCs in this manner could enhance the isolation and purification of a population of mesenchymal stem cells (MSCs) from easily obtainable adipose tissue. These stem cell populations from domestic animals, which make attractive models for transplantation studies, will be valuable for the evaluation of their efficacy in tissue regeneration applications in the future. These cells may also represent a population more easily reprogrammable during somatic cell nuclear transfer and thus expedite the development of transgenic animals for models and production of valuable pharmaceutical proteins.

Key words: Stem cells, Porcine, Adipose tissue, Cell cycle, Clonal expansion, Differentiation

1. Introduction

The expansion of our basic knowledge of stem cell biology will greatly impact many disciplines including development, cancer, and aging, as well as provide a foundation for the development of tissue engineering (1). An array of scientific disciplines have joined efforts to develop the techniques involved in tissue engineering for repairing, replacing, or regenerating diseased tissues and organs. With the advancement of this biotechnology, for example, scaffold biomaterials can be combined with a variety of stem cells to treat disease through cell-based therapies. Other cell therapeutic applications include bone marrow transplants; cartilage, tendon, ligament, and bone repair; treatment of vascular disease; and even the production of transgenic animals (1).

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Several applications involve the technique of cell transplantation. Embryonic stem cells have the capability to form biologically functional cell populations suitable for integration, response, and function within a diseased tissue. Embryonic stem cells produce these cell populations in response to signaling pathways induced by factors such as cytokines, transcription factors, and cell-to-cell interactions. Multiple cell populations derived from embryonic stem cells, including cardiomyocytes (2), insulin-secreting cells (3), and neuronal precursor cells (4, 5), have been successfully integrated within the host tissue and have displayed appropriate cell functions. For example, after transplantation into the spinal cords of myelin-deficient rats, glial precursor cells derived from embryonic stem cells successfully differentiated into astrocytes and oligodendrocytes capable of forming myelin at the site of injection and migrating several millimeters away from the injection site (4, 6). Candidate cells for such promising applications must meet several requirements to be eligible for classification as a stem cell population. Cells must have the capacity for self-renewal, must have long-term viability, and must have the ability to give rise to differentiated cell types to be considered "stem cells" (7). In addition to embryonic stem cells, a number of stem cell populations isolated from somatic tissues have met these criteria and are being evaluated for tissue engineering applications. ASCs from human liposuction aspirates have been described and are being investigated for such applications (8, 9). The availability of these cell populations from animals such as the pig will expedite the full investigation of these cells for efficacy and safety.

2. Materials

2.1. Cell Isolation

1. Sterile scalpel.
2. Autoclaved 100-ml beakers.
3. DPBS solution: Dulbecco's phosphate-buffered saline containing 200 U/ml of penicillin, 200 µg/ml of streptomycin, and 2.5 µg/ml of Fungizone.
4. Collagenase solution: 0.1% collagenase Type I (240 IU/ml) in DPBS with 1% bovine serum albumin (fraction V) (see Note 1).
5. Shaker incubator.
6. Dual filter system, comprised of one nylon net filter with a 20-µm pore size and one with a 80-µm pore size. The 80-µm filter is placed on top of the 20-µm filter in a syringe filter, and the assembled unit is autoclaved.
7. Centrifuge.

8. Hoechst stain: Hoechst 33342 (2 μg/ml) dissolved in water.
9. Expansion medium: Dulbecco's modified Eagle medium (DMEM) with high glucose (4,500 mg/L), 4.0 mM glutamine, and sodium pyruvate supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml streptomycin (pen/strep), and 2.5 μg/ml of Fungizone (see Note 2).

2.2. Cell Culture Maintenance

1. DPBS solution.
2. Enzymatic digestion medium: 0.25% trypsin–EDTA.
3. Expansion medium: DMEM with high glucose (4,500 mg/L), 4.0 mM glutamine, and sodium pyruvate supplemented with 10% FBS, pen/strep, and 2.5 μg/ml of Fungizone.

2.3. Clonal Isolation/ Establishment

1. DPBS solution.
2. Enzymatic digestion medium: 0.25% trypsin–EDTA.
3. Expansion medium.
4. Cloning rings.
5. 100-mm culture dishes.
6. 1.9-cm^2 culture dishes.
7. 9.62-cm^2 culture dishes.
8. 25-cm^2 culture flasks.
9. 75-cm^2 culture flasks.

2.4. Differentiation

1. DPBS solution.
2. Enzymatic digestion medium: 0.25% trypsin–EDTA.
3. Expansion medium.
4. Adipogenesis-inducing culture medium (days 1–4): DMEM supplemented with 3% FBS, pen/strep, 2.5 μg/ml of Fungizone, 10 ng/ml of insulin, 10^{-9} M dexamethasone, 250 μM isobutyl methylxanthine, and 0.2 mM indomethacin.
5. Adipogenesis-inducing culture medium (days 4 and beyond): DMEM supplemented with 3% FBS, pen/strep, 2.5 μg/ml of Fungizone, 10 ng/ml of insulin, and 10^{-9} M dexamethasone.
6. Chondrogenesis-inducing culture medium: DMEM supplemented with 10% FBS, pen/strep, 2.5 μg/ml of Fungizone, 6.25 μg/ml of insulin, 10 ng/ml of transforming growth factor-β and 10^{-9} M dexamethasone.
7. Osteogenesis-inducing culture medium: DMEM supplemented with 10% FBS, pen/strep, 2.5 μg/ml of Fungizone, 10 mM β-glycerophosphate, 50 ng/ml ascorbic acid, and 10^{-9} M dexamethasone (see Note 3).

8. Nile red staining solution: Stock solution, Nile red 1 mg/ml in DMSO. Working solution, 500 µl stock solution in 49.5 ml of 0.9% saline and 50 mg of polyvinyl pyrrolidone.
9. Safranin O staining solution: Working solution, 50 mg Safranin O in 50 ml of 0.9% saline.
10. Alizarin red S staining solution: Working solution, 1 g Alizarin red S in 45 ml of 0.9% saline and 5 ml of ammonium hydroxide (28–30%).
11. Cell fixation solution: 4% paraformaldehyde solution in DPBS without calcium and magnesium.

3. Methods

Stem cell applications for biomedical treatments continue to face many challenges concerning the originating source, expansion and culture, and differentiation. By definition, stem cells must be able to self-renew as well as differentiate into multiple tissue phenotypes. Adult stem cells can be found among differentiated tissues. Adult stem cells can renew themselves and, when removed from their niche, they are able to differentiate into the multiple cell types surrounding them in order to maintain and repair that tissue or organ. The development of an animal stem cell model will enhance important biomedical treatments, such as tissue engineering and cord blood stem cell transplants utilized in human patients.

Mesenchymal stem cells (MSCs) have been isolated from many different mesodermal tissues including bone marrow (8), fat (9, 10), and human cord blood (11). MSCs are pluripotent precursors that support hematopoiesis and formation of bone tissue, cartilage, and adipose tissue (8, 9, 12). MSCs have been isolated from various tissues based on their ability to adhere to plastic and their morphology similar to that of fibroblasts (13–17). However, hematopoietic cells contaminate MSCs, creating a heterogeneous population of cells in culture. An important characteristic used to distinguish MSCs from hematopoietic cells is their ability to support differentiation into several different cell types depending on culture conditions.

Human adipose tissue-derived adult stem cells (ASCs) are self-renewing and can be differentiated into multiple tissue lineages (8, 18). The following is a protocol developed for the isolation and culture of porcine and bovine ASCs. Growth characteristics including population doublings, cell cycle length, and proliferative capacities, as well as self-renewal and differentiation abilities have been determined for porcine ASCs cells using these methodologies.

3.1. Cell Isolation

1. Subcutaneous fat (approximately 10 g per pig) is harvested from the dorsal abdominal area at a local abattoir (see Note 4). Samples are transported on ice in DPBS solution.
2. The tissue is finely minced using sterilized scissors and washed with DPBS solution.
3. Each sample is digested with an equal volume of collagenase solution in a shaker incubator rotating at 200 rpm at 39°C for approximately 2 h.
4. The cell suspension is transferred into a 50-ml centrifuge tube and is centrifuged at 300 × *g* for 5 min, shaken vigorously for 30 s to promote separation of the stromal fraction, and centrifuged a second time with the same parameters (see Note 5).
5. After aspiration of floating adipocytes, the cell suspension is shaken once more for 30 s and then filtered through the dual filter system into a fresh 50-ml centrifuge tube.
6. Following filtration, the cell suspension is centrifuged at 300 × *g* for 5 min, the supernatant aspirated, and the pelleted stromal cells resuspended in 1 ml of expansion medium.
7. A 100-µl aliquot is removed and stained by the addition of 10 µl of Hoechst staining solution. The concentration of nucleated cells in the suspension is determined by counting in a hemocytometer under UV illumination.
8. Cells are plated at ~6,700 nucleated cells/cm^2 in 10 ml of expansion medium in a 75-cm^2 culture flask and incubated at 39°C under a 5% CO_2 in air humidified atmosphere.
9. Approximately 24 h after initial plating, expansion medium is removed and replaced with fresh medium to remove nonadherent cells.

3.2. Culture Maintenance

1. Fresh medium is supplied every 3–4 days.
2. At 70–90% confluence, cells are passaged by trypsinization, counted using a hemocytometer, and reseeded at a concentration of ~6,700 cells/cm^2 (see Note 6).
3. Cell cycle length is calculated based on the following equation: number of days in culture/population doublings. Sample data are presented in Fig. 1a.
4. Population doublings are calculated at each passage using the following equation: log (final concentration/initial concentration) × 3.33 (19). Sample data are shown in Fig 1b.
5. Cells are passaged until replicative senescence is observed (see Note 7).

3.3. Clonal Isolation/ Establishment

1. Cells are isolated according to the above procedure and plated from the initial culture (passage 0) at ~5 cells/cm^2 in 10 ml of expansion medium in a 10-cm diameter culture dish and

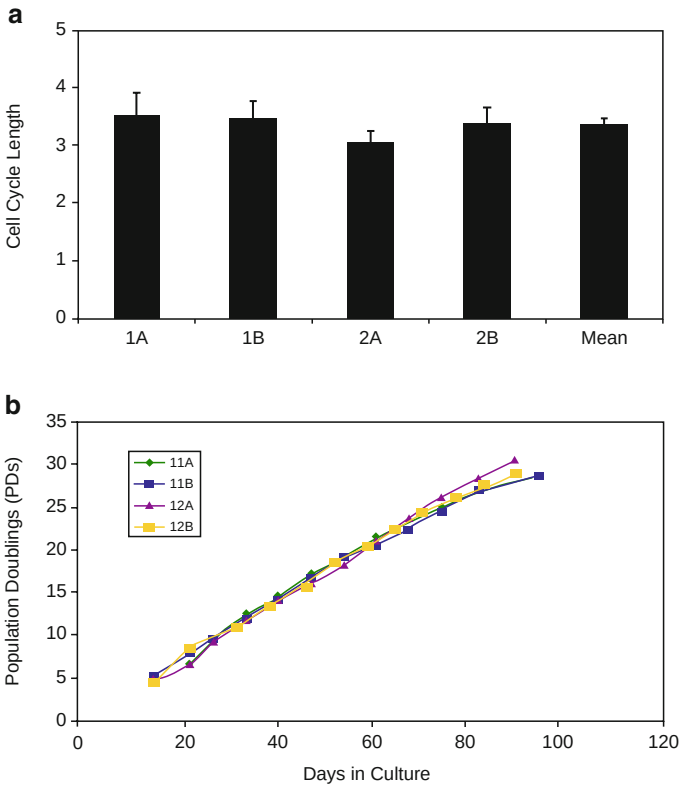

Fig. 1. Proliferative characteristics of undifferentiated stromal cells: 1 and 2 represent each cell line while (**a**) and (**b**) are replicates. (**a**) Average cell cycle lengths were recorded throughout the duration of culture. (**b**) Proliferative capacity indicates cells have not reached observed, replicative senescence.

incubated at 39°C under a 5% CO_2 in air humidified atmosphere.

2. Adherent cells form colonies after approximately 10 days in culture and are selected for ring cloning (passage 0) based on the criteria that colonies contain at least 100 cells and colonies are located a minimum of one microscopic field from all other colonies.
3. Cells are passaged by trypsinization and reseeded in a 1.9-cm^2 culture dish (passage 1) and at 70–90% confluence are passaged into a 9.62-cm^2 culture dish (passage 2), a 25-cm^2 culture flask (passage 3), and a 75-cm^2 culture flask (passage 4).

3.4. Differentiation

1. At 70–90% confluence (following passage 4 for clones), cells are passaged by trypsinization and cultured for 21 days in culture medium designed to induce differentiation. Culture media are changed every 3–4 days.
2. For adipogenesis, cells are cultured through day 4 in adipogenesis-inducing culture medium (days 1–4) and cultured

beyond day 4 in adipogenesis-inducing culture medium (days 4 and beyond).

3. For chondrogenesis, cells are cultured for the duration of differentiation in chondrogenesis-inducing culture medium.
4. For osteogenesis, cells are cultured for the duration of differentiation in osteogenesis-inducing culture medium.
5. After 21 days in culture, all three cell populations are histologically stained using the following stains: Nile red, which detects intracellular lipid accumulation used to determine adipocyte differentiation; Safranin O, which identifies glycosaminoglycans in the extracellular matrix of differentiated chondrocytes; and Alizarin red S, which detects mineralized calcium deposition common in osteocytes.
6. For staining, differentiation-inducing media are removed, the cells are washed twice with DPBS without calcium and magnesium, the fixation solution is added to each dish, and the dishes are stored at 4°C for 24 h.
7. The fixation solution is removed and the cells are washed with DPBS without calcium and magnesium four times. The respective working solution for each stain is added.
8. For cells stained with Nile red for adipocyte differentiation, cells should be incubated with stain for 24 h at room temperature protected from light. The stain is removed and the cells are washed once with distilled water. Stained cells are visualized under fluorescent microscopy with a filter set suitable for FITC illumination.
9. For cells stained with Safranin O for chondrocyte differentiation, cells should be incubated at room temperature for 10 min. The stain is removed, the cells are washed once with distilled water, and visualized with bright field or Hoffman contrast microscopy.
10. For cells stained with Alizarin red S for osteocyte differentiation, cells should be incubated at room temperature for 10 min. The stain is removed and cells washed once with distilled water and visualized with bright field or Hoffman contrast microscopy.
11. Cells stained for each differentiation condition are depicted in Fig 2.

4. Notes

1. Collagenase solution is prepared fresh with each use.
2. High-glucose DMEM with glutamine and sodium pyruvate is supplemented with FBS, pen/strep, and Fungizone, filter

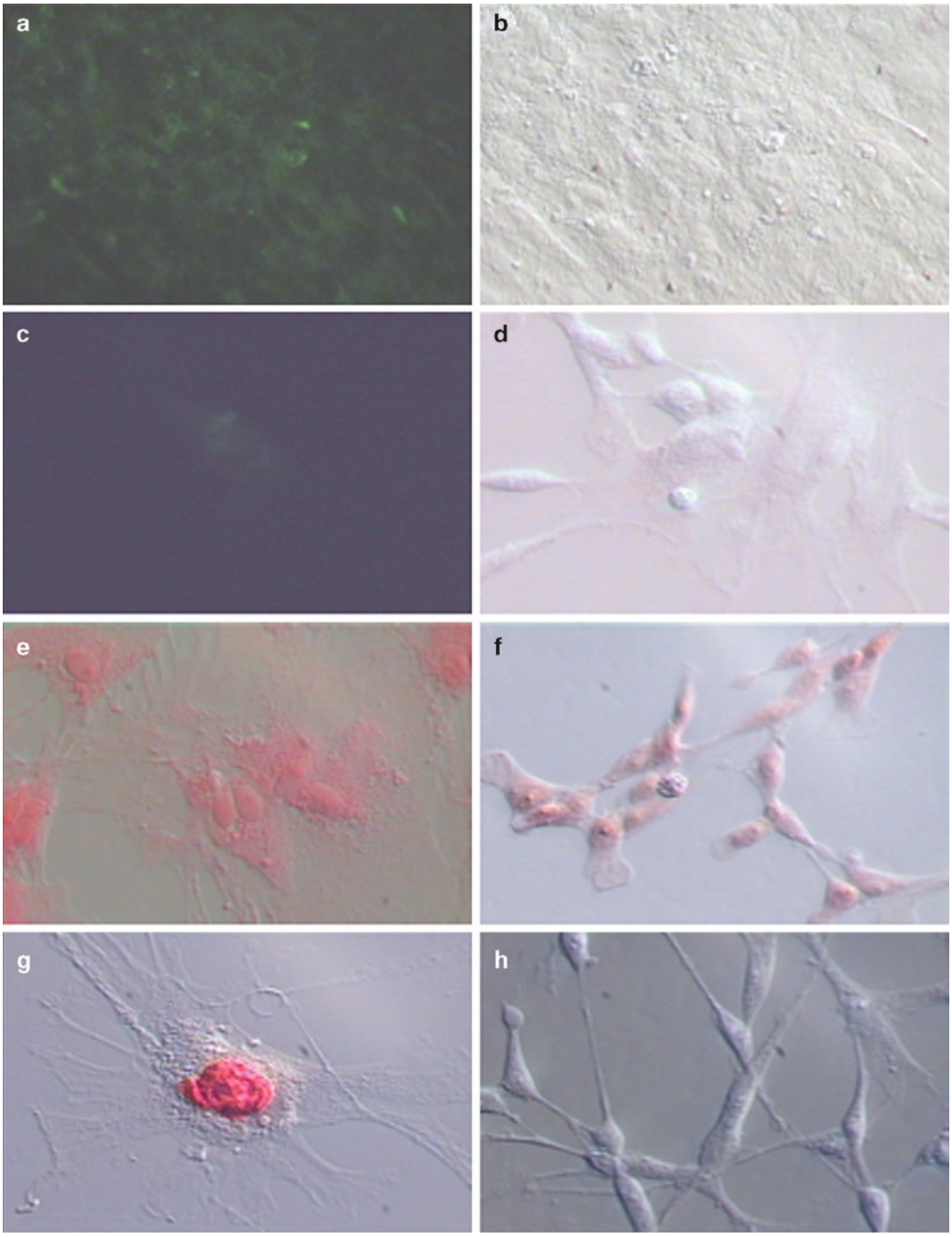

Fig. 2. Differentiation of P4 clones. Adipogenic differentiation stained positive with Nile red staining; viewed with a FITC filter (**a**) and Hoffman contrast (**b**). Undifferentiated stromal cells (control) demonstrated only slight Nile red staining ((**c**) FITC filter; (**d**) Hoffman contrast). Chondrogenic induction was detected by Safranin O in differentiated clones (**e**) and less intensely in undifferentiated cell populations (**f**). Osteocyte differentiation was observed only in induced cells (**g**) and not in undifferentiated stromal cells (**h**). Images (**a**)–(**f**) and (**h**) were magnified using a ×20 objective, while image (**g**) was magnified using a ×40 objective.

sterilized, stored at 4°C, and used for culture maintenance and clonal expansion for approximately 2 weeks.

3. Additives for differentiation inducing media are prepared as 100× stock solutions, stored at –20°C, and added to DMEM to prepare fresh differentiation media with each medium change.
4. Much smaller fat samples, as little as 1 g, can also be processed if desired. For samples as small as 1 g, 10 ml of collagenase solution is used in Step 3 of 3.1 Cell Isolation.
5. This remixing of the centrifuged cells by vigorous shaking is to complete the separation of stromal cells from the fat and is very important for a good yield of stromal cells.
6. Cells are passaged using a typical cold trypsinization procedure. Culture medium is removed and cells rinsed with calcium- and magnesium-free DPBS. Cold trypsin–EDTA solution is added to cover the cell surface and excess trypsin–EDTA is removed. Cells are incubated at 37°C until they begin to lift off the plastic, and the reaction is stopped by the addition of expansion medium.
7. Replicative senescence: cells cease to divide and are defined by a change in morphology from an elongated, spindle-like shape to a flattened, irregular shape with dispersed cytoplasm and the appearance of cytoplasmic vacuoles.

References

1. Merok, J.R. and J.L. Sherley. (2001) Breaching the kinetic barrier to in vitro somatic stem cell propagation. J Biomed Biotechnol 1: 25–27.
2. Klug, M.G., M.H. Soonpaa, G.Y. Koh, and L.J. Field. (1996) Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. J Clin Invest 98: 216–224.
3. Soria, B., E. Roche, G. Berna, T. Leon-Quinto, J.A. Reig, and F. Martin. (2000) Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. Diabetes 49: 157–162.
4. Brustle, O., K.N. Jones, R.D. Learish, K. Karram, K. Choudhary, O.D. Wiestler, I.D. Duncan, and R.D. McKay. (1999) Embryonic stem cell-derived glial precursors: A source of myelinating transplants. Science 285: 754–756.
5. McDonald, J.W., X.Z. Liu, Y. Qu, S. Liu, S.K. Mickey, D. Turetsky, D.I. Gottlieb, and D.W. Choi. (1999) Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat Med 5: 1410–1412.
6. Liu, S., Y. Qu, T.J. Stewart, M.J. Howard, S. Chakrabortty, T.F. Holekamp, and J.W. McDonald. (2000) Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc Natl Acad Sci USA 97: 6126–6131.
7. Tsai, R.Y., R. Kittappa, and R.D. McKay. (2002) Plasticity, niches, and the use of stem cells. Dev Cell 2: 707–712.
8. Gronthos, S., D.M. Franklin, H.A. Leddy, P.G. Robey, R.W. Storms, and J.M. Gimble. (2001) Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol 189: 54–63.
9. Gimble, J. and F. Guilak. (2003) Adipose-derived adult stem cells: Isolation, characterization, and differentiation potential. Cytotherapy 5: 362–369.
10. Friedenstein, A.J., J.F. Gorskaja, and N.N. Kulagina. (1976) Fibroblast precursors in

normal and irradiated mouse hematopoietic organs. Exp Hematol 4: 267–274.

11. Rodbell, M. (1966) Metabolism of isolated fat cells. II. The similar effects of phospholipase C (*Clostridium perfringens* α toxin) and of insulin on glucose and amino acid metabolism. J Biol Chem 241: 130–139.
12. Rodbell, M. and A.B. Jones. (1966) Metabolism of isolated fat cells. III. The similar inhibitory action of phospholipase C (*Clostridium perfringens* α toxin) and of insulin on lipolysis stimulated by lipolytic hormones and theophylline. J Biol Chem 241: 140–142.
13. Broxmeyer, H.E., G. Hangoc, S. Cooper, R.C. Ribeiro, V. Graves, M. Yoder, J. Wagner, S. Vadhan-Raj, L. Benninger, and P. Rubinstein. (1992) Growth characteristics and expansion of human umbilical cord blood and estimation of its potential for transplantation in adults. Proc Natl Acad Sci USA 89: 4109–4113.
14. Halvorsen, Y.C., W.O. Wilkison, and J.M. Gimble. (2000) Adipose-derived stromal cells – Their utility and potential in bone formation. Int J Obes Relat Metab Disord 24(Suppl 4): S41–S44.
15. Luria, E.A., A.F. Panasyuk, and A.Y. Friedenstein. (1971) Fibroblast colony formation from monolayer cultures of blood cells. Transfusion 11: 345–349.
16. Kassem, M., L. Mosekilde, and E.F. Eriksen. (1993) 1,25-Dihydroxyvitamin D3 potentiates fluoride-stimulated collagen type I production in cultures of human bone marrow stromal osteoblast-like cells. J Bone Miner Res 8: 1453–1458.
17. Rickard, D.J., M. Kassem, T.E. Hefferan, G. Sarkar, T.C. Spelsberg, and B.L. Riggs. (1996) Isolation and characterization of osteoblast precursor cells from human bone marrow. J Bone Miner Res 11: 312–324.
18. Guilak, F., K.E. Lott, H.A. Awad, Q. Cao, K.C. Hicok, B. Fermor, and J.M. Gimble. (2006) Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. J Cell Physiol 206: 229–237.
19. Arat, S., J. Gibbons, S.J. Rzucidlo, D.S. Respess, M. Tumlin, and S.L. Stice. (2002) In vitro development of bovine nuclear transfer embryos from transgenic clonal lines of adult and fetal fibroblast cells of the same genotype. Biol Reprod 66:1768–1774.

Chapter 8

Automated Isolation and Processing of Adipose-Derived Stem and Regenerative Cells

Kevin C. Hicok and Marc H. Hedrick

Abstract

The popularity of nonhematopoietic, adult tissue-derived stem and progenitor cells for use as a cellular research tool, and ultimately as a clinical therapeutic, has increased exponentially over the past decade. Almost all adult-derived stem/progenitor cells (autologous and allogeneic), with one exception, require at least some ex vivo expansion or further manipulation prior to use to satisfy efficacy and safety requirements for preclinical or clinical use. The principal reason is the relatively low frequency of these therapeutically valuable cells within any given adult tissue, except for adipose tissue, which has been shown to have at least two log greater concentrations of these progenitor cells. Therefore, use of autologous adipose-derived cells as both a research tool and cell therapeutic is feasible and has been shown to be both safe and efficacious in preclinical and clinical models of injury and disease. The development and utilization of automated processes and instrumentation such as Cytori Therapeutics' Celution® System to reduce variability and increase quality of the recovered cells is requisite for clinical use and preferred by basic researchers. Here, use of an automated, closed processing platform for isolation and concentration of adipose-derived stem and regenerative cells is described, including a profile of the isolated cells immediately prior to use, and commonly used methods to quantify and qualitatively assess the recovered cells.

Key words: Adipose-derived stem and regenerative cell, Closed system, Colony forming unit–fibroblast, Processing platform, Stromal vascular fraction cells

1. Introduction

Adult tissue-derived stem and progenitor cells are now well established as a therapeutic source for regenerative medicine. Numerous preclinical and clinical studies have been conducted using these cells obtained from many different tissues including adipose, bone marrow, skin, brain, liver, kidney, and skeletal and cardiac muscle (1–7). Both safety and efficacy are demonstrated, especially in the repair of tissues damaged by ischemia (2, 8, 9).

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However, with the exception of adipose tissue, the concentration of adult stem cells and other relevant progenitors is exceedingly rare, or else the procurement of the cells is complicated by comorbidity and limited access to the source tissue. For instance, the frequency of bone marrow-derived mesenchymal stem/stromal cells is described to be in the range of 0.001–0.0005% of isolated cells, depending on the age of the donor (10). Stem cells obtained from the major organs through biopsies are also relatively rare and collectively require ex vivo expansion to obtain an efficacious quantity for clinical use. Adipose tissue, on the other hand, contains at least two log higher concentrations of the cells that give rise to cultured adult stem cells, and the tissue is both abundant and easily acquired through comparatively low morbidity lipoaspiration procedures. Therefore, adipose tissue is uniquely poised to not only provide a more abundant source of cells for ex vivo expansion but also to provide an autologous source of valuable regenerative cells for immediate use.

Regardless of the intended application, isolating stem and regenerative cells from adipose poses some unique challenges. Multiple variables must be considered and empirically evaluated to obtain an optimal cell population. Likely the largest variable of all is the interpersonal difference in adipose composition at the cellular level. Using a standardized manual isolation procedure the range of recovered nucleated cell concentration normalized to volume of starting tissue among 266 female donors was 20,000–680,000 cells/mL. The mean ± standard deviation concentration of cells from this cohort was 230,000 ± 120,000 cells/mL of tissue (unpublished data, Cytori Therapeutics Inc.). Both inherent genetic and environmental factors contribute to the anatomical variation; however, a menagerie of other factors during the procurement and processing of the tissue contributes to the cumulative variation seen in the recovered cell concentration (see Table 1). Since overall variability in the isolation of adipose stem and regenerative cells is so large, automation and standardization of the controllable elements during processing is essential to obtain an acceptable outcome. Use of an automated cell isolation and enrichment platform such as the one described here serves to significantly reduce the amount of human subjectivity and error during the critical steps of cell isolation and enrichment. Furthermore, the instrument and supporting ancillary products for tissue collection and cell delivery were developed as a “Design In” technology yielding a platform suitable to support all stages of research from basic to preclinical to clinical. Optimization of techniques for adipose tissue acquisition prior to isolation, and implementation of verified methods for cell characterization afterwards, serve to further standardize the overall process. In this manner, an acceptable level of reproducibility in recovered cell quantity and quality is achievable.

Table 1
Multiple variables influence recovery of adipose-derived stem and regenerative cells for therapeutic use

Process step	Controllable variables	Uncontrollable variables
Tissue quality prior to collection	Anatomical depot	Donor sex, age, health status, vascular density
Tissue collection	Tissue harvesting method: Syringe/vacuum aspiration Ultrasound, Vaser, Bodyjet/Waterjet, etc. Cannula diameter and type Wetting solution composition Wait time prior to ADRC processing initiation	Amount of peripheral blood and wetting solution recovered with tissue Viability of cells after collection
Initial tissue washing	Geometry of washing chamber Type of wash solution Volume of wash solution Duration of wash solution	NA
Enzymatic release of cells	Enzyme solution composition Enzyme concentration Duration of exposure to enzyme Reaction temperature	Differences in donor tissue extracellular matrix quantity and content affect efficiency of cell release Presence of inhibitors of enzymes within tissue/cells
Enrichment and concentration of cells	Volume transferred to concentration chamber Centrifugation speed Centrifugation duration Chamber temperature	Amount of red blood cells released from intact vasculature
Cell resuspension	Volume and type of solution Type of mechanical perturbation used to resuspend cells Type of syringe/needle used to collect cells	Final viability of recovered cells Relative amounts of different cell subpopulations

1.1. Tissue Acquisition

Prior to the start of adipose-derived stem and regenerative cell (ADRC) isolation, a number of variables must be considered in order to optimize cell yield and quality. Donor dependent variables such as sex and health status have been reported to influence the quantity and quality of ADRCs (11–17). At the cellular level, these factors are known to influence both the relative abundance of vasculature and the predominant cell population within fat, the mature adipocyte. While these variables may be controlled when screening donors for research studies or manufacturing cells for

ex vivo applications, in the clinical setting where the cells will be used as a real-time therapeutic, this is not the case. Species-specific differences in the cellular composition and matrix content of adipose depots further complicate regenerative cell isolation strategies aimed at yielding populations of cells that are most similar to the composition of the human ADRCs. This is particularly important when justifying use of preclinical model data to rationalize initiation of human clinical trials. For example, rodent adipose tissue from the inguinal fat pad (commonly used as source tissue in preclinical studies) contains lymph nodes that must be removed to avoid overrepresentation of leukocyte cell populations. Failure to do so results in a noncomparable ADRC population to that of humans, which may confound data interpretation in regard to therapeutic safety, efficacy, and dosing.

In a clinical setting, donor-related variables may be used as inclusion or exclusion criteria for treatment, but for autologous real-time cell therapies, their effect on ADRC isolation is irrelevant (18). Therefore, identifying and understanding the effect of the controllable elements in adipose tissue collection have been the central focus in optimizing and automating ADRC isolation and enrichment. Within the aesthetic and plastic surgery community, it is well known that the type of force used to physically release the fat tissue, the instrumentation for tissue collection, the wetting or tumescent solution composition, and the anatomical site selected all may affect the quality of autologous fat used for grafting (19). It is logical to assume that these variables also influence ADRC quantity and quality. Traditionally, within the aesthetic surgery field, syringe-based lipoaspiration is the recommended method to obtain adipose tissue if cell viability is important. Yet, even relatively simple syringe-based tissue collection methods are reported to vary significantly depending on the size of syringe being used and the amount of force exerted (20). In our experience, other aspiration methods using vaser and laser technologies have been observed to adversely affect cell viability by exposing cells to unacceptable levels of sonic and heat energy (Fig. 1). Technologies that use focused hydraulic force to disrupt tissue and extracellular matrix prior to the aspiration process also alter the profile of the starting tissue by increasing the ratios of disrupted to intact tissue and of wetting solution to adipose tissue. Further, both the style and inner diameter of the tissue collection cannula are reported to affect adipose tissue quality and cell viability.

The composition of wetting solution used during tissue harvest can have a significant impact on automated tissue processing as well. Levels of epinephrine used influence vascular contraction and consequently the amount of contaminating peripheral blood within the tissue. High levels of lidocaine and the pH of the wetting solution both have been reported to affect cell viability (21).

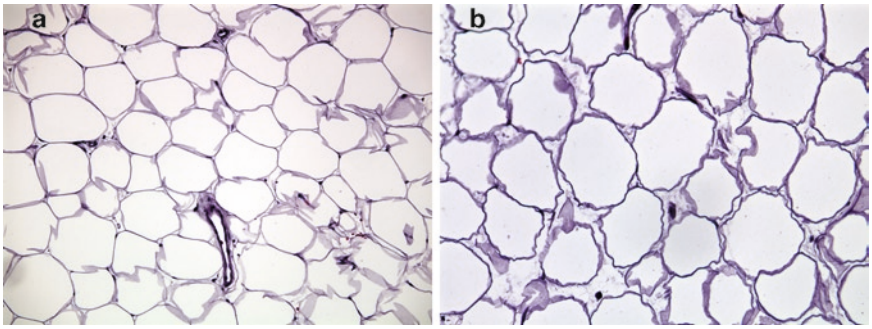

Fig. 1. Adipose tissue obtained by conventional vacuum-assisted lipoaspirate (**a**) or by a laser-based method (**b**) was washed twice with PBS, fixed with neutral-buffered formalin, and paraffin embedded. Eight-micrometer thick sections were cut, deparaffinized, and stained with hematoxylin and eosin according to standard histological methods. Note the increased area of intercellular spaces and adipocyte membrane ruffling (indicative of heat damage to the adipose tissue) in the section from laser-acquired tissue (**b**); whereas, vacuum-assisted tissue contained adipocytes that had smooth membranes and little intercellular space.

1.2. Automation and Standardization Using Cytori's Celution® System

The amount of inherent variability in cellular health and constitution prior to even acquiring adipose tissue demands implementation of standardizing methodology whenever and wherever possible to generate a system capable of meeting the rigorous requirements of regulatory approval, whether it be for GLP compliance in a research setting or to achieve safety and efficacy in the surgical suite. Cytori Therapeutics has developed an automated technology platform to do just that. For researchers from the early basic research stages of regenerative medicine to those ready to apply cell therapy in the clinic, Cytori's Celution® System has been developed to provide a qualitative and quantitatively consistent adipose-derived cell suspension that is available for immediate use or for cryogenic storage until it is needed in the future. As an investigative team transitions their discoveries into therapies, the Cytori's Celution® System provides all of the resources required to successfully acquire and process source tissue and to recover and deliver regenerative cells within the surgical suite.

An overview of how the Cytori's ADRC isolation technology functions is shown in Fig. 2. (1) Adipose tissue collected into 60-cc Toomey syringes is injected into the tissue injection port of the disposable sterile container capable of processing up to ~400 mL of aspirated tissue. (2) The tissue is prepared for enzymatic release of the ADRCs through a series of washing steps using Lactated Ringer's solution. The proprietary Celution System software algorithms customize the washing parameters for each tissue depending on starting mass and through monitoring of the wash solution via optical sensors as it is pumped to a waste solution collection bag. (3) After optimal washing of the starting tissue is achieved, a proprietary clinical-grade enzyme solution (Celase®) is reconstituted in Lactated Ringer's solution

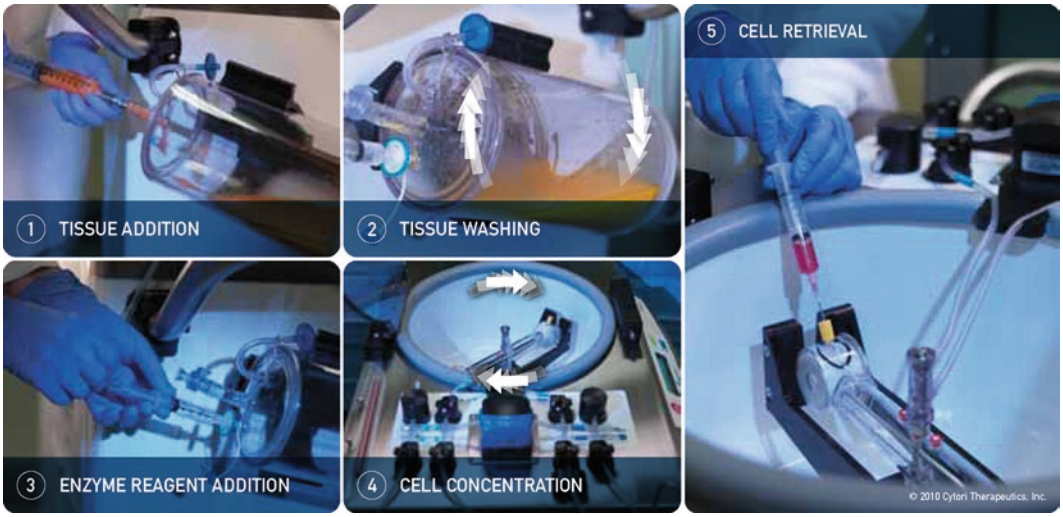

Fig. 2. Overview of Cytori's regenerative cell isolation technology.

and then injected into the container, where it is mixed with the tissue. The Celase™ solution in combination with software-regulated mechanical agitation optimally releases the stem and regenerative cell populations from surrounding extracellular matrix and from each other into the Ringer's solution and away from the buoyant lipid-containing mature adipocytes. Through this process, the ADRC population undergoes a twofold to threefold enrichment, since mature adipocytes comprise 50–60% of the cells within adipose tissue. (4) The released ADRCs are transported from the container into the microconcentration chamber where they are further collated in a series of wash and concentration steps. (5) The final output of ADRCs remains in 5 mL or less of Lactated Ringers, which is recovered using a syringe and blunt end needle. At this point, the cells are ready for further use.

At first appearance, the process appears relatively simple. The development and optimization of automated ADRC isolation and enrichment processes at each of these steps, however, is not trivial. Table 2 details some of the challenges that must be addressed. Broadly these process considerations can be assigned to three categories: those affecting safety, those affecting efficacy, and those that affect both.

All components of the Cytori's ADRC isolation equipment that come into contact with either the adipose tissue or cells must be composed of biocompatible materials. An extensive analysis of all fluidic pathway materials' biocompatibility was performed to ensure the highest levels of safety for not only the cells being isolated but for the system user as well. Furthermore, sterility and stability testing is performed on all of the consumable set components to ensure a safe, toxin free, and aseptic pathway is maintained during cell isolation and concentration.

Table 2
A multivariate optimization strategy required to develop an automated ADRC isolation and enrichment platform

Processing stage	Variable combinations evaluated
Washing	Number of washes Volume of wash solution to tissue Duration of agitation Separation of lipid and aqueous phases Temperature during wash steps
Enzymatic release of cells	Enzyme concentration Temperature during enzymatic release Volume of cell release solution Speed and duration of tissue perturbation during enzymatic processing Duration of aqueous and lipid phase separation Volume and repetition of washes after initial released cell removal for concentration
Cell concentration	Centrifuge time and speed Fluid column height Removal and addition speed of cell suspension and wash solution Fluid temperature

Another important parameter requiring optimization is the tissue preparatory wash steps. Combinatorial evaluations of wash solution composition, volume, wash time, method of perturbation, geometry of processing chamber, and subsequent fluid path design were performed to reduce the content of dead or dying nucleated cells and red blood cells prior to release of the ADRCs from the tissue.

Probably the most critical aspect of successful automated ADRC isolation is the identification of maximally safe and efficacious conditions for cell release from the adipose tissue. Unlike bone marrow, peripheral blood, or cord blood cell isolation in which stem cells are already suspended within the collected body fluid and can easily be concentrated or enriched through density gradient separation or magnetic immune-bead methods, the regenerative cells within adipose are released through enzymatic digestion of surrounding extracellular matrix and intercellular connecting proteins. Key elements to successful enzymatic release of ADRCs from adipose are the composition of enzyme(s) used, the concentration, or level of enzyme activity, and the duration of tissue exposure to the enzyme.

Similar to the issues associated with starting tissue composition, the makeup of the enzyme solution used to release ADRCs

Table 3
Commonly used commercial enzymes used to manually isolate cells from adipose

Enzyme name	Manufacturer	Protease composition	References
Liberase™ Blendzyme 1,2, 3	Roche	Type I/II collagenase and thermolysin (Various ratios)	(23) (24)
Sigma collagenase	Sigma/Aldrich	Type II collagenase, various contaminating proteases (thermolysin, clostripain)	(25)
Collagenase type I	Worthington	(Clostridiopeptidase A) caseinase, clostripain, and trypsin-like activity	(26)

from intact adipose affects automated isolation efficiency and safety. Historically, commercially available protease products (see Table 3) have been used by researchers to manually isolate cells from adipose (22–26). Because different isoforms of collagen compose the majority of the extracellular matrix within adipose, collagenase is an important enzymatic component used. However, different types of collagenase degrade different isoforms of collagen with varying degrees of efficiency. To address this limitation, different mixtures or "blends" of collagenase have been manufactured. Unless highly purified, these collagenase cocktails also contain various amounts of other contaminating proteases, which present a potential safety issue. Since collagenase alone is insufficient to digest all extracellular matrix proteins, much less all cell–cell adhesion proteins, some manufacturers have intentionally combined other proteases with collagenase in order to increase efficiency of cell release. Ultimately an empirical assessment must be made to identify the composition that provides the most consistent viable ADRC yield while maintaining an acceptable safety profile. Cytori's Celase™ solution is a clinical-grade, proprietary mixture of cGMP manufactured proteases demonstrated to be both safe and efficacious regardless of the Cytori cell isolation platform chosen. The composition of the enzyme has been rigorously evaluated to ensure purity of the product and limited variability between production lots. This has enabled optimization of automated enzymatic tissue processing parameters. Both incubation time and enzyme activity levels have been identified that provide the maximal rate of ADRC release while ensuring clinically safe levels of residual enzyme activity are achieved by the time the cells are removed from the machine. The development of an efficacious and clinically safe enzyme or enzymes for automated ADRC isolation poses a significant hurdle for those who wish to develop automated methodologies independent of those already available.

After cell release, ADRCs are moved from the tissue-processing container into the microenrichment chamber for concentration. The system takes advantage of fat cell's buoyancy in aqueous solution to separate the stem and regenerative cell fraction away from the mature adipocytes through a port in the bottom of the processing container. Optical sensors and proprietary software algorithms work in concert to identify when transport of released cells the enrichment chamber is complete. The microenrichment chamber performs a series of concentration and wash cycles that function to remove remaining cellular debris and importantly, reduce any remaining enzymatic activity to well below the dose determined to be unsafe. Both duration and speed of rotation during concentration steps within the microenrichment chamber are optimized to minimize cell damage while maximizing cell recovery.

After the ADRC isolation and enrichment process is completed, the cells are resuspended in the approximately 2.5 mL of Ringer's solution that remains within the collection recesses found at each end of the microenrichment chamber. During resuspension, the ADRCs should be drawn gently in and out of the transfer syringe several times. At this point, the ADRCs are ready for use directly, or for further ex vivo manipulation or study.

1.3. Methods Used to Quantify and Qualify Cells Obtained Using Celution Device

The three most basic and commonly evaluated parameters of automated adipose cell isolation and enrichment processes for research and development purposes are cell number, cell viability, and the capacity of the ADRCs to form adherent proliferative colonies. If one assumes the relative composition of the starting tissue is equivalent, then recovered cell number and viability give the most direct assessment of how changes to the device components or software algorithms affect the total ADRC output of the automated system. This is especially true in regard to examining the reproducibility of effect. However, one should not limit their assessment of automated output to just viable cell number since these data do not provide any information in regard to effect on a particular subpopulation of the ADRCs. Due principally to the historical development of the adipose-derived stem cell field, an assay originating from the bone marrow field (the colony forming unit–fibroblast [CFU-F] assay) was adopted as a straightforward evaluator for the subpopulation of ADRCs known to give rise the cultured adipose-derived adult stem cell population. It should be noted for those who hypothesize the adherent progenitor cells that give rise to the cultured adipose stem cells are major contributors to efficacy in their studies, that while the CFU-F assay is quite useful for examining the similarity of starting populations between test conditions, it has not been a strong correlate to outcome in preclinical studies. Therefore, further combinatorial characterization by flow cytometry, in vitro differentiation assays,

and ultimately functional verification in preclinical models comprise the gold standard criteria that must be met to truly validate efficacy.

The following two methods described here have been verified for use to quantitatively and qualitatively evaluate ADRCs derived from Cytori's cell enrichment systems.

2. Materials

2.1. Cell Count and Viability Determination Using the NucleoCounter®

1. NucleoCounter Automatic Cell Counter NC-100.
2. NucleoCassettes, PN M1293-0100.
3. Lysing buffer, reagent A; PN. P0820-5170.
4. Stabilizing buffer, reagent B; PN 0820-5180.
5. 1.5-mL microcentrifuge tubes.
6. 2–20-µL adjustable volume pipette.
7. 2–20-µL pipet tips.
8. 20–200-µL adjustable volume pipette.
9. 20–200-µL pipet tips.
10. 1× Phosphate-buffered saline (PBS) solution.
11. 100% Ethanol.
12. 70% Ethanol.
13. Kim Wipes™.

2.2. CFU–F Assay

1. DMEM/F-12 50/50, 1× with L-glutamine, 500-mL bottle.
2. Fetal bovine serum (FBS), heat inactivated, sterile filtered.
3. Antibiotic/Antimycotic 100× solution (ABAM), sterile, contains 10,000 U penicillin-G/mL; 10,000 µg streptomycin/mL; and 25 µg amphotericin B/mL.
4. 6-well tissue culture plates.
5. Pipet Aid.
6. 5-mL pipets, sterile, individually wrapped.
7. 10-mL pipets, sterile, individually wrapped.
8. 25-mL pipets, sterile, individually wrapped.
9. 0.5–10-µL adjustable volume pipette.
10. 10–100-µL adjustable volume pipette.
11. 100–1,000-µL adjustable volume pipette.
12. 0.1–10-µL sterile pipette tips.
13. 1–200-µL sterile pipette tips.
14. 100–1,000-µL sterile pipette tips.

15. 1.5-mL tubes, sterile.
16. 15 centrifuge tubes, sterile.
17. 50 centrifuge tubes, sterile.
18. Rack, tube holders.
19. 1× PBS, magnesium and calcium free.
20. 10% Formalin.
21. Gill hematoxylin III stain.

3. Methods

3.1. Sample Preparation for Cell Count and Viability Determination Using the NucleoCounter

1. Depending on the starting volume of adipose tissue processed, the concentration of cells recovered from Cytori's cell isolation and enrichment technology is usually greater than the maximal concentration allowed for use by NucleoCounter (2 million cells/mL). For example, 200 mL of tissue, which yields 250,000 cells/mL of tissue, would generate an ADRC output cell concentration of approximately 10 million cells/mL. Typically, diluting the ADRCs 1:10 in a diluent solution (Lactated Ringer's solution, PBS, physiological saline, etc.) prior to measurement is satisfactory, in this case yielding a sample of 1×10^6 cells/mL. A minimum of 50 µL of sample is required for loading cells into the NucleoCounter (see Note 1).

3.2. NucleoCounter Operation

1. Turn the instrument on by pressing the red ON/OFF button. When the unit has started successfully, the display should read "NucleoCounter Ready" and the green status indicator light should be illuminated. Data may be obtained directly from the display of the instrument or alternatively, the NucleoCounter can be connected to a computer by a USB connection and data collection and analysis software may be used. This method describes use of the NucleoCounter with NucleoView™ computer software.
2. If the computer is not already running, power up the computer and log on as user. Once the operating system is running, launch the NucleoView program (see Note 2).
3. To determine nonviable cell number acquire samples as follows: using a 20–200-µL pipette, collect a volume of thoroughly mixed ADRC sample into a microfuge tube (see Note 3). Either 0.5-mL or 1.5-mL volume tubes are suitable. It is recommended that at least 200 µL of cell suspension is acquired to ensure representative sampling.
4. Obtain a new NucleoCassette and place loading tip into the sample. Take care to fully immerse the loading tip of the

NucleoCassette into the sample, to avoid air bubbles in the flow system.

5. Press the loading piston gently with your thumb, until your thumb and the top of the piston are flush to the surface of the cassette.
6. After loading, release the piston (it will remain in place). Make sure no sample droplets are attached to the tip, to avoid contaminating the work surface, NucleoCounter instrument, or yourself.
7. To insert the cassette, flip the lid up or remove and set aside. Grip the cassette on either side of the piston barrel, but take care not to touch the measurement window.
8. Insert the cassette, beveled edge down, into the opening. Be sure to keep the sampling tip parallel to your hand, pointing toward your wrist.
9. Press the cassette down until you hear a click. The tip will lay parallel to the opening, along the body of the instrument.
10. Activate measurement by pressing the green "Run" button. Upon completion of the analysis, the result is shown on the display. The entire process takes approximately 30 s.
11. The results and the recorded fluorescent image of the sample will appear on the computer screen. Verify that the dilution factor and the file name for the sample are correct before running another sample.
12. When the process is finished, the cassette can be safely removed. Lift the lid and grab the cassette as before and remove the cassette.
13. Next the viable cell population of the sample is determined by performing the following steps. Mix the ADRC sample by gentle pipetting several times to obtain a homogenous suspension. Transfer 100 μL of the sample into a new microcentrifuge tube. Add to the sample 100 μL of NucleoCounter lysing buffer, Reagent A.
14. Close the sample tube and mix the sample and buffer thoroughly for 5 s, by inverting five to ten times. Add 100 μL of the NucleoCounter stabilization buffer to the mixture. Mix the sample and buffers thoroughly for 5 s as before.
15. Obtain a new NucleoCassette and place the loading tip into the sample. Again, take care to fully immerse the loading tip of the NucleoCassette into the sample, to avoid air bubbles in the flow system.
16. Load the sample into the NucleoCassette and the NucleoCassette into the NucleoCounter the same as described when determining the nonviable cell number.

3.3. NucleoCounter Data Acquisition and Analysis

1. Once the NucleoView software has completed the measurement of both nonviable and viable cell counting, a determination for the total viable cell count and viability can be made.
2. Select the Viability menu from NucleoView program.
3. Verify that the correct file name is selected for the both the total cell count and nonviable cell count, and the program will calculate the values for total viable cells/milliliter and viability. These values will be displayed at the bottom of the computer screen.
4. The reported numbers are then multiplied by the sample preparation dilution factor (if any) and by the volume (in milliliters) in which the ADRCs were resuspended to determine the total number of cells obtained by processing. The normalized viable ADRC number commonly used to compare interdonor results is obtained by dividing the total viable cell number by the starting volume of processed tissue (viable ADRCs/milliliter of processed lipoaspirate).

3.4. Preparation of Media and Samples for the CFU–F Assay

Prepare assay cell culture media in a biological safety cabinet (BSC) as follows:

1. Obtain an unopened 500-mL bottle of DMEM/F-12 media (stored at 4°C). Remove 55 mL of media and discard.
2. Add 50 mL of sterile-filtered fetal bovine serum and 5.0 mL of Antibiotic–Antimycotic 100× solution to the remaining 450 mL of media.
3. Carefully swirl media several times to ensure added reagents are mixed into media.
4. Label the bottle appropriately to indicate addition of the reagents and an expiration date 1 month following the date of preparation. Media should be stored between 2 and 8°C.
5. Prior to starting the assay, the viable cell concentration for each sample should have been determined using the NucleoCounter as described in the previous section (see Note 4). Samples are plated in triplicate wells on two plates at two concentrations (5,000 cells/well and 1,000 cells/well).
6. Obtain ADRC samples, refer to the recorded viable cell concentration, and calculate the fold dilution to obtain final plating densities of 5,000 and 1,000 cells/mL. To calculate how much sample is needed to obtain the minimum amount of cells required for the assay, use the following equations:
   (a) $(40{,}000 \text{ cells} \div \text{viable cell concentration}) \times 1{,}000 = X\,\mu L$ of ADRC sample
   Next, calculate how much DMEM/F-12 media diluent is needed:

(b) 24 mL – volume of ADRC sample needed = Y mL of media diluent (see Note 4).

7. Gently pipette the ADRC sample several times to ensure homogenous distribution prior to performing dilution steps (see Note 5). Eighteen milliliters of media containing ADRCs at a concentration of 1,667 cells/mL is required to plate three wells on two 6-well plates with 3 mL of sample per well.
8. Further dilute 4 mL of the remaining 1,667 cells/mL cell suspension with 16 mL of DMEM: F12 media to get a total volume of 20 mL cell suspension (at 333 cells/mL). Seed the 1,000 cells/well set of wells with 3 mL of cells per well.

3.5. CFU–F Assay Cell Culture

1. The six-well plate(s) are incubated at 37°C, 5% CO_2 in a humidified incubator chamber for 10–14 days.
2. Media is replaced with fresh DMEM:F12 media 5 days after initial plating and every 3–4 days after until the end of the assay.
3. Media should be warmed to at least room temperature prior to feeding cells.
4. Retrieve the cell culture plate(s) from the incubator and place inside the hood (see Note 6).
5. Inspect each plate macroscopically for any sign of microbial or fungal contamination, e.g., dark or black tiny speckles or cloudiness in the media.
6. If any macroscopic contamination is observed in a plate, set the plate aside until media changes are made to the remaining plates.
7. Continue changing media of the noncontaminated plate(s) as follows:
   (a) Vacuum aspirate all the media from all wells.
   (b) Refill each well with ~3 mL complete media.
   (c) Return the plate(s) to the 37°C CO_2 incubator.
8. For contaminated plate(s), follow the steps below:
   (d) Evaluate the suspected contaminated wells by microscopic observation to verify macroscopic observations.
   (e) If confirmed, add 2 mL of 100% beach into all of the contaminated well(s). Allow to sit for 10 min then aspirate liquid from the plate and discard into the biohazardous waste receptacle.
9. Check the confluency of the cell colonies by microscopic observation using an inverted phase microscope after 6–7 days of culture and every 2–4 days thereafter to ensure colonies are distinguishable (see Note 6).

3.6. Fixation and Staining of CFU–F Assays

1. Vacuum-aspirate media and discard into the liquid waste container.
2. Rinse each well of the assay plate by adding approximately 4 mL of PBS.
3. Aspirate the PBS from all wells. Discard the PBS into the liquid biohazard container.
4. Add approximately 1 mL of 10% neutral-buffered formalin to each well of the plate inside a fume hood or using a suitable fume extractor device. Place culture plate lid back onto each plate.
5. Let the plates sit for 15–20 min inside the fume hood.
6. Aspirate the formalin from each well and properly discard it into the formalin waste container.
7. Rinse the wells by adding 2–4 mL PBS to each well.
8. Aspirate PBS from all wells and discard.
9. Add approximately 1 mL hematoxylin Gill III formula to each well and incubate for 5–10 min at room temperature (see Note 7).
10. Aspirate all hematoxylin from all wells and rinse gently with tap water.
11. Blot off excess water by inverting and patting the plates onto a paper towel to dry the plate(s).

3.7. CFU–F Assay Data Acquisition and Analysis

1. After assay plates have been stained and dried, they are ready for evaluation under a bright-field inverted microscope. If the microscope is equipped with phase contrast optics, observation using phase contrast light enables better visualization of lightly stained cells.
2. Examine the entire surface of each sample-containing well using a 4× objective lens. A four-quadrant grid may be drawn on the bottom of each well using a light colored marker to help orient the observer during colony scoring.
3. Examples of CFU–F colonies are shown in Fig. 3. Ideal colonies contain 30 or more cells (see Note 8). When deciding whether a group of cells is one or two colonies, err on the conservative side and make the call as one colony (see Note 9).
4. For each plating density analyzed, determine the average number of colonies per well.
5. Calculate CFU–F frequency as follows:
   (a) CFU-F frequency (%) = (average CFU–F per well ÷ the number of cells plated in that well) × 100.
6. If incubation of the plates was stopped before 14 days, then this %CFU–F should be reported as a minimum percent frequency and the harvest day should be noted next to this

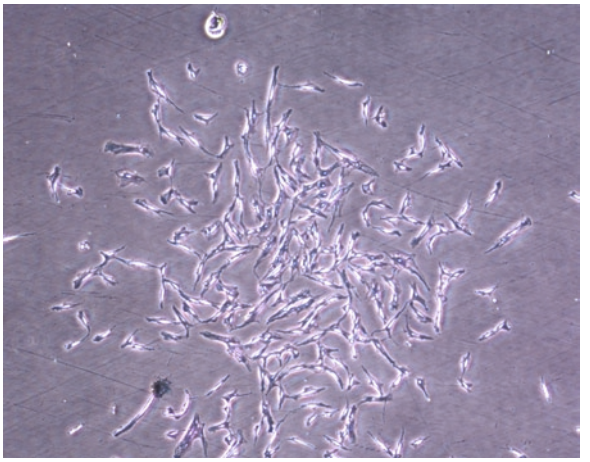

Fig. 3. Representative images of colony forming cell colonies originating from ADRCs. ADRC-derived CFU–F colonies are composed of cells with heterogeneous morphology and mobility. As a result, colonies appear as loosely organized clusters of cells.

minimum frequency value. For example, if there were an average of seven colonies per wells in the 5,000 cells/well set of wells, and the plates were taken down after 7 days, then the CFU-F frequency should be reported at ≥0.14% and the day that the plates were scored.

4. Notes

1. Two measurements are made for each sample (one to measure dead or dying cells and one to measure all nucleated cells. The number of viable cells is determined by subtracting the number of dead cells from the number of total cells measured. Each measurement uses at least 50 µL of sample and triple replicates are recommended, thus a minimum volume of 300 µL for each sample and six Nucleocassettes is required. To account for pipetting error and the potential need to recount the sample if it is out of range, an extra 300 µL of diluted sample is usually set aside.
2. Never close the NC Receiver. Data from the NucleoCounter can only be transferred when the NC Receiver is active.
3. Safety precaution: at a minimum, use latex/nitrile gloves and a lab coat when performing this procedure. Propidium iodide, the fluorescent dye used in the NucleoCounter may be mutagenic and/or carcinogenic. Never attempt to reuse the Nucleocassette, or to empty it once you have loaded it. Dispose all materials in contact with the chemical, e.g., tubes, pipet tips, Kimwipes, and gloves, in hazardous waste

container(s) located throughout the laboratory. If a Nucleocassette becomes damaged or begins to leak, any spill should be handled as potentially hazardous waste. Consult the propidium iodide MSDS in your laboratory for complete details and safety precautions prior to using the cassettes.

4. Ensure that there is a minimum of 40,000 cells per sample prior to continuing the CFU-F assay. If there are not 40,000 cells available in the sample, it cannot be used in this assay.
5. Resuspended ADRCs recovered from automated processing are typically concentrated at logs higher levels than what is required for the assay sample plating density and therefore a stepped dilution procedure should be used when diluting the cells to the final plating concentrations to avoid technical error resultant from pipetting very small volumes.
6. Do not move more than six plates into the hood at a time to avoid excessive periods of time during which cells are exposed to suboptimal temperature and gas exchange conditions.
7. Alternatively, crystal violet dye (0.1 g crystal violet [Sigma Aldrich] dissolved in 100 mL dH_2O + 0.25 mL glacial acetic acid) can be used to stain the cells if hematoxylin is not available. After fixation and PBS wash steps, add the 0.1% crystal violet solution for 30 min and then rinse three times with 5 mL dH_2O. Plates are then ready to be counted.
8. Ideally the number of cells within a colony should be greater than 32 cells (i.e., 5 cell doublings) to be considered a positive colony that is actively proliferating. When a sample CFU–F concentration exceeds 2–3%, however, the random distribution of mother cells across a well sometimes may generate colonies in close proximity to each other. Furthermore, daughter cells in a colony may migrate away and establish a secondary colony. When cell densities become too high, the ability to distinguish between colonies rapidly diminishes; therefore, a 16-cell minimum point may be more desirable if increased accuracy and sensitivity are desired. The following guideline is used in our laboratories:
   If there is at least one colony (containing at least 50 clustered cells) in at least five wells of the 1,000 cells/well set of wells and at least five colonies (containing at least 50 clustered cells) in at least five wells of the 5,000 cells/well set of wells, then the assay can be stopped before the cells become overconfluent. If colonies of ~32–60 cells are present and the largest colonies appear either close to or have already begun to merge together, incubation should be ceased and proceed to the next step. If no colonies of this size are noted at this time, observe semi-daily until these criteria are reached or until day 14 after plating is reached, whichever comes first.

If the assay is stopped before the 14-day time point, then the CFU–F frequency should be reported as a minimum percentage.

9. Colonies forming along the edge of the well are difficult to see especially if the wells are not completely dry. When deciding between one or two colonies along the edge of a well, choose one. There are often not as many cells as it first appears.

References

1. Sanz-Ruiz R, Fernández-Santos E, Domínguez-Muñoa M, Parma R, Villa A, Fernández L, Sánchez PL, Fernández-Avilés F (2009) Early translation of adipose-derived cell therapy for cardiovascular disease. *Cell Transplant* **18**, 245–54.
2. Cho HJ, Lee J, Wecker A, Yoon YS (2006) Bone marrow-derived stem cell therapy in ischemic heart disease. *Regen Med* **1**, 337–45.
3. Gil-Perotín S, Alvarez-Buylla A, García-Verdugo JM (2009) Identification and characterization of neural progenitor cells in the adult mammalian brain. *Adv Anat Embryol Cell Biol* **203**, 1–101.
4. Steindler DA (2007) Stem cells, regenerative medicine, and animal models of disease. *ILAR J* **48**, 323–38.
5. Kakinuma S, Nakauchi H, Watanabe M (2009) Hepatic stem/progenitor cells and stem-cell transplantation for the treatment of liver disease. *J Gastroenterol* **44**,167–72.
6. Humphreys BD, Bonventre JV (2007) The contribution of adult stem cells to renal repair. *Nephrol Ther* **3**, 3–10.
7. Schabort EJ, Myburgh KH, Wiehe JM, Torzewski J, Niesler CU (2009) Potential myogenic stem cell populations: sources, plasticity, and application for cardiac repair. *Stem Cells Dev* **18**, 813–30.
8. Quevedo HC, Hatzistergos KE, Oskouei BN, Feigenbaum GS, Rodriguez JE, Valdes D, Pattany PM, Zambrano JP, Hu Q, McNiece I, Heldman AW, Hare JM (2009) Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. *Proc Natl Acad Sci USA* **106**, 14022–7.
9. Alt E, Pinkernell K, Scharlau M, Coleman M, Fotuhi P, Nabzdyk C, Gehmert S, Song YH (2009) Effect of freshly isolated autologous tissue resident stromal cells on cardiac function and perfusion following acute myocardial infarction. *Int J Cardiol.* doi:10.1016/j.ijcard.2009.03.124 DOI:dx.doi.org.
10. Ahrens N, Tormin A, Paulus M, Roosterman D, Salama A, Krenn V, Neumann U, Scheding S (2004) Mesenchymal stem cell content of human vertebral bone marrow. *Transplantation* **78**, 925–9.
11. Ogawa R, Mizuno H, Watanabe A, Migita M, Hyakusoku H, Shimada T (2004) Adipogenic differentiation by adipose-derived stem cells harvested from GFP transgenic mice-including relationship of sex differences. *Biochem Biophys Res Commun* **319**, 511–7.
12. Dudas JR, Losee JE, Penascino VM, Smith DM, Cooper GM, Mooney MP, Jiang S, Rubin JP, Marra KG (2008) Leporine-derived adipose precursor cells exhibit in vitro osteogenic potential. *J Craniofac Surg* **19**, 360–8.
13. Aksu AE, Rubin JP, Dudas JR, Marra KG (2008) Role of gender and anatomical region on induction of osteogenic differentiation of human adipose-derived stem cells. *Ann Plast Surg* **60**, 306–22.
14. Girolamo LD, Lopa S, Arrigoni E, Sartori MF, Preis FW, Brini AT (2009) Human adipose-derived stem cells isolated from young and elderly women: their differentiation potential and scaffold interaction during in vitro osteoblastic differentiation. *Cytotherapy*, 1–11.
15. Zhu M, Kohan E, Bradley J, Hedrick M, Benhaim P, Zuk P (2009) The effect of age on osteogenic, adipogenic and proliferative potential of female adipose-derived stem cells. *J Tissue Eng Regen Med* **3**, 290–301.
16. Geer EB, Shen W (2009) Gender differences in insulin resistance, body composition, and energy balance. *Gend Med* **6** Suppl 1, 60–75.
17. Björntorp P (1996) The regulation of adipose tissue distribution in humans. *Int J Obes Relat Metab Disord* **20**, 291–302.
18. Frisbee JC (2007) Vascular dysfunction in obesity and insulin resistance. *Microcirculation* **14**, 269–71.
19. Butterwick KJ, Nootheti PK, Hsu JW, Goldman MP (2007) Autologous fat transfer:

an in-depth look at varying concepts and techniques. *Facial Plast Surg Clin North Am* **15**, 99–111.

20. Gonzalez AM, Lobocki C, Kelly CP, Jackson IT (2007) An alternative method for harvest and processing fat grafts: an in vitro study of cell viability and survival. *Plast Reconstr Surg* **120**, 285–94.
21. Keck M, Janke J, Ueberreiter K (2009) Viability of preadipocytes in vitro: the influence of local anesthetics and pH. *Dermatol Surg* **35**, 1251–7.
22. Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C (2008) Adipose-derived stem cells: isolation, expansion and differentiation. *Methods* **45**, 115–20.
23. Hoying JB, Boswell CA, Williams SK (1996) Angiogenic potential of microvessel fragments established in three-dimensional collagen gels. *In Vitro Cell Dev Biol Anim* **32**, 409–19.
24. Williams SK, McKenney S, Jarrell BE (1995) Collagenase lot selection and purification for adipose tissue digestion. *Cell Transplant* **4**, 281–9.
25. Fain JN (1975) Isolation of free brown and white fat cells. *Methods Enzymol* **35**, 555–61.
26. Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, Halvorsen YD, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM (2006) Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* **24**, 376–85.

Part II

Phenotyping Methods

Chapter 9

Methods for the Purification and Characterization of Human Adipose-Derived Stem Cells

Stan Gronthos and Andrew C.W. Zannettino

Abstract

Peripheral adipose tissue contains a population of clonogenic precursor cells referred to as adipose-derived stem cells (ASC) that retain the capacity to differentiate into multiple cell types including osteoblasts, adipocytes, chondrocytes, myocytes, and neuronal cells following ex vivo expansion. Recent studies have demonstrated that ASC are most likely derived from a perivascular niche within highly vascularised fat tissue, analogous to different mesenchymal cell populations identified in other tissues throughout the body. The following chapter describes techniques to prospectively isolate clonogenic ASC from adult human adipose tissue using antibodies directed against perivascular markers and methods to immunophenotypically characterize their ex vivo expanded progeny.

Key words: Bone marrow stromal stem cells, Mesenchymal stem cells, Magnetic-activated cell sorting, Fluorescence-activated cell sorting

1. Introduction

Extramedullary adipose tissue is a readily accessible tissue source for harvesting and propagating large quantities of multipotent mesenchymal stem cell-like populations known as adipose-derived stem cells (ASC) (1–7). The progeny of culture expanded ASC represent potential candidates for cell-based therapies designed to treat bone and cartilage defects/disorders, cardiovascular disease, neurological disorders, autoimmune diseases, as well as improve bone marrow (BM) and solid organ transplantation outcomes (8, 9). While the accessibility and quantity of ASC that can be recovered offers much appeal, the practical application and safety of using ex vivo expanded populations for clinical applications remains to be determined (10–12). However, what is clear is the need to better understand the basic properties of

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culture-expanded ASC in order to properly assess their growth, developmental potential, and capacity to modulate immune responses. An emerging area of research focus centres on the characterization of the stem cell niche and the identification of the microenvironmental factors that regulate immature MSC-like populations in situ. Further studies in this area are expected to help develop improved methodologies for cultivating safer and more potent ASC therapeutic cell preparations for regenerative medicine applications.

Thus far, the majority of studies have utilized plastic adherence for the selection of clonogenic ASC based on the methodologies used to isolate MSC-like populations from other tissue sources. The development of novel antibody reagents to positively select for BM-derived MSC within a background of haematopoietic cell populations has led to the characterization of the basic properties that define clonogenic MSC and their progeny (13–24). More recently, similar immunoselection strategies have been employed to identify different MSC-like populations present within various calcified and soft-tissue sources that appear to share many genotypic, phenotypic, and functional characteristics to those described for BM-derived MSC (25–27). Comparative analyses have demonstrated that some of these antibody reagents that react with antigens associated with perivascular cells (STRO-1, CD146, 3G5, alpha-smooth muscle actin) have a high affinity for selecting MSC-like populations from peripheral adipose tissue or identifying precursor populations in situ around blood vessels (25–30). Overall, these reports confirm a perivascular-like phenotype for ASC, similar to mesenchymal stem cell-like populations found in other tissues, including BM, dental pulp, periodontal ligament, skeletal muscle, pancreas, and placenta (18, 22, 25, 27, 31, 32). Moreover, several studies have implicated pericytes as the most likely candidate population representative of multipotent MSC-like populations identified in different tissues (22, 25, 31, 33, 34). The present chapter describes a method for generating highly purified preparations of clonogenic ASC from human extramedullary adipose tissue, based principally on their expression of perivascular/mesenchymal stem cell-associated markers (18, 22).

2. Materials

1. Collagenase I.
2. Dispase II.
3. 100 mM HEPES, pH 7.35.
4. Phosphate-buffered saline solution (PBS), pH 7.4.
5. Hanks balanced salt solution (HBSS).

6. HHF wash buffer: HBSS supplemented with 5% (v/v) fetal bovine serum (FBS) and 10 mM HEPES.
7. Blocking buffer: HHF supplemented with 5% (v/v) normal human AB serum.
8. Fetal bovine serum (FBS).
9. 40- and 70-μm Falcon cell strainers.
10. 50-ml Falcon tubes.
11. 4-ml polystyrene tubes.
12. 4-ml polypropylene tubes.
13. FACS Fix: 1% (v/v) formalin in PBS supplemented with 0.1 M d-glucose, 0.02% sodium azide.
14. Cell Counting Fluid: 0.4% Trypan Blue in PBS.
15. Alpha-Modification of Eagle's Medium (α-MEM).
16. Bovine serum albumin (Cohn fraction V).
17. L-Ascorbic-2-phosphate (100-mM stock).
18. L-Glutamine (200-mM stock).
19. Sodium pyruvate (100-mM stock).
20. β-mercaptoethanol (14-M stock).
21. Penicillin (5,000 U/ml) and streptomycin (5,000 μg/ml) 100× stock solution.
22. 0.5%Trypsin/EDTA solution.
23. T-25 and T-75 culture flasks.
24. 6-well culture plates.
25. 0.22-μm Ministart low protein-binding filters.
26. 1.8 ml Cryo-tubes.
27. Dimethyl sulphoxide (DMSO).
28. Paraformaldehyde.
29. Toluidine Blue.
30. Tween-20.
31. Biotinylated goat anti-mouse IgM (μ-chain specific) or anti-mouse IgG (γ-chain specific).
32. Goat anti-mouse IgM or anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC).
33. Streptavidin-conjugated FITC.
34. Polypropylene Falcon tubes, 4 and 14 ml.
35. Sheep anti-mouse IgG-conjugated or rat anti-mouse IgM-conjugated magnetic Dynabeads.
36. MPC-1 magnetic particle concentrator.
37. Eight-chamber plastic culture slides.
38. Prolong gold anti-fade mountant with DAPI.

3. Methods

The methods described below outline: (1) the isolation and enrichment of clonogenic adipose-derived stem cells (ASC) by magnetic bead and fluorescence-activated cell separation; (2) ex vivo expansion and cryopreservation of human ASC; and (3) immunophenotypic analysis of cultured human ASC.

3.1. Preparation of Single-Cell Suspensions from Human Adipose Tissue

1. Abdominal wall-derived adipose tissue can be obtained during routine adominoplasty following informed patient consent and with institutional human ethics clearance. All experimental working is carried out within a biohazard laminar flow hood. Aseptic technique is used throughout the procedure. The adipose tissue is separated from the skin and dermis crown and cut into small pieces using sterile surgical scalpels, then washed five times with PBS.
2. The tissue pieces are digested in a solution of collagenase type I (3 mg/ml in PBS) and dispase II (4 mg/ml in PBS) for 2–3 h at 37°C (approximately 1 mg tissue per 0.5 ml of enzyme solution). The enzyme-digested cell tissue preparation is then washed twice in HHF by centrifugation at $400 \times g$ for 10 min.
3. A single-cell suspension of adipose-derived cells is obtained by sequential separation through a 70-μm and then a 40-μm cell strainer. The cells are then washed in HHF by centrifugation at $400 \times g$ for 10 min.
4. Add 20 μl of cell suspension to 80 μl of 0.4% Trypan Blue solution and count the number of cells using a hemocytometer.

3.2. Fluorescence-Activated Cell Sorting for ASC

Fluorescence-activated cell sorting (FACS) can be used to selectively identify and isolate the ASC population. Following adipose tissue harvest and enzymatic digestion, we routinely recover approximately $2–3 \times 10^5$ cells from 1 mg of adipose tissue.

1. Prior to immunolabelling, adipose cells ($5–10 \times 10^6$) are resuspended in 0.5-ml blocking buffer and incubated on ice for approximately 30 min to reduce the possibility of Fc receptor-mediated binding of antibodies.
2. The cells are then incubated with primary antibodies reactive with ASC using either STRO-1 (mouse IgM anti-human MSC) (18, 35), CC9 (mouse IgG_{2a} anti-human CD146/MUC-18) (15, 22), 3G5 (mouse IgM anti-human pericyte) (22, 27), or STRO-4 (mouse IgG_1 anti-human heat shock protein-β) (36) for 1 h on ice at a concentration of 10 μg/ml for purified antibodies and one half dilution for hybridoma supernatants (18, 35) (see Note 1).

3. Separate tubes containing 2×10^5 cells are incubated with the relevant isotype-matched control mouse monoclonal antibodies used in this study: 1A6.12 (IgM), IB% (IgG_1), and 1A6.11 (IgG_{2b}) (kindly provided by Prof. L.K. Ashman, University of Newcastle, NSW, Australia) (see Note 2).
4. After washing with HHF, the cells are incubated with either goat anti-mouse IgM or IgG conjugated to FITC for 45 min on ice (see Note 3).
5. Single-colour FACS can be performed on any fluorescence-activated cell sorter fitted with a 250-mW argon laser emitting light at a wavelength of 488 nm able to detect FITC. Positive reactivity for each antibody was defined as the level of fluorescence greater than 99% of the isotype-matched control antibodies.
6. Primary cultures are initiated at a seeding density of $1–10 \times 10^4$ cells/cm^2 in α-MEM supplemented with 20% (v/v) FBS, 2 mM L-glutamine, 100 μM L-ascorbate-2-phosphate, 50 U/ml penicillin, 50 μg/ml streptomycin, and β-mercaptoethanol (5×10^{-5} M) (see Note 4).
7. After reaching 90% confluency, primary ASC can be subcultured by washing once with HBSS and liberated by enzymatic digestion by the addition of 3 ml of 0.5% Trypsin/EDTA solution per T-75 culture flask for 2–5 min at 37°C (see Note 5).
8. The single-cell suspension is then washed twice in growth medium containing as above with 10% FBS at $400 \times g$ for 10 min and reseeded into fresh culture flasks at a plating density of $0.5–1 \times 10^4$ cells/cm^2 in regular growth medium, α-MEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 μM L-ascorbate-2-phosphate, 50 U/ml penicillin, 50 μg/ml streptomycin, and β-mercaptoethanol (5×10^{-5} M) (see Note 6).

3.3. Magnetic Bead Isolation of ASC

Paramagnetic bead separation can be used as an alternative method to FACS to obtain purified preparations of human ASC.

1. Prior to immunolabelling, cells liberated from adipose tissue as described in Subheading 3.1 are resuspended in 0.5-ml blocking buffer and incubated on ice for approximately 30 min to reduce the possibility of Fc receptor-mediated binding of antibodies.
2. The cells are then incubated with primary antibodies reactive with ASC using either of the monoclonal antibodies described in Subheading 3.2.
3. Labelled cells are washed twice with PBS/1% bovine serum albumin ($400 \times g$ for 10 min) then incubated with either

sheep anti-mouse IgG-conjugated or rat anti-mouse IgM-conjugated magnetic Dynabeads (four beads per cell) for 40 min on a rotary mixer at 4°C. Cells binding to beads are removed using the MPC-1 magnetic particle concentrator according to the manufactures recommended protocol.

4. The STRO-1, STRO-4, CD146, or 3G5 bead-positive cells are then resuspended in regular growth medium described in Subheading 3.2.
5. Primary cultures are initiated and maintained as described in Subheading 3.2 (see Note 7).

3.4. Assessment of ASC Colony Efficiency

The expected incidence of CFU-F colony in human adipose tissue is approximately 400 CFU-F per 10^5 unfractionated cells plated.

1. Single-cell suspensions of adipose tissue are seeded into 6-well culture plates at 0.3, 1.0, and 3.0×10^4 cells per well in regular growth medium described in Subheading 3.2 with 20% (v/v) FBS. Cultures are set up in triplicate and incubated at 37°C in 5% CO_2 and >90% humidity for 12 days (see Note 8).
2. For enumeration, day 12 colonies are washed twice with PBS and then fixed for 20 min in 1% (w/v) paraformaldehyde in PBS.
3. The fixed cultures can then be stained with 0.1% (w/v) toluidine blue (in 1% paraformaldehyde solution) for 1 h then rinsed with tap water and allowed to dry. Aggregates of greater than 50 cells are scored as CFU-F using a dissecting light microscope. Colonies should be visually checked at day 10 to ensure that there is no overgrowth of cells making it difficult to enumerate individual colonies.

3.5. Flow Cytometric Analysis of ASC

To characterise the immunophenotype of ex vivo expanded ASC, flow cytometric analysis can be used to measure the expression of mesenchymal and non-mesenchymal stem cell-associated surface markers at different cell passages.

1. Adherent ex vivo expanded ASC at 70–80% confluency are washed once with HBSS and liberated by enzymatic digestion by the addition of 3 ml of 0.5% Trypsin/EDTA solution per T-75 culture flask for 5 min at 37°C. The single-cell suspension is then washed twice in HHF at $400 \times g$ for 10 min.
2. Cell count and assessment of viability is performed as described above.
3. Resuspend ASC for immunolabeling in 0.5-ml blocking buffer and incubate on ice for approximately 30 min to reduce the possibility of Fc receptor-mediated binding of antibodies.

4. Individual 4-ml round bottom polypropylene tubes containing 1×10^5 culture-expanded ASC are incubated with appropriate primary murine monoclonal IgG antibodies or isotype-matched controls at a concentration of 10 μg/ml for purified antibodies or one half diluted hybridoma supernatant for 1 h on ice. Wash the cells twice in 2 ml HHF as described above.
5. Incubate cells with appropriate secondary detection reagent goat anti-mouse IgG_1–FITC-conjugated antibody or anti-mouse IgM–FITC (1:50) or for 45 min on ice (see Note 9). Wash the cells twice in 2 ml HHF.
6. Resuspend cell pellet and add 0.5 ml FACS Fix solution to each tube to fix the cells.
7. Analyse the cells on any fluorescence-activated cell analyser fitted with a 250-mW argon laser (Fig. 1).

3.6. Immunofluorescence Staining of Cultured ASC

1. Staining of cultured adherent ASC can be performed using an eight-chamber plastic slide. Approximately 15×10^3 cells are seeded per chamber in regular growth medium and allowed to incubate at 37°C 5% CO_2 for 2–3 days.
2. Wash the cells three times with PBS.
3. Fix cells in 2% paraformaldehyde/PBS for 5 min at room temperature.
4. Wash cells three times in PBS + 0.5% Tween 20.
5. Incubate cells with blocking solution of 5% normal goat serum for 1 h at room temperature.
6. Samples are then incubated with either primary antibodies or relevant isotype control antibodies for 1 h at room temperature.
7. Following three washes in PBS + 0.5% Tween 20, the wells are incubated with the secondary detection antibodies, goat anti-mouse IgM–FITC or IgG–FITC at a 1:50 dilution, for 45 min at room temperature.
8. After washing in PBS + 0.5% Tween 20, the samples can be mounted with prolong gold anti-fade mountant containing DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) for nuclear staining (see Note 10).
9. The cells are viewed under a UV microscope such as Olympus BH2-RFCA fluorescence microscope (Fig. 2).

3.7. Cryopreservation of Ex Vivo Expanded ASC

1. Routinely, single-cell suspensions of culture expanded ASC are prepared by trypsin/EDTA digest as described above. The cells are then diluted and washed in cold HFF.
2. The cell pellet is resuspended at a concentration of 1×10^7 cells/ml in FBS and maintained on ice. An equal volume of freeze mix (20% DMSO in cold FBS) is then added gradually while gently mixing the cells to give a final concentration of

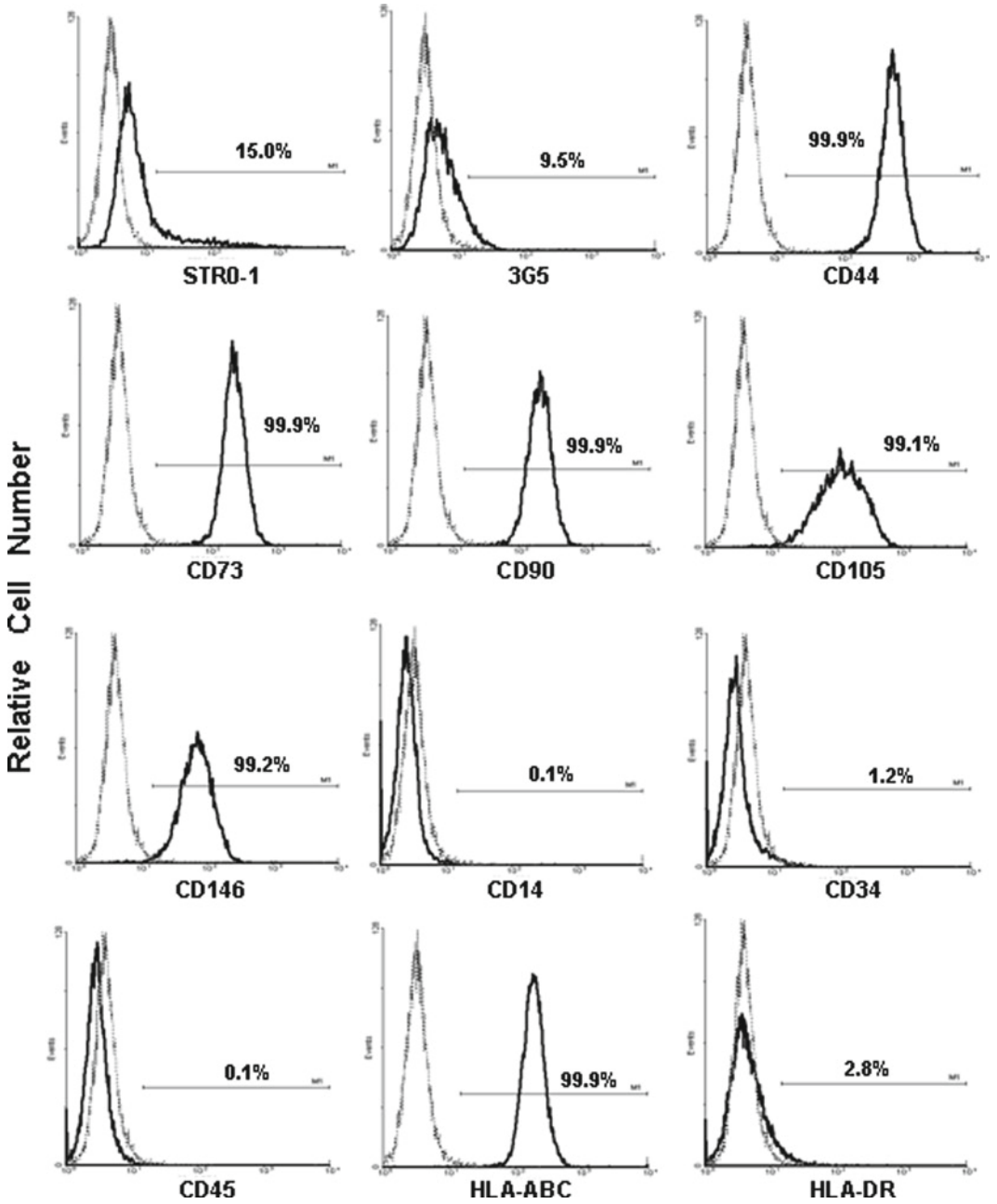

Fig. 1. Flow cytometric analysis of cell surface antigens. Representative histogram of flow cytometric analysis of ex vivo expanded ASC at cell passage 2 following trypsin/EDTA detachment. Isotype-matched control antibodies (*light line*) and test antibodies (*dark solid line*) were FITC conjugated. The *horizontal bars* indicate regions showing the percentage of positive cells above background fluorescence.

5×10^6 cells/ml in a 10% DMSO/FBS. One-millilitre aliquots are then distributed into 1.8-ml cryovials pre-cooled on ice, i.e., 1 ml/tube, then frozen at a rate of –1°C/min using a rate-control freezer (see Note 11).

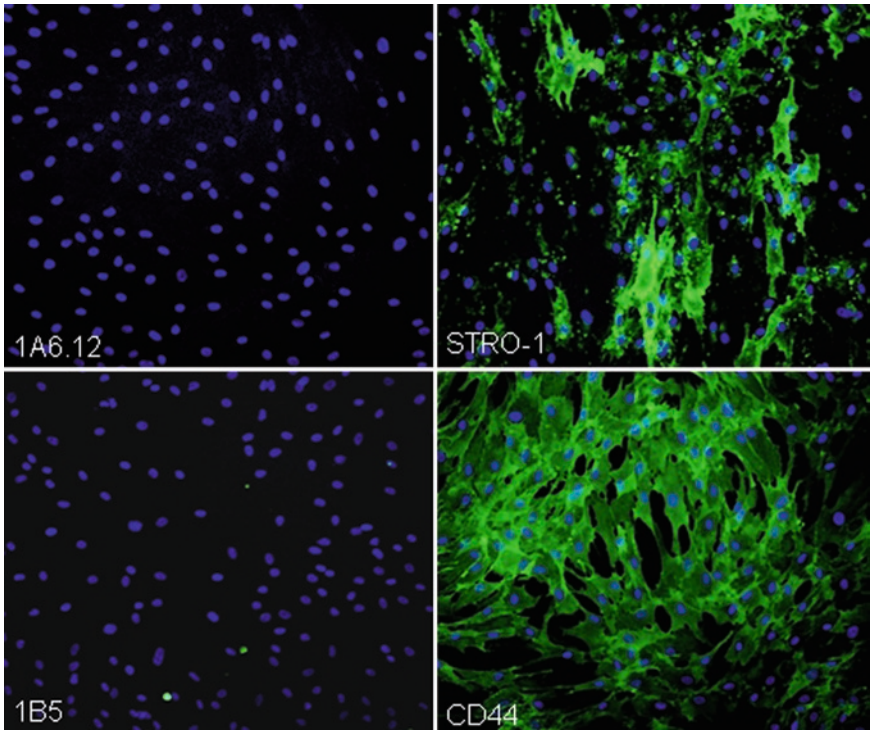

Fig. 2. In situ immunofluorescence staining of cultured ASC. Representative fields of ex vivo expanded (passage 2) ASC at 70% confluency, stained with FITC-conjugated antibodies to either STRO-1, CD44 (*green*), or isotype-matched controls (1A6.12 and 1B5), respectively. Nuclei were counterstained with DAPI (*blue*) (×200 magnification).

3. The frozen vials are then transferred to liquid nitrogen for long-term storage. Recovery of the frozen stocks is achieved by rapid thawing the cells in a 37°C water bath. The cells are then resuspended in cold HFF and spun at 280×*g* for 10 min.
4. To assess viability of the cells, prepare a 1:5 dilution in 0.4% Trypan Blue/PBS, and the number of cells determined using a haemocytometer. Typically this procedure gives viabilities between 80 and 90%.

4. Notes

1. The STRO-1 antibody can also be purchased commercially from R&D Systems Inc., Minneapolis, MN, USA.
2. Isotype negative control antibodies can also be purchased commercially from a variety of vendors including Becton Dickinson, CALTAG Laboratories, and R&D Systems.

3. Other fluorochrome-conjugated antibodies can also be used, such as phycoerythrin.
4. Variations in FBS batches can severely hamper the establishment of primary cultures and growth. Batch testing of FBS is highly recommended to ensure optimal establishment and growth of ASC.
5. Adherent cell cultures if overconfluent can be liberated using collagenase/dispase digestion. Wash the cells with PBS then incubate in a pre-warmed solution of collagenase I (3 mg/ml in PBS) and dispase II (4 mg/ml in PBS) approximately 1 ml/25 cm^2 surface area for 60 min at 37°C. Single-cell suspensions are then washed twice in HHF buffer.
6. Growth medium can be pre-made, sterilized using a 0.22-μm filter, and then stored at 4°C. If the medium is greater than a week old at 4°C, then fresh 2 mM L-glutamine should be added prior to use. Prior to culture, if cells appear to be clumping, pass them through a 70-μm cell strainer.
7. Prior to the first subculture, liberated ASC in cell suspension can be placed onto the MPC-1 magnetic particle concentrator to remove any cells that have engulfed the magnetic beads before reseeding into culture flasks with normal growth media.
8. Variations in FBS batches can affect colony cell number and size. Batch testing of FBS is highly recommended to ensure optimal growth conditions.
9. Other fluorochrome conjugates, such as Texas Red, can also be used to detect red fluorescence.
10. It is recommended that clear nail polish be used to seal around the glass coverslip to stop leakage of the mountant solution and movement of the coverslip.
11. The ASC can also be frozen using a Cryo 1°C freezing container "Mr. Frosty" by placing the container holding the cryotubes at –80°C overnight before transferring the cells into liquid nitrogen. For serum-free applications, ProFreeze solution containing a final concentration of 7.5% DMSO can be substituted for 10% FBS/DMSO freeze mix.

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References

1. Baglioni, S., Francalanci, M., Squecco, R., Lombardi, A., Cantini, G., Angeli, R., Gelmini, S., Guasti, D., Benvenuti, S., Annunziato, F., Bani, D., Liotta, F., Francini, F., Perigli, G., Serio, M., and Luconi, M. (2009) Characterization of human adult stem-cell populations isolated from visceral and subcutaneous adipose tissue, *FASEB J 23*, 3494–3505.
2. D'Andrea, F., De Francesco, F., Ferraro, G. A., Desiderio, V., Tirino, V., De Rosa, A., and Papaccio, G. (2008) Large-scale production of human adipose tissue from stem cells: a new tool for regenerative medicine and tissue banking, *Tissue Eng Part C Methods 14*, 233–242.
3. Gimble, J. M. and Guilak, F. (2003) Differentiation potential of adipose derived adult stem (ADAS) cells, *Curr Top Dev Biol 58*, 137–160.
4. Safford, K. M., Safford, S. D., Gimble, J. M., Shetty, A. K., and Rice, H. E. (2004) Characterization of neuronal/glial differentiation of murine adipose-derived adult stromal cells, *Exp Neurol 187*, 319–328.
5. Strem, B. M., Hicok, K. C., Zhu, M., Wulur, I., Alfonso, Z., Schreiber, R. E., Fraser, J. K., and Hedrick, M. H. (2005) Multipotential differentiation of adipose tissue-derived stem cells, *Keio J Med 54*, 132–141.
6. Wickham, M. Q., Erickson, G. R., Gimble, J. M., Vail, T. P., and Guilak, F. (2003) Multipotent stromal cells derived from the infrapatellar fat pad of the knee, *Clin Orthop Relat Res 412*, 196–212.
7. Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P., and Hedrick, M. H. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies, *Tissue Eng 7*, 211–228.
8. Nathan, S., Das De, S., Thambyah, A., Fen, C., Goh, J., and Lee, E. H. (2003) Cell-based therapy in the repair of osteochondral defects: a novel use for adipose tissue, *Tissue Eng 9*, 733–744.
9. Schaffler, A. and Buchler, C. (2007) Concise review: adipose tissue-derived stromal cells – basic and clinical implications for novel cell-based therapies, *Stem Cells 25*, 818–827.
10. Izadpanah, R., Kaushal, D., Kriedt, C., Tsien, F., Patel, B., Dufour, J., and Bunnell, B. A. (2008) Long-term in vitro expansion alters the biology of adult mesenchymal stem cells, *Cancer Res 68*, 4229–4238.
11. Rubio, D., Garcia-Castro, J., Martin, M. C., de la Fuente, R., Cigudosa, J. C., Lloyd, A. C., and Bernad, A. (2005) Spontaneous human adult stem cell transformation, *Cancer Res 65*, 3035–3039.
12. Yu, J. M., Jun, E. S., Bae, Y. C., and Jung, J. S. (2008) Mesenchymal stem cells derived from human adipose tissues favor tumor cell growth in vivo, *Stem Cells Dev 17*, 463–473.
13. Boiret, N., Rapatel, C., Veyrat-Masson, R., Guillouard, L., Guerin, J. J., Pigeon, P., Descamps, S., Boisgard, S., and Berger, M. G. (2005) Characterization of nonexpanded mesenchymal progenitor cells from normal adult human bone marrow, *Exp Hematol 33*, 219–225.
14. Deschaseaux, F., Gindraux, F., Saadi, R., Obert, L., Chalmers, D., and Herve, P. (2003) Direct selection of human bone marrow mesenchymal stem cells using an anti-CD49a antibody reveals their CD45med, low phenotype, *Br J Haematol 122*, 506–517.
15. Filshie, R. J., Zannettino, A. C., Makrynikola, V., Gronthos, S., Henniker, A. J., Bendall, L. J., Gottlieb, D. J., Simmons, P. J., and Bradstock, K. F. (1998) MUC18, a member of the immunoglobulin superfamily, is expressed on bone marrow fibroblasts and a subset of hematological malignancies, *Leukemia 12*, 414–421.
16. Gronthos, S., Graves, S. E., Ohta, S., and Simmons, P. J. (1994) The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors, *Blood 84*, 4164–4173.
17. Gronthos, S. and Simmons, P. J. (1995) The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro, *Blood 85*, 929–940.
18. Gronthos, S., Zannettino, A. C., Hay, S. J., Shi, S., Graves, S. E., Kortesidis, A., and Simmons, P. J. (2003) Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow, *J Cell Sci 116*, 1827–1835.
19. Joyner, C. J., Bennett, A., and Triffitt, J. T. (1997) Identification and enrichment of human osteoprogenitor cells by using differentiation stage-specific monoclonal antibodies, *Bone 21*, 1–6.
20. Miura, M., Chen, X. D., Allen, M. R., Bi, Y., Gronthos, S., Seo, B. M., Lakhani, S., Flavell, R. A., Feng, X. H., Robey, P. G., Young, M., and Shi, S. (2004) A crucial role of caspase-3 in osteogenic differentiation of bone marrow stromal stem cells, *J Clin Invest 114*, 1704–1713.

21. Quirici, N., Soligo, D., Bossolasco, P., Servida, F., Lumini, C., and Deliliers, G. L. (2002) Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies, *Exp Hematol 30*, 783–791.
22. Shi, S. and Gronthos, S. (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp, *J Bone Miner Res 18*, 696–704.
23. Simmons, P. J., Gronthos, S., Zannettino, A., Ohta, S., and Graves, S. (1994) Isolation, characterization and functional activity of human marrow stromal progenitors in hemopoiesis, *Prog Clin Biol Res 389*, 271–280.
24. Simmons, P. J. and Torok-Storb, B. (1991) Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1, *Blood 78*, 55–62.
25. Crisan, M., Yap, S., Casteilla, L., Chen, C. W., Corselli, M., Park, T. S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., Norotte, C., Teng, P. N., Traas, J., Schugar, R., Deasy, B. M., Badylak, S., Buhring, H. J., Giacobino, J. P., Lazzari, L., Huard, J., and Peault, B. (2008) A perivascular origin for mesenchymal stem cells in multiple human organs, *Cell Stem Cell 3*, 301–313.
26. Kalajzic, Z., Li, H., Wang, L. P., Jiang, X., Lamothe, K., Adams, D. J., Aguila, H. L., Rowe, D. W., and Kalajzic, I. (2008) Use of an alpha-smooth muscle actin GFP reporter to identify an osteoprogenitor population, *Bone 43*, 501–510.
27. Zannettino, A. C., Paton, S., Arthur, A., Khor, F., Itescu, S., Gimble, J. M., and Gronthos, S. (2008) Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo, *J Cell Physiol 214*, 413–421.
28. Gronthos, S., Franklin, D. M., Leddy, H. A., Robey, P. G., Storms, R. W., and Gimble, J. M. (2001) Surface protein characterization of human adipose tissue-derived stromal cells, *J Cell Physiol 189*, 54–63.
29. Khan, W. S., Tew, S. R., Adesida, A. B., and Hardingham, T. E. (2008) Human infrapatellar fat pad-derived stem cells express the pericyte marker 3G5 and show enhanced chondrogenesis after expansion in fibroblast growth factor-2, *Arthritis Res Ther 10*, R74.
30. Lin, G., Garcia, M., Ning, H., Banie, L., Guo, Y. L., Lue, T. F., and Lin, C. S. (2008) Defining stem and progenitor cells within adipose tissue, *Stem Cells Dev 17*, 1053–1063.
31. Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P. G., Riminucci, M., and Bianco, P. (2007) Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment, *Cell 131*, 324–336.
32. Seo, B. M., Miura, M., Gronthos, S., Bartold, P. M., Batouli, S., Brahim, J., Young, M., Robey, P. G., Wang, C. Y., and Shi, S. (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament, *Lancet 364*, 149–155.
33. Schor, A. M. and Canfield, A. E. (1998) Osteogenic potential of vascular pericytes, in *Marrow stromal cell culture* (Owen, M. E. and Beresford, J. N., Ed.), pp 128–148, Cambridge University Press, Cambridge, UK.
34. Doherty, M. J., Ashton, B. A., Walsh, S., Beresford, J. N., Grant, M. E., and Canfield, A. E. (1998) Vascular pericytes express osteogenic potential in vitro and in vivo, *J Bone Miner Res 13*, 828–838.
35. Gronthos, S. and Zannettino, A. C. (2008) A method to isolate and purify human bone marrow stromal stem cells, *Methods Mol Biol 449*, 45–57.
36. Gronthos, S., McCarty, R., Mrozik, K., Fitter, S., Paton, S., Menicanin, D., Itescu, S., Bartold, P. M., Xian, C., and Zannettino, A. C. (2009) Heat shock protein-90 beta (Hsp90ss) is expressed at the surface of multipotential mesenchymal precursor cells (MPC): generation of a novel monoclonal antibody, STRO-4, with specificity for MPC from human and ovine tissues, *Stem Cells Dev 18*, 1253–1262.

Chapter 10

Characterization of Human Adipose-Derived Stem Cells Using Flow Cytometry

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Abstract

One of the hallmark characteristics of human adipose-derived stem cells (hASCs) is their ability to differentiate into cells of mesenchymal lineages. It is also becoming apparent that ASCs can mediate a therapeutic benefit through cytokine, paracrine-driven mechanisms influencing apoptosis, angiogenesis, and potent anti-inflammatory responses. Although there is still no clear consensus on the antigen expression pattern that will define hASCs, a protocol is also presented for the flow cytometric analysis utilizing a series of antibody panels. The analysis of these surface epitope patterns can aide in the isolation and characterization of hASCs. Moreover, using this standardized antibody panel, direct comparisons can be made between ASCs isolated from various tissue sources, which will benefit the field by providing uniformity to the comparison process.

Key words: Adipose-derived stem cells, Characterization, Expansion, Human, Surface epitopes, Stem cells

1. Introduction

Adipose tissue is an abundant and accessible source of adult or somatic stem cells termed adipose-derived stem cells (ASCs). ASCs have generated a great deal of interest in the field of regenerative medicine for tissue engineering applications in last few years. Rodbell and colleagues first pioneered the procedures for the isolation of ASCs using rat epididymal fat (1–4). These methods were later adapted and refined for human tissues by multiple groups (5–8). The method relies on a combination of physical disruption of the tissue, collagenase digestion, and final selection of the ASCs based on their plastic adherence. This final step enriches the ASCs from the heterogeneous stromal vascular

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fraction (SVF) cells by ~30-fold (9). The plastic-adherent cell population isolated from collagenase digests of adipose tissue has been described by several different names in the literature, including adipose-derived stem/stromal cells (ASCs); adipose-derived adult stem (ADAS) cells, adipose-derived adult stromal cells, adipose-derived stromal cells (ADSC), adipose stromal cells (ASC), adipose mesenchymal stem cells (AdMSC), lipoblast, pericyte, pre-adipocyte, and processed lipoaspirate (PLA) cells. Due to significant confusion in the literature, the International Fat Applied Technology Society reached a consensus to adopt the term adipose-derived stem cells (ASCs) to identify the isolated, plastic-adherent, multipotent cell population.

The characterization of hASCs can be augmented with a battery of monoclonal antibodies and flow cytometry analyses to determine the presence or absence of particular cell surface proteins (Table 1). Unlike hematopoietic cells, however, there is little consensus on the antigen expression pattern that can precisely define ASCs. The utility of flow cytometric analysis of cell surface proteins lies in determining the type of cells obtained and establishing continuity of results among cell preparations and over time in culture. Due to the large number of antibodies needed to evaluate ASCs, a large number of cells is needed to complete the procedure. This problem can be alleviated in part using a panel of antibodies in each analysis thus reducing the number of cells needed from 30 million to 3 million per panel. The number of antibodies that can be mixed is determined by the type of flow cytometer, the number of channels that the instrument has available, the cross-reactivity of the antibodies with each other, and the availability of fluorochrome conjugates. The list of surface antigens that can be examined on ASCs is extensive. The protocol presented here includes the antibody panels that we commonly use for standard characterization of ASC and MSC preparations.

2. Materials

2.1. Reagents

1. Alpha Minimum Essential Medium (αMEM) with L-glutamine, without ribonucleosides or deoxyribonucleosides.
2. Fetal bovine serum (FBS), premium select, hybridoma-qualified, not heat-inactivated – selected from a screen of four to five lots as providing the most rapid growth of hASCs.
3. L-Glutamine (200 mM) in solution of 0.85% NaCl.
4. Penicillin G (10,000 U/mL) and streptomycin sulfate (10,000 μg/mL) in solution of 0.85% NaCl (*Optional*).
5. Phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+}, 1×.

Table 1
Descriptions of CD markers used for MSC updated flow cytometry panel

Marker	Description
CD3	OKT3, integral membrane glycoprotein that associates with T cell antigen receptor (TCR), and is required for TCR cell surface expression and signal transduction
CD11b	Integrin alpha M, Mac-1; Mediates adhesion to substrates by opsonization with iC3b and subsequent phagocytosis, neutrophil aggregation, chemotaxis
CD14	Lipopolysaccharide (LPS) receptor, monocyte differentiation antigen
CD19	Co-receptor with CD21; earliest B cell antigen in fetal tissue. Present on all B lymphocytes
CD29	Fibronectin receptor. Involved in cell–cell and cell–matrix activity
CD34	Cell–cell adhesion molecule and cell surface glycoprotein. Expressed on virtually all hematopoetic progenitor cells, including multipotential stem cells
CD36	Platelet GPIV or GPIIIb; thrombospondin receptor. Cell adhesion molecule in platelet adhesion and aggregation, platelet–monocyte and platelet–tumor cell interaction
CD44	Family of cell surface glycoproteins with isoforms generated by alternate splicing of mRNA. Important in epithelial cell adhesion to hyaluronate in basement membranes and maintaining polar orientation of cells; also binds laminin, collagen, and fibronectin
CD45	Leukocyte common antigen (LCA) expressed on all human white blood cells
CD49b	Very late antigen (VLA) alpha 2 chain – on T cells, a.k.a. GPIa/IIa when expressed on platelets
CD49c	Very late antigen (VLA) alpha 3 chain. Receptor for laminin, collagen, fibronectin, thrombospondin
CD49d	Reacts with the integrin alpha 4 chain. Mediates binding to VCAM-1
CD49f	Very late antigen (VLA) alpha 6 chain; laminin receptor
CD59	Protectin, regulates complement-mediated cell lysis by inhibiting formation of membrane attack complex (MAC)
CD73	Ecto-5'-nucleotidase
CD79a	MB-1, B-cell antigen receptor complex associated protein alpha-chain
CD90	Thy-1. Associated with multipotent progenitor activity

(continued)

Table 1 (continued)

Marker	Description
CD105	Endoglin, regulatory component of TGF-beta receptor complex; mediates cellular response to TGF-beta 1
CD106	VCAM-1; alpha 4 beta 1 ligand
CD117	c-kit, stem cell factor receptor
CD146	S-Endo, Muc18, and MCAM. Expressed in human endothelium
CD147	Neurothelin, extracellular matrix metalloproteinase inducer
CD166	Activated leukocyte cell adhesion molecule (ALCAM)
CD184	CXCR4, stromal cell-derived factor 1 (SDF-1). Receptor for the CXC chemokine SDF-1
CD271	Nerve growth factor receptor (NGFR)
c-Met	HGFR or hepatocyte growth factor receptor
HLA-1, ABC	The antigen corresponds to a monomorphic determinant of human HLA class I molecules. HLA-ABC is associated with β-2 microglobulin
HLA-2, DR, DP, DQ	All major histocompatibility Class II HLA-DR, DP, and most DQ antigens expressed on B cells, antigen-presenting cells and activated T cells
Isotype controls	Mouse IgG1/mouse IgG2a
PODXL	Podocalyxin or PCLP

6. 0.25% Trypsin and 1 mM ethylene diaminetetraacetic acid (EDTA) in Hanks' Balanced Salt Solution (HBSS).
7. 0.4% Trypan Blue in solution of 0.85% NaCl.
8. Ethanol (70%, 95%, 100%).
9. Isopropanol (100%).
10. Methanol (100%).
11. Deionized (DI) water.
12. Distilled water (dH_2O).
13. Neutral-buffered formalin, 10% (NBF).
14. Clear-rite 3 (Richard–Allan Scientific; catalog # 6901).
15. Permount Mounting Media (Fisher; catalog # SP15-500).
16. CD36 FITC IgG1 (Beckman–Coulter; catalog # IM0766).
17. CD34 PE IgG1 (Beckman–Coulter; catalog # IM1871).
18. CD19 ECD IgG1 (Beckman–Coulter; catalog # IM2708).

19. CD11b PeCy5 IgG1 (Beckman–Coulter; catalog # IM3611).
20. CD45 PeCy7 IgG1 (Beckman–Coulter; catalog # IM3548).
21. PODXL FITC IgG2a (MBL; catalog # M084-4).
22. CD166 PE IgG1 (Beckman–Coulter; catalog # A22361).
23. CD90 PeCy5 IgG1 (Beckman–Coulter; catalog # IM3703).
24. CD49b FITC IgG1 (Beckman–Coulter; catalog # IM1425).
25. CD105 PE IgG1 (Beckman–Coulter; catalog # A07414).
26. CD184 APC IgG2a (BD Biosciences; catalog # 555976).
27. CD3 PeCY7 IgG1 (Beckman–Coulter; catalog # 6607100).
28. CD147 FITC IgG1 (BD Biosciences; catalog # 555962).
29. CD49c PE IgG1 (BD Biosciences; catalog # 556025).
30. CD29 PeCy5 IgG1 (BD Biosciences; catalog # 559882).
31. CD59 FITC IgG1 (Beckman–Coulter; catalog # IM3457).
32. CD146 PE IgG2a (Beckman–Coulter; catalog # A07483).
33. CD79a PeCy5 IgG1 (Beckman–Coulter; catalog # IM3456).
34. HLA-Class I: ABC FITC IgG1 (BD Biosciences; catalog # 555552).
35. CD271 PE IgG1 (BD Biosciences; catalog # 557196).
36. CD49f PeCy5 IgG1 (BD Biosciences; catalog # 551129).
37. CD117 PeCY7 IgG1 (Beckman–Coulter, catalog # IM3698).
38. HLA-Class II: DR, DP, DQ FITC IgG1 (BD Biosciences; catalog # 555558).
39. CD73 PE IgG1 (BD Biosciences; catalog # 550257).
40. CD106 PeCy5 IgG1 (BD Biosciences; catalog # 551148).
41. HGF(c-Met) FITC IgG1 (EBioscience, catalog # 11-8858-71).
42. CD49d PE IgG1 (BD Biosciences, catalog # 555503).
43. CD14 ECD IgG2a (Beckman–Coulter, catalog # IM2707).
44. CD44 APC IgG2a (BD Biosciences, catalog # 559942).

2.2. Equipment

1. Biological Safety Cabinet, Class II (BSC).
2. Centrifuge with swinging bucket rotor, capable of holding various tube sizes up to 200 mL.
3. Incubator, water jacketed and humidified with 5% CO_2, maintained at 37°C.
4. Microscope, inverted, phase.
5. Vacuum aspiration source with tubing and waste container.
6. Five-color-capable flow cytometer.

2.3. Supplies

1. Sterile plastic disposable serological pipettes: 5 mL, 10 mL, 25 mL, and 50 mL.
2. Sterile plastic disposable pipettes for vacuum aspiration.
3. Single channel pipettors, air displacement, capable of accurately measuring from 10 to 1,000 µL.
4. Sterile aerosol-barrier pipette tips: 10, 20, 200 and 1,000 µL.
5. Sterile plastic disposable conical centrifuge tubes: 15 and 50 mL.
6. Plastic disposable snap-cap centrifuge tubes: 1.5 mL.
7. Costar 6-well TC-treated microplates.
8. Sterile 250-mL filter units 0.22-µm pore size.
9. Sterile 500-mL filter units, 0.22-µm pore size.
10. Sterile 1,000-mL filter units, 0.22-µm pore size.
11. Sterile tissue culture dishes/flasks: 15-cm diameter (145-cm^2) dishes, or T175 (175-cm^2) flasks.
12. Whatman #1 filter paper.
13. Disposable transfer pipettes: 3 mL.
14. 5-mL Polystyrene culture test tubes, 12×75 mm or tubes recommended by the flow cytometer manufacturer.

2.4. Solutions

Complete Culture Medium (*CCM*): 500 mL α-MEM, 100 mL FBS (final concentration ~16.5%), 6 mL L-glutamine (final concentration 2 mM), and 6 mL Penicillin G and streptomycin sulfate (final concentration 100 U/mL penicillin and 100 µg/mL streptomycin – *Optional)*.

Filter medium through 0.22-µm sterile filter unit. Divide into aliquots and store at 4°C for up to 2 weeks (see Note 1).

3. Methods

All supplies and reagents are sterile and the culture procedure should be performed in a tissue culture hood using aseptic techniques. All materials to be used in the biosafety cabinet (BSC) should be wiped down with 70% ethanol before bringing it in to the BSC. Lab coats and gloves should be worn when working with the cells.

3.1. Culture and Expansion of ASCs from Frozen Vials of Viable Cells

1. The cells are rapidly thawed at 37°C and immediately seeded in a 28-cm^2 or a 175-cm^2 plate in CCM.
2. Twenty-four hours after plating, aspirate the entire medium from the plates and replace with fresh CCM (see Notes 1 and 2).
3. The cells are maintained in a humidified tissue culture incubator at 37°C with 5% CO_2.

4. The medium is changed every second day until the cells reach 80–90% confluence, and then the cells can be harvested for flow cytometric analysis.
5. For harvesting viable ASCs, add a small volume (5–10 mL) of sterile warm PBS to the plates and allow PBS to remain on cells for 2 min.
6. Replace the PBS with 5 mL of trypsin/EDTA solution (0.5%). Incubate in incubator for 5–10 min. Verify under microscope that more than 90% of the cells have detached and then add 5 mL of CCM to allow the serum contained in the solution to neutralize the trypsin reaction.
7. Transfer the medium containing the suspended cells from the well to a sterile 50-mL tube.
8. Centrifuge at 1,200 rpm ($300 \times g$) for 5 min.
9. Aspirate the supernatant and suspend the cells in a small volume of stromal medium (~250 μL).
10. Proceed to cell counting by taking an aliquot of cells diluted in Trypan Blue (for a 1:8 dilution: add 12.5 μL of suspended cells to 87.5 μL of Trypan Blue). Count cells using the hemocytometer. After counting, cells can then be used for flow cytometry.

Viability of the cells can be checked using 0.4% Trypan Blue (Sigma). To 250 μL of Trypan Blue, add 150 μL HBSS and 100 μL cell suspension (dilution factor = 5). Wait 5–15 min. (If cells are exposed to Trypan Blue for extended periods of time, viable cells as well as nonviable cells may begin to take up the dye). Count cells in five large squares on one side of the hemocytometer and calculate the number of cells per milliliter using the formula:

$$\frac{\text{total cells counted} \times \text{dilution factor} \times 10^4}{\text{number of large squares counted}} = \#\ \text{cells/ml}$$

The four corner squares and the center square (1, 3, 5, 7, 9 on one side or 10, 12, 14, 16, 18 on the other side) in the figure below are considered the large squares.

$$\text{Total number of cells : number of cells/mL} \times \text{total volume (in mL)}$$

Nonviable cells will stain blue and live cells will be unstained. Count both blue and clear cells.

$$\text{Viability } (\%): \frac{\text{Total viable cells } (\text{unstained})}{\text{Total cells } (\text{stained and unstained})} \times 100$$

3.2. Staining Procedure for Surface Epitope Characterization of hASCs

1. Perform flow cytometer start up and quality control. The instrument procedure for startup and quality control should be performed as described by the manufacturer. This should include the analysis of fluorescent beads to validate the function

of the lasers, flow systems, and detection systems. Any problems encountered during this phase should be corrected before proceeding with the analysis of prepared samples.

2. Following the antibody manufacturer's recommendations, the appropriate volume of reagents should be dispensed into a series of eight 1.5-mL microfuge tubes (a panel) as follows:

   Tube 1: CD36 FITC, CD34 PE, CD19 ECD, CD11b PeCy5, CD45 PeCy7
   Tube 2: PODXL FITC, CD166 PE, CD90 PeCy5
   Tube 3: CD49b FITC, CD105 PE, CD184 APC, CD3 PeCy7
   Tube 4: CD147 FITC, CD49c PE, CD29 PeCy5
   Tube 5: CD59 FITC, CD146 PE, CD79a PeCy5
   Tube 6: HLA-Class I: ABC FITC, CD271 PE, CD49f PeCy5, CD117 PeCY7
   Tube 7: HLA-Class II: DR, DP, DQ FITC, CD73 PE, CD106 PeCy5
   Tube 8: HGF(c-Met) FITC, CD49d PE, CD14 ECD, CD44 APC
   Tube 9: Isotype control, IgG1/IgG2a
   Tube 10: Unstained control for background

   The tubes containing the antibody cocktails can be made ahead and stored at 4°C in the dark until needed.

3. Harvest cells and count using Trypan Blue or other method to determine viability. Resuspend cells in PBS at a final concentration of 1×10^6 viable cells/ml. Approximately 4×10^6 cells will be required to complete this protocol.

4. Aliquot between 25×10^4 and 5×10^5 cells per tube set up in step 3.2.2. Additionally, set up a 10th tube containing only cell suspension as a control for autofluorescence. Gently vortex to mix and incubate in the dark for 20 min at room temperature (RT).

5. Wash the cells by adding PBS to the 1.5-mL mark on each tube. Pellet the cells at $100 \times g$ for 3 min at RT. Remove the supernatant, resuspend the pellet in 1 mL PBS, and centrifuge again. Repeat one more time for a total of three washes.

6. Resuspend the final pellet in 500 µL PBS and gently vortex. Be sure no aggregates are present.

7. Using a transfer pipette, place the cell suspensions into the 12×75-mm culture test tubes (or recommended device) and analyze on the flow cytometer. Analyze the unlabeled cells (Tube 10) first, followed by the isotype control (Tube 9). Use the results of these two control tubes to set the gates

and analysis regions. Then read each of the antibody cocktail-labeled cells.

See Fig. 1 for typical FACS results and Table 2 for expected expression levels of the panels of antibodies on ASCs and an example of flow cytometry results performed on hASCs at passages 1–4 (P1–4).

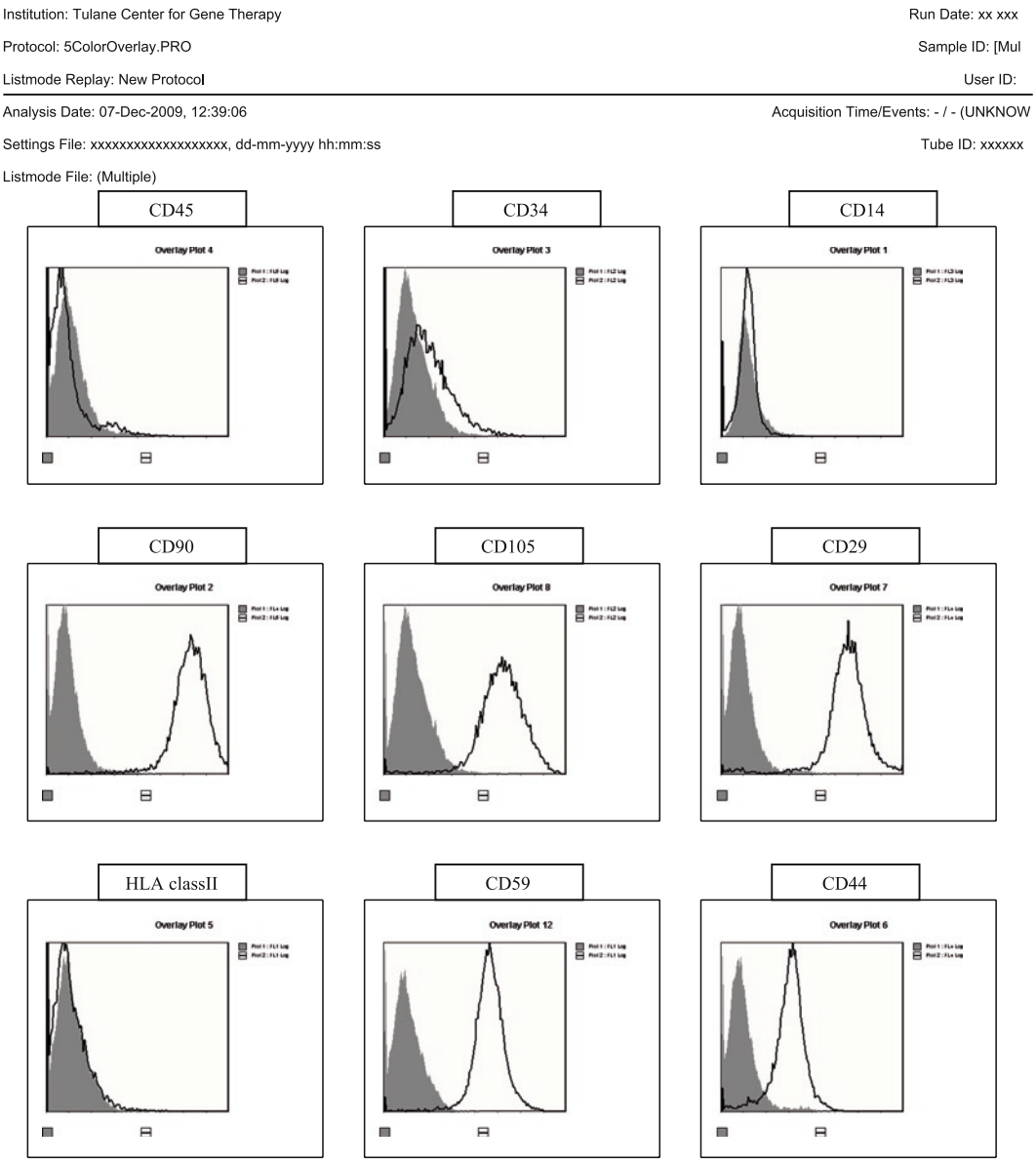

Fig. 1. Representative histogram plots for the detection of cell surface antigens on the presence of ASCs. For a full description, see Table 2.

Table 2
Surface epitope expression on hASCs at passage 2 for a single donor as determined by flow cytometry

	Antibody – Fluorochrome	Expected result
Protocol 1	CD36 – FITC	+
	CD34 – PE	+
	CD19 – ECD	–
	CD11b – PeCy5	–
	CD45 – PeCy7	–
Protocol 2	PODXL – FITC	+/–
	CD166 – PE	++
	CD90 – PeC45	+++
Protocol 3	CD49b – FITC	–
	CD105 – PE	++
	CD184 – APC	–
	CD3 – PeCy5	–
Protocol 4	CD147 – FITC	++
	CD49c – PE	++
	CD29 – PeCy5	++
Protocol 5	CD59 – FITC	++
	CD146 – PE	++
	CD79a – PeCy5	–
Protocol 6	HLA – abc – FITC	+
	CD271 – PE	+/–
	CD49f – PeCy5	+
	CD117 – PeCy7	–
Protocol 7	HLA – II – FITC	–
	CD73 – PE	++
	CD106 – PeCy5	–
Protocol 8	HGF – FITC	–
	CD49d – PE	+/–
	CD14 – ECD	–
	CD44 – APC	+

The expected level of antigen detection of the six hASC preparations. The unlabeled cells and the isotype controls should be negative
– Negative, +/– variable, + dimly positive, ++ moderately positive, +++ brightly positive

4. Notes

1. Place CCM in a 37°C water bath until use.
2. During the expansion phase in culture, replace the CCM media every third day.

References

1. Rodbell, M. (1964) Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis, *J. Biol. Chem. 239*, 375–380.
2. Rodbell, M. (1966) Metabolism of isolated fat cells. II. The similar effects of phospholipase C (*Clostridium perfringens alpha toxin*) and of insulin on glucose and amino acid metabolism, *J. Biol. Chem. 241*, 130–139.
3. Rodbell, M. (1966) The metabolism of isolated fat cells. IV. Regulation of release of protein by lipolytic hormones and insulin, *J. Biol. Chem. 241*, 3909–3917.
4. Rodbell, M. and Jones, A.B. (1966) Metabolism of isolated fat cells. The similar inhibitory action of phospholipase C (*Clostridium perfringens alpha toxin*) and of insulin on lipolysis stimulated by lipolytic hormones and theophylline, *J. Biol. Chem. 241*, 140–142.
5. Zuk, P.A., Zhu, M, Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P. and Hedrick, M.H. (2002) Human adipose tissue is a source of multipotent stem cells, *Mol. Biol. Cell. 13*, 4279–4295.
6. Engfeldt, P., Arner, P. and Ostman, J. (1980) Influence of adipocyte isolation by collagenase on phosphodiesterase activity and lipolysis in man, *J. Lipid Res. 21*, 443–448.
7. Deslex, S., Negrel, R., Vannier, C., Etienne, J. and Ailhaud, G. (1987) Differentiation of human adipocyte precursors in a chemically defined serum-free medium, *Int. J. Obes. 11*, 19–27.
8. Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Ailhaud, G., Negrel, R. and Pfeiffer, E.F. (1989) Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium, *J. Clin. Invest. 84*, 1663–1670.
9. Mitchell, J.B., McIntosh, K., Zvonic, S., Garrett, S., Floyd, Z.E., Kloster, A., Halvorsen, Y.D., Storms, R.W., Goh, B., Kilroy, G.S., Wu, X. and Gimble, J.M. (2006) The immunophenotype of human adipose derived cells: temporal changes in stromal- and stem cell-associated markers, *Stem Cells 24*, 376–385.

Chapter 11

Evaluation of Cellular and Humoral Immune Responses to Allogeneic Adipose-Derived Stem/Stromal Cells

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Abstract

Adipose-derived mesenchymal stem or stromal cells (ASCs) are poised for clinical use in an allogeneic setting. Although ASCs have been shown to be nonimmunogenic by several laboratories, it is advisable for the investigator to confirm this for ASCs used in their studies due to variations in ASC production and the animal models in which they are used. We describe here the use of the mixed lymphocyte reaction (MLR) assay to determine immunogenicity and suppression by ASCs in vitro as well as assessing T cell responses to allogeneic ASC transplantation in vivo. A flow cytometry assay to determine serum antibody titer to transplanted ASCs is also described.

Key words: Adipose-derived stem cells, Immune response, Cellular immunity, Humoral immunity, Mixed lymphocyte reaction, Allogeneic, Immune suppression, Immunogenicity, T cell priming

1. Introduction

Mesenchymal stem or stromal cells (MSCs) derived from bone marrow (BMSCs) and adipose tissue (ASCs) are nonimmunogenic when cultured with allogeneic T cells and suppressive for T cells stimulated with alloantigens and mitogens (1–10). As a consequence of their immunomodulatory activity in vitro, it was expected that allogeneic MSCs could be transplanted into immunocompetent allogeneic recipients without fear of rejection. Indeed, studies have shown evidence of MSC survival in allogeneic hosts by tracking experiments and functional studies demonstrating T cell tolerance and/or suppression in vivo (11–22). Although humoral responses have been detected in transplanted recipients (16, 17, 23–25), antibodies were determined in some studies to be specific for fetal bovine serum (FBS) proteins, a component of

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the MSC culture medium (17, 23, 24). It should be noted that some studies have indicated immune sensitization to allogeneic BMSC transplantation (25–29). These disparate results may indicate differences between labs in cell preparation, animal models, and assay systems used to determine immune sensitization.

The ability of these and other factors to affect cell immunogenicity makes it incumbent upon the investigator to assess the immunologic properties of MSCs produced in *their* laboratories and with *their* model system. This chapter will describe use of the mixed lymphocyte reaction (MLR) assay to obtain information regarding the immunogenic and suppressive properties of ASCs derived from humans and animals. We will also describe how to use the MLR to measure T cell responses to transplanted allogeneic ASCs in recipient animals. Finally, measurement of humoral antibody responses to transplanted ASCs by flow cytometry will be described.

2. Materials

All basic sterile plasticware (centrifuge tubes, pipettes, pipette tips, etc.) can be purchased from general lab suppliers.

In describing materials and methods for MLR assays, it is assumed that the investigator has experience with cell handling and culture. All operations must be performed using sterile technique in a biosafety cabinet. Tissue and blood products from humans and animals are potentially infectious and must be treated according to OSHA regulations and Universal Precautions for preventing transmission of bloodborne pathogens. Due to space constraints, basic protocols for working with cells will not be described. The reader is encouraged to consult Current Protocols in Immunology (John Wiley & Sons) or other protocol-driven books and articles for further information.

2.1. Culture Medium

1. Remove 15.5 mL medium from a 500-mL bottle of Iscove's Modified Dulbecco's Medium (IMDM) containing 25 mM HEPES and L-glutamine and discard.
2. Add 5 mL each of 100× nonessential amino acids, 100× sodium pyruvate, and 100× antibiotic/antimycotic. Add 0.5 mL 1,000× 2-mercaptoethanol.
3. Supplemented media is stored at 4°C in the dark. Media is stable under these conditions for 1 month.
4. Culture medium for human cells is produced by adding 5 mL human AB serum (Pel-Freez Biologicals, Rogers, AK) to 95 mL supplemented IMDM (final 5% serum concentration). Culture medium for animal cells is produced by adding 5 mL

homologous serum to 95 mL supplemented IMDM (final 5% serum concentration). Culture medium is stable for 2 weeks when stored at 4°C in the dark.

2.2. Responder Cells

1. Obtain human peripheral blood or a leukopack and purify peripheral blood mononuclear cells (PBMCs) by centrifugation over Ficoll–Hypaque density gradient. For rodent experiments, responder cells can be derived from lymphoid organs (lymph nodes, spleen) by processing the tissues into a single-cell suspension. For large-animal studies, responder cells can be derived from peripheral blood as described for human samples, using an appropriate density gradient suitable for the species involved.
2. Determine the number and percentage of viable cells by diluting cells in 0.4% Trypan Blue and counting live and dead cells on a hemocytometer. Cell viability should be above 90% for human and large-animal cells and above 70% for rodent cells. Dead cells can be removed from cell populations exhibiting lower viabilities on species-appropriate density gradients.
3. Pellet PBMCs by centrifugation and resuspend in culture medium at a concentration of 4×10^6 viable cells/mL.
4. If purified T cells are desired as responder cells, use negative selection techniques to remove non-T cells. For human T cell purification, we use magnetic beads coated with anti-mouse IgG antibodies to remove non-T cells that have been treated with mouse monoclonal antibodies. The T cell population should be at least 85% pure by flow cytometry as assessed using a fluorochrome-tagged anti-CD3 mAb. If cells are to be used fresh, resuspend them at 2×10^6 cells/mL in culture medium.
5. Excess cells can be cryopreserved for future studies. We use a cryopreservation medium consisting of 70% IMDM, 20% FBS, and 10% DMSO (all final concentrations).

2.3. Stimulator Cells

1. Methods used for the preparation of unfractionated responder cells are used for the preparation of stimulator populations.
2. ASCs can be obtained from actively growing cultures or cryopreserved populations.
3. Stimulator cells must be mitotically inactivated prior to cell culture. We have used a cesium 137 source gamma irradiator and an X-ray machine for this purpose. Cells should be irradiated with approximately 5,000 rad by X-ray or gamma radiation. If this is not possible, cells can be treated with mitomycin C (10 μg/mL for 1–2 h at 37°C) followed by extensive washing to remove the chemical. Mitomycin C is toxic and care must be exercised in using this chemical as well as its disposal.

Expect high cell losses due to toxicity and extensive cell washing.

4. Excess cells can be cryopreserved for future studies (see Subheading 2.2, item 5, above).

2.4. MLR Assay

1. Flat-bottom, low-evaporation 96-well microtiter plates.
2. ^{3}H-thymidine; Code ART 0178, 5–10 Ci/mmol, 1 mCi/mL, in aqueous sterilized solution (American Radiolabeled Chemicals, Inc., St. Louis, MO). Stock ^{3}H-thymidine is diluted 1:50 in culture medium before use.
3. Filtermats, plastic bags, scintillation fluid.

2.5. Antibody Assay

1. Round-bottom, 5-mL polystyrene tubes suitable for use with FACSCalibur flow cytometer.
2. Wash buffer: Dulbecco's PBS (DPBS) containing 0.5% BSA and 0.1% sodium azide.
3. Fluorochrome-conjugated goat anti-rat IgG or IgM secondary antibody. We use R-Phycoerythrin (RPE)-labeled antibodies.
4. 1% Paraformaldehyde is prepared by diluting a stock solution of 20% paraformaldehyde in DPBS.

3. Methods

Four methods are described in this section. The first two methods describe use of the MLR assay to determine immunogenicity and suppression by ASCs in vitro. The third method describes use of the MLR to evaluate T cell priming in animals after allogeneic transplantation of ASCs. The final section describes the measurement of antibodies to ASCs in these animals using a flow cytometry method.

3.1. MLR Assay: Evaluation of ASC Immunogenicity

The MLR is a quantitative assay used to measure the proliferative response of T cells to an allogeneic stimulator population. This section describes the use of the one-way MLR assay to evaluate the immunogenicity of human or animal ASCs when cultured with allogeneic T cells. The one-way MLR assay consists of responder cells reacting to a mitotically inactivated population of stimulator cells. This ensures that any cell proliferation detected in the cultures is due to responder T cells. The magnitude of the T cell response should be instructive in predicting the immune response to the population of ASCs after transplantation of these cells to an immunocompetent recipient.

3.1.1. Protocol

1. A typical 96-well plate setup for an immunogenicity assay is shown in Fig. 1. To evaluate ASCs from outbred species

A	$Resp_A$ + $xPBMC_A$ (5K)	$Resp_A$ + $xPBMC_A$ (10K)	$Resp_A$ + $xPBMC_A$ (20K)
B	$Resp_A$ + $xPBMC_B$ (5K)	$Resp_A$ + $xPBMC_B$ (10K)	$Resp_A$ + $xPBMC_B$ (20K)
C	$Resp_A$ + $xASC_C$ (5K)	$Resp_A$ + $xASC_C$ (10K)	$Resp_A$ + $xASC_C$ (20K)
D	$Resp_D$ + $xPBMC_D$ (5K)	$Resp_D$ + $xPBMC_D$ (10K)	$Resp_D$ + $xPBMC_D$ (20K)
E	$Resp_D$ + $xPBMC_B$ (5K)	$Resp_D$ + $xPBMC_B$ (10K)	$Resp_D$ + $xPBMC_B$ (20K)
F	$Resp_D$ + $xASC_C$ (5K)	$Resp_D$ + $xASC_C$ (10K)	$Resp_D$ + $xASC_C$ (20K)
G	$Resp_A$ Alone	$Resp_D$ Alone	xASC Alone (5K)
H	xASC Alone (10K)	xASC Alone (20K)	Blank

Fig. 1. Template for immunogenicity assay. Layout is shown for a human immunogenicity assay cultured in a 96-well microtiter plate with groups plated in quadruplicate wells. Shown are responder cells from two different donors ($Resp_A$, $Resp_D$) cultured with irradiated (x) autologous xPBMCs (*rows A, D*), allogeneic xPBMCs from donor B (*rows B, E*) and allogeneic ASCs from donor C (*rows C, F*). All stimulator populations are plated at three densities: 5,000, 10,000, and 20,000 cells/well. Control cultures of responder cells and ASCs cultured alone are displayed in *rows G and H.*

(including humans), responder cells from two different donors are typically cultured with three different stimulator populations (1) autologous cells (provides baseline control response); (2) allogeneic cells (provides positive control response); and (3) the ASC test population. If ASCs from inbred strains of animals are being evaluated, syngeneic and allogeneic stimulator cells may be obtained from any animal of the responder and ASC donor strains, respectively, since animals within a strain should be genetically identical.

2. Either unfractionated cells or purified T cells can be used as responder cells in the MLR (see Note 1). Responder populations, prepared fresh or thawed from cryopreserved material, are washed and resuspended in culture medium at 4×10^6 unfractionated cells/mL or 2×10^6 T cells/mL. Cells are added to the assay at 100 µL/well corresponding to 400,000 and 200,000 cells/well, respectively.
3. Each of the three stimulator populations is titrated in the MLR to provide dose–response data. Inactivated stimulator cells, prepared fresh or thawed from cryopreserved material, are resuspended in culture medium at 2×10^5 cells/mL. Two doubling dilutions are made of each population in culture medium to give cell concentrations of 1×10^5 cells/mL and 0.5×10^5 cells/mL, respectively. All three stimulator populations are added to the plates at 100 µL/well, which corresponds to 2×10^4, 10^4, and 5×10^3 stimulator cells/well.
4. The final culture volume per well is 250 µL: 100 µL responder cells, 100 µL stimulator cells, and 50 µL complete medium. In control wells containing only one cell population, add 150 µL culture medium.

5. Incubate the MLR cultures at 37°C in a humidified atmosphere of 5% CO_2 for 4–6 days. The optimal culture period should be determined in pilot experiments by the investigator as there are significant differences between species and the use of fresh versus cryopreserved cells.
6. Add 50 µL ^{3}H-thymidine diluted 1:50 in culture medium to each well (1 µCi/well) and continue incubation at 37°C in a humidified atmosphere of 5% CO_2 for an additional 18 h. Radioactivity must be handled according to the rules and regulations stipulated by the laboratory in which these materials are used. If the use of radioisotopes is not an option for measuring T cell proliferation, spectrophotometric methods offered by commercial vendors may be used.
7. Harvest the cells for scintillation counting to determine the amount of incorporated isotope. We use a Skatron Micro96 Cell Harvester. The cells are harvested on filtermats (see Note 2), placed in plastic bags with scintillation fluid, sealed with a plate sealer, and loaded into a cassette. The cassette is placed into a scintillation and luminescence counter for quantification. The amount of radioactivity incorporated is proportional to the degree of proliferation by responder T cells in the wells and is an indication of ASC immunogenicity.
8. Data analysis.

   The response to autologous/syngeneic (auto/syn) stimulator cells provides a baseline response with which the responses against the test ASCs are compared. If the response to ASCs is significantly higher than the response to the auto/syn control cells, the stem cell population is considered immunogenic.

   Statistical analysis can be performed several ways. One method is to calculate an upper threshold response to auto/syn stimulator cells by adding two standard deviations to the mean cpm response. Any value above this threshold has a statistical probability of less than 5% of falling within this group and is considered statistically significant (68–95–99.7 or empirical rule). Alternatively, the investigator can use analysis of variance or the Student's *t* test to test the significance of the difference between the auto/syn group and the test group.

   The response to allogeneic cells must be significantly different ($p < 0.05\%$) from the auto/syn group or the assay is not valid. Using human cells, we typically find responses to auto/syn PBMCs to be less than 500 cpm and responses to allogeneic PBMCs to be greater than 20,000 cpm. Responses deviating significantly from these values suggest modifications to the assay (see Notes 3 and 4).

   Proliferative responses of the various cell populations cultured alone should be less than 500 cpm.

Examples of graphical expression of immunogenicity data can be found in our published studies (4, 6).

9. Interpretation of results.

   There are common-sense guidelines for interpretation of MLR data in predicting the immunogenicity of an ASC population in vivo. A stem cell population that generates a T cell proliferative response that is not significantly different from the baseline response is considered nonimmunogenic and would not be expected to generate a T cell response in an immunocompetent recipient. Conversely, a stem cell population that generates a vigorous response similar to the positive control is immunogenic and would be expected to be rejected after transplantation. Interpretation of the gray area in between both extremes depends on the magnitude of the MLR response and where it lies on the continuum between the baseline and positive control responses. Unfortunately, a threshold value does not exist that can predict a rejection response requiring implementation of appropriate in vivo studies to determine immunogenicity.

   Multiple responder cell donors are recommended for human studies and outbred animal studies if the relationship (HLA disparity) between the responder donor and ASC donor is not known to avoid misinterpretation of negative results. Although unlikely, it is possible that the lack of response to the ASC population may be due to HLA similarity between responder and stimulator populations and not to a nonimmunogenic profile of the ASC population. The use of multiple responder donors diminishes this possibility. We normally use at least two different responder populations in this situation.

3.2. MLR Assay: Evaluation of Suppression by ASCs

The purpose of this assay is to determine whether ASCs can suppress alloreactive T cell proliferation in MLR cultures. The evaluation of suppression in multiple MLR cultures is encouraged to more accurately gauge the magnitude and variability of suppression.

3.2.1. Protocol

1. A typical configuration for a suppression assay is depicted in Fig. 2. MLR cultures can be set up in either the one-way or two-way (no irradiation of cells) configuration in 96-well microtiter plates. The setup is shown for testing the suppression by one ASC stem cell lot on two different MLR combinations.
2. The two-way MLR configuration is the easiest to utilize for this assay since the cells do not need to be mitotically inactivated. Two populations of unfractionated cells containing T cells from different donors are suspended in culture medium

A	MLR1 + ASC (5K)	MLR1 + ASC (10K)	MLR1 + ASC (20K)
B	MLR2 + ASC (5K)	MLR2 + ASC (10K)	MLR2 + ASC (20K)
C	MLR1	MLR2	ASC (5K)
D	ASC (10K)	ASC (20K)	Blank
E	Blank	Blank	Blank
F	Blank	Blank	Blank
G	Blank	Blank	Blank
H	Blank	Blank	Blank

Fig. 2. Template for suppression assay. Layout is shown is for a human suppression assay cultured in a 96-well microtiter plate with groups plated in quadruplicate wells. Shown are two different MLR combinations; e.g., MLR1 = $PBMC_A$ + $PBMC_B$; MLR2 = $PBMC_C$ + $PBMC_D$. MLRs are cultured with various numbers of ASCs ranging from 5,000 to 20,000 cells/well. MLRs cultured without ASCs serve as the baseline control response to which suppression will be measured. ASCs are cultured alone to provide a control response check to ensure that these cells are not contributing significantly to the coculture response.

at 5×10^6 cells/mL and mixed together in equal volumes sufficient for plating. The mixed cells are delivered to the MLR microtiter plate at 100 µL/well (5×10^5 cells/well).

3. ASCs, prepared fresh or thawed from cryopreserved material, are resuspended in culture medium at 2×10^5 cells/mL. Two doubling dilutions are made of each population in culture medium to give cell concentrations of 1×10^5 cells/mL and 0.5×10^5 cells/mL, respectively. All three dilutions of ASCs are added to the plates at 100 µL/well, which corresponds to 2×10^4, 10^4, and 5×10^3 ASCs/well. Higher numbers of ASCs should not be utilized in this assay (see Note 5).
4. Cultures consisting of MLR cells with no ASCs added are required for baseline control responses. Additional controls consist of ASCs cultured alone.
5. The final culture volume per well is 250 µL: 100 µL MLR cells, 100 µL ASCs, and 50 µL complete medium. In wells containing MLR cells or ASCs alone, add 150 µL culture medium.
6. Cell culture, ^{3}H-thymidine pulse, and cell harvest are performed as described for the immunogenicity assay (Subheading 3.1, steps 5–7; see Note 2).
7. Data analysis and interpretation.

The MLR response (no cells added) serves as the baseline response to which all other responses are compared. This response should be vigorous in order to accurately assess suppression; if not, the assay should be repeated after the MLR is optimized (see Note 4). The two-tailed Student's *t* test can be used to assess whether a putative suppressor cell population significantly enhances or suppresses the MLR response.

Results are typically expressed as percent suppression, which is calculated by the formula $(1-[stem\ cell+MLR\ cpm \div by\ MLR\ cpm]) \times 100$. The amount of suppression of MLR cultures by ASCs is variable. A range of 30–70% suppression is expected at the highest dose of ASCs. Suppression should decrease with the number of ASCs/well (see Note 6).

Graphical expression of data can be found in published studies (4, 6).

3.3. MLR Assay: T Cell Priming in Animal Models

In preclinical animal studies, the investigator can use the MLR assay to determine whether treatment with allogeneic ASCs induced T cell priming in recipient animals. T cells derived from an immunized animal will produce an enhanced proliferative response of higher magnitude to stimulator cells obtained from the ASC donor relative to a nonimmunized baseline response. In experiments where rats or mice are used, control animals treated with vehicle (or untreated) are required to produce a baseline response. In large-animal studies, prebleeds performed prior to ASC treatment can be used to establish baseline responses. We have used this assay to determine recipient sensitization to BMSCs derived from rats (15) and baboons (16) as well as ASCs derived from rats (17).

Prior to embarking on animal studies in which an immunological evaluation will be performed, the investigator should perform pilot studies to determine whether the donor/recipient combination under consideration will result in a MLR response of reasonable magnitude and consistency. If such a response cannot be elicited after attempts to optimize the assay (see Note 4), the investigator should be prepared to change strains/breeds of animals used in the experiment.

3.3.1. Protocol

1. Responder cells. Lymphocytes to be used as responder cells are harvested from animals at designated time points determined by the investigator. In rat and mouse models, lymphoid tissues (lymph nodes, spleens) need to be procured as a source of responder cells; lymph nodes that drain the site of ASC implantation are best. In our experience, freshly harvested cells produce optimal responses in MLR assays – cryopreserved rodent cells perform poorly. Lymphoid organs shipped overnight in cold PBS have produced adequate

responses when processed upon receipt (17). For large-animal studies, a prebleed and multiple bleedings at 2–4 week intervals following stem cell treatment are sufficient to perform a study. PBMCs can be purified and cryopreserved for assessment by MLR when a sufficient number of samples have been collected. After thawing, responder cells are suspended in culture medium containing homologous serum at 10× culture concentration and added to the plates at 100 µL/well. Although we have had success using responder cells at 3×10^5 cells/well, optimal cell numbers should be determined by pilot experiments.

2. Stimulator cells. Cell populations to be used as stimulators in the MLR can be spleen or lymph node cells (rats/mice) or PBMCs (large animals) derived from donors of the stem cells. ASCs should not be used as stimulators in the MLR since they are immunosuppressive. In rodent studies using inbred strains of animals as ASC donors and recipients, stimulator cells can be obtained from any animal of the donor strain since they are genetically identical. However, in large-animal studies that typically use outbred species, stimulator PBMCs must be obtained from the same animals from which the stem cells were derived. These same rules apply to the procurement of auto/syn cells to be used as controls in the MLR assay. For this reason, it will be necessary to obtain one large prebleed (or multiple smaller bleeds) from stem cell donors in large-animal studies. Once processed and washed, stimulator cells are irradiated (5,000 rad) and suspended in culture medium containing homologous serum at 10× culture concentration and added to the plates at 100 µL/well. Although we have used stimulator cells at 2×10^5 cells/well, optimal cell numbers should be determined by pilot experiments.
3. Specificity controls. In some studies, the investigator may wish to determine whether an effect on the MLR response is antigen specific. To control for specificity, third-party stimulator cells that do not share MHC antigens with the donor stem cells can be employed in the MLR assay.
4. Mitogenic controls. In order to gauge the overall health of responder T cells from individual animals, the proliferative response to a T cell mitogen (Con A, PHA, anti-CD3 antibody) is employed. If the responder populations derived from individual rats have similar numbers of healthy T cells, their proliferative responses to mitogen stimulation should be similar. Low responses by individual animals could indicate that problems occurred prior to or during the assay.
5. A typical configuration for a T cell priming assay is shown in Fig. 3. The setup is shown for testing four rats treated with vehicle (baseline control) and four rats treated with allogeneic ASCs.

A	Rat#1 RC-V + Medium	Rat#1 RC-V + xSyn Spl	Rat#1 RC-V + xAllo Spl
B	Rat#2 RC-V + Medium	Rat#2 RC-V + xSyn Spl	Rat#2 RC-V + xAllo Spl
C	Rat#3 RC-V + Medium	Rat#3 RC-V + xSyn Spl	Rat#3 RC-V + xAllo Spl
D	Rat#4 RC-V + Medium	Rat#4 RC-V + xSyn Spl	Rat#4 RC-V + xAllo Spl
E	Rat#5 RC-ASC + Medium	Rat#5 RC-ASC + xSyn Spl	Rat#5 RC-ASC + xAllo Spl
F	Rat#6 RC-ASC + Medium	Rat#6 RC-ASC + xSyn Spl	Rat#6 RC-ASC + xAllo Spl
G	Rat#7 RC-ASC + Medium	Rat#7 RC-ASC + xSyn Spl	Rat#7 RC-ASC + xAllo Spl
H	Rat#8 RC-ASC + Medium	Rat#8 RC-ASC + xSyn Spl	Rat#8 RC-ASC + xAllo Spl

Fig. 3. Template for T cell priming assay. Layout is shown for a rat T cell priming assay cultured in a 96-well microtiter plate with groups plated in quadruplicate wells. Shown is a simplified experiment in which four rats treated with vehicle (Rats 1–4) are compared with four rats treated with allogeneic ASCs (Rats 5–8). In the first four rows, responder cells from vehicle-treated rats (RC-V) are cultured in medium alone, with irradiated syngeneic spleen cells (xSyn Spl) or with irradiated allogeneic spleen cells derived from the ASC donor strain (xAllo Spl). In the last four rows, responder cells from rats treated with allogeneic ASCs (RC-ASC) are cultured under the same conditions.

Responder lymph node cells from each rat are cultured in medium alone, with syngeneic spleen cells, or with allogeneic spleen cells matched to the ASC donor in quadruplicate cultures. Specificity and mitogen controls are not shown.

6. Identical plates can be set up for harvest at a second time point to assess kinetics of the MLR response – one series of plates is usually harvested at an early time point (e.g., 3 days) and the other series of plates is harvested at a later, typically optimal time point (e.g., 7 days) determined by pilot studies.
7. To set up the MLR cultures, responder and stimulator cells are each added to the microtiter plate at 100 µL/well. Culture medium is added to each well to give a final volume of 250 µL/well. Cell culture, ^{3}H-thymidine pulse, and cell harvest are performed as described for the immunogenicity assay (Subheading 3.1, steps 5–7) with the exception of mitogen-stimulated cultures. These should be pulsed with ^{3}H-thymidine on day 2 and harvested on day 3.
8. Data analysis.
   The means and standard deviations of quadruplicate cultures are calculated for each time point. For each rat, the background response to syngeneic spleen cells is subtracted from the response to allogeneic cells and expressed as ΔCPM (see Note 7). The mean ΔCPM responses are then averaged for each group of rats ± standard error of the mean. The vehicle control group's response to stimulation with allogeneic donor cells serves as the baseline response with which the allogeneic ASC group is compared. The significance of differences between groups can be determined using analysis of variance

or the two-tailed Student's *t* test. Graphical expression of data can be found in published studies (15–17).

The investigator may wish to determine the percentage of animals in the ASC-treated group that is significantly different from the baseline control group, particularly in circumstances in which the means between the groups are not statistically significant due to high variability of response between ASC-treated animals. A threshold response can be calculated for the vehicle control group equal to the group mean ± 2 standard deviations. Individual responses falling outside the threshold are considered significantly different ($p < 0.05$, 68–95–99.7 or empirical rule).

9. Interpretation.

   If animals treated with allogeneic ASCs were primed to donor alloantigens, they should produce a MLR response that is significantly greater in magnitude than the vehicle control response. In a well-primed recipient, T cell proliferation would be expected to exhibit accelerated kinetics showing peak response at the early time point. The response of cells derived from vehicle-treated control animals to stimulation with allogeneic donor cells should be highest at the later time point.

   If animals treated with allogeneic ASCs were tolerized to donor alloantigens, they should produce an MLR response that is significantly lower in magnitude than the vehicle control response. The response to third-party stimulator cells would need to be determined in order to claim that tolerance was antigen specific.

   The overall T cell response to stimulation with PHA, Con A, or anti-CD3 antibody should not be significantly different between groups.

3.4. Flow Cytometry Assay: Humoral Antibody Response in Animal Models

Treatment with allogeneic MSCs can induce a humoral antibody response in recipient animals (16, 17, 23–25). To measure these antibodies, serial dilutions of serum samples obtained from recipient animals are added to donor ASCs and primary antibody binding is detected by adding fluorochrome-labeled secondary antibodies. Secondary antibodies specific for primary immunoglobulin subclasses can be used to distinguish IgM from IgG isotypes. Flow cytometry is performed to determine the mean fluorescence of binding that will be used to calculate an antibody titer to the ASCs. Bleeds from control animals treated with vehicle (or untreated) or prebleeds from large animals are required to establish baseline responses. We have used this assay to determine antibody titers to allogeneic ASCs in rats (17) and BMSCs in baboons (16).

3.4.1. Protocol

1. Obtain blood samples from animals in the study. For small animals, obtain at least 0.5 mL blood; for large animals, 2–3 mL is sufficient. Do not forget to collect prebleeds in large-animal studies. Allow the blood to clot, collect serum, aliquot, and freeze at −20°C or colder.
2. Prepare two series of tenfold serial dilutions of serum in wash buffer to give 1:10 and 1:100 dilutions. Add 20 µL of undiluted or diluted rat serum to 5 mL round-bottom tubes as indicated in Table 1.
3. A positive control serum or antibody should be incorporated into the assay to ensure that the assay is working properly. Antibodies specific for donor alloantigens or ASC-specific markers are good candidates. Appropriate dilutions to be used for labeling ASCs can be obtained from the product literature or through pilot studies.
4. To prepare ASCs as assay target cells, wash them in wash buffer and resuspend them at 3×10^6 cells/mL. Aliquot 80 µL of cells (2.4×10^5 cells) to each 5-mL tube. Note that this increases serum dilution by a factor of 5 so "Undil" becomes 1:5, "1:10" becomes 1:50, and "1:100" becomes 1:500.
5. Incubate tubes on ice for 30 min, wash twice with wash buffer, and resuspend cells in 100 µL of wash buffer containing 10 µg/mL isotype-specific fluorochrome-conjugated goat secondary antibodies: either anti-IgM antibody (tubes 1 and 5, Table 1) or anti-IgG antibody (tubes 2–4 and 6–8, Table 1). Alternatively, fluorochrome-conjugated goat anti-rat immunoglobulin antibody can be used to detect all subclasses of humoral antibodies that bind to donor ASCs.
6. The suspensions are incubated in the dark on ice for 30 min, washed, and fixed in 200 µL of 1% paraformaldehyde for a minimum of 15 min prior to acquisition on the flow cytometer. A minimum of 10,000 events are acquired for flow cytometry analysis. Mean fluorescence intensities (MFI) can be determined for each serum sample using CellQuest software.
7. Data analysis.
   For each antibody isotype, group means and standard errors for MFI are calculated for control and treatment groups. The significance of differences between groups can be determined using analysis of variance or the two-tailed Student's *t* test. Graphical expression of data can be found in published studies (17).
   Group serum titer for each isotype can be determined by comparing control and treatment means at each serum dilution. Titer is defined as the highest dilution of treatment serum that produces a MFI significantly higher than control

Table 1
Sample dilutions for the antibody assay (two samples are shown)

Tube number	Sample donor number	Sample dilution	Sample dilution after addition of ASCs	Secondary anti-IgM antibody	Secondary anti-IgG antibody
1	1	Undil	1:5	X	
2	1	Undil	1:5		X
3	1	1:10	1:50		X
4	1	1:100	1:500		X
5	2	Undil	1:5	X	
6	2	Undil	1:5		X
7	2	1:10	1:50		X
8	2	1:100	1:500		X

Undil undiluted

serum. Further serum titrations may be required to determine antibody titer if there is a significant response above baseline for the single IgM determination or if all three IgG serum dilutions are significantly greater than baseline.

The investigator may wish to determine the percentage of animals in the ASC-treated group that is significantly different from the baseline control group, particularly in circumstances in which the means between the groups are not statistically significant due to high variability of response between ASC-treated animals. A threshold response can be calculated for the control group equal to the group MFI mean + 2 standard deviations. Individual MFI responses falling above the threshold are considered to be statistically significant ($p < 0.05$, see Subheading 3.1.1.8 for empirical rule). Individual serum titers can also be calculated using this method.

8. Interpretation.

   Titer provides the investigator with a measurement of IgM and IgG antibody activity specific for ASCs based on quantity of antibody molecules in serum as well as their affinity for ASCs. Titer measurements are useful for comparisons between treatment groups; however, there is not a titer threshold that determines whether a serum antibody titer is biologically significant.

   This assay does not discriminate between a response to alloantigens, a response to an ASC-specific antigen, and a response to components of the cell culture medium that may be present

on ASC membranes. Additional experiments are required to determine antibody specificity; e.g., using other cell types expressing HLA class I and class II molecules from the donor animal as target cells to examine alloantigen specificity (16). ELISA has been used to verify antibodies specific for FBS proteins in animals (17, 23) and humans (24) treated with BMSCs.

The functional activity of antibodies specific for ASCs is not measured by this assay. To evaluate whether the antibodies are cytotoxic for ASCs, for example, additional studies employing complement can be performed and the viability of cells determined (17).

4. Notes

1. The decision to use unfractionated cells or purified T cells as responder cells is determined by the investigator. Unfractionated cells represent the entire complement of immune cells (including macrophages) allowing indirect presentation of alloantigens to T cells. By contrast, the use of purified T cells tests the ability of the ASC population to behave as an antigen-presenting cell requiring both the presentation of alloantigens on MHC molecules and the expression of costimulatory molecules. From a purely technical point of view, purified T cells produce a cleaner response with lower backgrounds to auto/syn cells and higher responses to allogeneic cells. However, the use of unfractionated cells as responders is perhaps more relevant in terms of replicating an in vivo response. In our experience, unfractionated human PBMCs produced a higher response to allogeneic BMSCs than purified T cells derived from the same donor (4).
2. Cocultures of T cells and ASCs may be difficult to harvest from 96-well microtiter plates due to the deposition of matrix in the wells, which can clog the filtermats and cause overflow of wells when the cell harvester is in "automatic" mode. To allow controlled harvest of cultures, we use the cell harvester in "manual" mode, being careful to aspirate all wells before delivering the next round of wash water.
3. High background responses (greater than 1,000 cpm) by responder cells cultured alone or cultured with auto/syn cells may be indicative of a mitogenic or antigenic component in the culture medium; e.g., FBS. If homologous serum was used, screening of other lots of serum is suggested. Long culture periods may also result in high background responses. The investigator should determine whether shorter culture periods can be used.

4. Low positive control responses to allogeneic cells (less than 10,000 cpm) may be increased by using different combinations of donors of responder and stimulator cells, shifting responder-to-stimulator cell ratios, increasing number of cells/well, changing serum used in the culture medium to a different lot or using FBS (may increase background), adjusting culture length, or using purified T cells as responder cells.
5. A major pitfall of this assay is distinguishing suppression from cell crowding effects. To diminish the effect of cell crowding, we limit the upper number of stem cells added per well to the number that results in cell confluence (approximately 2×10^4 cells/well for ASCs). The inclusion of a nonsuppressive control cell population in the assay can be used to determine nonspecific inhibition at various densities (6).
6. The ASC population should be assessed for Mycoplasma contamination if suppression does not decrease as the ASCs are diluted in the assay. Mycoplasma can interfere with ^{3}H-thymidine uptake by T cells thus providing a false indication of suppression.
7. Two background controls are performed in this assay: responder cells cultured in medium and with syngeneic cells. The rationale for two control groups is that responder cells often produce a larger response when cultured with syngeneic stimulators than in medium alone. A significant difference between control groups within certain animal treatment groups may alert the investigator to immune responses against antigens other than those expressed by ASCs. Whether this is due to a property of the syngeneic cells or to a possible autoimmune response is not currently clear.

References

1. Di Nicola, M., Carlo-Stella, C., Magni, M., Milanesi, M., Longoni, P.D., Matteucci, P., Grisanti, S., and Gianni, A.M. (2002) Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* **99**, 3838–43.
2. Le Blanc, K., Tammik, L., Sundberg, B., Haynesworth, S.E., and Ringden, O. (2003) Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol* **57**, 11–20.
3. Tse, W.T., Pendleton, J.D., Bever, W.M., Egalka, M.C., and Guinan, E.C. (2003) Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* **75**, 389–97.
4. Klyushnenkova, E., Mosca, J.D., Zernetkina, V., Majumdar, M.K., Beggs, K.J., Simonetti, D.W., Deans, R.J., and McIntosh, K.R. (2005) T cell responses to allogeneic human mesenchymal stem cells: immunogenicity tolerance, and suppression. *J Biomed Sci* **12**, 47–57.
5. Puissant, B., Barreau, C., Bourin, P., Clavel, C., Corre, J., Bousquet, C., Taureau, C., Cousin, B., Abbal, M., Laharrague, P., Penicaud, L., Casteilla, L., and Blancher, A. (2005) Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. *Br J Haematol* **129**, 118–29.

6. McIntosh, K., Zvonic, S., Garrett, S., Mitchell, J.B., Floyd, Z.E., Hammill, L., Kloster, A., Di Halvorsen, Y., Ting, J.P., Storms, R.W., Goh, B., Kilroy, G., Wu, X., and Gimble, J.M. (2006) The immunogenicity of human adipose derived cells: temporal changes in vitro. *Stem Cells* **24**, 1246–53.
7. Hoogduijn, M.J., Crop, M.J., Peeters, A.M.A., Van Osch, G.J.V.M., Balk, A.H.M.M., Ijzermans, J.N.M., Weimar, W., and Baan, C.C. (2007) Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. *Stem Cells Dev* **16**, 597–604.
8. Niemeyer, P., Kornacker, M., Melhorn, A., Seckinger, A., Vohrer, J., Schmal, H., Kasten, P., Eckstein, V., Soudkamp, N.P., and Krause, U. (2007) Comparison of immunological properties of bone marrow stromal cells and adipose tissue-derived stem cells before and after osteogenic differentiation in vitro. *Tissue Eng Part A* **13**, 111–21.
9. Wolbank, S., Peterbauer, A., Fahrner, M., Hennerbichler, S., van Griensven, M., Stadler, G., Redl, H., and Gabriel, C. (2007) Dose-dependent immunomodulatory effect of human stem cells from amniotic membrane: a comparison with human mesenchymal stem cells from adipose tissue. *Tissue Eng Part A* **13**, 1173–83.
10. Cui, L., Yin, S., Liu, W., Li, N., Zhang, W., and Cao, Y. (2007) Expanded adipose-derived stem cells suppress mixed lymphocyte reaction by secretion of prostaglandin E2. *Tissue Eng Part A* **13**, 1185–95.
11. Dai, W., Hale, S.L., Martin, B.J., Kuang, J.-Q., Dow, J.S., Wold, L.E., and Kloner, R.A. (2005) Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: short- and long-term effects. *Circulation* **112**, 214–23.
12. Gal, P., Necas, A., Planka, L., Kecova, H., Kren, L., Krupa, P., Hlucilova, J., Usvald, D. (2007) Chondrogenic potential of allogeneic mesenchymal stem cells transplanted without immunosuppression to regenerate physeal defect in rabbits. *Acta Vet Brno* **76**, 265–75.
13. Devine, S.M., Cobbs, C., Jennings, M., Bartholomew, A., Hoffman, R. (2003) Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood* **101**, 2999–3001.
14. Le Blanc, K., Frassoni, F., Ball, L., Locatelli, F., Roelofs, H., Lewis, I., Lanino, E., Sundberg, B., Bernardo, M.E., Remberger, M., Dini, G., Egeler, R.M., Bacigalupo, A., Fibbe, W., and Ringdén, O. (2008) Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* **371**, 1579–86.
15. Li, Y., McIntosh, K., Chen, J., Zhang, C., Gao, Q., Borneman, J., Raginski, K., Mitchell, J., Shen, L., Zhang, J., Lu, D., and Chopp, M. (2006) Allogeneic bone marrow stromal cells promote glial-axonal remodeling without immunologic sensitization after stroke in rats. *Exp Neurol* **198**, 313–25.
16. Beggs, K.J., Lyubimov, A., Borneman, J.N., Bartholomew, A., Moseley, A., Dodds, R., Archambault, M.P., Smith, A.K., and McIntosh, K.R. (2006) Immunologic consequences of multiple high-dose administration of allogeneic mesenchymal stem cells to baboons. *Cell Transplant* **15**, 711–21.
17. McIntosh, K.R., Lopez, M.J., Borneman, J.N., Spencer, N.D., Anderson, P., and Gimble, J.M. (2009) Immunogenicity of allogeneic adipose-derived stem cells in a rat spinal fusion model. *Tissue Eng Part A* **15**, 2677–86.
18. Zappia, E., Casazza, S., Pedemonte, E., Benvenuto, F., Bonanni, I., Gerdoni, E., Giunti, D., Ceravolo, A., Cazzanti, F., Frassoni, F., Mancardi, G., and Uccelli, A. (2005) Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T cell anergy. *Blood* **106**,1755–61.
19. Zhou, H.P., Yi, D.H., Yu, S.Q., Sun, G.C., Cui, Q., Zhu, H.L., Liu, J.C., Zhang, J.Z., and Wu, T.J. (2006) Administration of donor-derived mesenchymal stem cells can prolong the survival of rat cardiac allograft. *Transplant Proc* **38**, 3046–51.
20. Itakura, S., Asari, S., Rawson, J., Ito, T., Todorov, I., Liu, C.-P., Sasaki, N., Kandeel, F., and Mullen, Y. (2007) Mesenchymal stem cells facilitate the induction of mixed hematopoietic chimerism and islet allograft tolerance without GVHD in the rat. *Am J Transplant* **7**, 336–46.
21. Gerdoni, E., Gallo, B., Casazza, S., Musio, S., Bonanni, I., Pedemonte, E., Mantegazza, R., Frassoni, F., Mancardi, G., Pedotti, R., and Uccelli, A. (2007) Mesenchymal stem cells effectively modulate pathogenic immune response in experimental autoimmune encephalomyelitis. *Transplantation* **83**, 783–90.
22. Augello, A., Tasso, R., Negrini, S.M., Cancedda, R., and Pennesi, G. (2007) Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis. *Arthritis Rheum* **56**, 1175–86.

23. Spees, J.L., Gregory, C.A., Singh, H., Tucker, H.A., Peister, A., Lynch, P.J., Hsu, S.C., Smith, J., and Prockop, D.J. (2004) Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. *Mol Ther* **9**, 747–56.
24. Sundin, M., Ringdén, O., Sundberg, B., Nava, S., Götherstrom, C., and Le Blanc, K. (2007) No alloantibodies against mesenchymal stromal cells, but presence of anti-fetal calf serum antibodies, after transplantation in allogeneic hematopoietic stem cell recipients. *Haematologica* **92**, 1208–15.
25. Poncelet, A.J., Vercruysse, J., Saliez, A., and Gianello, P. (2007) Although pig allogeneic mesenchymal stem cells are not immunogenic in vitro, intracardiac injection elicits an immune response in vivo. *Transplantation* **83**, 783–90.
26. Wang, Y., Chen, X., Armstrong, M.A., and Li, G. (2007) Survival of bone marrow-derived mesenchymal stem cells in a xenotransplantation model. *J Orthop Res* **25**, 926–32.
27. Nauta, A.J., Westerhuis, G., Kruisselbrink, A.W., Lurvink, E.G.A., Willemze, R., and Fibbe, W.E. (2006) Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloblative setting. *Blood* **108**, 2114–20.
28. Eliopoulos, N., Stagg, J., Lejeune, L., Pommey, S., and Galipeau, J. (2005) Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. *Blood* **106**, 4057–65.
29. Tolar, J., O'Shaughnessy, M.J., Panoskaltsis-Mortari, A., McElmurry, R.T., Bell, S., Riddle, M., McIvor, R.S., Yant, S.R., Kay, M.A., Krause, D., Verfaille, C.M., and Blazar, B.R. (2006) Host factors that impact the biodistribution and persistence of multipotent adult progenitor cells. *Blood* **107**, 4182–8.

Chapter 12

Adipose-Derived Stromal Cells: Cytokine Expression and Immune Cell Contaminants

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Abstract

The present method describes an immunoselection/depletion approach to isolate the native human adipose tissue-derived progenitor cells that are free from endothelial cells and immune cells by the use of magnetic nanobeads and microbeads coupled to antibodies. Moreover, methods to isolate and to analyse the distinct cell populations that constitute the microenvironment of the human adipose tissue progenitor cells, i.e. mature adipocytes, endothelial cells, and macrophages, are mentioned.

Key words: Adipose tissue, Obesity, Growth factors, Inflammation, Flow cytometry, Real-time PCR, Immunoselection

1. Introduction

Human adipose tissue is composed of adipocytes that are the metabolic active cells and a stromal vascular fraction (SVF) that contains several cell populations. The culture and the in vitro expansion of the adherent cells present in the SVF, defined as adipose stromal cells (ASCs) showed that such cells display mesenchymal stromal cell-like properties that have extensive proliferative capacity associated with adipogenic, chondrogenic and osteogenic potentials (1). Moreover, several approaches have demonstrated that they exhibit in vivo the capacity to promote the neovascularization of ischemic tissues (2–4). Such proangiogenic effects were thought to originate from the endothelial cell differentiation of the ASCs and/or through paracrine effects via the secretion of proangiogenic factors and cytokines. Therefore,

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human ASCs represent a source of adult stem/progenitor cells of therapeutic interest in the field of regenerative medicine.

The high number of stromal cells within the freshly isolated SVF is compatible with the use of non-expanded isolated native cells. However, the native SVF of the human adipose tissue is composed of distinct cell subsets. Indeed, based on several cell surface antigen expression and flow cytometry analyses, several cell populations are identified: the CD34+/CD31+ endothelial cells, the CD34+/CD31– progenitor cells (2, 5), the CD14+ myeloid cells (6) and cells negative for CD34, CD31 and CD14 (mainly mast cells, lymphocytes and minor cell populations). This chapter describes methods for isolating and analysing native purified human adipose tissue progenitor cells free from immune cell and endothelial cell contaminants by the use of magnetic nanobeads and microbeads associated with flow cytometry and real-time PCR analyses. Moreover, such a protocol allows one to isolate and to characterize the other cell types present in the microenvironment of the progenitor cells (mature adipocytes, endothelial cells and immune cells) and therefore to analyse the potential impact of such a microenvironment on the human adipose tissue native progenitor cells.

2. Materials

2.1. Isolation of the Stromal Vascular Fraction

1. Phosphate-buffered saline (PBS): without $MgCl_2$ and $CaCl_2$.
2. Bovine serum albumin (BSA): 7.5% sterile solution and free fatty acid fraction V.
3. Collagenase type 1: 250 U/mL in PBS with 2% BSA. pH is adjusted to 7.4 and the solution sterilized with 0.22-μm filters, and then kept at –20°C in 50-mL Falcon tube aliquots.
4. DNAse 1 from lyophilized bovine pancreas (sterile): reconstituted at 10 mg/mL in sterile water (stock solution) and used at a final concentration of 10 μg/mL.
5. Krebs Ringer Bicarbonate Buffer Hepes (KRBHA): 1.8 g/L glucose and without $CaCl_2$ and $NaCO_3$, supplemented with 0.1% free fatty acid BSA.
6. Endothelial Cells Basal Medium (ECBM): supplemented with penicillin and streptomycin 100× penicillin/streptomycin solution.
7. Fœtal calf serum (FCS).
8. 100 mM sterile-filtered ethylene diamine tetra acetic acid (EDTA).

9. Erythrocyte Lysis Buffer (ELB): 155 mM NH_4Cl, 0.1 mM EDTA, 5.7 mM K_2HPO_4 at pH 7.3 and sterilized by 0.22-μm filtering.
10. Trypan blue solution.

2.2. Cell Isolation

1. Easysep buffer: PBS, 2 mM EDTA, 2% FCS.
2. Human CD34, CD14 and CD3 Easysep Selection Kit.
3. Stem cell magnet.
4. Human CD31 microbeads, Dynal.
5. Dynal Eppendorf tube magnet.
6. Dynal Buffer: PBS, 0.1% BSA.

2.3. Flow Cytometry Analyses

1. FACS buffer: PBS, 0.5% BSA, 2 mM EDTA.
2. Fluorescent-labeled antibodies isotype control.
3. Fluorescent-labeled antibodies.

2.4. Real-time PCR Analyses

1. Rneasy Mini Kit.
2. RNA lysis buffer (RLT/DTT): supplemented with 2.5 mM dithiothreitol.
3. Qiazol lysis reagent.
4. SuperScript II Reverse Transcriptase.
5. 5× First-strand buffer.
6. 10 mM dNTP solution.
7. 500 μg/mL Random primers.
8. 2× TaqMan Universal PCR Master Mix.
9. 96-well reaction plates.
10. TaqMan probes.

3. Methods

3.1. Collection and Preparation of the Human Adipose Tissue

Cells from the stromal vascular fraction (SVF) can be isolated from lipoaspirate or dermolipectomy from several anatomical locations (see Notes 1 and 2). The preparation and isolation are performed under sterile conditions.

For lipoaspirate, ~200 mL of lipoaspirate is transferred on a plastic sieve. The tissue fragments are washed several times with PBS until an erythrocyte-free eluate is obtained. The tissue is then transferred into a 1,000-mL sterile culture flask. For dermolipectomy, the lobules of adipose tissue, dissected from blood vessels and extracellular matrix, are transferred into several 50-mL Falcon tubes and roughly minced with scissors. The minced tissue pieces (200 mL) are then transferred into a 1,000-mL sterile culture flask.

3.2. Isolation of the Stromal-Vascular Fraction

1. Add one volume (200 mL) of the pre-warmed collagenase solution supplemented with DNAse 1 (Fig. 1) (see Notes 3 and 4).
2. Resuspend the adipose tissue by shaking the flask.
3. Incubate in a shaking water bath at 37°C under rapid shaking (200 rpm) for 30 min for lipoaspirate or for 60 min for dermolipectomy.
4. Filter the digested tissue on a sterile nylon sieve (250-µm mesh) to remove undigested conjunctive tissue, rinse the sieve with PBS and spin the filtrate for 30 s at 100 × *g* at room temperature. The floating mature human adipocytes are carefully removed with a 50-mL pipette and washed in a 50-mL Falcon tube at 37°C three times with KRBHA volume to volume.
5. The remaining digestion medium is centrifuged for 10 min at 300 × *g* at room temperature.
6. Discard the supernatant.
7. Resuspend the pellet in nine volumes of ELB pre-warmed at 37°C and homogenize for 5 min by pipeting up and down.
8. Filter the cell suspension on successively 100 µm, 70 µm and 40 µm cell strainers.
9. Centrifuge for 10 min at 250 × *g* at room temperature and re-suspend the pellet in 10 mL Easy Sep Buffer.

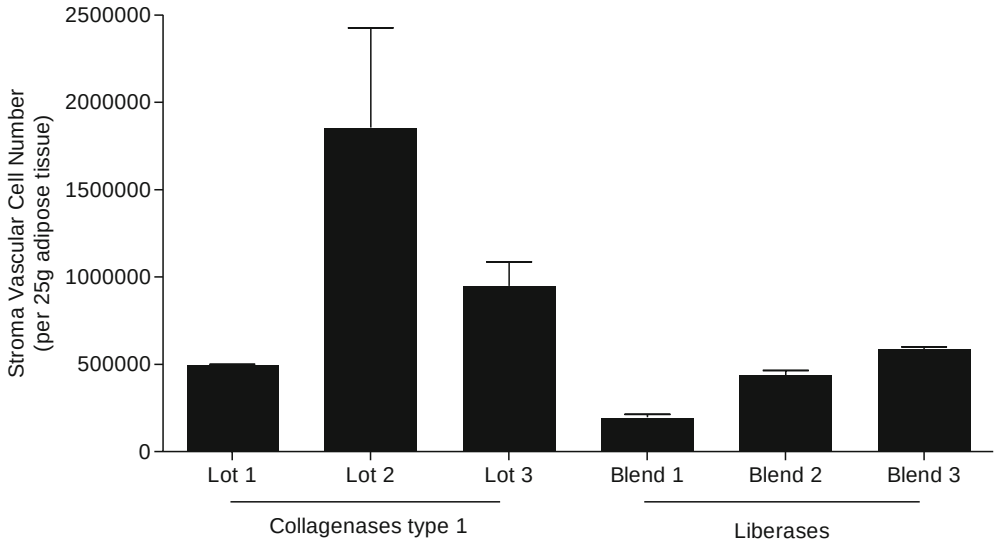

Fig. 1. The human adipose tissues (25 g) obtained from plastic surgery (lipoaspirate) are digested under constant shaking with 25 mL of distinct batches and blends of collagenase or liberase (250 U/mL PBS/2% BSA) for 45 min. After centrifugation, the number of viable stromal vascular cells is calculated with Trypan blue staining. The results are means ± SEM of cell numbers obtained with two distinct tissues.

10. Centrifuge for 10 min at 250 × *g* at 4°C to eliminate the ELB residual and re-suspend the pellet in 1 mL cold Easy Sep Buffer (see Notes 5 and 6).
11. Count the number of viable cells with Trypan blue staining (exclusion method).
12. Perform flow cytometry analyses to determine the immune cells contaminants present in the SVF (Fig. 2).

3.3. Flow Cytometry Analyses of the SVF

1. Prepare the stromal vascular cells at a concentration of 10^5 cells in 100 µL FACS buffer.
2. Pipet 5 µL of each fluorescent-labeled isotype control in one tube (FITC, PE, PerCP) (see Notes 7 and 8).
3. Pipet 10 µL of the specific fluorescent-labeled antibodies in another tube.
4. Add 100 µL of the stromal vascular cell solution to each tube.
5. Incubate 30 min on ice in the dark.
6. Wash with 2 mL of PBS.
7. Centrifuge at 300 × *g*, 10 min at 4°C.
8. Discard the supernatant.
9. Resuspend the cell pellet in 0.5 mL of PBS.
10. Perform the analysis with the flow cytometer and calculate the percentage of immune cell contaminants within the SVF.

3.4. Isolation of the CD34+ Cells from the SVF

1. Start with a maximum of 4×10^7 SVF cells in 1 mL of Easysep buffer (see Note 9).

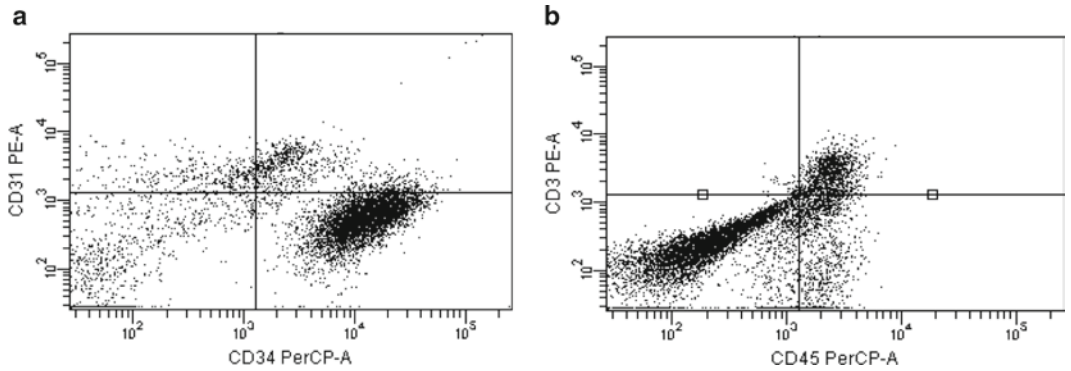

Fig. 2. Representative dot plots of the SVF of the human adipose tissue. (**a**) Dot plot obtained with the antibodies anti-CD34–PerCP and anti-CD31–PE showing in the lower right quadrant the progenitor cells CD34+/CD31– and in the upper right quadrant the endothelial cells CD34+/CD31+. (**b**) Dot plot obtained with the antibodies anti-CD3–PE and anti-CD45–PerCP showing the immune cells in both right quadrant.

2. Add 200 µL of CD34 Easysep Positive selection cocktail. Mix well by pipetting up and down and incubate for 15 min at room temperature with an intermediate mix every 5 min.
3. Add 100 µL of nanobeads, mix gently, avoiding bubbles, and incubate for 10 min at room temperature with intermediate mix every 5 min.
4. Separate the solution into two tubes (650 µL in each tube) and bring the volume up to 2.5 mL with Easysep buffer.
5. Introduce the tubes into the Easysep magnets for 5 min.
6. Collect the CD34-negative cell fraction by keeping the 50-mL Falcon tube within the magnet and turning it upside down to drain off the supernatant into a fresh tube.
7. Remove the tube from the magnet and wash with 2.5 mL Easysep buffer.
8. Homogenize by pipetting up and down.
9. Repeat step 8 four times.
10. Centrifuge the CD34+ and the CD34– cell fraction at 300 × *g* for 10 min at 4°C.
11. Count the number of viable cells with Trypan blue staining.
12. Re-suspend the CD34+ cells in 1 mL of Dynal buffer for the depletion of the CD31+ cells.

3.5. Depletion of the CD31+ Endothelial Cells Present in the CD34+ Cells

1. Transfer the CD34+ cell fraction into a 15-mL Falcon tube.
2. Add 100 µL of prewashed CD31 microbeads (see Notes 10 and 11).
3. Incubate at 4°C under constant shaking for 20 min.
4. Bring the volume up to 12 mL with Dynal buffer, homogenize and apply 1 mL of the cell suspension in each Eppendorf tube (12) placed in front of two Dynal Eppendorf tube magnets.
5. Incubate for 1 min.
6. Carefully collect (avoid touching the beads) the supernatant from each tube into a 50-mL Falcon tube.
7. Wash the Eppendorf tubes again with 1 mL of cold Dynal buffer.
8. Place the Eppendorf tubes in front of the magnet and incubate for 1 min.
9. Carefully collect the supernatants into the 50-mL Falcon tube.
10. Centrifuge the Falcon tube containing the CD34+/CD31– progenitor cells at 300 × *g* for 10 min at 4°C and re-suspend the cell pellet in ECBM/0.1% BSA before counting (see Note 12).

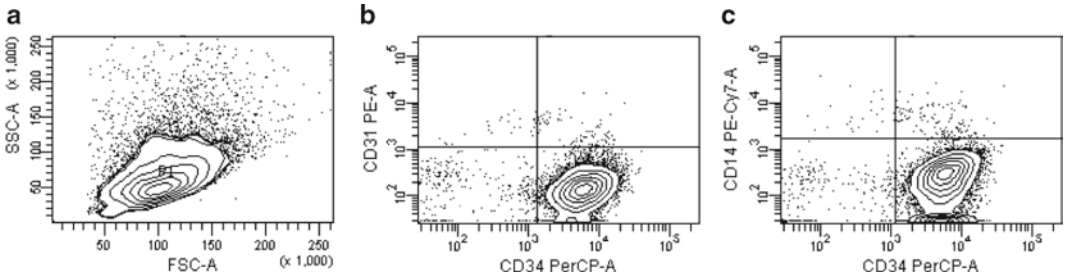

Fig. 3. Representative density plots of the isolated progenitors cells obtained by flow cytometry analyses. (**a**) Density plot of the CD34+CD31- size (FSC) and granularity (SSC). (**b**) Density plot using CD34-PerCP and CD31-PE. (**c**) Density plot using CD34-PerCP and CD14-PE-Cy7 (*gray line*: isotype control; *black line*: specific antibody).

11. Perform flow cytometry analyses to determine the purity of the CD34+/CD31– cell fraction (Fig. 3) (see Note 13).

3.6. Isolation of the CD14+ Cells from the CD34– Cells

1. Prepare the CD34– cell fraction at a concentration lower than 10^8 cells/mL in Easysep buffer.
2. Add 100 µL of the CD14+ Easysep Positive selection cocktail. Mix well by pipetting up and down and incubate for 15 min at room temperature with an intermediate mix every 5 min.
3. Add 50 µL of nanobeads, mix gently, avoiding bubbles, and incubate for 10 min at room temperature with an intermediate mix every 5 min.
4. Bring the volume up to 2.5 mL with Easysep buffer.
5. Introduce the tube into the Easysep magnet for 5 min.
6. Collect the CD14-negative cell fraction by keeping the 50-mL Falcon tube within the magnet and turning it upside down to drain off the supernatant into a fresh tube.
7. Remove the tube from the magnet and wash with 2.5 mL Easysep buffer.
8. Homogenize by pipetting up and down.
9. Repeat step 8 four times.
10. Centrifuge the CD14+ and the CD14– cell fraction at $300 \times g$ for 10 min at 4°C.
11. Count the number of viable cells with Trypan blue staining.

3.7. Real-time PCR Analyses

Add 350 µL RLT/DTT to 5×10^5 cells and perform the RNA extraction in the RNeasy Mini Spin column as described in the kit instructions (see Note 14).

3.7.1. RNA Extraction of the SVF Cell Population

3.7.2. RNA Extraction of Mature Adipocytes

1. Freeze mature adipocytes in Qiazol reagent (volume to volume) at –80°C.
2. Allow the adipocyte/Qiazol solution to thaw at 37°C.

3. Vortex vigorously for 15–20 s.
4. Incubate the sample at room temperature for 5–10 min.
5. Centrifuge at 5,000 × *g* for 1 min.
6. Discard the lipid upper phase.
7. Add one volume of chloroform (for five volumes of the remaining phase) to extract the remaining lipids and vortex vigorously for 15 s.
8. Incubate the tube at room temperature for 2–3 min.
9. Centrifuge at 5,000 × *g* for 10 min.
10. Transfer the upper aqueous phase to a new tube and add one volume of 70% ethanol and mix thoroughly by vortexing.
11. Perform the RNA extraction in the RNeasy Mini Spin column as described in the kit.

3.7.3. Real-time PCR Analysis

1. Add to 0.5 µg total RNA, in 1× first-strand buffer, 200 U superScript II Reverse Transcriptase, 0.5 mM DNTP, 10 mM DTT, and 50 ng of the Random Primers, in a final volume of 20 µL.
2. Incubate for 10 min at 25°C, followed by 50 min at 42°C.
3. Stop the reaction by heating for 15 min at 70°C. The cDNA obtained is chilled on ice (if used the same day) or stored at –20°C until use.
4. Perform the amplification reaction in duplicate on 15 ng (5 µL) of the cDNA samples using 1× TaqMan Universal PCR Master Mix and the TaqMan probes for cytokines in a final volume of 20 µL in 96-well reaction plates in a GeneAmp 7500 detection system under the conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min (see Note 15) (Fig. 4).

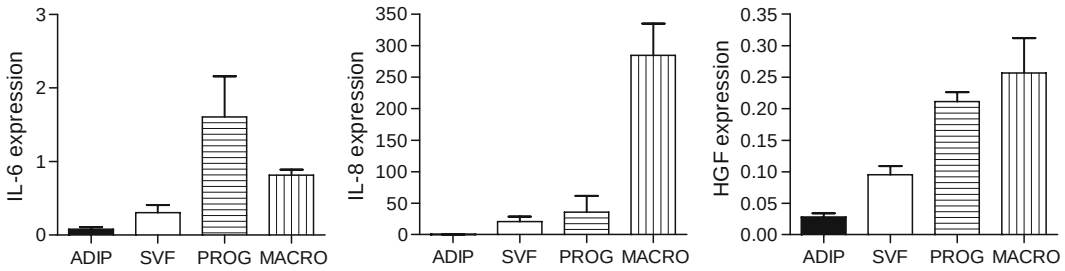

Fig. 4. Real-time PCR analysis of various cytokines and factors (interleukin (IL)-6, IL-8 and hepatocyte growth factor (HGF)) performed on the native human mature adipocytes (ADIP), cells from the SVF (SVF), isolated progenitor cells CD34+/CD31– (PROG) and macrophages (MACRO) from the human adipose tissues. Values are means ± SEM normalized to 18 S RNA expression from at least five distinct cell preparations.

4. Notes

1. Visceral human adipose tissue contains more immune cells than subcutaneous adipose tissue (7).
2. The percentage of immune cells contaminants within the SVF increases with the body mass index of the patients (6–8).
3. Traditional collagenase is a crude and variable fermentation by-product of *Clostridium histolyticum*. Liberase enzymes are blends of purified enzymes. In order to get reproducible results, the same lots of collagenase or the same type of liberase must be used. Figure 1 shows the yields of viable stromal vascular cells obtained from the same amount of adipose tissue digested in distinct collagenases lots and liberase blends with the same activity.
4. DNase is necessary to disrupt the DNA that is released as a consequence of tissue digestion, which would otherwise result in a non-homogeneous and gelatinous cell suspension that is difficult to process further.
5. If all of the extraction protocol cannot be performed in the same day, the SVF can be kept overnight under constant shaking at 4°C in ECBM supplemented with 0.1% BSA and 2 mM EDTA.
6. Stromal vascular cells can be frozen in liquid nitrogen. We obtained a good viability of the cells when the SVF was frozen in ECBM supplemented with 45% FCS and 10% dimethylsulfoxide (DMSO) (81 ± 5% cell recovery compared with freshly harvested cells, n = 3 distinct tissues). Moreover, the flow cytometry analyses performed on cryopreserved and freshly harvested stromal vascular cells showed that the cryopreservation led to an enrichment of the SVF in the CD34+ cells together with a reduction in the CD14+ cells (1.4-fold more CD34+ and 0.7-fold less CD14+ cells in the cryopreserved compared with freshly harvested SVF, $n = 3$ tissues).
7. The cells from the SVF are highly auto-fluorescent. Moreover, due to the heterogeneity in the cell populations, they exhibit high heterogeneity in size (Forward Scatter) and granularity (Side Scatter). It is thus necessary to carefully set up the cytometer settings and to perform the compensation steps prior to begining multiple labeling.
8. The choice of antibodies for flow cytometry analyses must consider several points. Due to the high autofluorescence in the FL1 channel, FITC-labeled antibodies must be chosen for cell surface markers that are expressed at high levels. The clones for the CD34 and CD14 antibodies used for the FACS analysis must be distinct from the ones used for the cell immunoselection.

9. It is important to keep all buffers at 4°C to avoid non-specific labeling and cell contamination.
10. Since the Dynal CD31 beads are microbeads, they are attracted by a lower magnetic field than the Easysep CD34 nanobeads. Therefore, using the Dynal magnet adapted for microbeads, it is possible to selectively attract the cells that are both covered by the CD31 microbeads and the CD34 nanobeads but not the cells covered only by the CD34 nanobeads.
11. Before use, beads must be washed three times (on the magnet) in Dynal buffer (1 mL of buffer for 100 µL of beads) to remove residual ethanol.
12. To eliminate contaminant beads, replace the progenitor cells suspension on the magnet.
13. When the aim of the study is to obtain pure human adipose tissue progenitor cells free from endothelial cells and immune cell contaminants, the isolation protocol may stop at this step.
14. When starting with 5×10^5 cells; we usually obtained 0.1 µg/µL total RNA.
15. For the ribosomal RNA control (18 S rRNA), the PCR mixture contained 5 µL of 4× primers and fluorogenic probe mix (Applied Biosystems) and 10 µL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems).

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References

1. Gimble J, Guilak F. (2003) Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* 5:362–369.
2. Miranville A, Heeschen C, Sengenes C, Curat CA, Busse R, Bouloumie A. (2004) Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation* 110:349–355.
3. Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Penicaud L, Casteilla L. (2004) Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. *Circulation* 109:656–663.
4. Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, Pell CL, Johnstone BH, Considine RV, March KL. (2004) Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 109:1292–1298.
5. Sengenes C, Lolmede K, Zakaroff-Girard A, Busse R, Bouloumie A. (2005) Preadipocytes in the human subcutaneous adipose tissue display distinct features from the adult mesenchymal and hematopoietic stem cells. *J Cell Physiol* 205:114–122.
6. Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R, Bouloumie A. (2004) From blood monocytes to adipose tissue-resident

macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* 53:1285–1292.

7. Duffaut C, Zakaroff-Girard A, Bourlier V, Decaunes P, Maumus M, Chiotasso P, Sengenes C, Lafontan M, Galitzky J, Bouloumie A. (2009) Interplay between human adipocytes and T lymphocytes in obesity. CCL20 as an adipochemokine and T lymphocytes as lipogenic modulators. *Arterioscler Thromb Vasc Biol* 29:1608–14.
8. Curat CA, Wegner V, Sengenes C, Miranville A, Tonus C, Busse R, Bouloumie A. (2006) Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin. *Diabetologia* 49:744–747.

Chapter 13

Gel-Based and Gel-Free Proteomic Technologies

Peter Scherp, Ginger Ku, Liana Coleman, and Indu Kheterpal

Abstract

Proteomics refers to the analysis of expression, localization, functions, posttranslational modifications, and interactions of proteins expressed by a genome at a specific condition and at a specific time. Mass spectrometry (MS)-based proteomic methods have emerged as a key technology for unbiased systematic and high-throughput identification and quantification of complex protein mixtures. These methods have the potential to reveal unknown and novel changes in protein interactions and assemblies that regulate cellular and physiological processes. Both gel-based (one-dimensional [1D] gel electrophoresis, two-dimensional [2D] polyacrylamide gel electrophoresis, 2D difference in-gel electrophoresis [DIGE]) and gel-free (liquid chromatography [LC], capillary electrophoresis) approaches have been developed and utilized in a variety of combinations to separate proteins prior to mass spectrometric analysis. Detailed protocols for global proteomic analysis from adipose-derived stem cells (ASCs) using two central strategies, 2D-DIGE-MS and 2D-LC-MS, are presented here.

Key words: Adipose-derived stem cells, Proteomics, Mass spectrometry, Two-dimensional difference in-gel electrophoresis, Liquid chromatography, iTRAQ™

1. Introduction

Adipose-derived stem cells (ASCs) have been shown to differentiate into adipocytes, chondrocytes, osteoblasts, and myoblasts under appropriate lineage-specific culturing conditions (1–3). Understanding and controlling differentiation properties of stem cells in *in vitro* studies are necessary to develop stem cell therapies for treatment of diseases. Due to the small correlation between gene and protein expression, this challenging task requires identification and mapping of stem cell specific proteins, differentiation-specific intracellular and secreted proteins, changes in protein levels and posttranslational modifications, and proteins involved in signal transduction driving differentiation. Proteomics is a

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large-scale study of protein properties including expression, subcellular localization, posttranslational modifications, protein–protein interactions, and pathways under a specific condition and at a specific time. Despite the remarkable advancements in stem cell biology and proteomic technologies, a limited number of studies have applied proteomic methods for evaluation of ASCs (4–9).

Mass spectrometry (MS)-based proteomics has emerged as the central technology for analyzing and characterizing proteins to understand cellular and physiological processes (10). MS-based proteomic methods allow an unbiased systematic investigation of a large number of proteins without the need for antibodies with high affinity and specificity and have the potential to reveal unknown and novel changes in protein interactions and assemblies that regulate cellular and physiological processes (10–12). A number of strategies using gel electrophoresis, capillary electrophoresis, and liquid chromatography (LC) have been applied for separation of polypeptides prior to MS analysis (13, 14). These strategies have been complemented by the development of novel MS instrumentation, methods for data acquisition, and bioinformatics tools for interpretation of large sets of MS data (15–17). A majority of the protein separation and MS tools are modular and can be adapted in a variety of ways to answer biological questions (18). Two of the most commonly employed MS-based quantitative proteomic strategies, two-dimensional (2D) difference in-gel electrophoresis (DIGE) and 2D-LC, are described in detail in this chapter.

2D-gel electrophoresis is still the most widely used method in proteomic studies and is the only technique that can be applied for parallel quantitative expression profiling of large sets of complex protein mixtures (>1,000 proteins) (19–21). In the 2D-DIGE approach, protein mixtures are labeled (at either lysines or cysteines) with spectrally resolvable CyDye fluorophores (Cy2, Cy3, and Cy5).These dyes are matched for mass and charge, so proteins labeled with any of these fluorophores comigrate on a 2D-gel (22).The samples are pooled together and separated using two consecutive techniques: isoelectric focusing (IEF), which discriminates proteins based on their isoelectric point, followed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), which discriminates proteins based on their apparent molecular weight (19–21). Two experimental samples (labeled with Cy3 and Cy5) and a pooled internal standard (labeled with Cy2) comprised of equal amounts of protein from each sample are included on every gel. Each gel is scanned at three different wavelengths, corresponding to the excitation and emission wavelengths of each of the three fluorophores. The abundance of each protein spot is measured as a ratio to its corresponding spot present in the internal standard. The pooled internal standard run on every gel allows an accurate comparison of protein abundance

between various samples run on different gels (22). The spots showing statistically significant changes are marked for further analysis. For protein identification by MS, parallel preparative 2D-gels are run and post-stained with a fluorescent dye such as SYPRO® Ruby. The gel images using post-staining methods are matched with images obtained using covalent CyDye fluorophores. Spots of interest are excised, digested with trypsin, and further analyzed using MS methods. We have used the 2D-gel electrophoresis approach to characterize proteome and secretome of ASCs before and after differentiation (5, 9). More recently, we have utilized the DIGE approach to study human skeletal muscle cell cultures (23) and adipose tissue (unpublished data).

A gel-free approach utilizing 2D-LC in conjunction with isobaric tagging of proteins for relative and absolute quantification (iTRAQ™) is also presented below. The iTRAQ™ reagents produce diagnostic low-mass MS/MS signature ions (17, 24, 25), and the intensity of these signature ions is compared to obtain relative protein concentrations. Currently, up to eight isobaric tags are available, allowing quantitative proteomic analysis of up to eight samples simultaneously. The amine specificity of these reagents results in labeling of all peptides, providing broad proteomic coverage. In this approach, individual protein samples are denatured, reduced, alkylated, and proteolyzed with trypsin in solution prior to any separation. Each trypsin-digested sample is labeled with a unique iTRAQ™ reagent. Labeled peptide mixtures are pooled at equimolar concentrations for further separation, identification, and quantification via LC-tandem mass spectrometry (MS/MS).The labeled pooled peptide mixture is fractionated using strong cation exchange chromatography (SCX) in the first dimension. Peptides in each SCX fraction are further separated by reverse-phase (RP) LC prior to MS/MS analysis. The advantage of this approach is that, after proteolysis and labeling, samples are analyzed together, resulting in higher speed and lower coefficients of variance. Moreover, a pooled internal standard can be included in each analysis for relative quantification across large numbers of samples. This approach has been successfully applied toward stem cell biology (26–28). Comparative analyses of gel-based and gel-free proteomic technologies have demonstrated that both of these methods have advantages for analyzing complex protein samples and should therefore be seen as complementary rather than exclusive (29, 30).

2. Materials

Unless otherwise stated, all solutions were prepared in deionized water (18.2 MΩ conductivity).

2.1. Protein Extraction

1. ReadyPrep™ Sequential Extraction Reagent 3 (Bio-Rad) lysis buffer: 5 M urea, 2 M thiourea, 2% (w/v) ultrapure 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate hydrate (CHAPS), 2% (w/v) SB 3–10, 0.2% Bio-Lyte3/10, 40 mM Tris.
2. 100 mM Phenylmethanesulfonylfluoride (PMSF).
3. *n*-Dodecyl-b-d-maltoside.
4. 2 mM Tributylphosphine (TBP).
5. Benzonase.
6. Cell wash solution: 10 mM Tris and 250 mM sucrose, pH 7.
7. Orbital shaker.
8. Inverted microscope with 20× and 40× phase contrast objectives.
9. Cell scraper.
10. Digital Sonifier with 3-mm microtip.
11. 1 M Dithiothreitol (DTT).

2.2. Protein Quantification: Bradford Protein Assay

1. Protein Assay Dye Reagent Concentrate (Bio-Rad, Cat No. 500-0006).
2. QuickStart™ bovine serum albumin (BSA) standard solutions (Bio-Rad).
3. 96-Well microtiter plates (flat bottom, 96 well, clear, PS, nonsterile).
4. Microtiter plate spectrophotometer.

2.3. 2D-DIGE

2.3.1. Protein Precipitation

1. UPPA Protein Concentrate™ Kit-Micro (G Biosciences): UPPA-I, UPPA-II, OrgoSol buffer, UPC-wash, SEED solution.
2. Gel-loading pipette tips.
3. CyDye labeling buffer: 30 mM Tris, 7 M urea, 2 M thiourea, and 4% CHAPS. Adjust to pH 8.5 using 1 M HCl.

2.3.2. Protein Labeling

1. pH paper strips (pH 7–14).
2. 50 mM NaOH, pH > 9.5.
3. Alcohol wipes.
4. *N*,*N*-Dimethylformamide (DMF; anhydrous, 99.8%). Store in a dessicator for <3 months.
5. 1-mL syringe with 20-gauge needle.
6. CyDye DIGE fluorophores, minimal dyes Cy2, Cy3, and Cy5; 5 nmol each (GE Healthcare). Store at –80°C.
7. 10 mM L-Lysine.

8. DeStreak Rehydration Solution (GE Healthcare, Cat. No. 17-6003-19).
9. Bio-Lyte 3/10, 100× ampholytes (20% ampholyte solution, Bio-Rad).

2.3.3. Isoelectric Focusing

1. ReadyStrip™ IPG strips pH 3–10 NL, 24 cm (Bio-Rad).
2. Forceps.
3. Isoelectric focusing (IEF) 24-cm tray with electrodes (Bio-Rad).
4. IPG strip equilibration/storage trays with lid (Bio-Rad).
5. Mineral oil.
6. Protean® IEF cell (Bio-Rad) (see Note 1).
7. Thick blot paper.
8. Electrode wicks.
9. 15 mM DTT.

2.3.4. SDS-PAGE

1. Ettan™ DALT*six* gel caster (GE Healthcare).
2. DALT separator sheets (GE Healthcare).
3. DALT filler sheets (GE Healthcare).
4. Ettan™ DALT*six* Large Vertical System (GE Healthcare): electrophoresis unit, gel caster, power supply, upper buffer chamber (UBC), lower buffer chamber (LBC) with ceramic heat exchanger, Multi Temp™ III Thermostatic Circulator (see Note 1).
5. DALT blank cassette inserts (GE Healthcare).
6. Low-fluorescent glass plates with spacers (GE Healthcare).
7. 95% Ethanol.
8. 60-mL plastic syringe barrel with neoprene rubber tubing attached.
9. Ring stand with clamp.
10. Hemostat.
11. 30% Acrylamide/Bis solution (Bio-Rad).
12. 1.5 M Tris, pH 8.8.
13. Sodium dodecyl sulfate (SDS; MW 288.38 g/mol).
14. Ammonium persulfate (APS).
15. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED).
16. 1-Butanol, saturated with deionized water (50 mL of 1-butanol with 10 mL of deionized water).
17. 10× SDS-PAGE electrophoresis running buffer: 250 mM Tris, 1.92 M glycine, and 1% SDS.

18. Equilibration buffer: 6 M urea, 2% SDS, 0.375 M Tris, pH 8.8, and 20% glycerol.
19. Reduction buffer: 1% DTT in equilibration buffer.
20. Alkylation buffer: 2.5% iodoacetamide (IAA) in equilibration buffer.
21. Thick blot paper.
22. Gel stand.
23. Two forceps.
24. 50-mL pipette (see Note 2).
25. 0.75-mm spacer.
26. Ready Prep™ overlay agarose (Bio-Rad).
27. Beaker.
28. Thermometer.
29. Electrode wicks.
30. Low molecular weight (LMW) markers (GE Healthcare, Cat. No. 17-0446-01).
31. Orbital shaker.

2.3.5. Image Acquisition and Analysis

1. Fluorescence scanner or imaging system equipped with appropriate excitation and emission filters for Cy2, Cy3, and Cy5 dyes and SYPRO® Ruby protein stain.
2. 2D-gel analysis software.

2.3.6. Total Protein Detection with SYPRO® Ruby

1. Gel-staining trays.
2. Fixation/Destain Solution: 10% ethanol and 7% glacial acetic acid in deionized water.
3. SYPRO® Ruby protein gel-stain (Bio-Rad).
4. Gel-cutting sheets.
5. One-gallon plastic storage bags.
6. Aluminum foil.
7. Orbital shaker.

2.3.7. Spot Excision and In-Gel Digestion

1. EXQuest™ Spot Cutter (Bio-Rad).
2. Microtiter plates.
3. Microtiter plate-sealing film.
4. Heating block.
5. 100 mM ammonium bicarbonate buffer.
6. 100% acetonitrile (LC/MS grade).
7. Reduction solution: 10 mM DTT in 100 mM ammonium bicarbonate buffer.

8. Alkylation solution: 55 mM IAA in 100 mM ammonium bicarbonate buffer.
9. Extraction solution: 1% formic acid and 2% acetonitrile in LC/MS-grade water.
10. Trypsin.
11. Trypsin resuspension solution: 50 mM acetic acid (Promega).

2.4. 2D-LC-MS/MS

All solvents for 2D-LC-MS/MS are prepared using LC/MS-grade water unless otherwise specified.

2.4.1. Protein Desalting, Digestion, and iTRAQ™ Reagent Labeling

1. Acetone (chilled to –20°C).
2. Gel-loading pipette tips.
3. iTRAQ™ 4-plex kit (Applied Biosystems, Cat. No. 4352135): iTRAQ™ Reagent vials, Triethylammoniumbicarbonate buffer (dissolution buffer), 2% sodium dodecyl sulfate (SDS; denaturing agent), 50 mM Tris (2-carboxyethyl) phosphine (TCEP; reducing agent), 200 mM methyl methane thiosulfonate (MMTS; cysteine-blocking reagent), and 100% ethanol.
4. Digital Sonifier with 3-mm microtip.
5. Trypsin.
6. Trypsin resuspension buffer: 50 mM acetic acid.
7. pH paper strips (pH 7–14).

2.4.2. Strong Cation Exchange Chromatography

1. SCX column: PolySulfoethyl A (2.1 × 50 mm, 3 µm, 200 Å, Nest Group, Cat. No. 052SE0502).
2. SCX guard column: PolySulfoethyl (2.1 × 10 mm, 5 µm, 200 Å, Javelin®, Nest Group, Cat. No. PJ22GCSE0502).
3. HPLC system equipped with pumps, autosampler, UV/VIS detector, and fraction collector.
4. Solvent A: 10 ammonium formate, 20% acetonitrile, adjusted with formic acid to pH 3.
5. Solvent B: 600 mM ammonium formate, 20% acetonitrile, adjusted with formic acid to pH 3.
6. Solvent C: 100% LC/MS-grade water.
7. Solvent D: 40 mM EDTA, disodium, dihydrate.

2.4.3. RPLC-MS/MS

1. nanoAcquity UltraPerformance LC® (Waters Corp.) equipped with a Symmetry® C18 trap column (5 µm, 180 µm × 20 mm, Waters Corp.) and a BEH C18 analytical column (1.7 µm, 75 µm × 100 mm, Waters Corp.).
2. Quadrupole time-of-flight mass (Q-TOF) spectrometer (SYNAPT HDMS™, Waters Corp.) interfaced to the nano

AcquityUPLC® via a nano-electrospray ionization source. The LC-MS/MS system includes MassLynx™ data acquisition and ProteinLynx Global Server™ 2.3 data analysis software packages.

3. Solvent A: 0.1% (v/v) formic acid in LC/MS-grade water.
4. Solvent B: acetonitrile (LC/MS grade) with 0.1% formic acid.

3. Methods

2D-DIGE: The 2D-DIGE approach utilizes fluorescent labeling of proteins followed by separation using IEF and SDS-PAGE. The 2D-DIGE technology uses either highly sensitive saturation labeling with cysteine-reactive cyanine fluorescent dyes (CyDyes) or minimal labeling with lysine-reactive cyanine fluorescent dyes. Saturation labeling is very sensitive, allowing labeling of as little as 5 μg of protein (22, 31), and is ideal for samples limited in quantity. Minimal labeling with lysine-reactive dyes is used for protein mixtures extracted from cell cultures where sample is not limited. The detailed methods presented below use lysine-reactive minimal labeling. Using minimal labeling, <5% of the proteins in the sample are labeled; however, presence of the dye can change protein mobility, especially of low molecular weight proteins. Thus, the same gels are further poststained with fluorescent stains such as SYPRO® Ruby to facilitate spot excision. The excised gel plugs are destained, reduced, alkylated, and digested with trypsin. The peptides are then extracted from gel plugs and analyzed using RPLC-MS/MS.

2D-LC-MS/MS: Detailed methods for 2D LC-MS/MS methods in conjunction with labeling with four iTRAQ™ tags for protein quantification are presented below. The principle of the ITRAQ™ method is based on the use of isobaric tags consisting of a reporter group (e.g., 114–117 Da for iTRAQ™ 4plex kit), a balancer group (31–28 Da, respectively) and a peptide reactive group (Fig. 1). During the labeling procedure, the reporter and balancing group are covalently bound to peptides. The reporter groups result in diagnostic low mass (m/z 114–117) MS/MS signature ions. The intensity of these reporter ions is compared to obtain relative protein concentrations in up to four samples simultaneously.

Individual experimental samples are labeled with four individual iTRAQ™ reagents, pooled together, and separated either using an on-line or off-line 2D-LC approach prior to MS analysis. In an on-line approach, SCX and RP columns are connected back to back, and peptides are “bumped” off the SCX at various salt

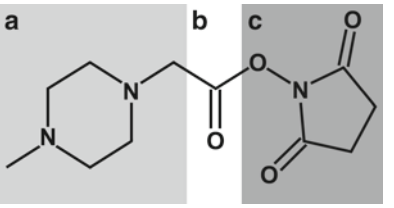

Fig. 1. Chemical structure of the iTRAQ™ Reagent consisting of the reporter group (**a**), the balancing group (**b**), and the amine specific reactive group (**c**). The different masses of the individual reporter groups (114–117) are compensated by the balancing groups to yield identical total mass of 145 Da for the reporter and balancing group (**a** and **b**).

concentrations and injected directly into the RP column, offering speed and automation. In an off-line approach, pooled peptide samples are first separated by charge using SCX chromatography. Each fraction is then further analyzed individually on a RPLC-MS/MS system as described below. This approach does not allow complete automation; however, it allows multiple injections of fractions containing more complex mixtures of peptides, thus increasing the number of protein identifications. Furthermore, mass/charge boundaries of the precursor ions in multiple runs can be limited (mass segmentation) to increase the number of peptides analyzed.

3.1. Protein Extraction

3.1.1. Lysis Buffer Preparation

1. Reconstitute ReadyPrep™ Sequential Extraction Reagent 3 by adding 6.3 mL of deionized water to the reagent bottle and dissolve completely, yielding 10 mL of lysis buffer (see Note 3).
2. Add 500 µL of 100 mM PMSF to 10 mL of lysis buffer.
3. Add 200 mg of *n*-dodecyl-b-D-maltoside to 10 mL of lysis buffer for a final concentration of 2%.
4. Aliquot lysis buffer in microfuge tubes (1 mL per tube). Aliquots can be stored at –80°C for 6 months if not all of the solution is needed immediately.
5. Add 25 µL of 2 mM TBP and150 U of benzonase to 1 mL of lysis buffer immediately before use.

3.1.2. Cell Lysis

1. Remove all media from the cell culture dish by decanting.
2. Rinse cells in each culture dish three times with 1.5 mL of cell wash solution to remove media.
3. Decant off and remove by pipetting as much wash solution as possible.
4. After the final wash, add 1 mL of lysis buffer to each culture dish.
5. Incubate samples on the orbital shaker at low speed for 20 min at room temperature.

6. Visualize cells under microscope to verify cell lysis.
7. Scrape remaining cells off the dish using a cell scraper and transfer cell lysate into a microfuge tube using a 1-mL pipette. If necessary, cell lysates from multiple dishes can be combined (see Note 4).
8. Sonicate samples in 20-s bursts four times and incubate samples on ice for 20 s in between each burst. Avoid longer cooling as salts in lysis buffer will precipitate.
9. Incubate samples for 45 min at room temperature.
10. Add 50 µL of 1 M DTT per 1 mL of lysis buffer into each tube.
11. Centrifuge cell lysates for 30 min at 20,800 × *g* to pellet insoluble cell components.
12. Transfer supernatant into a new microfuge tube and store at −80°C until further use.

3.2. Protein Quantification: Bradford Protein Assay

The protocol described here is used to measure total protein concentration after cell lysis and prior to labeling with CyDyes and iTRAQ™ Reagents in DIGE and 2D-LC-MS/MS protocols, respectively. Appropriate negative controls are specified in the sections below.

1. Prepare 20 mL of Bradford dye solution by diluting 4 mL of Protein Assay Dye Reagent Concentrate with 16 mL of deionized water. This is sufficient to fill 96 0.2-mL wells.
2. Dilute protein samples in deionized water in microfuge tubes and mix. Negative control (lysis buffer containing benzonase, DTT, and TBP) is diluted according to protein samples. Deionized water is used as a blank. We generally make 25-fold and 50-fold dilutions of each sample. Each dilution is run in triplicate.
3. BSA standards at concentration of 0.25 mg/mL to 1 mg/mL are used directly from the BSA standard solution kit. These standards can be further diluted in deionized water in order to accurately measure lower protein concentrations. Each standard is run in triplicate.
4. In a 96-well microtiter plate, pipette 200 µL of Bradford dye solution into an appropriate number of wells. Add 10 µL of blank, BSA standards, negative control, or cell lysis samples to individual wells in triplicate, respectively.
5. Mix and incubate at room temperature for 5 min.
6. Measure absorbance using the spectrophotometer at 595 nm.
7. Calculate the protein concentrations.

3.3. 2D-DIGE

3.3.1. Protein Precipitation

1. Aliquot 150 µg of each protein sample into a microfuge tube (see Notes 5 and 6).
2. Add 450 µL of UPPA I buffer (150 µL of UPPA I buffer per 50 µg of protein) to each sample, and vortex to mix.
3. Incubate at 4°C for 15 min.
4. Add 450 µL of UPPA II (150 µL of UPPA II buffer per 50 µg of protein) to each sample, and vortex to mix.
5. Centrifuge at 20,800 × *g* for 10 min at 4°C.
6. Remove and discard supernatant without disturbing the pellet (protein).
7. Centrifuge at 20,800 × *g* for 30 s at 4°C.
8. Remove any remaining supernatant using gel-loading tips and discard.
9. Add 60 µL of UPC-Wash (40 µL of UPC-wash per 300 µL of UPPA I) to the pellet, and vortex to mix.
10. Centrifuge samples at 20,800 × *g* for 10 min at 4°C.
11. Remove the supernatant (wash solution) and add sufficient volume (usually 25 µL) of deionized water to cover the pellet. Vortex to mix.
12. Chill OrgoSol buffer from the UPPA Protein Concentrate kit at –20°C for at least 1 h before use.
13. Add 1 mL of OrgoSol buffer and 5 µL of SEED solution (the volume of OrgoSol buffer must be at least tenfold in excess of water added in step 11, adjust if necessary). Vortex to mix.
14. Incubate samples at –20°C for 15 min. Vortex samples for 20–30 s.
15. Repeat step 14.
16. Centrifuge samples at 20,800 × *g* for 10 min at 4°C.
17. Remove and discard the supernatant without disturbing the pellet.
18. Air-dry pellets until they become translucent.
19. Resuspend each pellet in 15 µL of CyDye labeling buffer.
20. Determine the protein concentration using the Bradford Protein Assay as described above using the CyDye labeling buffer as negative control.

3.3.2. Protein Labeling

1. Centrifuge samples briefly and check the pH by spotting a small aliquot of samples onto pH paper.
2. If necessary, adjust the pH to 8.5 using 0.5 µL increments of 50 mM NaOH (see Note 7).
3. Prepare pooled internal standard by combining 25 µg of protein from each sample.

4. Aliquot 50 µg of each sample and 50 µg of pooled internal standard separately into individual 1.5 mL microfuge tubes (see Notes 5 and 6).
5. Allow lyophilized CyDye DIGE fluorophores to equilibrate to room temperature. Spin CyDye vials briefly to make sure that the entire solid is at the bottom of the tube. Place CyDye vials on ice.
6. Wipe the top of the DMF stock container (injection vial with rubber cap) with an alcohol wipe. Using a 1-mL syringe equipped with a 20-gauge needle, remove 30 µL of DMF and transfer into a microfuge tube.
7. Transfer 5 µL of DMF from the microfuge tube to 5 nmol of each CyDye vial, yielding a stock solution of 1 nmol/µL of CyDye.
8. Vortex each CyDye vial vigorously to assure complete dissolution. Spin down the CyDye solution for 30 s. The solution is now ready to use and can be stored at –80°C for 3 months in the dark.
9. Add 400 pmol of Cy2, Cy3, and Cy5 to 50 µg pooled internal standard, protein sample 1, and protein sample 2, respectively.
10. Vortex and spin samples briefly.
11. Incubate samples on ice for 30 min in the dark (see Note 8).
12. Add 1 µL of freshly prepared 10 mM L-lysine to stop the labeling reaction. Vortex and spin briefly.
13. Incubate samples on ice for 10 min in the dark.
14. If necessary, samples can be stored after CyDye labeling for up to 3 months at –80°C in the dark.
15. Prepare DeStreak solution containing 1% ampholytes by mixing 25 µL of Bio-Lyte 3/10 20% ampholyte stock solution with 475 µL of DeStreak Rehydration solution for each IPG strip.
16. Pool Cy2, Cy3, and Cy5 samples to be run simultaneously on one gel and adjust to a final volume of 450 µL using DeStreak buffer with 1% ampholytes.
17. Incubate samples at room temperature for 1 h in the dark.
18. Centrifuge samples at 20,800 × *g* for 10 min at room temperature.
19. Protein samples are now ready for IEF.

3.3.3. Isoelectric Focusing

1. Load each CyDye-labeled sample (as obtained in the section above) across the length of an individual channel of a clean IEF tray. Avoid introducing any air bubbles during the transfer.

If air bubbles are present in the tray, use the pipette tip to remove bubbles without removing the supernatant.

2. Hold IPG strip with forceps on one end and remove the protective plastic backing using a second set of forceps. Gently lay the gel side of the strip in the IEF channel containing the CyDye sample (see Note 9). Repeat this procedure for as many IPG strips as needed (one strip per IEF channel).
3. Cover IEF tray with aluminum foil and incubate strips in the IEF tray at room temperature for 30 min.
4. Using a 1-mL transfer pipette, completely overlay each IPG strip with mineral oil (ca. 5 mL per channel) to prevent dehydration.
5. Cover the IEF tray with the lid and place tray into the Protean® IEF cell. Make sure that the IPG gel strip makes contact with the electrode wire (see Note 9).
6. Perform active rehydration at 50 V and 20°C. Samples must rehydrate for at least 22 h.
7. After active rehydration is completed, pour off excess mineral oil, remove individual strips using forceps, and rinse with deionized water using a straw squirt bottle to remove as much oil as possible.
8. Blot IPG strips between two blotting paper strips moistened with deionized water.
9. Place IPG strips into channels of a fresh IEF tray.
10. Add 8 µL of 15 mM DTT solution to an electrode wick (one wick per IPG strip).
11. Place a wick with DTT between each IPG gel strip and the negative electrode wire of the IEF tray. Make sure that the ends of the gel on the IPG strip are placed above the electrodes (see Note 10).
12. Wet another set of electrode wicks with deionized water and place between each IPG gel strip and the positive electrode wire.
13. Cover strips with mineral oil without introducing air bubbles.
14. Cover the IEF tray with the lid and place tray into the Protean IEF cell. Cover the IEF cell with aluminum foil.
15. Perform IEF using the following protocol. Step 1: 150 V, ramp rapid, 45 min; step 2: 250 V, ramp rapid, 2 h; step 3: 500 V, ramp linear, 1 h; step 4: 1,000 V, ramp linear, 4 h; step 5: 2,000 V, ramp linear, 1 h; step 6: 5,000 V, ramp linear, 1 h; step 7: 9,000 V, ramp linear, 5 h; step 8: 10,000 V, ramp rapid, 70,000 Vh; step 9: 150 V, ramp rapid, 12 h. IEF is completed at the end of step 8. The Protean IEF cell can be stopped at any time during step 9.

16. The positive electrode wicks will accumulate blue dye from the DeStreak buffer. In order to avoid saturation, replace all positive electrode wicks moistened with deionized water every hour until all of the blue dye is absorbed. Absorption of the blue dye takes about 5–7 h after starting IEF.
17. After IEF is completed, pour off excess mineral oil; remove individual IPG strips using forceps and rinse strips with deionized water using a straw squirt bottle. Blot strips between two blotting paper strips moistened with deionized water.
18. Strips can be stored in an IEF storage tray at –80°C until SDS-PAGE.
19. To store, place IPG strip gel side up in an IEF storage tray. Place lid on tray, and wrap with stretchable sealing film and aluminum foil.

3.3.4. SDS-PAGE: Assembling Gel Caster Unit

1. Clean caster, glass plates, separation sheets, and all other gel caster unit components with 95% ethanol and lint-free wipes.
2. Assemble all six individual cassettes using glass plates and spacers and place them into the Ettan™ DALT*six* gel caster with thin separation sheets between each cassette. If less than six gels are used, fill the unused positions with DALT blank cassette inserts. To fill the remaining space between the last gel cassette and the caster, insert first a thin separator sheet followed by thicker (1-mm) filler sheets. Seal the caster by placing the foam rubber gasket in the groove of the gel caster and close the caster by locking the removable front panel. For detailed instructions, please refer to the manual for Ettan™ DALT*six* electrophoresis system.
3. Stand caster up to make sure the top of the cassettes are flush with the back of the caster. Cover with plastic wrap.
4. In order to simplify pouring the polyacrylamide solution into the caster, we built a funnel-like contraption by attaching a 50-cm-long neoprene rubber tube to the barrel of a 60-mL syringe.
5. The free end of the rubber tubing is placed into the filling channel in the back plate of the caster and the syringe barrel is clamped into a ring stand about 10–20 cm above the caster. The neoprene tubing is clamped off with a hemostat. When using the filling channel, make sure to seal the gel caster by plugging the barbed fitting in the faceplate.

3.3.5. SDS-PAGE: Gel Casting and Pouring

1. In a 1-L vacuum flask, prepare polyacrylamide solution by combining 220 mL of 30% acrylamide/bis acrylamide solution, 137.5 mL of 1.5 M Tris (pH 8.8), 192.5 mL of deionized water, and 5.5 mL of 10% SDS. Mix by gently swirling the flask.

2. Degas the polyacrylamide solution at room temperature for 2 h by applying a vacuum.
3. While degassing the polyacrylamide solution, prepare 6 mL of 10% APS.
4. Once degassing of the polyacrylamide solution is complete, add 248 µL of TEMED to the polyacrylamide solution in the vacuum flask. Gently mix without introducing air bubbles.
5. Add 5 mL of freshly prepared 10% APS to the polyacrylamide/TEMED solution. Gently mix without introducing air bubbles.
6. Fill the gel caster with polyacrylamide solution by pouring it into the 60-mL syringe barrel and opening the hemostat. Make sure the other end of the neoprene tubing is tightly fitted in the loading channel of the caster.
7. Refill the syringe barrel continuously with polyacrylamide solution without introducing air bubbles, as polyacrylamide gels with air inclusions cannot be used (see Note 11).
8. Fill the gel cassette to about 2 cm from the top of the glass plates.
9. Overlay gels with 1 mL of water-saturated butanol to obtain a uniform and straight gel. This allows the IPG strip to lay flat on the polymerized gel.
10. Cover the caster with plastic wrap and allow gels to polymerize for 2 h.
11. After polymerization, decant butanol and rinse gels with deionized water until all butanol is removed.
12. Prepare 500 mL of casting buffer by mixing 125 mL of 1.5 M Tris (pH 8.8), 5 mL of 10% SDS, and 370 mL of deionized water.
13. Pour casting buffer on top of gels, cover with plastic wrap to prevent dehydration, and allow gels to polymerize overnight.
14. Remove all gel cassettes from the gel caster, rinse outer surfaces with deionized water, and clean all surfaces with lint-free wipes.

3.3.6. SDS-PAGE: Gel Electrophoresis

1. Prepare 1× SDS-PAGE electrophoresis running buffer by diluting 10× SDS-PAGE with deionized water.
2. Fill Ettan™ DALT*six* lower buffer chamber (LBC) tank with 1× running buffer and set temperature to 4°C.The LBC holds about 4.3 L of 1× running buffer.
3. Place upper buffer chamber (UBC) in a sufficiently large pan filled with 1× running buffer to wet the seals. Cover the pan with plastic wrap until ready to use.

4. Prepare equilibration, reduction, and alkylation buffers (see Note 12). Allow DTT and IAA to dissolve completely in reduction and alkylation buffers, respectively.
5. Allow frozen IPG strips to equilibrate to room temperature.
6. Add 8 mL of reduction buffer to each strip. Incubate on orbital shaker at low speed at room temperature for 15 min.
7. After incubation, remove reduction buffer and rinse strips with deionized water.
8. Add 8 mL of alkylation buffer to each strip. Incubate on orbital shaker at low speed at room temperature for 15 min.
9. After incubation, remove alkylation buffer and rinse strips with deionized water.
10. Gel tops of the SDS gels must be straight to accommodate IPG strip. Do not use gels containing air inclusions or irregularities. Rinse the top of gels with deionized water followed by a rinse with 1× running buffer. Remove running buffer and place gels in stand (see Note13).
11. Heat agarose in a microwave in 10 s bursts until it is liquefied. Place liquefied agarose and a thermometer in a beaker containing warm water. Allow temperature of agarose to reach 25–30°C.
12. Using forceps, remove IPG strip from the storage tray while keeping track of the "+" and "–" orientation of the gel strip. Immerse IPG strip into the 50-mL modified pipette filled with1× running buffer for 20 s.
13. Blot IPG strip between two blotting paper strips moistened with deionized water.
14. Overlay the top of the gel with agarose using a 1-mL transfer pipette. Be careful not to introduce air bubbles.
15. Carefully and quickly position the IPG strip with its gel side facing forward between the long and short glass plate. The acidic ("+") side of the IPG strip is placed on the left side of the glass plate.
16. Using a 0.75-mm spacer, gently push the IPG strip between the glass plates through the liquid overlay agarose until the entire length of the strip makes full contact with the surface of the polyacrylamide gel. Be careful to only touch the plastic backing of the IPG strip, not the gel. Do not introduce air bubbles.
17. Wet electrode wicks with 8 µL of LMW marker and place on acidic ("+") end of the IPG strip. Refill the top of the gels with agarose.
18. Allow the agarose on top of the gels to solidify for 10 min.

19. Repeat these steps until all IPG strips are incorporated into their gels. Reheat agarose to liquefy if necessary.
20. Place gels into gel cassette holder and place cassette holder into LBC (see Note 14).
21. Remove the UBC from 1× running buffer and push it onto the gel cassette holder making a tight seal.
22. Adjust the volume of 1× running buffer in the LBC to the maximum fill line.
23. Dilute 10× SDS-PAGE Running Buffer to 3× running buffer with deionized water.
24. Fill the UBC with 3× running buffer to the same height as LBC to establish hydrostatic balance. The UBC holds about 2.5 L of 3× running buffer.
25. Place the lid onto the Ettan™ DALT*six* tank and cover the tank with aluminum foil to protect the samples from light.
26. Set the MultiTemp III temperature to 4°C. Program the power supply with the following settings: step 1: 10 mA/gel, 80 V and 1 W/gel for 1 h; step 2: 12 mA/gel, 150 V and 2 W/gel for 15–17 h. (see Note 15). Continue electrophoresis until bromophenol blue dye front has reached the bottom of the gels. Turn off the power supply. Remove the UBC first, then the gel cassette containing the gels.

3.3.7. Gel Image Acquisition

1. Remove gels from the gel electrophoresis unit while leaving them in the glass plates. Gels are imaged using a fluorescence imaging system with three-color detection capabilities.
2. After imaging, wrap gels in aluminum foil and store at 4°C (see Note 16).
3. Analyze gel images using 2D-gel analysis software. Routine statistical analyses available within the 2D-gel analysis software are used to identify differentially regulated spots.

3.3.8. Total Protein Detection with SYPRO® Ruby

1. After 2D-gel electrophoresis, remove gels from the glass plates and place into a staining tray (see Note 17).
2. Incubate each gel in 800 mL of fixation solution at room temperature for 30 min (see Note18).
3. Remove fixation solution and add 500 mL of SYPRO® Ruby Stain to each gel. Incubate for at least 3 h on orbital shaker at low speed at room temperature.
4. After staining, wash gels in 800 mL of destain solution for 30 min on orbital shaker at low speed at room temperature.
5. Incubate gels in deionized water for at least 2 h (see Note 19).
6. Image gels using the fluorescence imaging system.

7. For storage, place each gel on a clean gel-cutting sheet and place the gel into a gallon-size plastic storage bag. Add 100 mL of destain solution to the bag. Seal and wrap bag with aluminum foil.
8. The wrapped gels are stored at 4°C until spot excision.

3.3.9. Spot Excision and In-Gel Digestion

1. Match SYPRO® Ruby gel images with CyDye images and select spots of interest for further analysis.
2. Excise selected protein spots. We use an EXQuest™ Spot Cutter, but a spot cutter from any manufacturer can be utilized.
3. Place individual gel plugs into separate wells of a 96-well microtiter plate and place on a heating block (40°C).
4. Remove all excess liquid from each well.
5. Destain gel plugs by adding 50 µL of 100 mM ammonium bicarbonate buffer and 50 µL of acetonitrile to each well. Incubate for 10 min, and then remove liquid by pipetting.
6. Repeat step 5.
7. Dehydrate gel plugs by adding 50 µL of acetonitrile to each well. Incubate for 10 min and remove liquid by pipetting.
8. Allow excess acetonitrile to evaporate for 10 min.
9. Reduce proteins by adding 50 µL of reduction solution to each well. Incubate at room temperature for 30 min.
10. Alkylate the proteins by adding 50 µL of alkylation solution. Incubate for 20 min.
11. Add 100 µL of acetonitrile to each well, incubate for 5 min, and remove all liquid by pipetting.
12. Wash each gel plug by adding 50 µL of 100 mM ammonium bicarbonate buffer. Incubate for 10 min and remove liquid by pipetting.
13. Wash each gel plug by adding 50 µL of acetonitrile to each well. Incubate for 5 min and remove all liquid by pipetting.
14. Repeat step 13 two more times.
15. Evaporate off excess acetonitrile.
16. Prepare a 100 ng/µL trypsin stock solution by dissolving 20 µg of trypsin in 200 µL of 50 mM acetic acid.
17. Prepare a working solution of 6 ng/µL of trypsin in 50 mM ammonium bicarbonate buffer.
18. Add 25 µL of trypsin working solution (6 ng/µL) to each well and cover the microplate.
19. Seal the microtiter plate with sealing film and incubate at 37°C for 6 h.

20. Extract peptides from gel plugs by adding 30 µL of extraction solution.
21. Cover the microplate and incubate at room temperature for 30 min.
22. Transfer the supernatant of each well into a well of a new microtiter plate.
23. Repeat extraction two more times by adding 12 µL of extraction solution to each well and incubating for 30 min at room temperature.
24. Supernatants from all three extractions are pooled.
25. Load 1–10 µL of tryptic digest onto a Q-TOF instrument for LC-MS/MS analysis as described in the LC-MS/MS analysis section (see Subheading 3.4.5) below.

3.4. 2D-LC-MS/MS

3.4.1. Protein Desalting

1. Determine the protein concentration of samples in lysis buffer using Bradford Protein Assay (*see* Subheading 3.2).
2. Aliquot 200 µg of protein from each sample into 2-mL microfuge tubes. Chill sample tube to 4°C.
3. Add six volumes of 100% acetone (chilled to –20°C) to the cold sample (see Note 20).
4. Incubate samples overnight at –20°C to precipitate proteins.
5. Centrifuge samples at 2,600 × *g* at 4°C for 5 min.
6. Remove supernatant from microfuge tubes without disturbing the pellet. A long-tapered gel-loading tip helps to remove as much supernatant as possible. Do not dry samples.
7. Resuspend protein pellets in 20 µL of iTRAQ™ dissolution buffer (see Note 21).
8. Perform the Bradford Protein Assay as described above to determine the protein concentration in each sample in dissolution buffer. Use dissolution buffer as a negative control.

3.4.2. Protein Digestion

1. Aliquot 100 µg of protein from each sample into individual microfuge tubes. It is important to use an equal amount of protein from each sample (see Note 22).
2. Adjust the total volume of each sample to 20 µL with iTRAQ™ dissolution buffer if necessary. If more than 20 µL of sample volume is used, then recalculate the volumes of the subsequent solutions accordingly (see Note 23).
3. Add 1 µL of 2% SDS per 20 µL of sample to each tube (see Note 24).Vortex and spin samples.
4. Add 2 µL of 50 mM TCEP per 20 µL of sample to each tube. Vortex and spin samples.

5. Incubate samples at 60°C for 1 h. If urea was added to dissolve the protein pellet, then incubate samples at 37°C for 1 h (see Note 25).Vortex and spin samples.
6. Add 1 µL of 200 mM MMTS per 20 µL of sample to each tube. Vortex and spin samples.
7. Incubate samples at room temperature for 30 min.
8. Prepare 1 µg/µL of trypsin by dissolving 20 µg of lyophilized trypsin in 20 µL of resuspension buffer.
9. Add 10 µL of trypsin solution to each protein sample (enzyme to protein ratio 1:10 w/w).
10. Vortex, centrifuge, and incubate samples at 37°C overnight.

3.4.3. iTRAQ™ Reagent Labeling

1. The pH of the samples after digestion with trypsin must be >7. Adjust the pH of samples with dissolution buffer if necessary.
2. Concentrate samples to approximately 25 µL using a Speedvac concentrator (see Note 26).
3. Equilibrate iTRAQ™ Reagent vials (114–117) to room temperature and spin to bring the solution to the bottom of the tube.
4. Add 70 µL of 100% ethanol to each iTRAQ™ Reagent vial. Vortex and centrifuge.
5. Transfer content of one iTRAQ™ Reagent vial to one sample tube (Fig. 2). Vortex and spin.

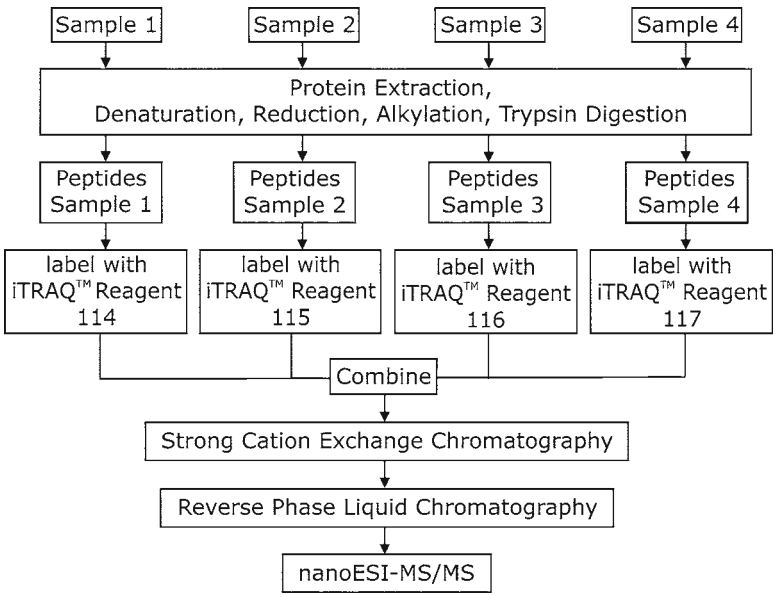

Fig. 2. A sample preparation workflow for iTRAQ™ Reagent labeling. Proteins are extracted, denatured, reduced, alkylated, and digested with trypsin. Resulting peptides are labeled sample specific with iTRAQ™ Reagents and separated by SCX and RPLC prior to MS analysis.

6. Incubate samples at room temperature for 1 h.
7. After incubation, add 100 µL of LC/MS-grade water to each sample to stop the labeling reaction.
8. Incubate at room temperature for 30 min.
9. Combine the four iTRAQ™ Reagent-labeled samples into one microfuge tube. Each tube can be further rinsed with LC/MS-grade water to ensure recovery of the entire protein digest.
10. Concentrate the sample to ca. 25 µL using the Speedvac concentrator.
11. Add 100 µL of LC/MS-grade water to the tube, vortex, spin, and concentrate sample to ~25 µL.
12. Repeat step 11 once and concentrate samples to ~5 µL.
13. Adjust volume to 125 µL with Solvent A for SCX chromatography.

3.4.4. Strong Cation Exchange Chromatography

1. Prepare and degas all solvents immediately before use.
2. Flush the SCX column for 75 min using 100% Solvent C at a flow rate of 0.2 mL/min.
3. Condition the column using the following method: 20 min, 0–100% Solvent B; 5 min, 100% Solvent B; 20 min, 100% Solvent A. Repeat this sequence for a total of three times at a flow rate of 0.2 mL/min.
4. The system can be flushed with 100% Solvent D for 24 h at a flow rate of 0.1 mL/min to passivate all metal surfaces in the HPLC system as well as the column.
5. Condition the SCX column using 100% Solvent B for 75 min followed by 100% Solvent A for 75 min at a flow rate of 0.2 mL/min (see Note 27) prior to loading samples.
6. Load 100 µL of iTRAQ™ Reagent-labeled sample onto the SCX column and separate peptides using the following gradient at a flow rate of 0.2 mL/min: 0–30 min, 0–40% Solvent B; 30–45 min, 40–100% Solvent B; 45–50 min, isocratic 100% Solvent B; 50–65 min, 100–0% Solvent B; 65–80 min, isocratic 100% Solvent A (see Notes 28 and 29).
7. Absorbance is monitored at 280 nm, and a representative chromatogram of an iTRAQ™ Reagent-labeled sample is presented in Fig. 3.
8. Collect fractions at 1-min intervals in a microtiter plate.
9. Concentrate all fractions to ~5 µL using a Speedvac concentrator.

3.4.5. LC-MS/MS Analysis

1. Reconstitute each SCX fraction to 30 µL using 100% LC/MS-grade water (see Note 30).

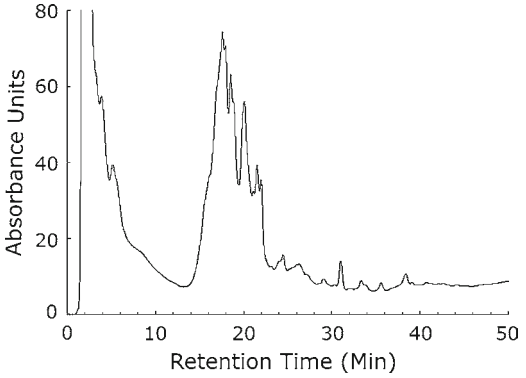

Fig. 3. A SCX chromatogram of a mixture of iTRAQ™ Reagent-labeled tryptic peptides. Forty-five fractions were collected from 2 to 46 min.

2. RP chromatographic separation of each fraction is carried out using a nanoflow ultraperformance LC at a flow rate of 300 nL/min (see Notes 28 and 31).
3. Load each SCX fraction (1–10 µL) onto the trap column and wash with 1% solvent B at 5 µL/min for 15 min to remove any salts from SCX.
4. Start the chromatographic separation using the following gradient: 0–5 min, 1–10% Solvent B; 5–60 min, 10–30% Solvent B; 60–65 min, 30–80% Solvent B; 65–68 min, isocratic at 80% Solvent B; 68–70 min, 80–1% Solvent B; and 70–85 min, isocratic at 1% Solvent B.
5. The separated peptides are introduced into the MS via a nanospray emitter.
6. The MS is operated in the positive-ion mode with a resolution of 10,000 full-width half-maximum using a source temperature of 150°C, capillary voltage of 3.5 kV, cone voltage 35 V, and extraction cone voltage of 2 V (see Note 31). A representative chromatogram obtained after RP separation of one of the SCX fractions is presented in Fig. 4a.
7. Operate the Q-TOF MS in an automatic data-dependent acquisition mode. In this mode, MS TOF scans are acquired from m/z 400 to 1,600, and up to three precursors are selected for MS/MS from m/z 50 to 1,600, using data-dependent acquisition and rolling collision energy applied to promote fragmentation. All of the data acquisition is performed automatically using Mass Lynx™ software (Waters Corp.). Figure 4b presents a MS/MS spectrum of a precursor ion with m/z 624.32 detected at 25.1 min.
8. Smooth and centroid raw MS and MS/MS spectra from the Q-TOF System using ProteinLynx Global Server™ (PLGS) software (Waters, Corp.) for identification and quantification between samples.

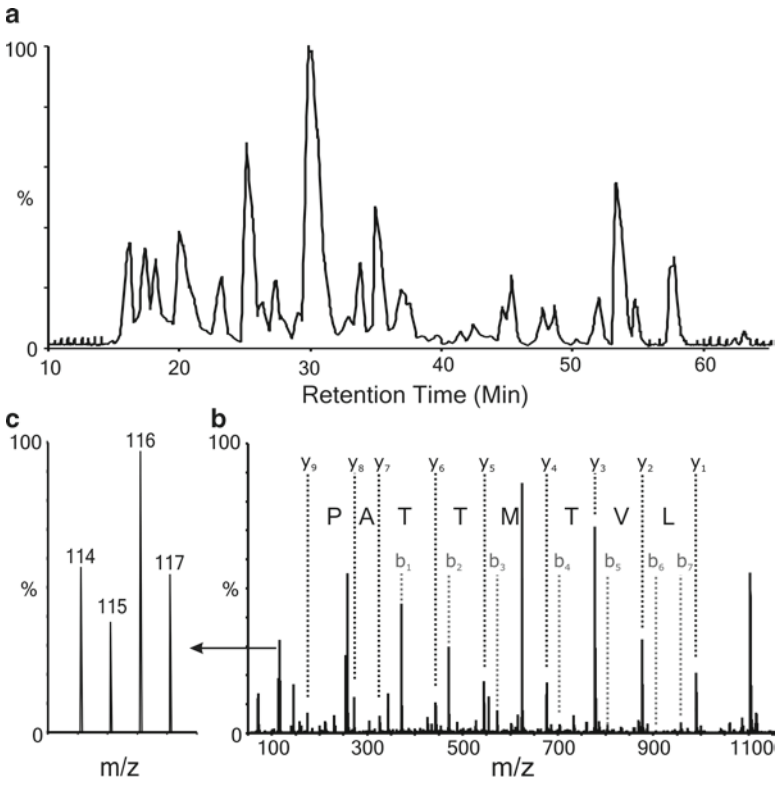

Fig. 4. (**a**) RPLC-MS chromatogram of peptides eluted in fraction 18 during SCX as presented in Fig. 3. (**b**)The tandem mass spectrum of parent ion (*m/z* 624.32) eluted at 25.1 min during RPLC presented in (**a**). The peptide sequence derived from the membrane primary amine oxidase (Swiss Prot Accession Number Q16853) is listed on the spectrum. (**c**) Ratios of the signature iTRAQ™ reporter ions at *m/z* 114–117 in the tandem mass spectrum are measured for peptide quantification.

9. Perform database searching using PLGS to compare experimental MS/MS spectra of peptides with those predicted from a sequence database for peptide identification (see Note 32). The matched sequence of the MS/MS spectrum is included in Fig. 4b. We generally use SwissProt database, but any database of choice can be selected. Multiple peptide identifications are assembled into a global protein identification list.
10. iTRAQ™ Reagents produce diagnostic low mass MS/MS signature ions at *m/z* 114, 115,116, and 117 in the MS/MS spectrum (Fig. 4c). Compare intensities of these signature ions to obtain relative protein concentrations. The ratios of these diagnostic low-mass MS/MS signature ions are also calculated in PLGS software. The ratio of diagnostic ions in Fig. 4c was determined to be 1:0.7:1.7:1 (114:115:116:117).
11. After rendering of PLGS data, the results are exported and integrated in a spreadsheet.

4. Notes

1. We have used IEF and SDS-PAGE electrophoresis equipment from Bio-Rad and GE Healthcare, respectively. These experiments can be performed with instrumentation and reagents from other manufacturers as well.
2. In order to wash the IPG strips prior to assembly on the polyacrylamide gel, we modified a 50-mL plastic serological pipette by excising 3 cm from the back of the pipette and closing the tip with stretchable sealing film. This modified pipette is filled with 1× running buffer and acts as a reservoir to immerse the full length of the IPG strips in running buffer.
3. Upon adding water, the solution will cool down significantly. Do not use until buffer is completely dissolved and the solution is clear.
4. Pipette tips may get clogged during cell transfer. Alternatively, cutting the first 2–3 mm of a 1-mL pipette tip can aid in pipetting larger fragments of cell debris.
5. The amount of protein required in this step depends on the number of biological and technical replicates to be analyzed. An equal amount of protein from all biological replicates is pooled together to generate a pooled internal standard. The amount of protein and reagents listed in this protocol are needed for running one 2D-DIGE gel consisting of two samples and a pooled internal standard.
6. We generally load 50 μg of each protein sample and 50 μg of the pooled internal standard on each gel (150 μg total protein per gel). Our protein yield after protein precipitation is generally 50%. Therefore, the protocol includes 150 μg of starting protein mixture per sample.
7. The pH of the NaOH solution drops over time; thus, it is critical to check that pH is greater than 9.5 prior to use.
8. CyDyes are light sensitive; hence, avoid prolonged light exposure. Cover samples with aluminum foil during all incubation steps.
9. During IEF focusing, it is important to avoid introduction of any air bubbles. If air bubbles are present, gently move IPG strip using forceps to push out air bubbles. Be careful not to damage the gel strip.
10. DTT keeps proteins from forming disulfide bonds during IEF. DTT is placed on only the basic (negative electrode) side of the strip.
11. The gel solution needs to be poured quickly as it will begin to polymerize within 5–10 min after addition of APS.

12. The equilibration buffer can be stored at –20°C. The equilibration buffer will be cloudy until it has reached room temperature.
13. Any unused gels can be stored at 4°C for up to 1 week wrapped in plastic wrap and moist paper towels to prevent dehydration.
14. If running less than six gels, unoccupied slots are filled with blank cassettes.
15. For quality control and troubleshooting purposes, it is generally best to record current, voltage, and power throughout the gel run.
16. These gels are good for only a few days since the proteins are not fixed.
17. Generally, preparative gels containing 200 μg of pooled internal standard are run to facilitate MS analysis. Since we are using minimal labeling, and ≤5% of the protein sample is labeled with CyDyes, we stain these gels with SYPRO® Ruby after imaging of CyDyes is completed.
18. Once gels are fixed, they can be stored at 4°C until spot excision.
19. The gels shrink in fixing solution. Incubation in water will allow them to expand to their original size.
20. Free amines, detergents, and DTT present in the lysis buffer interfere with labeling, cysteine blocking, and trypsin digestion. Acetone precipitation removes these interfering substances.
21. The protein pellet may not dissolve completely. Sonication using a 3-mm tip can aid in dissolving the pellet. Also, 6 M urea can be added. However, it is important that the urea concentration must not exceed 1 M in the final sample solution.
22. It is important to use equal amount of protein from each sample for accurate peptide quantification using iTRAQ™. The exact amount of protein used is based on the amount of sample available and the experiment. We generally use 50–100 μg of each sample for global proteomics experiments.
23. The iTRAQ™ Reagent labeling method listed here is the general method used in our laboratory. We recommend consulting the user manual for iTRAQ™ labeling reagents for complete details on possible variations and compatibility of chemicals.
24. It is important that SDS concentration is <0.05% during trypsin digestion.
25. The urea will degrade at temperatures above 40°C and lead to precipitation of protein.

26. iTRAQ™ Reagents will hydrolyze in the presence of excess water. Therefore, the aqueous component must be below 40% of the total sample volume during the labeling reaction. Seventy microliters of organic solvent is added for labeling, thus we concentrate samples to ~25 μL.
27. It is generally best to run a blank and a quality control sample such as a tryptic digest of BSA to ensure the performance of the system prior to analyzing iTRAQ™ Reagent-labeled samples.
28. We are using an off-line 2D-LC approach where pooled peptide samples are first separated by charge using SCX chromatography. Each fraction is then further analyzed individually on an RPLC-MS/MS system.
29. For long-term storage of the column, flush column with a solution of 80% acetonitrile with 20% LC/MS-grade water. The column can then be removed from the system and stored at 4°C.
30. The fractions from SCX can be further diluted prior to LC-MS/MS if necessary. Dilution factors vary based on the amount of total protein utilized in the experiment, the amount of peptides in each fraction, and the sensitivity of the mass spectrometer used for analysis. We typically dilute these fractions further by a factor of 10–30 and perform multiple RPLC-MS/MS analyses.
31. Although we have used a LC-Q-TOF MS from Waters Corp., any LC-MS/MS system and associated software can be used for sample analysis and protein identification.
32. We use PLGS 2.3 (Waters Corp.) for data processing and database searching. Alternatively, Peaks 5.0 software (Bioinformatics Solutions) or Mascot (Matrix Science) together with iTRAQ™ quantification software such as i-Tracker (32) or Multi-Q (33) can be used.

References

1. Zuk, P. A., Zhu, M., Ashjian, P., De Ugarte, D. A., Huang, J. I., Mizuno, H., Alfonso, Z. C., Fraser, J. K., Benhaim, P., and Hedrick, M. H. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279–95.
2. Gimble, J. and Guilak, F. (2003) Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* **5**, 362–9.
3. Bunnell, B. A., Estes, B. T., Guilak, F., and Gimble, J. M. (2008) Differentiation of adipose stem cells. *Methods Mol Biol* **456**, 155–71.
4. Celis, J. E., Moreira, J. M., Cabezon, T., Gromov, P., Friis, E., Rank, F., and Gromova, I. (2005) Identification of extracellular and intracellular signaling components of the mammary adipose tissue and its interstitial fluid in high risk breast cancer patients: toward dissecting the molecular circuitry of epithelial-adipocyte stromal cell interactions. *Mol Cell Proteomics* **4**, 492–522.
5. DeLany, J. P., Floyd, Z. E., Zvonic, S., Smith, A., Gravois, A., Reiners, E., Wu, X. Y., Kilroy, G., Lefevre, M., and Gimble, J. M. (2005) Proteomic analysis of primary cultures of

human adipose-derived stem cells – modulation by adipogenesis. *Mol Cell Proteomics* **4**, 731–40.

6. Kim, W. S., Park, B. S., Kim, H. K., Park, J. S., Kim, K. J., Choi, J. S., Chung, S. J., Kim, D. D., and Sung, J. H. (2008) Evidence supporting antioxidant action of adipose-derived stem cells: protection of human dermal fibroblasts from oxidative stress. *J Dermatol Sci* **49**, 133–42.
7. Noel, D., Caton, D., Roche, S., Bony, C., Lehmann, S., Casteilla, L., Jorgensen, C., and Cousin, B. (2008) Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials. *Exp Cell Res* **314**, 1575–84.
8. Roche, S., Delorme, B., Oostendorp, R. A., Barbet, R., Caton, D., Noel, D., Boumediene, K., Papadaki, H. A., Cousin, B., Crozet, C., Milhavet, O., Casteilla, L., Hatzfeld, J., Jorgensen, C., Charbord, P., and Lehmann, S. (2009) Comparative proteomic analysis of human mesenchymal and embryonic stem cells: towards the definition of a mesenchymal stem cell proteomic signature. *Proteomics* **9**, 223–32.
9. Zvonic, S., Lefevre, M., Kilroy, G., Floyd, Z. E., DeLany, J. P., Kheterpal, I., Gravois, A., Dow, R., White, A., Wu, X., and Gimble, J. M. (2007) Secretome of primary cultures of human adipose-derived stem cells: modulation of serpins by adipogenesis. *Mol Cell Proteomics* **6**, 18–28.
10. Cravatt, B. F., Simon, G. M., and Yates, J. R., 3rd (2007) The biological impact of mass-spectrometry-based proteomics. *Nature* **450**, 991–1000.
11. Devarajan, P. (2007) Proteomics for biomarker discovery in acute kidney injury. *Semin Nephrol* **27**, 637–51.
12. Merchant, M. L. and Klein, J. B. (2007) Proteomics and diabetic nephropathy. *Semin Nephrol* **27**, 627–36.
13. Conrotto, P. and Souchelnytskyi, S. (2008) Proteomic approaches in biological and medical sciences: principles and applications. *Exp Oncol* **30**, 171–80.
14. Lambert, J. P., Ethier, M., Smith, J. C., and Figeys, D. (2005) Proteomics: from gel based to gel free. *Anal Chem* **77**, 3771–87.
15. Abu-Farha, M., Elisma, F., Zhou, H., Tian, R., Asmer, M. S., and Figeys, D. (2009) Proteomics: from technology developments to biological applications. *Anal Chem* **81**, 4585–99.
16. Malmstrom, J., Lee, H., and Aebersold, R. (2007) Advances in proteomic workflows for systems biology. *Curr Opin Biotechnol* **18**, 378–84.
17. Ong, S. E. and Mann, M. (2005) Mass spectrometry-based proteomics turns quantitative. *Nat Chem Biol* **1**, 252–62.
18. Fournier, M. L., Gilmore, J. M., Martin-Brown, S. A., and Washburn, M. P. (2007) Multidimensional separations-based shotgun proteomics. *Chem Rev* **107**, 3654–86.
19. O'Farrell, P. H. (1975) High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250**, 4007–21.
20. Gorg, A., Weiss, W., and Dunn, M. J. (2004) Current two-dimensional electrophoresis technology for proteomics. *Proteomics* **4**, 3665–85.
21. Wittmann-Liebold, B., Graack, H. R., and Pohl, T. (2006) Two-dimensional gel electrophoresis as tool for proteomics studies in combination with protein identification by mass spectrometry. *Proteomics* **6**, 4688–703.
22. Marouga, R., David, S., and Hawkins, E. (2005) The development of the DIGE system: 2D fluorescence difference gel analysis technology. *Anal Bioanal Chem* **382**, 669–78.
23. Kheterpal, I., Coleman, L., Ku, G., Wang, Z. Q., Ribnicky, D., and Cefalu, W. T. (2010) Regulation of insulin action by an extract of *Artemisia dracunculus* L. in primary human skeletal muscle culture – a proteomics approach. *Phytother Res* **24**, 1278–84.
24. Aebersold, R. and Mann, M. (2003) Mass spectrometry-based proteomics. *Nature* **422**, 198–207.
25. Gygi, S. P., Rist, B., Griffin, T. J., Eng, J., and Aebersold, R. (2002) Proteome analysis of low-abundance proteins using multidimensional chromatography and isotope-coded affinity tags. *J Proteome Res* **1**, 47–54.
26. Salim, K., Kehoe, L., Minkoff, M. S., Bilsland, J. G., Munoz-Sanjuan, I., and Guest, P. C. (2006) Identification of differentiating neural progenitor cell markers using shotgun isobaric tagging mass spectrometry. *Stem Cells Dev* **15**, 461–70.
27. Griffiths, S. D., Burthem, J., Unwin, R. D., Holyoake, T. L., Melo, J. V., Lucas, G. S., and Whetton, A. D. (2007) The use of isobaric tag peptide labeling (iTRAQ) and mass spectrometry to examine rare, primitive hematopoietic cells from patients with chronic myeloid leukemia. *Mol Biotechnol* **36**, 81–9.
28. Seshi, B. (2006) An integrated approach to mapping the proteome of the human bone marrow stromal cell. *Proteomics* **6**, 5169–82.

29. Thon, J. N., Schubert, P., Duguay, M., Serrano, K., Lin, S., Kast, J., and Devine, D. V. (2008) Comprehensive proteomic analysis of protein changes during platelet storage requires complementary proteomic approaches. *Transfusion* **48**, 425–35.
30. Wu, W. W., Wang, G. H., Baek, S. J., and Shen, R. F. (2006) Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D gel- or LC-MALDITOF/TOF. *J Proteome Res* **5**, 651–58.
31. Greengauz-Roberts, O., Stoppler, H., Nomura, S., Yamaguchi, H., Goldenring, J. R., Podolsky, R. H., Lee, J. R., and Dynan, W. S. (2005) Saturation labeling with cysteine-reactive cyanine fluorescent dyes provides increased sensitivity for protein expression profiling of laser-microdissected clinical specimens. *Proteomics* **5**, 1746–57.
32. Shadforth, I. P., Dunkley, T. P., Lilley, K. S., and Bessant, C. (2005) i-Tracker: for quantitative proteomics using iTRAQ. *BMC Genomics* **6**, 145.
33. Lin, W. T., Hung, W. N., Yian, Y. H., Wu, K. P., Han, C. L., Chen, Y. R., Chen, Y. J., Sung, T. Y., and Hsu, W. L. (2006) Multi-Q: a fully automated tool for multiplexed protein quantitation. *J Proteome Res* **5**, 2328–38.

Part III

Differentiation Methods: In Vitro and In Vivo Applications

Chapter 14

Adipogenic Differentiation of Adipose-Derived Stem Cells

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Abstract

The primary physiological function of adipose-derived stem cells (ASCs) is to differentiate into adipose tissue. It is now possible to isolate, expand, and cryopreserve ASC from adipose depots of many animal species. These ASC can be induced to undergo adipogenic differentiation in vitro by exposure to a cocktail of chemical agents or inductive growth factors. The current chapter describes methods to induce adipogenesis and to quantify this differentiation process in vitro.

Key words: Adipose-derived stem cells, Adipogenesis, Basic fibroblast growth factor, Bone morphogenetic protein, CCAAT/enhancer binding protein α, Cyclic adenosine monophosphate, Epidermal growth factor, Glucocorticoid receptor, Indomethacin, Insulin, Isobutylmethylxanthine, Peroxisome proliferator-activated receptor γ2, Rabbit serum

1. Introduction

There is abundant evidence that adipose-derived stem cells (ASCs) are responsible for regenerating adipocyte numbers in situ (1, 2). In vivo, mature adipocytes in human adipose depots have been estimated to display a half-life of 240–465 days based on mass spectroscopy measures of heavy water labeling (3) and a 10% turnover rate per year based on ^{14}C analyses (4). ASCs isolated from adipose depots in multiple species are readily available as outlined in the chapters of this textbook. The ASCs can serve as a tool for evaluating the mechanism(s) underlying adipogenesis from a molecular biological and pharmacological perspective. Furthermore, cryopreserved ASCs can be stored long term with minimal loss of differentiation potential, providing investigators with increased flexibility for experimental design. Adipogenic differentiation is induced in part through small molecules.

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These include agents capable of elevating cyclic adenosine monophosphate (cAMP) levels, such as forskolin and isobutylmethylxanthine (IBMX) or ligands of the nuclear hormone receptor family members, such as rosiglitazone and dexamethasone, agonists of the peroxisome proliferator-activated receptor γ2 (PPARγ2) and the glucocorticoid receptor (GR), respectively (5). The presence of peptide hormones, such as insulin, bone morphogenetic proteins (BMP), and basic fibroblast growth factor (bFGF), can further stimulate this process (6–8). This chapter will highlight a generalized protocol to promote ASC adipogenesis in vitro and provide qualitative and quantitative assays for monitoring this process.

2. Materials

2.1. Cells

Adipose-derived stem cells (ASC) derived from subcutaneous or visceral depots from human or other species. These can be freshly isolated or cultured cells thawed from cryopreserved stocks.

2.2. Supplies

1. Tissue culture flasks and plates.
2. Pipets and disposable reagents for medium preparation and distribution.
3. Filter units for medium preparation (0.2 μm).
4. Fetal bovine serum.
5. Rabbit serum.
6. Dulbecco's Modified Eagles Medium (DMEM)/Ham's F-12 Medium.
7. Antibiotic/antimycotic solution (100×).
8. Biotin.
9. Dexamethasone (water soluble).
10. d-Pantothenate.
11. Human insulin (cell culture grade).
12. Isobutylmethylxanthine (IBMX).
13. Dimethylsulfoxide.
14. Rosiglitazone (or alternative PPARγ ligand such as ciglitazone, pioglitazone, troglitazone).
15. Phosphate-buffered saline (PBS).
16. Oil Red O.
17. Trypsin.
18. RT-PCR primers and reagents.

19. RNA isolatin reagents.
20. Adipokine (adiponectin, leptin) ELISA kits.

2.3. Equipment

1. Inverted microscope – Nikon Eclipse TS100 with Epi-Fluorescence Attachment (Mercury Lamp Illuminator model name: C-SHG) (Nikon Instruments Incorporation, Melville, NY) and equipped with a camera photometric cool-snap (Nikon).
2. MetaMorph Imaging Software (Universal Imaging Corporation).
3. Water bath (37°C).
4. Centrifuge.
5. Biosafety hood.
6. CO_2 incubator.
7. Rocking platform.
8. Aspirator.
9. Plate reader for optical density measurements.
10. Hemocytometer.

2.4. Media Stock Solution (see Note 1)

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

1. *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× 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.
2. *Differentiation medium*: In advance, prepare and aliquot the following stock solutions and store frozen at –20°C until required.

   66 mM stock solution of biotin (2,000-fold concentration) dissolved in 1 N sodium hydroxide.

   34 mM stock solution d-pantothenate (2,000-fold concentration) dissolved in water.

   1 mM dexamethasone (1,000-fold concentration) dissolved in water or ethanol depending on its formulation.

   250 mM stock solution of IBMX (1,000-fold concentration) dissolved in dimethyl sulfoxide.

   200 μM stock solution of human insulin (2,000-fold concentration) dissolved in PBS.

   5 mM stock solution of rosiglitazone or equivalent PPARγ agonist dissolved in dimethyl sulfoxide.

   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-panthothenate, 5 μM rosiglitazone (or equivalent PPARγ2 ligand), 1 μM dexamethasone, 200 nM human insulin. Use this solution within 2 weeks of its preparation.

3. *Adipocyte maintenance medium*: This solution is prepared in an identical manner as Differentiation medium except that it does not contain either the IBMX or the PPARγ agonist; these two stock solutions should be omitted. Use this solution within 2 weeks of its preparation.
4. *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 stock solution. At the time of use, take 6 mL of Oil Red O stock solution. Add 4 mL of distilled water. Let stand 1 h at room temperature before use. Use this solution within 24 h of its preparation.

3. Methods

3.1. Adipocyte Differentiation

1. Plate fresh or thawed, cryopreserved ASCs (see Note 2) at a density of 5×10^3 or 10^4 cells/cm^2 (see Notes 3 and 4). When the cells reach between 80 and 90% confluence, the preadipocytes are induced to differentiate. Aspirate the medium, add a small volume (about 1.5 mL for a single well of a 6-well plate) of prewarmed PBS + 1% antibiotic to wash the cells, and then remove the PBS by aspiration (see Note 5). Next, add the differentiation medium.
2. 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 Notes 6 and 7). Then add a volume (2.5–3 mL for a 6-well plate) of adipocyte medium.
3. The adipocyte medium will be changed every 3 days until mature adipocytes are obtained (Day +9 to +12 differentiation for human ASC) (see Notes 8 and 9).

3.2. Fixation of Cells

1. After 12 days of differentiation, the cells can be fixed either using 10% formalin solution or 4% paraformaldehyde.
2. After removing the medium and washing the cells with PBS, immerse the cells in the fixative solution: 10% formalin or 4% paraformaldehyde for 30 min or 10 min, respectively.
3. Remove the fixative before staining (fixed cells can be stored at 4°C for as long as several months, although proceeding to

stain immediately is recommended since there is a risk of sample dehydration over time).

3.3. Oil Red O Staining

1. Add 50 µL Oil Red O to each well for 15 min at room temperature.
2. Rinse three times or more with 50 µL distilled water. The rinse should become completely clear (no red coloring). Do not rinse with a volume larger than 50 µL. 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.
3. At this stage, take photomicrographs of the stained cultures for records or publication purposes. To enhance the image, the cell nuclei can be counterstained with hematoxylin/eosin; however, do not do so if you intend to quantify the Oil Red O staining as outlined in the next steps.

3.4. Oil Red O Quantification

1. Elute the stain from the cells by adding 50 µL isopropanol per well. Elution is immediate.
2. Read the OD_{540} 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. Normalize the Oil Red O stain based on the number of cells per well.
3. Wells of ASCs maintained under identical culture conditions but left unstained can be harvested by trypsin digestion and the cell number calculated by Trypan Blue exclusion and cell count determination using a hemocytometer.

3.5. Adipocyte-Specific Gene and Protein Upregulation

1. Adipogenic induction also can be evaluated by lineage-specific gene and protein expression. To confirm adipogenesis, upregulation of adipogenic-specific genes can be examined by RT-PCR for the expression of aP2, LPL, leptin, etc. (a list of the human adipocyte-specific genes and their primer sequences are available in Table 1). The mRNA levels should be normalized relative to a "housekeeping" gene control, such as β-actin, cyclophilin B, or 18S RNA.
2. Alternatively, the induction of secreted adipokine proteins can be determined in conditioned medium using ELISA assays available from many commercial vendors. The levels of adiponectin or leptin in the conditioned medium should be normalized to the cell number. Representative wells of undifferentiated or adipocyte-differentiated ASCs should be harvested as outlined in Subheading 3.4, step 3 above for cell counts.

Table 1
Human adipogenic mRNA primers

aP2 (Fatty acid binding protein 4)	5′-TGGTTGATTTT CCATCCCAT-3′	5′-TACTGGGCCA GGAATTTGAT-3′	150 bp
Adiponectin	5′-GGCCGTGAT GGCAGAGAT - 3′	5′-TTTCACCGATG TCTCCCTTAGG-3′	88 bp
LPL	5′-GAGATTTCTC TGTATGGCACC-3′	5′-CTGCAAATGA GACACTTTCTC-3′	276 bp
Leptin	5′-GGCTTTGGCCC TATCTTTTC-3′	5′-GCTCTTAGAG AAGGCCAGCA-3′	325 bp
PPAR γ2	5′-GCTGTTATGGG TGAAACTCTG-3′	5′-ATAAGGTGGA GATGCAGGTTC-3′	325 bp

4. Notes

1. Before purchase, the fetal bovine serum should be assayed to test for its ability to support adipogenesis.
2. My cryopreserved ASCs do not differentiate as well as they did when freshly isolated; what happened to them? We have observed this in some, but not all, lots of human ASCs; however, we can only speculate as to the cause. We have found that supplementing the stromal medium with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) at concentrations of 1 ng/mL will reduce this problem. Both growth factors promote the proliferation of the human ASCs and, together, enhance the adipogenic response (6).
3. Does the size of the well or flask change the adipogenic process? Anecdotally, we have seen that adipogenesis is more robust in small-diameter plates. Furthermore, the adipogenesis is most pronounced in the center of a well or immediately adjacent to the wall of the well. While we can only speculate on the mechanism accounting for these observations, the same lot of ASC can show lower adipogenesis when plated in a large-diameter as compared with a small-diameter plate under otherwise identical culture conditions.
4. Can I change the seeding density? Some groups recommend seeding at a higher density of 30×10^3 ASC/cm^2. This allows the culture to reach confluence after an overnight incubation; adipogenic induction can commence the following day.
5. My adipocytes are detaching. Do not dry the well when changing the medium since adipocytes tend to float when new medium is added.

6. My medium is turning yellow! 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 ($pH \leq 6$), indicating the need for an immediate change of the medium.
7. Some groups recommend allowing the ASC to remain in adipogenic differentiation medium for 7 days without changing the medium. This reduces the effort and cost involved. Not everyone washes the cultures with PBS + antibiotic/antimycotic, and this is not a critical step. Whatever you do, do not allow the ASCs to go dry during the medium changing process as this may reduce the adhesion of the cell sheet to the plastic culture surface.
8. Some groups recommend maintaining the ASC in adipocyte maintenance medium for 7 days without changing; this procedure follows the 7-day adipogenic differentiation medium incubation described in Note 7 above. Again, this longer incubation period reduces costs and effort. Furthermore, eliminating additional manipulation of the cultures has the potential to reduce the risk of bacterial or yeast contamination.
9. My cells do not differentiate very well. The differentiation process is donor 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 culture from older people. To further enhance adipogenesis, the following alternatives are proposed:
   (a) You may try different PPARγ agonists (troglitazone, pioglitazone, indomethacin, among others).
   (b) 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 and this may increase differentiation, particularly in ASC derived from nonhuman species (9, 10).
   (c) 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. Martin RJ, Hausman GJ, Hausman DB (1998) Regulation of adipose cell development in utero. Proc Soc Exp Biol Med **219**(3):200–10.
2. Hausman GJ, Hausman DB (2006) Search for the preadipocyte progenitor cell. J Clin Investig **116**(12):3103–7.
3. Strawford A, Antelo F, Christiansen M, Hellerstein MK (2004) Adipose tissue triglyceride turnover, de novo lipogenesis, and cell proliferation in humans measured with 2H2O. Am J Physiol Endocrinol Metab **286**(4): E577–88.
4. Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, Blomqvist L, Hoffstedt J, Naslund E, Britton T, Concha H, Hassan M, Ryden M, Frisen J, Arner P (2008) Dynamics of fat cell turnover in humans. Nature **453**:783–7.
5. 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.
6. Hebert TL, Wu X, Yu G, Goh BC, Halvorsen YD, Wang Z, Moro C, Gimble JM (2009) Culture effects of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) on cryopreserved human adipose-derived stromal/stem cell proliferation and adipogenesis. J Tissue Eng Regen Med **3**(7):553–61.
7. Bowers RR, Lane MD (2007) A role for bone morphogenetic protein-4 in adipocyte development. Cell Cycle **6**(4):385–9.
8. 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.
9. 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.
10. Vidal MA, Kilroy GE, Lopez MJ, Johnson JR, Moore RM, Gimble JM (2007) Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells. Vet Surg **36**(7):613–22.

Chapter 15

Three-Dimensional Culture Systems to Induce Chondrogenesis of Adipose-Derived Stem Cells

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Abstract

Stem cells can easily be harvested from adipose tissue in large numbers for use in tissue-engineering approaches for cartilage repair or regeneration. In this chapter, we describe in vitro tissue-engineering models that we have used in our laboratory for the chondrogenic induction of adipose-derived stem cells (ASC). In addition to the proper growth factor environment, chondrogenesis requires cells to be maintained in a rounded morphology in three-dimensional (3D) culture, and thus properties of the biomaterial scaffold also play a critical role in ASC differentiation. Histologic and immunohistologic methods for assessing chondrogenesis are also presented. In general, 10–12 weeks are required to assess ASC chondrogenesis in these model systems.

Key words: Adipose-derived stem cells, Chondrogenesis, Tissue engineering, Alginate, Agarose, Pellet culture

1. Introduction

Damage to articular cartilage, either through trauma or degenerative joint disease, poses tremendous challenges to the medical community, as cartilage exhibits little to no intrinsic repair capabilities. Much effort has gone into the development of methods to promote regeneration and/or repair of damaged tissue through microfracture of the subchondral bone, abrasion arthroplasty, or the transplantation of autologous or allogeneic osteochondral grafts (1–11). While the early results from these clinical procedures have been somewhat encouraging, complete regeneration and repair of cartilage has yet to be achieved, and long-term outcomes show little difference between the microfracture technique and autologous chondrocyte implantation (12). The most

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common form of arthritis is osteoarthritis, which results in a degeneration of the articular surfaces of cartilage. With direct and indirect costs of treatment of osteoarthritis escalating to greater than $65 billion annually (13), approaches that result in regeneration of cartilage and improved patient outcomes are welcomed. To this end, one promising approach for cartilage tissue regeneration is through a tissue-engineering approach employing adipose-derived stem cells (ASCs), as these cells can be autologously harvested in large numbers and have demonstrated the ability by many investigators to be capable of chondrogenic differentiation (14–25). This protocol presents methods that have been used by us and others to induce chondrogenesis of ASCs in 3D culture systems using either pellet culture or encapsulation in hydrogels such as agarose or alginate. These culture systems maintain the cells in a rounded shape, which appears to be critical for chondrogenesis and prevention of a fibroblastic phenotype.

2. Materials

2.1. Cells

ASCs derived from subcutaneous adipose liposuction aspirates (see Chapter 2) (See Notes 1, 2 and 6.).

2.2. Supplies

2.2.1. Alginate Bead Culture

1. Alginate.
2. Sodium chloride.
3. Sodium citrate trisodium salt dihydrate.
4. Calcium chloride.
5. Sterile syringe filter (0.22 mm).
6. 150-mL 0.22-μm filter system.
7. 50-mL Polypropylene centrifuge tubes, sterile.
8. 24-well plate, with lid, flat bottom, ultra-low attachment surface.
9. Micro stir bars (12.7 mm × 3 mm).

2.2.2. Agarose Culture

1. Type VII low-melting point agarose.
2. Phosphate-buffered saline.
3. Sterile syringe filter (0.22 mm).
4. 15-mL Polypropylene centrifuge tubes, sterile.
5. 24-well plate, with lid, flat bottom, Ultra-low attachment surface.
6. Micro stir bars (12.7 mm × 3 mm).
7. Multiple needle gauges for drawing and dispensing agarose.
8. Mold for agarose gel. Note: molds vary from a simple petri dish, wells of a specific size plate, or a gel tray to a complex

custom-designed mold with a more complex, or specifically defined shape for its intended purposes (26–28).

9. Skin biopsy punches of desired size (used for punching specific-sized constructs for culture).

2.2.3. Pellet Culture

1. 15-mL polypropylene centrifuge tubes, sterile.

2.2.4. Chondrogenic Induction

1. Dulbecco's Modified Eagles Medium–high glucose (DMEM-HG).
2. ITS + supplement.
3. Dexamethasone.
4. L-Ascorbic acid 2-phosphate sesquimagnesium salt.
5. Penicillin/streptomycin.
6. Transforming growth factor (TGF) β-3.
7. Bone morphogenetic protein (BMP)-6.
8. Siliconized 200-μL pipette tips.
9. Siliconized 0.6-mL Snap-Cap microtubes.
10. 15-mL Polypropylene centrifuge tubes, sterile.
11. 50-mL Polypropylene centrifuge tubes, sterile.

2.2.5. dsDNA Quantitation

1. PicoGreen dsDNA Quantitation kit (Invitrogen) or equivalent.

2.2.6. Fixation

1. 16% Paraformaldehyde.
2. Sodium cacodylate, trihydrate.
3. Barium chloride.

2.2.7. Histology

1. Xylene.
2. Tissue-embedding medium (paraffin).
3. Histology cassettes.
4. Microscope slides.
5. Safranin-O.
6. Fast Green FCF.
7. Weigert hematoxylin solution.
8. Differentiation solution.
9. Mounting solution.

2.2.8. Immunohistochemistry

1. Immunohistochemical staining kit.
2. Type II collagen antibody.
3. Type I collagen antibody.
4. Type X collagen antibody.
5. 2-B-6 Chondroitin-4-sulfate antibody.

6. 3-B-3 Chondroitin-6-sulfate antibody.
7. Xylene.
8. AEC substrate–chromagen.
9. Pepsin.
10. Chondroitinase ABC.
11. PAP pen.
12. Methanol.
13. Hydrogen peroxide.
14. Goat serum.
15. GVA Mounting Medium (Invitrogen) or equivalent.

2.3. Equipment

1. Centrifuge.
2. Water bath shaker.
3. Microplate reader (for fluorescence-based dsDNA quantitation).
4. Microtome.
5. Hot plate with temperature control.

2.4. Stock Solutions

1. Cell differentiation: incomplete chondrogenic medium (Table 1): may be stored at 4°C in the dark for up to 6 weeks. Complete chondrogenic medium: growth factors are thawed and added freshly to incomplete chondrogenic medium just before use. Three different growth factor combinations have been used successfully to promote ASC chondrogenesis

Table 1
Incomplete chondrogenic medium

Component	Concentration
DMEM-HG	~89%
Fetal bovine serum	10%
ITS+	6.25 µg/mL bovine insulin 6.25 µg/mL transferrin 6.25 µg/mL selenous acid 5.33 µg/mL linoleic acid 1.25 mg/mL BSA
Dexamethasone	100 nM
Penicillin/streptomycin/ Fungizone:	1× (100 µg/mL streptomycin, 100 U/mL penicillin, 250 ng/mL Fungizone)

Table 2
Growth factor combinations for ASC chondrogenesis[a]

Options	Growth factors[a]	Final concentration	References
1	TGF-β1	10 ng/mL	(14, 16, 23)
2	BMP-6	500 ng/mL	(25, 32)
3	TGF-β3 BMP-6	10 ng/mL 10 ng/mL	(33)

[a] Siliconized tips and tubes should be used when working with growth factors. This will minimize binding of the growth factors to the pipettes and tubes and therefore ensure greater accuracy for the experiment

(Table 2). Additionally, 37.5–50 μg/mL L-ascorbic acid 2-phosphate is added freshly to overcome the instability of L-ascorbic acid 2-phosphate in culture medium. Stock 100x L-ascorbic acid 2-phosphate can be made in DMEM-HG and stored in frozen aliquots for up to 6 weeks at −20°C.

2. Alginate culture: 1.2% (w/v) alginate solution in 150 mM saline solution. Heat on a hot plate and stir thoroughly. Care should be taken that the solution is not boiled or that it is not overly heated such that excess evaporation occurs. Filter sterilize using a 0.22-μm filter and store at 4°C. Alternatively, use aseptic technique in a sterile hood to avoid the need for filter sterilization.
   Calcium chloride (102 mM) solution in dH_2O: filter sterilize using a 0.22-μm filter and store working solution (102 mM) at 4°C.
   Saline (150 mM) solution in dH_2O: filter sterilize using a 0.22-μm filter and store at 4°C.
3. Agarose culture (2% (w/v) type VII agarose in D-PBS): Heat on a hot plate or in the microwave, taking care not to allow excess evaporation to take place during heating. Once the agarose is in solution, quickly filter sterilize the solution using a 0.22-μm syringe filter. Note that if excessive cooling occurs, it will not be possible to sterilize the agarose in this manner. If this is the case, reheat the solution and reattempt the filter sterilization. Maintain the sterilized agarose at ~39 ± 2°C for encapsulation of cells.
4. Papain digestion solution: 125 μg/mL papain, 0.1 M sodium phosphate ($NaH_2PO_4 \cdot H_2O$), 5 mM EDTA ($C_{10}H_{16}N_2O_8$), and 5 mM cysteine hydrochloride ($HSCH_2CH(NH_2)COOH \cdot HCl$) in dH_2O. Note: EDTA requires a pH > 8.0 to go into solution. Therefore increase the pH to 8.0 to dissolve all components and then titrate the solution to pH of 6.5 with HCl prior to use.

5. Sample fixative for histology/immunohistochemistry: 4% paraformaldehyde, 100 mM sodium cacodylate; if alginate is being fixed, add 50 mM $BaCl_2$ to irreversibly cross-link the alginate matrix. Titrate the solution with 1 N sodium hydroxide to a pH of 7.4. Caution: paraformaldehyde and sodium cacodylate are considered carcinogenic. Only handle these reagents in a biosafety level 3 cabinet with the sash adjusted to the proper height to provide maximum protection to the user.

3. Methods

3.1. Pellet Culture (2 h to Set Up on Day of Experiment)

1. Following trypsinization of the cells, split desired number of cells (250,000 per pellet) to 15-mL tubes designated for either negative control or chondrogenic conditions (See Note 3.).
2. Resuspend cells in either incomplete (control) or complete chondrogenic medium at a density of 500,000 cells/mL.
3. Pipette 0.5 mL of cell suspension into 15-mL polypropylene conical tubes. This yields 250,000 ASCs per tube.
4. Centrifuge at 300 × *g* at 21°C for 5 min to form a pellet at the bottom of the tube.
5. Loosen the tops of the conical tubes for gas exchange. Incubate cultures at 37°C and 5% CO_2.
6. The following day, the pellets will become rounded in the bottom of the tube (Fig. 1a).
7. Every other day, for the duration of the experiment, prepare complete chondrogenic medium by adding growth factors

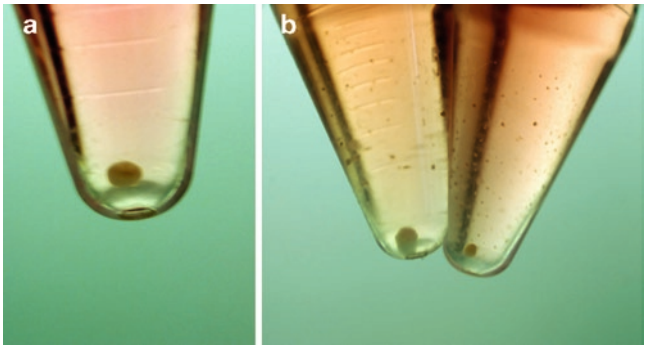

Fig. 1. (**a**) ASC pellet 24 h after centrifugation in the bottom of a 15-mL conical tube. (**b**) *Left*: chondrogenic and *Right*: control pellets after 8 weeks of in vitro culture. Note: magnifications differ between (**a**) and (**b**). Adapted from Estes BT, Diekman BO, Gimble JM, Guilak F. Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. Nat Protoc. 2010 Jul;5(7):1294-311. PubMed PMID: 20595958 (34).

and L-ascorbic acid 2-phosphate and exchange medium. Typical durations are 2, 4, 6, and 8 weeks.

8. At each medium exchange, agitate tube gently to ensure the pellet has not adhered to the wall of the tubes. See Fig. 1b for an example of chondrogenic- vs. control-induced ASC pellets after 8 weeks in culture.
9. To harvest, remove the chondrogenic medium and wash the pellets once with D-PBS. Fix the pellet in the paraformaldehyde solution for immunohistochemistry and histology or digest the pellet in a papain solution for biochemical analysis.
10. Incomplete chondrogenic medium (Table 1) can be used as a negative control. Complete chondrogenic medium (Table 2) with ASCs known to be capable of chondrogenesis may be used as a positive control.

3.2. Making Alginate Beads (4 h to Set Up on Day of Experiment)

1. Warm alginate and $CaCl_2$ to 37°C prior to encapsulation of ASCs.
2. Resuspend ASCs in 1.2% alginate solution at 5×10^6 cells/mL in a 50-mL conical tube. Mix thoroughly by pipetting without creating bubbles, or mix with the use of micro stir bars using a magnetic stirring plate. Note: if the latter technique is used for mixing, a volume of greater than 700 µL is required to avoid making bubbles (Fig. 2) (See Note 3).
3. Draw 1 mL of solution into tip of a 1-mL pipette. Note: if larger beads are desired, the tips of the pipettes may be cut with a scalpel (Fig. 3a) prior to pipetting to increase the size of the alginate drops.
4. Dispense by tilting the pipette sideways and slowly pipetting cell suspension such that one drop falls from the pipette tip

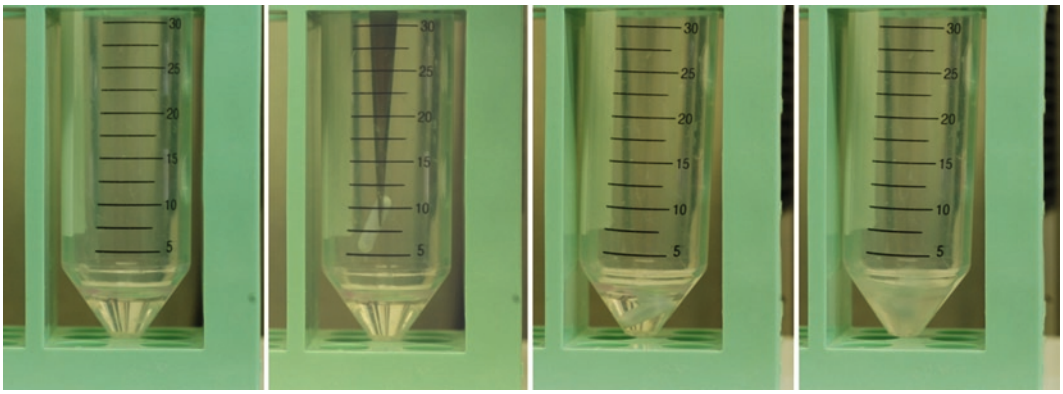

Fig. 2. (**a**) ASC pellet in alginate before mixing, (**b**) micro stir bar introduced to alginate, (**c**) micro stir bar prior to mixing, and (**d**) micro stir bar during mixing. Note that no bubbles are introduced in 2 mL of solution using this method. Note: as little as ~700 mL can be used with this method without introducing bubbles.

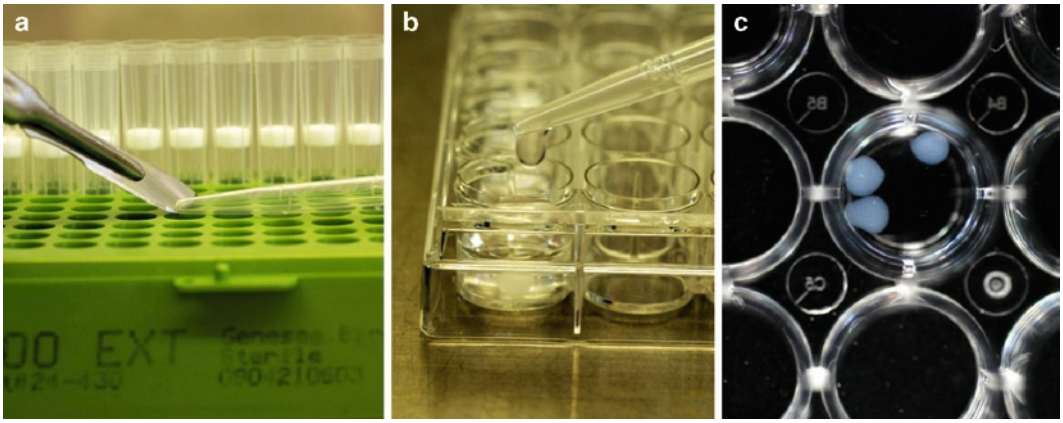

Fig. 3. (**a**) Preparation of pipette tip for making alginate beads – the size of the bead can be controlled depending on where the tip is cut. (**b**) Dropping alginate beads into 102 mM $CaCl_2$, and (**c**) cross-linked alginate beads in a 24-well plate.

into 1 mL of prewarmed $CaCl_2$. Typically, three drops of alginate are added to each well of a low-attachment surface 24-well plate (See Note 5). Note: the alginate bead diameter will vary from ~4 to 5 mm and will contain approximately 275,000 cells. Five to six wells can be filled per milliliter of alginate (see Fig. 3b, c).

5. Incubate the alginate beads at 37°C for 5 min to allow Ca^{2+} cations to fully diffuse through the alginate and cross-link the alginate cell suspension.
6. Pipette off the $CaCl_2$ solution. Note: aspiration can be used, but care must be taken to avoid suction pressure on the alginate beads.
7. Wash the beads with 1.5 mL incomplete chondrogenic medium at 37°C for 15 min. Pipette off incomplete medium and repeat for an additional 15 min at 37°C.
8. Replace incomplete medium with complete chondrogenic medium with 1 mL/well in a 24-well plate. Note: typically 1 mL of medium is used for every 800,000–1,000,000 cells. A typical culture in one well of a 24-well plate contains three beads (Fig. 3c).
9. Incubate at 37°C, 5.0% CO_2. Every other day for the duration of the experiment, prepare complete chondrogenic medium by adding growth factors and L-ascorbic acid 2-phosphate and exchange medium. Typical durations are 2, 4, 6, and 8 weeks.
10. Incomplete chondrogenic medium can be used as a negative control. Complete chondrogenic medium with ASCs known to be capable of chondrogenesis may be used as a positive control.

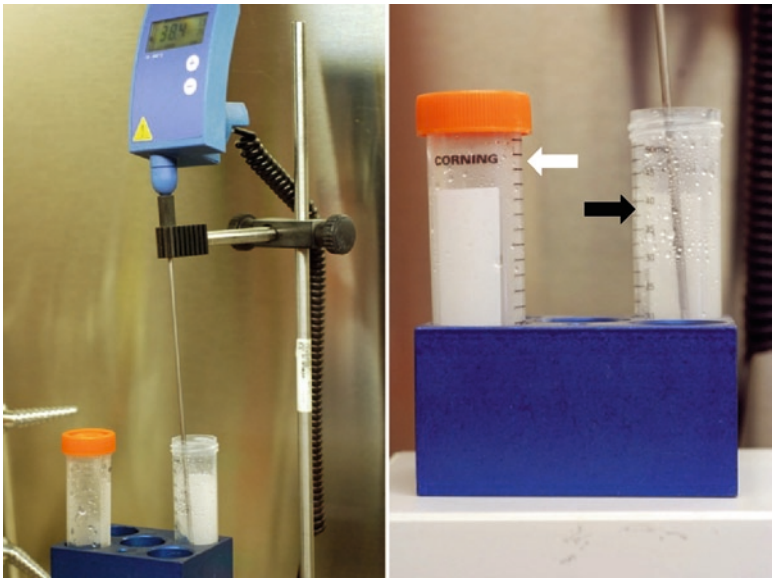

Fig. 4. Setup for agarose/ASC construct preparation. Magnetic stirrer with hot plate is configured with temperature feedback control: agarose temperature control solution (*black arrow*) and sterile agarose to be used for experiment (*white arrow*).

3.3. Making Agarose Discs (4 h to Set Up on Day of Experiment)

1. Molten agarose should be maintained at 39 ± 2°C prior to encapsulation of ASCs. A magnetic stirrer with hot plate with feedback control may be used to precisely control the temperature (e.g., Fig. 4).
2. Resuspend ASCs in the 2% agarose solution (temperature = ~39°C) (See Note 4) at 5×10^6 cells/mL in a 50-mL conical tube. Mix thoroughly by pipetting without creating bubbles, or mix with the use of micro stir bars using a magnetic stirrer with hot plate (e.g., same process as used with alginate in Fig. 2). Note: if the latter technique is used for mixing, a volume of greater than 700 µL is required to avoid making bubbles. Work quickly while keeping the tube on the hot plate as much as possible during cell mixing to avoid premature gelation (See Note 3).
3. Quickly draw the molten agarose/cell suspension into a syringe using a 14-gauge needle or larger and add to ASC pellet (Fig. 5a). Dispense agarose into desired mold for gelation using an ~18-gauge needle (Fig. 5b).
4. Allow agarose to gel for 10 min at room temperature (RT) in mold.
5. The gel (e.g., Fig. 5c) may be cut with a sterile biopsy punch if desired (Fig. 6a–c). Typically, we have cut our agarose slabs with 6-mm-diameter biopsy punches.

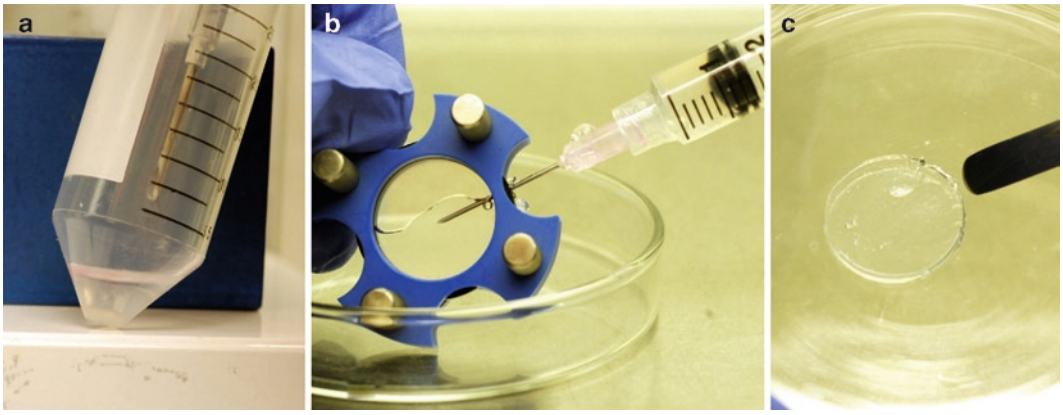

Fig. 5. Preparation of agarose cultures. (**a**) Dispensing 2% agarose with a 14-gauge needle onto an ASC pellet. (**b**) Injecting molten agarose into custom mold. (**c**) Agarose/ASC gel after 10 min gelation at RT.

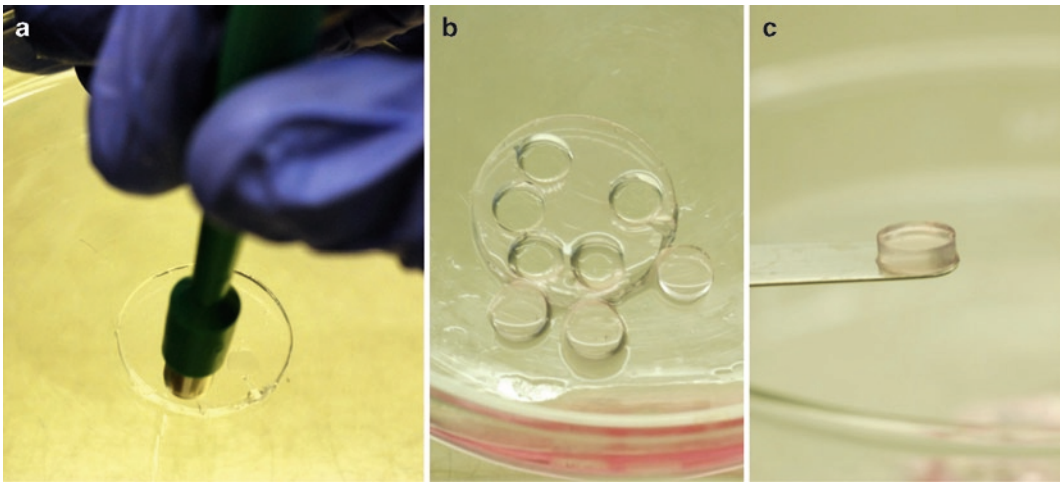

Fig. 6. (**a**) Punching 6-mm-diameter discs with a skin biopsy punch. (**b**, **c**) ASC-seeded agarose discs (6-mm diameter) after punching.

6. Add 1 mL (or appropriate volume) of complete chondrogenic medium or control medium (i.e., incomplete medium) at 1 mL/well in a 24-well plate. Note: typically 1 mL of medium is used for every 800,000–1,000,000 cells. Figure 7 shows 6-mm-diameter ASC-seeded agarose discs in a 24-well plate (See Note 5).
7. Incubate at 37°C, 5.0% CO_2. Every other day for the duration of the experiment, prepare complete chondrogenic medium by adding growth factors and L-ascorbic acid 2-phosphate and exchange medium. Typical durations are 2, 4, 6, and 8 weeks.
8. Incomplete chondrogenic medium can be used as a negative control. Complete chondrogenic medium with ASCs known to be capable of chondrogenesis may be used as a positive control.

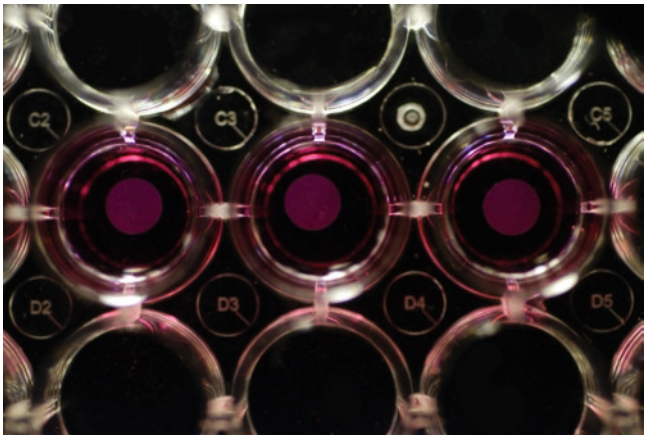

Fig. 7. ASC-seeded agarose discs (6-mm diameter) in a 24-well plate in 1 mL of medium/well.

3.4. Total dsDNA Determination

1. Samples (See Note 3) are harvested at the appropriate time points and are digested in 1 mL of papain solution (for alginate beads, three beads/well = one construct) for 15–18 h at 65°C. If desired, digested samples can be stored at –20°C and then thawed for analysis. Note: If wet weight is desired for normalization, weigh the samples before digestion in papain.
2. Determine total DNA per construct using the PicoGreen dsDNA quantitation kit per the instructions from the manufacturer. Total dsDNA can then be used, for example, to normalize for total glycosaminoglycan and collagen content (methods not presented herein).

3.5. Paraffin Embedding and Sectioning (1 h to Fix Samples Overnight, 1 Day to Embed Samples, 15 min/sample for Sectioning, Several Days for Staining)

1. Immediately following the culture period, place each construct (three beads = one construct for alginate beads) in 20 mL of the paraformaldehyde solution. Fix for 4 h at RT or overnight at 4°C.
2. Dehydrate constructs with 30% EtOH for 30 min, 50% EtOH for 30 min, 70% EtOH for 30 min (note: store long term in 70% if not continuing processing), 80% EtOH for 30 min, 100% EtOH for 30 min, followed by one additional 100% EtOH wash (note: can store overnight at 100% EtOH).
3. Clear constructs by removing 50% of solution and replacing with xylene yielding a final concentration of 50% EtOH/50% xylene. Incubate 30 min at RT. Replace this 1:1 mixture with 100% xylene and incubate 30 min at RT. Exchange 100% xylene and incubate for an additional 30 min at RT. Note: ensure that constructs are translucent. If not clear, continue processing with xylene washes until constructs become clear to translucent.

4. Embed the constructs in paraffin by removing half of the xylene and replacing it with paraffin such that the final concentration is 50% xylene/50% paraffin. Incubate at 60°C for 1 h. Note: work quickly with the paraffin to ensure that the paraffin does not have time to solidify. If the paraffin solidifies, incubation times must increase to properly infiltrate the construct with paraffin. Replace 1:1 mixture with 100% paraffin and incubate at 60°C for 1 h; replace paraffin again with 100% paraffin and incubate again at 60°C for 1 h. Note: avoid incubation with molten paraffin for longer times than are needed for complete infiltration as excessive time at 60°C may denature and obscure detection of epitopes needed for immunohistochemistry. Place in embedding tray in desired orientation (attempt to get all three alginate beads on the same plane for subsequent sectioning) and allow to harden overnight.
5. Cut sections 6–10 μm in thickness with a microtome and place in a 45–50°C waterbath.
6. We recommend placing sections on Superfrost®/Plus Microscope Slides (Fisher Scientific or equivalent) by using slides to remove from waterbath. Allow slides to dry overnight in a 37°C slide warmer. Note: these recommended slides have been surface treated for improved adherence of tissue sections to slides during processing.

3.6. Safranin-O/Fast Green

1. For Safranin-O/Fast Green staining,[1] deparaffinize sections in xylene three times for 3 min each and rehydrate in 100% EtOH twice for 5 min, 95% EtOH once for 2 min, and 70% EtOH once for 2 min followed by a wash in tap water for 30 s.
2. Stain in Weigert hematoxylin solution for 8 min and then wash in tap water for 3 min by repeatedly dipping slides.
3. Differentiate in differentiation solution for 30 s (until sections turn a blue hue) and then wash in tap water for 10 min by repeatedly dipping slides (until sections turn a blue hue).
4. Stain in 0.02% (w/v) aqueous Fast Green for 3 min, rinse for approximately 10 s in 1% acetic acid, dip the slides in tap water briefly, and then remove excess water from the slide.
5. Stain in 0.1% (v/v) aqueous Safranin-O (See Note 7) for up to 5 min maximum, rinse in 100% EtOH to remove extra red staining on the slide.
6. Dehydrate in 95% EtOH twice for 5 min, followed by 100% alcohol twice for 5 min before clearing in xylene three times for 2 min and mounting with appropriate mounting solution.

[1] Nuclei are stained black or dark blue. Bone, muscles (collagen) are stained green, and cartilage is stained orange or red.

3.7. Immunohistochemistry

1. For immunohistochemistry, we recommend use of Zymed Histostain® Plus Broad Spectrum or equivalent.
2. Deparaffinize by washing slides three times for 2 min/wash in xylene followed by dipping the slides in 100% EtOH for two washes of 2 min each. After slide dries, circle the section with the PAP pen.
3. Rehydrate slides: 95% EtOH for two washes of 2 min each; 80% EtOH for one wash of 2 min; 50% EtOH for one wash of 2 min; and D-PBS for one wash of 5 min.
4. Quench endogenous peroxidase activity by submerging slides in 1 part 30% H_2O_2 to 9 parts methanol for 10 min before washing slides in D-PBS: three washes of 2 min each.
5. For antigen retrieval, add sufficient pepsin to completely cover each tissue section. Incubate at RT for 5 min. Wash slides in D-PBS: three washes of 2 min each.
6. If using antibodies for labeling chondroitin sulfate epitopes, apply chondroitinase ABC to each section and incubate at RT for 20 min. If not labeling for chondroitin sulfate, proceed to blocking step. Wash slides in D-PBS: three washes of 2 min each. Block sections with serum blocking agent. Add enough reagent to completely cover tissue section (2–3 drops). Incubate at RT for 30 min (ensure sections do not dry). Blot excess serum from bottom of inclined slide (do not rinse).
7. Dilute primary antibody in nonimmune serum or 10% goat serum. Note: use recommended dilutions from the manufacturer of the antibody. Add antibody onto (+) staining sections, and blocking serum onto (−) control sections. Incubate at RT for 1 h or overnight at 4°C in a large plastic petri dish lined with wet filter paper to keep slides moist. Wash slides in D-PBS: three washes of 2 min each.
   Apply appropriate secondary antibody to cover tissue sections (two drops). Incubate at RT for 10 min. Wash slides in D-PBS: three washes of 2 min each. Apply enough enzyme conjugate to cover tissue sections (two drops). Incubate at RT for 10 min. Wash slides in D-PBS: three washes of 2 min each. Add enough substrate chromagen (e.g., AEC) mixture to cover tissue (two drops). Incubate at RT for 20 min. Gently dip sections in dH_2O (leave wet).
8. Counterstain (optional, stains cell nuclei). Add 1–2 drops hematoxylin to counterstain nuclei. If slides are not counterstained proceed to wash step with dH_2O. Note: for tissue-engineering studies, hematoxylin can sometimes obscure the intracellular and pericellular detection of matrix proteins and therefore should be used with caution. Incubate at RT for

5 min. Wash with tap H_2O. Wash slides in D-PBS for 30 s or until slides turn a blue hue. Wash slides in dH_2O: three washes for 2 min each (let slides remain in H_2O until coverslips are placed). Mount slides by applying mounting medium to one slide at a time and place coverslips. Note: do not allow slides to dry before placing mounting medium.

3.8. Final Comments

This protocol primarily focuses on 3D culture systems for ASCs with primary qualitative assessment tools histology and immunohistochemistry to ascertain the degree of chondrogenesis. Other techniques, even though they are beyond the scope of this current protocol, may be appropriate for evaluating chondrogenesis. These assays include, but are not limited, to the following:

1. Dimethyl-methylene blue assay (DMB) to determine glycosaminoglycan content of the constructs (15).
2. Hydroxyproline assay to determine total collagen content of the constructs (15).
3. Quantitative PCR (qPCR) to evaluate transcription of growth factors and extracellular matrix proteins, which can provide early data on the differentiation profile of the ASCs (25, 28, 29).
4. Mechanical testing to ascertain the development of functional mechanical properties of the constructs during chondrogenesis (15, 30).

Finally, this protocol presents three different 3D culture conditions, which have been used by many groups to successfully differentiate ASCs along a chondrogenic lineage. The reader is reminded, however, that these are simple model systems that have been employed, but many other scaffolds have also been described and used successfully for these same purposes and should be considered when selecting a model culture system for chondrogenesis (29–31).

4. Notes

1. Isolation and expansion conditions for ASCs are extremely important (28). The user should familiarize himself/herself with the various techniques and procedures before proceeding with the chondrogenic differentiation protocols presented herein.
2. Depending on culture conditions, donor, cell passage, and serum used, it is not uncommon for a percentage of ASCs to undergo apoptosis or necrosis during culture. For this reason,

we recommend monitoring the dsDNA content as a function of time throughout the culture period.

3. The user is encouraged to maintain samples at day 0 for dsDNA content in addition to histologic and immunohistologic examination. These samples are valuable reference points for the evaluation of the efficacy of chondrogenic differentiation.
4. Care should be taken when encapsulating cells in agarose as too high of a temperature can result in immediate reduction in cell viability. We closely monitor the temperature of the agarose using a temperature probe in a control centrifuge tube, subjected to identical environmental conditions, and also containing an equal amount of agarose as the experimental, sterile agarose. This ensures knowledge of the precise temperature of the experimental agarose solution at all time points during cell encapsulation (Fig. 4).
5. Ultra-low attachment culture plates are used as ASCs that become detached from the matrix will attach to the bottom of standard tissue culture plastic and begin to proliferate. Within days, these cells will overgrow the surface of the well and become a significant variable in the experiment. Use of ultra-low attachment plates will not allow the cells to attach, and the cells can therefore be removed upon medium exchange. An alternate to using purchased ultra-low attachment plates is to coat the wells of standard tissue culture plastic with a thin layer of sterile agarose prior to the start of the experiment.
6. If the cells do not differentiate well, it may be due to a myriad of factors including donor, age of donor, and the specific lot of serum used. Consider combining lots of ASCs from different donors to minimize variation and screening additional serum lots to ascertain their effect on chondrogenesis. Using different growth factor conditions may also facilitate chondrogenesis (see Table 2).
7. Fresh Safranin-O should always be used for GAG staining, as old (or expired) solutions will result in reduced or no staining of the negatively charged matrix.
8. Safranin-O should not be used with alginate sections as the negatively charged alginate matrix will be heavily stained by the Safranin-O.
9. For immunohistochemistry, the user may consider increasing antibody specificity with an alternate secondary antibody as the secondary antibody supplied in immunohistochemistry kits are often broad spectrum in nature. (e.g., Sigma, B7151 diluted 1:400 in D-PBS with 1% BSA could be used as an alternate antibody).

Acknowledgments

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References

1. Aichroth, P.M., Patel, D.V., and Moyes, S.T. (1991). A prospective review of arthroscopic debridement for degenerative joint disease of the knee. *Int Orthop*, **15**, 351–55.
2. Aubin, P.P., Cheah, H.K., Davis, A.M., and Gross, A.E. (2001). Long-term followup of fresh femoral osteochondral allografts for posttraumatic knee defects. *Clin Orthop Relat Res*, S318–327.
3. Baumgaertner, M.R., Cannon, W.D., Jr., Vittori, J.M., Schmidt, E.S., and Maurer, R.C. (1990). Arthroscopic debridement of the arthritic knee. *Clin Orthop Relat Res*, **253**, 197–202.
4. Denoncourt, P.M., Patel, D., and Dimakopoulos, P. (1986). Arthroscopy update #1. Treatment of osteochondrosis dissecans of the knee by arthroscopic curettage, follow-up study. *Orthop Rev*, **15**, 652–657.
5. Emmerson, B.C., Gortz, S., Jamali, A.A., Chung, C., Amiel, D., and Bugbee, W.D. (2007). Fresh osteochondral allografting in the treatment of osteochondritis dissecans of the femoral condyle. *Am J Sports Med*, **35**, 907–914.
6. Friedman, M.J., Berasi, C.C., and Fox, J.M. (1984). Preliminary results with abrasion arthroplasty in the osteoarthritic knee. *Clin Orthop Relat Res*, **182**, 200–205.
7. Ghazavi, M.T., Pritzker, K.P., Davis, A.M., and Gross, A.E. (1997). Fresh osteochondral allografts for post-traumatic osteochondral defects of the knee. *J Bone Joint Surg Br*, **79**, 1008–1013.
8. Johnson, L.L. (2001). Arthroscopic abrasion arthroplasty: a review. *Clin Orthop Relat Res*, S306–S317.
9. Kish, G., Modis, L., and Hangody, L. (1999). Osteochondral mosaicplasty for the treatment of focal chondral and osteochondral lesions of the knee and talus in the athlete. Rationale, indications, techniques, and results. *Clin Sports Med*, **18**, 45–66, vi.
10. Steadman, J.R., Briggs, K.K., Rodrigo, J.J., Kocher, M.S., Gill, T.J., and Rodkey, W.G. (2003). Outcomes of microfracture for traumatic chondral defects of the knee: average 11-year follow-up. *Arthroscopy*, **19**, 477–484.
11. Steadman, J.R., Rodkey, W.G., and Rodrigo, J.J. (2001). Microfracture: surgical technique and rehabilitation to treat chondral defects. *Clin Orthop Relat Res*, S362–S369.
12. Knutsen, G., Drogset, J.O., Engebretsen, L., Grontvedt, T., Isaksen, V., Ludvigsen, T.C., Roberts, S., Solheim, E., Strand, T., and Johansen, O. (2007). A randomized trial comparing autologous chondrocyte implantation with microfracture. Findings at five years. *J Bone Joint Surg Am*, **89**, 2105–2112.
13. Jackson, D.W., Simon, T.M., and Aberman, H.M. (2001). Symptomatic articular cartilage degeneration: the impact in the new millennium. *Clin Orthop Relat Res*, S14–S25.
14. Awad, H.A., Halvorsen, Y.C., Gimble, J.M., and Guilak, F. (2003). Effects of transforming growth factor beta1 and dexamethasone on the growth and chondrogenic differentiation of adipose-derived stromal cells. *Tissue Eng*, **9**, 1301–1312.
15. Awad, H.A., Wickham, M.Q., Leddy, H.A., Gimble, J.M., and Guilak, F. (2004). Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials*, **25**, 3211–3222.
16. Erickson, G.R., Gimble, J.M., Franklin, D.M., Rice, H.E., Awad, H., and Guilak, F. (2002). Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. *Biochem Biophys Res Commun*, **290**, 763–769.
17. Guilak, F., Lott, K.E., Awad, H.A., Cao, Q., Hicok, K.C., Fermor, B., and Gimble, J.M. (2006). Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. *J Cell Physiol*, **206**, 229–237.
18. Halvorsen, Y.C., Wilkison, W.O., and Gimble, J.M. (2000). Adipose-derived stromal

cells – their utility and potential in bone formation. *Int J Obes Relat Metab Disord*, **24(Suppl 4)**, S41–S44.

19. Huang, C.Y., Reuben, P.M., D'Ippolito, G., Schiller, P.C., and Cheung, H.S. (2004). Chondrogenesis of human bone marrow-derived mesenchymal stem cells in agarose culture. *Anat Rec A Discov Mol Cell Evol Biol*, **278**, 428–436.
20. Safford, K.M., Hicok, K.C., Safford, S.D., Halvorsen, Y.D., Wilkison, W.O., Gimble, J.M., and Rice, H.E. (2002). Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochem Biophys Res Commun*, **294**, 371–379.
21. Wickham, M.Q., Erickson, G.R., Gimble, J.M., Vail, T.P., and Guilak, F. (2003). Multipotent stromal cells derived from the infrapatellar fat pad of the knee. *Clin Orthop Relat Res*, **412**, 196–212.
22. Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P., and Hedrick, M.H. (2002). Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell*, **13**, 4279–4295.
23. Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P., and Hedrick, M.H. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng*, **7**, 211–228.
24. Wang, D.W., Fermor, B., Gimble, J.M., Awad, H.A., and Guilak, F. (2005). Influence of oxygen on the proliferation and metabolism of adipose derived adult stem cells. *J Cell Physiol*, **204**, 184–191.
25. Estes, B.T., Wu, A.W., and Guilak, F. (2006). Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. *Arthritis Rheum*, **54**, 1222–1232.
26. Mauck, R.L., Soltz, M.A., Wang, C.C., Wong, D.D., Chao, P.H., Valhmu, W.B., Hung, C.T., and Ateshian, G.A. (2000). Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. *J Biomech Eng*, **122**, 252–260.
27. Hung, C.T., Lima, E.G., Mauck, R.L., Takai, E., LeRoux, M.A., Lu, H.H., Stark, R.G., Guo, X.E., and Ateshian, G.A. (2003). Anatomically shaped osteochondral constructs for articular cartilage repair. *J Biomech*, **36**, 1853–1864.
28. Estes, B.T., Diekman, B.O., and Guilak, F. (2008). Monolayer cell expansion conditions affect the chondrogenic potential of adipose-derived stem cells. *Biotechnol Bioeng*, **99**, 986–995.
29. Diekman, B.O., Estes, B.T., and Guilak, F. (2009). The effects of BMP6 overexpression on adipose stem cell chondrogenesis: interactions with dexamethasone and exogenous growth factors. *J Biomed Mater Res A*, **93**, 994–1003.
30. Cheng, N.C., Estes, B.T., Awad, H.A., and Guilak, F. (2009). Chondrogenic differentiation of adipose-derived adult stem cells by a porous scaffold derived from native articular cartilage extracellular matrix. *Tissue Eng A*, **15**, 231–241.
31. Moutos, F.T., Freed, L.E., and Guilak, F. (2007). A biomimetic three-dimensional woven composite scaffold for functional tissue engineering of cartilage. *Nat Mater*, **6**, 162–167.
32. Estes, B.T., Wu, A.W., Storms, R.W., and Guilak, F. (2006). Extended passaging, but not aldehyde dehydrogenase activity, increases the chondrogenic potential of human adipose-derived adult stem cells. *J Cell Physiol*, **209**, 987–995.
33. Hennig, T., Lorenz, H., Thiel, A., Goetzke, K., Dickhut, A., Geiger, F., and Richter, W. (2007). Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6. *J Cell Physiol*, **211**, 682–691.
34. Estes BT, Diekman BO, Gimble JM, Guilak F. Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. Nat Protoc. 2010 Jul;5(7):1294–311. PubMed PMID: 20595958.

Chapter 16

Neural Differentiation of Human Adipose Tissue-Derived Stem Cells

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Abstract

While adult stem cells can be induced to transdifferentiate into multiple lineages of cells or tissues, their plasticity and utility for human therapy remains controversial. In this chapter, we describe methods for the transdifferentiation of human adipose tissue-derived stem cells (ASCs) along neural lineages using in vitro and in vivo systems. The in vitro neural differentiation of ASCs has been reported by several groups using serum-free cytokine induction, butylated hydroxyanisole (BHA) chemical induction, and neurosphere formation in combination with the cytokines, such as brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor (bFGF). For in vivo neurogenic induction, ASCs are treated with BDNF and bFGF to form neurospheres in vitro and then delivered directly to the brain. In this chapter, several detailed protocols for the effective neurogenic induction of ASCs in vitro and in vivo are described. The protocols described herein can be applied to further molecular and mechanistic studies of neurogenic induction and differentiation of ASCs. In addition, these methods can be useful for differentiating ASCs for therapeutic intervention in central nervous system disorders.

Key words: Adipose tissue-derived mesenchymal stem cells, Transdifferentiation, Neurogenesis, Reprogramming, Neurosphere

1. Introduction

Tissue-specific stem cells, commonly termed adult stem cells, are derived from specific organs such as brain, gut, lung, liver, adipose tissue, and bone marrow in humans (1). It has become evident that these stem cells persist in several adult tissues, although they represent a rare population localized in small niches (2). Among tissue-specific stem cells, human adipose tissue-derived stem cells (ASCs) differentiate along multiple mesenchymal lineages, as well as the neural lineage (2–6). In addition, ASCs display self-renewal capacity and long-term viability (7). Thus, ASCs have emerged as

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a promising tool for therapeutic applications for regenerative medicine in tissue engineering as well as cell and gene therapy due to their multipotency and accessibility (3). However, the specific environmental cues that serve to induce the proliferation of ASCs by cytokines and growth factors are not well understood. Brain-derived neurotrophic factor (BDNF), interleukin (IL)-6, and vascular endothelial growth factor (VEGF) are some of the primary cytokines secreted by ASCs in culture (2–4). Together, these factors are known to act in synergy with other cytokines in vitro to promote the proliferation of multipotent hematopoietic progenitor cells and rat spinal cord-derived neural progenitor cells (NPCs) (8, 9). Together, these factors are known to act in synergy with other cytokines in vitro to promote the proliferation of multipotent hematopoietic progenitor cells and rat spinal cord-derived NPCs (10–12).

Although the classic definition of stem cell plasticity specifies the ability of cells to differentiate into a variety of cell lineages, the term is also currently applied to the ability of a given cell type to reciprocally de-differentiate, re-differentiate, and/or trans-differentiate in response to specific stimuli (28–29). ASC reprogramming also represents the withdrawal of cells from a given differentiated state into a stem cell-like state, a process that precedes reentry into the cell cycle (13) and transdifferentiation into different lineage of mature cell or tissue, including the neural lineages. Several cytokines have been associated with adult neurogenesis, and have been demonstrated to mediate neurogenesis after brain injuries such as trauma and ischemic damage. After ischemia in the adult brain, the regenerative process requires a close association between angiogenesis and neurogenesis at the lesion site (14, 15). Thus, cytokines or growth factors are strong candidates as injury-induced mitotic and neurogenic factors that are able to modulate the recovery of traumatic lesions in the brain.

Transdifferentiation of ASCs involves reprogramming. Cell reprogramming is characterized by the reversed differentiation of cells from a lineage-specific differentiated phenotype into a more embryonic stem cell-like phenotype; this is associated with pluripotency and reentry into the cell cycle. Transdifferentiation of ASCs can be identified by changes in cell morphology, chromatin remodeling, and gene expression. Ultimately, the reprogrammed ASC has the capability of differentiating into various cell types (4, 15). Before transdifferentiation into the neural lineage, ASCs transit through a more primitive stem cell state after exposure to various physical factors such as low oxygen or electric stimuli, or after exposure to a novel antioxidant that has been associated with cell de-aging and neural lineage differentiation. Previously, several groups hypothesized that the antioxidants selenium and 4-(3,4-dihydroxy-phenyl)-derivative (DHP-d), a botanical extract with powerful reactive oxygen specie (ROS) scavenger function,

were capable of inducing de-aging, cell reprogramming, improved proliferation, and differentiation of adult stem cells or ASCs into mesodermal, ectodermal, and endodermal lineages (1, 16). On the basis of these observations, we proposed that the combination of a natural antioxidant, VEGF, BDNF, and basic fibroblast growth factor (bFGF) would enhance cell reprogramming and transdifferentiation and improve the maintenance or induction of stem and/or precursor cells and mature neurons at a high ratio. Consistent with this, adult somatic stem cells have the capacity to participate in the regeneration of different tissues (17, 18), suggesting that restrictions on differentiation are not completely irreversible and can be reprogrammed with de-differentiation and trans-differentiation processes (16).

In this chapter, several detailed protocols for effective neurogenic induction of ASCs in vitro and in vivo are described. The protocols described herein can be applied to further molecular and mechanistic study of neurogenic induction and full differentiation of ASCs in vitro. In addition, these methods can be useful for differentiating ASCs for therapeutic intervention in central nervous system disorders (19, 20).

The approaches can be summarized in three basic procedures:

1. Neurogenic induction of cultured ASC cells in vitro.
2. Evaluation of neurogenic potency and efficiency.
3. In vivo neurogenic induction of ASC cells in the fetal mouse brain.

2. Materials

2.1. Preparation of ASC for Neural Differentiation

1. 0.075% Collagenase type 1A.
2. α-MEM, 10% fetal bovine serum (FBS), Hank's buffered salt solution (HBSS).
3. 100 U/mL penicillin, 100 μg/mL streptomycin.

2.2. Neuronal Induction: Serum-Free Induction

1. 2% B27 supplement.
2. Neurobasal media (NB).
3. 2 mM L-Glutamine.
4. 20 ng/mL Epidermal growth factor (EGF).
5. 20 ng/mL Basic fibroblast growth factor (bFGF).
6. Poly-D-lysine (PDL).
7. Laminin.
8. Chamber slide.
9. 10 ng/mL BDNF.

2.3. Neuronal Induction: Chemical

1. DMEM, DMEM/F12.
2. 200 mM Butylated hydroxyanisole (BHA).
3. 5 mM KCl.
4. 2 μM Valproic acid.
5. 10 μM Forskolin.
6. 1 μM Hydrocortisone.
7. Insulin.
8. 1 mM β-mercaptoethanol (BME).
9. 2% Dimethylsulfoxide (DMSO).
10. 200 μM Indomethacin.
11. 0.5 mM Isobutylmethylxanthine (IBMX).

2.4. Immunocytochemistry

1. 4% Paraformaldehyde in PBS.
2. 5% normal serum from the host of the secondary antibody.
3. Triton X-100.
4. Primary antibodies – see Table 1.

2.5. Western Blot Analysis

1. RIPA lysis buffer.
2. Sample buffer.
3. 10% SDS-PAGE reducing gels.
4. PVDF membrane.

Table 1
Common markers used to confirm neuronal induction

Cell marker	Specificity	Company	Dilution	Cat. #
Nestin	Neural stem cell marker	Chemicon	1:200	AB5922
NeuN	Early neuronal	Chemicon	1:100	MAB377
β-tubulin III	Early neuron	Chemicon	1:500	MAB1637
Tau	Mature neuronal	Abcam	1:50	Ab3931
MAP2	Mature neuronal	Sigma	1:500	M4403
NF-70	Mature neuronal	Chemicon	1:100	MAB5294
Trk-A	Neuronal	Chemicon	1:100	AB5372
Vimentin	Early glial	Sigma	1:50	V4630
GFAP	Mature glial (astrocyte)	Dako	1:500	Z0334
GalC	Oligodendrocyte lineage	Chemicon	1:100	MAB342
O4	Immature and mature oligodendrocytes	Chemicon	1:100	MAB345

5. 5% Non-fat dry milk.
6. Primary antibodies – see Table 1.
7. Western blot detection reagents.

2.6. Reverse Transcriptase (RT)-PCR

1. Trizol reagent.
2. RNase-free DNase.
3. Reverse transcription system.
4. Taq DNA polymerase.

2.7. Real-Time PCR

1. First-strand cDNA synthesis and qRT-PCR kits.
2. DNA master SYBR Green real-time PCR kit.

2.8. In Vivo Neural Differentiation Assay of ASC (Mouse Fetal Brain)

1. Animals.
   Postnatal ICR newborn mice (~3 days of age).
2. Cell transplantation.
   1. Stereotaxic apparatus.
   2. Microinjector controller.
   3. Microinjection needle.
   4. CFDA.
   5. HBSS, pH 7.4.
   6. ASCs (5×10^4–1×10^5 cells/animal).
3. Detection of engrafted cell in in vivo animal tissue.
   1. Confocal microscopy or fluorescence microscopy.
   2. Microtome.
   3. PDL-coated slide glass.
   4. Cover glass.
   5. Mountin g solution.
   6. Pap pen.
4. Detection of engrafted cells by immunohistochemistry.
   1. 4% Paraformaldehyde in PBS.
   2. 5% Normal goat serum.
   3. Triton X-100.
   4. Primary antibodies – see Table 1.
   5. Rabbit or mouse source secondary antibodies.
5. RT-PCR analysis of neurogenic differentiation of engrafted cells.
   1. Homogenizer.
   2. Trizol reagent.
   3. RNase-free DNase.

4. Reverse transcription system.
5. Taq DNA polymerase.

6. Western blot analysis of engrafted cells.
   1. RIPA lysis buffer.
   2. Sample buffer.
   3. 10% SDS–PAGE reducing gels.
   4. PVDF membrane.
   5. 5% Nonfat dry milk.
   6. Primary antibodies – see Table 1.
   7. Western blotting detection reagents.

3. Methods

3.1. Isolation of ASC

The Institutional Review Board approved all protocols involving human subjects. Adipose tissue samples were obtained from individuals undergoing elective abdominoplasty after obtaining informed consent. The ASCs are isolated according to the methods described (21).

1. Digest adipose tissues at 37°C for 30 min with 0.075% type IA collagenase.
2. Neutralize the enzyme activity with α-modified Eagle's medium (α-MEM) containing 10% FBS.
3. Centrifuge the cell suspension at 1,200×*g* for 10 min to pellet.
4. Suspend the pellet in red blood cell (RBC) lysis buffer and incubate for 10 min at room temperature to lyse the contaminating RBCs, if necessary.
5. Collect the stem cell pellet and incubate overnight at 37°C/5% CO_2 in 10% FBS containing α-MEM medium.
6. Plate the cell suspension for 24 h and wash nonadherent cells away with media.
7. Following incubation, wash the tissue culture plates to remove any residual nonadherent cells and maintain at 37°C/5% CO_2 in the control medium.
8. When the monolayer of adherent cells reach a level of 50–60% confluence, trypsinize and subculture the cells at a concentration of 2,000 cells/cm^2 in α-MEM containing 10% FBS.

3.2. Neural Differentiation In Vitro

To induce neuronal differentiation for murine and human ASCs, the cells at passage 2–5 are grown to 50–60% confluency, at which point, cell differentiation is initiated. Subconfluent cultures of ASCs are maintained in DMEM/10% FBS. The procedures are

modified from various published induction protocols (22, 23). For the induction of neural differentiation through neurosphere and cytokine methods, cells are incubated in a neurobasal medium (NB) supplemented with B27 containing 20 ng/mL bFGF and 20 ng/mL EGF for 4–7 days. The culture density of the spheroid bodies is maintained at 20–50 cells/cm^2 to prevent self-aggregation. Then, neurospheres derived from the cells are layered and cultured further on PDL–laminin double-coated well chamber slide for 7 days.

3.2.1. Serum-Free Induction Protocol

1. ASCs are plated on low-attachment plastic tissue culture plates at 1×10^5 cells/cm^2 in neurosphere-induction medium composed of NB supplemented with 2% B27, 2 mM L-glutamine, 20 ng/mL EGF, and 20ng/mL bFGF. After 5–7 days, the formation of three-dimensional spheres will be observed (see Note 1).
2. Neurospheres are layered on PDL–laminin double-coated chamber slides.
3. Spheres are cultured and maintained for 10 days in NB media containing the 2% B27 supplement and 10 ng/mL recombinant human (rh)-BDNF, 2 mM L-glutamine, and 1% penicillin/streptomycin.
4. During differentiation, 70% of the media is replaced every 3 days (see Note 2).

3.2.2. Chemical Induction (Three Protocols)

Chemical Induction Protocol 1

1. Plate the ASC suspension in DMEM and 10% FBS.
2. Remove the growth medium from the cells at 50–60% confluence.
3. Add preinduction medium composed of DMEM, 10 ng/mL bFGF, and 10 ng/mL EGF for 24 h.
4. After this preinduction, cells are washed with PBS buffer.
5. Medium is replaced with neural induction medium composed of DMEM, 1% N2 supplement, 200 mM BHA, 5 mM KCl, 2 μM valproic acid, 10 μM forskolin, 1 μM hydrocortisone, and 5 μg/mL insulin.
6. Media is changed every 3 days for 2 weeks.

Chemical Induction Protocol 2

1. Plate the ASC suspension in DMEM and 10% FBS.
2. Remove the basal medium from the cells at 50–60% confluence.
3. Add preinduction medium composed of DMEM, 20% FBS, and 1 mM β-mercaptoethanol (BME) for 24 h.

4. Remove the preinduction medium.
5. Wash the cells with PBS buffer.
6. Replace buffer with neural induction medium composed of DMEM, 2% DMSO, and 200 mM BHA for up to 9 h and analyze by immunocytochemistry.

Chemical Induction Protocol 3

1. Plate the ASC suspension in DMEM/10% FBS.
2. Remove the basal medium from the cells at 50–60% confluence.
3. Wash the cells with PBS buffer.
4. Add neural induction media composed of DMEM, 10% FBS, 1% antibiotic/antimycotic, 5 μg/mL insulin, 200 μM indomethacin, and 0.5 mM IBMX.
5. Replace with fresh neural induction medium every 3 days, for a total differentiation time of 2 weeks.

3.3. Confirmation of Neural Differentiation

Most studies rely only upon morphological changes and/or neural marker gene expression at the mRNA or protein level by immunocytochemistry or Western blot. Previous studies have performed electrophysiological analysis on potential MSC-derived neurons (24, 25).

3.3.1. Immunocytochemistry

To determine the expression of the neural markers, the cells are incubated with primary antibodies against neural epitopes (e.g., anti-TuJ1 and anti-GFAP). The details are as follows:

1. After neural induction, fix the cells with 4% paraformaldehyde for 15 min at room temperature.
2. Incubate for 30 min in blocking solution (PBS, 5% normal host serum, and 0.1% Triton X-100).
3. Incubate with each primary antibody at 4°C overnight and subsequently with appropriate secondary antibody for 1 h at room temperature.
4. Visualize staining under a fluorescent microscope.

3.3.2. Western Blot Analysis

1. Rinse the neuronal induced and noninduced ASCs in PBS buffer.
2. Scrape the cells off and transfer the lysate to a microcentrifuge tube. Centrifuge for 30 s at 15,000 × *g* and discard the supernatant.
3. Add lysis buffer to whole cell lysate.
4. Lyse the cells further by sonication.
5. Centrifuge at 14,000 × *g* for 30 min at 4°C and collected the supernatant.

6. Dissolve protein samples (50 μg each) in sample buffer, boil for 5 min, and separate on 10% SDS–PAGE reducing gels.
7. Electroblot proteins to PVDF membrane.
8. Block the membranes in 5% nonfat dry milk/PBS for 1 h and incubate with primary antibody.
9. Reveal antibody binding by incubation with an appropriate secondary antibody on the second day.
10. Incubate the membranes with Western blotting detection reagents and image using a digital imaging system.

3.3.3. Reverse Transcription (RT): Polymerase Chain Reaction (PCR)

1. Total RNA was extracted from neuronal induced ASCs by TRIzol reagent.
2. Treat with RNase-free DNase to remove any residual genomic DNA.
3. For RT-PCR analysis, 2 μg aliquots of total cellular RNA are subjected to cDNA synthesis with avian myeloblastosis virus (AMV) reverse transcriptase and oligo (dT) 15 primer.
4. The cDNA in 2 μL of the reaction mixture is amplified with Taq DNA polymerase and 10 pmol each of sense and antisense primers.
5. The thermal cycle profile is as follows: 35 cycles at 94°C for 30 s, 55–62°C (depending on the primers used) for 30 s, and 72°C for 45 s.

3.4. Neural Differentiation In Vivo

3.4.1. Animal Care

The experimental evaluation of neurogenic potency of ASCs in vivo in the mouse brain is important (26, 27). For this, adult female ICR mice weighing 30 g or approximately 5-week-old are used. All animal procedures require approval by the Institutional Animal Care and Use Committee, Institutional Review Board, and/or the ethics committee. Before initiating the experiment, mice are anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg). The animal is placed on a heating pad, and a core body temperature is maintained at 38 ± 0.2°C during the operation.

3.4.2. Cell Preparation for Transplantation into Newborn Mouse Brain

1. ASC preparations (1×10^5 cells/animal).
2. Label cells using fluorescent dye (CFDA or CM-Dil) according to manufacturer's instructions.
3. Confirm both the fluorescent labeling and viability of the ASCs by Trypan blue exclusion method and fluorescence microscopy of labeled, viable cells.
4. Suspend the labeled cells in 10 μL of HBSS or PBS buffer.
5. Ten microliters of cell suspension will be implanted into ICR mouse (3–4 days after birth) brain using microsyringe.

3.4.3. Detection of Engrafted Cell in In Vivo Brain Tissue

1. Recover the transplants 6 weeks later, fix with 4% formalin for 24 h, and transfer the tissue into 30% sucrose solution for 2 days.
2. Embed tissue on OCT compound and transfer into a –80°C freezer for longer than 6 h.
3. Slice 10-μm-thick tissues on a microtome, attach sections to PDL-coated glass slides, and dry for 24 h.
4. Detect fluorescent-labeled engrafted cells with direct fluorescence microscopy.

3.4.4. Immunohistochemistry for Evaluation of Neurogenic Potency of Engrafted Cells

1. Fix the frozen sections for 30 min in 4% paraformaldehyde.
2. Wash the sections three times in PBS and block nonspecific binding with 10% normal horse serum (see Note 3).
3. Incubate the sections overnight at 4°C with the following antibodies: anti-GFAP, anti-Tuj, and anti-NF160 (see Note 4).
4. After extensive washing in PBS, incubate the cells for 30 min with FITC- or Texas Red-conjugated secondary antibodies (see Note 5).
5. Evaluate the specimens by fluorescence microscopy.
6. Visualize colocalization of CM-Dil/engrafted cells with several neural lineage markers via confocal microscopy.
7. For the relative quantification of NF160- or TuJ-positive cells, conduct area counts of the striatum.

3.4.5. Functional Evaluation of ASCs in In Vivo Brain Through Electrophysiological Recording

Hippocampal slice preparation

1. After induction of in vivo neurogenesis of ASCs, mouse brain hippocampal slices are prepared from donor ICR mice. The mice are rapidly decapitated under ketamine and xylazine anesthesia.
2. The brain is quickly removed and immersed in oxygenated (95% O_2; 5% CO_2), ice-cold artificial cerebrospinal fluid (ACSF). The composition of the ACSF is (in mM) 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 25 glucose, 3 pyruvic acid, and 1 ascorbic acid, which is continuously gassed with 95% O_2 and 5% CO_2 at 34°C for at least 1 h before being used for the recording.
3. Three or four coronal slices (300-μm thick) containing the hippocampus are cut from the brain with a vibrating tissue slicer.
4. For recordings, a slice is transferred to a submersion-type recording chamber continuously perfused with the 95% O_2/5% CO_2-saturated ACSF at ~34–35°C.
5. Individual cells are viewed with a microscope fitted with a 40× water-immersion objective and differential interference

contrast optics. The injected CM-Dil-labeled ASCs are identified by their fluorescence signal under a microscope equipped with an appropriate filter cube and visualized using differential interference contrast video microscopy (see Note 6).

3.4.6. Whole-Cell Patch-Clamp Recordings

1. Whole-cell patch-clamp recordings are made using a patch-clamp amplifier.
2. The recording pipettes are pulled from borosilicate capillaries (1.5-mm OD, 1.0-mm ID) using a micropipette puller.
3. The resistance of the pipette should be 4–6 MΩ when it is filled with a solution containing (in mM): potassium gluconate, 130.0; $MgCl_2$, 1.0; HEPES, 10.0; EGTA, 10.0; $CaCl_2$, 1.0; and adenosine triphosphate-Mg, 4.0 that is adjusted to pH 7.25 with 1 M KOH (290–320 mOsm).
4. The slice is then placed in a glass-bottomed recording chamber and fixed with a grid of parallel nylon threads supported by a U-shaped stainless steel weight.
5. The solutions are perfused at a rate of 3–4 mL/min at 34–35°C.
6. A tight GΩ seal is obtained on the identified neuron. Recordings begin ~5 min after whole-cell access is obtained and the current reaches a steady state. Series resistances typically range from 15 to 30 mΩ, which is estimated directly from the amplifier.
7. Data is digitized by a Digidata device.
8. The recording is abandoned if the input resistance changes more than 15% during the recording.

4. Notes

1. The culture density of the spheroid bodies should be maintained at 10–20 cells/cm^2 to prevent self-aggregation.
2. Analyze the cultures of cells at intervals from 3 h to 14 days following neuronal induction to determine whether ASC cells display changes in cellular morphology or antigen expression.
3. For immunohistochemical analysis of the frozen tissues, nonspecific binding is blocked with 10% normal horse serum and the tissues are not washed for the next step of primary antibody reaction.
4. For immunofluorescence analysis of differentiated cells or tissues, dilutions of specific antibodies should be optimized. The optimum dilutions of antibodies are different between different cell and tissue types.

5. Controls in which the primary antibodies are omitted or replaced with irrelevant IgG should result in no detectable staining.
6. For electrophysiological analysis of brain tissue, mice are rapidly decapitated under ketamine and xylazine anesthesia and the brains are quickly removed and immersed in oxygenated, ice-cold artificial cerebrospinal fluid (ACSF) to reduce cell death in the brain slices.

References

1. Kim JH, Lee MR, Kim JH, Jee MK, Kang SK. 2008. Selenium induces improvement of stem cell behaviors in human adipose-tissue stromal cells via SAPK/JNK and stemness acting signals. Stem Cells **26**:2724–2734.
2. Kang SK, Putnam LA, Ylostalo J, Popescu IR, Dufour J, Belousov A, Bunnell BA. 2004. Neurogenesis of Rhesus adipose stromal cells. J Cell Sci **15**:4289–4299.
3. Kang SK, Putnam L, Dufour J, Ylostalo J, Jung JS, Bunnell BA. 2004. Expression of telomerase extends the lifespan and enhances osteogenic differentiation of adipose tissue-derived stromal cells. Stem Cells **22**:1356–1372.
4. Kim JH, Jee MK, Lee SY, Han TH, Kim BS, Kang KS, Kang SK. 2009. Regulation of adipose tissue stromal cells behaviors by endogenic Oct4 expression control. PLoS One **4**:e7166.
5. Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM, Rice HE. 2002. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun **294**:371–379.
6. Krampera M, Marconi S, Pasini A, Galie M, Rigotti G, Mosna F, Tinelli M, Lovato L, Anghileri E, Andreini A, Pizzolo G, Sbarbati A, Bonetti B. 2007. Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. Bone **40**:382–390.
7. Tseng PY, Chen CJ, Sheu CC, Yu, CW, Huang YS. 2007. Spontaneous differentiation of adult rat marrow stromal cells in a long-term culture. J Vet Med Sci **69**:95–102.
8. Kang SK, Yeo JE, Kang KS, Phinney DG. 2007. Cytoplasmic extracts from adipose tissue stromal cells alleviates secondary damage by modulating apoptosis and promotes functional recovery following spinal cord injury. Brain Pathol. **17:**263–275.
9. Kang SK, Shin MJ, Jung JS, Kim YG, Kim CH. 2006. Autologous adipose tissue-derived stromal cells for treatment of spinal cord injury. Stem Cells Dev. **15:**583–594.
10. Rosenstein K. 2003. Liquidation of a pharmacy's prescription drug inventory. Health Law Can **24**:18–27.
11. Lennmyr F, Ata KA, Terent A. 1998. Expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1) following permanent and transient occlusion of the middle cerebral artery in the rat. J Neuropathol Exp Neurol **57**:874–882.
12. Zhu Y, Jin K, Greenberg DA. 2003. Vascular endothelial growth factor promotes proliferation of cortical neuron precursors by regulating E2F expression. FASEB J **17**:186–193.
13. Grafi G. 2004. How cells dedifferentiate: a lesson from plants. Dev Biol **268**:1–6.
14. Yourey PA, Gohari S, Alderson RF. 2000. Vascular endothelial cell growth factors promote the in vitro development of rat photoreceptor cells. J Neurosci **20**:6781–6788.
15. Grafi G, Ben-Meir H, Avivi Y, Moshe M, Dahan Y, Zemach A. 2007. Histone methylation controls telomerase-independent telomere lengthening in cells undergoing dedifferentiation. Dev Biol **306**:838–846.
16. Jee MK, Kim JH, Han YM, Jung SJ, Kang KS, Kim DW, Kang SK. 2010. DHP-derivative and low oxygen tension effectively induces human adipose stromal cell reprogramming. PLOS ONE **9**:5(2):e9026.
17. Akimoto T, Nonaka T, Harashima K, Sakurai H, Ishikawa H, Mitsuhashi N. 2004. Radicicol potentiates heat-induced cell killing in a human esophageal cancer cell line: the Hsp90 chaperone complex as a new molecular target for enhancement of thermosensitivity. Int J Radiat Biol **80**:483–492.
18. Ng DC, Bogoyevitch MA. 2000. The mechanism of heat shock activation of ERK mitogen-activated protein kinases in the interleukin

3-dependent proB cell line BaF3. J Biol Chem **275**:40856–40866.

19. Qian DX, Zhang HT, Ma X, Jiang XD, Xu RX. 2009. Comparison of the Efficiencies of three neural induction protocols in human adipose stromal cells. Neurochem Res. **35**(4): 572–579.
20. Padovan CS, Jahn K, Birnbaum T, Reich P, Sostak P, Strupp M, Straube A. 2003. Expression of neuronal markers in differentiated marrow stromal cells and CD133+ stem-like cells. Cell Transplant **12**:839–848.
21. Kim YJ, Kim HK, Cho HH, Bae YC, Suh KS, Jung JS. 2007. Direct comparison of human mesenchymal stem cells derived from adipose tissues and bone marrow in mediating neovascularization in response to vascular ischemia. Cell Physiol Biochem **20**:867–876.
22. Hermann A, Gastl R, Liebau S, Popa MO, Fiedler J, Boehm BO. 2004. Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. J Cell Sci **1**:4411–4422.
23. Ashjian PH, Elbarbary AS, Edmonds B, DeUgarte D, Zhu M, Zuk PA, Lorenz HP, Benhaim P, Hedrick MH. 2003. In vitro differentiation of human processed lipoaspirate cells into early neural progenitors. Plast Reconstr Surg **111**:1922–1931.
24. Woodbury D, Reynolds K, Black IB. 2002. Adult bone marrow stromal stem cells express germLine, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J Neurosci Res **69**:908–917.
25. 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**:4279–4295.
26. Franco Lamberta AP, Fraga Zandonai A, Bonatto D, Cantarelli Machado D, Pegas Henriques JA. 2009. Differentiation of human adipose-derived adult stem cells into neuronal tissue: Does it work? Differentiation **77**:221–228.
27. Hofstetter CP, Schwarz EJ, Hess D, Widenfalk J, El Manira A, Prockop DJ, Olson L. 2002. Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. Proc Natl Acad Sci USA **99**:2199–2204.
28. Goodell MA. 2003. Stem-cell "plasticity": befuddled by the muddle. Curr Opin Hematol **10**:208–213.
29. Wagers AJ, Weissman IL. 2004. Plasticity of adult stem cells. Cell **116**:639–648.

Chapter 17

Osteogenic Differentiation Strategies for Adipose-Derived Mesenchymal Stem Cells

Robert Jan Kroeze, Marlene Knippenberg, and Marco N. Helder

Abstract

Adipose stem cell preparations, either obtained as a freshly isolated so-called stromal vascular fraction (SVF) or as cells cultured to homogeneity and then referred to as adipose stem cells (ASCs), have found widespread use in a broad variety of studies on tissue engineering and regenerative medicine applications, including bone repair.

For newcomers within the field, but also for established research laboratories having up to 10 years of expertise in this research area, it may be convenient to strive for, and use consensus protocols (1) for studying the osteogenic differentiation potential of ASC preparations in vitro, and (2) for osteogenic induction regimes for in vivo implementation. To assist in achieving this goal, this chapter describes various step-by-step osteogenic differentiation protocols for adipose-derived stem cell populations (SVF as well as ASCs) currently applied within our laboratory, with particular emphasis on protocols aimed at intra-operative use. The protocols describe the use of inducing compounds, including the bone morphogenetic proteins (BMPs), 1,25-dihydroxyvitamin-D3, and polyamines, as well as methods and parameters for evaluating the level of differentiation achieved.

We would appreciate receiving feedback on the protocols described; this will facilitate the development of consensus protocols, which in turn will allow better comparison of data sets generated by different research groups. This continuing standardization, which might be reported on at international meetings like those of IFATS (http://www.IFATS.org), might be of benefit for the whole ASC research community.

Key words: Adipose tissue, Stem cells, Differentiation, Osteogenic, Tissue engineering

1. Introduction

The discovery of adult stem cells in adipose tissue has changed the perspective of bone tissue engineering in our group. At first, we established a stem cell bank by using waste adipose tissue after liposuction or resection surgeries, complying with the VU Medical

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Center ethical committee guidelines. Thereafter, we developed the so-called "one-step surgical procedure" (OSP) based on the large relative (per gram of adipose tissue) and absolute yields in stem cell numbers, compared with bone marrow tissue samples (1). This surgical procedure allows us to harvest adipose tissue, process it to obtain a single-cell suspension (stromal vascular fraction [SVF]) and directly re-implant this SVF into the patient, all within 2.5–3 h, thus fitting in one surgery, for bone tissue-engineering purposes. With regard to the aforementioned time restrictions, we performed experiments on differentiation of adipose stem cells (ASC) towards the osteogenic lineage with a triggering stimulus of only 15 min (2). Furthermore, since attachment of cells to biomaterials is crucial for the later implantation, we use rather short attachment times (10–60 min). We thus conducted the majority of the in vitro experiments to fit within the OSP. We will discuss in this chapter the various experimental settings we have used to differentiate ASCs towards the osteogenic lineage. While some of these experiments are tailored for the OSP, other experiments are used to obtain more insight into the biology of these stem cell preparations. It needs to be kept in mind that some experiments were performed on goat adipose tissue mesenchymal stem cells (MSCs) and others on their human counterparts, both in vitro and in vivo. In order to clarify this difference, we will use the following symbols after each subheading to indicate under what settings the experiments were performed: OSP (*), goat-MSCs (†), human-MSCs (‡), in vitro (+), and in vivo (++).

2. Materials

2.1. Items

1. The procurement of SVF from human and/or goat adipose tissue is outside the scope of this paper. For detailed information *see* refs. (2, 3), respectively.
2. For dissolving powders, we use milli-Q® water (mQ) obtained through a 0.2-µm filter instead of distilled water.
3. If appropriate, both the stock concentration (s.c.) and the final concentration (f.c.) of materials are indicated.

2.2. Cell Culture

1. Culture flasks, culture plates.
2. Dulbecco modified Dulbecco's Modified Eagle's Medium (DMEM 31885) containing the following substances to obtain "DMEM^{++}":
   - *Fetal-calf serum (FCS)*: Filter through a 0.2-µm filter. Add in a 1:10 ratio to the culture medium (f.c. 10%).

- *Streptomycin sulfate (Fw 1457)*: Add 2.5 g streptomycin sulfate to 50 ml mQ. Filter using a 0.2-µm filter, and store at –20°C in working aliquots (s.c. 50 mg/ml mQ). Add to culture medium in a 1:100 ratio (f.c. 500 µg/ml).
- *Penicillin G-sodium salt (Fw 356.4)*: Add 3 g penicillin G to 50 ml mQ. Filter using 0.2-µm filter, and store at –20°C in working aliquots (s.c. 60 mg/ml mQ). Add to culture medium in a 1:100 ratio (f.c. 600 µg/ml).
- *Amphotericin B (Fungizone)*: Store at –20°C in working aliquots (s.c. 250 µg/ml). Add amphotericin B to culture medium in a 1:100 ratio (f.c. 2.5 µg/ml).

3. Trypsinizing solution to trypsinize the cells upon confluency: Phosphate-buffered saline (PBS) containing 0.25% trypsin and 0.1% ethylenedinitrilotetraacetic acid disodium salt dihydrate (EDTA). Dissolve 1 g Trypsin 1:250 (1,120 BAEE U/mg) (porcine pancreas), in 400 ml mQ using a magnetic stirrer (f.c. 0.25%). Subsequently, dissolve 0.4 g EDTA (Mw 372.24 g/mol), in this solution (f.c. 0.1%). Store at –20°C in working aliquots (see Note 1).
4. Dimethyl sulfoxide (DMSO) 100%. Dilute DMSO 100% at a 1:5 ratio using $DMEM^{++}$ (s.c. 1: 20%). Store at 4°C for 1 week maximum. Dilute DMSO/$DMEM^{++}$ solution 1:1 with cell-containing $DMEM^{++}$ solution (f.c. 10%, see Subheading 3.1).

2.3. Osteogenic Media

In our studies, we use various osteogenic induction factors, in either continuous or short incubation regimes. These will be described in Subheading 3.2. For the use of dexamethasone in osteogenic media (see Note 2), the induction medium always contains standard culture medium to which is added:

- *Glycerol-2-phosphate disodium salt hydrate (ß-GP, Mw 216.06 g/mol)*: Dissolve 50 mg ß-GP in 10 ml DMEM and sterilize using a 0.2-µm filter (s.c. 5 mg/ml). Store in working aliquots at –20°C. Add to medium in a 1:100 ratio (f.c. 50 ng/ml).
- *2-Phospho-l-ascorbic acid trisodium salt (vitamin C, Mw 322.05)*: Dissolve 2.5 mg in 2.5 ml mQ, filter through a 0.2-µm filter (s.c. 1 mg/ml) and add to medium in a 1:200 ratio (f.c. 50 µg/ml). Make fresh for every medium change and keep powder in the dark (see Note 3).

2.3.1. Growth Factor*,†,‡,+,++ (2, 4)

Recombinant human bone morphogenetic protein (BMP)-2 dissolved in mQ containing bovine serum albumin fraction V (BSA): Dissolve 1 mg of BSA in 2 ml mQ, sterilize through a 0.2-µm filter, and add 1 ml to 10 µg of protein (s.c. 10 µg/ml). Store at –20°C in working aliquots (see Note 4). Add to culture medium in a 1:1,000 to 1:100 ratio (f.c. 10 ng/ml to 100 ng/ml, respectively) (see Note 5).

2.3.2. Polyamines†,‡,+ (5, 6)*

Spermine tetrahydrochloride (Mw 348.18 g/mol), f.c. 3–30 µM: Dissolve 1.04 mg spermine in 10 ml DMEM (Invitrogen, s.c. 300 µM). Store at −20°C in working aliquots. Add to culture medium in a 1:100 or 1:10 ratio (f.c. 3 µM and 30 µM, respectively).

2.3.3. Vitamin D_3†,‡,+ (7)

1α,25-Dihydroxyvitamin-D_3 (Mw 416.6 g/mol) in ethanol 100%: Dissolve 10 µg in 800 µl ethanol (s.c. 12.5 µg/ml = 30 µM). Store at −80°C in working aliquots. Add to culture medium in a 1:3,000 ratio (f.c. 4.2 ng/ml = 10 nM). This dilution factor avoids potential adverse effects of the vehicle (ethanol) in cell cultures. Keep in the dark, it is light sensitive.

2.4. Osteogenic Differentiation Analysis

2.4.1. Alkaline Phosphatase (ALP) Activity

1. To determine the ALP activity:

   Substrate solution 4-nitrophenyl phosphate disodium salt: Dissolve 18.56 mg 4-nitrophenyl phosphate disodium salt (Mw 371.15 g/mol) (f.c. 5 mM) in a 10-ml buffer solution at pH 10.3 (see below).

   Buffer solution: 5 nM $MgCl_2 \bullet 6H_2O$ and 0.5 M 2-amino-2-methyl-1-propanol (AMP): Dissolve 100 mg $MgCl_2 \bullet 6H_2O$ (Mw 203 g/mol, Merck) in 50 ml mQ using a magnetic stirrer. Add 1 ml of 6 M hydrochloric acid (HCl) to this solution. Add 5 ml stock solution 37% HCL (~12 M, Merck) to 5 ml mQ in a fume hood to obtain 10 ml of 6 M HCL. Add 4.45 g AMP (Mw 89 g/mol, Fluka) to the 51 ml solution, while stirring. Adjust to pH 10.3 with droplets of 6 M HCl using a pH gage. Supplement with mQ up to 100 ml. Store at 4°C.

   Stop solution sodium hydroxide (NaOH): Dissolve 3 g NaOH (Mw 40 g/mol) in 250 ml mQ (f.c. 0.3 M) and store sealed at room temperature (RT).

   Standard curve 4-nitrophenol: Add 13.9 mg 4-nitrophenol (Mw 139 g/mol) to 10 ml buffer solution (s.c. 10 mM). Dilute the 10-mM stock solution by tenfold with the buffer solution, to obtain 1 ml of 1 mM 4-nitrophenol in buffer solution (=f.c.). Use a 96-well ELISA plate, U-bottom, and microplate reader to assay.

2. To determine the amount of protein, use a bicinchoninic acid (BCA) protein assay reagent kit with a 96-well ELISA plate, U-bottom, and microplate reader.

3. To determine the amount of DNA, use a CyQUANT Cell Proliferation Assay Kit with a 96-well ELISA plate, U-bottom, and microplate reader.

2.4.2. ALP Staining

1. 1× PBS.
2. 4% Formaldehyde.
3. *Tris(hydroxymethyl)aminomethane buffer*: 0.1 M Tris-AM (Mw 121.14 g/mol) at pH 10. Dissolve 2.42 g of Tris in

100 ml mQ using a magnetic stirrer (s.c. 0.2 M). Adjust pH to 10 using droplets of 6 M HCL. Dilute with mQ in a 1:1 ratio (f.c. 0.1 M).

4. *0.1 M Tris buffer containing 0.1 M NaCl and 0.05 M MgCl2•6H2O*: 0.2 M NaCl (Mw 58.44 g/mol). Dissolve 1.17 g NaCl in 100 ml mQ using a magnetic stirrer (s.c. 0.2 M). When dissolved, add 0.1 M $MgCl_2$ (Mw 203 g/mol): Dissolve 2 g $MgCl_2•6H_2O$ in the abovementioned 100 ml of 0.2 M NaCl solution while stirring (s.c. 0.1 M). Add 0.2 M Tris buffer (s.c.) 1:1 with the abovementioned solution containing 0.2 M NaCl and 0.1 M $MgCl_2•6H_2O$. This will result in an 0.1 M Tris buffer containing 0.1 M NaCl and 0.05 M $MgCl_2•6H_2O$.
5. 4-Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, di-sodium salt (NBT/BCIP): Supplied stock solution comprised of 18.75 mg/ml NBT (Mw 817.7 g/mol) and 9.4 mg/ml BCIP (Mw 433.6 g/mol) in 67% DMSO (v/v).
6. 100% Methanol.

2.4.3. Colony Forming Unit Assay (CFU)–ALP

CFU

1. One 6-well plate.
2. 1× PBS.
3. 4% Formaldehyde.
4. *0.2% Toluidine blue solution in 0.2% borax in mQ*: Dissolve 1 g of toluidine blue and 1 g of borax in 100 ml mQ on a hot plate at 100°C using a magnetic stirrer. After cooling the solution, filter using paper filter (595½), (s.c. 1% toluidine blue and 1% borax). Before staining make a 1:5 dilution (f.c. 0.2% toluidine blue and 0.2% borax).

CFU-ALP (*see* Subheading 2.3.2)

1. One 6-well plate.
2. Osteogenic medium.
3. 1× PBS.
4. 4% Formaldehyde.
5. 0.1 M Tris-AM.
6. 0.1 M Tris containing 0.1 M NaCl and 0.05 M $MgCl_2•6H_2O$.
7. NBT/BCIP.
8. 100% Methanol.

2.4.4. Alizarin Red Staining

1. *2% Alizarin red-S*: Dissolve 2 g of Alizarin red-S in 100 ml mQ using a magnetic stirrer. Adjust the pH to 5.5 using

droplets of 0.1% ammonium hydroxide (NH_4OH) while constantly measuring the pH.

2. *0.1% Ammonium hydroxide*: Dilute ammonia (NH_3, 37%, Merck) to a 1:370 ratio using mQ to obtain a f.c. of 0.1% NH_4OH.
3. 96 and 30% Ethanol.
4. *0.2% Toluidine blue*: Dissolve 1 g of toluidine blue in 100 ml distilled water on a hot plate at 100°C using a magnetic stirrer. After cooling the solution, filter using a paper filter (s.c. 1%). Before staining, make a 1:5 dilution (f.c. 0.2%).
5. 0.2-μm filter.

2.4.5. Bone Sialo-Protein (8) and Osteonectin (9) Immunohistochemical Staining

1. 1× PBS.
2. 4% Paraformaldehyde.
3. 1× PBS containing 1% BSA and 0.1% saponin (from quillaja bark) (= PBS++).
4. 1:400 dilution of Alexa Fluor® 488 goat anti-rabbit IgG.
5. *Propidium iodide (PI)*: Dissolve 1 mg/ml PI (1.5 mM) 1:200 in 1× PBS to obtain an final concentration of 7.5 μM.
6. *Bone sialo-protein (BSP) antibody*: 1:20 dilution of normal goat serum and 1:200 dilution of a rabbit antibody against human BSP.
7. *Osteonectin antibody*: 1:20 dilution of normal goat serum and 1:200 dilution of a rabbit antibody against osteonectin.
8. Negative control antibodies:
   (a) 1:1,000 non-immune mouse serum.
   (b) 1:1,000 non-immune rabbit serum.

2.4.6. Real-Time Polymerase Chain Reaction

1. RNA: isolated using Trizol® reagent according to the manufacturer's instructions.
2. 20 ml reaction mix consisting of 5 U Transcriptor Reverse Transcriptase (RT).
3. 0.08 A_{260} units random primers.
4. 1 mM of each dNTP.
5. 1× concentrated Transcriptor RT reaction buffer.
6. LightCycler − 480 SYBR green I Master reaction mix.
7. Primers used for real-time PCR for human adipose tissue mesenchymal stem cells (hAT-MSC) are listed in Table 1.
8. Primers used for real-time PCR for goat AT-MSCs are uploaded at: http://www.STEGA.nl.

Table 1
Primers used for real-time PCR for hAT-MSCs (4, 7)

Housekeeping genes	Oligonucleotide sequence	Product size (bp)	Preferred time points
18S forward (4) *18S reverse*	5′-gtaacccgttgaaccccatt-3′ 5′-ccatccaatcggtagtagcg-3′	151	All time points
YWHAZ forward (12) *YWHAZ reverse*	5′-cactacccaacaccaagaca-3′ 5′-ctggtttccctacagctgat-3′	229	All time points
HPRT forward (12) *HPRT reverse*	5′-gctgacctgctggattacat-3′ 5′-cttgcgaccttgaccatct-3′	260	All time points
Target genes			
Runx-2 forward (4) *Runx-2 reverse*	5′-atgcttcattcgcctcac-3′ 5′-actgcttgcagccttaaat-3′	156	0, 4 days
OPN forward (4) *OPN reverse*	5′-ttccaagtaagtccaacgaaag-3′ 5′-gtgaccagttcatcagattcat-3′	181	0, 4, 14 days
OCN forward (4) *OCN reverse*	5′-agccaccgagacaccatgaga-3′ 5′-ctcctgaaagccgatgtggtc-3′	288	0, 4, 14 days
COL1A1 forward (13) *COL1A1 reverse*	5′-tccaacgagatcgagatcc-3′ 5′-aagccgaattcctggtct-3′	195	0, 4, 14 days

ALP alkaline phosphatase, *OPN* osteopontin, *OCN* osteocalcin, *COL1A1* collagen1A1

3. Methods

3.1. Cell Culture and Storage

After seeding SVF, media are refreshed after 24 h to remove cell debris and the non-adhering cell fraction, but otherwise media are changed twice per week. When cells reach 70–80% confluency, cells are trypsinized and either stored in liquid N_2 in a DMSO solution or cultured for another passage. For liquid nitrogen storage, cells are frozen in a 1-ml CryoTube™ whereby 0.5 ml of 20% DMSO/DMEM^{++} is added to 0.5 ml of the cell-containing culture medium (DMEM^{++}). Cells are always cultured in a humidified incubator at 37°C and 5% CO_2.

3.2. CFU-F Assay and CFU-F ALP

1. Prepare two 6-well plates in which the SVF is diluted tenfold across both rows, resulting in an upper row containing 10^4 and a lower row containing 10^3 nucleated SVF cells.
2. For the CFU-fibroblast (CFU-F) assay, the time of culture until fixation is generally between 10 and 20 days, depending

on the amount of cells and the growth kinetics of the colonies, which is donor dependent (merging of colonies should be avoided) (see Note 6).

At the appropriate time point,

1. Remove the medium
2. Wash the cells with 1× PBS
3. Fix with 4% paraformaldehyde for 10 min
4. After removal of paraformaldehyde, wash once with 1× PBS, and add 0.2% toluidine blue solution in borax buffer for 1 min
5. Excess stain should be removed using distilled water
6. Air dry (rinse properly, since dried-in toluidine blue stains can interfere with colony counting)
7. Count the colonies

CFU-F ALP

Cells of the duplicate 6-well plate will be processed in a CFU-ALP assay.

1. Culture the 6-well plate in normal medium for 7 days (to obtain colonies and to remove contaminating cells)
2. After 7 days, add osteogenic medium for 2 weeks
3. Perform ALP staining (see Subheading 3.3).

3.3. Induction of Osteogenic Differentiation

Various protocols exist for induction of osteogenic differentiation, of which, some examples will be given below. The induction media always consist of standard culture medium ($DMEM^{++}$) to which ß-GP and 2-phospho-l-ascorbic acid are added (see Subheading 2.2).

3.3.1. Osteogenic Induction with BMP-2 (Continuous Stimulation)

1. Add the BMP-2 stock solution (see Subheading 2.2) to $DMEM^{++}$/β-GP/ascorbate culture medium in a 1:100 ratio (f.c. 100 ng BMP-2/ml) (see Note 7)
2. Add this medium to the cells (either in suspension prior to plating, or on plated and attached cells)
3. Refresh media every 2–3 days
4. At the appropriate time point, process for osteogenic differentiation analysis (see Subheading 3.3).

3.3.2. Osteogenic Induction with BMP-2 (Short-Term Stimulation; see Fig. 1)

1. Add the BMP-2 stock solution (see Subheading 2.2) to $DMEM^{++}$/β-GP/ascorbate culture medium in a 1:500 ratio (f.c. 20 ng BMP-2/ml) (see Note 7).
2. Resuspend the pelleted SVF in 250 µl $DMEM^{++}$/β-GP/ascorbate culture medium. Add an equal volume of the

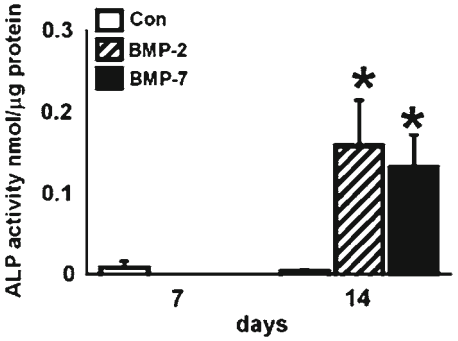

Fig. 1. Effect of 10 ng/ml BMP-2 and BMP-7 on the number of cells, protein content, and ALP activity by gAT-MSCs. Freshly isolated gAT-MSCs were treated for 15 min with 10 ng/ml BMP-2 or BMP-7 followed by a post-incubation period of 7 or 14 days without growth factor. BMP-2 and BMP-7 up-regulated ALP activity by gAT-MSCs at day 14. Values are means ± SEM of three to five cultures. *BMP* bone morphogenetic protein, *gAT-MSCs* goat adipose tissue-derived mesenchymal stem cells, *ALP* alkaline phosphatase. *Significant effect of growth factor, $p < 0.05$ (Reprinted with permission from ref. (2).).

BMP-2-containing medium (see previous step) to the cell suspension (f.c. BMP-2: 10 ng/ml). Mix well.

3. Incubate the cell suspension for 15 min at 37°C.
4. Wash the cells twice with 1× PBS to remove excess BMP-2.
5. Plate the cells in DMEM^{++}/β-GP/ascorbate culture medium without BMP-2 for in vitro analysis, or use for in vivo cell implantation studies (one-step surgical procedure (1)) directly.
6. For in vitro studies: refresh media every 2–3 days.
7. At the appropriate time point, process for osteogenic differentiation analysis (see Subheading 3.3).

3.3.3. Osteogenic Induction with 1,25-Dihydroxyvitamin-D_3 (Continuous Stimulation)

1. Switch off the light in the safety cabinet, since 1,25-dihydroxyvitamin-D_3 is light sensitive
2. Add the 1,25-dihydroxyvitamin-D_3 stock solution (see Subheading 2.2) to DMEM^{++}/β-GP/ascorbate culture medium in a 1:3,000 ratio (f.c. 10 ng/ml)
3. Add this medium to the cells (either in suspension prior to plating, or on plated and attached cells)
4. Refresh media every 2–3 days
5. At the appropriate time point, process for osteogenic differentiation analysis (see Subheading 3.3)

3.3.4. Osteogenic Induction with the Polyamine Spermine (Short-term Stimulation)$^{*,\dagger,\ddagger,+}$ (5, 6)*

1. Add the spermine stock solution (see Subheading 2.2) to DMEM^{++}/β-GP/ascorbate culture medium in a 1:50 or 1:5 ratio (f.c. 6 μM and 60 μM, respectively).

2. Resuspend the pelleted SVF in 250 µl DMEM^{++}/β-GP/ascorbate culture medium. Add an equal volume of the spermine-containing medium (see previous step) to the cell suspension (f.c. spermine: 3 and 30 µM, respectively). Mix well.
3. Incubate the cell suspension for 30 min at 37°C.
4. Wash the cells twice with 1× PBS to remove excess spermine.
5. Plate the cells in DMEM^{++}/β-GP/ascorbate culture medium without spermine for in vitro analysis, or use for in vivo cell implantation studies (one-step surgical procedure (1)) directly.
6. For in vitro studies: refresh media every 2–3 days.
7. At the appropriate time point, process for osteogenic differentiation analysis (see Subheading 3.3).

3.4. Osteogenic Differentiation Analysis

3.4.1 ALP Activity

Preparation of cell lysate:

1. Remove culture medium
2. Wash with PBS twice
3. Add 300–600 µl of MiliQ
4. Incubate for 10 min
5. Scrape cells off with cell scraper
6. Transfer into 1.5-ml Eppendorf tube
7. Sonicate in bath for 5 min (see Note 8)
8. Centrifuge 10 min at 600×*g* at RT
9. Freeze at –20°C until further use

Protein assay:

BCA, according to manufacturer's protocol (http://www.piercenet.com).

1. Make a protein concentration standard curve (0–400 µg/ml).
2. Make "color solution" by adding solution "B" to solution "A" in a 1:50 ratio.
3. Pipette 40 µl per sample (cell lysate) and 40 µl per standard (in the same lysis solution as the sample, here mQ) into a 96-well plate in duplicates. Use row 1 and 2 for standards, A1 and A2 contain the highest concentration and H1 and H2 contain lysis solution only.
4. Add 160 µl of "color solution" to both the sample as the standard.
5. Incubate for 30 min at 60°C.
6. Determine colorimetric values directly by spectrophotometry, reading at 562 nm.

ALP activity in the cell lysate:

1. Make a standard curve (0, 0.05, 0.1, 0.2, 0.4, 0.5, 0.75 mM).
2. Pipette 40 µl per sample (cell lysate) and 40 µl per standard (in the same lysis solution as the sample, here mQ) into a 96-well plate in duplicates. Use row 1 and 2 for standards, A1 and A2 contain the highest concentration and H1 and H2 contain lysis solution only.
3. Add 60 µl mQ to both the sample as the standard.
4. Add 100 µl substrate solution to both the samples and standards.
5. Incubate for 60 min at 37°C (see Note 9).
6. Terminate reaction with 100 µl stop solution.
7. Determine colorimetric values directly by spectrophotometry, reading at 405 nm.

DNA content in the cell lysate:

1. Make two color solutions by adding 5 µl Component A (CompA) to 2 ml mQ (CompA 1, f.c. 1:400) and the second solution by adding 5 µl CompA to 1 ml mQ (CompA 2, f.c. 1:200).
2. Make a DNA concentration standard curve (0–600 ng/ml) by adding 4 µl of DNA stock solution to 396 µl of CompA 1 (= 1,000 ng/ml). From this concentrated solution, pipet various amounts into a black 96-well plate and add CompA 1 (see Note 10). Use row 1 and 2 for standards, A1 and A2 contain the highest concentration and H1 and H2 contain lysis solution only.
3. Pipette 50 µl per sample (cell lysate) and 100 µl per standard (in the same lysis solution as the sample, here MilliQ) into the well of a 96-well plate in duplicates (see Note 11). Before measurement, pipet 50 µl of CompA 2 into each of the 50-µl cell lysate samples.
4. Determine colorimetric values directly by spectrophotometry, reading at 520 nm.

Preferred time points:

1. 4 days
2. 14 days and later

3.4.2. ALP Staining

1. Remove culture medium
2. Wash with PBS twice
3. Fix cells in 4% paraformaldehyde for 15 min at 4°C
4. Wash with PBS twice
5. Incubate with 0.1 M Tris buffer at pH 9.5 for 10 min at RT

6. Stain cells using NBT/BCIP in a 1:50 dilution with 0.1 M Tris buffer containing 0.1 M NaCl and 0.05 M $MgCl_2$ for 30 min
7. Rinse with aquadest
8. Incubate in 100% methanol for 5 min to remove non-specific background staining

Preferred time points:

1. 14 days and later

3.4.3. Alizarin Red Staining

When counterstaining for cartilaginous tissue, perform steps 1–5. If not, start the staining protocol at step 6.

1. Filter toludine blue solution through a 0.2-μm filter
2. Stain the slides/wells for 5–10 min
3. Rinse in 30% ethanol
4. Rinse in distilled water
5. Air dry
6. Place the slides/wells on a hot section (54°C)
7. Pipet alizarin red-S on the slide/well and incubate for 60–180 s
8. Remove the staining solution by jets of distilled water
9. Remove the water by jets of ethanol 96%
10. Put back on hot plate and air dry

Preferred time points:

1. 21 days and later

3.4.4. BSP and Osteonectin Immunohistochemical Staining

We visualize all the staining in culture wells and in 3D scaffolds using (confocal) microscopy.

For the staining of 3D scaffolds, we use plastic labtek™ chamberslides™ for containment of the various solutions during the staining procedure and since glass interferes with confocal lasers. For each step, 150–200 μl of the appropriate solution is generally sufficient to cover the scaffold, but the amount is greatly dependent on the scaffold size.

BSP:

1. Wash scaffolds/slides once with PBS and once with PBS/1% BSA/0.1% saponin (=PBS++)
2. Add normal goat serum 1:20 in PBS++ for 10 min at RT
3. Remove normal goat serum
4. Add a 1:200 dilution of a rabbit antibody against human BSP in PBS++ and add a 1:1,000 dilution of non-immune rabbit serum in PBS++ to the negative controls. Incubate for 1 h at RT on a shaker and subsequently overnight at 4°C

5. Wash with PBS++ three times
6. Add a 1:400 dilution of Alexa Fluor® 488 goat anti-rabbit IgG in PBS++ for 1 h at RT. Keep in the dark
7. Wash with PBS three times
8. Add 7.5–15 μM propidium iodide in PBS for 15 min in the dark
9. Wash with PBS three times
10. Keep samples in the dark until analysis

Osteonectin:

1. Wash scaffolds/slides once with PBS and once with PBS++
2. Add normal goat serum 1:20 in PBS++ for 10 min at RT
3. Remove normal goat serum
4. 1:200 dilution of a rabbit antibody against osteonectin in PBS++ and add a 1:1,000 dilution of non-immune rabbit serum in PBS++ to the negative controls. Incubate for 1 h at RT on a shaker and subsequently overnight at 4°C
5. Wash with PBS++ three times
6. Add a 1:400 dilution of Alexa Fluor® 488 goat anti-rabbit IgG in PBS++ for 1 h at RT. Keep in the dark
7. Wash with PBS three times
8. Add 7.5–15 μM propidium iodide in PBS for 15 min in the dark
9. Wash with PBS three times
10. Keep samples in the dark until analysis

Preferred time points:

1. 21 days and later

3.4.5. Real-Time Polymerase Chain Reaction

Total RNA is isolated according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). cDNA synthesis is performed using 0.5–1 μg total RNA in a 20-ml reaction mix consisting of five units transcriptor reverse transcriptase (RT), 0.08 A260 units random primers, 1 mM of each dNTP, and 1× concentrated Transcriptor RT reaction buffer. Real-time PCR reactions are performed using the LightCycler 480 SYBR green I Master reaction mix according to the manufacturer's instructions in a LightCycler 480, and relative expressions of a housekeeping gene (18S) and target genes (runx-2, OPN, and COL1A1) are determined. Primers used for real-time PCR are listed in Table 1. Values of target gene expression are normalized for 18S gene expression.

3.5. Summary

The aim of this chapter is to describe various osteogenic differentiation protocols for adipose-derived stem cells currently applied within our laboratory. It has to be kept in mind that the development and implementation of protocols is always subject to ongoing optimizations, and may vary even within the same laboratory to some degree between researchers. In addition, it is important to realize that in particular in deviations from normal circumstances, e.g., when seeding SVF or cultured ASC preparations on scaffold materials, conditions and outcome may differ from those normally observed. For example, scaffold properties such as hydrophobicity/hydrophilicity, surface roughness, or sterilization history may affect the differentiation pathway, time frames, and other parameters thought to be stable under "regular" circumstances. This implies that, even though this review aims to provide some more or less standardized protocols for osteogenic induction of ASCs, it might be that still some minor optimizations have to be performed for a specific research environment. However, even though minor local differences may exist, we hope to have contributed to some degree to a standardization of current osteogenic induction protocols. We would appreciate receiving feedback on the protocols described; this will facilitate the development of consensus protocols, which in turn will allow better comparison of data sets generated by different research groups. This continuing standardization, which might be reported on in international meetings like those of IFATS (http://www.IFATS.org), might be of benefit for the whole ASC research community.

4. Notes

1. Depending on the size of the culture flask used, various amounts of the trypsinizing solution are used: 0.5 ml for 25 cm^2, 1 ml for 75 cm^2 and 2 ml for 175 cm^2.
2. Zuk and colleagues (10, 11) described induction media containing 100 nM dexamethasone, reflecting conditions also used for induction of bone marrow-derived MSCs. However, in our hands, this concentration resulted in massive cell death of ASCs. When this concentration was lowered tenfold, i.e., to 10 nM, this toxicity was no longer observed, but the appearance of the cell cultures was still different from the cultures induced via other osteogenic growth factors; the cells were highly flattened, were hardly visible under phase-contrast microscopy, and required extremely long trypsin incubation times to release them from the tissue culture plastic. Moreover, release occurred in sheets, and not as individual cells.

Our laboratory therefore decided not to pursue dexamethasone-mediated osteogenic differentiation.

3. Literature describes different concentrations of ascorbic acid, ranging from 10 to 100 μg/ml. In our experimental experience, we found that these varying concentrations may have their own effect on the differentiation state of the ASC cultures. If the purpose is comparing non-differentiation ($DMEM^{++}$ only) vs. optimal osteogenic induction conditions ($DMEM^{++}$/β-GP/ascorbate, plus optimal growth factor concentration), one might choose a high concentration (50–100 μg/ml) of ascorbic acid. However, when the purpose is to strictly determine the effect of the growth factor, i.e., by comparing $DMEM^{++}$/β-GP/ascorbate medium *without* and *with* optimal growth factor concentration, one should lower the ascorbic acid concentrations (33 μg/ml, 50 μg/ml maximally), to avoid increase of basal differentiation levels in the non-growth factor-containing ("control") cultures, which might hamper drawing the appropriate conclusions.
4. According to the product information, the lyophilized protein is stable for at least 2 years at –20°C. Reconstituted BMP-2 is stable for at least 3 months stored at –20°C. Make working aliquots and prevent repeated freeze/thaw cycles.
5. The BSA functions to avoid "sticking" of the BMP-2 to the tube wall. Although medium containing 10% FCS may have similar properties, we have not used this option, since other compounds within the FCS may have negative effects on the BMP-2 activity and/or activity.
6. Morphology of colonies is remarkably different between species; goat ASC colonies are nicely round and relatively homogeneous in size, whereas the human colonies are more disperse, not clearly delineated, and much more variable in size.
7. This procedure is applicable to both freshly isolated SVF and cultured cells. In our experience, lower concentrations (10 and 40 ng BMP-2/ml) did not result in consistent and reproducible osteogenic induction. In high concentrations (1 μg/ml and higher), especially in the short-term induction protocols, we observed toxic effects on the cells.
8. In our group, results of DNA content that were more consistent in samples were obtained by analyzing the supernatant after centrifugation of the sample *without sonication*. Thereafter, the sample is sonicated and further processed for analysis of protein content and ALP activity.
9. The amount of substrate converted by ALP is time dependent, therefore we maintain an incubation period of 1 h.

10.

Concentration (ng/ml)	DNA1,000 ng/ml stock solution (µl)	CompA1 (1:400) (µl)
600	150	100
400	100	150
200	50	200
100	25	225
50	12.5	237.5
0	0	250

11. For data analysis, correct for the difference in sample volume (50 µl) and standard volume (100 µl).

References

1. Helder MN, Knippenberg M, Klein-Nulend J, Wuisman PI. (2007) Stem cells from adipose tissue allow challenging new concepts for regenerative medicine. Tissue Eng; 13(8):1799–1808.
2. Knippenberg M, Helder MN, Zandieh DB, Wuisman PI, Klein-Nulend J. (2006) Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells. Biochem Biophys Res Commun; 342(3):902–908.
3. Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C. (2008) Adipose-derived stem cells: isolation, expansion and differentiation. Methods; 45(2):115–120.
4. Knippenberg M, Helder MN, Doulabi BZ, Bank RA, Wuisman PI, Klein-Nulend J. (2009) Differential effects of bone morphogenetic protein-2 and transforming growth factor-beta1 on gene expression of collagen-modifying enzymes in human adipose tissue-derived mesenchymal stem cells. Tissue Eng Part A; 15(8):2213–2225.
5. Tjabringa GS, Vezeridis PS, Zandieh-Doulabi B, Helder MN, Wuisman PI, Klein-Nulend J. (2006) Polyamines modulate nitric oxide production and COX-2 gene expression in response to mechanical loading in human adipose tissue-derived mesenchymal stem cells. Stem Cells; 24(10):2262–2269.
6. Tjabringa GS, Zandieh-Doulabi B, Helder MN, Knippenberg M, Wuisman PI, Klein-Nulend J. (2008) The polymine spermine regulates osteogenic differentiation in adipose stem cells. J Cell Mol Med; 12(5A):1710–1717.
7. Knippenberg M, Helder MN, Doulabi BZ, Semeins CM, Wuisman PI, Klein-Nulend J. (2005) Adipose tissue-derived mesenchymal stem cells acquire bone cell-like responsiveness to fluid shear stress on osteogenic stimulation. Tissue Eng; 11(11–12): 1780–1788.
8. Bianco P, Fisher LW, Young MF, Termine JD, Robey PG. (1991) Expression of bone sialoprotein (BSP) in developing human tissues. Calcif Tissue Int; 49(6):421–426.
9. Termine JD, Kleinman HK, Whitson SW, Conn KM, McGarvey ML, Martin GR. (1981) Osteonectin, a bone-specific protein linking mineral to collagen. Cell; 26(1 Pt 1):99–105.
10. Zuk PA, Zhu M, Mizuno H et al. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng; 7(2):211–228.
11. Zuk PA, Zhu M, Ashjian P et al. (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell; 13(12):4279–4295.
12. Jurgens WJ, Van Dijk A, Zandieh-Doulabi B et al. (2009) Freshly isolated stromal cells from the infrapatellar fat pad are suitable for a one-step surgical procedure to regenerate cartilage tissue. Cytotherapy; 11(4):1–13.
13. Knippenberg M, Helder MN, de Blieck-Hogervorst JM, Wuisman PI, Klein-Nulend J. (2007) Prostaglandins differentially affect osteogenic differentiation of human adipose tissue-derived mesenchymal stem cells. Tissue Eng; 13(10):2495–2503.

Chapter 18

Generation of Adipose Stromal Cell-Derived Hepatic Cells

Joseph C. Ruiz

Abstract

The demand for primary human hepatocytes to test the toxicity of new drug candidates and to develop cell therapies for liver disease far exceed the number of hepatocytes that can be isolated from donated tissues. Less than 700 whole livers per year are available for research applications. The ability to utilize nonhepatic progenitor cells, such as adipose stromal cells (ASCs), to generate derivatives that mimic primary human hepatocytes would enable the scale-up production of cell products for bioartifical liver-assist devices, cell therapy, and drug discovery applications. ASC hepatogenesis is a rapidly evolving field with improved protocols continually being reported in the literature. In this chapter, current and effective protocols for the expansion, hepatic differentiation, and functional characterization of ASC-derived hepatic cells are outlined. Two major features distinguish optimized methodologies: (a) cytokine-mediated "reprogramming" of mesenchymal ASCs to enable transdifferentiation into endodermal cell lineages, and (b) treatment with sequential media formulations containing factors/extracellular matrices that mimic the temporal expression profiles seen during fetal liver development. Criteria for success are acquisition of hepatic functional activities, such as albumin/urea production and p450 CYP activities, at levels that approach those observed in primary human hepatocyte controls.

Key words: Hepatocytes, Hepatic function, Cytochrome p450, Liver development, Drug discovery, Cell therapy

1. Introduction

Primary human hepatocytes represent optimal cell models for assessing the toxicity of new drug candidates (1) and well as novel cell therapeutics for liver disease (2). The major limitation for the use of primary human liver cells is that the vast majority of hepatocytes do not have proliferative capabilities. Thus, the number of cells needed for cell therapy and drug discovery applications far exceeds the number of cells available from donated livers, and cannot provide a renewable resource for repeat studies. For example, up to three billion hepatocytes are transplanted in human cell

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therapy studies (2). As an alternative to transplantation, extracorporeal devices containing at least ten billion hepatocytes have been used as bioartifical liver devices (3). Lastly, while the numbers of hepatocytes are required for large-scale toxicology screens are less than those required for cell therapy, the finite number of hepatocytes that can be isolated from a single donor limits the number of screens that can be conducted on a specific donor genotype. The ability to use progenitor cell populations with extensive proliferative capacities, such as ASCs, to generate cells that closely recapitulate the hepatic phenotype will address the growing demand for a renewable source of hepatic cells for drug discovery and cell therapy applications.

ACSs have extensive proliferative capacities. For example, from one T75 flask of primary stromal vascular fraction (SVF) cells (two to three million cells per confluent flask derived from ~10 ml of lipoaspirate), 80 million ASCs can be generated by passage 1, 3.2 billion ASCs by passage 2, and more than 120 billion ASCs by passage 3 (1:40 passage). ASCs can be expanded at least four passages without apparent effects on the stem cell expression profile (4). Moreover, ASCs survive cryopreservation; we routinely achieve viabilities exceeding 95% after cryopreservation. Therefore, ASCs provide a renewable source of progenitor cells with extraordinary expansion capacities.

Improved protocols for the hepatic differentiation of ASC are continually being published (please see (5) for a review of the field). The protocol outlined in this chapter represents the optimal protocol based on published results. The key requirements for inducing effective in vitro hepatic differentiation include withdrawal from cell cycle, commitment (i.e., reprogramming) to endodermal cell lineage pathways, and sequential exposures to cytokine/extracellular matrix environments that approximate those present in the developing fetal liver. Gene expression and mutational studies in mouse and zebrafish have revealed the interaction of a variety of signaling pathways that modulate early liver development (6, 7). Among these include activin, BMP, FGF, and EGF signaling pathways. The most effective protocols incorporate these factors in the differentiation regimen (5, 8–11).

Establishing benchmark functional parameters is essential for determining the efficacy of hepatic differentiation. Cell functionality is determined by measuring parameters accepted by the field as indicative of healthy hepatocyte function: albumin secretion, ureagenesis, and cytochrome p450 basal or induced activities. We do not use hepatic gene expression to determine cell function, but rather use expression as a rapid in-house screen for evidence of hepatic differentiation. Two additional nonquantitative assays for hepatic function will be presented: glycogen storage and the uptake/clearance of the organic anion, indocyanine green (ICG) (12). Normalizing hepatic functional data (i.e., albumin and urea

production, CYP activities) will be important so that the efficacy of independent differentiation experiments can be properly assessed. At least three replicate samples are analyzed for each assay. Data is recorded as activity per milligram protein for each replicate ($n = 3$, data collated as means ± SE). The ratio of the normalized data from differentiated to undifferentiated cultures will be calculated as fold-induction. As a positive control for all assays, we suggest the use of adult primary hepatocytes, which can be obtained from commercial cell distributers.

2. Materials

2.1. Cell Culture

1. Expansion medium: Dulbecco's Modified Eagle's medium (DMEM) low glucose and MCDB-201 at a 60:40 ratio supplemented with 10% fetal bovine serum (FBS), 1× ITS (insulin, transferrin, selenium) mix, 10^{-9} M dexamethasone, 10^{-4} M ascorbic acid 2-phosphate, 10 ng/ml epidermal growth factor (EGF), penicillin/streptomycin, and 2 mM glutamax™-1.
2. Cryopreservation medium: eight parts Expansion medium: one part FBS: one part dimethyl sulfoxide (DMSO).
3. Differentiation media (four sequential media treatments):
   (a) Preinduction medium: DMEM low glucose containing penicillin/streptomycin, and 2 mM glutamax™-1;
   (b) Commitment medium: Preinduction medium supplemented with 20 ng/ml EGF, 20 ng/ml Activin A, and 10 ng/ml basic fibroblast growth factor (bFGF) *or* Preinduction medium supplemented with 20 ng/ml EGF, 10 ng/ml bone morphogenic protein 4 (BMP4), and 10 ng/ml bFGF (either commitment media can be used);
   (c) Differentiation medium: Preinduction medium supplemented with 20 ng/ml hepatic growth factor (HGF), 0.61 g/L nicotinamide, 5% KnockOut™ serum replacement; and
   (d) Maturation medium: Preinduction medium supplemented with Oncostatin M, 1× ITS, and 2% KnockOut™ serum replacement.
4. TrypLE™ Express.
5. Uncoated T75 flasks; collagen I-coated T75 flasks and 12-well dishes.
6. Fibronectin and collagen III diluted in phosphate-buffered saline (PBS) for coating T75 flasks.

2.2. Expression Analysis

1. RNA isolation: RNeasy minikit.
2. cDNA synthesis: Superscript III first-strand synthesis supermix, RNAse OUT™ Recombinant ribonuclease inhibitor.
3. PCR analysis: GoTaq green mastermix.
4. PCR primers: For the detailed list of additional human-specific markers of hepatic differentiation, see Schmelzer et al. (13). Forward and reverse primers are mixed at 50 ng/μl each.
   (a) Albumin: 188-base pair product.
   Forward primer: GTGGGCAGCAAATGTTGTAA
   Reverse primer: TCATCGACTTCCAGAGCTG
   (b) α-Fetoprotein: 148-base pair product.
   Forward primer: ACCATGAAGTGGGTGGAATC
   Reverse primer: TGGTAGCCAGGTCAGCTAAA
   (c) Transferrin: 152-base pair product.
   Forward primer: CTACACAGGCGCTTTCAGGT
   Reverse primer: TACCATCAAGGCACAGCAAC
   (d) Dipeptylpeptidase 4: 237-base pair product.
   Forward primer: TCCATATCCAAAGGCAGGAG
   Reverse primer: TCTTTCTTGTGTTGCCCATG
   (e) α1-anti-trypsin: 143-base pair product.
   Forward primer: GGACCTCTGTCTCGTCTTGG
   Reverse primer: GCTCTGATTTGGGGTTGTGT
   (f) p450 CYP3A4: 187-base pair product.
   Forward primer: GCCTGGTGCTCCTCTATCTA
   Reverse primer: GGCTGTTGACCATCATAAAAGC
   (g) GAPDH (positive control): 173-base pair product.
   Forward primer: ATGTTCGTCATGGGTGTGAA
   Reverse primer: GTCTTCTGGGTGGCAGTGAT.

2.3. Cell Staining

1. Glycogen staining: Periodic acid–Schiff base (PAS) kit.
2. Indocyanine green (ICG) uptake.
3. Albumin detection: Human albumin ELISA kit.
4. Urea detection: QuantiChromtm urea assay kit. Ammonium chloride.
5. P450 CYP activity: P450-Glo™ Assays; P450-Glo™ CYP1A1 Assay; P450-Glo™ CYP2C9 Assay; P450-Glo™ CYP3A4 Assay. Rifampicin dissolved in DMSO. White opaque 96-well luminometer plates.

2.4. Protein Determination

1. *RC DC* Protein assay kit II.
2. Protein lysis buffer: 0.05 M Tris pH 8.0, 0.15 M sodium chloride, and 1% Triton X-100.

3. Methods

Here we describe (1) the expansion of established ASCs; (2) a sequential hepatic differentiation protocol; and (3) a number of assays routinely used to determine the extent of hepatic differentiation including: expression of hepatic genes; glycogen storage; ICG uptake and clearance; albumin and urea production; and p450 cytochrome activity. ASC-derived hepatic cells can also be transplanted into immunocompromised mice (e.g., SCID mice) to provide in vivo functional data (8, 14), but these protocols are beyond the scope of this review.

3.1. Expansion of Adipose Stromal Cells

1. T75 flasks used for expansion are coated with a solution of 5 μg/ml fibronectin in PBS for a minimum of 2 h at 37°C, after which time, the solution is removed by aspiration.
2. Once an ASC population is established (see Note 1), the cells are lifted off the flasks by a 5-min treatment with TrypLE™ Express at 37°C, and plated at a density of 10^3 cells/cm^2 on fibronectin-coated flasks (1:40 split per passage). The cells are propagated to ~80% confluence before passaging. In an 80% confluent T75 flask, we routinely obtain ~three million ASCs. If all the cells are expanded, up to 120 million ASCs can be obtained with one additional passage, 4.8 billion cells with two passages, and 192 billion cells with three passages. Cells are not propagated beyond passage 4.
3. Cells are cryopreserved beginning at passage 1. Cells are centrifuged at $200 \times g$ to pellet the ASCs, resuspended in cryopreservation medium at a density of 3×10^6 cells/ml, and slowly cooled to −80°C overnight before long-term storage in liquid nitrogen. To reestablish cultures, the cells are quickly thawed at 37°C, pelleted at $200 \times g$, resuspended in Expansion Medium, and seeded into four T75 fibronectin-coated flasks. We routinely achieve postthaw viabilities exceeding 95%.

3.2. Hepatic Differentiation

1. T75 flasks used for expansion are coated with a solution of 2 μg/ml collagen III in PBS for a minimum of 2 h at 37°C, after which time, the solution is removed by aspiration.
2. ASCs expanded on fibronectin-coated T75 flasks are collected and seeded onto collagen III-coated T75 flasks at a density of 25,000 cells/cm^2 (approximately three million cells).

3. When the cells attain 80% confluency (see Note 2), the cells are switched to Preinduction medium, which is serum-free, for 1 day to induce cell cycle withdrawal. This is defined as day 1 of the differentiation process.
4. The growth-arrested ASCs are then propagated in Commitment medium for 2 days to induce cells to enter endodermal cell lineage pathways. This is defined as day 2 of the differentiation process (see Note 3).
5. The ASCs are then propagated in Differentiation medium for 7 days to induce the initial stages of hepatic differentiation. This is defined as day 4 of the differentiation process.
6. On the fourth to sixth day of propagation in Differentiation medium, the cells are lifted off the T75 flask with TrypLE™ Express and seeded onto collagen I-coated flasks at a density of 65×10^3 cells/cm^2 (approximately five million cells). Cells can be passaged at any point of the differentiation process with no loss of yield (see Note 4).
7. In addition to seeding the cells onto T75 flasks, the cells are seeded onto a minimum of three 12-well collagen I-coated dishes at a density of 65×10^3 cells/cm^2 (approximately three million cells per 12-well dish) (see Note 5). The 12-well dishes are used for all the hepatic functional assays beginning 7 days after the Maturation medium step (functional assays outlined below).
8. After the seventh day of Differentiation medium treatment, the cultures are switched to Maturation medium to allow the cells to attain hepatic functional activities. The cultures are maintained in Maturation medium for the duration of the hepatogenic process. This is defined as day 11 of the differentiation process. Cultures can be propagated in Maturation medium for at least 3 weeks (see Note 6).

3.3. Expression Analysis

1. Beginning at day 18 of the differentiation process (7 days after propagation in Maturation medium) and every 7 days thereafter, the cells in one well are lysed using 350 µl of RNA lysis buffer from the RNeasy Minikit. Lysates can be stored at –80°C until all desired time points are collected. The protocol outlined below is not intended to provide quantitative expression data, but rather to provide data on the extent of differentiation. Alternatively, qPCR can be utilized as to generate quantitative expression data.
2. Total RNAs are purified using the manufacturer's instructions. RNA yields are not determined as the cell densities in each well are comparable.
3. Two microliters of the purified RNA preparation is used to synthesize cDNA using the Superscript III First-strand

Synthesis Supermix according to the manufacturer's instructions (with the inclusion of RNAse OUT™ Recombinant Ribonuclease Inhibitor).

4. One microliter of the cDNA preparation is used as template for PCR amplification of hepatic markers of differentiation using GoTaq Green Mastermix. In each individual amplification reaction, 50 ng of each primer pair is used. The cycling conditions are as follows: 94°C for 2 min, then 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min (35 cycles), with a final 72°C extension step for 2 min before cooling to 4°C. One-fifth of the PCR reaction is fractionated through a 2% agarose gel in TAE buffer to visualize the products.
5. RNAs collected from undifferentiated ASCs will serve as a negative control for hepatic gene expression; RNAs from primary human hepatocytes, obtained from a commercial cell distributer, will provide positive controls for hepatic gene expression (culture conditions suggested by the distributor should be followed) (see Note 7).
6. As noted in the introduction, expression analysis is not utilized to define hepatic function, but only to provide rapid evidence that the ASCs are progressing along the differentiation pathway. Alternatively, qRT-PCR can be used to obtain quantitative expression data (13).

3.4. Cell Staining

1. Glycogen storage and uptake and subsequent release of the organic anion, ICG, are indicators of hepatic function that can be readily visualized by microscopy.
2. To detect glycogen storage in differentiated cells, three wells in the 12-well dish are fixed with methanol for 2 min, then stained using the Periodic acid–Schiff base (PAS) kit according to the manufacturer's instructions, except that the recommended counterstain with hematoxylin is omitted (see Note 8). Glycogen will be revealed by a purple histological stain under bright field optics; glycogen-free cells will remain unstained. Glycogen-positive cells can be detected as early as day 18 of the differentiation protocol.
3. Indocyanine green (ICG) is suspended DMSO at 5 mg/ml, and diluted in medium at 1 mg/ml for cell staining. Live cells are incubated with 1 mg/ml ICG diluted in medium for 30 min at 37°C. The live cultures are washed three times with medium then the clearance of intracellular ICG from the cells is documented by fluorescence microscopy over the next 6 h.
4. Undifferentiated ASCs seeded in 12-well dishes will serve as negative controls for PAS and ICG staining. Primary human hepatocytes will provide positive controls for glycogen storage and ICG uptake/release (see Note 7).

3.5. Albumin Detection

1. Spent media from three wells of the 12-well dishes ($n = 3$) is collected after a 3-day exposure to cells (i.e., for triplicate measurements of albumin production), and is stored at –20°C until all desired samples are collected. Primary human hepatocytes are used for positive controls for albumin production. We begin collecting spent media 10 days after the initiation of the differentiation process (see Note 9).
2. The level of albumin production is determined using the human albumin ELISA kit according to the manufacturer's instructions.
3. Undifferentiated ASCs seeded in 12-well dishes serve as negative controls for albumin production.

3.6. Urea Detection

1. Individual wells are propagated in medium containing 6 mM NH_4Cl for 24 h before collecting the spent media. The spent media from three wells of the 12-well dish ($n = 3$) can be stored at –20°C until all desired samples are collected. Primary human hepatocytes are used as positive controls for urea production. We begin collecting spent media 10 days after the initiation of the differentiation process (see Note 9).
2. The level of urea production, after the addition of NH_4Cl, is determined using the QuantiChrome™ Urea Assay kit according to the manufacturer's instructions.
3. Undifferentiated ASCs seeded in 12-well dishes serve as negative controls for urea production.

3.7. P450 CYP Activity

1. The following protocol outlines the determination of basal levels of P450 CYP1A1, CYP2C9, and CYP3A4 activities using P450-Glo™ Assays. Three wells ($n = 3$) from the 12-well dish are used for the measurements. We typically begin screening for CYP activity approximately 4 weeks into the differentiation process (see Note 9).
2. In Preinduction media, prepare 100 μM CYP1A1 substrate, Luciferin-CEE; 100 μM CYP2C9 substrate, Luciferin-H; and 50 μM CYP3A4 Luciferin-PFBE (see Note 10).
3. Maturation media is aspirated from the wells and replaced with 500 μl of the CYP substrates diluted in the Preinduction media.
4. Three wells are also incubated with Preinduction media without substrates to serve as a control for background luciferin emission.
5. Primary human hepatocytes obtained from a cell distributor are tested for CYP activity in parallel as positive controls for the P450-GLO™ assays.

6. The substrates are incubated with the cells for 3 h at 37°C, and then collected into 1.5-ml microfuge tubes for storage at –80°C until all desired samples are collected.
7. Detection of the luciferin produced by CYP activity is determined after incubation with the Luciferin Detection Reagent according to the manufacturer's instructions in 96-well white opaque luminometer plates, and the signal detected using a Luminometer.
8. Optionally, inducible CYP2C9 and CYP3A4 activities can be determined in parallel as follows. Three additional wells for each CYP assay are incubated with 25 μM rifampicin 24 h prior to determining P450-GLO™ activities as outlined in steps 2–7.
9. Undifferentiated ASCs seeded in 12-well dishes serve as negative controls for p450 CYP activity.

3.8. Protein Determination

1. At the conclusion of the differentiation experiments (to be determined by the investigator), protein lysates are prepared from the individual wells that were used to collect the albumin, urea, and CYP functional data.
2. Place 500 μl of Protein Lysis Buffer in each well, incubate 5 min at room temperature, and store the lysate in 1.5-ml microfuge tubes.
3. For protein determination, dilute the lysates 1:10 in water to dilute the concentration of Triton X-100 to 0.1% (to prevent interference with the Protein Determination Assay), and use the *RC DC* Protein Assay Kit II according to the manufacturer's instructions.

3.9. Normalizing Functional Data

1. As noted in the introduction, normalizing hepatic functional data (i.e., albumin and urea production, CYP activities) will be important so that the efficacy of independent differentiation experiments can be properly assessed.
2. Three replicate samples will be analyzed for each assay. These data will be recorded both as activity per milligram protein contained in each respective sample ($n = 3$; data collated as means ± SE).
3. In addition, the ratio of the normalized data from differentiated:undifferentiated cultures will be calculated as fold-induction.
4. As a positive control for all assays, the functional data obtained from adult primary hepatocytes will provide an indication on how closely ASC-derived hepatic cells approach "normal" functional activities. It is important to note, however, that each lot of human hepatocytes is derived from a unique donor

with a unique genotype and lifestyle, which will result in significant variations in functional capacity. Nonetheless, despite this caveat, an indication that ASCs acquire multiple hepatic functional properties at levels that approach those observed in primary human hepatocytes would indicate effective in vitro hepatogenesis.

4. Notes

1. Since ASCs are derived from human donors, variability in expansion and differentiation capabilities is to be expected. While the establishment of ASCs is beyond the scope of this chapter, we validate the immunophenotype of ASCs before initiating differentiation experiments (e.g., CD44, CD73, CD90, and CD105 positive; and CD14 and CD45 negative (4)).
2. It is essential that differentiation is initiated before confluence is attained (~80% confluence), as some of the cells will continue to divide during the Preinduction medium step. By the end of the preinduction step, the plates should be 100% confluent; cell density is key for efficient hepatic differentiation.
3. Either Activin A-based or BMP4-based Commitment media can be used at this step.
4. Since Differentiation media contains 5% KnockOut™ serum replacement, the cells will proliferate. The cells are passaged and reseeded at the indicated cell densities on BD-Biocoat™ flasks/plates not only to provide a matrix that is optimal for hepatocytes, but also to maintain optimal cell densities. The cells can be dissociated from the plates and reseeded at the suggested densities as needed to maintain 100% confluent flasks/plates. Once the cultures are transferred into Maturation medium (containing 2% KnockOut™ serum replacement), the cells display minimal proliferative capabilities.
5. The 12-well dishes to be used for hepatic functional analyzes can be seeded at any time during the Maturation media step as well, if needed.
6. Development of new media formulations that promote long-term stability of ASC-derived hepatic cells (i.e., greater than 3 weeks) is still in progress as is the development of cryopreservation media for ASC-derived hepatic cells.
7. It is important that primary human hepatocytes be used controls for the hepatic functional assays not only to serve as positive controls for the assays but to serve as the standard (benchmark) for "normal" hepatic function for direct comparison to the functional activities of ASC-derived hepatic

cells. While it preferable that fresh primary human hepatocytes be used, cryopreserved human hepatocytes can be used to lower costs.

8. The hematoxylin step is eliminated to increase the contrast between the glycogen-positive purple-stained and unstained (transparent) cells under bright-field optics.
9. For consistency, the same wells are sampled, respectively, for the albumin, urea, and P450-Glo™ assays at defined time points during the differentiation process. Thus, the data will indicate the temporal profile of the functional values for specific cell populations (i.e., cultures within a specific well).
10. It is essential that substrates that have been validated for the use in live cells be used: P450-Glo™ CYP1A1 Assay; P450-Glo™ CYP2C9 Assay; P450-Glo™ CYP3A4 Assay.

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References

1. LeCluyse, E. L. (2001) Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. *Eur. J. Pharm. Sci.* **13**, 343–368.
2. Fox, I. J. and Chowdhury, J. R. (2004) Hepatocyte transplantation. *Am. J. Transplant.* **4 Suppl 6,** 7–13.
3. Sauer, I. M., Neukirchen, W., Rahimi, G., Grunheck, F., Hescheler, J., and Wartenberg, M. (2003) Extracorporeal liver support based on primary human liver cells and albumin dialysis – treatment of a patient with primary graft non-function. *J. Hepatol.* **39**, 649–653.
4. Mitchell, J. B., Mitchell, J. B., McIntosh, K., Zvonic, S., Garrett, S., Floyd, Z. E., et al. (2006) Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* **24,** 376–385.
5. Snykers, S., De Kock, J., Rogiers, V., and Vanhaecke, T. (2009) In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. *Stem Cells* **27,** 577–605.
6. Huang, H., Ruan, H., Aw, M. Y., Hussain, A., Guo, L., Gao, C., et al. (2008) Mypt1-mediated spatial positioning of Bmp2-producing cells is essential for liver organogenesis. *Development* **135**, 3209–3218.
7. Rossi, J. M., Dunn, N. R., Hogan, B. L., and Zaret, K. S. (2001) Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes Dev.* **15,** 1998–2009.
8. Banas, A., Teratani, T., Yamamoto, Y., Tokuhara, M., Takeshita, F., Osaki, M., et al. (2009) Rapid hepatic fate specification of adipose-derived stem cells and their therapeutic potential for liver failure. *J. Gastroenterol. Hepatol.* **24,** 70–77.

9. Bonora-Centelles, A., Jover, R., Mirabet, V., Lahoz, A., Carbonell, F., Castell, J.V., et al. (2009) Sequential heaptogenic trans-differentiation of adipose tissue-derived stem cells: relevance of different extracellular signaling molecules, transcription factors involved and expression of new key marker genes. *Cell Transplant*. **18,** 1319–1340.
10. Campard, D., Lysy, P. A., Najimi, M., and Sokal, E. M. (2008) Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. *Gastroenterology* **134,** 833–848.
11. Snykers, S., Vanhaecke, T., Papeleu, P., Luttun, A., Jiang, Y., Vander Heyden, Y., et al. (2006) Sequential exposure to cytokines reflecting embryogenesis: the key for in vitro differentiation of adult bone marrow stem cells into functional hepatocyte-like cells. *Toxicol. Sci*. **94,** 330–341.
12. Agarwal, S., Holton, K. L., and Lanza, R. (2008) Efficient differentiation of functional hepatocytes from human embryonic stem cells. *Stem Cells* **26,** 1117–1121.
13. Schmelzer, E., Wauthier, E., and Reid, L.M. (2006) The phenotypes of pluripotent human hepatic progenitors. *Stem Cells* **24,** 1852–1858.
14. Seo, M. J., Suh, S. Y., Bae, Y. C., and Jung, J. S. (2005) Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. *Biochem Biophys Res Commun*. **328,** 258–264.

Chapter 19

Adipose Stem Cell Differentiation into Smooth Muscle Cells

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Abstract

The differentiation of adipose-derived stem cells (ASCs) into functional smooth muscle cells has received limited investigation. Various methodologies for both in vitro and in vivo differentiation is described. In vitro differentiation is obtained by either chemical or mechanical stimulation, and is determined by expression of smooth muscle cell markers. In vivo differentiation studies include animal models of cardiovascular disease and one study with urinary bladder reconstruction. The ease of obtaining an abundant number of ASCs render this cell population useful for potential vascular therapies that require autologous smooth muscle cells.

Key words: Adipose-derived stem cells, Smooth muscle cells

1. Introduction

Stem cells have great potential for use in tissue repair and regeneration. The recent discovery and characterization of multilineage cells from adipose tissue has been met with a great deal of excitement in the fields of tissue engineering and regenerative medicine. This chapter focuses on recent literature describing the isolation, characterization, and smooth muscle differentiation potential of adipose-derived stem cells (ASCs).

Mature blood vessel walls have a complex three-dimensional structural organization that reflects compartments of highly differentiated cells and their associated extracellular matrix. The endothelial cells (ECs) that line the lumen are separated from the smooth muscle cells (SMCs)-containing medial layer by a sub-endothelial extracellular matrix and the internal elastic lamina. Medial SMCs are arranged in concentric lamellar layers that are separated by an elastic fiber-rich matrix. Fibroblasts of the outer collagen-rich adventitial layer are, in turn, separated from media

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SMCs by the outer elastic lamina (1, 2). Recent studies have demonstrated that adult bone marrow stromal cell populations have the potential to differentiate into cells characteristic of blood vessels (3–6). Liu et al. have isolated SMC progenitors from bone marrow (BM-SMPC) and generated tissue-engineered vascular grafts that, 5 weeks after implantation into the jugular veins of five lambs, demonstrated circumferential cell alignment and extensive collagen production (7).

The ability of ASCs to differentiate into SMCs has not been as widely studied, but the abundance and easy harvesting of ASCs compared with bone marrow progenitor cells make them a possibly more preferable candidate as a cell source for SMCs. ASC to SMC differentiation has been initiated with in vitro studies determining which chemical factors added to the culture medium induce smooth muscle cell markers. For a recent review on these studies, see de Villiers et al. (8). Rodriguez et al. (9) examined the capacity of ASCs to differentiate into phenotypic and functional SMCs for repair of the urinary tract. The authors reported genetic expression of all SMC markers and increased protein expression of SMC-specific α-actin, calponin, caldesmon, SM22, myosin heavy chain, and smoothelin when ASCs were cultured in smooth muscle-inductive medium (medium MCDB 131 from Sigma supplemented with 1% fetal bovine serum (FBS) and 100 U/mL heparin) for 6 weeks. In addition, ASC-derived smooth muscle-like cells were reported to exhibit the ability to contract and relax in response to pharmacologic agents (9). Abderrahim-Ferkoune et al. reported that the overexpression of aortic carboxypeptidase-like protein (ACLP) promotes transdifferentiation of established mouse preadipocyte clonal line 3T3-L1 cultured in β-mercaptoethanol (BME) and ascorbic acid (AA) into smooth muscle-like cells, which express smooth muscle (SM)-specific markers such as SM22α, SM α-actin, SM-MHC, and caldesmon (see Note 1) (10). Kim et al. have demonstrated that addition of angiotensin II (Ang II) to human ASC cultures caused increased SMC-specific gene expression, induced the secretion of transforming growth factor (TGF) β_1 from the cells, and induced contraction in response to 60 mM KCl (11). Yang et al. added 50 ng/mL platelet-derived growth factor (PDGF)-BB to SMC induction media along with 5 ng/mL TGF β_1 to increase SMC markers in ASCs (12). Chen et al. recently demonstrated smooth muscle-like differentiation of rat ASCs using BME (13). Some other chemical components tested in vitro for smooth muscle differentiation of ASCs have been sphingosine 1-phosphate and thromboxane A(2) (14–17).

There have been multiple reports of using ASCs as a cell therapy for repairing SMC-containing tissue within animal models of cardiovascular disease and one study with urinary bladder reconstruction. For a review summarizing studies using ASCs as a cell

therapy for treating the cardiovascular system, specifically acute or chronic ischemic limbs after femoral artery ligation within a mouse model, *see* Madonna et al. (18). Traktuev et al. cocultured ASCs with human microvascular ECs within Matrigel in vitro and demonstrated enhanced network assembly into cords with ASCs stabilizing the abluminal walls (19). This work demonstrates the potential for ASCs to be used in treating vascular disease or generating tissue-engineered vascular grafts. Jack et al. pretreated human ASCs with a SMC-inductive media (consisting of MCDB131 media supplemented with 1% FBS and 100 U/mL heparin) for 6 weeks before seeding on poly-lactic-co-glycolic acid (PLGA) scaffolds for bladder repair (20). The scaffolds replaced the removal of half of nude rat bladders and throughout 12 weeks compliance and capacity were maintained only in the animals with scaffolds seeded with SMC-induced ASCs and not in the unseeded scaffolds.

It has been well documented that cells and tissues in their native environment are constantly subjected to various forms of mechanical stimulation (such as compression, tension, and shear) that are necessary for their normal development and functions (21–23). In vivo, the arterial vessel wall is continuously exposed to mechanical stresses. While vascular ECs are exposed to shear stress due to the nature of blood flow, vascular SMCs are subjected to significant cyclic mechanical strain in the circumferential direction throughout the cardiac cycle (24). These mechanical stresses are likely to influence the physiology of these cells by contributing to regulation of growth and phenotypic state of these cells. Work within our laboratory published by Lee et al. has demonstrated cyclic strain in combination with TGF 1 stimulation to inhibit proliferation of human ASCs and cause the F-actin cytoskeleton of the cells to align perpendicularly to the direction of strain (see Note 2) (25).

In summary, several protocols have been recently published describing the differentiation of ASCs into SMCs, and these protocols are based on both chemical and mechanical stimulation methods.

2. Materials

1. Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and gentamycin.
2. Dexamethasone.
3. Collagenase (type II).
4. Hank's solution.

5. Double-layer gauze.
6. Erythrocyte lysis buffer.
7. Centrifuge tubes.
8. CyQUANT assay.
9. Fatty acid-free BSA.
10. β-mercaptoethanol (BME).
11. Retinoic acid (RA).
12. Transforming growth factor (TGF)-β_1.
13. Platelet-derived growth factor (PDGF)-BB.
14. Normal donkey serum (NDS).
15. Normal goat serum (NGS).
16. Triton-X.
17. Phosphate-buffered saline (PBS)–Tween 20.
18. 4′,6-Diamidino-2-phenylindole, dihydrochloride (DAPI).
19. 10% SDS PAGE gel.
20. Monoclonal antibody specific for α-SMA (Clone 1A4).
21. Paraformaldehyde.
22. Protease inhibitor cocktail.
23. Detergent-based lysis buffer.

3. Methods

Adipose-derived stem cells (ASCs) were isolated from human subcutaneous adipose tissue harvested during elective abdominoplasty and cultured as previously described (26, 27).

1. The subcutaneous adipose tissue was minced and then digested in Hank's Balanced Salt Solution (HBSS) (see Note 3) containing 1 mg/mL type II collagenase and 3.5% fatty acid-free BSA in a 37°C shaking water bath until the mixture was homogeneous (see Note 4).
2. The digested tissue was filtered through a double-layered gauze filter (350 μm) and centrifuged at 1,000 rpm (216×*g*) for 10 min (see Note 5).
3. After centrifugation, the resulting cell pellet was resuspended within Erythrocyte Lysis Buffer, vortexed, and centrifuged at 1,000 rpm (216×*g*) for 10 min (see Note 6).
4. Lastly, the cells were resuspended in regular cell culture media (1:1 DMEM/F12, supplemented with 10% FBS, dexamethasone, and antibiotics) (see Note 7) and plated at a density of 5×10^4 cells/cm^2.

5. Cell culture media was changed every 2 days until confluence. Low-passage cells (<P4) from female donors (between the ages of 31 and 51 years) were utilized for these experiments, and were thawed and cultured in regular plating medium for 24 h before the cells were lifted and seeded either on 22 × 22-mm glass coverslips in 6-well polystyrene plates (for immunocytochemical staining) or in 100-mm tissue culture dishes (for either mRNA or protein collection).
6. At confluence, ASCs were exposed to media with reduced serum (DMEM/F12, 5% FBS, 1% P/S, without dexamethasone) supplemented with one of the following chemicals or growth factors (see Note 8): 50 μM β-mercaptoethanol (BME) and 0.3 mM ascorbic acid (AA); 1 μM retinoic acid (RA); 1 μM RA and 0.5 mM dibutyryl-cyclic adenosine monophosphate (d-cAMP); 1 ng/mL and 10 ng/mL TGF-β_1; or 10 ng/mL PDGF-BB.
7. The ASCs were treated with fresh differentiation media every 2 days for the duration of 7 and 14 days.
8. Controls were ASCs cultured in plating medium with 5% serum.
9. At the end of each time point, glass coverslips were fixed for immunostaining, whereas cell lysates were collected for Western blot analysis.

3.1. Immunofluorescence Staining

At the termination of each experiment, cells were immunostained to identify protein expression.

1. Cells were fixed in 2% paraformaldehyde for 15 min at room temperature and immunostained according to standard immunocytochemistry protocols.
2. After washing twice with PBS, 0.1% Triton-X made in PBS was applied to the cells for 15 min, and then nonspecific binding was blocked using NDS or NGS.
3. A monoclonal antibody specific for α-SMA was applied for 60 min at room temperature, followed by incubation with fluorescent-tagged secondary antibody for 60 min.
4. After subsequent washes with PBS, stained cells were viewed by fluorescent microscopy.
5. To provide a quick assessment of cell distribution, 0.6 μg/mL DAPI was used to stain the nuclei of cells.
6. Protein expression was qualitatively observed through the inspection of immunofluorescent images (see Note 9).

3.2. Western Blot Analysis

Cell lysates were collected at the termination of each experiment from each culture condition to quantify protein expression.

1. Cells were washed with PBS, then 300 μL detergent-based lysis buffer and protease inhibitor cocktail (1:100 dilution) were added to each 100-mm dish for collection of total cellular protein.
2. Equal amounts of protein (10 μg) from each sample were loaded into a 10% SDS PAGE gel for gel electrophoresis.
3. The separated proteins were transferred to a nitrocellulose membrane, and the membrane was blocked in 5% nonfat milk/PBS–Tween 20 solution at 4°C overnight, followed by the application of a monoclonal antibody specific for α-SMA in 5% nonfat milk/PBS–Tween 20.
4. After incubation at room temperature for 1–1.5 h, the secondary antibody in 5% nonfat milk/PBS–Tween 20 was applied for 1 h, and the membrane was washed three times with 0.01% PBS/Tween 20 for 10 min after each antibody application.
5. The proteins on the nitrocellulose membrane were detected with the ECL Plus detection system, according to the manufacturer's protocol.
6. The resulting protein bands were quantified by volume summation of image pixels with NIH ImageJ 1.30v.

4. Notes

1. SM22α, SM α-actin, SM-MHC, caldesmon, and smoothelin are well-characterized SMC markers and demonstrate the contractile ability of differentiated progenitor cells (8). Smooth muscle α-actin is an early SMC marker, whereas SM-MHC and smoothelin are highly restricted to contractile, functional SMCs. Smoothelin can distinguish SMCs from myofibroblasts.
2. This work utilized the FlexCell system using settings of only 10% strain at 1 Hz for 7 days. However, these parameters could be varied in order to optimize mechanical strain to further enhance SMC differentiation of ASCs.
3. It is important to note that collagenase should be added to HBSS specifically instead of using PBS.
4. Digestion of tissue is highly dependent on the starting consistency of the donor tissue. Starting with whole adipose tissue will require a longer digestion time, whereas lipoaspirate digests more quickly. Within approximately 30 min, the tissue should be mostly digested. Digestion should be considered complete when only smaller (approximately <5 mm in diameter) tissue pieces remain.

5. The gauze filtering step to remove leftover connective tissue can be performed here at this step OR at the end of the process, right before plating.
6. It is important to ensure thorough vortexing of the cells within the erythrocyte lysis buffer solution to completely lyse all erythrocytes.
7. Also, an antifungal/antimycotic component such as Amphotericin B (up to 0.125 μg/mL) can be used to prevent yeast contaminations that can commonly occur from processing of human adipose tissue.
8. Work within our laboratory published by Lee et al. demonstrated addition of TGF β_1 to be the more dominant factor in SM α-actin expression when ASCs were treated under different combinations of these factors (28). PDGF-BB and d-cAMP significantly reduced SM α-actin expression within this work.
9. Proper antibody dilutions are necessary for minimizing protein expression signal within control undifferentiated cells compared with differentiated cells. Our laboratory has observed some normal expression of early SMC markers such as SM α-actin within cultured undifferentiated ASCs.

References

1. Sidawy, A. N. (1997) Peptide growth factors and their role in the proliferative diseases of the vascular system. New York: Futura.
2. Moses, M. A., Klagsbrun, M., and Shing, Y. (1995) The role of growth factors in vascular cell development and differentiation. *Int Rev Cytol* **161,** 1–48.
3. Arakawa, E., Hasegawa, K., Yanai, N., Obinata, M., and Matsuda, Y. (2000) A mouse bone marrow stromal cell line, TBR-B, shows inducible expression of smooth muscle-specific genes. *FEBS Lett* **481**, 193–6.
4. Arakawa, E., Hasegawa, K., Irie, J., Ide, S., Ushiki, J., Yamaguchi, K., Oda, S., and Matsuda, Y. (2003) l-ascorbic acid stimulates expression of smooth muscle-specific markers in smooth muscle cells both in vitro and in vivo. *J Cardiovasc Pharmacol* **42**, 745–51.
5. Kinner, B., Zaleskas, J. M., and Spector, M. (2002) Regulation of smooth muscle actin expression and contraction in adult human mesenchymal stem cells. *Exp Cell Res* **278**, 72–83.
6. Wang, D., Park, J. S., Chu, J. S., Krakowski, A., Luo, K., Chen, D. J., and Li, S. (2004) Proteomic profiling of bone marrow mesenchymal stem cells upon transforming growth factor beta1 stimulation. *J Biol Chem* **279**, 43725–34.
7. Liu, J. Y., Swartz, D. D., Peng, H. F., Gugino, S. F., Russell, J. A., and Andreadis, S. T. (2007) Functional tissue-engineered blood vessels from bone marrow progenitor cells. *Cardiovasc Res* **75**, 618–28.
8. de Villiers, J. A., Houreld, N., and Abrahamse, H. (2009) Adipose derived stem cells and smooth muscle cells: implications for regenerative medicine. *Stem Cell Rev Rep* **5,** 256–65.
9. Rodriguez, L. V., Alfonso, Z., Zhang, R., Leung, J., Wu, B., and Ignarro, L. J. (2006) Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cells. *Proc Natl Acad Sci U S A* **103**, 12167–72.
10. Abderrahim-Ferkoune, A., Bezy, O., Astri-Roques, S., Elabd, C., Ailhaud, G., and Amri, E. Z. (2004) Transdifferentiation of preadipose cells into smooth muscle-like cells: role of aortic carboxypeptidase-like protein. *Exp Cell Res* **293**, 219–28.
11. Kim, Y. M., Jeon, E. S., Kim, M. R., Jho, S. K., Ryu, S. W., and Kim, J. H. (2008) Angiotensin II-induced differentiation of

adipose tissue-derived mesenchymal stem cells to smooth muscle-like cells. *Int J Biochem Cell Biol* **40**, 2482–91.

12. Yang, P., Yin, S., Cui, L., Li, H., Wu, Y., and Liu, W. (2008) Experiment of adipose derived stem cells induced into smooth muscle cells. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* **22**, 481–86.
13. Chen, R. B., Liu, J. H., Rao, K., Pan, Y. G., Wang, T., Wang, S. G., and Ye, Z. Q. (2009) Differentiation of rat adipose-derived stem cells into smooth-muscle-like cells in vitro. *Zhonghua Nan Ke Xue* **15**, 425–30.
14. Nincheri, P., Luciani, P., Squecco, R., Donati, C., Bernacchioni, C., Borgognoni, L., Luciani, G., Benvenuti, S., Francini, F., and Bruni, P. (2009) Sphingosine 1-phosphate induces differentiation of adipose tissue-derived mesenchymal stem cells towards smooth muscle cells. *Cell Mol Life Sci* **66**, 1741–54.
15. Yun, D. H., Song, H. Y., Lee, M. J., Kim, M. R., Kim, M. Y., Lee, J. S., and Kim, J. H. (2009) Thromboxane A(2) modulates migration, proliferation, and differentiation of adipose tissue-derived mesenchymal stem cells. *Exp Mol Med* **41**, 17–24.
16. Kim, M. R., Jeon, E. S., Kim, Y. M., Lee, J. S., and Kim, J. H. (2009) Thromboxane a(2) induces differentiation of human mesenchymal stem cells to smooth muscle-like cells. *Stem Cells* **27**, 191–9.
17. Jeon, E. S., Park, W. S., Lee, M. J., Kim, Y. M., Han, J., and Kim, J. H. (2008) A Rho kinase/myocardin-related transcription factor-A-dependent mechanism underlies the sphingosylphosphorylcholine-induced differentiation of mesenchymal stem cells into contractile smooth muscle cells. *Circ Res* **103**, 635–642.
18. Madonna, R., Geng, Y. J., and De Caterina, R. (2009) Adipose tissue-derived stem cells. Characterization and potential for cardiovascular repair. *Arterioscler Thromb Vasc Biol.* **29**(11), 1723–1729
19. Traktuev, D. O., Merfeld-Clauss, S., Li, J., Kolonin, M., Arap, W., Pasqualini, R., Johnstone, B. H., and March, K. L. (2008) A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. *Circ Res* **102**, 77–85.
20. Jack, G. S., Zhang, R., Lee, M., Xu, Y., Wu, B. M., and Rodriguez, L. V. (2009) Urinary bladder smooth muscle engineered from adipose stem cells and a three dimensional synthetic composite. *Biomaterials* **30**, 3259–70.
21. Lee, A. A., Delhaas, T., Waldman, L. K., MacKenna, D. A., Villarreal, F. J., and McCulloch, A. D. (1996) An equibiaxial strain system for cultured cells. *Am J Physiol* **271**, C1400–8.
22. Terracio, L., Tingstrom, A., Peters, W. H., 3rd, and Borg, T. K. (1990) A potential role for mechanical stimulation in cardiac development. *Ann N Y Acad Sci* **588,** 48–60.
23. Vandenburgh, H. H., and Karlisch, P. (1989) Longitudinal growth of skeletal myotubes in vitro in a new horizontal mechanical cell stimulator. *In Vitro Cell Dev Biol* **25**, 607–16.
24. Davis, M. J., Wu, X., Nurkiewicz, T. R., Kawasaki, J., Davis, G. E., Hill, M. A., and Meininger, G. A. (2001) Integrins and mechanotransduction of the vascular myogenic response. *Am J Physiol Heart Circ Physiol* **280**, H1427–33.
25. Lee, W. C., Maul, T. M., Vorp, D. A., Rubin, J. P., and Marra, K. G. (2007) Effects of uniaxial cyclic strain on adipose-derived stem cell morphology, proliferation, and differentiation. *Biomech Model Mechanobiol* **6**, 265–73.
26. Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P., and Hedrick, M. H. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* **7**, 211–28.
27. Zuk, P. A., Zhu, M., Ashjian, P., De Ugarte, D. A., Huang, J. I., Mizuno, H., Alfonso, Z. C., Fraser, J. K., Benhaim, P., and Hedrick, M. H. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279–95.
28. Lee, W. C., Rubin, J. P., and Marra, K. G. (2006) Regulation of alpha-smooth muscle actin protein expression in adipose-derived stem cells *Cells Tissues Organs* **183**, 80–6.

Chapter 20

Endothelial and Cardiac Regeneration from Adipose Tissues

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Abstract

For a long time, adipose tissue was only considered for its crucial role in energy balance and associated diseases. The discovery of the presence of immature cells highlights a putative role for these tissues as reservoirs of therapeutic cells. Indeed, since fat pads can be sampled by liposuction under local anesthesia in adult patients, adipose tissue represents a promising source of regenerative cells, particularly in cardiovascular regeneration. Indeed among other potentials, we and others have demonstrated the great angiogenic properties of adipose-derived stromal cells (ASCs) and the existence of peculiar cells, at least in mice, that are able to spontaneously give rise to functional cardiomyocytes. This review deciphers the different steps necessary to isolate, characterize, and manipulate such striking cells.

Key words: Adipose tissue, Endothelial cells, Cell therapy, Regenerative medicine, Angiogenesis, Heart, Stems cells, Cardiomyocytes, Mesenchymal cells

1. Introduction

Adipose tissue is now well recognized as a highly plastic tissue. Indeed, fat masses have a great and reversible capacity for expansion, which appears to be permanent throughout adult life. Adipose tissue enlargement is the result of adipocyte hypertrophy and the recruitment and differentiation of regenerative precursors located in the stroma vascular fraction (SVF). The SVF represents the heterogeneous cell population surrounding adipocytes in fat tissue. Cultured SVF cells, named adipose-derived stromal cells (ASCs), were classically used to investigate preadipocyte differentiation into mature adipocytes (1). However, this fraction was also reported to be a very convenient and non-restrictive source of multipotent stem cells that are able to differentiate into other cells of the mesenchymal lineage, including osteoblasts, chondrocytes,

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and myoblasts (2, 3). We and others have also shown that ASCs participate in the revascularization of the ischemic hindlimb (4, 5). The proangiogenic properties of ASCs are sustained by their ability to differentiate into endothelial cells and to secrete potent angiogenic factors such as vascular endothelial growth factor (VEGF) and leptin (6, 7). The angiogenic properties of ASCs can be enhanced with pre-conditioning during the culture process (8). However, some immature cells from the SVF differentiate spontaneously, at least in mice, into cells with properties of cardiomyocytes (9). Therefore, the SVF and ASCs represent a promising source of regenerative cells, particularly in cardiovascular regeneration. In this chapter, we describe methods to study ASC endothelial and cardiac differentiation.

1.1. Endothelial Differentiation

Several in vitro methods have been developed to study angiogenesis of ASCs. In our laboratory, we have used methylcellulose, a semi-solid media, known to reveal endothelial potential in progenitors (10). We observed that ASCs cultured in methylcellulose form a network of branched tube-like structures (5). Moreover, most of these cells express specific endothelial markers such as CD31 and von Willebrand Factor (vWF). The use of a simple liquid culture medium to reveal the angiogenic potential of ASCs is also possible when the cells are placed on an extracellular matrix-coated plate. We and others (data not published) have developed a technique of culture of ASCs on fibronectin- or gelatin-coated plates in the presence of a liquid medium supplemented with angiogenic factors such as VEGF and/or insulin growth factor (IGF) (4). Under these conditions, after 10 days of culture, ASCs develop cobblestone areas and tube-like structures as well as specific endothelial marker expression.

In vivo evaluation of ASC pro-angiogenic properties is assessed in our laboratory by using the mouse model of ischemic hindlimb developed by Silvestre et al. (11). In this model, mouse, or human ASC transplantation improves angiogenesis in the ischemic mouse hindlimb as shown by the increase of angiographic scores and cutaneous blood flow. These cells induce neovascularization in mouse ischemic muscle partly through endothelial differentiation (5). Indeed, antibodies directed specifically against the human CD31 isoform stain numerous CD31-positive cells into functional vessels of immunodeficient mice hindlimb injected with human ASCs. No CD31-positive cells are detected in non-injected contra-lateral hindlimbs.

1.2. Cardiac Differentiation

Mouse SVF cells from adipose tissue reveal multiple differentiation potentials in vitro. A convenient procedure for evidencing the co-existence of various differentiated cells in culture is to plate SVF cells into a semi-solid culture medium in order to allow the clonogenic differentiation and maintenance of non-adherent cells.

The presence of distinct cell morphologies can be observed after 2–3 weeks of SVF cell culture in methylcellulose.

Among the clusters that develop in methylcellulose, we identified spontaneously contractile cells that were characterized to be functional cardiac-like cells. Such cells express various cardiac genes (Nkx2.5, GATA4, MEF2c) and proteins (MLC-2v, cTnT, sarcomeric α-actinin) and display cardiac ultrastructures as demonstrated using electron microscopy. Some cells present a pacemaker activity that can be modulated by pharmacological agents (β-adrenergic agonists, cholinergic agonists, and respective antagonists) (9).

In vivo, the administration of adipose-derived cardiogenic cells in a mouse model of acute myocardial infarction highlights that these cells are capable of cardiogenesis as assessed by the identification of injected cells expressing GFP along with cardiac markers such as cTnT or MLC-2v. Cell administration is, moreover, associated with the induction of angiogenesis, the improvement of ventricular function and limitation of left ventricle remodeling (12). To date, identical culture procedures have not showed evidence of equivalent cells from human SVF, even though cardiac-like cells were obtained from rabbit adipose tissue after 5 aza-cytidine treatment (13).

2. Materials

2.1. Cell Isolation

1. Digestion buffer: Dulbecco's Modified Eagle's Medium F-12 (DMEM/F-12) is supplemented with 2% bovine serum albumin (BSA) and 2 mg/ml collagenase A.
2. Erythrocyte lysis buffer: ammonium chloride solution.
3. Phosphate-buffered saline (PBS).
4. 25- and 100-μm nylon filters.

2.2. Cell Culture for Endothelial Differentiation

SVF Culture

1. Culture medium: Minimum Essential Medium alpha (MEMα) supplemented with 10% newborn calf serum (NCS) and amphotericin (0.25 μg/ml), streptomycin (0.1 mg/ml) and penicillin (100 U/ml).
2. Solution of trypsin–EDTA 0.05%.

Methylcellulose

3. Methylcellulose medium with recombinant cytokines (Methocult GF M3534 for mice ASCs and Methocult GF H4534 for human ASCs).
4. Plastic culture dishes, 35 mm.
5. 37% Formaldehyde solution.

Angiogenic Liquid Medium

6. Culture medium: MEMα supplemented with 10% NCS.
7. VEGF is dissolved at 10 μg/ml in PBS supplemented with 0.1% BSA and stored in single-use aliquots at –20°C.
8. Gelatin (2%) is diluted to 0.1% in PBS.
9. 48-well tissue culture plates.

2.3. ASC Transplantation in Ischemic Hindlimb in Mice

1. Animals: Seven-week-old male C57Bl6N or nu/nu mice housed in a controlled environment (12 h light/dark cycle at 21°C) with free access to water and standard chow diet.
2. Anesthesia: Xylazine, ketamine.
3. Surgical procedure: Dissecting scissors, 6/0 nylon for ligature, and 8/0 nylon for suture.
4. Functional evaluation of the model: heating plate, laser Doppler.
5. Structural evaluation of the model: Barium sulfate; X-ray transducer; Isopentane solution; Superfrost® plus slides; Tris Buffer Saline (TBS); Anti-human SMA α1; Anti-mouse CD31 antibody; goat and horse serum; Alexa-fluor 594 goat anti-mouse antibody; Alexa-fluor 594 donkey anti-rat antibody; DAPI solution (4,6-diamidino-2-phenylindole).

2.4. Cardiac Differentiation

1. Methylcellulose medium with recombinant cytokines (Methocult GF M3534).
2. Plastic culture dishes, 35 mm.
3. 37% Formaldehyde solution.

2.5. Cardiogenic Cell Amplification

1. Spontaneous beating clusters of cardiac-like cells selected from methylcellulose culture of SVF.
2. Leica DMIL inverted microscope.
3. Plastic culture dishes, 35 mm.
4. Culture medium: BHK21 supplemented with 10% fetal bovine serum, 10^{-4} M β-mercaptoethanol, 2 mM glutamine, 1 mM pyruvate, 0.1 mM non-essential amino acid, 0.25 μg amphotericin, 100 U/ml penicillin G and 100 μg/ml streptomycin.
5. Gelatin (2%) is diluted to 0.1% in PBS.

2.6. FACS Analysis

1. Non-adherent rounded cells and elongated contractile cells in cardiogenic liquid medium BHK21.
2. Solution of trypsin–EDTA 0.05%.
3. Directly fluorescent-conjugated anti-mouse monoclonal antibodies (mAb) or non-conjugated monoclonal antibody Troponin T cardiac.

4. Fluorescent anti-mouse IgG secondary antibody.
5. PBS supplemented with 2% fetal bovine serum.
6. 5-ml Polystyrene round-bottom tube.
7. Fluorescence analysis cell sorter FACS Calibur.
8. Cell quest software.
9. 37% Formaldehyde solution.
10. PBS containing 1% BSA and 0.5% saponin.

2.7. Immunostaining for Endothelial and Cardiac Markers

Endothelial-like differentiation in methylcellulose

1. Kit Dako S2001.
2. Goat serum.
3. Triton X-100.
4. Rabbit IgG anti-human vWF antibody 5.7 g/l.
5. Horseradish peroxidase (HRP) goat anti-rabbit $F(ab')_2$ 0.8 mg/ml.
6. AEC Substrate Chromogen (3-amino-9-ethylcarbazole).

Endothelial-like differentiation in angiogenic liquid medium

7. Mouse anti-human CD31 antibody (clone JC70A).
8. Alexa-fluor 488 goat anti-mouse antibody.
9. Tween 20.
10. 300 nM DAPI solution in water.

In vivo endothelial differentiation

11. Mouse anti-human CD31 antibody (clone JC70A).
12. BCIP (5-bromo-4-chloro-3-indolyl phosphate)/NBT (nitro blue tetrazolium) detection kit.

Cardiac-like differentiation in methylcellulose

13. Primary antibodies against myosin light chain (MLC-2v), sarcomeric α-actinin, MEF2C, alpha-myosin heavy chain (α-MHC), titin, and cardiac troponin T (clone 13-11), rabbit antibody against connexin 43, purified mouse, and rabbit IgG isotype controls.
14. Secondary antibody Alexa-fluor 546-, Alexa-fluor 594-conjugated anti-mouse, or anti-rabbit IgG.
15. 3.7% Formaldehyde in PBS buffer, 1% BSA PBS buffer, 0.3% Triton X-100 PBS buffer.
16. Nuclear stain: 300 nM DAPI solution in water.
17. Fluorescent mounting medium.

3. Methods

3.1. Cell Isolation

A number of similar methods for isolating adipose tissue cells are reported in the literature. However, the number and composition of SVF cells can change according to the protocol used. In our laboratory, we use the cell isolation protocol described below.

1. Inguinal adipose tissue from mice and human adipose tissue from dermolipectomy are cut into small pieces (see Note 1).
2. Adipose tissues are digested at 37°C in DMEM/F-12 containing 2% BSA and 2 mg/ml collagenase A for 45 min (see Note 2).
3. After elimination of undigested fragments by filtration through 100- and 25-μm filters, mature adipocytes are separated from other cells by centrifugation (600 × *g*, 10 min, see Note 3).
4. Erythrocyte cells are eliminated by incubation with erythrocyte lysis buffer for 5 min at 4°C (see Note 4).
5. After washing with PBS and centrifugation (600 × *g*, 10 min), pellets of SVF cells are obtained (see Note 5).

3.2. In Vitro Endothelial-like Differentiation

SVF culture

1. SVF cells are plated at a density of 4×10^3 cells/cm^2 in MEMα supplemented with 10% NCS and amphotericin (0.25 μg/ml), streptomycin (0.1 mg/ml) and penicillin (100 U/ml).
2. After 24 h, all non-adherent cells are removed by PBS washing.
3. Adherent cells are then expanded to reach confluence. Media are changed every 3 days.
4. Confluent SVF cells or ASCs are trypsinized and the cells are counted with a cell counter.

Methylcellulose

5. ASCs are plated at a density of 1×10^4 cells in 1.5 ml methylcellulose in 35-mm dishes.
6. Cell culture is performed for 10 days to develop an endothelial-like morphology such as branched alignments and tube-like structures.
7. Methylcellulose is then eluted from the wells by washing with PBS and fixed in 3.7% formaldehyde for 10 min at 4°C (see Note 6).
8. Cells are stored at 4°C in PBS until immunostaining to detect specific endothelial markers (see Subheading 3.6.1).

Angiogenic liquid medium

The angiogenic liquid medium to permit ASC endothelial differentiation and described below, with some modifications, was originally described by Miranville et al. (4).

9. 48-Well plates are coated with 200 µl per well of gelatin 0.1% freshly prepared. Plates are placed at 37°C for 1 h. Excess liquid gelatin is removed.
10. ASCs are plated at a density of 1×10^5 cell/cm^2 in MEMα medium 10% NCS (see Note 7).
11. After 24 h, cells are washed with PBS and cultured in MEMα 10% NCS supplemented with 10 ng/ml VEGF. The medium is changed every 3 days (see Note 8).
12. After 10 days of culture, ASCs develop cobblestone areas and tube-like structures characteristic of endothelial cell morphology.
13. Cells are washed with PBS, fixed with 3.7% formaldehyde for 10 min at room temperature and stored at 4°C in PBS until immunostaining (see Subheading 3.6.2 and Note 9).

3.3. ASC Transplantation in Ischemic Hindlimb in Mice

3.3.1. Surgical Procedure

Mice undergo surgery to perform unilateral hindlimb ischemia as described by Silvestre et al. (11). Animals (eight animals per group) are anesthetized by isoflurane 2% inhalation. Exposure is obtained by performing an incision in the skin overlying the middle portion of the hindlimb. A ligature is performed on the femoral artery, 0.5 cm from the bifurcation of the saphenous and popliteal arteries. The overlying skin is closed using a surgical suture (see Note 10) (Fig. 1).

3.3.2. ASC Preparation for In Vivo Injection

Confluent ASCs are washed twice with warmed PBS, then trypsined and washed twice again to eliminate all traces of serum,

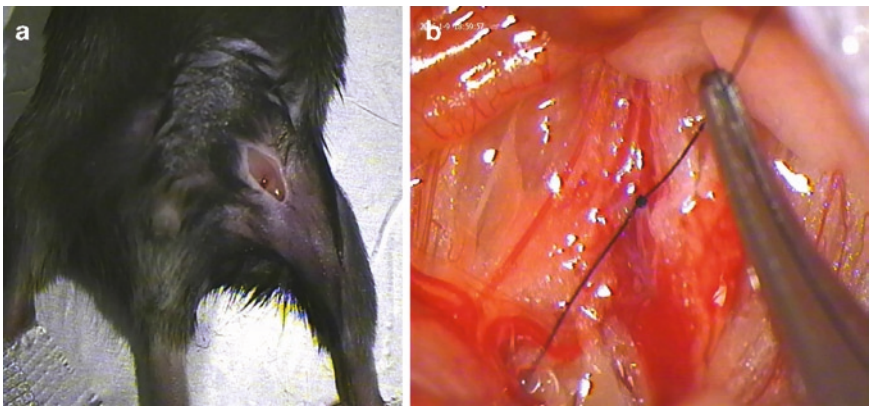

Fig. 1. Surgical procedure. (**a**) Incision in the left hindlimb of a C57Bl6N mouse. (**b**) Ligature of the femoral artery.

antibiotics, or antifungals in the serum-free ASC culture medium without supplementation with any antibiotics or antifungals (control medium). Cells are then counted and collected in this control medium in order to obtain 1×10^6 ASCs per 75 µl (see Note 11).

3.3.3. ASC Transplantation in Ischemic Hindlimb in Mice

Five hours after mice surgery, 1×10^6 ASCs are administered by intramuscular injection at three different sites (gastrocnemius, gracilis, and quadriceps, respectively, 25 µl per injection) of the ischemic leg of an isoflurane-anesthetized mouse. Control mice receive the same injections of the control ASC culture medium described previously. Mice are then housed under specific pathogen-free conditions and functional analyses are performed 15 days after the onset of ischemia (see Note 12).

3.3.4. Functional Evaluation of the Model

Laser Doppler perfusion imaging is performed to provide evidence for ischemia-induced changes in vascularization, as described by Couffinhal et al. (14). Mice are anesthetized by isoflurane inhalation. Excess hair on the limbs is removed with depilatory cream before imaging, and mice are placed on a heating plate at 37°C for 15 min before measurement in order to minimize temperature variation. To account for variables such as ambient temperature and light, cutaneous blood flow is measured three times and expressed as a ratio of ischemic to non-ischemic leg (see Note 13) (Fig. 2).

3.3.5. Structural Evaluation of the Model with Microangiography

Vessel density can be evaluated using high-definition microangiography 15 days post-ischemia as described originally by Silvestre et al. (15) with little modification (Fig. 3).

1. Mice are anesthetized by intraperitoneal administration of xylazine (7.5 mg/kg) and ketamine (75 mg/kg).

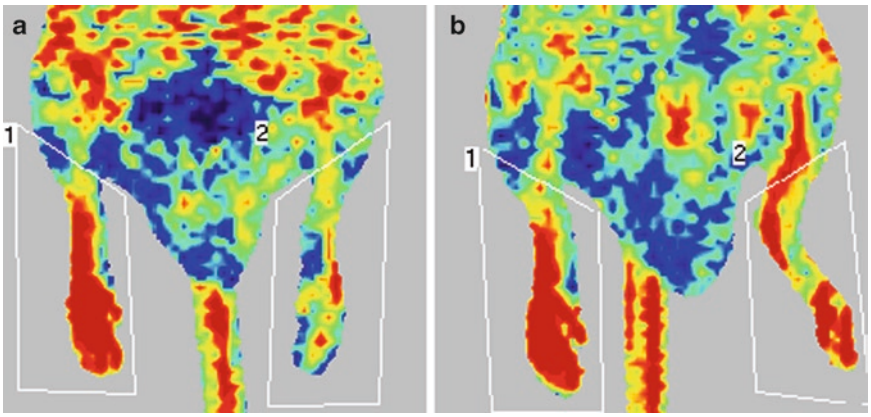

Fig. 2. Laser Doppler perfusion imaging. (**a**) Left hindlimb ischemia in control mouse. (**b**) Recovery of foot perfusion in ASC-transplanted mouse.

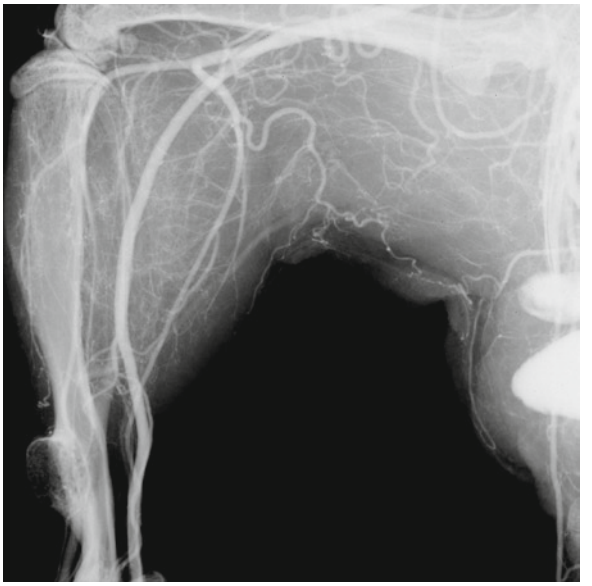

Fig. 3. Example of a microangiograph.

2. A contrast medium (barium sulfate 1 g/ml) is injected through a catheter introduced into the abdominal aorta (see Note 14).
3. A radiograph of both hindlimb regions is then acquired by a digital X-ray transducer (see Note 15).
4. The quantification area is delineated by the location of the ligature of the femoral artery, the knee, the edge of the femur and the external limit of the leg.
5. Vessel density is then expressed as a percentage of pixels per image occupied by vessels in the quantification zone.

3.3.6. Structural Evaluation of the Model with Capillary Density

Microangiographic analysis can be completed by assessment of capillary densities in ischemic versus non-ischemic muscles as previously described (15). Moreover, the arterioles versus capillaries ratio can be assessed by immunostaining with specific antibodies directed against smooth muscle actin α1 or CD31, respectively.

1. Ischemic and non-ischemic muscles are dissected and progressively frozen in isopentane solution cooled by liquid nitrogen.
2. Sections (7 μm) are first incubated for 30 min in PBS containing 5% BSA at room temperature and then 1 h with either a mouse monoclonal antibody directed against human smooth muscle actin α1 (dilution 1:50) to identify arterioles, or with a rat monoclonal antibody directed against CD31 (dilution 1:50) to identify capillaries (see Note 16).

3. Sections are then washed four times for 5 min with TBS.
4. Arterioles are revealed with a fluorescent Alexa-fluor 594 goat anti-mouse antibody (dilution 1:500) incubated for 30 min (see Note 17).
5. Capillaries are revealed with a fluorescent Alexa-fluor 594 donkey anti-rat antibody (dilution 1:500) incubated for 30 min (see Note 17).
6. Sections are then washed four times for 5 min with TBS.
7. Sections are then incubated for 2 min with DAPI solution (dilution 1:10,000) to stain nuclei.
8. Capillary density is analyzed from three different sections of gastrocnemius muscle, and five different randomly chosen fields are used in each section.

3.4. In Vitro Cardiac Differentiation

3.4.1. Spontaneous Cardiac-like Differentiation in Methylcellulose

1. Isolated SVF cells from adipose tissue are directly plated in semisolid methylcellulose medium at 3×10^4 cells/1.5 ml of methylcellulose in a 35-mm dish without previous cell expansion or culture selection (see Note 18).
2. SVF cells plated in methylcellulose are observed every 2 days under an inverted phase-contrast microscope.
3. From 6 days of culture, clones of rounded cells together with small tube cells emerge.
4. From the latter, some rounded cells independently start a contractile activity at days 11–14 after plating. Within a few days, myotube-like structures appear, grow in size, and proliferate, and are still surrounded by some of the rounded cells.
5. After 20–30 days, the areas give rise to a cohesive group of cells, with the presence of branching fibers and shared tight connections. At this time, the entire area beats at a single rate (see Note 19).
6. Methylcellulose is then eluted from the wells by washing with PBS and fixed in 3.7% formaldehyde for 10 min at 4°C (see Note 6).
7. Cells are stored at 4°C in PBS until immunostaining to detect specific cardiac markers (see Subheading 3.7) (Fig. 4).

3.4.2. Process to Highly Improve Culture of Adipose-Derived Cardiomyogenic Cells in Liquid Medium

1. Culture primary SVF cells in methylcellulose until emergence of adipose-derived cardiomyogenic cells (AD-CMG). These cells are selected and picked up under an inverted microscope before plating (1,500 cells/cm^2) into 30-mm culture dishes coated with 0.1% gelatin and cultured in BHK21 medium supplemented with 10% fetal bovine serum, 10^{-4} M β-mercaptoethanol, 2 mM glutamine, 1 mM pyruvate, 0.1 mM non-essential amino acid, 0.25 μg/ml amphotericin, 100 U/ml penicillin G and 100 μg/ml streptomycin.

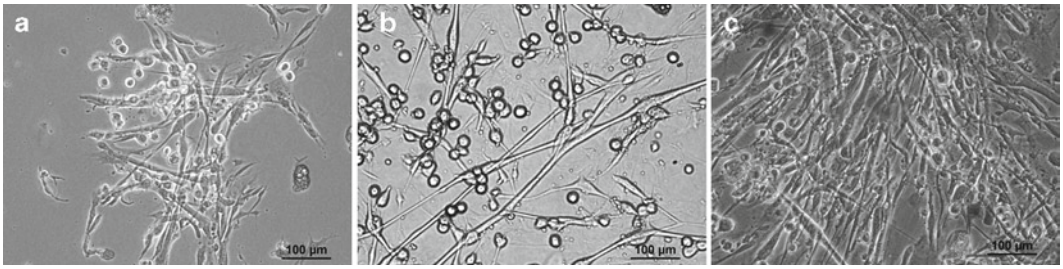

Fig. 4. Different stage of spontaneous emerging cardiac-like cells in methylcellulose (**a**, **b**) or liquid medium (**c**).

2. Change medium every 3 days.
3. Two distinct morphologies of AD-CMG can be identified in BHK21 culture: rounded cells that are non-adherent and elongated contractile cells that are adherent onto the plastic dish.
4. Cell expansion is performed by harvesting cells suspended in the culture medium every 3 days, centrifuging for 5 min at 600×*g* and plating into new cultured dishes coated with 0.1% gelatin and culturing in BHK21 medium (see Notes 6 and 20).
5. The culture is stopped when the morphology of cardiac cells appears, at which time the cells are washed with PBS and fixed in 3.7% formaldehyde for 10 min at 4°C.
6. Cells are stored at 4°C in PBS until immunostaining to detect specific cardiac markers (see Subheading 3.7).

3.5. FACS Analysis

3.5.1. Direct Immunofluorescent Staining for Receptors and Other Cell Surface Antigens

1. Cells suspended in BHK21 liquid medium are harvested, centrifuged for 5 min at 600×*g* and resuspended in 5-ml polystyrene round-bottom tubes with 100 µl of staining buffer (PBS with 0.2% fetal calf serum) at 5×10^6 cells/ml.
2. Adherent elongated cells are washed twice with warmed PBS, trypsinized, and then centrifuged for 5 min at 600×*g* and resuspended in 5-ml polystyrene round-bottom tubes with 100 µl of staining buffer (PBS with 0.2% fetal calf serum) at 5×10^6 cells/ml.
3. Cells are incubated in 100 µl of staining buffer containing a pre-titrated, optimal concentration of a fluorescent monoclonal antibody specific for a receptor or with an immunoglobulin (Ig) isotype-matched control for 30 min at 4°C in the dark (see Notes 21 and 22).
4. After incubation, cells are washed once with 1 ml of staining buffer, then centrifuged to pellet the cells (500×*g* for 5 min), and then the supernatant is removed.
5. Cells are resuspended in 250 µl of PBS.
6. Data acquisition and analysis is then performed on a fluorescence analysis cell sorter (Fig. 5).

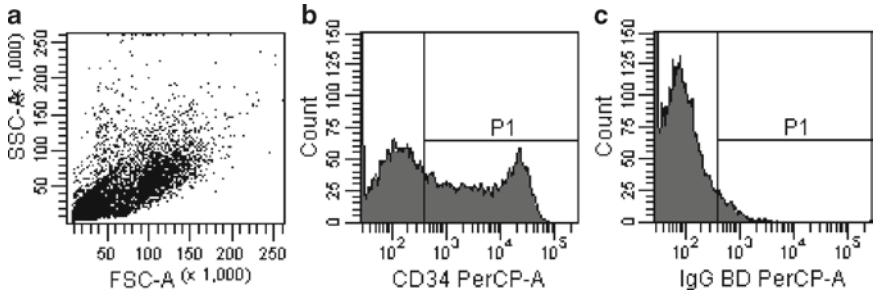

Fig. 5. FACS analysis of the cardiomyocyte-like cells. (**a**) Representative profile of cell population according to cell size and granulosity criteria. (**b**) Representative mono-color FACS analysis using CD34 Per-CP antibody. (**c**) Signals for non-specific fluorescence with isotype IgGs are presented in the *left panel.*

3.5.2. Indirect Immunofluorescent Mono-staining for Intracytoplasmic Antigen

1. After recovering cells as previously described, fix the cells for 10 min at 4°C with 3.7% formaldehyde followed by permeabilization with PBS containing 1% BSA and 0.5% saponin for 20 min at room temperature.
2. Cells are incubated in 100 µl of staining buffer containing a pre-titrated, optimal concentration of a non-fluorescent monoclonal antibody specific for an intracytoplasmic protein or with an Ig isotype-matched control for 30 min at 4°C.
3. After incubation, cells are washed once with 1 ml of staining buffer, then pelleted by centrifugation (500 × *g* for 5 min) and the supernatant removed.
4. Cells are resuspended and incubated in 100 µl of staining buffer containing a fluorescent anti-Ig secondary antibody for 30 min at 4°C in the dark. After the incubation, cells are washed once with 1 ml of staining buffer, pelleted by centrifugation (500 × *g* for 5 min) and the supernatant is removed.
5. Cells are resuspended in 250 µl of PBS. Data acquisition and analysis is then performed on a fluorescence analysis cell sorter.

3.5.3. Acquisition and Analysis Controls

1. Immunoglobulin isotype controls: To extract meaningful conclusions from experiments involving immunofluorescent staining, it is recommended that Ig isotype-matched controls be run in the same experiment at the same concentration as the antigen-specific antibodies.
2. Autofluorescence controls: Autofluorescence results from fluorescent emissions occurring when intracellular materials are excited at the same wavelength as the fluorescent probes used for staining. To determine the baseline fluorescence, controls that include only unstained cells can be used.
3. Compensation controls: Electronic compensation is needed to correct the spectral overlap of fluorescent emissions when

multiple fluorescent probes excited by a single wavelength are used. Cell samples stained with individual fluorescent probes are compared with cells labeled with both fluorescent probes to determine the level of fluorescence signal overlap and to establish proper compensation.

3.6. Immunostaining for Endothelial Markers

3.6.1. Endothelial-like Differentiation in Methylcellulose

Immunostaining is done at room temperature with gentle shaking (Fig. 6).

1. PBS is removed from the well containing formaldehyde fixed cells as described in Subheading 3.2, step 2.
2. Endogenous peroxidases are blocked with Dako kit S2001.
3. Cells are washed three times during 5 min in PBS.
4. Non-specific sites are blocked during 1.5 h with PBS containing 3% goat serum and Triton 0.3X.
5. Blocking solution is removed and cells are incubated for 1 h with rabbit anti-human vWF antibody (dilution 1:400 in PBS with 3% goat serum and Triton 0.3X).
6. Primary antibody is removed and cells are washed four times during 5 min in PBS with 3% goat serum and Triton 0.3X.
7. Wash buffer is removed and cells are incubated for 1 h with HRP goat anti-rabbit antibody (dilution 1:100 in PBS with 3% goat serum and Triton 0.3X).
8. Secondary antibody is removed and cells are washed two times for 5 min in PBS with 3% goat serum and Triton 0.3X.
9. Cells are washed two times for 5 min in PBS.
10. Wash buffer is removed and cells are incubated for 5 to 25 min with AEC substrate chromogen kit.

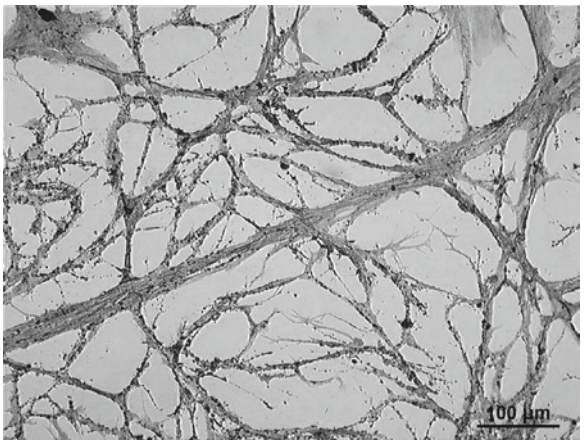

Fig. 6. Culture of ASCs in methylcellulose form a network of branched tube-like structures. Most of these cells express specific endothelial markers, such as von Willebrand Factor.

11. Cells are washed with distilled water and wells are filled with 600 µl PBS until photographs are taken under an inverted microscope.

3.6.2. Endothelial-like Differentiation in Angiogenic Liquid Medium

Immunostaining is directly performed in 48-well tissue culture plates at room temperature, under gentle shaking, and in a minimum volume of 200 µl per well (Fig. 7).

1. PBS is removed from the wells containing fixed cells.
2. Non-specific sites are blocked during 30 min with PBS containing 2% BSA.
3. Blocking solution is removed and cells are incubated for 2 h with mouse anti-human CD31 antibody (dilution 1:10 in PBS with 2% BSA).
4. Primary antibody is removed and cells are washed four times during 10 min in PBS with 0.2% Tween 20.
5. Wash buffer is removed and cells are incubated for 1 h with Alexa-fluor 488 goat anti-mouse antibody (dilution 1:200 in PBS with 2% BSA).
6. Secondary antibody is removed and cells are washed four times during 10 min in PBS with 0.2% Tween 20.
7. Wash buffer is removed and cells are incubated for 2 min with DAPI solution (dilution 1:10,000)
8. DAPI solution is removed and wells are filled with 600 µl PBS. Photos are taken using an inverted fluorescent microscope.

3.6.3. In Vivo Endothelial Differentiation

To assess the ability of injected human ASCs to incorporate new blood vessels in immunodeficient mice subjected to femoral artery

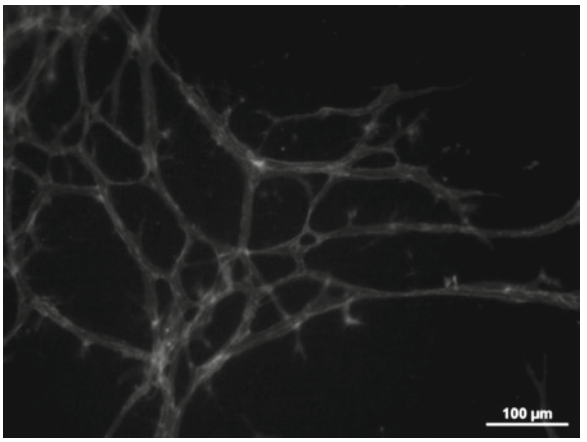

Fig. 7. Anti-CD31 immunostaining on endothelial-like differentiation of ASCs in angiogenic liquid medium.

occlusion, immunohistochemistry experiments are performed using an antibody specific to human CD31 that does not cross-react with mouse CD31.

1. Non-specific sites of frozen tissue sections (7 μm) are blocked with PBS with 2% BSA for 30 min.
2. Slides are then incubated for 1 h with the anti-human CD31 antibody.
3. Immunostaining is visualized using BCIP/NBT detection.

3.7. Immunofluorescence for Cardiac Markers

1. Remove PBS from the dish containing paraformaldehyde-fixed cells as described in Chapter 3, Subheading 3.4, steps 1 and 2.
2. Non-specific sites are blocked during 1 h with PBS containing 1% BSA.
3. Cells are incubated for 1 h in 0.3% Triton X-100/PBS buffer with primary antibody against MLC-2V (1:200), sarcomeric α-actinin (1:500), titin (1:100), troponin T (1:100), connexin 43(1:50). Negative controls are performed with purified mouse IgG or rabbit IgG.
4. After washing, a secondary antibody Alexa-fluor 546 or Alexa-fluor 594 (1:500)-conjugated anti-mouse or anti-rabbit (IgG) is applied for an additional 60 min at room temperature.
5. Secondary antibody is removed and cells are washed three times during 5 min in PBS.
6. Wash buffer is removed and cells are incubated for 2 min with DAPI solution (dilution 1:10,000).
7. DAPI solution is removed, the plate is washed with water and mounted with fluorescent mounting medium. A stained preparation can be stored for several months in the dark at 4°C.
8. Fluorescence images are obtained with a confocal microscope or with a fluorescent wide-field microscope.
9. For confocal microscopy, to improve resolution and signal-to-noise ratio, images are restored using Huygens software and viewed using Imaris software (Fig. 8).

4. Notes

1. During sampling, connective tissue should be carefully dissected and discarded.
2. The incubation time with digestion buffer is of great importance since it can influence the quality of cells selected and affect the surface markers of the cells and therefore their potential.

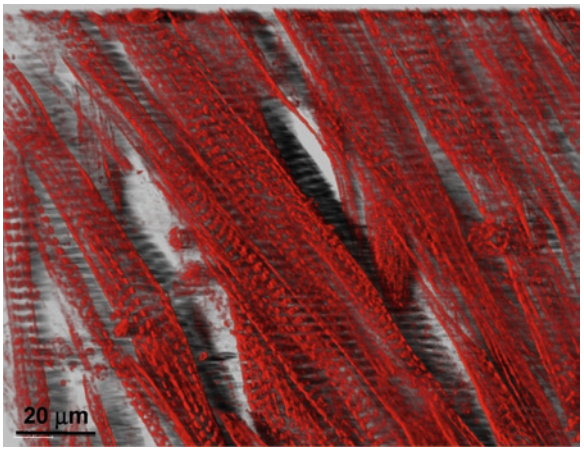

Fig. 8. MLC-2V immunostaining of cardiac-like cells visualized with a confocal microscope.

3. Filters can be adjusted on top of 50- or 20-ml syringes with cut tips and tightly scotch taped to the body of the syringe. After centrifugation, carefully remove the upper layer containing mature adipocytes and delivered oil. It is advisable to change tubes after each operation to get rid of all oil residues.
4. Erythrocyte lysis buffer should not remain too long in contact with other cells that can be injured.
5. After lysis, pellets logically appear less red, inversely to the supernatant, which gains a reddish coloration.
6. Gently remove methylcellulose by pipetting PBS up and down very slowly in order to prevent cell clotting.
7. Carefully place drops of cell suspension in the middle of and all around the well to prevent the gelatin from getting away.
8. In order to observe the VEGF effect, a well without VEGF should be prepared.
9. To prevent cell clots, carefully wash cells by pipetting PBS on the well wall and not directly on cells.
10. Precision requires a stereomicroscope and a cold lamp to prevent tissues from drying out. Ligature is possible on the left or right femoral artery. Mice often try to remove the suture. The surgical procedure should be adapted according to the mouse stem; compared with nu/nu mice, C57Bl6N mice have a more complicated network of vessels and microvessels, allowing some collaterals to bypass the ligature and cancel out its effects. It is then necessary to have a larger surgical procedure: after ligation of the proximal end of the femoral artery, the digital portion of the saphenous artery is also ligatured

and the artery and all side-branches are dissected freely. Then the femoral artery and attached side-branches are excised.

11. Counting has to be done after washing because of the great loss of cells during the wash steps. Confluent ASCs in a 75-cm^2 flask should provide approximately 1×10^6 cells.
12. It can be necessary to thoroughly homogenize the cell suspension before pipetting to prevent any obstruction of the needle. Insulin syringes can be used for the injection into the hindlimb; carefully and slowly remove the needle from the muscle to avoid any leakage of the cell suspension outside of the muscle.
13. Mice should be placed on a green carpet for better absorbance of the red laser light around the mouse and to prevent reflection. The room temperature must be stable and not too cold to prevent vasoconstriction during measuring. One solution is to place the Laser Doppler itself in a cooled chamber. Anesthesia can also be performed with xylazine/ketamine. Nevertheless, it is important to use a heating plate because mice tend to get cold during anesthesia.
14. Clinical barium sulfate solution for digestive radiography can also be used; it contains excipients that improve its quality and prevent particles from agglutinating. Nevertheless, since the barium sulfate suspension rapidly dissociates, it is important to mix it very well before pipetting. Catheter introduction into the abdominal aorta requires a stereoscopic microscope. Cardiac injection in the left ventricle yields the same result. Overpressure is prevented by cutting right atrium.
15. X-ray transducers used for dental radiography are suitable for this application as well as Faxitron MX-20 radiography systems.
16. The use of a solvent-resistant pen with hydrophobic film allows one to reduce the volume of antibody suitable for covering the specimen on the slide.
17. Operate in the dark to prevent fluorochrome degradation under light exposure.
18. Before plating cells, methylcellulose is stabilized at 37°C. Cells are gently resuspended in methylcellulose to prevent bubble formation.
19. The emergence of contracting clones is independent of the presence of 5-aza-2′-deoxycytidine: cardiac-like clusters appear when any adipose deposit is tested. However, the percentage of beating clones counted at day 20 varies according to the location of the adipose tissue used to prepare SVF cells; from 0.02 to 0.07% of plated naïve SVF cells purified from interscapular and inguinal deposits, respectively.

20. Non-adherent rounded cells can be easily harvested, without using trypsin, by collecting the culture medium and centrifugation. From this observation and to avoid the use of trypsin, we performed a large amount of cell expansion by successive collecting and plating of cells suspended in the culture medium.
21. Each plating is considered one passage. Primary culture of SVF cells in methylcellulose remained necessary to identify the cardiomyogenic cells as a characteristic morphology and then selecting and isolating them.
22. In cases of multicolor staining, other fluorescent antibodies directed against various cell surface antigens can be added at the same time with the receptor-specific antibody.

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References

1. Bjorntorp, P., Karlsson, M., Pertoft, H., Pettersson, P., Sjostrom, L., Smith, U. (1978) Isolation and characterization of cells from rat adipose tissue developing into adipocytes. *J Lipid Res.* **19**, 316–324.
2. Zuk, P. A., Zhu, M., Ashjian, P., De Ugarte, D. A., Huang, J. I., Mizuno, H., et al. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell.* **13**, 4279–4295.
3. Gimble, J. M., Katz, A. J., Bunnell, B. A. (2007) Adipose-derived stem cells for regenerative medicine. *Circ Res.* **100**, 1249–1260.
4. Miranville, A., Heeschen, C., Sengenes, C., Curat, C. A., Busse, R., Bouloumie, A. (2004) Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation.* **110**, 349–355.
5. Planat-Benard, V., Silvestre, J. S., Cousin, B., Andre, M., Nibbelink, M., Tamarat, R., et al. (2004) Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. *Circulation.* **109**, 656–663.
6. Rehman, J., Traktuev, D., Li, J., Merfeld-Clauss, S., Temm-Grove, C. J., Bovenkerk, J. E., et al. (2004) Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation.* **109**, 1292–1298.
7. Bouloumie, A., Drexler, H. C., Lafontan, M., Busse, R. (1998) Leptin, the product of Ob gene, promotes angiogenesis. *Circ Res.* **83**, 1059–1066.
8. Carriere, A., Ebrahimian, T. G., Dehez, S., Auge, N., Joffre, C., Andre, M., et al. (2009) Preconditioning by mitochondrial reactive oxygen species improves the proangiogenic potential of adipose-derived cells-based therapy. *Arterioscler Thromb Vasc Biol.* **29**, 1093–1099.
9. Planat-Benard, V., Menard, C., Andre, M., Puceat, M., Perez, A., Garcia-Verdugo, J. M., et al. (2004) Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. *Circ Res.* **94**, 223–229.

10. Gehling, U. M., Ergun, S., Schumacher, U., Wagener, C., Pantel, K., Otte, M., et al. (2000) In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood*. **95**, 3106–3112.
11. Silvestre, J. S., Mallat, Z., Tamarat, R., Duriez, M., Tedgui, A., Levy, B. I. (2001) Regulation of matrix metalloproteinase activity in ischemic tissue by interleukin-10: role in ischemia-induced angiogenesis. *Circ Res.* **89**, 259–264.
12. Leobon, B., Roncalli, J., Joffre, C., Mazo, M., Boisson, M., Barreau, C., et al. (2009) Adipose-derived cardiomyogenic cells: in vitro expansion and functional improvement in a mouse model of myocardial infarction. *Cardiovasc Res.* **83**, 757–767.
13. Rangappa, S., Fen, C., Lee, E. H., Bongso, A., Sim, E. K. (2003) Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes. *Ann Thorac Surg.* **75**, 775–779.
14. Couffinhal, T., Silver, M., Zheng, L.P., Kearney, M., Witzenbichler, B., Isner, J. M. (1998) Mouse model of angiogenesis. *Am J Pathol.* **152(6)**:1667–1679.
15. Silvestre, J. S., Mallat, Z., Duriez, M., Tamarat, R., Bureau, M. F., Scherman, D., Duverger, N., Branellec, D., Tedgui, A., Levy, B. I. (2000) Antiangiogenic effect of interleukin-10 in ischemia-induced angiogenesis in mice hindlimb. *Circ Res.* **87(6)**: 448–452.

Chapter 21

Epithelial Differentiation of Human Adipose-Derived Stem Cells

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Abstract

The versatile differentiation potential of adipose-derived stem cells (ASC) into cells of mesodermal, entodermal, and ectodermal origin places these cells at the forefront of cell-based therapies and cell transplantation. Epithelial differentiation of ASC may either be initiated by direct cell–cell or cell–matrix contacts, by chemical factors like retinoic acid, or via secreted cellular factors like cytokines, interleukins, or growth factors included in conditioned media.

This protocol describes methods to induce the in vitro differentiation of ASC from human adipose tissue into the epithelial lineage, and describes the methods used to verify this induced differentiation. We present two differentiation protocols based on either retinoic acid or conditioned medium of cultured epithelial cells.

Key words: Adipose-derived stem cells (ASC), Epithelial, Differentiation, Mesenchymal stem cells (MSC), Conditioned media, Retinoic acid, Epithelial cells, Renal tubular cells

1. Introduction

Ideal stem cells for use in therapeutic approaches should not be involved in ethical conflict; their isolation should pose only minimal harm for the patient or donor; and they must be available in high cell numbers, proliferate in culture, and differentiate into a broad spectrum of lineages. Adult adipose-derived stem cells (ASC) fulfill these prerequisites. ASC have characteristics very similar to bone marrow-derived mesenchymal stem cells (1–4), and are accessible, and easy and abundant to harvest from human adipose tissue aspirates (5, 6). They are isolated by their capacity to adhere to culture dish plastic and can be expanded in culture while maintaining their multilineage differentiation potential (6–8).

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ASC have reduced immunogenic properties and also an immunosuppressive potential (9), which make them attractive for stem cell therapies.

During organogenesis, epithelial structures (like the renal tubular epithelium) emerge from mesenchymal cells (10). In organ repair processes, similar mechanisms may regulate cellular reorganization and differentiation. Epithelial differentiation of ASC may either be initiated by direct cell–cell or cell–matrix contacts, by chemical factors like retinoic acid (11), or via secreted cellular factors like cytokines, interleukins, or growth factors included in conditioned media (CM) (12). This protocol describes the in vitro differentiation of ASC from human adipose tissue into the epithelial lineage, and describes the methods used to verify this induced differentiation. Based on our previously published findings, we present two differentiation protocols based on either retinoic acid or CM of cultured epithelial cells (11, 12).

2. Materials

2.1. Liposuction Aspirates

Human adipose-derived adult mesenchymal stem cells (ASC) were isolated from subcutaneous adipose tissue samples obtained from liposuction aspirates from patients undergoing cosmetic liposuction, as described in the chapter of Yu et al.

2.2. Supplies

1. Cell culture flasks, 25 and 75 cm^2.
2. Cell culture chambers, Permanox or Glass.
3. 0.2-µm filter units.
4. 50-ml conical tubes.
5. 2-ml tubes.
6. All-trans retinoic acid (ATRA).
7. Antibodies: anti-Cytokeratin 18, anti-zona occludens protein 1, Vimentin, smooth muscle actin, CD90.
8. Saponin.
9. Human IgG preparation (hIgG).
10. Slide mounting: Eukitt; Glycerine–gelatine.

2.3. Equipment

1. Inverted cell microscope: Zeiss Axiovert 10.
2. Cell culture equipment: Clean bench (Laminar flow), CO_2 incubator.
3. Fluorescence microscope: Zeiss Axiolab equipped with a camera.
4. Flow cytometer: BD FACScalibur.
5. Western blot Mini Protean 3 System.
6. Semidry blotting apparatus.

2.4. Media and Buffers

All supplements are filtered through a 0.22-μm filter unit. All supplements should be p.a. grade and cell culture tested.

1. *Standard culture medium*: Add 50 ml of fetal bovine serum to 450 ml of Dulbecco's modified Eagle's medium (DMEM) (Sigma No. D6046) with a physiologic glucose concentration (100 mg/dl). Every lot of fetal calf serum (FCS; e.g., PAA) should be screened for its ability to support both cell proliferation and adipocyte differentiation.
2. *Differentiation medium 1*: Prepare a 10 mM stock solution of ATRA (2,000-fold concentration) dissolved in dimethyl sulfoxide. Aliquot this stock solution and store frozen at –80°C until required. Store for a maximum of 1 year. Add ATRA to standard culture medium aiming for a final concentration of 5 μM prior to use. Use this solution immediately after its preparation. Do not store the differentiation medium.
3. *Differentiation medium 2 (Conditioned medium [CM])*: Produce CM by culturing epithelial cells in pure Medium 199 (M199) with physiologic glucose concentration and without serum (see Subheading 3.2). Store CM at 4°C for a maximum of 24 h or freeze for long-time storage at –20°C. If using other epithelial cells than the TEC described here, use pure basal medium (without FCS) for conditioning. Always use unconditioned medium as a control. This control medium should be processed in the same way as your CM – therefore, put the control medium into the CO_2 incubator for 4 days in a culture flask without cells.
4. *Medium sterility test procedure*: Sterility of the medium should be tested in an aliquot from each bottle prior to use. Incubate this aliquot in a well of a culture plate for 24–48 h in a cell culture incubator and examine the plate for evidence of contamination using a phase contrast microscope. If contaminated, discard this charge of medium.
5. *Lysis buffer for Western blotting*: 10 mM Tris-buffered saline pH 7.4, 0.1% SDS, 0.1% Tween 20, 0.5% Triton X100, 150 mM NaCl, 10 mM EDTA, 1 M urea, 10 mM *N*-ethylmaleimide, 4 mM benzamidine, and 1 mM phenylmethylsulfonyl-fluoride. Add the proteases immediately before using the buffer, do not store the buffer with added proteases.
6. *Standard sample buffer (3×) for Western blotting*: 62.5 mM Tris–HCl pH 6.8, 30% glycerol, 6% SDS, 6% β-mercatoethanol, 0.01% bromophenol blue.
7. *Blocking buffer for Western blotting*: 10 mM Tris-buffered saline pH 7.4, 0.1% Tween 20, and 3% nonfat powdered milk.

8. *Blocking buffer for fluorescence microscopy*: phosphate-buffered saline (PBS) containing 5% goat serum and 5 mg/ml hIgG.
9. *Antibody solution for fluorescence microscopy*: PBS containing 1% goat serum and 1 mg/ml hIgG.
10. *Permeabilization solution for intracellular flow cytometry*: PBS containing 0.5% BSA, 5.3 mM EDTA, and 0.03% saponin.

3. Methods

All of the following procedures are performed sterile in a clean bench. All solutions used should be preheated to 37°C in a water bath.

3.1. Culture and Expansion of ASC

Do not use pre-coated flasks for the culture of ASC. For ASC expansion, culture the cells in standard culture medium (see Subheading 2.4). Culture without antibiotics should be preferred. Cultured ASC should be tested for their phenotype. Cultured ASC are $CD29^+$, $CD44^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD166^+$, and $CD11b^-$, $CD14^-$, and $CD45^-$ (1, 11, 13). Use cells in the second to fifth passage for the experiments. In the expansion phase, passage ASC at 70–80% of confluency, do not culture the cells to confluence. Check the differentiation potential of cultured ASC (see Note 1).

3.2. Media Conditioning

We produce CM for the differentiation of ASC from cultured subconfluent human renal tubular epithelial cells (TEC). TEC were separated from renal tissue as described previously (14, 15). TEC used to produce CM should be in second to fifth passage (see Note 2).

Culture epithelial cells in Medium 199 (M199) with 10% fetal bovine serum, but without antibiotics. Use M199 with a physiologic glucose concentration (100 mg/dl).

1. Passage confluent cells by trypsination (split ratio 1:3). Use trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA) (1 mM) until cells detach – check by microscopic examination.
2. Wash cells three times with PBS when they reach subconfluence (70–80%) and change medium to serum-free M199 for 4 days. Thus, CM is produced by non-confluent TEC, which could be regarded as injured cells (see Note 3). We add 4 ml of the medium to a 25-cm^2 culture flask (i.e., 160 µl/cm^2 growth area).
3. After 4 days of conditioning, centrifuge the medium at 300 × *g* for 5 min and filter through a 0.22-µm filter.

4. Estimate glucose content of CM after conditioning by a routine method and substitute glucose to 100 mg/dl. Alternatively add 100 mg/dl glucose – we have found that almost no residual glucose is in the medium after 4 days of conditioning.
5. Quality control of CM: Measure medium osmolarity after conditioning with a cryometric technique using a micro-osmometer. Measure the medium pH value after conditioning by a standard routine method using a pH meter (see Note 4).
6. Use CM directly after conditioning to induce epithelial differentiation of ASC or store at 4°C a maximum of 24 h (see Note 5).

3.3. Induction of Epithelial Differentiation

Differentiation medium 1

1. Culture ASC in appropriate culture flasks for the later verification method (see Note 6).
2. Wash subconfluent cultures of ASC twice with PBS.
3. Transfer differentiation medium 1 directly onto ASC cultures.
4. Culture ASC for 14 days and replace CM every 3–4 days by fresh differentiation medium. Do not split the cells within the differentiation phase.
5. As a control, use pure DMEM medium and culture ASC within this medium for 14 days.

Differentiation medium 2 (CM)

6. Culture ASC in appropriate culture flasks for the later verification method (see Note 6).
7. Wash subconfluent cultures of ASC twice with PBS.
8. Transfer CM directly onto ASC cultures. Use a minimum of 5 ml CM for a 75-cm^2 flask (66 µl/cm^2 growth area) to ensure that the cells are fully covered with medium.
9. Culture ASC for 14 days and replace CM every 3–4 days by fresh CM. Do not split the cells within the differentiation phase.
10. As a control, use pure basal medium (M199 without conditioning) and culture ASC within this medium for 14 days.

3.4. Verification of Epithelial Differentiation

Assess morphological changes in your cultures over the whole differentiation procedure by phase contrast microscopy. Undifferentiated ASC display an elongated "fibroblastoid" phenotype and grow in multiple layers, whereas the morphology of differentiated ASC changes to a more epithelial-like round shape and the cells grow in as a monolayer.

Use the following methods to show induction of cytokeratin 18 (CK18) and zona occludens protein 1 (ZO1) expression, and the decrease/disappearance of mesenchymal markers (vimentin, smooth muscle actin [smA], CD90) (summarized in Table 1).

Table 1
Methods used to verify increases or decreases in marker expression of characteristic antigens

	CK18	ZO1	smA	Vimentin	CD90
Western blot	✓	✓	✓	✓	–
Immunofluorescence	✓	✓	–	✓	–
Flow cytometry	✓	–	–	–	✓

Abbreviations: cytokeratin 18 (CK18), zona occludens protein 1 (ZO1), smooth muscle actin (smA)

Western blot analysis

1. Wash cells twice with ice-cold PBS.
2. Lyse ASC using a lysis buffer and collect by scraping.
3. Centrifuge at 18,000 × *g* for 10 min and take the supernatant to determined protein content. This is a critical step for loading equal amounts of protein amounts into every sample (see Note 7).
4. Add an appropriate amount of dH_2O aiming for a volume of 40 µl in every sample and add 20 µl of 3× standard sample buffer. Heat the samples at 95°C for 10 min and load equal volumes of your samples (60 µl) on the electrophoresis gel.
5. Perform electrophoresis on a 6–8% SDS polyacrylamide gel.
6. Transfer the separated proteins to an Immobilon transfer membrane (Millipore) by semi-dry blotting.
7. Block the membranes for 1 h in blocking buffer in order to block non-specific binding sites.
8. Incubate the membrane with an antibody against CK18 (resulting in a 45-kDa band), ZO-1 (220 kDa), α-smooth muscle actin (smA, 42 kDa), or vimentin (58 kDa), and by a secondary antibody (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG) overnight at 4°C (see Note 8).
9. Use the enhanced chemiluminescence system to make the specific protein bands visible and record on a radiographic film. Perform densitometric evaluation by using ImageJ 1.36 (see Note 9).

Fluorescence microscopy

10. For immunofluorescence staining, culture ASC in 8-well chambered slides (see Note 6). Use epithelial cells to verify cytokeratin and ZO-1 staining.

11. Rinse cells three times with PBS and fix with ice-cold methanol/acetone (1:1) for 5 min.
12. Block unspecific binding sites in blocking buffer for 20 min.
13. Apply primary antibody (approximately 4–10 μg/ml diluted in antibody solution) without washing and incubate for 30 min at 37°C with gentle shaking (see Note 10).
14. Wash the cells twice with PBS and incubate with a Cy3- or FITC-conjugated goat-anti-mouse IgG secondary antibody (diluted in antibody solution) for 30 min at 37°C. Add 0.5 μg/ml 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) as a nuclear stain directly into your secondary antibody solution.
15. Mount the slides in mounting medium and examine using a fluorescence microscope (see Note 11). Calculate cytokeratin-positive cells versus -negative cells in 10–20 visual fields in an appropriate magnification (40× or 63×).

Flow cytometry

16. After induction of differentiation, detach ASC from cell culture surface by 0.05% trypsin and 0.02% EDTA. Incubate ASC only for a short time in trypsin/EDTA – check cell detachment in a microscope, knock the cell culture flask carefully on the desk to accelerate detachment. Add medium with 10% FCS immediately after cells are detached.
17. Centrifuge ASC for 5 min at 300 × *g*, count the cells using a hemacytometer, and pipet 10^5 ASC in an appropriate tube for your flow cytometer.
18. Resuspend ASC in permeabilization solution for intracellular staining and incubate for 30 min. When staining for the mesenchymal marker CD90, skip this step.
19. Incubate with a primary antibody for 30 min on ice. Mix well. If using an antibody directed against cytokeratin 18, perform this step in the presence of 0.03% saponin.
20. After washing and centrifugation, incubate ASC with a FITC-labeled secondary antibody (Goat anti-mouse IgG) for 20 min in the dark. Add 0.03% saponin when staining an intracellular antigen. Mix well.
21. Fix the cells in PBS containing fresh 1% paraformaldehyde for 10 min. The cells can be stored at 4°C in the dark for up to 1 week.
22. Analyze 10^4 labeled ASC using a FACScan flow cytometer. Data analysis can be performed subsequently with WinMDI 2.8. Use negative controls without antibodies or with isotype controls.

3.5. Summary

Although pre-clinical and clinical studies demonstrate the effectiveness of ASC in cell therapy for the treatment of several diseases, many questions still remain. What are the mechanisms of action during engraftment, homing, and differentiation? In vitro models could provide reliable insights in the mechanisms involved in stem cell differentiation. Knowledge of these mechanisms and the cellular interactions involved, together with a growing understanding of the mechanisms underlying organ regeneration and repair (e.g., in the heart, kidney, lung), should help to exploit the biological potential of administered stem cells for new therapeutic approaches.

4. Notes

1. Check the in vitro differentiation potential of your cultured ASC into other described lineages by specific media (5, 7, 8). In brief, induce osteogenic differentiation of ASC by osteogenic medium containing ascorbic acid (50 μM), β-glycerophosphate (10 mM), dexamethasone (0.1 μM), and 15% FCS for 12–14 days. Assess the osteogenic phenotype by calcium hydroxyapatite staining according to von Kossa staining. Induce adipogenic differentiation by adipogenic medium containing insulin (10 μM), dexamethasone (1 μM), isobutyl-methylxanthin (1 mM), indomethacine (200 μM), and 10% FCS for 12–14 days. Show accumulation of lipid droplets in intracellular vacuoles indicating adipogenic differentiation by Oil-Red-O staining.
2. If you use other epithelial cells rather than the here-described TEC, test whether the CM you have produced with these cells is able to induce ASC differentiation. This requires four critical steps:
   (a) Determination of an optimal time for conditioning
   (b) Adequate replenishment of glucose after conditioning
   (c) Proof of the growth signals secreted by epithelial cells
   (d) Proof of the initiation of differentiation steps by a common signal transduction pathway, changes in cell morphology, and differentiation marker expression.
3. Option: Additional time-course experiments to evaluate the best conditioning time can be done if using epithelial cells other than TEC. Test different conditioning time points for its ability to induce signal transduction in ASC (e.g., by immunoblotting for MAPK [ERK1/2] phosphorylation). As negative controls, use media not in contact with epithelial cells.

4. In our preparations, medium osmolarity and pH value remained nearly unchanged after conditioning. Osmolarity after conditioning was 295 ± 7.1 mosm/L. In pure, unconditioned medium 199 it was 284 ± 5.8 mosml/L. The medium pH value of CM was 7.34 ± 0.05, whereas in pure medium 199, the pH was 7.44 ± 0.06. Nevertheless, check medium osmolarity and pH, and discard CM if there is any irregularity.
5. Option: Check CM for its ability to induce signal transduction in ASC cultures. Incubate CM with ASC for 5, 10, or 15 min and perform an immunoblot for MAPK (ERK1/2) phosphorylation. Compare with control medium that had not been in contact with epithelial cells.
6. For fluorescence microscopy, culture ASC in chambered slides. There are different advantages of these culture slides: (1) you need very low amounts of CM during the differentiation (approximately 100 µl/well) and (2) you need a very low amount of the antibody to perform staining. We culture ASC in 25-cm² flasks (flow cytometry) with 4 ml CM, or 75-cm² flasks (Western blot) with 12 ml CM.
7. Load 5–10 µg total protein on a 8% gel for the detection of CK18, vimentin, or smA. Load 35 µg of total protein on a 6% gel for ZO1 detection. Load lysates of epithelial cells as controls.
8. Incubate the antibody in the appropriate solution: 10 mM Tris-buffered saline pH 7.4, 0.1% Tween 20, and 0.1% nonfat powdered milk for the antibodies against CK18, smA, or vimentin; 10 mM Tris-buffered saline pH 7.4, 0.1% Tween 20, and 3% bovine serum albumin for the anti-ZO1 antibody.
9. Do not forget to strip your blots and check the protein loading by a housekeeping control (e.g., beta-actin). Strip your membrane in a solution containing 1 M Tris pH 6.8, 1% SDS, and 1% β-mercaptoethanol for 30 min at 50°C. Wash the membrane for 30 min with 10 mM Tris-buffered saline pH 7.4, 0.1% Tween 20, and 0.1% nonfat powdered milk. Then, wash the membrane in 1 M Tris-buffered saline pH 7.4, and begin the new immunostaining with your blocking solution.
10. Do not forget controls without primary antibody and with an isotype-matched control to exclude autofluorescence or nonspecific fluorescence of your secondary antibody.
11. If using Eukitt for mounting slides, use glass chambered slides rather than Permanox slides. Eukitt is better for long-time storage of the slides, but induces a deformation of the Permanox slides. As an alternative, mount Permanox slides with glycerin–gelatin.
12. *Troubleshooting.* What if the ASC do not differentiate well into the epithelial lineage?

(a) Check your ASC culture conditions and media used during cell expansion.

(b) Check the differentiation potential of your ASC into adipocytes (and a second differentiation pathway).

(c) Check another ASC preparation – the differentiation of ASC is dependent on patient, donor, and liposuction side.

(d) Test a longer differentiation time – incubate your ASC for 20–28 days in the presence of ATRA or CM. Do not forget to change the differentiation medium every 3–4 days.

References

1. Gronthos S, Franklin DM, Leddy HA et al (2001) Surface protein charactzerization of human adipose-derived stromal cells. J Cell Physiol 189:54–63.
2. De Ugarte DA, Morizono K, Elbarbary A et al (2003) Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs 174:101–109.
3. Wagner W, Wein F, Seckinger A et al (2005) Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol 33:1402–1416.
4. Kern S, Eichler H, Stoeve J et al (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord or adipose tissue. Stem Cells 24:1294–1301.
5. Zuk PA, Zhu M, Mizuno H et al (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228
6. Zuk PA, Zhu M, Ashjian P et al (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295
7. Strem BM, Hicok KC, Zhu M et al (2005) Multilineage differentiation of adipose tissue-derived stem cells. Keio J Med 54:132–141.
8. Gimble JM, Guilak F (2003) Differential potential of adipose derived adult stem (ADAS) cells. Curr Top Dev Biol 58:137–160
9. Lombardo E, DelaRosa O, Mancheño-Corvo P et al (2009) Toll-like receptor-mediated signaling in human adipose-derived stem cells: implications for immunogenicity and immunosuppressive potential. Tissue Eng Part A 15:1579–1589
10. Stuart RO, Nigam SK (1995) Development of the tubular nephron. Semin Nephrol 15:315–326.
11. Brzoska M, Gauer S, Geiger H et al (2005) Epithelial differentiation of human adipose tissue-derived adult stem cells. Biochem Biophys Res Commun 330:142–150
12. Baer PC, Missler C, Bereiter-Hahn J et al (2009) Conditioned medium from renal tubular epithelial cells initiates differentiation of human mesenchymal stem cells. Cell Prolif 42:29–37
13. Mitchell JB, McIntosh K, Zvonic S, et al (2006) Immunophenotype of human adipose-derived cells: Temporal changes in stromal-associated and stem-cellassociated markers. Stem Cells 24:376–385
14. Baer PC, Nockher WA, Haase W et al (1997) Isolation of proximal and distal tubule cells from human kidney by immunomagnetic separation. Kidney Int 52:1321–1331
15. Baer PC, Bereiter-Hahn J, Schubert R et al (2006) Differentiation status of human renal proximal and distal tubular epithelial cells in vitro: Differential expression of characteristic markers. Cells Tissues Organs 184:16–22

Chapter 22

Screening for Epigenetic Target Genes that Enhance Reprogramming Using Lentiviral-Delivered shRNA

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Abstract

Small molecules will need to be identified and/or developed that target protein classes limiting reprogramming efficiency. A specific class of proteins includes epigenetic regulators that silence, or minimize expression, of pluripotency genes in differentiated cells. To better understand the role of specific epigenetic modulators in reprogramming, we have used shRNA delivered by lentivirus to assess the significance of individual epi-proteins in reprogramming pluripotent gene expression.

Key words: Reprogramming, shRNA

1. Introduction

Direct reprogramming of somatic cells to induced pluripotent stem (iPS) cells has been demonstrated by viral transduction of exogenous transcription factors (1–12). These initial demonstrations have provided valuable insight into molecular mechanisms of somatic cell reprogramming and raised the possibility that alternative strategies could be developed on an industrial scale to produce pluripotent cells without using embryos or genetic manipulations.

Recent reports have demonstrated epigenetic differences between fully reprogrammed induced pluripotent stem (iPS) cells and partially reprogrammed cells (13). For example, in fully reprogrammed iPS cells, complete demethylation of key pluripotency gene promoters was observed, while incomplete demethylation characterized partially reprogrammed cells. Partially reprogrammed cells could be fully reprogrammed by treatment with

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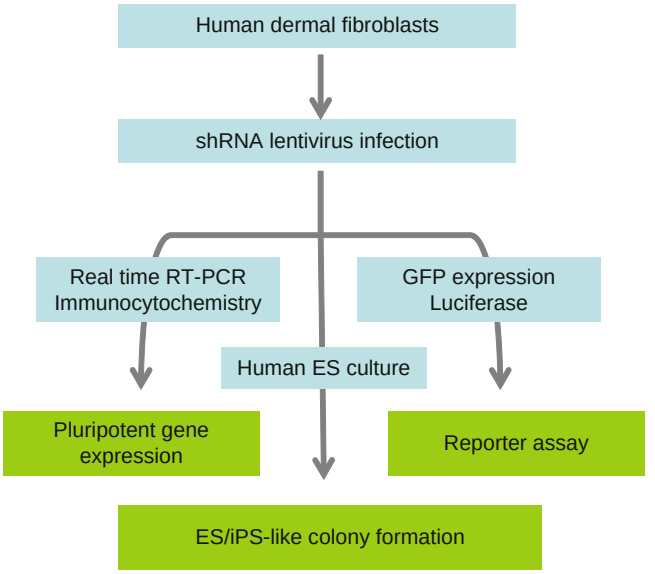

Fig. 1. Strategies for screening epigenome-related target genes using shRNA lentivirus.

the demethylating agent 5-azaC, suggesting small molecule epi-drugs could significantly impact the reprogramming process (14, 15). Identification of specific epigenetic regulators controlling pluripotent gene expression will provide a basis to rationally develop highly specific small molecule epi-drugs that will be critical to develop a purely chemical approach to reprogram a differentiated nucleus to a less differentiated state. In order to identify key targets, we have developed a shRNA screen in combination with colony morphology to identify epigenetic targets that maintain a differentiated state. RNA interference by shRNA provides specific knockdown of epi-genes. The use of a lentiviral vector and polybrene enhances delivery of the shRNA and, combined with green fluorescent protein (GFP), allows visualization. Together, these provide a valuable approach to identifying key proteins regulating pluripotent gene expression. Our strategy for screening epigenome-related target genes is outlined in Fig. 1.

2. Materials

1. Buffers and reagents.
   (a) Trypsin/EDTA solution (0.025% Trypsin and 0.75 mM EDTA without Ca^{2+} and Mg^{2+} in PBS).
   (b) PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4).
   (c) Buffers for RNA isolation and purification.
   (d) Fixative solution (ethanol:acetic acid:H_2O = 7:2:1).

(e) Antibody dilution solution (10% FBS in PBS with 0.2% saponin).

2. Primary human fibroblasts supplemented with Fibroblasts Growth Medium (see Note 1).
3. BJ fibroblasts (ATCC, www.atcc.org).
4. UV-irradiated DR4 embryonic fibroblasts (ATCC, www.atcc.org).
5. Variant human ES cell harboring hO ct4-GFP reporter supplemented with Knockout Serum Replacement (KSR) hESC medium (see Note 2), human bFGF, Dulbecco's Modified Eagle Medium (high glucose), DMEM/F12 with GlutaMax, porcine skin gelatin.
6. mTeSR1 medium (Stem Cell Technology, Vancouver, Canada) (see Note 2) and Matrigel-coated cell culture plate (BD Biosciences, www.bdbiosciences.com).
7. SMARTvector shRNA lentivirus (Thermo Scientific, www.thermofisher.com) (see Note 3).
8. piPSC-hOct4, piPSC-hNanog overexpression lentivirus (System Biosciences, www.systembio.com) (see Note 3).
9. Polybrene (hexadimethrine bromide), Valproic acid, 5-aza-deoxycytidine, Zeocin (Sigma, St. Louis, MO).
10. Pluripotent gene specific TaqMan primers and probes (Applied Biosystems, www.appliedbiosystems.com) (see Note 6).
11. Trozol reagent and RNA isolation and purification kits (Invitrogen, Carlsbad, CA).

3. Methods

3.1. Culturing Primary Human Preadipocytes, Skeletal Muscle Cells, and Lung and Dermal Fibroblasts

Human preadipocytes, skeletal muscle cells, and lung and dermal fibroblast cells are primary cells derived from a single donor of normal human subcutaneous adipose tissues, fetal skeletal muscle, adult lung, and breast or fetal skin (18 weeks gestation), respectively. Doubling times of human primary cells are around 28–32 h and fibroblasts can be cultured and propagated for less than ten population doublings without losing their morphologic characteristics and function. Each lot is tested for its ability to attach and spread on tissue cultureware and potential biological contaminations including HIV-1, Hepatitis B and C virus, Mycoplasma, bacteria, yeast, and fungi (see Note 1).

1. Primary human preadipocytes, skeletal muscle cell, lung fibroblasts, and dermal fibroblast cells can be purchased from Cell Applications. BJ fibroblasts can be purchased from ATCC. Open cryopreserved cell packages immediately upon arrival and keep in liquid nitrogen storage tanks until use.

2. Clean the biological safety cabinet with HEPA-filtered laminar airflow with 70% alcohol and warm complete cell culture medium at 37°C in a water bath before removing vials from the liquid nitrogen storage tank.
3. Remove the cryopreserved vials of primary human preadipocytes, skeletal muscle cells, and lung and dermal fibroblasts from the liquid nitrogen storage tank.
4. Release trapped liquid nitrogen inside the cell vials by turning the vial cap a quarter turn, then tighten the cap again.
5. Thaw the cells quickly by placing the vials in a 37°C water bath with gentle agitation for 1–2 min. The medium color should turn pink as the cells are thawing.
6. Take the vial out of the water bath, decontaminate the vial exterior with 70% alcohol, and wipe it dry in a sterile biological safety cabinet. With the exception of centrifugation, the remaining steps should be performed in a sterile biological safety cabinet.
7. Remove the vial cap carefully and, by gentle pipetting, transfer the cells into a conical centrifuge tube containing 5 mL of complete cell culture medium.
8. Centrifuge the conical tube at 220 × *g* for 5 min at room temperature to pellet the cells.
9. Aspirate the supernatant from the tube completely, without disturbing the cell pellet, and resuspend the cells in 10 mL of warmed cell culture medium by gently pipetting to break up the clumps.
10. Transfer the cell suspension into a T-75 flask and place in a 37°C, 5% CO_2 humidified incubator overnight.
11. Change the culture medium every other day and subculture the cells when the cells reach 90% confluency.
12. Remove the Trypsin/EDTA solution and Trypsin neutralization solution from the refrigerator and leave them at room temperature before subculture.
13. Remove the medium from the culture flasks by aspiration, wash the flasks with 5 mL of HBSS, and remove the solution by aspiration.
14. Add 6 mL of Trypsin/EDTA solution into the T-75 flask and rock the flask gently to ensure the solution covers all the cells.
15. Remove the Trypsin/EDTA solution immediately and monitor the trypsinized cells under a microscope until the cells become rounded (usually about 2–4 min at room temperature).
16. Release the trypsinized cells from the culture surface by hitting the side of the flask and add 5 mL of trypsin-neutralizing solution to inhibit further enzyme activity.

17. Transfer the cell suspension from the flask to a sterile conical centrifuge tube and rinse the flask with an additional 5 mL of trypsin-neutralization solution and collect the suspension into the same conical centrifuge tube.
18. Centrifuge the conical tube at $220 \times g$ for 5 min at room temperature to pellet the cells.
19. Aspirate the supernatant from the tube completely, without disturbing the cell pellet, and resuspend the cells in 5 mL of warmed cell culture medium by gently pipetting to break up the clumps.
20. Count the cells with a hemocytometer or cell counter.
21. Inoculate 2×10^5 cells in 10 mL of culture medium into a T-75 flask ($2–3 \times 10^3$ cells/cm^2 surface area). A 1:3 split is recommended to maintain human primary cell cultures.

3.2. shRNA Lentiviral Infection

RNA interference through siRNA or recently developed shRNA provides specific gene knockdown in vitro and in vivo. However, the efficiency of gene silencing is dependent on the delivery system and host cell tropism. The use of a retroviral or lentiviral vector dramatically enhances efficiency of transfection into a wide range of mammalian cell types in culture compared with traditional chemical delivery systems. Furthermore, additional selection and visualization is possible by adding antibiotic resistance genes and GFP, respectively, to the viral vector. To determine transfection efficiency of different origins of fibroblast cultures, ten transfection units of GAPDH-targeting shRNA lentiviral particles were infected into primary cultures from human subcutaneous adipose tissue (Preadipocytes), lung (HLF), skeletal muscle (SkM), and skin (HDF). Epi-gene specific interference shRNA lentivirus generated from the SMARTvector™ with non-target control (S-005000-01) and human GAPDH targeting shRNA (S-001000-01) were purchased from Dharmacon (Thermo Scientific, www.thermofisher.com). SMARTvector™ shRNA lentivirus co-express markers for GFP and puromycin selection. An average titer for Dharmacon's shRNA lentivirus was $1–6 \times 10^8$ transfection units (TU)/mL (see Note 4). Cryopreserved lentiviral particles were stored at –70°C until use.

1. A day before lentiviral infection, seed low passage (around 3–5 passages) human primary cells in multiwell cell culture plates at 3.5×10^4 cells/mL (0.5 mL/24-well cluster, 1 mL/12-well cluster, 2 mL/6-well cluster) and incubate the cells in a 37°C, 5% CO_2 humidified incubator overnight. On the next day, cells should be about 60–70% confluent.
2. The next day, thaw the shRNA lentivirus on ice, spin down, and keep it on ice (see Note 5).
3. Prepare polybrene stock to a concentration of 10 mg/mL with PBS.

4. Infect the target cells with an appropriate amount of lentivirus (MOI 5 or 10) in a half volume of complete growth medium containing 10 μg/mL of polybrene (for example, 1 mL of medium for a 6-well cluster) and incubate for 3–4 h in a 37°C, 5% CO_2 humidified incubator.
5. Three to four hours later, add the remaining half volume of complete medium without polybrene (for example, 1 mL of medium for a 6-well cluster) and incubate overnight in a 37°C, 5% CO_2 humidified incubator.
6. The next day, remove the viral supernatant and add the complete growth medium to the cells and incubate overnight in a 37°C, 5% CO_2 humidified incubator.
7. GFP can be detected 24–48 h after lentiviral infection using a fluorescent microscope. Further selection can be accomplished using puromycin (see Note 7), and cells can be subcultured when confluent (usually 72 h after infection).

Figure 2a illustrates that more than 80% of HDF cells were positive for turboGFP™ expression as a transfection marker in contrast to preadipocyte and SkM cells, which resulted in less than 5% transfection yield. Polybrene is a polycation that increases binding between the viral capsid and the cellular membrane. The optimal concentration of polybrene varies with cell type and may need to be empirically determined (we do not see any toxicity up to 10 μg/mL for tested human primary fibroblasts cultures). When the same MOI of lentivrus was infected with 10 μg/mL of polybrene, the transfection efficiency was increased further, except for skeletal muscle cells and preadipocytes, which show very low tropism (Fig. 2a). Expression of GFP can be detected 2 days after shRNA lentiviral infection and increased continuously (Fig. 2b). Expression of GAPDH mRNA from HDF (Fig. 2c) suggests that greater than 80% of GAPDH expression was knocked down after 5 days of GAPDH-targeting shRNA lentiviral infection. Further knockdown of GAPDH expression was obtained with puromycin selection (3 μg/mL) (Fig. 2c), resulting in more than 95% of cells expressing turboGFP™ as assessed by fluorescence microscopy (Fig. 2b, puromycin).

3.3. Isolation of RNA from Cultured Cells

1. Rinse cultured cells with PBS (500 μL/well in a 24-well plate; 2 mL/well in a 6-well plate) then collected in 300 μL of Trizol (Invitrogen).
2. The lysed cells in Trizol may be stored at −80°C or processed using the PureLink RNA Mini Kit.
3. Incubate the samples at room temperature for 5 min to allow complete dissociation of nucleoprotein complexes.
4. Add 60 μL of chloroform (200 μL chloroform/1 mL Trizol used). Shake tube vigorously (by hand, do not vortex) for 15 s.
5. Incubate at room temperature for 2–3 min.

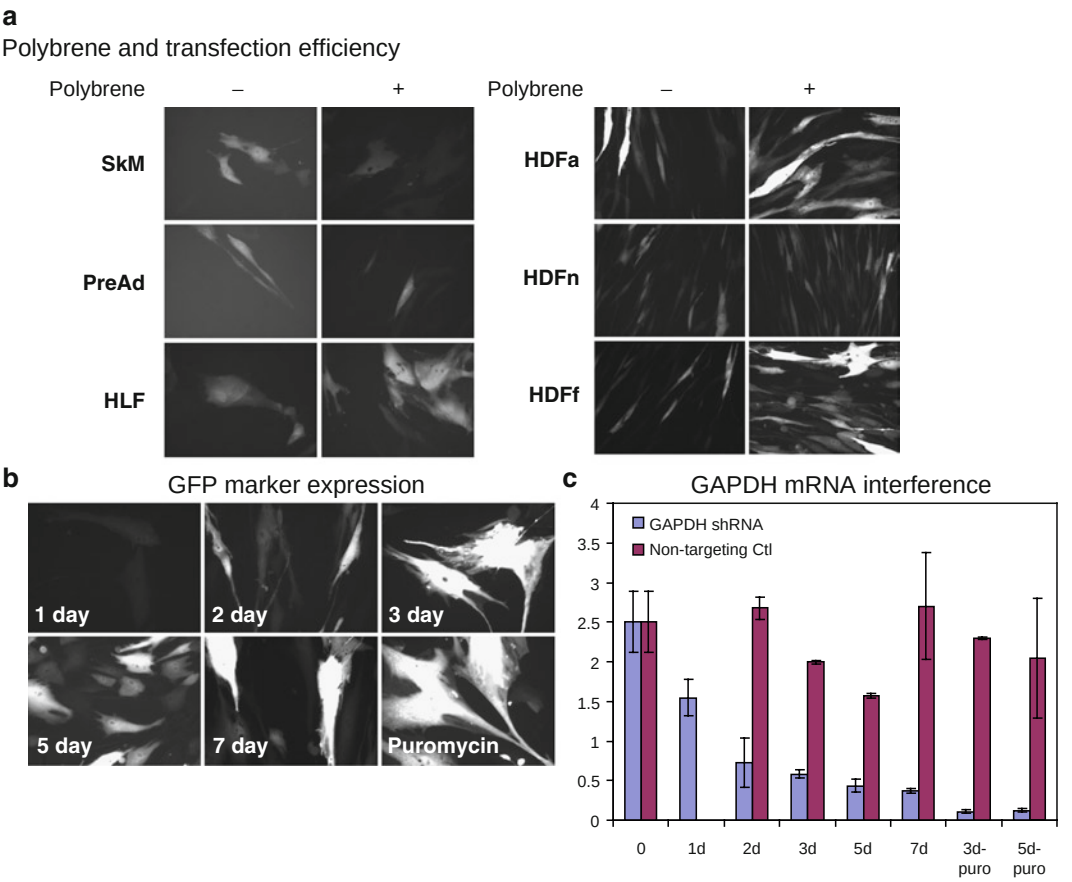

Fig. 2. (**a**) Infection of lentiviral shRNA (GAPDH) into human primary cultures with and without polybrene (2 μg/mL). Expression of turboGFP from lentiviral vector was visualized at 4 days after lentiviral infection using fluorescent microscopy. *SkM* human skeletal muscle cells, *PreAd* human preadipocytes, *HLF* human lung fibroblasts, *HDFa* human adult dermal fibroblasts, *HDFn* human neonatal dermal fibroblasts, *HDFf* human fetal dermal fibroblasts. (**b**) Expression of turboGFP was visualized up to 7 days. *Puromycin* puromycin selection (2 μg/mL) for 5 days. (**c**) The level of GAPDH mRNA was analyzed by real-time RT-PCR. *Puro* puromycin selection (2 μg/mL).

6. Centrifuge the samples at 8,000 × *g* for 15 min. The mixture will separate into phases: a lower, pink phenol–chloroform phase, a white interphase, and a clear, aqueous upper phase containing the RNA.
7. Carefully transfer ~150 μL of the colorless upper phase to a new RNase-free tube. It is very important not to collect any of the lower phase!
8. Add an equal volume of 70% ethanol to obtain a final ethanol concentration of 35%. Vortex the sample to mix well. Invert the tube to disperse any precipitate that may have formed after adding the ethanol.
9. Transfer the entire volume (up to 700 μL at a time) to a spin cartridge on a collection tube provided in the kit.

10. Centrifuge at 8,000 × *g* for 15 s at room temperature. Discard the flow-through.
11. Add 350 µL of Wash Buffer I to the spin cartridge and centrifuge at 8,000 × *g* for 15 s. Discard the flow-through.
12. Add 80 µL of DNase I solution, prepared as described below, to the center of each spin cartridge. Use Invitrogen PureLink DNase kit (see Note 8).

Component	Volume
RNase-free water	62 µL
10× DNase buffer	8 µL
DNase I (1 U/µL)	10 µL
Final volume	80 µL

13. Incubate for 15 min at room temperature.
14. Add 350 µL of Wash Buffer I to the spin cartridge and centrifuge at 8,000 × *g* for 15 s. Discard the flow-through.
15. To prepare Wash Buffer II, add 60 µL of 100% ethanol to the container as described on the label.
16. Add 500 µL of Wash Buffer II to the spin cartridge and centrifuge at 8,000 × *g* for 15 s. Discard the flow-through.
17. Repeat step 16 once.
18. Discard the collection tube and insert the spin cartridge in a new RNase-free tube for RNA recovery.
19. Add 50 µL of RNase-free water to the center of the spin cartridge and incubate at room temperature for 1 min.
20. Centrifuge the spin cartridge for 2 min at 8,000 × *g* for 15 s. If the RNA will be used within a few hours, store on ice. Otherwise, store RNA samples at −80°C for longer storage.

3.4. Reverse Transcription of RNA to cDNA

1. Vortex each sample well to evenly suspend the RNA, then place 1.5 µL of the sample on a NanoDrop Spectrophotometer to measure the concentration of RNA (in nanograms per microliter) in each sample.
2. 500 ng of RNA is used in the reverse transcription reaction. Calculate the quantity of sample required by dividing 500 ng by the concentration in nanograms per microliter. This will give you the number of microliters of sample that contain 500 ng of RNA.
3. Subtract the number from step 2 from 25. This is the amount of water that you add to bring the volume up to 25 µL.
4. To a 96-well plate, add the volumes of water required for each sample (calculated in step 3) in consecutive wells.

5. Vortex the samples well, then add the required volume of sample as calculated in step 2 to the corresponding well containing the water.
6. Cover the plate with a plastic cover and place in the BioRad iCycler thermocycler at 65°C for 10 min.
7. Chill the plate on ice for ~10 min or until cool.
8. While the plate is in the thermocycler, prepare the RT Master Mix, as described below.

Component	Volume
10× RT buffer	5 µL
25× dNTP	2 µL
10× Random primers	5 µL
Multiscribe reverse transcriptase	2.5 µL
RNase inhibitor	0.5 µL
Water	10 µL
Final volume/well	25 µL

9. Once plate is cool, add 25 µL of the RT Master Mix per well, bringing the final reaction volume up to 50 µL.
10. To perform the reverse transcription, place the plate in a thermocycler for:
    (a) 25°C for 10 min
    (b) 37°C for 2 h
    (c) Hold at 4°C.
11. If the RNA will be used within a few hours, store on ice. Otherwise, store cDNA samples at –80°C for longer storage.

3.5. Quantitative Real-Time PCR

1. Dilute the samples 1:5.
   (a) Add 40 µL of RNase-free water to a new 96-well plate.
   (b) Using a multichannel pipet, homogenize the samples well and add 10 µL to the corresponding well in the 96-well plate.
   (c) Cover and store on ice until use.
2. Prepare the standard curve in the wells immediately following the samples.

15 ng	Add 25 µL of Positive Control to 25 µL RNase-free water
3 ng	Add 10 µL of 15 ng Standard to 40 µL RNase-free water
0.6 ng	Add 10 µL of 3 ng Standard to 40 µL RNase-free water
0.12 ng	Add 10 µL of 0.6 ng Standard to 40 µL RNase-free water
0.024 ng	Add 10 µL of 0.12 ng Standard to 40 µL RNase-free water

3. A higher volume of samples and standards may be required if you are looking at a large number of genes. Check your calculations carefully to ensure there will be enough.
4. Each sample and standard are run twice on the real time plate to ensure accuracy. To calculate the amount of Reagent Master Mix you will need, use the following equation: 2 (# samples + # standards) + 5 extras = Total # (with room for error). Multiply this total # by each of the values listed in the table in Step 5. Add this up and this will give you the total volume of Master Mix needed for the experiment.
5. Prepare the Reagent Master Mix

Component	Volume
Primer/probe from ABI	0.5 µL
Water	1.5 µL
Universal Taqman Mix from ABI	5.0 µL
Total volume	7 µL

6. Using a 2–20-µL multichannel pipet (from Rainin), homogenize the samples and standards well. Add 3 µL of each sample/standard twice onto a 384-well plate, switching tips between each sample/standard.
7. Vortex and spin down the master mix. Use an electronic pipet to add 7 µL of the master mix to the samples.
8. Cover with PCR-compatible optical adhesive film and store in the dark at 4°C for up to 48 h until use.

3.6. iPS Colony Formation and Human ES Cell Culture

It has been demonstrated that epigenetic components including DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) play an important role in regulating transcription of development-related genes as well as reprogramming somatic cells. Since human dermal fibroblasts exhibited the best lentiviral transfection efficiency in our previous experiments (Fig. 2a), we investigated the effects of shRNA-induced knockdown of DNMT1 and HDAC gene expression on pluripotency gene expression in this cell type. As shown in Fig. 3a, the expression of DNMT1 mRNA (open circle) was diminished by as much as 50–60% compared with the control level after 5 days of DNMT1-targeted shRNA lentiviral infection. During this process, the expression of Oct4 (closed circle) was increased (Fig. 3a, left). Additional subculture with puromycin selection resulted in the formation of multiple colonies exhibiting positive staining for Oct4, Sox2, and SSEA4 proteins (Fig. 3b). Similar to the colonies from previous reports that are missing either Klf4 or cMyc (8), these colonies failed to proliferate and continuously grow under hES culture condition. We reasoned that the inhibition of a single

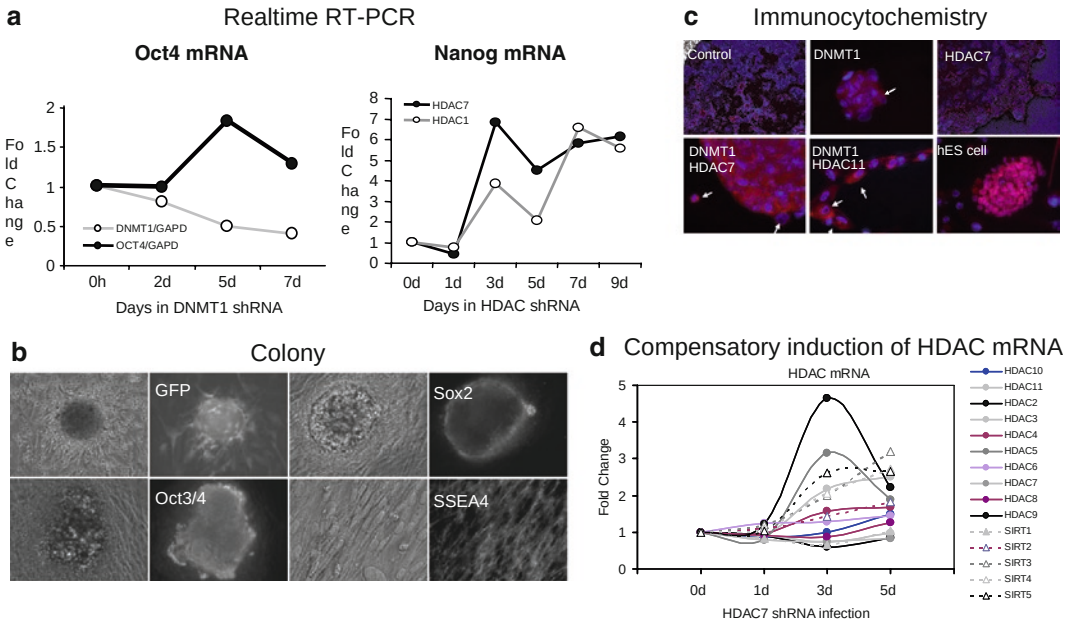

Fig. 3. (**a**) Real-time RT-PCR. Induction of Oct4 and Nanog mRNA in human dermal fibroblasts during shRNA-induced gene knockdown of epigenome-related target genes (DNMT1, HDAC7, and HDAC11). Target gene expression was analyzed using real-time qRT-PCR and normalized by GAPDH expression. (**b**) Colony formation. Expression of turboGFP, Oct4, Sox2, and SSEA4 from colony formation was visualized by immunocytochemistry using specific antibodies and green fluorescence (turboGFP). (**c**) Immunocytochemistry. Oct4 protein expression in human dermal fibroblasts with shRNA interference of DNMT1 and/or HDACs compared with noninfected control cells. *Arrows* indicate nuclear staining for Oct4. *Blue* DAPI, *Red* Oct4. hES cells were obtained from Invitrogen. (**d**) Compensatory induction of HDAC mRNA by HDAC7 shRNA lentiviral infection. Target gene expression was analyzed using real-time qRT-PCR and normalized by GAPDH expression.

DNMT is not sufficient to activate the whole reprogramming processes. This provided a rationale to test the inhibition of HDACs and combinations of DNMTs and HDACs. In the same manner, human dermal fibroblast cultures were infected with HDAC7- and HDAC11-targeted shRNA lentivirus. Gene expression of Oct4 increased slightly from HDAC7 and HDAC11 gene knockdown (<2-fold; data not shown), but Nanog gene expression was significantly upregulated within 3 days after infection and this effect was maintained consistently thereafter (Fig. 3a, right). Immunocytochemistry also demonstrated induction of Oct4 protein in the nucleus of human dermal fibroblast cells with DNMT1- and/or HDAC-targeted shRNA infection (Fig. 4c).

Because hyperacetylated chromatin is transcriptionally active and hypoacetylated chromatin is transcriptionally repressed, histone acetylation plays an important role in the regulation of gene expression. HDACs control the level of acetylated chromatin, thus participate in a series of pathways during cell growth and development. Three classes of HDACs have been identified, however, the function of the individual HDACs is currently still under investigation. Interestingly, we have observed compensatory mechanisms of HDACs during HDAC7 gene knockdown. As

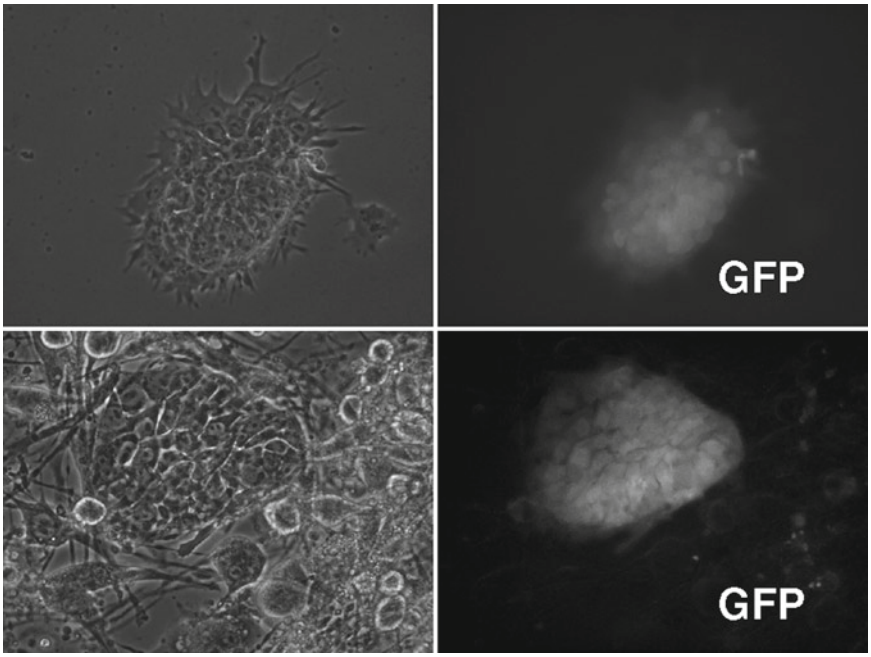

Fig. 4. Human ES cells cultured on Matrigel (*top*) and a feeder cell layer (*bottom*). BG01V/hOG cells, a variant hES cells harboring the hOct4-GFP reporter from Invitrogen, were cultured on Matrigel with mTeSR1 medium (StemCell Technologies) or a feeder cell layer with hES culture medium (Invitrogen). GFP was visualized under a fluorescent microscope.

shown in Fig. 3d, expression of HDAC9, HDAC5, HDAC11, SIRT4, and SIRT5 increases dramatically with 50% knockdown in HDAC7 gene expression. Although, remarkably, we have not observed compensatory induction with HDAC11 gene knockdown (data not shown), compensatory expression for these same HDACs was induced by DNMT1 gene knockdown (data not shown). Double infection with DNMT1 and HDACs (HDAC7 or HDAC11) shRNA lentivirus produced further increased Oct4 protein levels in human dermal fibroblast cultures (Fig. 3c) as well as Sox2 protein (data not shown).

1. For the preparation of human ES culture medium (mTeSR1 medium, Stem Cell Technology, Vancouver, Canada) for ES cell culture on Matrigel, thaw mTeSR1 5× supplement at 4°C overnight, and add 100 mL of 5× supplement to 400 mL of basal medium and mix well. Complete mTeSR medium is stable up to 2 weeks at 4°C, and up to 6 months at –20°C. For the preparation of human ES culture medium using Knockout Serum Replacement (KSR; Invitrogen) for ES cell culture on feeder cell layer, thaw KSR, and add 20 mL of 5× KSR to 79 mL of DMEM/F12 with glutamax, 1 mL of 10 mM non-essential amino acids, 100 μL of 55 mM 2-mercaptoethanol, and 40 μL of bFGF (10 μg/mL). This complete hES culture medium is stable up to 1 week at 4°C.

2. For the preparation of Matrigel plates (BD Biosciences), thaw BD Matrigel matrix on ice until liquid. BD Matrigel matrix should be aliquoted and frozen down at −70°C (good for 6 months). Add thawed BD Matrigel matrix to the ice-cold DMEM/F12 and mix well and immediately apply 1 mL diluted Matrigel solution to culture plates (1 mL/well of 6-well plates). Swirl the plate to evenly cover the well and leave the plate at room temperature for at least 1 h. Then, gently remove excess BD Matrigel solution from the plates just before use.
3. For the preparation of feeder cell layers, 2 days before human ES cell culture, plate 3×10^4 cells/cm^2 of mitotically inactivated MEFs (ATCC, SCRC-1045.2) with complete DMEM medium containing 10% FBS and antibiotics. The next day, replace medium with serum knockout human ES culture medium (Invitrogen).
4. When human dermal fibroblast cells infected with shRNA lentivirus reach confluence, split the cells into prepared Matrigel plates or feeder cell layers by a 1:6 split.
5. Two days after the split, change the medium with mTeSR or serum knockout medium for Matrigel plates or feeder cell layers, respectively.
6. Change the medium every day until hES cell-like colonies (homogenous clusters of cells with high nuclear/cytoplasmic ratio and prominent nucleoli that have pushed aside the surrounding feeder cells) start to appear, as shown in Fig. 4.
7. Pick the colony and expand the cells on Matrigel plates or feeder cell layers.

   To passage human ES cells, aspirate medium and rinse with prewarmed DMEM/F12. Add an appropriate amount of dispase (for example, 1 mL/well of 6-well plate) at a concentration of 1 mg/mL (for stem cell application). Place at 37°C for exactly 7 min and remove the dispase solution. After gently rinsing two to three times with 2 mL of DMEM/F12, add 2 mL of complete human ES culture medium and scrape the colonies off with a cell scraper. Transfer the detached cell aggregates to a 15-mL tube, rinse, and collect with 2 mL of additional human ES culture medium. Make 1:6–1:10 splits.
8. Place the plates in a 37°C, 5% CO_2 humidified incubator.

3.7. Immunocytochemistry

1. Plate the cells on Nunc Lab-Tek chamber slides (Sigma–Aldrich) and incubate overnight in a 37°C, 5% CO_2 humidified incubator.
2. Rinse briefly with ice-cold PBS and add fixative solution (ethanol:acetic acid:H_2O = 7:2:1).

3. Incubate for 10 min at room temperature, and rinse three times with 10% FBS in PBS solution for 5 min each.
4. Add an appropriate dilution of primary antibody (1:100–1:500 dilution) in 10% FBS in PBS with 0.2% saponin.
5. Incubate for 1 h at room temperature with gentle rocking.
6. Rinse three times with 10% FBS in PBS for 5 min each, and add an appropriate dilution of fluorescent (Alexa Fluor)-conjugated secondary antibody in 10% FBS in PBS with 0.2% saponin.
7. Incubate for 1 h at room temperature with gentle rocking (keep in the dark).
8. Rinse three times with 10% FBS in PBS for 5 min each.
9. Rinse with PBS and save in mounting media with DAPI (Vectorshield) with a cover glass (keep in the dark until observed).

3.8. Reporter Assay for Small Molecule Screening

Although forced overexpression of defined transcription factors (Oct4, Sox2, cMyc, and Klf4 or Oct4, Sox2, Nanog, and Lin28) can reprogram somatic cells to induced pluripotent stem (iPS) cells, the application of current iPS cells in regenerative medicine is hampered by the use of viral delivery systems and tumor-related iPS factors such as cMyc and Klf4. Toward the latter challenge, Feng and colleagues have recently shown that two nuclear orphan receptors (Esrrβ and Esrrγ) target genes involved in self-renewal and pluripotency can substitute for cMyc and Klf4 to mediate reprogramming of mouse embryonic fibroblasts (16). Furthermore, chemical complementation of Klf4 using 5 μM kenpaullone, a broad-range protein kinase inhibitor, gave rise to iPS cells indistinguishable from mouse ES cells (17). Together with small molecules that induce epigenetic modifications described previously (VPA, 5-azaC, TSA, and SAHA) (14), these findings suggest that chemical compounds can replace the viral-delivered transcription factors to achieve safely reprogrammed somatic cells. Therefore, development of screening methods for inducing expression of pluripotent genes should be beneficial to produce reprogrammed somatic cells that are capable of directed differentiation into multiple lineages derived from patient somatic cells. For small molecule screening, sensitive and rapid in vitro assessments are keys for successfully identifying specific reprogramming agents and the decision to move forward with induction studies to validate reprogramming of somatic cells. GFP and luciferase have been widely used as reporters to identify small molecules and cell signaling for transcriptional regulation of specific genes using mammalian cell culture system. Either a GFP or luciferase reporter system under the control of Oct4 or Nanog binding sites (Fig. 5a) will provide a sensitive and rapid method

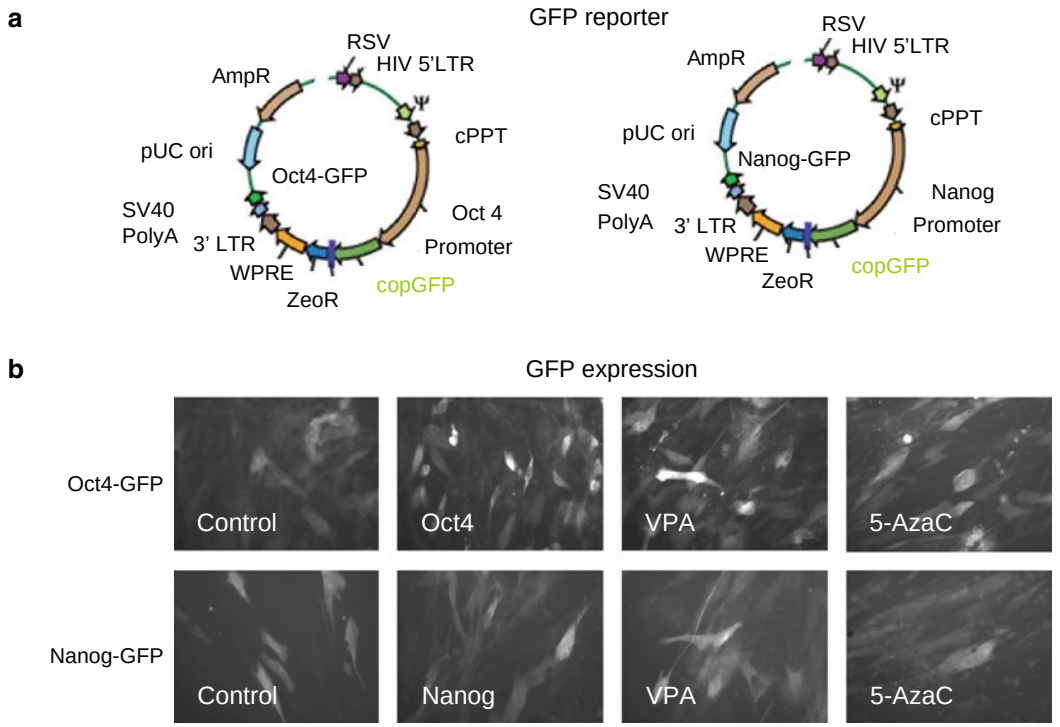

Fig. 5. (**a**) Plasmid maps for Oct4 or Nanog promoter-driven GFP are shown. (**b**) GFP expression. Lentivirus harboring Oct4–GFP or Nanog–GFP reporters were infected into human dermal fibroblasts and cells were treated with an HDAC inhibitor (VPA) or a DNMT inhibitor (5-azaC), or Oct4 and Nanog overexpression lentivirus. GFP expression was visualized using a fluorescent microscope.

for high-throughput screening of small molecules for the induction of pluripotent gene expression. Since a zeocin resistance selection marker is available, stable cell lines can be generated for consistency. When human dermal fibroblasts that harbor Oct4–GFP or Nanog–GFP were treated with lentiviruses that overexpress Oct4 or Nanog, and chemical inhibitors for HDAC (valproic acid) and DNMT (5-aza-deoxycytidine), expression of GFP increased compared with no treatment (Fig. 5b).

4. Notes

1. Although human primary fibroblasts are tested for potential biological contaminations, human cells should be handled as potentially biohazardous materials under at least Biosafety Level 2 containment. Details of precautions associated with biosafety levels can be found online at http://bmbl.od.nih.gov/sect3bsl2.htm. At this level, precautions against the biohazardous materials in question are minimal, most likely

involving gloves and working under a biosafety cabinet with air flow connected to outside of the building. Decontamination procedures for this level are similar in most respects to modern precautions against everyday microorganisms (i.e., washing one's hands with antibacterial soap, washing all exposed surfaces of the lab with disinfectants, 10% bleach solution, and 70% ethanol). In a lab environment, all materials used for cell cultures are decontaminated via autoclave. Laboratory personnel should have specific training in the procedures conducted in the laboratory and should be supervised by a scientist with general training in microbiology or a related science.

2. Follow the general guidelines to store medium (4°C), 5× supplements (–20°C), and bFGF at proper conditions. With proper storage conditions, most 5× supplements last 6–12 months. Complete hESC medium can be stored at 4°C for up to 1 week; preheat the medium to 37°C before use. mTeSR1 medium is developed to maintain undifferentiated hES and iPS cells with serum- and feeder-free conditions. BD Matrigel is a culture matrix that maintains hES and iPS cells without direct contact with feeder cell layers. Complete mTeSR1 medium can be stored at 4°C for up to 2 weeks or is stable when frozen at –20°C for 6 months. Do not preheat complete mTeSR1 medium before use, instead, bring the required amount of complete mTeSR1medium to room temperature.
3. Lentiviruses are a subset of retroviruses that have the ability to integrate into host chromosomes and to infect nondividing cells. For safety purposes, commercially available lentiviral vectors have replication genes removed. Therefore, the risk is considered low for conversion to a replication-competent state. However, as stated above, universal precautions are needed at all times when handling lentivirus and lentivirus-transfected cells under a class II biological safety cabinet. In addition, nonpackaging control cells (i.e., HEK293) should be transfected and monitored for replication-competent lentivirus (RCL) according to the manufacturer's recommendation. Materials exposed to shRNA lentiviral particles should be bleached and the working area should be washed with 70% alcohol after use. Bleached dry materials should be autoclaved separately.
4. Transducing unit (TU, or multiplicity of infection [MOI]) of lentivirus is the ratio of transducing particles to cells. Lentiviral titers (TU/mL) are determined by PCR amplification (indirect method) or direct counting of infected cells by measuring the expression of internal markers (SMARTvector shRNA lentivirus include GFP and lentiviral overexpression from SBI include red fluorescent protein [RFP]). PCR-based indirect

titering methods could include incomplete viral particles, therefore, it is necessary to measure the actual viral TUs (for the shRNA targeting) and expression levels (for the lentiviral overexpression) using specific target cells before use.

5. Under –70°C storage conditions, lentiviral particles are stable for at least 6 months. Repeated freeze–thaw cycles should be avoided as this can negatively affect viral titer. It is highly recommended to thaw lentiviruses on ice, aliquot into smaller volumes, and immediately return them to –70°C. Alternatively, thawed viral particles can be maintained at 4°C for approximately 1 week without significant loss in titer (37°C overnight incubation usually loses 90% of viral titers).
6. Search for primers and probes for TaqMan RealTime RT-PCR at: https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=ABGEKeywordSearch&catID=600689.
7. SMARTvector shRNA lentivirus contains a puromycin resistance gene that allows selection of lentivirus-transduced cells using the antibiotic puromycin that inhibits protein synthesis. Optimal concentration of puromycin varies depending on cell type (1–10 μg/mL) and usually takes 2 weeks to complete selection.
8. In order to minimize the effect of genomic DNA contamination during RT-PCR, DNase treatment is necessary to analyze target gene expressions having very low levels of mRNA, such as stem cell markers in somatic cells.

References

1. Lowry, W.E., et al., *Generation of human induced pluripotent stem cells from dermal fibroblasts.* Proceedings of the National Academy of Sciences of the United States of America, 2008. 105: p. 2883–2888.
2. Maherali, N., et al., *Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution.* Cell Stem Cell, 2007. 1(1): p. 55–70.
3. Meissner, A., M. Wernig, and R. Jaenisch, *Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells.* Nature Biotechnology, 2007. 25(10): p. 1171–1181.
4. Okita, K., T. Ichisaka, and S. Yamanaka, *Generation of germline-competent induced pluripotent stem cells.* Nature, 2007. 448(7151): p. 313–317.
5. Okita, K., M. Nakagawa, H. Hyenjong, T. Ichisaka, and S. Yamanaka, *Generation of mouse induced pluripotent stem cells without viral vectors.* Science, 2008. 322(5903): p. 949–953.
6. Park, I., P.H. Lerou, R. Zhao, H. Huo, and G.Q. Daley, *Generation of human-induced pluripotent stem cells.* Nature Protocols, 2008. 3(7): p. 1180–1186.
7. Park, I.H., N. Arora, H. Huo, N. Maherali, T. Ahfeldt, A. Shimamura, C. Cowan, M.W. Lensch, K. Hochedlinger, and G.Q. Daley, *Disease-specific induced pluripotent stem cells.* Cell, 2008. 134: p. 1–10.
8. Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* Cell, 2006. 126(4): p. 663–676.
9. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. 131: p. 861–872.
10. Aoi, T., K. Yae, M. Nakagawa, T. Ichisaka, K. Okita, K. Takahashi, T. Chiba, and S. Yamanaka, *Generation of pluripotent stem cells*

from adult mouse liver and stomach cells. Science, 2008. 321(5889): p. 1–4.

11. Wernig, M., et al., *In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state*. Nature, 2007. 448(7151): p. 318–324.
12. Yu, J., M.A. Vodyanik, K.S. Otto, J.A. Bourget, J.L. Frane, S. Tian, J. Nie, G.A. Jonsdottir, V. Ruotti, R. Stewart, I.I. Slukvin, and J.A. Thomson, *Induced pluripotent stem cell lines derived from human somatic cells.* Science, 2007. 318(5858): p. 1917–1920.
13. Mikkelsen, T.S., J. Hanna, X. Zhang, M. Ku, M. Wernig, P. Schorderet, B.E. Bernstein, R. Jaenisch, E.S. Lander, and A. Meissner, *Dissecting direct reprogramming through integrative genomic analysis.* Nature, 2008. 454(7200): p. 49–55.
14. Huangfu, D., R. Maehr, W. Guo, A. Eijkelenboom, M. Snitow, A.E. Chen, and D.A. Melton, *Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds.* Nature Biotechnology, 2008. 26(7): p. 795–797.
15. Huangfu, D., K. Osafune, R. Maehr, W. Guo, A. Eijkelenboom, S. Chen, W. Muhlestein, and D.A. Melton, *Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2.* Nature Biotechnology, 2008. 26(11): p. 1269–1275.
16. Feng, B., J. Jiang, P. Kraus, J-H. Ng, J-C.D. Heng, Y. Chan, L-P. Yaw, W. Zhang, Y-H. Loh, J. Han, V.B. Vega, V.C. Rataboul, B. Lim, T. Lufkin, and H-H. Ng, *Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb.* Nature Cell Biology, 2009. 11(2): p. 197–203.
17. Lyssiotis, C.A., R.K. Foreman, J. Staerk, M. Garcia, D. Mathur, S. Markoulaki, L.L. Lairson, J. Hanna, B.D. Charette, L.C. Bouchez, M. Bollong, C. Kunick, C.Y. Cho, A. Brinker, P.G. Schultz, and R. Jaenisch, *Reprogramming of murine fibroblasts to induced pluripotent stem cells with chemical complementation of Klf4.* Proceedings of the National Academy of Sciences of the United States of America, 2009. 106(22): p. 8912–8917.

Part IV

Bioengineering, Manufacturing, and Clinical Technologies

Chapter 23

Adipogenic Differentiation of Human Adipose-Derived Stem Cells on 3D Silk Scaffolds

Jennifer H. Choi, Evangelia Bellas, Gordana Vunjak-Novakovic, and David L. Kaplan

Abstract

Current treatment modalities for soft tissue defects due to various pathologies and trauma include autologous grafting and the use of commercially available fillers. However, these treatment methods are associated with a number of limitations, such as donor site morbidity and volume loss over time. As such, improved therapeutic options are needed. Tissue engineering techniques offer novel solutions to these problems through development of bioactive tissue constructs that can regenerate adipose tissue with an appropriate structure and function. The recent advances in the derivation and characterization of hASCs have led to numerous studies of soft tissue reconstruction. In this chapter, we discuss methods in which our laboratory has used hASCs and silk scaffolds for adipose tissue engineering. The use of naturally occurring and clinically acceptable materials such as silk protein for tissue-engineering applications poses advantages with respect to biocompatibility and mechanical and biological properties.

Key words: hASC, Adipose tissue, Silk, Scaffold, Lipogenesis, Lipolysis

1. Introduction

The utilization of 3D adipose tissue systems to study different physiological states provides several advantages over traditional 2D culture studies. The 3D environment provides a scaffold structure that functions as a physiologically like microenvironment, and thereby promotes cell attachment and migration, and cell–cell and cell–matrix interactions (1). Therefore, regenerative medicine strategies, and, more specifically, adipose tissue engineering, provide promising avenues for the study of adipose tissue in both the normal and diseased states.

Human adipose-derived stem cells (hASCs) are widely available, abundant, and easily accessible through procedures such as

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liposuction and biopsies (2). Remarkable progress has been made with respect to the use of hASCs with natural and synthetic scaffolds, in vitro and in vivo, and for various time frames. In addition to exploring hASC viability and differentiation capacity on various materials (3–10), the contributions of hASCs toward vascularization (11–14) and wound healing (15) have been investigated.

Silk is a versatile protein biomaterial for various tissue-engineering applications (16–19), with favorable characteristics such as low immunogenicity, slow degradation, absence of bioburdens, and strong mechanical properties (20–25). Additionally, we recently demonstrated the ability to engineer vascularized adipose tissue in vitro by cocultivating adipocytes derived from hASCs with endothelial cells in silk scaffolds (26). The ability to tailor silk with respect to biodegradation, mechanical strength, and ability to undergo surface modifications provides strong rationale for its use as a scaffold for soft tissue regeneration (27). Critically, this protein-based biomaterial offers sustained morphological and architectural support, from months to years in vivo during slow degradation, providing soft tissue reconstruction support without loss of size and shape with time until tissue regeneration occurs in vivo.

2. Materials

2.1. Cell Culture and Lysis

1. Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin–fungizone (PSF).
2. 0.25% Trypsin–EDTA.
3. Biotin is dissolved in water at 33 mM (1,000×), stored in aliquots at –20°C, and added to medium as required.
4. Pantothenate is dissolved in water at 17 mM (1,000×), stored in aliquots at –20°C, and added to medium as required.
5. Dexamethasone is dissolved in water at 1 mM (1,000×), stored in aliquots at –20°C, and added to medium as required.
6. Human recombinant insulin is dissolved in water at 1 mM (1,000×), stored in aliquots at 4°C, and added to medium as required.
7. Thiazolidinedione (TZD) is dissolved in dimethyl sulfoxide (DMSO) at 5 mM (1,000×), stored in aliquots at –20°C, and added to medium as required.
8. 3-Isobutyl-1-methylxanthine (IBMX) is dissolved in absolute ethanol at 500 mM (1,000×), stored in aliquots at –20°C, and added to medium as required.
9. DNA cell lysis buffer: 10 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mg proteinase K.

10. Intracellular triglyceride lysis buffer: sodium dodecyl sulfate (SDS) buffer (0.1% w/v) in water.
11. ASC expansion media: DMEM/F-12 supplemented with 10% FBS and 1% PSF.
12. Adipogenic induction medium: DMEM/F-12 supplemented with 3% FBS, 1% PSF, 33 μM biotin, 17 μM pantothenate, 1 μM dexamethasone, 1 μM insulin, 5 μM TZD, and 500 μM IBMX.
13. Adipogenic maintenance media: induction media minus TZD and IBMX.
14. Surgical microscissors.

2.2. Silk Scaffold Preparation

1. *Bombyx mori* silkworm cocoons are purchased from Tajima Shoji Co., LTD. (Naka-ku, Yokohama, Japan).
2. All water used is ultrapure grade.
3. 0.2 M Sodium carbonate in water.
4. 9.3 M LiBr solution in water.
5. 3.3.5-kDa Molecular weight cutoff dialysis cassette.
6. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP).
7. Sieve salt particles to obtain the 500–600-μm range.
8. Teflon cylindrical molds.
9. Dermal biopsy punches.

2.3. Assay Kits

1. Quant-iTTM PicoGreen® dsDNA Reagent and kits.
2. Serum Triglyceride Determination kit.
3. Quantikine® Human Leptin Immunoassay.

2.4. Oil Red O Staining

1. Scaffold fixative: 2 M sucrose in water (see Note 1).
2. Staining fixative: 10% buffered neutral formalin.
3. Oil Red O solution: 0.700 g Oil Red O powder in 200 mL isopropranol. Make a 60% working solution with 0.2-μm filtered Oil Red O solution in phosphate-buffered saline (PBS).
4. Glycerol jelly mounting medium.

2.5. Cultureware

1. Non-tissue cultured-treated 6-well plates.
2. Glass-bottom dishes.

3. Methods

Mature adipocytes, the predominant cell type in the adipose tissue, contain microscopic intracellular lipid droplets, which are the first visible signs of adipose tissue formation. Oil Red O staining

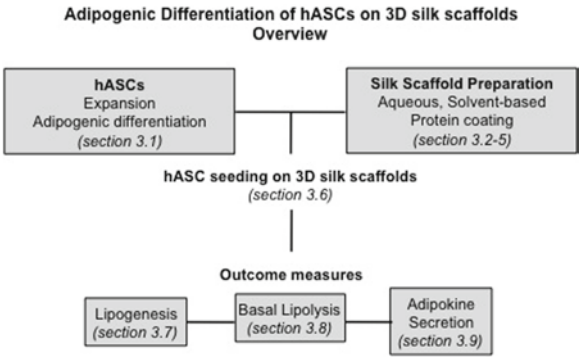

Fig. 1. Overview of adipogenic differentiation of hASCs on 3D silk scaffolds, with the methods discussed in this chapter.

and enzymatic assays are conducted to visualize and quantify the intracellular lipid, respectively (7, 14, 28–31). As adipose tissue is recognized as a vital endocrine organ, the engineered tissue's ability to secrete appropriate adipokines, such as leptin, has also been widely investigated (28, 32, 33). Another key measure of adipose tissue function is its sensitivity to hormone stimulation. Standard assays involve measurements on glucose uptake or glycerol and fatty acid release following stimulation with lipogenic (e.g., insulin) or lipolytic (e.g., catecholamine) hormones (5, 29, 33, 34).

In this section, we discuss detailed protocols for culture of adipogenic cells derived from hASCs on 3D silk scaffolds. In addition to hASC and 3D scaffold preparation, we include protocols for characterization of adipose tissue outcomes. See Fig. 1 for an overview of the methods discussed in this chapter.

3.1. hASC Expansion and Adipogenic Differentiation

1. Prepare ASC expansion media.
2. Quickly thaw frozen hASCs in a 37°C water bath, add to the appropriate volume of expansion media, and allow cells to attach overnight.
3. The following day, aspirate all medium and replace with fresh expansion medium.
4. Exchange media after 3 days.
5. Prepare adipogenic induction medium.
6. Once cells are confluent, aspirate media, wash cells with PBS 2×, and add an appropriate volume of adipogenic induction medium.
7. Prepare adipogenic maintenance media.
8. Following 1-week induction, exchanging medium 2×, exchange induction media with adipocyte maintenance media.

3.2. Silk Biomaterial Preparation

1. Cut *Bombyx mori* silkworm cocoons into small pieces. Discard worm.
2. Boil 5 g of cocoons in 2 L of 0.2 M sodium carbonate for 20 min to degum and remove as much sericin as possible (see Note 2).
3. Rinse the silk fibroin in 1 L of ultrapure water three times for 20 min each time.
4. Dry overnight.
5. Solubilize the dry silk in 9.3 M lithium bromide at 60°C until dissolved (see Note 3), so that the final concentration is 20% w/v.
6. Load the silk solution into a 3.5-kDa molecular weight cutoff dialysis cassette in ultrapure water (see Note 4).
7. Exchange the water a total six times over 3 days.

3.3. Aqueous-Based Silk Scaffold Preparation

1. Dilute the aqueous solution to 6% w/v (see Note 5) using ultrapure water.
2. Pour the silk fibroin solution into a Teflon cylindrical mold and add 500–600-μm NaCl particles to the solution. Use 2 g of NaCl for every milliliter of solution.
3. Cover the mold and leave at room temperature for 2–3 days. Gently tap the mold to ensure even packing of the NaCl particles.
4. Remove the lid and place the scaffolds into ultrapure water to leach out the NaCl particles.
5. Exchange the water three times per day for 2 days.
6. Cut the scaffolds to the desired size using a biopsy punch and autoclave to sterilize (see Note 6) (see Fig. 2).

3.4. Solvent-Based Silk Scaffold Preparation

1. Lyophilize the aqueous silk solution for 2–3 days or until dry.
2. Dissolve the lyophilized silk in HFIP to yield a 17% w/v silk–HFIP solution.
3. Cover the solution immediately, seal with Parafilm, and keep at room temperature for 2–3 days until fully dissolved.
4. To cast the scaffolds, 3.4 g of NaCl particles is used for each milliliter of silk–HFIP solution; leave at room temperature to dry for 2 days.
5. Uncover the scaffolds to allow for HFIP evaporation for 1 day at room temperature and then place in methanol for 1 day.
6. Remove the scaffolds from the methanol and allow to dry.
7. Transfer to Ultrapure water to allow the salt to leach out over 2 days.

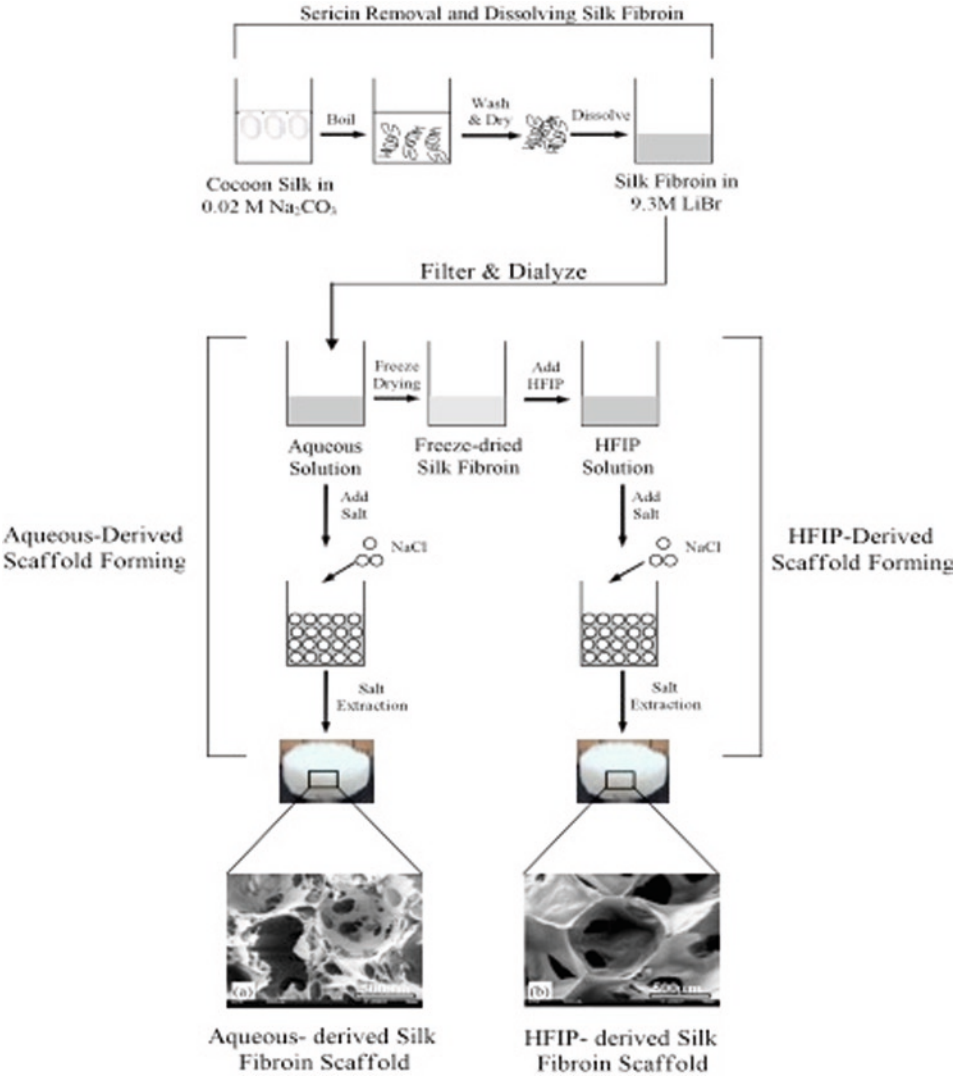

Fig. 2. Schematic of 3D silk fibroin scaffold formation. Processing methods for both aqueous-derived and HFIP-derived silk scaffolds are shown. Reproduced from Biomaterials with permission from Elsevier (27).

8. The scaffolds are cut and sterilized in the same way as the aqueous based scaffolds (see section 3.3) (see Fig. 2).

3.5. Protein Coating

1. Coating solutions were prepared by dissolving VEGF and/or laminin in sterile distilled water at a concentration of 10 μg/mL.
2. Incubate the sterile scaffolds for 15 min in the coating solution, allow to dry to adsorb proteins, and repeat two more times.

3.6. hASC Seeding on 3D Scaffolds

1. One day prior to cell seeding, presoak autoclaved silk scaffolds in adipogenic maintenance media in 37°C, 5% CO_2, 95% relative humidity (see Note 7).

2. The following day, gently aspirate all media from culture wells and from within scaffolds using a 1,000-mL pipette tip (see Note 8). Place each scaffold in a well of a sterile, non-tissue culture-treated plate.
3. Detach differentiated adipocytes from culture flasks by incubating 4 mL of trypsin–EDTA per T185 culture flask for 5 min at 37°C, 5% CO_2, 95% relative humidity. Add media (2× trypsin volume), collect and centrifuge for 10 min at 300×*g* in 4°C.
4. Following centrifugation, carefully aspirate media from cell pellet, resuspend in 5 mL media, and proceed to count cells using standard protocols via hemocytometer. Centrifuge again.
5. For each 8-mm×4-mm (diameter×height) cylindrical silk scaffold, a cell volume of 60 μL is necessary, in which 1×10^6 cells are added per scaffold. Carefully pipette 60 μL cells drop-by-drop onto scaffold.
6. Place culture plates in incubator, and allow cells to attach for 2–3 h.
7. After 2–3 h, carefully add appropriate volume of adipocyte maintenance media to each well such that scaffolds are completely submerged.
8. The following day, carefully transfer seeded scaffolds to new non-tissue culture-treated plates with autoclaved tweezers (see Note 9). Add fresh media.
9. Exchange media every 3 days. See Fig. 3 for a confocal microscopy image of adipogenic-differentiated hASCs seeded on 3D silk scaffolds.

3.7. Measuring Lipogenesis of 3D Adipocyte-Seeded Scaffolds

1. Intracellular triglyceride can be quantified by harvesting scaffolds at various time points in 200 μL intracellular triglyceride lysis buffer, and storing in 1.5-mL Eppendorf tubes at –20°C until time of assay.
2. Thaw samples at room temperature, and chop scaffolds using autoclaved sterile surgical microscissors. Sonicate scaffolds briefly.
3. Centrifuge chopped scaffolds at 15,700×*g* for 10 min at 4°C.
4. Collect supernatant and proceed to assay according to manufacturer's protocols using TG quantification kit.
5. Accumulated lipid can also be visualized using Oil Red O staining. Fix scaffolds in 2 M sucrose overnight for histology. Embed scaffolds in OCT medium and freeze over dry ice, and store in –90°C. Frozen scaffolds can then be sectioned (10-μm sections) using a cryostat and stained.

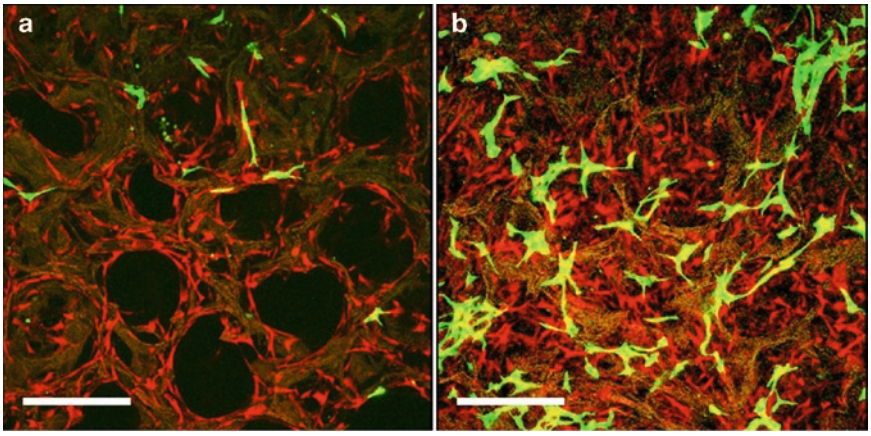

Fig. 3. Confocal microscopy images of tomato red-transduced hASCs in coculture with green fluorescence protein-transduced endothelial cells in porous 3D aqueous silk fibroin scaffolds at day 4 (**a**) and 8 (**b**). *Scale bar* 375 μm. Reproduced from Tissue Engineering with permission from Mary Ann Liebert (26).

6. For Oil Red O staining, fix frozen sections in 10% buffered neutral formalin for 15 min, and rinse with distilled water. Pipette working solution onto frozen sections and incubate for 20 min. Following this, rinse slides with distilled water, and counterstain if desired. Cover using glass cover slips and glycerin jelly. Image with phase contrast microscope. An example image of a section from adipocyte-seeded silk scaffolds is shown in Fig. 4.

3.8. Measuring Basal Lipolysis of 3D Adipocyte-Seeded Scaffolds

1. Levels of basal lipolysis can be measured by quantifying secreted glycerol from spent media samples.
2. Collect spent media samples at desired time points, and store at −80°C.
3. At time of assay, thaw samples, centrifuge at 15,700 × *g* for 10 min at 4°C. Collect supernatants.
4. Detected glycerol can be measured (540 nm) using the TG kit described earlier, however excluding the hydrolysis step. Glycerol levels detected in media samples from blank scaffolds should also be subtracted from all media samples.

3.9. Measuring Leptin Secretion from 3D Adipocyte-Seeded Scaffolds

1. Levels of leptin secretion can be measured as an indicator of mature adipocyte differentiation.
2. Collect spent media samples at the desired time point, and store at −80°C.
3. At time of assay, thaw samples and centrifuge at 15,700 × *g* for 10 min at 4°C. Collect supernatants.

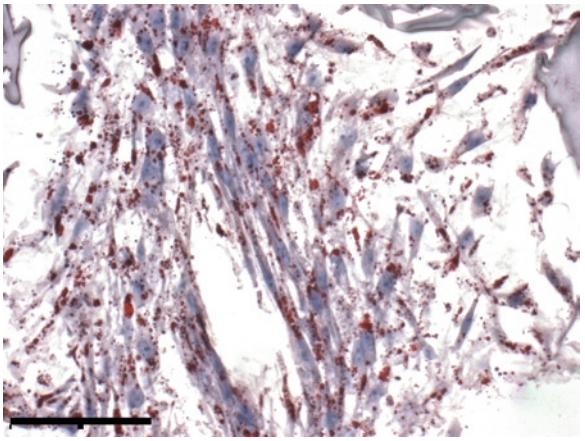

Fig. 4. Oil Red O phase contrast images of differentiated hASCs on porous 3D aqueous silk fibroin scaffolds. *Scale bar* 500 μm; original magnification ×20.

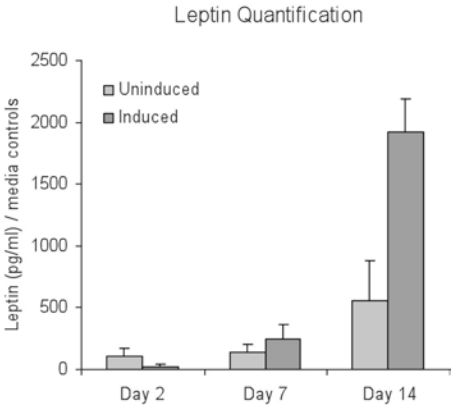

Fig. 5. Leptin secretion measured in undifferentiated hASC/endothelial cocultures (uninduced) and differentiated hASC/endothelial cocultures (induced). Induced cocultures secreted significantly more leptin than uninduced cocultures at day 14 ($*p < 0.05$) and increased secretion at days 7 and 14 ($+p < 0.05$), indicative of the presence of more mature adipocytes. Uninduced cocultures increased leptin secretion at day 14 as well ($++p < 0.05$). Reproduced from Tissue Engineering with permission from Mary Ann Liebert (26).

4. Leptin can then be detected using a quantitative sandwich enzyme immunoassay technique (ELISA) for human leptin. See Fig. 5 for example leptin measurements taken from adipogenic cells derived from hASCs in 3D aqueous silk scaffolds.

4. Notes

1. Prepare 2 M sucrose solution while stirring, slowly adding water to obtain final volume. It may take up to an hour for the sucrose to fully dissolve.
2. Publications from our lab have used varying boiling times. However, for our purposes, we chose to boil for 20 min. This boiling time yielded consistent batches of scaffolds, whereas longer boiling times do not always produce robust scaffolds.
3. Dissolving the silk in LiBr usually takes between 1 and 4 h. It is generally better to pack the dry silk into a beaker that just fits all the silk, and then slowly pour the LiBr solution over the top.
4. The silk solution will be viscous, and shearing of the solution may prematurely induce β-sheet formation. Use the largest needle diameter possible when loading and unloading the dialysis cassette.
5. The concentration was calculated by drying 1 mL of silk solution in a preweighed weigh boat and placing into a 60°C oven till fully dry and then measuring.
6. The scaffolds can be sterilized by autoclaving or by ethanol sterilization, however, degradation properties may be altered by the sterilization technique.
7. When presoaking scaffolds, due to the hydrophobicity exhibited by the protein structure, be sure to gently submerge the scaffolds into the media to ensure that all sides of the scaffold are wetted.
8. Aspirate media from scaffolds right before cell seeding to prevent completely drying scaffolds out.
9. Transferring scaffolds to new plates ensures that any nutrient-competing cells that may have attached onto the culture plate during seeding are eliminated.

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References

1. Gomillion CT and Burg KJ (2006) Stem cells and adipose tissue engineering. *Biomaterials* 27(36): p. 6052–63.
2. Dubois SG, *et al.* (2008) Isolation of human adipose-derived stem cells from biopsies and liposuction specimens. *Methods Mol Biol* 449: p. 69–79.
3. Choi YS, *et al.* (2006) Adipogenic differentiation of adipose tissue derived adult stem cells in nude mouse. *Biochem Biophys Res Commun* 345(2): p. 631–7.
4. Clavijo-Alvarez JA, *et al.* (2006) A novel perfluoroelastomer seeded with adipose-derived stem cells for soft-tissue repair. *Plast Reconstr Surg* 118(5): p. 1132–42; discussion 1143–4.
5. Flynn LE, Prestwich GD, Semple JL, Woodhouse KA (2008) Proliferation and differentiation of adipose-derived stem cells on naturally derived scaffolds. *Biomaterials* 29(12): p. 1862–71.
6. Hong L, Peptan IA, Colpan A, Daw JL (2006) Adipose tissue engineering by human adipose-derived stromal cells. *Cells Tissues Organs* 183(3): p. 133–40.
7. Uriel S, *et al.* (2008) The role of adipose protein derived hydrogels in adipogenesis. *Biomaterials* 29(27): p. 3712–9.
8. Hong L, *et al.* (2007) 17-Beta estradiol enhances osteogenic and adipogenic differentiation of human adipose-derived stromal cells. *Tissue Eng* 13(6): p. 1197–203.
9. Mischen BT, *et al.* (2008) Metabolic and functional characterization of human adipose-derived stem cells in tissue engineering. *Plast Reconstr Surg* 122(3): p. 725–38.
10. Wall ME, Bernacki SH, Loboa EG (2007) Effects of serial passaging on the adipogenic and osteogenic differentiation potential of adipose-derived human mesenchymal stem cells. *Tissue Eng* 13(6): p. 1291–8.
11. Amos PJ, *et al.* (2008) IFATS collection: The role of human adipose-derived stromal cells in inflammatory microvascular remodeling and evidence of a perivascular phenotype. *Stem Cells* 26(10): p. 2682-90.
12. Flynn L, Prestwich GD, Semple JL, Woodhouse KA (2008) Adipose tissue engineering in vivo with adipose-derived stem cells on naturally derived scaffolds. *J Biomed Mater Res A* 89(4): p. 929–41.
13. Hemmrich K, *et al.* (2005) Implantation of preadipocyte-loaded hyaluronic acid-based scaffolds into nude mice to evaluate potential for soft tissue engineering. *Biomaterials* 26(34): p. 7025–37.
14. Lin SD, Wang KH, Kao AP (2008) Engineered adipose tissue of predefined shape and dimensions from human adipose-derived mesenchymal stem cells. *Tissue Eng Part A* 14(5): p. 571–81.
15. Altman AM, *et al.* (2008) IFATS Series: Human adipose-derived stem cells seeded on a silk fibroin-chitosan scaffold enhance wound repair in a murine soft tissue injury model. *Stem Cells* 27(1):p. 250–8.
16. Lovett M, *et al.* (2007) Silk fibroin microtubes for blood vessel engineering. *Biomaterials* 28(35): p. 5271–9.
17. Meinel L, *et al.* (2004) Engineering bone-like tissue in vitro using human bone marrow stem cells and silk scaffolds. *J Biomed Mater Res A* 71(1): p. 25–34.
18. Vunjak-Novakovic G, Meinel L, Altman G, Kaplan D (2005) Bioreactor cultivation of osteochondral grafts. *Orthod Craniofac Res* 8(3): p. 209–18.
19. Wang Y, Blasioli DJ, Kim HJ, Kim HS, Kaplan DL (2006) Cartilage tissue engineering with silk scaffolds and human articular chondrocytes. *Biomaterials* 27(25): p. 4434–42.
20. Altman GH, *et al.* (2003) Silk-based biomaterials. *Biomaterials* 24(3): p. 401–16.
21. Horan RL, *et al.* (2005) In vitro degradation of silk fibroin. *Biomaterials* 26(17): p. 3385–93.
22. Jin HJ, Fridrikh SV, Rutledge GC, Kaplan DL (2002) Electrospinning *Bombyx mori* silk with poly(ethylene oxide). *Biomacromolecules* 3(6): p. 1233–9.
23. Kim UJ, Park J, Kim HJ, Wada M, Kaplan DL (2005) Three-dimensional aqueous-derived biomaterial scaffolds from silk fibroin. *Biomaterials* 26(15): p. 2775–85.
24. Meinel L, *et al.* (2005) The inflammatory responses to silk films in vitro and in vivo. *Biomaterials* 26(2): p. 147–55.
25. Nazarov R, Jin HJ, Kaplan DL (2004) Porous 3-D scaffolds from regenerated silk fibroin. *Biomacromolecules* 5(3): p. 718–26.
26. Kang JH, Gimble JM, Kaplan DL (2009) In vitro 3D model for human vascularized adipose tissue. *Tissue Eng Part A* 15(8): p.2227–36
27. Wang Y, Kim HJ, Vunjak-Novakovic G, Kaplan DL (2006) Stem cell-based tissue engineering with silk biomaterials. *Biomaterials* 27(36): p. 6064–82.
28. Kang X, Xie Y, Kniss DA (2005) Adipose tissue model using three-dimensional cultivation of preadipocytes seeded onto fibrous polymer scaffolds. *Tissue Eng* 11(3–4): p. 458–68.

29. Stosich MS, *et al.* (2007) Vascularized adipose tissue grafts from human mesenchymal stem cells with bioactive cues and microchannel conduits. *Tissue Eng* 13(12): p. 2881–90.
30. Vashi AV, *et al.* (2008) Adipose differentiation of bone marrow-derived mesenchymal stem cells using Pluronic F-127 hydrogel in vitro. *Biomaterials* 29(5): p. 573–9.
31. Weiser B, *et al.* (2008) In vivo development and long-term survival of engineered adipose tissue depend on in vitro precultivation strategy. *Tissue Eng Part A* 14(2): p. 275–84.
32. Fischbach C, *et al.* (2004) Three-dimensional in vitro model of adipogenesis: Comparison of culture conditions. *Tissue Eng* 10(1–2): p. 215–29.
33. Vermette M, *et al.* (2007) Production of a new tissue-engineered adipose substitute from human adipose-derived stromal cells. *Biomaterials* 28(18): p. 2850–60.
34. Kim J, Saidel GM, Kalhan SC (2008) A computational model of adipose tissue metabolism: Evidence for intracellular compartmentation and differential activation of lipases. *J Theor Biol* 251(3): p. 523–40.

Chapter 24

A First Approach for the Production of Human Adipose Tissue-Derived Stromal Cells for Therapeutic Use

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Abstract

Adipose tissue-derived stromal cells (ASCs) are promising tools for the new therapeutic field of regenerative medicine. Many research teams are intent on producing these cells for therapeutic purposes. The cell production must follow strict rules for safety and for constant quality of the cell product to ensure a reliable effect in patients. These rules are grouped under the generic term Good Manufacturing Practices. In this chapter, we describe the general concepts of ASC production for therapeutic use, explaining new terms such as traceability and qualification. We also introduce general requirements for the installation, equipment, material, and staff for the cell production. Then, we outline a general strategy for building a cell culture process. Finally, as an example, we describe the use of CellStack™ chambers and specific tube sets that allow for producing cells beginning with the stromal vascular fraction under near-closed conditions.

Key words: Adipose tissue-derived stromal cells, Good manufacturing practices, Safety, Traceability, Qualification, Regenerative medicine

1. Introduction

Thanks to their remarkable properties of differentiation and tissue repair, human adipose tissue-derived stromal cells (ASCs) could be good therapeutic material for a number of diseases (1). However, they must be produced safely for clinical trials evaluating their therapeutic potential as "cell drugs." Whatever the cell stage, the production of cells for therapeutic use must adhere to strict rules as defined by national or international regulations and by common guidelines for cells and drugs. In the European community, ASCs are considered advanced-therapy medicinal products, as

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defined by the European regulation n°1394/2007 (2), which is based on European guidelines (3). In the United States, ASCs are considered human cell, tissue, and cellular and tissue-based products and fall under the Code of Federal Regulation (CFR), title 21, part 1271 (4). Additionally, the US Food and Drug Administration recently released for public comment the "Guidance for Industry: Current Good Tissue Practice and Additional Requirements for Manufacturers of Human Cells, Tissues and Cellular and Tissue-based Products" (5). This guideline is in addition to the previously published "Guidance for Industry: Regulation of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps) – Small Entity" (6). Regulatory authorities of other countries have issued similar guidelines. When beginning to produce ASCs, investigators must keep in mind these regulations to perfect a technique that will be acceptable to the relevant legal authorities.

Because the stromal vascular fraction (SVF) extracted from adipose tissue is a rich source of ASC, some companies propose the use of SVF without purification or amplification by culture. They have devised equipment that can automatically and safely extract SVF. This solution could have some clinical application with autologous cells (7); however, the use of SVF is not convenient for allogeneic applications because of the presence of immune cells. Furthermore, its use is not workable when the clinical effect requires more than ten million ASCs or a pure population for tissue engineering. Moreover, the companies have optimized and secured the use of the equipment for using SVF, and little can be done to adapt their protocols for clinical trials. For these reasons, in this chapter, we focus on the general concepts of the production of ASCs by culture. We give examples of culture methods of this cell source that can be used in regenerative medicine.

1.1. General Concepts

The "philosophy" of the rules for the use of therapeutic cells can be summarized by the Latin "Primum non nocere," or "First, do no harm." Thus, safety must be kept in mind when a team builds a protocol for the production of ASCs. The corollary of security is traceability and qualification. These parameters must be applied at all times during the production process, for the handling of biological materials (harvesting, culture media, additives, etc.), the equipment, the production environment, and staff.

Traceability involves the verification of the chronological interrelation of the uniquely identifiable entities. Traceability allows for verifying the history, location, or application of an item by its documentation. For a biological compound, the origin, batch number, and quality controls (QCs), for example, are recorded; for a technician, the technician's certification, the tasks the technician can perform, and other training (internal or external) are documented.

Qualification is establishing confidence that the production environment, equipment, material, and staff can consistently operate within established limits and tolerance. Of course, qualification does not mean the same things for the equipment or staff members.

In general, everything that could influence the cell production process and thus the final product must be considered, especially the environment, equipment, devices, and materials used for culture and the staff.

1.1.1. The Production Environment

Production of cells according to good manufacturing practice (GMP) rules requires clean rooms. These rooms are built to restrain the number of particles in suspension in the air and to avoid contamination. Clean rooms are classified according to the number and size of particles. The US Federal Standard 209E, although cancelled in 2001, is still in use, although the International Organization for Standardization standard (ISO 14644-1) must be used (8). For example, a laminar flow hood must be class 100 according to US 209E and class 5 for the ISO (see Table 1). The rooms must have particle filters and a system of air circulation to maintain the particle number as low as required and to ensure a positive pressure to avoid particle entry. The ceiling, walls, and floor should be constructed to avoid particle accumulation (smooth, without roughness, no release of particles) and be easy to clean and disinfect. For example, the floor must be built of a resistant material (against shocks or chemicals) without joints, and the electrical outlets must be inside the wall. The access to the clean rooms must be controlled by a system of locks. This avoids leakage of the positive pressure in the clean room. The equipment must be easy to clean and emit a low level of particles. For example, the benches could be stainless steel

Table 1
United States Federal Standard 209E: clean room standards

Class	Maximum particles/ft³					ISO equivalent
	≥0.1 m	≥0.2 m	≥0.3 m	≥0.5 m	≥5 m	
1	35	7	3	1		ISO 3
10	350	75	30	10		ISO 4
100		750	300	100		ISO 5
1,000				1,000	7	ISO 6
10,000				10,000	70	ISO 7
100,000				100,000	700	ISO 8

that is resistant, emits no particles, and allows for extensive cleaning. The room must be qualified in terms of particle number, air flow rate, surface and air bacterial contamination, and ability to remove excess particles according to ISO 14644-2 and 14698 (9, 10). The clean room must be qualified before its use (operational qualification) to ensure that all target parameter values are reached. Then, it must be qualified during activity (performance qualification). The control values during the operation of the room depend on the level of the activity. Of course, qualification must be completed after the filtration system is inspected or after other activities that may affect the fitness of the room.

1.1.2. The Equipment

In addition to the characteristics described for the environment, all equipment must pass operational and performance qualifications. For example, for a centrifuge, the operational qualification, done when the equipment is received, should ensure that the electrical power required is compatible with the specifications. Likewise, the actual number of rotations per minute should correspond to the required number of rotations per minute. The performance qualification should ensure that the expected results are reproducibly obtained with the centrifuge (i.e., separation of the SVF from mature adipocytes, density gradient separation). The equipment must be well maintained and parameters checked regularly according to the apparatus specifications. For example, in our maintenance plan, the laminar flow hood is checked every 6 months (particles, and surface and air bacterial contamination). In general, use of a specialized company is preferable for regular qualification of the equipment because of the technical nature of the process.

1.2. Devices and Material

In general, requirements for culture devices are not a problem because the devices are manufactured by well-established companies. However, the devices must have been manufactured according GMP rules, and the manufacturer must be able to provide a quality certificate that at least mentions the batch number, controls and results of control testing, and date of expiration. All biological products that are in contact with the cells at any phase of the process must be considered. Indeed, they could be the vector of a toxic agent or a contamination. Although perhaps restrictive, the French regulation identifies such biological products as "annex therapeutic products," with specific legislation similar to that of drugs (11). In France, companies wanting to distribute such products must provide a dossier that includes all information about the origin of the raw material, the manufacturing process, proof of efficacy, results of toxicity studies, traceability, and QCs in order to obtain an authorization from the regulatory agency, which is not required in other countries, even in Europe. However, investigators devising a process of cell production must take into

account the specificity of biological products. In fact, they must check all the items we cited previously. Each lot of product used must be qualified to maintain the quality of the process. For example, for collagenase, the testing could include comparing three to five digestions of adipose tissue with the actual lot to ensure that defined parameters (i.e., cell yield, phenotype, and viability) are within the limits of acceptance. It is preferable to buy a product produced according to clinical GMP (cGMP) guidelines.

1.3. The Staff

Everyone involved in the cell production must be well trained and have appropriate qualifications. Each staff member must have a good knowledge of the quality assurance and have defined tasks. However, each staff member must understand the general structure of the process in which they participate. For academic production structures or small enterprises, to ensure an efficient team, each member should be involved in the development of the process, from the research and development phase to the final version. Continuing education is an important parameter as well.

1.4. The Process

Simple rules may be followed for the design of the cell production process. The process should be preferably defined before any preclinical experimentation to ensure the highest value of those experiments, but, in general, the process comes from the research laboratory protocol, when the great majority of the experiments were performed. The challenge is adapting the research protocol so that it is transposable to cGMP conditions of production while ensuring the maintenance of the therapeutic properties of the cells. Thus, the production process must be built according to a precise plan.

Each step should be validated by well-chosen experiments aiming to verify the properties of the cells. The simplest process has the best chance of success. In a first step, the team must ensure that the research protocol for the cell culture is described with the highest level of detail, preferably as a flowchart. Each step must be identified, and the equipment (centrifuge, flow hood, incubator, etc.), the material (medium, additives, plastic ware, etc.), and the methods must be documented. After the analysis of the protocol, crucial steps are identified because they constitute a milestone (i.e., tissue digestion) or because of specific risk (i.e., filtration of the SVF). Similarly, materials of crucial importance must be documented (i.e., collagenase, basal medium, detachment enzyme). The next step often consists in simplifying the protocol by eliminating, for example, manipulations posing a security problem for the cells (i.e., complex manipulation under open circuits) or products that will not be able to have sufficient quality by cGMP standards. For the culture medium, the fewest additives added is preferable to manage the traceability and QC

assessments. QCs and release criteria (RC) must be defined and their confidence limits established.

Once every simplification, change, and addition is determined, a plan is developed. Preferably, each change to the research protocol is accompanied by a validation step (in vitro and/or in vivo testing depending on the level of the changes). This part of the development of the process has the double aim of verifying that each step does not change the therapeutic properties of the cells and, possibly, adapting confidence limits of the QCs or the RC. QCs must be distinguished from tests that are performed during the development process. The latter tests could be destructive for the cell batch or necessitate stopping the procedure because it takes too long to obtain the results (i.e., in vivo testing, differentiation protocols). In general, such tests must be avoided in QC.

QC testing is globally performed at three steps of the process: the starting material (tissue harvesting), during the production process, and at the end to release the cell product or not. Often, QC testing must fulfill contradictory objectives: being discriminating and fast enough to allow passage to the next step and not destructive for the product. QC evaluation must be performed by staff not in charge of the process. QC testing of the starting material must be distinguished from the validation of the donor. The later ensures that the harvested tissue can be used (e.g., serological tests, detection of diseases that alter ASCs). Because of the nature of the harvested tissue (fragment of adipose tissue), QC testing of the starting material is simple and not too discriminating (e.g., weight, aspect). In fact, deciding to begin the culture is based on QC testing of the SVF. In our production process, at this step, QC testing involves cell count and viability, phenotype (CD34, CD45, and mesenchymal marker levels), and microbiological tests. QC testing during the culture allows for the next step. Controls during culture can include visually monitoring the cells during the culture and, when cells are detached, cell counting, cell viability, phenotype, and microbiological testing, and, eventually, colony-forming unit fibroblast evaluation. If the cell product must be released soon after the end of the culture, the QC testing must be quick but reliable. For cryopreservation of cells, QC testing could also include longer tests such as karyotype or functional assays (i.e., differentiation).

RC are established from the results of the QC testing at the end of the culture and from some QC results of previous steps. For example, QC testing for microbiological contamination needs a culture period (12 days in France), so the results of all the tests performed during the culture may be take into account for the decision to release the cell product or not. In our experience, direct examination of the final cell suspension for bacteria results in too many false positives. A decision algorithm must be created

to help the investigator who decides to release the cell product. The algorithm allows for prioritizing RC and defining the mandatory criteria and those that can be replaced by criteria from an additional analysis in case of anomalies.

Standard operating procedures (SOPs) must be written during the development process and approved by its end. They must describe all steps of the cell culture process, including what to do in cases of anomalies, the equipment, the devices and the materials to be used, and the conditions of storage; when and how to harvest samples for the QC testing; and what records to keep (traceability). SOPs could be easily developed from the culture protocols we provide in the Methods section (Subheading 3).

Finally, all processes (except the administration of the cells) must be tested in at least five procedures with real conditions. This step will allow for a test run of the different steps, to check practicability in real time and to assess the frequency by which the cell product passed the RC testing. In our development plan, if one out of five "mock" procedures fail to pass the RC, we perform two additional procedures. A process cannot be validated if more than 20% of the procedures fail to pass the RC.

2. Materials

2.1. Culture Devices

1. *CellStack*™ (Corning): These culture chambers are convenient for large-scale production of cells. They have two large necks that could be used with standard plastic pipettes (development phase). Caps are equipped with filters and could be replaced by caps with a connecting system that is included. The proposed surfaces range from 675 to 27,000 cm^2 (1 floor, 2, 5, 10, or 40 floors), so up-scaling is easy.
2. *Culture sets*: Three types of sets exist, one for the culture initiation (Fig. 1), one for the medium exchange (Fig. 2), and one for cell detachment (Fig. 3). The sets can be directly connected to the CellStack™ chamber and to the medium bag or the wash solution bag. They include a connecting port by which additives can be injected through a 0.22-μm filter. Thus, they allow for almost all manipulations to be performed in a near-closed system.

2.2. Plasticware

1. *Cell strainer*: The strainer is available in three meshes: 40, 70, and 100 μm. We use the classical 100-μm size. The strainer can be placed on top of various 50-ml tubes.
2. *Tubes* (TPP): 50-ml polypropylene.
3. *Syringes*: 1, 2, 10, and 60 ml.

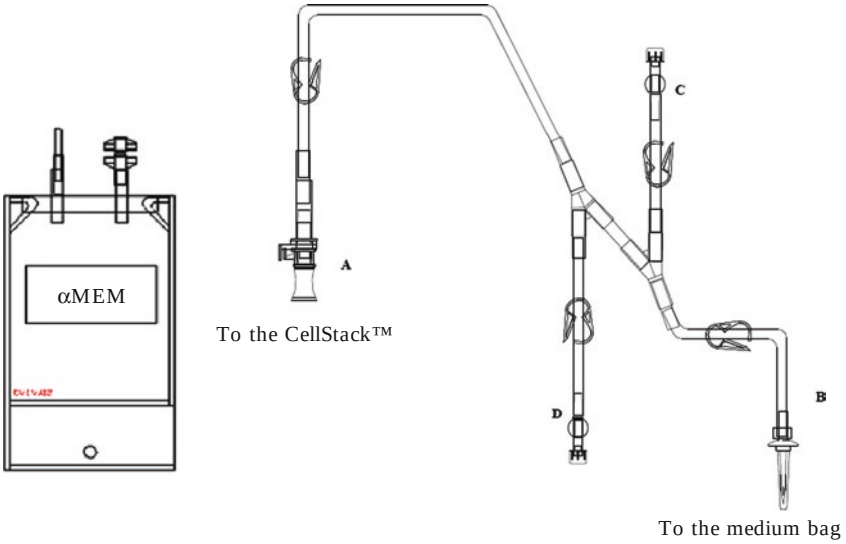

Fig. 1. Seeding set with a connecting system to the culture vessel (**a**), one spike to connect the culture medium bag (**b**), and two ports to inject additives (**c**, **d**).

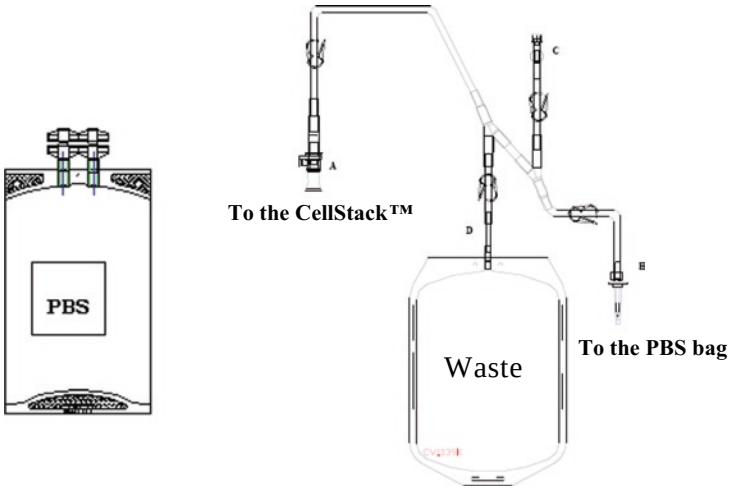

Fig. 2. Medium exchange set with an attached waste bag, a connecting system to the culture vessel, one spike to connect the culture medium bag, and one port to inject additives.

2.3. Culture Medium and Solutions

1. *Alpha MEM in bags*: According to the chosen size of the CellStack™ chambers, they can be 270 or 500 ml.
2. *Phosphate-buffered saline* (*PBS*): The wash solution for cells.
3. *Ciprofloxacin*: This is a good antibiotic and the injectable solution is convenient for cell culture. It presents all the guaranties of a registered drug.
4. *Human albumin*: We use a 4% injectable solution.
5. *Serum*: Depending on the application, fetal calf serum (from Australia or New Zealand) or human AB serum or platelet

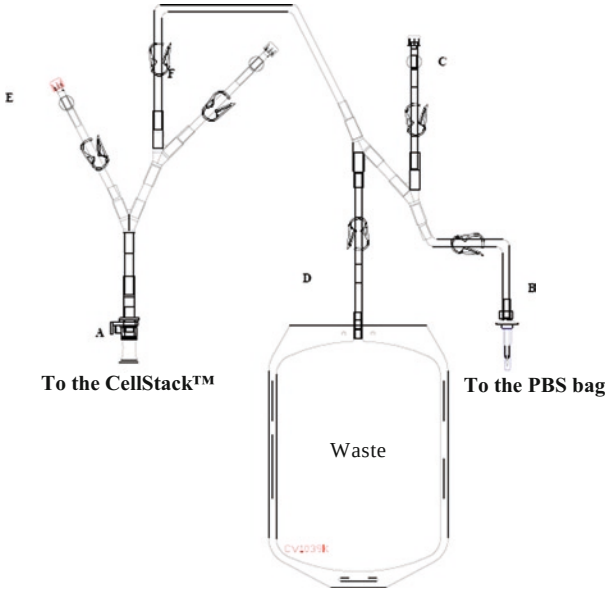

Fig. 3. Cell detachment set with an attached waste bag, a connecting system to the culture vessel, one spike to connect the PBS bag, and ports to inject trypsin and collect the cells.

growth factors-enriched plasma could be used. However, a human product is preferred to avoid xenogenic proteins.

6. *Complete medium*: Alpha MEM plus serum plus ciprofloxacin (10 µg/ml). If human plasma is added, 1 UI of heparin must be added to the medium.

2.4. Enzymes

1. *Collagenase NB6*: This collagenase is prepared according to GMP standards. A solution of 15 UPZ/ml is aliquoted and frozen.
2. *Trypsin–EDTA*: Must be irradiated.

2.5. Other Reagents

1. *Vitalyse*™: To eliminate red blood cells for counting.
2. *Trypan blue solution*: For cell viability assessment.

3. Methods

All the manipulations must be performed under a laminar flow hood, in a clean room.

3.1. Obtaining the SVF

Fat tissue from liposuction is cleared from "fat oil" and red blood cells during the surgical procedure (centrifugation) and sent in syringes to a cell-engineering laboratory.

1. The fat tissue is transferred in 50-ml sterile tubes (10 g per tube). The collagenase solution (final concentration

0.4 U PZ/ml) is then added at 34 ml per tube (diluent = alpha MEM + ciprofloxacin 10 µg/ml). The tubes are locked hermetically and agitated in a culture incubator at 37°C for 45 min (see Note 1).

2. Enzymatic digestion is stopped by adding 10 ml of complete medium (CM). The contents of each tube are distributed in two additional tubes, and 24 ml of CM is added in each tube.
3. After homogenization, the digested suspension is filtered on sterile cell strainers placed on top of 50-ml sterile tubes. The tubes are centrifuged for 10 min at $600 \times g$, at ambient temperature.
4. The supernatant is eliminated by pipetting, and the SVF is suspended in 20 ml CM. A 2-ml aliquot is taken for QC.

3.2. Culture of the SVF

1. A culture-initiating set is connected to a 1,270-cm² CellStack™ chamber (branch A, Figs. 1 and 4a). The alpha MEM bag is connected to the culture initiating set (branch B), and serum and ciprofloxacin are added to the medium through a 0.22-µm filter connected to a port (branch D, Fig. 4b). The cell suspension (5×10^6 cells) is transferred to a syringe and injected in the medium bag through the second port (branch C, see Note 2). The cell suspension is then transferred to the CellStack™ chamber by gravity (Fig. 4c, see Note 3). The culture initiating set is disconnected from the culture chamber that is closed. The culture chamber is placed in an incubator at 37°C in an atmosphere saturated with moisture and 5% CO_2.
2. Twenty-four hours after the culture initiation, a medium exchange set is connected to the culture vessel (branch A, Fig. 2), and a PBS bag is connected to the set (branch B). The nonadherent cells are gently suspended by agitation of the CellStack™ chamber. The medium and nonadherent cells are eliminated in the waste bag, by gravity (Fig. 5). The container is washed once with PBS (see Note 4). The set is disconnected from the CellStack™ chamber. A new medium exchange set is connected to the culture chamber, and CM is

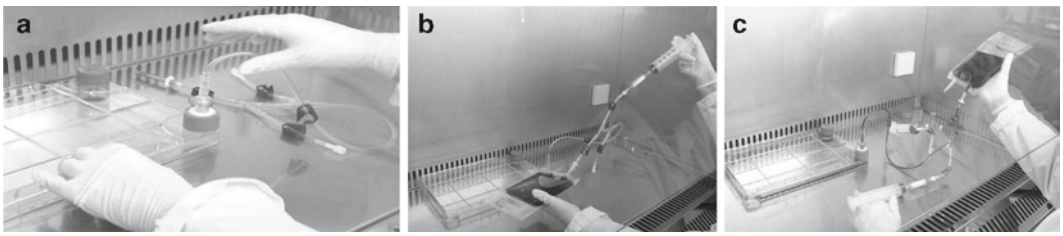

Fig. 4. Pictures of the culture initiation process. (**a**) The seeding set is connected to the CellStack™ chamber using a special connecting system included in the cap, (**b**) additives are added to the αMEM through a 0.22-µm sterile filter, (**c**) complete medium with the cells is introduced in the culture vessel by gravity.

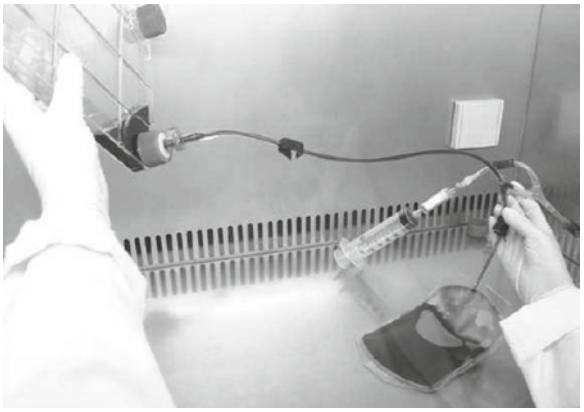

Fig. 5. Elimination of the medium and nonadherent cells, by gravity, in the waste bag.

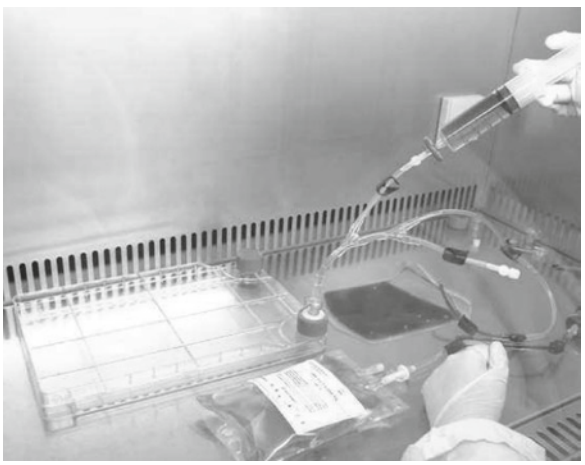

Fig. 6. Detachment phase of the process, the trypsin solution is injected in the CellStack™ chamber using a 60-ml syringe.

prepared as described previously. The CM is then added by gravity to the CellStack™ chamber. The culture container is placed in the incubator. The medium is changed completely on days 4 and 6 according to the same technique, except for washing with PBS (see Note 5).

3. On day 8, the cells are recovered as follows. A detachment set is connected to the CellStack™ chamber (branch A, Fig. 3), and a PBS bag is connected to the set with use of the available spike (branch B). The CM is transferred to the waste bag, by gravity. The CellStack™ chamber is washed once with PBS, which is eliminated in its own bag. Irradiated trypsin (50 ml) is added to the culture chamber (branch E, Fig. 6). After the chamber rests for 5 min at ambient temperature, the action of the enzyme is inhibited by the addition of 75 ml CM (see Note 6).

4. The cells are recovered in a bag connected to the set (branch C); a 2-ml aliquot is taken to perform QC.
5. The cells (7.5×10^6 cells) are seeded in three 1,270-cm² CellStack™ chambers by means of three culture initiation sets as described previously. The medium is changed completely on days 11 and 13 of culture. After each medium change, the culture chambers are placed in the incubator.

3.3. Preparation of the Cellular Product

1. On day 14, the cells of the three CellStack™ chambers are recovered as described in chapter 3.2 point 3 and 4. All three cellular suspensions are pooled in a bag, and 300 ml PBS is added to the cells (through connecting tubing). The bag is centrifuged for 10 min at $500 \times g$. The supernatant is eliminated in a transfer bag after sterile connection (see Note 7). The cells are suspended in 20 ml human albumin. An aliquot of 2 ml is taken for QC.

3.4. Controls

1. *Controls for the starting material*: The fat tissue is weighed on a precision balance. Harvested material must weigh >5 g (the required cell number to seed a CellStack™ chamber will never be reached).
2. *Controls for the SVF*: Cell counting and viability by use of Vitalyse™ and Trypan blue exclusion. The proportion of living cells is calculated (number of living cells/total number of cells). The preparation is rejected if the total number of cells is $<6 \times 10^6$ cells or if the percentage of alive cells is <70%, or both. Phenotype analysis (flow cytometry, CD34, CD45, CD14, CD73, and CD90 levels) and bacteriological controls are carried out. If the percentage of $CD34^+$ cells is <5%, the preparation is rejected. The sample must not be contaminated.
3. *Controls at day 8*: Cell counting, viability, phenotype, and bacteriological controls are performed as described previously. With viability <70%, percentage of $CD45^+$ cells > 20% or cell number $< 8 \times 10^6$ cells, the process is stopped.
4. *Controls for the final product*: Cell counting, viability, phenotype, and bacteriological controls are performed as described previously. With viability < 70%, percentage of $CD45^+$ cells > 5% or percentage of $CD73^+/CD90^+$ cells < 80%, the cell product is not released.

4. Notes

1. We use the MACSmix™ apparatus for constant agitation of the tubes. The apparatus can be easily placed in the incubator and holds four 50-ml tubes.

2. The tubing used for the cell transfer must be rinsed with CM to not lose cells. The CM is aspirated with the syringe that contained the cells and then put into the CellStack™ chamber. This maneuver is performed at least two times.
3. The culture chambers have two floors; thus, the cell suspension must be distributed equally in the two levels. The CellStack™ chamber is placed on its longest side to wait for the equalization of the level of the medium in the two compartments, then the CellStack™ chamber is delicately rotated back to the horizontal position.
4. At this step, as in the others, the technician must verify the clamps so as not to mix solutions.
5. Attached cells must be examined at every change of the medium. However, only the lower floor of the CellStack™ chamber can be observed under a microscope.
6. The "old" CM (medium in the waste bag) can be used to stop the trypsin activity. The medium will be largely diluted when the cells are reseeded. We have validated this point.
7. A blood press is mandatory to eliminate the supernatant from the bag. Because the cell pellet is fragile, this operation must be carried out with great caution.

References

1. Gimble, J.M., Katz, A.J., and Bunnel, B.A. (2007) Adipose-derived stem cells for regenerative medicine. *Circ. Res.* **100**, 1249–1260
2. http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:324:0121:0137:EN:PDF
3. http://ec.europa.eu/enterprise/pharmaceuticals/eudralex/vol4_en.htm
4. http://www.access.gpo.gov/nara/cfr/waisidx_08/21cfr1271_08.html
5. http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/ucm062693.htm
6. http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/ucm073366.htm
7. Lendeckel, S., Jodicke, A., Christophis, P., Heidinger, K., Wolff, J., Fraser, J.K., Hedrick, M.H., Berthold, L., and Howaldt, H.P. (2004) Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. *J. Craniomaxillofac. Surg.* **32**, 370–373
8. http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=25052
9. http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=25008
10. http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=25015
11. http://droit-finances.commentcamarche.net/legifrance/511589-produits-therapeutiques-annexes

Chapter 25

Viral Transduction of Adipose-Derived Stem Cells

Patricia A. Zuk

Abstract

Increasing numbers of regenerative approaches now involve use of adult stem cells, like the bone marrow MSC or the adipose-derived ASC. With their ease of in vitro manipulation and successful tissue integration in vivo, the ASC makes an attractive candidate for gene delivery in vivo using viral-based gene therapy strategies. As such, this chapter describes methods for the transduction of human ASCs with two popular types of recombinant viruses: adenovirus and lentivirus.

Key words: Adipose-derived stem cells, ASCs, Adult stem cells, Lentivirus, Adenovirus, Viral transduction

1. Introduction

In the pursuit of optimizing transfection efficiencies and improving expression of the desired transgene, many cell biologists have turned to viral-based systems. Exploiting the natural ability of the virus to enter a cell, viruses "hijack" the host cell taking over the host's replication/transcription/translation cycle and directing the expression of their genetic material, often over that of the host. Some viruses are even capable of physically inserting their genetic material into the host's genome, incorporating the genes of that virus into the host cell for its life span. As such, mammalian viruses are a powerful method for expressing recombinant proteins or creating siRNA constructs for genetic knockdown.

In the field of stem cell biology, two viral-based systems have become popular: adenovirus and lentivirus.

1.1. Adenovirus

Adenoviruses, of the family *Adenoviridae*, are medium-sized, non-enveloped viruses with double-stranded linear DNA as their

Jeffrey M. Gimble and Bruce A. Bunnell (eds.), *Adipose-Derived Stem Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 702, DOI 10.1007/978-1-61737-960-4_25,

genome. Comprised of 5′ and 3′ inverted repeats, the early genes E1A/B, E2A, E2B, E3, and E4 and the late genes L1 through L4, the adenoviral genome ranges from 26 to 45 kbp. Today's commercial kits employ homologous recombination using adenoviral vectors based on those initially described by Bett et al. 1994 (1) (i.e., *pAdEasy*). In these systems, "shuttle" vectors lacking the E1 and E3 regions are removed allowing for the insertion of up to 8 kb of foreign DNA under the control of a eukaryotic promoter like the CMV promoter. Since the E1 and E3 regions are critical for viral replication, these viral regions must be provided in *trans*-through transfection of a packaging cell line, like HEK293 cells, with the recombinant adenoviral vector. Upon transfection, the HEK293 cells are capable of producing mature virion particles. However, while the virion particles contain the desired recombinant gene, they still lack the E1/E3 region and are thus referred to as *replication deficient*, owing to their inability to replicate in cell lines outside of HEK293 cells.

Transduction of mammalian cells with recombinant adenoviruses has been described extensively in the literature (see Note 2). In addition, adenoviruses have also become a popular method for altering the phenotype of adult stem cells. Intravenous delivery of MSCs overexpressing PLGF has been found to functionally improve rodents in a cerebral ischemic model, suggesting that adenoviruses may be able to provide some level of neuroprotection (2). In the cardiovascular field, adenoviral introduction of a HIF1a mutant into bone marrow MSCs has been shown to promote their differentiation to cardiomyocytes (3) and provide protection against apoptosis to mature cardiac myocytes (4). MSCs overexpressing VEGF splice variants via adenoviral transduction show both increased proliferation and expression of genes associated with endothelial differentiation (5), making it possible for these cells to be used as gene therapy vehicles for the treatment of ischemic heart disease (6). Adenoviral transduction of MSCs is also being explored as a means of treating cancer, with MSCs being used to elevate IL12 level in invasive gliomas (7), to deliver uPA antagonists in osteolytic tumor models, and to target increased expression of NK4, an HGF antagonist, in lung tumors (8).

In the field of ASC biology, adenoviruses have also been used to promote both tissue development and healing in a wide variety of animal models. The applicability of ASCs as gene delivery vehicles using adenovirus was first explored by Morizono et al. (9). Since then, the possible uses of adenovirally transduced ASCs are rapidly expanding. Work by both the Peterson and the Dragoo research teams have reported increased production of recombinant human BMP2 through adenoviral constructs and the healing of bony defects in rodents (10–12). Increased osteogenic potential of ASCs has also been reported upon adenoviral-mediated overexpression of BMP4, BMP7, and Runx2 (13–15). The

regenerative potential of adenovirally transduced ASCs is not limited to bone as enhanced chondrogenesis has also been observed in these cells (16, 17). Based on previous work in MSCs, the homing properties of ASCs to intracerebral gliomas have been explored (18) in the hopes that ASCs could be used as gene delivery vehicles for the treatment of certain types of cancers. In a similar vein, improvement of neurologic deficits may also be possible with the report that neurologic function is improved upon intracerebral transplantation of adenovirally transduced ASCs overexpressing BDNF (19). Interestingly, ASCs also appear to be affected by wild-type adenoviruses, lacking any recombinant genetic manipulation. Transduction of human ASCs with human adenovirus type 36 (*Ad-36*) has been shown to induce their commitment and differentiation and result in enhanced lipid accumulation. Exposure of human adipose tissue explants to Ad-36 also resulted in increased glucose uptake and enhanced expression of several adipogenic genes, suggesting that adenoviral infections may have a causative link to obesity (20, 21). Therefore, there may be some applicability of wild-type adenoviruses in altering ASC potential.

1.2. Lentivirus

A member of the *Retroviridae* family, lentivirus is a genus of slow viruses (*"lenti"* = slow), characterized by a long incubation period. Five serogroups of lentivirus are recognized, each reflecting the vertebrate hosts with which they are associated (primates, sheep/goats, horses, cats, and cattle). The primate lentiviruses are distinguished by their use of the CD4 protein as a receptor for internalization and include the human immunodeficiency virus (HIV).

Lentiviruses are enveloped, RNA viruses replicated in the host cell via the enzyme *reverse transcriptase*. Once the DNA, with its three open reading frames of *gag*, *pol*, and *env*, is produced, it then integrates into the host genome as a provirus via the *integrase* enzyme. The host machinery is then used for the replication of the provirus, the production of viral structural proteins and viral RNA and its subsequent assembly into new virion particles (for a review, see ref. (22)). Early forms of lentivirus for use in mammalian gene expression protocols were based on the murine leukemia virus (MLV). Today, third-generation lentiviral vectors, produced by Dull et al. 1998 (23) and based on the genome of the HIV-1 virus, are also available as easy-to-use commercial kits. Similar to adenoviral systems, lentiviral "shuttle" vectors are created through the removal of critical viral genes (gag, pol, and env), allowing for the insertion of the foreign DNA sequence – often through homologous recombination by bacteria. Upon liposome-mediated transfection of 293 T cells with the shuttle vector and a "packaging mix" of plasmids containing the HIV gag, pol, and rev genes, together with a viral envelope gene (e.g., from VSV), mature, replication-deficient virion particles are produced.

Like adenoviruses, the use of recombinant lentiviruses to transduce adult stem cells has grown in popularity. MSCs genetically engineered via lentivirus to deliver enhanced levels of dystrophin complement the genetic defect in Duchenne muscular dystrophy myotubes (24). Continuing this work in ASCs, Goudenege and colleagues confirm a substantial increase in ASC-derived muscle fibers upon transplantation of virally transduced ASCs delivering MyoD (25). The combination of lentivirus and ASCs has not been limited to skeletal muscle, as ASCs can also be engineered via lentivirus to deliver enhanced levels of HGF to acute myocardial infarcts, resulting in improved left ventricle function (26). Improvement of function or treatment of disease has also spawned the use of lentiviral-transduced MSCs for the delivery of a variety of genes in numerous models of human disease/injury, including angiopoeitin 1 to acute lung injuries (27), beta-glucuronidase to NOD-SCID mice, and osteoprogerin to a model of myeloma bone disease (28). Finally, in addition to the use of lentiviruses for gene delivery, a popular use of these viruses is now for knockdown studies via RNA interference (RNAi). Virally based vector systems, again commercially available, facilitate the expression of artificial RNA molecules called short hairpin RNA (shRNA). Behaving similarly to siRNA, these shRNA molecules trigger the RNAi response and are capable of silencing desired mRNA sequences (29, 30). While the use of these systems in ASCs is relatively new, lentiviral-mediated knockdown of the osteogenic transcription factor Tbx3 has recently been described (31). As such, this technique may become a powerful tool for determining the molecular mechanisms behind ASC differentiation.

2. Materials

2.1. Reagents for the Harvest and Culture of the ASC

1. Sterile phosphate-buffered saline (PBS).
2. Collagenase type IA.
3. High-glucose DMEM containing 4.5 g/L glucose, 1.0 mM sodium pyruvate, 4.0 mM L-glutamine.
4. 10% Fetal bovine serum (FBS).
5. 10,000 IU Penicillin and 10,000 µg/mL streptomycin solution.

2.2. Basic Tissue Culture Reagents for the Expansion of the ASC Culture

1. *ASC control medium*: High-glucose DMEM, 10% FBS, 100 IU penicillin, 100 µg/mL streptomycin.

2.3. Tissue Culture Reagents for the Expansion of HEK293 and 293 T Cells

1. *HEK293 growth medium*: High-glucose DMEM, 10% FBS, 100 IU penicillin, 100 μg/mL streptomycin.
2. *293 T growth medium*: High-glucose DMEM, 10% FBS, 100 IU penicillin, 100 μg/mL streptomycin. Prior to amplification, grow until 90% confluent.

2.4. Commercial Systems for Production of Recombinant Adenovirus or Lentivirus

1. Adenoviral commercial systems: AdEasy Adenoviral Vector System, RAPAd® Adenoviral Expression Systems, ViraPower™ Adenoviral Expression Systems (see Note 1).
2. Lentiviral commercial systems: ViraPower™ Lentiviral Expression Systems, OMICs Link, Lenti-X Lentiviral Expression System, ViraSafe™ Universal Lentiviral Expression System (see Note 1).

2.5. Reagents for Titering of Lentivirus

1. HT1080 fibrosarcoma cells.
2. Antibiotics for selection of transduced cells.
3. 1% Crystal violet solution in 10% ethanol.
4. Alternative approach: Commercial kit for lentivirus titering (e.g., UltraRapid Lentiviral Titering Kit).

2.6. Reagents for Titering of Adenovirus

1. HEK293 cells.
2. 4% Agarose solution.
3. Plaquing medium: DMEM, 2% FBS.
4. Alternative approach: Commercial kit for adenovirus titering (e.g., AdEasy Viral Titer Kit).

2.7. Infection of ASCs with Lentivirus

1. 10 μg/mL Polybrene (hexadimethrine bromide) stock (see Note 6).
2. ASC control medium.
3. Titered viral lysate or supernatant (see Subheading 2.5).

2.8. Infection of ASCs with Adenovirus

1. ASC control medium.
2. Titered viral lysate or supernatant (see Subheading 2.6).

2.9. Confirmation of Transgene Expression

1. Reagents for Western blotting or ELISA analysis.

3. Methods

3.1. Amplification and Titering of Recombinant Adenoviral Constructs Prior to ASC Transduction

Most adenoviral expression systems available today result in replication-deficient adenoviruses (i.e., through E1/E3 deletions). Therefore, amplification of these recombinant adenoviruses must be done using HEK293 cells. The HEK293 cell line is a stable cell line created through the transformation of human embryonal kidney cells (HEK) with sheared human adenoviral type 5 DNA (32, 33).

Specifically, the HEK293 cell line stably expresses the E1A adenoviral gene critical for viral replication. Infection of HEK293 cells in culture will be evident by the "lifting" of the cells off the plate surface, indicating that the cells are loaded with viral particles. Alternatively, continued culturing of these cells will result in their lysis (denoted as the gradual turning of the growth medium to yellow) and the release of the viral particles into the supernatant. For infection of ASCs in vitro, ASCs may be infected with the viral supernatants. Use of infected ASCs in vivo will require the amplification and purification of the viral particles.

1. Amplify the HEK293 cells in HEK293 growth medium.
2. Aspirate the medium and add DMEM/5% FBS, containing the desired recombinant adenovirus. Add enough medium to just cover the cells. For subsequent use in ASCs in vitro, crude viral lysates or viral supernatants can be used rather than purified viral particles.
3. Incubate the cells for 1 h at 37°C with gentle rocking every 10 min.
4. Add additional 293 growth medium and incubate the cells until all the cells have lifted off the plate (approximately 2 days). Collect the cells and supernatant. Store the supernatant at 4°C. Viral supernatants may be stored up to 4°C for 1 month without significant loss of activity. Pellet the cells and freeze/thaw to produce a crude viral lysate. Store the crude lysate at –80°C. Alternatively, cells may be cultured until the cells lyse (approximately 5 days). Collect the supernatant and store as above.
5. Titer the viral supernatant using a conventional HEK293 plaquing assay (34). Commercial kits for adenoviral titering may also be used (see Subheading 2.6).

3.2. Transduction of ASCs with Recombinant Adenovirus

1. On the day before transduction, harvest and count the ASC population (see Note 2). Into a 6-well tissue culture dish, plate 1×10^5 cells/well in ASC control medium and incubate overnight at 5% CO_2 and 37°C. The cells should be 70–80% confluent at the time of transduction.
2. On the day of transduction, dilute the titered viral stock in ASC control medium in order to prepare a series of viral supernatants in which the multiplicity of infection (MOI) ranges from 0.0 to 50.0. MOIs may be increased to as high as 200 to optimize posttransduction expression (see Note 3). To improve efficiency, keep the volume of the viral supernatant as low as possible. For a 6-well dish, 1.0 mL/well is sufficient. Once prepared, DO NOT vortex the viral supernatants.
3. Remove the culture media from the wells and gently mix the viral supernatant by pipetting up and down. Gently overlay

the cells in each well with 1.0 mL of the adenovirus supernatant at the desired MOI.

4. Incubate the cells with the viral supernatant at 37°C and 5% CO_2 overnight. For the first few hours, gently rock the plate every 10 min to ensure the plate is evenly covered. Another 1.0 mL of ASC control medium may be added after 3–4 h incubation if concerns about media volume during the overnight incubation arise.
5. Add an equal volume of normal growth medium. Culture the ASCs for 24–48 h before analysis. Expression typically will be evident after 48 h, but a 24-h posttransduction period may be sufficient depending on the gene.
6. Harvest the transduced cells and assay for the desired overexpressed protein using your method of choice (e.g., Western blot).

3.3. Amplification of Recombinant Lentiviral Constructs Prior to ASC Transduction

As with adenoviruses, today's lentiviral expression systems produce replication-deficient viruses. Therefore, amplification of recombinant lentiviruses is done in HEK293 cells. The required 293 cell line is a modified line that stably expresses the SV40 large T antigen-HEK293T cells (or 293 T cells). High-level and constitutive expression of the large T antigen, under the control of the CMV promoter, has been shown to maximize viral production in 293 cells (35). The 293 T cell line also contains the neomycin gene (under the SV40 enhancer/promoter) for antibiotic selection and the creation of stable cell lines. Unlike amplification of adenoviruses, amplification of lentivirus in 293 T cells *does not* produce any overt, cytotoxic effects such as nonadherence and cell lysis. However, the viral supernatants will contain the lentiviral particles. For transduction in vitro, transduction may be infected with viral supernatants. Use of transduction in vivo will require the amplification and purification of the viral particles prior to the infection of the ASC.

1. Amplify the 293 T cells in 293 T growth medium.
2. Aspirate the medium and add DMEM/10% FBS containing the desired recombinant lentiviral lysate. Since 293 T cells are very susceptible to lentiviral infection, an MOI range of five to ten particles per cell will be sufficient. Add enough medium to just cover the cells. For subsequent in vitro use in ASCs, crude viral lysates or viral supernatants can be used rather than purified viral particles.
3. Incubate the cells overnight at 37°C.
4. The next day, add additional 293 T growth medium and incubate the cells for an additional 48–72 h. Collect the supernatant and store at 4°C. Viral supernatants may be stored up to 4°C for 1 month.

5. Titer the viral supernatant using a suitable cell line or a commercial titering kit (see Subheading 2.5).

3.4. Transduction of ASCs with Recombinant Lentiviruses

Like recombinant adenoviruses, recombinant lentiviral constructs are capable of infecting both dividing and nondividing primary and cultured cell lines. Since the ASC is a dividing cell line, lower MOIs in the range of 1.0–3.0 viral particles per ASC are usually effective. Control lentiviral plasmids, such as those containing a lacZ or GFP gene, should be used initially to help to determine the transduction efficiency and MOI range for the ASC.

Because infection of cells with lentiviruses using commercial lentiviral expression systems results in stable integration of the lentiviral genome into the host (containing an antibiotic resistance gene), stable transduction cell lines can be created after infection by culturing the transduction in the presence of the appropriate antibiotic (e.g., puromycin, neomycin) (see Note 7 and 8). Antibiotic concentrations for ASC selection will need to be empirically determined.

1. On the day before transduction, harvest and count the ASC population. Into a 6-well tissue culture dish, plate 1×10^5 cells/well in complete growth medium and incubate overnight at 5% CO2 and 37°C. The cells should be 70–80% confluent at the time of transduction.
2. On the day of transduction, dilute the titered viral stock in ASC control medium in order to prepare a series of viral supernatants in which the MOI ranges from 0.0 to 10.0 (see Note 3 and 4). To improve efficiency, keep the volume of the viral supernatant as low as possible. For a 6-well dish, 1.0 mL/well is sufficient. Once prepared, DO NOT vortex the viral supernatants.
3. Remove the culture media from the wells and gently mix the viral supernatant by pipetting up and down. Gently overlay the cells in each well with 1.0 mL of the lentivirus supernatant at the desired MOI.
4. If desired, polybrene at a final concentration of up to 10.0 μg/mL can be added to improve infection efficiency. To maximize ASC transduction, 5.0 μg/mL polybrene is usually sufficient (see Note 6).
5. Incubate the cells overnight at 37°C and 5% CO_2. For the first few hours, gently rock the plate every 10 min to ensure the plate is evenly covered. Additional ASC control medium (another 1.0 mL) may be added prior to overnight incubation if concerns about medium volume and ASC viability arise.
6. Remove the transduction medium the next day and replace with normal control medium (see Note 5). Culture cells for an additional 24–48 h. Harvest the transduced cells and assay for the desired overexpressed protein using your method of choice (e.g., Western blot).

7. If desired, creation of stable ASC lines can be achieved via selection of transduced ASCs for antibiotic resistance (see Note 8). For this, continue culturing transduced ASC cultures with ASC control medium containing the appropriate antibiotic. To empirically determine antibiotic concentration for ASC selection, create a series of ASC control medium preparations containing a range of antibiotic. Culture *nontransduced ASCs* in these media preparations and determine the minimum concentration of antibiotic that results in over 90% ASC cell death within 7–10 days after addition of antibiotic.

4. Notes

1. *Commercial kits for recombinant viruses*: Today, most recombinant viruses (adenoviral and lentiviral) are produced using commercial viral expression systems (Subheading 2.4). Titering of these viruses can also be performed using commercial kits (see Subheadings 2.5 and 2.6). These systems are relatively easy to use and reliably result in the production of the desired recombinant virus ready to infect the desired mammalian cell. The resulting recombinant virus is replication deficient, making it capable of use in typical BSL2 laboratories. However, amplification of these replication-deficient viruses requires the infection of specific lines of HEK293 cells.
2. *Terminology*: The terms infection and transduction are frequently used in viral protocols. Infection applies to the situation where viral replication occurs and infectious progeny are created. Transduction applies to the situation where no viral replication occurs and no viral progeny are generated. For example, HEK293 cells are infected; ASCs are transduced.
3. *Multiplicity of Infection (MOI)*: In order to obtain optimal expression of the desired gene, the viral construct must be transduced into the ASC using a suitable MOI. MOI is defined as the ratio of infectious particles (i.e., number of viral particles) to infection target (i.e., the target cell being infected). For example, an MOI of 1 means 1 viral particle potentially infecting one target cell.

   In general, MOI correlates with the number of integration events of the viral genome into the ASC genome. Typically, integration increases linearly as the MOI increases. A number of factors can affect the optimal MOI, includinsg the transduction efficiency, the nature of the introduced gene, and the cell being transduced (i.e., dividing vs. nondividing). Both adenoviruses and lentiviruses are capable of infecting dividing and nondividing cells. In general, nondividing cells will

require higher MOIs. As such, a range of MOIs should be examined for each viral construct that is made. A good starting range of MOIs for both adenoviruses and lentiviruses is 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 viral particles per ASC. However, adenoviral transduction of ASCs may require higher MOIs to achieve efficient transgene expression. As such, the adenoviral MOI range may be increased to as high as 200. It is important to determine the optimal MOI for each recombinant virus as insufficient MOIs will not give adequate transduction rates in ASCs while MOIs at too high of a level will result in toxicity. Control viral shuttle plasmids, such as those containing a lacZ or GFP gene, may be used initially to help to determine the transduction efficiency and MOI range for the ASC.

The protocols outlined in this chapter describe the infection of a set number of ASCs in a 6-well dish. Smaller or larger tissue culture dishes with lower and higher densities of ASCs can be used. However, as the number of cells in the well changes, the number of viral particles in the viral supernatant must be adjusted so that the desired MOI is maintained. For example, to prepare 1.0 mL of viral supernatant such that 1×10^4 total ASCs will be infected at an MOI of 10, 1×10^5 viral particles/mL will be required. Therefore, the viral stock should be diluted such that 1×10^5 viral particles are used to make the 1.0 mL viral supernatant. To prepare 1.0 mL of viral supernatant such that 1×10^3 ASCs will be transduction at this same MOI, 1×10^4 viral particles should be added per milliliter.

4. *Presence of serum*: The presence of FBS (10%) or antibiotics (1%) does not appear to affect the efficiency of transduction with lentivirus or adenovirus. Therefore, infection can be performed in the presence of ASC control medium containing 10% FBS (see Subheadings 3.2 and 3.4). However, if concerned about the presence of serum, adenoviral and lentiviral transductions of ASCs can be performed in the presence of low serum (e.g., 0.5%) or serum-free DMEM without any deleterious effects on the cell. If serum-free or low-serum DMEM is used for the transduction, additional ASC control medium may be added after 4–6 h of transduction.
5. *Transduction time*: Determining overall transduction times for ASCs will require some trial and error. Transduction times as low as 1 and 4 h have been reported for adenovirus and lentivirus, respectively. However, the protocols given in this chapter suggest transduction times of overnight (see Subheadings 3.2 and 3.4). Overnight transduction times do not appear to affect the viability of the ASC and may optimize transgene expression.

6. *Use of polybrene*: The addition of polybrene (hexadimethrine bromide) may help improve the efficiency of lentiviral transduction of ASC. Polybrene is a cationic polymer that improves viral entry by neutralizing the charge repulsion between virions and sialic acid on the cell surface (36). Generally, no more than 5.0 μg/mL final concentration is required. However, concentrations of up to 10.0 μg/mL are well tolerated by the ASC (Subheading 3.4).
7. *Integration into the ASC genome*: The use of recombinant lentiviruses allows for a high level of recombinant protein expression in the host cell (i.e., the ASC); more so then what may be experienced with adenoviral-based systems (23, 35). Incorporation of the recombinant lentiviral genome into the host cell's genome (22) also allows for the stable, long-term expression of the desired recombinant protein. Therefore, cellular progeny will contain the recombinant gene. However, viral integration may result in genetic instability due to insertional mutation of the ASC genome or uncontrolled growth if inserted into key regions controlling cell division. High-level protein expression is also possible with adenovirus infection. However, because they do not integrate into most mammalian cells, recombinant adenoviruses will not cause insertional mutation of the ASC genome. However, eventual loss of the recombinant adenovirus will result as the number of viral particles in the ASC is "diluted out" with each successive round of mitosis.
8. *Creation of stable cell lines*: Commercial shuttle vectors for lentivirus creation contain antibiotic resistance genes that allow for the creation of stable cell lines after integration into the host genome. The appropriate antibiotic concentration will need to be empirically determined in ASCs (see Subheading 3.4).

References

1. Bett, A. J., Haddara, W., Prevec, L., and Graham, F. L. (1994) An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc Natl Acad Sci U S A* **91**, 8802–6.
2. Liu, H., Honmou, O., Harada, K., Nakamura, K., Houkin, K., Hamada, H., and Kocsis, J. D. (2006) Neuroprotection by PlGF gene-modified human mesenchymal stem cells after cerebral ischaemia. *Brain* **129**, 2734–45.
3. Wang, Y., Feng, C., Xue, J., Sun, A., Li, J., and Wu, J. (2009) Adenovirus-mediated hypoxia-inducible factor 1alpha double-mutant promotes differentiation of bone marrow stem cells to cardiomyocytes. *J Physiol Sci* **59**, 413–420.
4. Wang, Y., Sun, A., Xue, J., Feng, C., Li, J., and Wu, J. (2009) Bone marrow derived stromal cells modified by adenovirus-mediated HIF-1alpha double mutant protect cardiac myocytes against CoCl2-induced apoptosis. *Toxicol In Vitro* **23**, 1069–75.
5. Lin, H., Shabbir, A., Molnar, M., Yang, J., Marion, S., Canty, J. M., Jr., and Lee, T. (2008) Adenoviral expression of vascular endothelial growth factor splice variants differentially regulate bone marrow-derived mesenchymal stem cells. *J Cell Physiol* **216**, 458–68.

6. Gao, F., He, T., Wang, H., Yu, S., Yi, D., Liu, W., and Cai, Z. (2007) A promising strategy for the treatment of ischemic heart disease: Mesenchymal stem cell-mediated vascular endothelial growth factor gene transfer in rats. *Can J Cardiol* **23**, 891–8.
7. Hong, X., Miller, C., Savant-Bhonsale, S., and Kalkanis, S. N. (2009) Antitumor treatment using interleukin-12-secreting marrow stromal cells in an invasive glioma model. *Neurosurgery* **64**, 1139–46; discussion 46–7.
8. Kanehira, M., Xin, H., Hoshino, K., Maemondo, M., Mizuguchi, H., Hayakawa, T., Matsumoto, K., Nakamura, T., Nukiwa, T., and Saijo, Y. (2007) Targeted delivery of NK4 to multiple lung tumors by bone marrow-derived mesenchymal stem cells. *Cancer Gene Ther* **14**, 894–903.
9. Morizono, K., De Ugarte, D. A., Zhu, M., Zuk, P. A., Elbarbary, A., Ashjian, P. H., Benhaim, P., Chen, I. S., Hedrick, M. H. (2003) Multilineage cells from adipose tissue as gene delivery vehicles. *Hum Gene Ther* **14**, 59–66.
10. Dragoo, J. L., Choi, J. Y., Lieberman, J. R., Huang, J., Zuk, P. A., Zhang, J., Hedrick, M. H., and Benhaim, P. (2003) Bone induction by BMP-2 transduced stem cells derived from human fat. *J Orthop Res* **21**, 622–9.
11. Dragoo, J. L., Lieberman, J. R., Lee, R. S., Deugarte, D. A., Lee, Y., Zuk, P. A., Hedrick, M. H., and Benhaim, P. (2005) Tissue-engineered bone from BMP-2-transduced stem cells derived from human fat. *Plast Reconstr Surg* **115**, 1665–73.
12. Peterson, B., Zhang, J., Iglesias, R., Kabo, M., Hedrick, M., Benhaim, P., and Lieberman, J. R. (2005) Healing of critically sized femoral defects, using genetically modified mesenchymal stem cells from human adipose tissue. *Tissue Eng* **11**, 120–9.
13. Lin, L., Shen, Q., Wei, X., Hou, Y., Xue, T., Fu, X., Duan, X., and Yu, C. (2009) Comparison of osteogenic potentials of BMP4 transduced stem cells from autologous bone marrow and fat tissue in a rabbit model of calvarial defects. *Calcif Tissue Int* **85**, 55–65.
14. Zhang, X., Yang, M., Lin, L., Chen, P., Ma, K. T., Zhou, C. Y., and Ao, Y. F. (2006) Runx2 overexpression enhances osteoblastic differentiation and mineralization in adipose – derived stem cells in vitro and in vivo. *Calcif Tissue Int* **79**, 169–78.
15. Yang, M., Ma, Q. J., Dang, G. T., Ma, K., Chen, P., and Zhou, C. Y. (2005) In vitro and in vivo induction of bone formation based on ex vivo gene therapy using rat adipose-derived adult stem cells expressing BMP-7. *Cytotherapy* **7**, 273–81.
16. Feng, G., Wan, Y., Balian, G., Laurencin, C. T., and Li, X. (2008) Adenovirus-mediated expression of growth and differentiation factor-5 promotes chondrogenesis of adipose stem cells. *Growth Factors* **26**, 132–42.
17. Jin, X., Sun, Y., Zhang, K., Wang, J., Shi, T., Ju, X., and Lou, S. (2007) Ectopic neocartilage formation from predifferentiated human adipose derived stem cells induced by adenoviral-mediated transfer of hTGF beta2. *Biomaterials* **28**, 2994–3003.
18. Lamfers, M., Idema, S., van Milligen, F., Schouten, T., van der Valk, P., Vandertop, P., Dirven, C., and Noske, D. (2009) Homing properties of adipose-derived stem cells to intracerebral glioma and the effects of adenovirus infection. *Cancer Lett* **274**, 78–87.
19. Kang, S. K., Lee, D. H., Bae, Y. C., Kim, H. K., Baik, S. Y., and Jung, J. S. (2003) Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. *Exp Neurol* **183**, 355–66.
20. Rogers, P. M., Mashtalir, N., Rathod, M. A., Dubuisson, O., Wang, Z., Dasuri, K., Babin, S., Gupta, A., Markward, N., Cefalu, W. T., and Dhurandhar, N. V. (2008) Metabolically favorable remodeling of human adipose tissue by human adenovirus type 36. *Diabetes* **57**, 2321–31.
21. Pasarica, M., Mashtalir, N., McAllister, E. J., Kilroy, G. E., Koska, J., Permana, P., de Courten, B., Yu, M., Ravussin, E., Gimble, J. M., and Dhurandhar, N. V. (2008) Adipogenic human adenovirus Ad-36 induces commitment, differentiation, and lipid accumulation in human adipose-derived stem cells. *Stem Cells* **26**, 969–78.
22. Buchschacher, G. L., Jr., and Wong-Staal, F. (2000) Development of lentiviral vectors for gene therapy for human diseases. *Blood* **95**, 2499–504.
23. Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., and Naldini, L. (1998) A third-generation lentivirus vector with a conditional packaging system. *J Virol* **72**, 8463–71.
24. Goncalves, M. A., de Vries, A. A., Holkers, M., van de Watering, M. J., van der Velde, I., van Nierop, G. P., Valerio, D., and Knaan-Shanzer, S. (2006) Human mesenchymal stem cells ectopically expressing full-length dystrophin can complement Duchenne muscular dystrophy myotubes by cell fusion. *Hum Mol Genet* **15**, 213–21.
25. Goudenege, S., Pisani, D. F., Wdziekonski, B., Di Santo, J. P., Bagnis, C., Dani, C., and Dechesne, C. A. (2009) Enhancement of

myogenic and muscle repair capacities of human adipose-derived stem cells with forced expression of MyoD. *Mol Ther* **17**, 1064–72.

26. Zhu, X. Y., Zhang, X. Z., Xu, L., Zhong, X. Y., Ding, Q., and Chen, Y. X. (2009) Transplantation of adipose-derived stem cells overexpressing hHGF into cardiac tissue. *Biochem Biophys Res Commun* **379**, 1084–90.
27. Xu, J., Qu, J., Cao, L., Sai, Y., Chen, C., He, L., and Yu, L. (2008) Mesenchymal stem cell-based angiopoietin-1 gene therapy for acute lung injury induced by lipopolysaccharide in mice. *J Pathol* **214**, 472–81.
28. Rabin, N., Kyriakou, C., Coulton, L., Gallagher, O. M., Buckle, C., Benjamin, R., Singh, N., Glassford, J., Otsuki, T., Nathwani, A. C., Croucher, P. I., and Yong, K. L. (2007) A new xenograft model of myeloma bone disease demonstrating the efficacy of human mesenchymal stem cells expressing osteoprotegerin by lentiviral gene transfer. *Leukemia* **21**, 2181–91.
29. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J., and Conklin, D. S. (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* **16**, 948–58.
30. Yu, J. Y., DeRuiter, S. L., and Turner, D. L. (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci U S A* **99**, 6047–52.
31. Lee, H. S., Cho, H. H., Kim, H. K., Bae, Y. C., Baik, H. S., and Jung, J. S. (2007) Tbx3, a transcriptional factor, involves in proliferation and osteogenic differentiation of human adipose stromal cells. *Mol Cell Biochem* **296**, 129–36.
32. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36**, 59–74.
33. Harrison, T., Graham, F., and Williams, J. (1977) Host-range mutants of adenovirus type 5 defective for growth in HeLa cells. *Virology* **77**, 319–29.
34. Tollefson, A. E., Kuppuswamy, M., Shashkova, E. V., Doronin, K., and Wold, W. S. (2007) Preparation and titration of CsCl-banded adenovirus stocks. *Methods Mol Med* **130**, 223–35.
35. Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A* **93**, 11382–8.
36. Davis, H. E., Rosinski, M., Morgan, J. R., and Yarmush, M. L. (2004) Charged polymers modulate retrovirus transduction via membrane charge neutralization and virus aggregation. *Biophys J* **86**, 1234–42.

Chapter 26

Use of Adipose-Derived Stem Cells in High-Throughput Screening to Identify Modulators of Lipogenesis

Y. Reneé Lea-Currie, David J. Duffin, and Benjamin M. Buehrer

Abstract

Drug discovery efforts have an increasing focus on functional cell-based screening to identify compounds that modulate targets presented in a relevant format. Historically, immortalized cell lines have been used in primary and secondary screens due to their ease of manipulation, transformation, and propagation. However, more researchers are using primary cells that present their drug targets in their natural context. Human primary cell isolation and propagation procedures have become efficient enough to provide these cells in the necessary scale for early stage drug discovery. Adult human stem cells provide an opportunity for investigating multiple pathways of differentiation, development, regeneration, and toxicity using a single cell source and type. Adipose-derived stem cells (ASCs) are an attractive adult human primary stem cell for drug discovery due their abundance in adipose tissue, ease of isolation, and propagation in culture. They can be expanded in high numbers and retain their unique properties to differentiate into multiple lineages. In this chapter, we describe a protocol to identify modulators of human ASC lipogenesis following partial differentiation to adipocytes.

Key words: Adipocyte, High-throughput screening, Drug discovery, Cell-based assay, ASC

1. Introduction

High-throughput screening is the mainstay of drug discovery efforts in large pharmaceutical companies, biotechnology companies, and academic laboratories. Often these screens are focused on well-defined biochemical targets using in vitro assays that can be miniaturized to allow rapid testing of hundreds of thousands of compounds. After confirming the primary active compound, significant chemical optimization may be performed to generate a compound series to test in relevant disease models, including cell-based systems. It is at this point that many series fail due to cytotoxicity, cell impermeability, or other cell-related issues. This

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failure can result in a large amount of wasted time and effort if the compound series are unable to be utilized. Problems such as these have led researchers to use cell-based assays earlier in the drug discovery process, including primary screens.

Cell-based activity assays can provide a robust screening system that presents the drug target in a more relevant context (1, 2). Many of these approaches employ engineered immortalized cell lines that (over)express the target of interest or carry a reporter construct that is easily monitored (3). These cell-based assay systems are not only specifically constructed to enhance screening parameters such as signal:noise and signal:background, but also have the added benefit of the low cost of these immortalized cell lines. Nevertheless, these systems do not necessarily represent the normal biological context of the target. Often, cell lines are used that do not normally express the target to lower the background signal; this enhances screening capabilities but may present the target without necessary cofactors, interacting proteins, or signaling components. One way to overcome these drawbacks is to use primary cells containing the target of interest.

Recent advances in primary cell isolation, propagation, and characterization have made high-quality human primary cells readily available for drug discovery (4). Human hepatocytes have been used in drug development and toxicology work for decades, however, more cells, including stem cells, are now widely available for use in the discovery process. Human primary cell-based assays present their own set of limitations and drawbacks that must be identified before use as a screening tool (5, 6). However, the overwhelming potential of stem cells to treat diseases, or be used in tissue engineering and regeneration has increased both the use of primary cells, in general, and stem cells, specifically, to identify modulators of differentiation and self-renewal (7–11).

Human adipose-derived stem cells (ASCs) are an adult stem cell of choice for drug discovery applications due to their relative abundance and ease of isolation for large-scale screening studies. ASCs can be readily expanded to produce screening quantities of cells and are amenable to high-throughput screening in 96-, 384-, and 1,536-well plates. They can be formatted to identify modulators of differentiation, propagation, toxicity, and induced pluripotency (12). ASCs can also be isolated from donors of varying demographics or disease state to identify compounds active in a specific end-point disease model, such as type 2 diabetes or obesity. We have successfully formatted ASCs differentiated to adipocytes in 96- and 384-well plates for many functional assays, including triglyceride accumulation, lipolysis, glucose uptake, NF-κB induction, and 11β-HSD1 activity.

In this chapter, we focus on identifying modulators of lipogenesis in ASCs that have been partially induced to adipocytes as an example of how ASCs can be used as a drug discovery

platform. We also describe some of the parameter testing necessary to establish a human primary cell-based screen.

2. Materials

2.1. Cell Culture

1. Preadipocyte Medium (PM-1): Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (1:1 v/v) containing HEPES (pH 7.4), fetal bovine serum, and antibiotics (Zen-Bio, Inc., RTP, NC).
2. Adipocyte Maintenance Medium (AM-1, Zen-Bio): Preadipocyte medium containing reduced serum, biotin, pantothenate, insulin, and dexamethasone.
3. Adipocyte differentiation medium (DM-2, Zen-Bio): Adipocyte medium with IBMX and PPAR gamma agonist.
4. ASCs (ASC-F-SL, Zen-Bio) are isolated from human subcutaneous adipose tissue as previously described (13, 14) and cryopreserved for plating (see Note 1).
5. Tumor necrosis factor (TNF)-alpha and DMSO.
6. 384-Well clear tissue culture-treated plates.

2.2. Differentiation and Cytotoxicity Assays

1. Phosphate-buffered saline (PBS; Zen-Bio).
2. Automation including Cell Washer and PlateMate 2×3 for higher-throughput cell washing and dispensing compound treatments.
3. Triglyceride assay kit (Zen-Bio): Containing Wash Buffer, Lysis buffer, Reagent B (lipase), glycerol Reagent A, 1 mM glycerol standard, and standards diluent.
4. Plate reader capable of measuring absorbance at 540, 570, and 600 nm in 384-well plates, such as a Synergy 2.
5. Cell Titer Blue Cell Viability Reagent.

3. Methods

3.1. Plating ASCs in 384-Well Format

1. Human cryopreserved preadipocytes are thawed rapidly in a 37 °C water bath and transferred to a sterile conical bottom centrifuge tube containing PM-1. The cells are collected by centrifugation at 1,200 rpm (282×*g*)/20 °C/5 min. The medium is aspirated and the pelleted cells suspended in an appropriate volume of PM-1 to give about 1×10^6 cells/ml. Cell count and viability should be verified using a hemocytometer or other cell-counting device.

2. Preadipocytes are plated at a density of 6×10^3 cells per well in 50 µl PM-1 using 384-well plates and placed in a 37 °C humidified 5% CO_2 incubator overnight (see Note 2).
3. Remove all PM-1 and replace with 50 µl of 40% DM-2 (40 DM-2:60 PM-1) for all treatment wells. On each plate, positive control wells contain 50 µl of 100% DM-2; vehicle control wells contain 50 µl of 40% DM-2. One plate contains the remaining controls: uninduced control wells contain 50 µl PM-1; and negative control wells contain 50 µl of 40% DM-2. Plates are placed back into the 37°C humidified 5% CO_2 incubator for 7 days without further media exchange.

3.2. Treatment of ASCs with Compound

1. Thaw frozen compound stocks (in DMSO) already formatted in 96-well plates. Compound stocks should be prepared such that final dilution results in a DMSO concentration equal to or less than 0.25% (see Note 3).
2. Remove 30 µl DM-2 (or PM-1) from each well of the 384-well plates. Using an automated liquid handler (e.g., PlateMate 2x3), dilute the compounds in AM-1 to give a 267-fold dilution (see Note 4).
3. Add 40 µl of AM-1 containing diluted compounds to cells in a 384-well plate in quadruplicate using an automated liquid handler. This results in a final 400-fold dilution of the treatment compounds. On each plate, the positive and vehicle controls contain 40 µl of AM-1 + DMSO concentration present in diluted compounds. Forty microliters of PM-1 + the same DMSO concentration as above is added to the uninduced control wells. The negative control wells are treated with 40 µl of AM-1 + 10 ng/ml TNFα. The plates are placed back into the 37°C humidified 5% CO_2 incubator for 7 days without further media exchange.

3.3. Assessment of Lipid Accumulation

1. Using an automated cell washer, gently wash cells one time with PBS (see Note 5).
2. Prepare the Reagent B by adding an appropriate amount of room temperature deionized water to the reagent powder and gently invert. Use a pipette to ensure that the powder is completely dissolved. Keep at room temperature and store in a light-protected bottle.
3. Using an automated liquid handler, add 5 µl Lysis Buffer to each well and then place plates into a 37°C incubator for 20 min. Add 45 µl Wash Buffer to each well using the PlateMate and tap plates to mix Wash Buffer and Lysis Buffer.
4. Add 6.7 µl of Reagent B (lipase) to each well and incubate plates at 37°C for 2 h (see Note 6).

5. Prepare the Reagent A by adding the appropriate volume of room temperature deionized water per bottle and gently invert. Use a pipette to ensure that the powder is completely dissolved. Keep at room temperature.
6. Transfer 12.5 µl of each lipase-treated lysate to a new clear-bottom 384-well plate containing 12.5 µl Wash Buffer (two-fold dilution). At this time, prepare a glycerol standard curve ranging from 3 to 200 µM by twofold stepwise dilution of a 1 mM glycerol stock. Transfer 25 µl of the standards to a clear-bottom 384-well plate and add 25 µl of Glycerol Reagent A to all wells (samples, controls, and standards). Incubate at room temperature for 15 min and read absorbance at 540 nm.
7. The amount of total triglyceride is determined based on comparison of sample values with the standard curve. There is a 1:1 molar ratio between the amount of glycerol detected and cellular triglyceride content.

3.4. Assessment of Cytotoxicity

1. Prepare a duplicate set of treated cell plates using the protocols outlined in Subheadings 3.1 and 3.2. Include wells containing only medium without cells for background subtraction. On the day of the cytotoxicity assay, remove 25 µl of conditioned media from each well so that the remaining well volume is 25 µl.
2. Add 5 µl of Cell Titer Blue reagent and tap plates to mix. Place plates in the 37°C humidified 5% CO_2 incubator for 2 h.
3. Remove plates from the incubator and using a plate reader read absorbance at 570 nm with a reference wavelength of 600 nm. The reference absorbance value is subtracted from the 570 nm value for each well. Subtract the 570 – 600 nm value for the blank (no cell) wells from each value to determine the overall absorbance value. Low values reflect cytotoxicity.

3.5. Data Analysis: Triglyceride Assay

1. Generate a standard curve using the absorbance values from the glycerol standards by subtracting the absorbance value from the blank well from the remaining standards.
2. A linear curve fit can be determined from the resulting absorbance values, from which the concentration of liberated glycerol for each sample can be determined. Be sure to account for any dilution of the sample, such as the twofold dilution in Subheading 3.3, step 6 above.
3. The concentration of total triglyceride per sample is equal to that of glycerol. Values greater than the 200 µM standard may reflect interference with the assay reagents.

4. Average the values for each quadruplicate and calculate the standard deviation. Calculate the coefficients of variation (CVs) for each quadruplicate by: 100 × standard deviation ÷ average. CVs greater than 25% may indicate a failure in the assay or simply be reflective of low average values (inhibition).

3.6. Assay Development and Validation

The selected assay must be sensitive to weak modulators to be useful as a primary cell-based screen. In our lipogenesis assay, 40% DM-2 was selected for the screening assay based on dilution studies from 0 to 100% DM-2 medium. We determined that 40% DM-2 medium gave a robust response that could be modulated by inhibitors and activators of lipogenesis. Figure 1 shows an example of the range of activity expected in the assay.

Compounds are often dissolved in 100% DMSO so it is necessary to determine the maximal tolerated dose of DMSO to identify the highest feasible concentration for screening. An example of such a test is shown in Fig. 2. Increasing concentrations of DMSO in AM-1 decreased the total amount of triglyceride accumulated during the maturation of differentiated ASCs. Based on these results and our intention of testing the highest possible concentration of test compounds, we selected a 400-fold dilution, which would generate a final concentration of 0.25% DMSO.

After determining the conditions for both the differentiation and treatment of ASCs, the assay was validated using control treatments. TNFα effectively inhibits ASC adipocyte differentiation when incubated during the differentiation phase. It also inhibits the increase in lipogenesis that occurs during the maturation phase, in which we are testing the extracts. An example of TNFα inhibition of triglyceride accumulation is shown in Fig. 1. As shown in Fig. 1, 100% DM-2 increases triglyceride accumulation

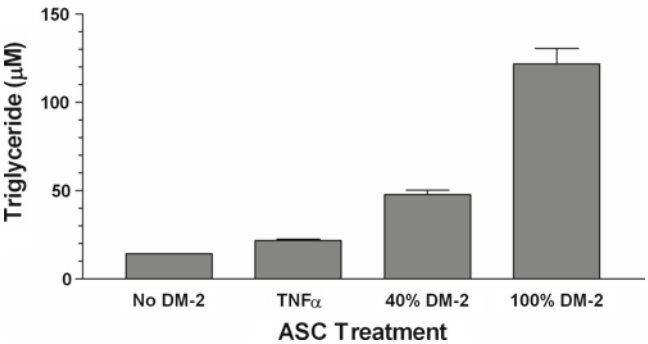

Fig. 1. Triglyceride accumulation in response to lipogenic modulators. ASCs were treated with no DM-2, 40% DM-2, or 100% DM-2 during differentiation. ASCs differentiated with 40% DM-2 were treated with 10 ng/ml TNFα in AM-1. Triglyceride increased with 100% DM-2 and decreased with TNFα treatment. Values are reported as average micromolar triglyceride and standard deviation of the mean.

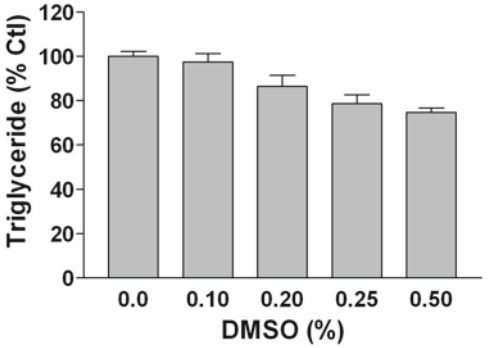

Fig. 2. DMSO effect on triglyceride accumulation. ASCs were differentiated with 40% DM-2 and treated with increasing concentrations of DMSO in AM-1. Triglyceride accumulation was determined after maturation.

significantly over 40% DM-2. Z′ was calculated according to Zhang et al. (15) comparing 40% DM-2 with both TNFα and 100% DM-2 responses and determined to be 0.61 and 0.55, respectively. These values ($Z' > 0.4$) suggest that the assay is robust enough to identify both inhibitors and activators of triglyceride accumulation. However, primary cell-based assays have considerable plate-to-plate and day-to-day variation, sometimes requiring more lenient exclusion criteria (5).

3.7. Hit Identification

Primary human whole cell screens generally have higher CVs than other cell-based and in vitro screening assays. The increased variance points to the critical need to include appropriate controls on each plate to determine the cutoff values for follow-up analysis. Our results from testing 290 samples in this assay is shown in Fig. 3, where the vehicle control values are set to 100% and the cutoff values representing three standard deviations from the vehicle control are shown as dotted lines. Most of the active samples significantly decreased the level of accumulated triglyceride, whereas a single sample increased triglyceride accumulation. Decreases in cellular function are commonly due to cytotoxicity instead of a specific mode of action by the test samples, so it is necessary to test all primary actives for cytotoxic activity.

Once the true actives are confirmed as not cytotoxic, then further analysis testing is required. Typically, dose response analyses of the active compounds, both in triglyceride accumulation and cytotoxicity are performed to determine if there is a significant difference between the two activities. Fig. 4 shows two similar samples that display a separation of cytotoxicity and inhibition of triglyceride accumulation. Subsequent analysis should be performed with either resupplied or newly synthesized material to verify the nature of the active sample.

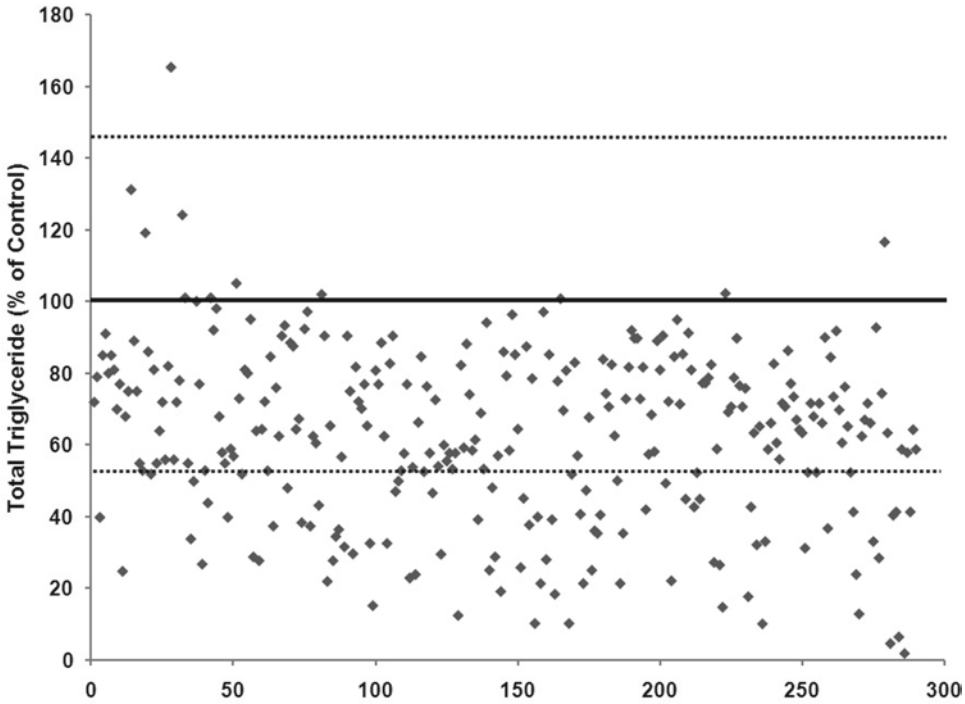

Fig. 3. Example of primary screening data using 290 test samples. ASCs were differentiated with 40% DM-2 and treated with 0.25% test samples in AM-1. Total triglyceride was determined and plotted as percent of the 40% DM-2 vehicle control. *Dashed lines* indicate three standard deviations from the control and provide a cutoff for potential actives.

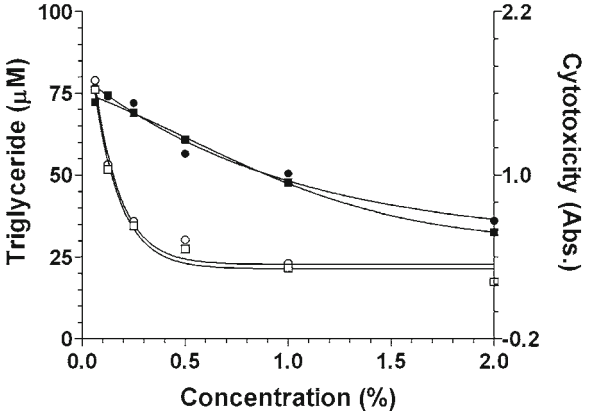

Fig. 4. Dose response of triglyceride and cytotoxicity for two samples. ASCs were differentiated with 40% DM-2 and treated with increasing concentrations of test samples in AM-1. Total triglyceride was determined after maturation (*open symbols*). Cytotoxicity was determined on duplicate samples (*solid symbols*).

4. Notes

1. To remove some of the donor to donor variability associated with human primary cells, we prepared a multidonor lot of ASCs consisting of five female donors with similar ages and

body mass index (BMI). Individual variation can lead to misidentification or masking of active compounds during a drug screen; the use of a multidonor lot produces a response averaged over the five unique donors.

2. Cell number was optimized based on the triglyceride signal generated compared with the total cell number required for the screen. We chose 6×10^3 cells/well because it gave a robust signal while not using too many cells for the entire screen.
3. We format the plates to have compounds within the inner 60 wells of a 96-well plate, because we do not assay cells seeded in the outer wells due to edge effects and differential evaporation.
4. Our dilution was carried out in two steps using deep-well blocks: 10 µl compounds into 490 µl AM-1 (1:50); 60 µl of dilution into 258 µl of AM-1 (1:265, final).
5. Set up the cell washer for incomplete aspiration, leaving a small amount of liquid in the wells. Add 60 µl of PBS and aspirate.
6. Two hours is sufficient time for the breakdown of triglyceride to glycerol and fatty acids. Longer incubations can be used, but are not required. At this point, the plates may be stored at –80°C for use at a later point, however, care must be taken to ensure the integrity of the plates.

References

1. Gasparri, F., Sola, F., Bandiera, T., Moll, J., and Galvani, A. (2008) High-content analysis of kinase activity in cells. *Comb Chem High Throughput Screen* **11**, 523–36.
2. Minor, L. K. (2008) Label-free cell-based functional assays. *Comb Chem High Throughput Screen* **11**, 573–80.
3. Wunder, F., Kalthof, B., Muller, T., and Huser, J. (2008) Functional cell-based assays in microliter volumes for ultra-high throughput screening. *Comb Chem High Throughput Screen* **11**, 495–504.
4. Goldbard, S. (2006) Bringing primary cells to mainstream drug development and drug testing *Curr Opin Drug Discov Devel* **9**, 110–6.
5. Dunne, A., Jowett, M., and Rees, S. (2009) Use of primary human cells in high-throughput screens *Meth Mol Biol* **565**, 239–57.
6. Maddox, C. B., Rasmussen, L., and White, E. L. (2008) Adapting cell-based assays to the high throughput screening platform: problems encountered and lessons learned. *JALA Charlottesv Va* **13**, 168–73.
7. Crook, J. M. and Kobayashi, N. R. (2008) Human stem cells for modeling neurological disorders: accelerating the drug discovery pipeline. *J Cell Biochem* **105**, 1361–6.
8. Eglen, R. M., Gilchrist, A., and Reisine, T. (2008) An overview of drug screening using primary and embryonic stem cells. *Comb Chem High Throughput Screen* **11**, 566–72.
9. Fang, Y. Q., Wong, W. Q., Yap, Y. W., and Orner, B. P. (2007) Stem cells and combinatorial science. *Comb Chem High Throughput Screen* **10**, 635–51.
10. Nirmalanandhan, V. S. and Sittampalam, G. S. (2009) Stem cells in drug discovery, tissue engineering, and regenerative medicine: emerging opportunities and challenges. *J Biomol Screen* **14**, 755–68.
11. Zhang, X. Z., Li, X. J., Ji, H. F., and Zhang, H. Y. (2009) Impact of drug discovery on stem cell biology. *Biochem Biophys Res Commun* **383**, 275–9.
12. Sun, N., Panetta, N. J., Gupta, D. M., Wilson, K. D., Lee, A., Jia, F., Hu, S., Cherry, A. M.,

Robbins, R. C., Longaker, M. T., and Wu, J. C. (2009) Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proc Natl Acad Sci U S A* **106**, 15720–15725.

13. Halvorsen, Y. D., Bond, A., Sen, A., Franklin, D. M., Lea-Currie, Y. R., Sujkowski, D., Ellis, P. N., Wilkison, W. O., and Gimble, J. M. (2001) Thiazolidinediones and glucocorticoids synergistically induce differentiation of human adipose tissue stromal cells: biochemical, cellular, and molecular analysis. *Metabolism* **50**, 407–13.
14. Aust, L., Devlin, B., Foster, S. J., Halvorsen, Y. D., Hicok, K., du Laney, T., Sen, A., Willingmyre, G. D., and Gimble, J. M. (2004) Yield of human adipose-derived adult stem cells from liposuction aspirates. *Cytotherapy* **6**, 7–14.
15. Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* **4**, 67–73.

Chapter 27

Preservation Protocols for Human Adipose Tissue-Derived Adult Stem Cells

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Abstract

The development of simple but effective storage protocols for adult stem cells will greatly enhance their use and utility in tissue-engineering applications. There are three primary storage techniques, freezing (cryopreservation), drying (anhydrobiosis), and freeze drying (lyophilization), each with its own advantages and disadvantages. Cryopreservation has shown the most promise but is a fairly complex process, necessitating the use of chemicals called cryoprotective agents (CPAs), freezing equipment, and obviously, storage in liquid nitrogen. Preservation by desiccation is an alternative that attempts to reproduce a naturally occurring preservative technique, namely, the phenomenon of *anhydrobiosis* and requires the use of high (and possibly, toxic) concentration of CPAs as well as disaccharides (sugars). Lyophilization works by first cryopreserving (freezing) the material and then desiccating (drying) it by the process of sublimation or the conversion of ice (solid) to water vapor (gas phase). The purpose of this chapter is to present a general overview of these storage techniques and the optimal protocols/results obtained in our laboratory for long-term storage of adult stem cells using freezing storage and drying storage.

Key words: Cryopreservation, Anhydrobiosis, Desiccation, Flow cytometry, Histochemical analysis, Adipogenesis, Osteogenesis, Apoptosis

1. Introduction

Multiple studies report that primary preadipocytes derived from stromal vascular fraction (SVF) of human adipose tissue, although able to differentiate into adipocytes under appropriate conditions, decrease their ability to differentiate after multiple passages; this coincides with a prolonged doubling time and replicative senescence (1–11). These variable results, indicating the genotype and phenotype may not remain stable during growth and passage of adipose tissue-derived adult stem cells (ASCs), suggest that passaged cells for higher generations may not be suitable for regenerative medical

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therapies. Moreover, serial passaging exposes cell cultures to the repeated risk of contamination by environmental microorganisms. These shortcomings associated with in vitro stem cell expansion can be partly alleviated by proper long-term storage (suspended animation) of SVF of human adipose tissue or passage 1 (P1) ASCs. The three primary options of long-term storage of cells, macromolecules, proteins, and other biosystems, as shown in Fig. 1, are:

1. *Freezing*: Starting at Point A (room temperature [RT]), a reduction/lowering in temperature will lead to phase change/freezing (formation of ice, line demarcating liquid and solid phase), and subsequent lowering of temperature below the glass transition temperature (T_g) will lead to glass formation (line demarcating solid and amorphous (glass) phase) and low-temperature stable storage (Point B).
2. *Desiccation*: Starting at Point A, a progressive increase in solute concentration, by the process of drying or evaporating the liquid in the system, will also lead to glass formation and shelf-temperature stable storage (Point C).
3. *Freeze Drying*: Starting at Point A, the temperature is first reduced, leading to the formation of ice, and then the solute concentration is progressively increased by sublimation, leading to glass formation and refrigeration stable storage (Point D).

Note the differences between the three processes, freezing requires a substantial reduction in temperature (~150°C),

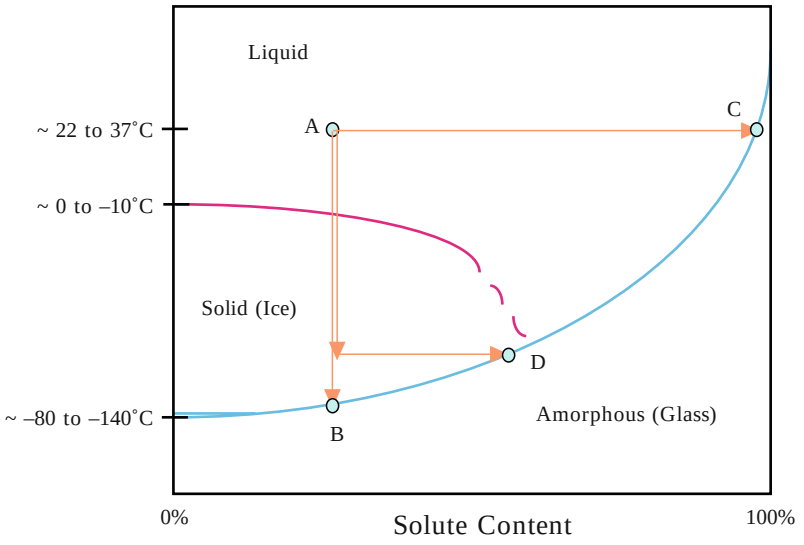

Fig. 1. Schematic representation of the processes relating to freezing (Point A–B), desiccation (Point A–C), and freeze drying (Point A–D) in a generic phase diagram. The transition from liquid to solid phase is delineated by the downward sloping line while the transition from either the liquid or the solid phase to the amorphous phase is shown as an upward sloping line. The temperature (°C) experienced by the cells is shown on the *y*-axis while the percentage of solute content is shown on the *x*-axis.

desiccation requires a substantial increase in solute concentration (~70%), while freeze drying requires both a moderate decrease in temperature (~60°C) and a moderate increase in solute concentration (~30%). Correspondingly, the damage mechanisms associated with the three processes are also different:

1. Freezing storage, typically, has (12–17):
   (a) Osmotic injury or toxic injury due to the addition of chemicals (called cryoprotective agents [CPAs]) that are needed to alleviate the freezing injury.
   (b) Chilling injury associated with the reduction of temperature from RT to the phase change (ice formation) temperature.
   (c) Cooling injury associated with the formation of extracellular and intracellular ice.
   (d) Thawing injury associated with the increase in temperature from liquid nitrogen storage to RT.
2. Desiccation storage, typically, has (18–25):
   (a) Solute effects injury or exposure to progressively increasing (and toxic) concentrations of solutes/salts.
   (b) Precipitation (or crystallization) injury and the associated damage to cellular membranes and intracellular components.
   (c) Long processing times, leading to hypoxia and cell death before the cells can be stabilized and properly stored.
   (d) Rehydration injury associated with the reconstitution (or addition) of solvents to the desiccated extracellular and intracellular matrix.
3. Freeze-drying storage, typically, has, as described above, a combination of the damage mechanisms associated with freezing storage and desiccation storage (22, 26–33):
   (a) Osmotic or toxic injury due to the addition of CPAs.
   (b) Chilling injury.
   (c) Cooling injury.
   (d) Precipitation (or crystallization) injury, although this is somewhat mitigated by the fact that this process occurs at subzero temperatures.
   (e) Rehydration injury (and the associated thawing injury).

And finally, the advantages/disadvantages associated with the three processes are also different:

1. Freezing storage, typically, has shown the most promise of post-storage cell recovery and is widely used, but requires the use of costly freezing and low-temperature storage equipment.

2. Desiccation storage is yet to be shown as being an efficacious technique for mammalian cells but has the advantage of ambient cell storage and ambient temperature processing.
3. Freeze-drying storage requires the use of costly and ill-developed equipment and is also not widely used due to the low recovery of cell survival poststorage.

Thus, this chapter will present the optimal protocols/results obtained in our experience for long-term storage of ASCs using freezing storage (34–38) and drying storage (39). A more biophysically based approach to the development of ASC cryopreservation protocols is presented elsewhere (40–42).

1.1. Freezing Storage of Adipose Tissue-Derived ASCs

The use of low temperatures to preserve (and store) biomaterials has long been known to humankind and, once frozen, these biomaterials (most commonly, fruits and vegetables) can endure storage at very low temperatures for a very long period of time. Obviously, the higher the subzero storage temperature, the shorter the time that these substances can be successfully stored. The primary reasons for the success of freezing storage are:

1. No thermally driven reactions occur in aqueous systems at the commonly used storage temperature of liquid nitrogen (approximately −160°C).
2. Liquid water does not exist below −130°C. The crystalline and glassy states that do exist have a very high viscosity (>10^3 poise) such that diffusion is insignificant over less then geological time spans (43).

Choice of CPAs: Starting with a serendipitous observation by Polge and colleagues in the 1940s, the use of chemicals, denoted as CPAs, in the freezing media has been shown to be a prerequisite for the successful outcome of a freezing storage process (44). Chemicals that exhibit and/or confer some form of cryoprotection range from low molecular weight permeating solutes like dimethylsulfoxide (DMSO), glycerol, ethylene glycol (EG), etc., to high molecular weight nonpermeating solutes like polyvinylpyrrolidone (PVP), hydroxyethylstarch (HES), methylcellulose (MC), etc. (45–48). The exact nature and the extent of cryoprotection offered by these chemicals to a specific cell type is still a matter of conjecture and is ill understood (49–52). But the explanations range from:

1. Enhanced colligative properties of solutions with CPAs (53–55).
2. Modifying the cell membrane transport properties (41, 56–62).

3. Preventing denaturing of membranes by elevated concentrations of extracellular salts at low temperatures (63–66).
4. Forming a protective coating on sensitive plasma membranes (67–70).
5. The prevention of seeding of the supercooled intracellular water and, thus, the formation of damaging intracellular ice (71–73).
6. The ability of the CPAs to alter the physical properties of solutions during freezing rate rather than in any direct effects on cellular membranes (74–81).

Thus, the choice and the composition of an optimal cryopreservation media is an area of intense research in the field of cryobiology and is not, as yet, completely understood. Our experiments with SVF and P1 ASCs were conducted with the media listed later in this chapter (36–38).

1.2. Desiccation Storage of ASCs (39)

An optimized desiccation procedure gives significant advantages over standard cryopreservation protocols because the process of desiccation is simpler, quicker, and typically less toxic protectants are needed; additionally, storage conditions are less stringent and the logistics of transportation are greatly simplified. As a first step for developing desiccation (drying storage) procedures for ASCs, we have measured the postrehydration membrane integrity (PRMI) of two passages, passage 0 (P0) and passage 1 (P1), of human ASCs (39).

Process of Drying: The damage to biological systems during drying is primarily due to the changes in the physical state of membrane lipids and changes in the structure of sensitive proteins (82–86).

1. In nature, a variety of organisms, including artic frog, salamanders, tardigrades and nematodes bacteria, yeast, fungi, and rotifers have been shown to survive extreme dehydration and dry conditions due to the presence of sugars (87–89).
2. Studies on the drying preservation of both prokaryotes and eukaryotes have revealed that nonreducing sugars, such as trehalose, sucrose, and maltose, protect liposomes from the adverse effect of drying (82–92).
3. Recent studies also suggest that mammalian cells, including human primary foreskin fibroblasts, 3T3 murine fibroblasts, human mesenchymal stem cells (hMSCs), corneal epithelial cells, and mouse spermatozoa, can tolerate a drying process in the presence of either intracellular or extracellular trehalose (92–102).

2. Materials

2.1. Freezing Experiments

Where appropriate, materials should be sterile and all manipulations should be performed using a laminar flow bench (fume hood). Adequate safety equipment (gloves, face shield) will be used during all liquid nitrogen-handling procedures.

1. Sterile plastic dishes, pipets, and micropipets.
2. –80°C Freezer.
3. 37°C CO_2 incubator.
4. Cell digestion/isolation, culture, and differentiation media (see text below for further details).
5. Diluent: Dulbecco's Modified Eagle Medium (DMEM), DMEM-F12, fetal calf serum (FCS), and human serum (HS).
6. Cryoprotectants (CPAs): DMSO (see Note 6), MC, PVP. All cryoprotectant solutions should be prepared using DMEM supplemented with either FCS or HS.
7. Cryovials, such as 2-ml plastic cryotubes.
8. Commerically available and programmable controlled-rate freezer (CRF) able to cool at rates of 1–40°C/min to –80°C; see Note 2.
9. Directional solidification stage (DSS), capable of controlled cooling rates ranging from 1–40°C/min to –80°C.
10. Type-T hypodermic thermocouples and precision temperature data logger.
11. Liquid nitrogen storage dewars (long-term storage containers).
12. 37°C Warm water bath.
13. Glass microslides with central grooves.
14. Warm copper block maintained at 50°C.
15. Fluorescence-Activated Cell Sorting (FACS) machine and associated chemicals (Etoposide, hydrogen peroxide, Annexin V–FITC/PI kit, propidium iodide [PI]). Cell Quest software to acquire the FACS data.

2.2. Desiccation Experiments

Where appropriate, materials should be sterile and all manipulations performed using a laminar flow bench (fume hood).

1. Convective drying chamber.
2. Pressure regulator.
3. Glass microslides with central grooves.
4. Chemicals: D-PBS, HBS, stromal media, trehalose, and glycerol.

5. 37°C CO_2 incubator.
6. Vacuum/suction device and plastic bags.
7. Cell viability kits (SYBR-14 and PI).
8. Light microscope with FITC and Texas Red filter cubes.

3. Methods

3.1. Collection and Isolation of ASCs

The process of ASC isolation (and culture/expansion/differentiation) from human liposuction aspirate (adipose tissue) is described elsewhere (103–112) and is only briefly described below:

1. Obtain liposuction aspirate from a plastic surgeon.
2. Wash tissue and collagenase digest at 37°C with continuous shaking for 1 h.
3. Centrifuge to obtain the SVF pellet; see Note 12.
4. Suspend SVF in culture medium containing 10% FBS at equivalent of 1 ml of tissue/ml of media – see Note 3.
5. Seed flasks at a density of 0.156 ml tissue digest/cm^2 surface area as P0. Incubate at 37°C, 5% CO_2.
6. Wash cultures to remove nonadherent cells after 24 h of incubation.
7. Feed cultures every 2–3 days.
8. Harvest P0 by trypsin/EDTA digestion when it reaches 80–90% confluency (mean, 5.5 days).
9. Seed P1 and subsequent passages of undifferentiated ASCs at a density of 5×10^3 cells/cm^2 and maintain according to steps 6 and 7.
10. Harvest P1 by trypsin/EDTA digestion when it reaches 80–90% confluency (mean, 5.5 days) – see Note 1.

3.2. Preparation of Freezing Media

PVP and MC are individually autoclaved at 121°C for 30 min before being added to DMEM; see Note 4. The DMEM-PVP solutions and DMEM-MC solutions are prepared by dissolving weighted PVP and MC in DMEM at RT and the solutions are then stored overnight at 4°C to obtain a homogeneous preparation. We find concentrations above 1% MC and 40% PVP to be highly viscous and hard to handle and hence, do not use them (36–38).

Freezing Media for SVF of Adipose Tissue (those with * are sub-optimal). The measured post-freeze–thaw viability is shown for each media. The detailed freeze–thaw procedures are described in Subheading 3.3. Additional (and corresponding) data on post-thaw apoptotic and necrotic cells is presented elsewhere (37).

1. DMEM with 80% FCS and 10% DMSO: 65.3 ± 3.3%
2. DMEM with 80% HS and 10% DMSO: 61.9 ± 2.4%
3. *DMEM with 0% DMSO: 11.4 ± 7.4%
4. DMEM with 2% DMSO: 60.1 ± 4.4%
5. DMEM with 4% DMSO: 62.8 ± 4.5%
6. DMEM with 6% DMSO: 65.8 ± 2.4%
7. DMEM with 8% DMSO: 72.9 ± 3.3%
8. DMEM with 10% DMSO: 69.8 ± 2.5%
9. *DMEM with 1% MC: 37.3 ± 4.1%
10. *DMEM with 1% MC and 10% FCS: 22.2 ± 8.3%
11. *DMEM with 1% MC and 10% HS: 20.0 ± 6.8%
12. DMEM with 1% MC and 10% DMSO: 66.0 ± 2.1%
13. DMEM with 10% PVP: 54.6 ± 1.7%
14. DMEM with 10% PVP and 10% FCS: 52.3 ± 5.7%
15. DMEM with 10% PVP and 40% FCS: 49.7 ± 6.7%
16. DMEM with 10% PVP and 80% FCS: 58.6 ± 3.2%
17. DMEM with 10% PVP and 10% HS: 53.9 ± 5.5%

Freezing Media for P1 ASCs (those with * are sub-optimal). The measured post-freeze–thaw viability is shown for each media. Additional (and corresponding) data on post-thaw apoptotic and necrotic cells is presented elsewhere (36, 38).

1. DMEM with 80% FCS and 10% DMSO: 84.1 ± 7.7%
2. DMEM with 80% HS and 10% DMSO: 82.5 ± 8.3%
3. *DMEM with 0% DMSO: 25.3 ± 5.7%
4. DMEM with 2% DMSO: 83.8 ± 5.3%
5. DMEM with 4% DMSO: 80.6 ± 3.1%
6. DMEM with 6% DMSO: 86.0 ± 4.6%
7. DMEM with 8% DMSO: 85.6 ± 2.8%
8. DMEM with 10% DMSO: 87.9 ± 2.9%
9. *DMEM with 1% MC: 46.8 ± 7.6%
9. *DMEM with 1% MC and 10% FCS: 41.5 ± 7.6%
10. *DMEM with 1% MC and 10% HS: 39.8 ± 7.6%
11. DMEM with 1% MC and 10% DMSO: 79.8 ± 2.8%
12. *DMEM with 1% PVP: 5.7 ± 0.5%
13. DMEM with 5% PVP: 49.3 ± 8.0%
14. DMEM with 10% PVP: 69.7 ± 8.2%
15. DMEM with 20% PVP: 55.5 ± 11.0%
16. *DMEM with 40% PVP: 4.6 ± 1.3%

17. DMEM with 5% PVP and 10% HS: 53.2 ± 8.7%
18. DMEM with 10% PVP and 10% HS: 72.1 ± 8.1%
19. DMEM with 20% PVP and 10% HS: 61.8 ± 12.5%
20. DMEM with 5% PVP and 10% FCS: 55.4 ± 11.0%
21. DMEM with 10% PVP and 10% FCS: 69.2 ± 6.7%
22. DMEM with 20% PVP and 10% FCS: 64.3 ± 6.4%
23. DMEM with 10% PVP and 40% FCS: 70.5 ± 3.5%
24. DMEM with 10% PVP and 80% FCS: 72.5 ± 2.5%

3.3. Process of Adding CPAs and Cooling to Liquid Nitrogen Temperatures and Thawing to RT

1. Suspend the SVF or P1 ASCs at a concentration of 1.0×10^6 cells/ml in the various cryopreservation medium, described above; see Note 5.
2. Place the cells in an ethanol-jacketed closed container overnight at –80°C.
3. The temperature/time history experienced by the cells in the ethanol-jacketed container can be measured using type-T hypodermic needle thermocouples. Thermocouple voltages can be read by a precision temperature data logger and transferred to a personal computer for further reduction and data analysis. Representative cooling rates experienced by the cells in the ethanol-jacketed container placed in a –80°C freezer are shown in Fig. 2; see Note 14.
4. Note that the exact magnitude of the cooling rates experienced by the cells in the ethanol-jacketed container is a function of the cryopreservation media.
5. Store the cells in liquid nitrogen vapor and/or immersed in liquid nitrogen; see Notes 7–9, and 13.
6. Rapidly thaw individual cryovials of cells in a 37°C water bath (1–2 min of agitation); see Note 10.
7. Resuspend the cells in stromal culture media, and seed into the separate wells of a 6-well plate for a 24-h incubation period at 37°C; see Note 11.

3.4. Evaluation of Frozen/Thawed Cells

The post-thaw viability and functionality of the cells was analyzed using flow cytometry (113) and histochemical staining for adipogenic and osteogenic differentiability (35, 105, 106, 108–110).

Cell Viability and Apoptosis/Necrosis Assessment

1. A well-established Annexin V apoptosis assay was analyzed by quantitative flow cytometry.
2. The chemically induced apoptotic control was cells incubated in fresh medium enriched with 40 mM Etoposide for 24 h. Apoptosis is characterized by phosphatidylserine (PS) translocation from the inner leaflet to the outer leaflet of the lipid

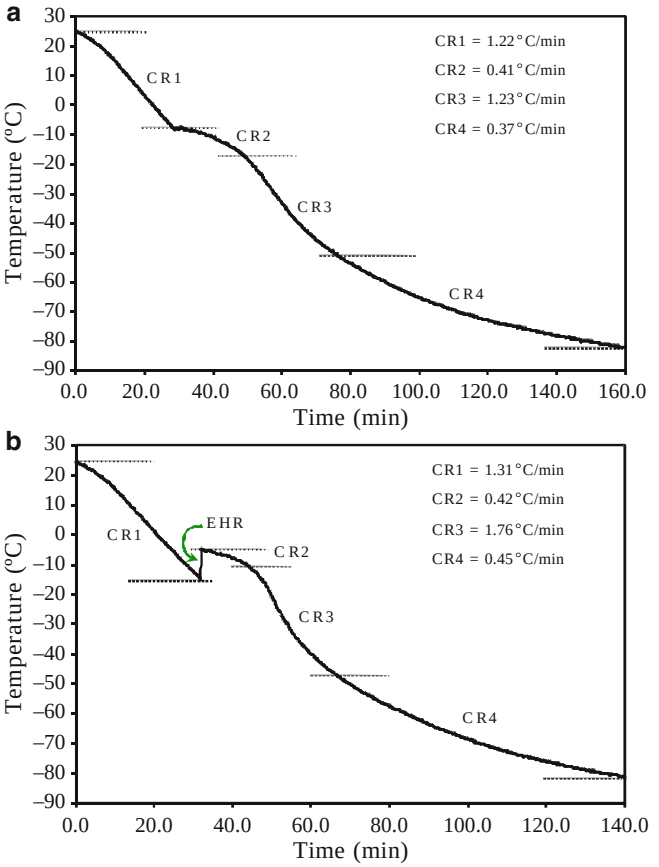

Fig. 2. Representative cooling rates experienced by the P1 ASCs in the ethanol-jacketed closed container placed in the −80°C freezer with 80% FCS + 10% DMSO in DMEM (**a**) and 10% PVP in DMEM (**b**). The measurements were measured using a type-T thermocouple coupled with a precision temperature data logger. The plot suggests that the cells are subjected to different cooling rates at different time points in different freezing media within the ethanol-jacketed container. For media with 80% FCS + 10% DMSO in DMEM, the ice nucleation was observed around −8 (±1.1)°C and subsequently, a cooling rate of ~0.4°C/min was imposed to a temperature of approximately −18°C. The cooling rates experienced by the cells then further drops to ~1.2°C/min until approximately −50°C and then to ~0.4°C/min, before reaching approximately −80°C (**a**). Alternatively, for media with 10% PVP in DMEM, there was significant super cooling before any ice nucleation was observed around −17 (±2.3)°C. Due to the enthalpic heat release (EHR), the sample temperature was abruptly raised to −5°C and a subsequent cooling rate of ~0.4°C/min was imposed to a temperature of −10°C. The cooling rates experienced by the cells then further drops to 1.8°C/min until approximately −50°C and then to ~0.4°C/min, before reaching approximately −80°C (**b**).

bilayer, while the cell membrane remains intact. Annexin V-positive cells correspond to cells that have experienced PS translocation.

3. The necrotic control was cells incubated in fresh medium with 5 mM hydrogen peroxide (H_2O_2) for 24 h. PI staining

of the cells indicates that the integrity of the cell membrane has been compromised and is used to distinguish living and early apoptotic cells from necrotic cells.

4. The no-treatment control was cells in fresh medium, free from inducing agents.
5. For each treatment, detached and attached cells were pooled, harvested by trypsinization (0.25% trypsin), washed with 10 ml of culture medium, and resuspended in 100 µl of 1× Annexin-binding buffer (included in Annexin V–FITC/PI kit).
6. Approximately 100 µl of the cell suspension was mixed with 8 µl of Annexin V–FITC and 8 µl of 100 µg/ml PI and incubated in the dark at RT for 15 min.
7. Liquid volume was removed by centrifugation and aspiration, and the cells were resuspended by gentle vortexing in 300 µl of 1× Annexin-binding buffer and subsequently analyzed on the flow cytometer.
8. Apoptotic analyses for ASCs were performed on a FACS Caliber flow cytometer utilizing 488-nm laser excitation and fluorescence emission at 530 nm (FL1) and >575 nm (FL3).
9. Forward and side scatter measurements were made using linear amplification, and all fluorescence measurements were made with logarithmic amplification. A total of 2×10^4 cells per sample were acquired using Cell Quest software.
10. Quadrant analysis was performed on the fluorescence dotplot to quantify the percentage of live, necrotic, and apoptotic cell populations (36–38, 113). The fluorescent dotplots show three cell populations: live (Annexin V–FITC negative and PI negative [Annexin V^- and PI^-]) necrotic (Annexin V–FITC positive and PI positive [Annexin V^+ and PI^+]), and apoptotic (Annexin V–FITC positive and PI negative [Annexin V^+ and PI^-]). The quadrants positions were placed according to the no-treatment control and 5 mM H_2O_2 necrotic control (Fig. 3).

Adipogenesis

11. Confluent cultures of ASCs were induced to adipogenesis by replacing the medium with an adipocyte induction cocktail containing DMEM/F-12 Ham's with 3% FBS, 33 µM biotin, 17 µM pantothenate, 1 µM bovine insulin, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), 5 µM rosiglitazone, and 100 U penicillin + 100 µg streptomycin + 0.25 µg fungizone.
12. After 72 h, the adipocyte induction medium was replaced with adipocyte maintenance media, which contains the same components as the induction medium except IBMX and rosiglitazone.

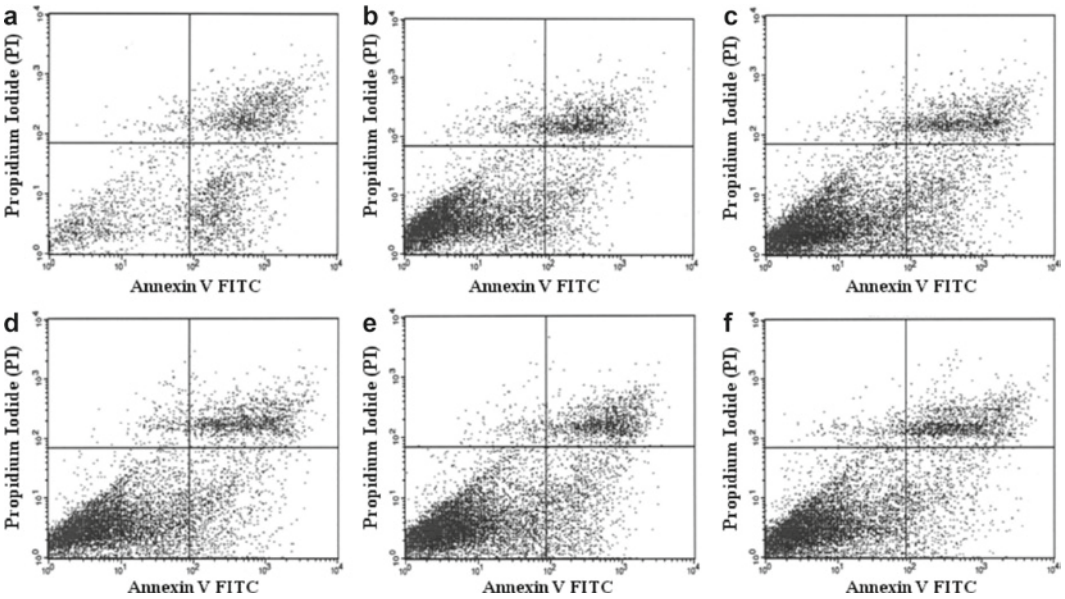

Fig. 3. Characteristic flow cytometer fluorescence dotplots showing FACS analysis of SVF cells frozen/thawed in the presence various concentrations (0, 2, 4, 6, 8, or 10%) of DMSO in DMEM. Corresponding plots for other media and P1 ASCs are available in the literature (36, 38). (**a–f**) represent 0, 2, 4, 6, 8, or 10% DMSO in DMEM, respectively. The fluorescent dotplots show three cell populations: live (Annexin V–FITC negative and PI negative [Annexin V^- and PI^-]), necrotic (Annexin V–FITC positive and PI positive [Annexin V^+ and PI^+]), and apoptotic (Annexin V–FITC positive and PI negative [Annexin V^+ and PI^-]). The quadrants positions were placed according to the no-treatment control, 40 μm Etoposide apoptotic control, and 5 mM H_2O_2 necrotic control. Reprinted with permission from (37).

13. Induced cells were maintained in culture for 9 days, with adipocyte maintenance medium replacement every 3 days. Upon the ninth day, the cultures were washed twice with prewarmed PBS and fixed in formalin at 4°C. Adipocyte quantification was determined by staining neutral lipids with Oil Red O (Fig. 4).

Osteogenesis

14. Confluent cultures of ASCs were induced to osteogenesis by replacing the medium with an osteogenic induction cocktail containing DMEM/F-12 Ham's, 10% FBS, 10 mM β-glycerophosphate, 50 μg/ml sodium ascorbate 2-phosphate, and 100 U penicillin + 100 μg streptomycin + 0.25 μg fungizone.
15. The induced cells were fed fresh osteogenic induction media every 3 days for 3 weeks. The cultures were then washed with 0.9% sodium chloride solution and fixed in 70% ethanol.
16. Osteoblast quantification was determined by Alizarin red staining for calcium phosphate (Fig. 4).

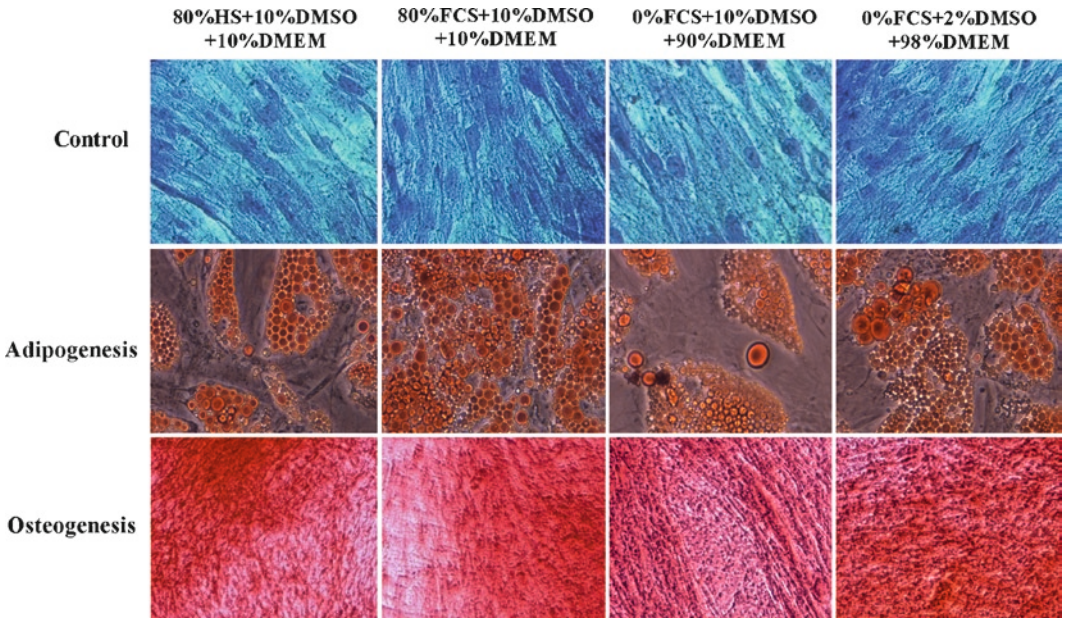

Fig. 4. Representative phase contrast photomicrographs of P1 ASCs cultured under untreated (*first row*; Toluidine blue staining), adipogenic (*second row*; Oil Red O staining), or osteogenic (*third row*; alizarin red staining) conditions. Corresponding plots for other media and SVF cells are available in the literature (37, 38). Adipogenic cultures were stained with Oil Red O 14 days after induction while osteogenic cultures were stained with alizarin red after 21 days of culture. Images in column 1 represent cells that were cryopreserved in media containing 80% FCS with 10% DMSO in DMEM. Images in column 2 represent cells that were cryopreserved in media containing 80% HS with 10% DMSO in DMEM. Images in column 3 represent cells that were cryopreserved in media containing 0% FCS with 10% DMSO in DMEM. And finally, images in column 4 represent cells that were cryopreserved in media containing 0% FCS with 2% DMSO in DMEM. Reprinted from (36).

3.5. Some Observations on the Effect of Various Cryoprotectants on the Post-freeze–Thaw Response of SVF of Adipose Tissue

1. The data suggests that the choice of the serum (HS or FCS) does not significantly alter the SVF cell survival when frozen/thawed in 10% DMSO and DMEM.
2. The highest percentage of post-thaw survival was achieved with a concentration of 8% DMSO (~73%), the post-thaw survival values obtained with DMSO concentrations of 2, 4, 6, and 10% are comparable and are ~60%, ~63%, ~66%, and ~70%, respectively. The only significant differences in the data were found when the cells were frozen with DMSO and without DMSO, i.e., even the presence of 2% DMSO was significant and this extremely low concentration was able to cryoprotect SVF cells.
3. Replacing DMSO with MC as the CPA lead to a significant reduction (~50%) in the post-thaw cell viability of SVF cells. Intriguingly, the addition of either HS or FCS with MC is extremely deleterious to post-thaw cell viability.
4. DMSO is more effective in preserving cell viability than MC, i.e., the maximum percentage of cell survival obtained with

MC (~37%) is still only half of that obtained with media containing various concentrations of DMSO (~60–70%).

5. The presence or the absence of FCS did not significantly alter the post-thaw viability results for SVF cells frozen in the presence of 10% PVP.
6. The percentage of post-thaw cell viability (~55%) obtained with 10% PVP and DMEM is comparable to that obtained (~64%) with control media (10% DMEM with 10% DMSO and either 80% FCS or 80% HS) and also comparable with the values obtained with DMSO and DMEM (~65%).

3.6. Some Observations on the Effect of Various Cryoprotectants on the Post-freeze–Thaw Response of P1 ASCs

1. The viability of ASCs cryopreserved in the control media containing 80% serum with 10% DMSO in DMEM was ~83%.
2. The highest percentage of post-thaw cell survival was found for cells frozen in DMEM with 2–10% DMSO (~80–88%) and the lower values were found for cells frozen in DMEM with either 40% PVP or 1% PVP (~5%), DMEM with 0% DMSO (~25%), and in DMEM with 1% MC and either 10% HS or 10% FCS (~40%).
3. Freezing P1 ASCs in the absence of DMSO was detrimental to cell survival.
4. The addition of 1% MC significantly lowers the cell viability, i.e., the post-thaw cell viability with 1% MC in DMEM (~47%) is significantly lower than that obtained with DMEM containing 1% MC and 10% DMSO (~80%).
5. The use of 1% and 40% PVP in DMEM caused a dramatic loss in cell viability (~5%) whereas the use of 10% PVP produced a maximum viability of ~65%. Although the viability of ASCs decreased when the percentage of PVP was increased from 10% to 20%, the data analysis revealed that this increase is statistically not significant (>95% confidence level).
6. The ASCs frozen/thawed in the presence of PVP (and in the absence of both DMSO and serum) displayed similar morphology and growth/differentiation characteristics when compared with the ASCs cryopreserved in 80% serum with 10% DMSO in DMEM (Fig. 4).

An important (and unique) feature of our ASC freezing storage protocols is the development of a cryopreservation media without serum (and without DMSO) that simplifies their use and application in in vivo tissue-engineering applications.

3.7. Effect of Freezing Device on the Post-freeze–Thaw Response of ASCs

1. The effect of directional cooling on the immediate post-thaw membrane integrity of ASCs was investigated using a directional solidification stage (DSS) and a commercially available controlled-rate freezer (CRF) – Fig. 5.

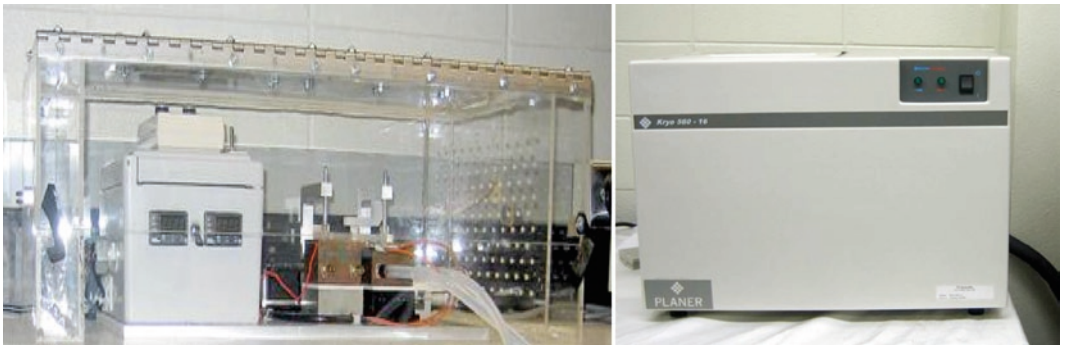

Fig. 5. Photographs of the custom-built directional solidification stage (DSS) on the *left-hand side* and the commercially available controlled-rate freezer (CRF) on the *right-hand side* (source: "Bioengineering Laboratory, Department of Mechanical Engineering, Louisiana State University, Baton Rouge, LA"). As described in the text, the choice of the freezing device has a significant impact on the post-thaw viability of ASCs.

2. P0, P1, and P2 ASCs were cooled at 1°C/min, 5°C/min, 20°C/min, or 40°C/min to an end temperature of −80°C in the presence and absence of a CPA (DMSO). For experiments in the presence of DMSO, the cell suspensions were gently mixed with 10% DMSO as a cryoprotective solution (CPA), i.e., a one-step addition of CPA. The samples were equilibrated in the cryoprotective solution for ~10 min before the DSS and CRF experiments were performed.
3. After freezing to −80°C, the samples were thawed at a rate of 200°C/min and the ability of the frozen/thawed ASCs to exclude fluorescent dyes was assessed.

DSS Experiments

4. The DSS built by Rubinsky and Ikeda (114) consisted of hot and cold temperature copper bases (set at T_h and T_c, respectively) separated by a distance (D_{gap}).
5. Freezing was achieved by placing the sample on a glass slide and moving the glass slide from the hot base to the cold base at a known velocity (V). The cooling rate (B) experienced by the sample is then described as: $B = \frac{T_h - T_c}{D_{gap}} \bullet V$.
6. For the DSS freezing experiments, ~100 µl of ASC suspension was placed in a grooved microslide and traversed between the precisely controlled copper blocks at a constant velocity. Note that the first copper block was held at +4°C and the second at −80°C. The samples were then cooled at a nominal cooling rates of 1°C/min, 5°C/min, 20°C/min, or 40°C/min to an end temperature of −80°C. Due to interactions

with the ambient atmosphere the actual cooling rates experienced by the cells between 0 and –40°C were ~1.1°C/min, ~5.2°C/min, ~10.6°C/min, ~21.7°C/min, and ~43.6°C/min, respectively (34).

7. All the glass microslides had a circular milled well, perpendicular to the direction of microslide travel, within which the cell suspension was placed. The well was 3.2-mm wide (in the direction of travel) and of 1-mm depth (into the microslide surface).
8. The thawing rate of ~200°C/min was achieved by quickly bringing the bottom of the glass slide into contact with a warm copper block maintained at 50°C.

CRF Experiments

9. A programmable CRF machine.
10. The samples were loaded into a 6-well cell culture cluster.
11. The cluster was then loaded into the cryo-machine (which was precooled to +4°C) and kept for 1 min for equilibration. The samples were then cooled at either 1, 5, 20 or 40°C/min to an end temperature of –80°C.
12. The thawing rate 200°C/min was obtained by removing the 6-well cluster from the CRF machine and quickly bringing the bottom of the cluster into contact with a warm water bath maintained at 37°C (42).

3.8. Some Observations on the Effect of Freezing Device on the Post-freeze–Thaw Response of ASCs

1. The viability of cell suspensions was measured using calcein AM and PI (115).
2. A 10-µl sample of cell suspension was removed from the microslide and incubated with 2 µmol/L calcein AM and 3 µmol/L PI for 15–30 min at 37°C.
3. After incubation, cells were scored as either live or dead under a fluorescent microscope.
4. Viability measurements of cells taken directly from the stock suspension without freezing were determined before and after every freeze–thaw experiment as a control; all experimental viability values were normalized to the average control viability.
5. A comparison of the P1 ASCs post-freeze viability in the presence and absence of DMSO obtained using the DSS and the CRF is shown in Fig. 6. Corresponding data for P0 and P2 cells are available elsewhere (34).
6. The post-freeze viability of ASCs is significantly higher (confidence level >95%) when they are frozen in the presence of DMSO than in its absence.

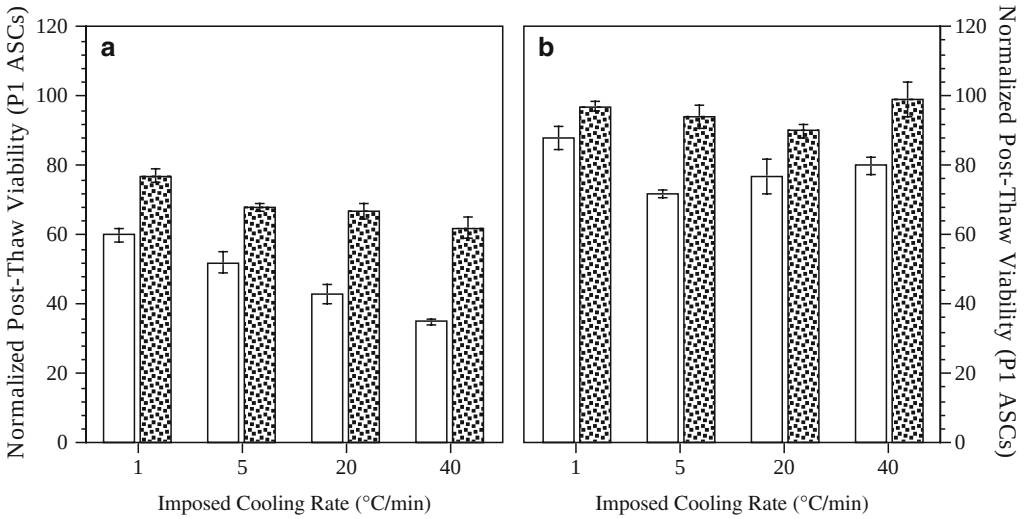

Fig. 6. Comparison of post-thaw viability of P1 ASCs frozen in the absence (**a**) and presence (**b**) of 10% (v/v) DMSO is shown. In each figure, the post-thaw viability obtained using the DSS (*unfilled columns*) and CRF (*filled columns*) is also shown. The error bars represent standard deviation in the data ($n = 18$). In both the figures, the immediate post-thaw ASC membrane integrity is shown on the *y*-axis while the cooling rate imposed on the sample (°C/min) is shown on the *x*-axis. Redrawn from (34).

7. A cooling rate of 1°C/min results in the highest post-thaw cell survival for ASCs frozen using either device.
8. The post-freeze viability of ASCs is uniformly higher (confidence level >95%) when they are frozen using the CRF as opposed to using the DSS. This significant reduction in cell viability for the cells frozen using the DSS might be related to previously postulated mechanical/damaging interactions between the ice crystals and the cell membranes (116–119).

3.9. Convective Drying, Storage, and Rehydration Process

1. For all drying experiments, the ASC concentration was adjusted to be 1×10^6 cells/ml.
2. Before drying, 1 ml stock solution of containing 100 mM trehalose and 768 mM glycerol was added to 1 ml of ASC suspension. Thus, the final concentration of trehalose and glycerol in the drying media was 50 mM and 384 mM, respectively.
3. Prior to conducting the convective drying experiments described below, the ASCs were incubated at 37°C, 5% CO_2, and 100% relative humidity (RH) for 30 min.
4. A convective drying stage similar to the one described earlier (102) was used in the present study to achieve controlled drying of P0 and P1 ASCs; see Note 15.

5. The drying chamber was designed to hold three glass microslides, with each grooved microslide capable of holding a 10-μl ASC droplet.
6. To ensure different drying rates, the flow of nitrogen gas was controlled by adjusting the exhaust pressure using a pressure regulator. The different drying rates are denoted as slow (with regulator pressure of 275 kPa), moderate (205 kPa), and rapid (140 kPa).
7. After drying ASCs on the stage for 30 min, the ASCs were placed in a 6-well plate:
   (a) Stored at ambient temperature.
   (b) In plastic bags at ambient temperature.
   (c) In a vacuum sealed bag at ambient temperature; see Note 16.
8. After storing the ASC samples for 48 h, they were rehydrated with 1 ml of D-PBS and 1 ml of stromal media. The mixture of the cell suspension and the media were allowed to equilibrate briefly ~5 min, and then placed in an incubator at 37°C, 5% CO_2, and 100% RH; see Note 17.
9. After 48 h, the cells were removed and their membrane integrity was assessed using fluorescent nucleic acid dyes (SYBR-14 and PI).
10. SYBR-14 (live cell stain) and PI (dead cell stain) were prepared fresh, daily in Hepes-buffered saline (HBS) and used at final concentrations of 100 nM and 600 nM, respectively (120).
11. Between 300 and 500 cells/sample were scored in each assay using a light microscope at 200× magnification and FITC and Texas Red filter cubes.
12. The average cell survival, based on the ability of the rehydrated cell to exclude dyes, was defined as the ratio of the live cells to the total cells in the field of view.

3.10. Some Observations on the Effect of Drying Rates and Storage Conditions on the PRMI of ASCs

1. For P0 ASCs the optimal drying and storage conditions are slow drying and vacuum storage, respectively, and ~37% of rehydrated ASCs are able to exclude dyes when slow dried and stored in vacuum.
2. For P0 ASCs dried either at a moderate or rapid drying rate, the percentage of PRMI is independent of the storage condition.
3. For P1 ASCs at a given storage condition, the percentage of PRMI is independent of the drying rate, with the highest values (~12–14%) being obtained for vacuum-stored samples (Fig. 7).

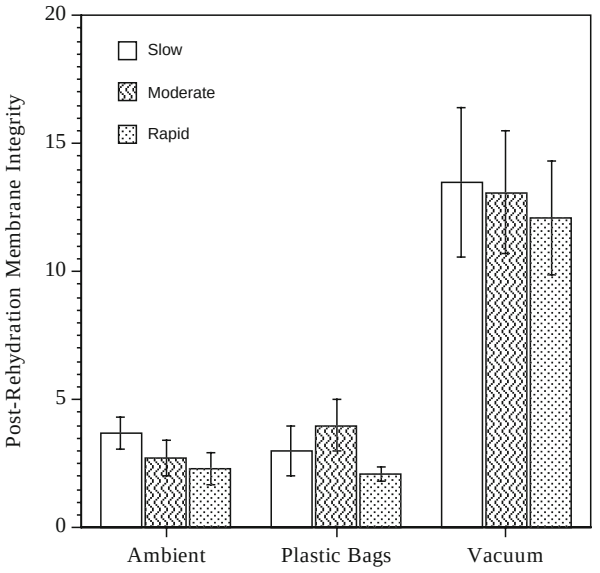

Fig. 7. A comparison of the PRMI for P1 ASCs. Corresponding data for P0 ASCs is available in Mittal and Devireddy (39). The three storage conditions: (1) at ambient temperature, (2) in plastic bags at ambient temperature, and (3) in vacuum-sealed plastic bags at ambient temperature, are represented on the *y*-axis while the PRMI is shown on the *x*-axis. For each storage condition, the PRMI is shown for the three drying rates studied: slow, moderate, and rapid. The errors bars represent standard deviation in the data ($n = 6$). Redrawn from (39).

4. For P1 ASCs, the percentage of PRMI for samples stored in either ambient environment or in plastic bags (~2–4%) are significantly smaller than vacuum-stored samples (~12–14%).
5. The maximum percentage of PRMI obtained is significantly smaller for P1 ASCs (~14%) when compared with P0 ASCs (~37%).

4. Notes

Notes on Freezing Experiments

1. The outcome of any cryopreservation procedure will depend on the quality of the prefrozen sample. Therefore, cells harvested for cryopreservation should be at their optimum viability to ensure maximal survival during freezing and after thawing.
2. Commercially available programmable CRFs are costly ($20,000). Cheaper alternatives might be a –80°C freezer.

3. Tissue culture media are highly species specific. Cryoprotectants and growth media components must be of high purity (analar-grade and spectroscopic grade for DMSO).
4. Where possible, cryoprotectant mixtures should be sterilized by filtration or for high-viscosity solutions by autoclaving.
5. The concentration, duration, and temperature of the addition of the CPAs is a critical factor in the success of the whole procedure. Care should be taken to minimize the time of exposure to CPAs. Once the cells have been prepared for freezing, they should be frozen as soon as possible.
6. DMSO freely penetrates the skin and can be irritating to the eyes and skin.
7. Storage in the vapor phase of the liquid nitrogen is recommended. However, storage in the liquid phase does prevent the possibility of inadvertent warming.
8. Extreme caution should be exercised while removing the samples from the liquid phase as the penetration of liquid nitrogen into defective containers can cause excessive pressure (and possibly, explosive) buildup of gas in a confined space during the thawing process. To reduce this possibility, cryovials should be allowed to equilibrate in the vapor phase before being transferred to a warm water bath for thawing. It might also be advisable to place the cryovials in a "closed metal container" (to contain any exploded material for a simpler decontamination procedure).
9. The storage temperature needs to be maintained below –139°C.
10. To retain maximum viability, cells should be thawed rapidly and uniformly, but carefully, so that the maximum temperature does not exceed the normal temperature range.
11. After thawing it is necessary to remove or slowly dilute the cryoprotectant to prevent osmotic shock. DMSO will evaporate from the medium at 37°C.
12. Minimal g forces need to be applied during centrifugation.
13. Storage time does not influence the viability of the stored material. Therefore, the whole duration of low-temperature storage can be as little as 1 h, greatly reducing the length of the whole procedure.
14. Physical factors influencing the success of a cryopreservation procedure include the imposed cooling rate, the composition of the freezing media, the time of exposure to the cryoprotectants, the nature and sequence of the cryoprotectant addition and removal procedures, the storage temperature, and the thawing process. All of these variables need to be carefully controlled during the freezing process to ensure an optimal outcome.

15. The drying process needs to be carefully controlled as well as the final moisture content in the desiccated sample. Unfortunately, the traditional methods of measuring moisture content, such as gravimetric and infrared moisture absorption, are limited in sensitivity and reproducibility. Our measurements suggest that approximately 1–5% of the moisture needs to be retained by the cells for optimal desiccation storage.
16. Storage in vacuum is recommended. Since it is impossible to maintain the cells in absolute vacuum, the duration of the storage does have an impact on the final quality of the desiccated sample.
17. The stability of the desiccated sample is not yet clear and further studies are needed.

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References

1. De Ugarte, D. A., Morizono, K., Elbarbary, A., Alfonso, Z., Zuk, P. A., Zhu, M., Dragoo, J. L., Ashjian, P., Thomas, B., Benhaim, P., Chen, I., Fraser, J., and Hedrick, M. H. (2003) Comparison of multi-lineage cells from human adipose tissue and bone marrow, *Cells Tissues Organs* **174**, 101–109.
2. Kang, S. K., Putnam, L., Dufour, J., Ylostalo, J., Jung, J. S., and Bunnell, B. A. (2004) Expression of telomerase extends the lifespan and enhances osteogenic differentiation of adipose tissue-derived stromal cells, *Stem Cells* **22**, 1356–1372.

3. Mitchell, J. B., McIntosh, K., Zvonic, S., Garrett, S., Floyd, Z. E., Kloster, A., Di Halvorsen, Y., Storms, R. W., Goh, B., Kilroy, G., Wu, X., and Gimble, J. M. (2006) Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers, *Stem Cells* **24**, 376–385.
4. Rubio, D., Garcia-Castro, J., Martin, M. C., de la Fuente, R., Cigudosa, J. C., Lloyd, A. C., and Bernad, A. (2005) Spontaneous human adult stem cell transformation, *Cancer Res* **65**, 3035–3039.
5. Wall, M. E., Bernacki, S. H., and Loboa, E. G. (2007) Effects of serial passaging on the adipogenic and osteogenic differentiation potential of adipose-derived human mesenchymal stem cells, *Tissue Eng* **13**, 1291–1298.
6. Gimble, J. M., Katz, A. J., and Bunnell, B. A. (2007) Adipose-derived stem cells for regenerative medicine, *Circ Res* **100**, 1249–1260.
7. Zhang, F. B., Li, L., Fang, B., Zhu, D. L., Yang, H. T., and Gao, P. J. (2005) Passage-restricted differentiation potential of mesenchymal stem cells into cardiomyocyte-like cells, *Biochem Biophys Res Commun* **336**, 784–792.
8. Tsutsumi, S., Shimazu, A., Miyazaki, K., Pan, H., Koike, C., Yoshida, E., Takagishi, K., and Kato, Y. (2001) Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF, *Biochem Biophys Res Commun* **288**, 413–419.
9. Zeng, X., and Rao, M. S. (2007) Human embryonic stem cells: long term stability, absence of senescence and a potential cell source for neural replacement, *Neuroscience* **145**, 1348–1358.
10. Zimmermann, S., and Martens, U. M. (2008) Telomeres, senescence, and hematopoietic stem cells, *Cell Tissue Res* **331**, 79–90.
11. Piacibello, W., Gammaitoni, L., and Pignochino, Y. (2005) Proliferative senescence in hematopoietic stem cells during ex-vivo expansion, *Folia Histochem Cytobiol* **43**, 197–202.
12. Pegg, D. E. (1987) Mechanisms of freezing damage, *Symp Soc Exp Biol* **41**, 363–378.
13. Day, J. G. (2007) Cryopreservation of microalgae and cyanobacteria, *Methods Mol Biol* **368**, 141–151.
14. Pegg, D. E. (2007) Principles of cryopreservation, *Methods Mol Biol* **368**, 39–57.
15. Bischof, J. C., and Rubinsky, B. (1993) Large ice crystals in the nucleus of rapidly frozen liver cells, *Cryobiology* **30**, 597–603.
16. Toner, M., Cravalho, E. G., Stachecki, J., Fitzgerald, T., Tompkins, R. G., Yarmush, M. L., and Armant, D. R. (1993) Nonequilibrium freezing of one-cell mouse embryos. Membrane integrity and developmental potential, *Biophys J* **64**, 1908–1921.
17. Venkatasubramanian, R. T., Grassl, E. D., Barocas, V. H., Lafontaine, D., and Bischof, J. C. (2006) Effects of freezing and cryopreservation on the mechanical properties of arteries, *Ann Biomed Eng* **34**, 823–832.
18. Schill, R. O., Mali, B., Dandekar, T., Schnolzer, M., Reuter, D., and Frohme, M. (2009) Molecular mechanisms of tolerance in tardigrades: new perspectives for preservation and stabilization of biological material, *Biotechnol Adv* **27**, 348–352.
19. Franca, M. B., Panek, A. D., and Eleutherio, E. C. (2007) Oxidative stress and its effects during dehydration, *Comp Biochem Physiol A Mol Integr Physiol* **146**, 621–631.
20. Liang, Y., and Sun, W. Q. (2002) Rate of dehydration and cumulative desiccation stress interacted to modulate desiccation tolerance of recalcitrant cocoa and ginkgo embryonic tissues, *Plant Physiol* **128**, 1323–1331.
21. Hoekstra, F. A., Golovina, E. A., and Buitink, J. (2001) Mechanisms of plant desiccation tolerance, *Trends Plant Sci* **6**, 431–438.
22. Allison, S. D., Randolph, T. W., Manning, M. C., Middleton, K., Davis, A., and Carpenter, J. F. (1998) Effects of drying methods and additives on structure and function of actin: mechanisms of dehydration-induced damage and its inhibition, *Arch Biochem Biophys* **358**, 171–181.
23. Setlow, P. (1995) Mechanisms for the prevention of damage to DNA in spores of Bacillus species, *Annu Rev Microbiol* **49**, 29–54.
24. Crowe, L. M., and Crowe, J. H. (1992) Anhydrobiosis: a strategy for survival, *Adv Space Res* **12**, 239–247.
25. Clegg, J. S., Seitz, P., Seitz, W., and Hazlewood, C. F. (1982) Cellular responses to extreme water loss: the water-replacement hypothesis, *Cryobiology* **19**, 306–316.
26. Yu, J., and Anchordoquy, T. J. (2009) Synergistic effects of surfactants and sugars on lipoplex stability during freeze-drying and rehydration, *J Pharm Sci* **98**, 3319–3328.
27. Jiang, W., and Schwendeman, S. P. (2008) Stabilization of tetanus toxoid encapsulated in PLGA microspheres, *Mol Pharm* **5**, 808–817.
28. Santivarangkna, C., Kulozik, U., and Foerst, P. (2008) Inactivation mechanisms of lactic

acid starter cultures preserved by drying processes, *J Appl Microbiol* **105**, 1–13.

29. Stoner, G. D., Chen, T., Kresty, L. A., Aziz, R. M., Reinemann, T., and Nines, R. (2006) Protection against esophageal cancer in rodents with lyophilized berries: potential mechanisms, *Nutr Cancer* **54**, 33–46.
30. Hansen, R. K., Zhai, S., Skepper, J. N., Johnston, M. D., Alpar, H. O., and Slater, N. K. (2005) Mechanisms of inactivation of HSV-2 during storage in frozen and lyophilized forms, *Biotechnol Prog* **21**, 911–917.
31. Arakawa, T., Prestrelski, S. J., Kenney, W. C., and Carpenter, J. F. (2001) Factors affecting short-term and long-term stabilities of proteins, *Adv Drug Deliv Rev* **46**, 307–326.
32. Milano, M. T., and Bernhard, W. A. (1999) The influence of packing on free radical yields in solid-state DNA: film compared to lyophilized frozen solution, *Radiat Res* **152**, 196–201.
33. Kreilgaard, L., Frokjaer, S., Flink, J. M., Randolph, T. W., and Carpenter, J. F. (1998) Effects of additives on the stability of recombinant human factor XIII during freeze-drying and storage in the dried solid, *Arch Biochem Biophys* **360**, 121–134.
34. Fuller, R., and Devireddy, R. V. (2008) The effect of two different freezing methods on the immediate post-thaw membrane integrity of adipose tissue derived stem, *Int J Heat Mass Transfer* **51**, 5650–5654.
35. Goh, B. C., Thirumala, S., Kilroy, G., Devireddy, R. V., and Gimble, J. M. (2007) Cryopreservation characteristics of adipose-derived stem cells: maintenance of differentiation potential and viability, *J Tissue Eng Regen Med* **1**, 322–324.
36. Thirumala, S., Gimble, J., and Devireddy, R. V. (2010) Evaluation of methylcellulose and dimethylsulfoxide as the cryoprotectants in a serum free freezing media for cryopreservation of adipose derived adult stem cells, *Stem Cells Dev* **19**, 513–522.
37. Thirumala, S., Gimble, J., and Devireddy, R. V. (2010) Cryopreservation of stromal vascular fraction of adipose tissue in a serum free freezing media, *J Tissue Eng Regen Med* **4**, 224–232.
38. Thirumala, S., Wu, X., Gimble, J., and Devireddy, R. V. (2010) Evaluation of polyvinylpyrollidone (PVP) as a cryoprotectant for adipose derived adult stem cells (ASCs), *Tissue Eng Part C Methods* **16**, 783–792.
39. Mittal, S., and Devireddy, R. V. (2008) Desiccation tolerance of adult stem cells in the presence of trehalose and glycerol, *Open Biotechnol J* **2**, 211–218.
40. Devireddy, R. V., Thirumala, S., and Gimble, J. M. (2005) Cellular response of adipose derived passage-4 adult stem cells to freezing stress, *J Biomech Eng* **127**, 1081–1086.
41. Thirumala, S., Gimble, J. M., and Devireddy, R. V. (2005) Transport phenomena during freezing of adipose tissue derived adult stem cells, *Biotechnol Bioeng* **92**, 372–383.
42. Thirumala, S., Zvonic, S., Floyd, E., Gimble, J. M., and Devireddy, R. V. (2005) Effect of various freezing parameters on the immediate post-thaw membrane integrity of adipose tissue derived adult stem cells, *Biotechnol Prog* **21**, 1511–1524.
43. Mazur, P. (1984) Freezing of living cells: mechanisms and implications, *Am J Physiol* **247**, C125–C142.
44. Polge, C., Smith, A. U., and Parkes, A. S. (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures, *Nature* 164, 666.
45. McGrath, J. J. (1997) Quantitative measurement of cell membrane transport: technology and applications, *Cryobiology* **34**, 315–334.
46. Anchordoguy, T. J., Cecchini, C. A., Crowe, J. H., and Crowe, L. M. (1991) Insights into the cryoprotective mechanism of dimethyl sulfoxide for phospholipid bilayers, *Cryobiology* **28**, 467–473.
47. Anchordoguy, T., Carpenter, J. F., Loomis, S. H., and Crowe, J. H. (1988) Mechanisms of interaction of amino acids with phospholipid bilayers during freezing, *Biochim Biophys Acta* **946**, 299–306.
48. Yu, Z. W., and Quinn, P. J. (1994) Dimethyl sulphoxide: a review of its applications in cell biology, *Biosci Rep* **14**, 259–281.
49. Fahy, G. M., Lilley, T. H., Linsdell, H., Douglas, M. S., and Meryman, H. T. (1990) Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms, *Cryobiology* **27**, 247–268.
50. Fahy, G. M. (2010) Cryoprotectant toxicity neutralization, *Cryobiology* 60, S45–S53.
51. Fahy, G. M., Wowk, B., Wu, J., and Paynter, S. (2004) Improved vitrification solutions based on the predictability of vitrification solution toxicity, *Cryobiology* **48**, 22–35.
52. Fahy, G. M. (1986) The relevance of cryoprotectant "toxicity" to cryobiology, *Cryobiology* **23**, 1–13.
53. Farrant, J. (1969) Is there a common mechanism of protection of living cells by polyvinylpyrrolidone and glycerol ding freezing?, *Nature* **222**, 1175–1176.

54. Hey, J. M., and MacFarlane, D. R. (1998) Crystallization of ice in aqueous solutions of glycerol and dimethyl sulfoxide 2: ice crystal growth kinetics, *Cryobiology* **37**, 119–130.
55. Shaw, J. M., Kuleshova, L. L., MacFarlane, D. R., and Trounson, A. O. (1997) Vitrification properties of solutions of ethylene glycol in saline containing PVP, Ficoll, or dextran, *Cryobiology* **35**, 219–229.
56. Alapati, R., Stout, M., Saenz, J., Gentry, G. T., Jr., Godke, R. A., and Devireddy, R. V. (2009) Comparison of the permeability properties and post-thaw motility of ejaculated and epididymal bovine spermatozoa, *Cryobiology* **59**, 164–170.
57. Hagiwara, M., Choi, J. H., Devireddy, R. V., Roberts, K. P., Wolkers, W. F., Makhlouf, A., and Bischof, J. C. (2009) Cellular biophysics during freezing of rat and mouse sperm predicts postthaw motility, *Biol Reprod* **81**, 700–706.
58. Alapati, R., Goff, K., Kubisch, H. M., and Devireddy, R. V. (2008) Water transport in epididymal and ejaculated rhesus monkey (*Macaca mulatta*) sperm during freezing, *Cryobiology* **57**, 182–185.
59. Kardak, A., Leibo, S. P., and Devireddy, R. (2007) Membrane transport properties of equine and macaque ovarian tissues frozen in mixtures of dimethylsulfoxide and ethylene glycol, *J Biomech Eng* **129**, 688–694.
60. Li, G., Saenz, J., Godke, R. A., and Devireddy, R. V. (2006) Effect of glycerol and cholesterol-loaded cyclodextrin on freezing-induced water loss in bovine spermatozoa, *Reproduction* **131**, 875–886.
61. Pinisetty, D., Huang, C., Dong, Q., Tiersch, T. R., and Devireddy, R. V. (2005) Subzero water permeability parameters and optimal freezing rates for sperm cells of the southern platyfish, *Xiphophorus maculatus*, *Cryobiology* **50**, 250–263.
62. Thirumala, S., Huang, C., Dong, Q., Tiersch, T. R., and Devireddy, R. V. (2005) A theoretically estimated optimal cooling rate for the cryopreservation of sperm cells from a live-bearing fish, the green swordtail *Xiphophorus helleri*, *Theriogenology* **63**, 2395–2415.
63. Meryman, H. T. (1974) Freezing injury and its prevention in living cells, *Annu Rev Biophys Bioeng* **3**, 341–363.
64. Meryman, H. T. (1956) Mechanics of freezing in living cells and tissues, *Science* **124**, 515–521.
65. Meryman, H. T. (2007) Cryopreservation of living cells: principles and practice, *Transfusion* **47**, 935–945.
66. Connor, W., and Ashwood-Smith, M. J. (1973) Cryoprotection of mammalian cells in tissue culture with polymers; possible mechanisms, *Cryobiology* **10**, 488–496.
67. Holt, W. V., and North, R. D. (1994) Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane damage in cryopreserved ram spermatozoa, *Biol Reprod* **51**, 414–424.
68. Barrios, B., Perez-Pe, R., Gallego, M., Tato, A., Osada, J., Muino-Blanco, T., and Cebrian-Perez, J. A. (2000) Seminal plasma proteins revert the cold-shock damage on ram sperm membrane, *Biol Reprod* **63**, 1531–1537.
69. Rodgers, F. G., and Davey, M. R. (1982) Ultrastructure of the cell envelope layers and surface details of *Legionella pneumophila*, *J Gen Microbiol* **128**, 1547–1557.
70. De Leeuw, F. E., De Leeuw, A. M., Den Daas, J. H., Colenbrander, B., and Verkleij, A. J. (1993) Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing, *Cryobiology* **30**, 32–44.
71. Barnard, T. (1987) Rapid freezing techniques and cryoprotection of biomedical specimens, *Scanning Microsc* **1**, 1217–1224.
72. Hirsh, A. G., Williams, R. J., and Meryman, H. T. (1985) A novel method of natural cryoprotection: intracellular glass formation in deeply frozen populus, *Plant Physiol* **79**, 41–56.
73. Rall, W. F., Mazur, P., and McGrath, J. J. (1983) Depression of the ice-nucleation temperature of rapidly cooled mouse embryos by glycerol and dimethyl sulfoxide, *Biophys J* **41**, 1–12.
74. Elliott, R., Szleifer, I., and Schick, M. (2007) A microscopic model calculation of the phase diagram of ternary mixtures of cholesterol and saturated and unsaturated phospholipids, *Methods Mol Biol* **398**, 303–317.
75. Fahy, G. M. (1980) Analysis of "solution effects" injury. Equations for calculating phase diagram information for the ternary systems NaCl-dimethylsulfoxide-water and NaCl-glycerol-water, *Biophys J* **32**, 837–850.
76. Guignon, B., Aparicio, C., Otero, L., and Sanz, P. D. (2009) Prediction of ice content in biological model solutions when frozen under high pressure, *Biotechnol Prog* **25**, 454–460.
77. Kleinhans, F. W., and Mazur, P. (2007) Comparison of actual vs. synthesized ternary phase diagrams for solutes of cryobiological interest, *Cryobiology* **54**, 212–222.

78. Morris, G. J., Goodrich, M., Acton, E., and Fonseca, F. (2006) The high viscosity encountered during freezing in glycerol solutions: effects on cryopreservation, *Cryobiology* **52**, 323–334.
79. Watson, P. F., and Duncan, A. E. (1988) Effect of salt concentration and unfrozen water fraction on the viability of slowly frozen ram spermatozoa, *Cryobiology* **25**, 131–142.
80. Rall, W. F., Mazur, P., and Souzu, H. (1978) Physical-chemical basis of the protection of slowly frozen human erythrocytes by glycerol, *Biophys J* **23**, 101–120.
81. Shepard, M. L., Goldston, C. S., and Cocks, F. H. (1976) The H2O-NaCl-glycerol phase diagram and its application in cryobiology, *Cryobiology* **13**, 9–23.
82. Wolkers, W. F., Tablin, F., and Crowe, J. H. (2002) From anhydrobiosis to freeze-drying of eukaryotic cells, *Comp Biochem Physiol A Mol Integr Physiol* **131**, 535–543.
83. Crowe, L. M. (2002) Lessons from nature: the role of sugars in anhydrobiosis, *Comp Biochem Physiol A Mol Integr Physiol* **131**, 505–513.
84. Crowe, J. H., Carpenter, J. F., and Crowe, L. M. (1998) The role of vitrification in anhydrobiosis, *Annu Rev Physiol* **60**, 73–103.
85. Crowe, J. H., Hoekstra, F. A., and Crowe, L. M. (1992) Anhydrobiosis, *Annu Rev Physiol* **54**, 579–599.
86. Crowe, J. H., and Crowe, L. M. (1982) Induction of anhydrobiosis: membrane changes during drying, *Cryobiology* **19**, 317–328.
87. Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., and Crowe, L. M. (1995) Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying, *Appl Environ Microbiol* **61**, 3592–3597.
88. Leslie, S. B., Teter, S. A., Crowe, L. M., and Crowe, J. H. (1994) Trehalose lowers membrane phase transitions in dry yeast cells, *Biochim Biophys Acta* **1192**, 7–13.
89. Guo, N., Puhlev, I., Brown, D. R., Mansbridge, J., and Levine, F. (2000) Trehalose expression confers desiccation tolerance on human cells, *Nat Biotechnol* **18**, 168–171.
90. Potts, M. (2001) Desiccation tolerance: a simple process? *Trends Microbiol* **9**, 553–559.
91. Potts, M. (1994) Desiccation tolerance of prokaryotes, *Microbiol Rev* **58**, 755–805.
92. Conrad, P. B., Miller, D. P., Cielenski, P. R., and de Pablo, J. J. (2000) Stabilization and preservation of *Lactobacillus acidophilus* in saccharide matrices, *Cryobiology* **41**, 17–24.
93. Gordon, S. L., Oppenheimer, S. R., Mackay, A. M., Brunnabend, J., Puhlev, I., and Levine, F. (2001) Recovery of human mesenchymal stem cells following dehydration and rehydration, *Cryobiology* **43**, 182–187.
94. Puhlev, I., Guo, N., Brown, D. R., and Levine, F. (2001) Desiccation tolerance in human cells, *Cryobiology* **42**, 207–217.
95. Chen, T., Acker, J. P., Eroglu, A., Cheley, S., Bayley, H., Fowler, A., and Toner, M. (2001) Beneficial effect of intracellular trehalose on the membrane integrity of dried mammalian cells, *Cryobiology* **43**, 168–181.
96. McGinnis, L. K., Zhu, L., Lawitts, J. A., Bhowmick, S., Toner, M., and Biggers, J. D. (2005) Mouse sperm desiccated and stored in trehalose medium without freezing, *Biol Reprod* **73**, 627–633.
97. Eroglu, A., Bailey, S. E., Toner, M., and Toth, T. L. (2009) Successful cryopreservation of mouse oocytes by using low concentrations of trehalose and dimethylsulfoxide, *Biol Reprod* **80**, 70–78.
98. Buchanan, S. S., Gross, S. A., Acker, J. P., Toner, M., Carpenter, J. F., and Pyatt, D. W. (2004) Cryopreservation of stem cells using trehalose: evaluation of the method using a human hematopoietic cell line, *Stem Cells Dev* **13**, 295–305.
99. Erdag, G., Eroglu, A., Morgan, J., and Toner, M. (2002) Cryopreservation of fetal skin is improved by extracellular trehalose, *Cryobiology* **44**, 218–228.
100. Eroglu, A., Toner, M., and Toth, T. L. (2002) Beneficial effect of microinjected trehalose on the cryosurvival of human oocytes, *Fertil Steril* **77**, 152–158.
101. Eroglu, A., Russo, M. J., Bieganski, R., Fowler, A., Cheley, S., Bayley, H., and Toner, M. (2000) Intracellular trehalose improves the survival of cryopreserved mammalian cells, *Nat Biotechnol* **18**, 163–167.
102. Bhowmick, S., Zhu, L., McGinnis, L., Lawitts, J., Nath, B. D., Toner, M., and Biggers, J. (2003) Desiccation tolerance of spermatozoa dried at ambient temperature: production of fetal mice, *Biol Reprod* **68**, 1779–1786.
103. Guilak, F., Lott, K. E., Awad, H. A., Cao, Q., Hicok, K. C., Fermor, B., and Gimble, J. M. (2006) Clonal analysis of the differentiation potential of human adipose-derived adult stem cells, *J Cell Physiol* **206**, 229–237.
104. DeLany, J. P., Floyd, Z. E., Zvonic, S., Smith, A., Gravois, A., Reiners, E., Wu, X., Kilroy,

G., Lefevre, M., and Gimble, J. M. (2005) Proteomic analysis of primary cultures of human adipose-derived stem cells: modulation by Adipogenesis, *Mol Cell Proteomics* **4**, 731–740.

105. Hicok, K. C., Du Laney, T. V., Zhou, Y. S., Halvorsen, Y. D., Hitt, D. C., Cooper, L. F., and Gimble, J. M. (2004) Human adipose-derived adult stem cells produce osteoid in vivo, *Tissue Eng* **10**, 371–380.
106. Safford, K. M., Safford, S. D., Gimble, J. M., Shetty, A. K., and Rice, H. E. (2004) Characterization of neuronal/glial differentiation of murine adipose-derived adult stromal cells, *Exp Neurol* **187**, 319–328.
107. Aust, L., Devlin, B., Foster, S. J., Halvorsen, Y. D., Hicok, K., du Laney, T., Sen, A., Willingmyre, G. D., and Gimble, J. M. (2004) Yield of human adipose-derived adult stem cells from liposuction aspirates, *Cytotherapy* **6**, 7–14.
108. Awad, H. A., Wickham, M. Q., Leddy, H. A., Gimble, J. M., and Guilak, F. (2004) Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds, *Biomaterials* **25**, 3211–3222.
109. Gimble, J. M., and Guilak, F. (2003) Differentiation potential of adipose derived adult stem (ADAS) cells, *Curr Top Dev Biol* **58**, 137–160.
110. Gimble, J., and Guilak, F. (2003) Adipose-derived adult stem cells: isolation, characterization, and differentiation potential, *Cytotherapy* **5**, 362–369.
111. Safford, K. M., Hicok, K. C., Safford, S. D., Halvorsen, Y. D., Wilkison, W. O., Gimble, J. M., and Rice, H. E. (2002) Neurogenic differentiation of murine and human adipose-derived stromal cells, *Biochem Biophys Res Commun* **294**, 371–379.
112. Gronthos, S., Franklin, D. M., Leddy, H. A., Robey, P. G., Storms, R. W., and Gimble, J. M. (2001) Surface protein characterization of human adipose tissue-derived stromal cells, *J Cell Physiol* **189**, 54–63.
113. Thirumala, S., Forman, J. M., Monroe, W. T., and Devireddy, R. (2007) Freezing and post-thaw apoptotic behaviour of cells in the presence of palmitoyl nanogold particles, *Nanotechnology* **18**, 195104.
114. Rubinsky, B., and Ikeda, M. (1985) A cryomicroscope using directional solidification for the controlled freezing of biological mat, *Cryobiology* **22**, 55–68.
115. Garner, D. L., and Johnson, L. A. (1995) Viability assessment of mammalian sperm using SYBR-14 and propidium iodide, *Biol Reprod* **53**, 276–284.
116. Takamatsu, H., and Rubinsky, B. (1999) Viability of deformed cells, *Cryobiology* **39**, 243–251.
117. Takamatsu, H., Takeya, R., Naito, S., and Sumimoto, H. (2005) On the mechanism of cell lysis by deformation, *J Biomech* **38**, 117–124.
118. Wolfe, J., and Bryant, G. (1999) Freezing, drying, and/or vitrification of membrane-solute-water systems, *Cryobiology* **39**, 103–129.
119. Rubinsky, B. (2000) Cryosurgery, *Annu Rev Biomed Eng* **2**, 157–187.
120. Devireddy, R. V., Swanlund, D. J., Roberts, K. P., and Bischof, J. C. (1999) Subzero water permeability parameters of mouse spermatozoa in the presence of extracellular ice and cryoprotective agents, *Biol Reprod* **61**, 764–775.

Chapter 28

Soft Tissue Reconstruction

J. Peter Rubin and Kacey G. Marra

Abstract

The potential of adipose-derived stem cells (ASCs) in clinical applications of soft tissue regeneration is immense. This chapter discusses the isolation and characterization of human ASCs, expansion in vitro, and relevant in vivo models for adipose tissue engineering.

Key words: Adipose tissue, Mesenchymal stem cells, Reconstruction

1. Introduction

Soft tissue reconstruction remains one of the most significant challenges for plastic surgeons. A lack of soft tissue can occur due to trauma, congenital diseases (e.g., in Rombergh disease (1) or Poland syndrome (2)), or oncologic surgery. Transplantation of autologous adipose tissue has been used for soft tissue reconstruction for the last century. Neuber was among the first to report his findings regarding the use of autologous fat transplantation (3). Current strategies involve tissue transplantation, including composite tissue flaps. The use of autologous composite tissue flaps containing an intact vascular network is more successful in respect to adipose grafting but this technique results in a donor site defect and, potentially, the need for multiple, complex, costly surgical procedures (4, 5). The clinical outcome of adipose tissue transplantation remains unpredictable as there is a graft resorption due to a lack of vascularization (6). Adipose tissue is highly vascularized with extensive capillary networks surrounding adipocytes; therefore, it is imperative that the newly transplanted adipocytes have access to a vascular source (7–9).

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Furthermore, there is a constant resorption rate that can vary from 20 to 90% over time (10–15). Immediately after harvesting of the fat graft, the suctioned aspirate can be composed of a wide range of viable fat: 10–90% (16). It has been hypothesized that the number of viable adipocytes at the time of transplantation correlates with the ultimate fat graft survival volume (17, 18). However, recently scientists have recently considered the influence of undifferentiated cells, e.g., adipose-derived stem cells (ASCs), normally found in fatty tissue as a variable that can affect graft integration (16).

ASCs are the precursor cells that are contained within the stromal-vascular fraction of enzymatically digested adipose tissue. ASCs have the ability to differentiate into many different cell types, such as osteoblasts (19, 20), chondrocytes (21, 22), myocytes (19, 23, 24), neuronal-like cells (25, 26), cardiovascular cells (27–30), hepatocytes (31–33), and adipocytes (19, 34–38). ASCs are also capable of dividing and renewing for extended periods of time (39). Of particular interest to reconstructive surgery is the ability of ASCs to undergo facile differentiation into the mesenchymal phenotype, particularly adipose tissue.

2. Materials

1. Dulbecco's Modified Eagle's Medium (DMEM/F12).
2. Fetal bovine serum.
3. Penicillin/Streptomycin.
4. Gentamycin.
5. Dexamethasone.
6. Collagenase solution: type II collagenase, M8B10274 (0.1%), with bovine serum albumin (BSA; 3.5%) in 1× Hank's solution.
7. Human regular medium: DMEM and DMEM/F12 at a 1:1 ratio, 10% fetal bovine serum, 0.1 mM penicillin, 0.06 mM streptomycin, 0.1 mM dexamethasone, and gentamycin sulfate (10 mg/L).
8. Erythrocyte lysis buffer.
9. Centrifuge tubes.
10. CyQUANT assay.
11. Fatty acid-free BSA.
12. PKH26.
13. Hematoxylin and Eosin.
14. PBS.
15. Formalin.

3. Methods

3.1. Adipose Tissue Removal

1. Adipose tissue (100–200 g) is harvested from the superficial abdominal depots of females or males undergoing elective abdominal reduction surgery.
2. The patient age range is 40–60 years old and all are healthy according to clinical examination and laboratory tests. The University of Pittsburgh Institutional Review Board (IRB) approved the procedure of collecting the samples of adipose tissue.
3. Samples are not pooled, with each experiment using cells from different patients.

3.2. ASC Isolation

1. Abdominal adipose tissue is placed in sterile 50-mL conical tubes.
2. A collagenase solution is sterile filtered then added at a 3:1 ratio of solution to adipose tissue for digestion.
3. This solution is then filtered through double-layer gauze (4 × 4 in.), to remove unprocessed debris.
4. The resulting filtered solution is centrifuged at 9,200 × *g* for 10 min at 20°C. The supernatant is removed and the pellet is resuspended in 10 mL of erythrocyte lysis buffer (see Note 1).
5. Resuspended pellets are vortexed to lyse RBCs and debris, and then recentrifuged at 9,200 × *g* for 10 min at 20°C.
6. The supernatant is then removed and the pellet resuspended in 10 mL of human regular media.
7. After 6 h, the ASCs are adherent to the plates and then washed extensively with PBS to remove any contaminants.
8. Cells are passaged at confluency, and characterized as previously described by our laboratory (40, 41).

3.3. ASC Culture

1. Plating media is changed three times a week, and continued until the ASCs reach approximately 80% confluence (see Note 2).

3.4. Measurement of Proliferation

1. Approximately 2.5×10^3 cells from each depot are seeded in triplicate into 24-well plates.
2. After 48 and 96 h, the plates are then frozen at –80°C for a minimum of 24 h.
3. The plates are then thawed to room temperature and a CyQUANT® cell proliferation assay kit is used to quantify cell number.
4. Fluorescence is measured at 480-nm excitation and 520-nm emission.
5. A standard curve is created and cell number obtained.

3.5. In Vivo Model

A common in vivo model for assessment of human ASCs for soft tissue reconstruction is described.

1. A dorsal subcutaneous injection is made in 6-week-old male athymic nude mice (see Note 3).
2. Each injection is 1 mL in volume and performed through a hypodermic syringe (see Note 4).
3. For histological evaluation, animals are sacrificed, and excised gel implants are rinsed in PBS and fixed in 10% buffered formalin at 37°C.
4. The gel explants and surrounding tissues are dehydrated in a graded series of alcohols at 37°C, and embedded in paraffin.
5. The transverse paraffin is sectioned through the center of gel implants at 5-μm thickness, and histologically processed using hematoxylin and eosin (H & E) stains.
6. Identification of implanted ASCs can be achieved through a variety of techniques, described in the next section.

3.6. Identification of ASCs

Labeling of ASCs prior to animal implantation can occur using:

1. Viral transfection with green fluorescent protein (GFP).
2. Cell membrane dyes such as PKH26.
3. Nuclear dyes such as CMFDAse.
4. Gender mismatch (e.g., implantation of male cells into female animals and identification of the Y chromosome via FISH).

4. Notes

1. Erythrocyte lysis buffer is composed of 154 mM NH_4Cl, 10 mM $KHCO_3$, <1 mM EDTA in water, and sterile filtered.
2. It is important to avoid overconfluence as the ASCs may spontaneously differentiate into adipocytes.
3. Various delivery systems can be used, such as fibrin glue, collagen gels, or hyaluronic acid.
4. Time points can range from 1 day to 1 year. Typically, researchers use 4, 8, or 12 weeks as end-points, as it is important to demonstrate long-term adipose retention.

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References

1. Finch, G. D. and Dawe, C. J. (2003) Hemiatrophy. *J Pediatr Orthop* **23**, 99–101.
2. Fokin, A. A. and Robicsek, F. (2002) Poland's syndrome revisited. *Ann Thorac Surg* **74**, 2218–25.
3. Neuber, F. (1893) Fettransplantation. *Bericht uber die Verhandlungen der Deutscht Gesellsch Chir* **22**, 66.
4. Shenaq, S. M. and Yuksel, E. (2002) New research in breast reconstruction: adipose tissue engineering. *Clin Plast Surg* **29**, 111–12.
5. Langstein, H. N. and Robb, G. L. (1999) Reconstructive approaches in soft tissue sarcoma. *Semin Surg Oncol* **17**, 52–65.
6. Peer, L. A. (1956) The neglected free fat graft. *Plast Reconstr Surg* **18**, 233–50.
7. Crandall, D., Hausman, G., and Kral, J. (1997) A review of the microcirculation of adipose tissue: anatomic, metabolic, and angiogenic perspectives. *Microcirculation* **4**, 211–32.
8. Sierra-Honigmann, M. R., Nath, A. K., Murakami, C., García-Cardeña, G., Papapetropoulos, A., Sessa, W. C., Madge, L. A., Schechner, J. S., Schwabb, M. B., Polverini, P. J., and Flores-Riveros, J. R. (1998) Biological action of leptin as an angiogenic factor. *Science* **11**, 1683–86.
9. Patrick, C. W. J. (2001) Tissue engineering strategies for adipose tissue repair. *Anat Rec* **263**, 361–66.
10. Nguyen, A., Pasyk, K. A., Bouvier, T. N., Hassett, C. A., and Argenta, L. C. (1990) Comparative study of survival of autologous adipose tissue taken and transplanted by different techniques. *Plast Reconstr Surg* **85**, 378–86.
11. Boyce, R. G., Nuss, D. W., and Kluka, E. A. (1994) The use of autogenous fat, fascia, and nonvascularized muscle grafts in the head and neck. *Otolaryngol Clin North Am* **27**, 39–68.
12. Matsudo, P. K. and Toledo, L. S. (1988) Experience of injected fat grafting. *Aesthetic Plast Surg* **12**, 35–38.
13. Rohrich, R. J., Sorokin, E. S., and Brown, S. A. (2004) In search of improved fat transfer viability: a quantitative analysis of the role of centrifugation and harvest site. *Plast Reconstr Surg* **113**, 391–95.
14. Jackson, I. T., Simman, R., Tholen, R., and DiNick, V. D. (2001) A successful long-term method of fat grafting: recontouring of a large subcutaneous postradiation thigh defect with autologous fat transplantation. *Aesthetic Plast Surg* **25**, 165–69.
15. Boschert, M., Beckert B. W., Puckett, C. L., and Concannon, M. J. (2002) Analysis of lipocyte viability after liposuction. *Plast Reconstr Surg* **109**, 761–65.
16. Coleman, S. R. (2006) Structural fat grafting: more than a permanent filler. *Plast Reconstr Surg* **118**, 108S–20S.
17. Peer, L. A. (1955) Cell survival theory versus replacement theory. *Plast Reconstr Surg* **16**, 161–68.
18. Gonzalez, A. M., Lobocki, C., Kelly, C. P., and Jackson, I. T. (2007) An alternative method for harvest and processing fat grafts: an in vitro study of cell viability and survival. *Plast Reconstr Surg* **120**, 285–94.
19. Zuk, P. A., Zhu, M., Ashjian, P., De Ugarte, D. A., Huang, J. I., Mizuno, H., Alfonso, Z. C., Fraser, J. K., Benhaim, P., and Hedrick, M. H. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279–95.
20. Gabbay, J. S., Heller, J. B., Mitchell, S. A., Zuk, P. A., Spoon, D. B., Wasson, K. L., Jarrahy, R., Benhaim, P., and Bradley, J. P. (2006) Osteogenic potentiation of human adipose-derived stem cells in a 3-dimensional matrix. *Ann Plast Surg* **57**, 89–93.
21. Jin, X., Sun, Y., Zhang, K., Wang, J., Shi, T., Ju, X., and Lou, S. (2007) Ectopic neocartilage formation from predifferentiated human adipose derived stem cells induced by adenoviral-mediated transfer of hTGF beta2. *Biomaterials* **28**, 2994–3003.
22. Xu, Y., Balooch, G., Chiou, M., Bekerman, E., Ritchie, R. O., and Longaker, M. T. (2007) Analysis of the material properties of early chondrogenic differentiated adipose-derived stromal cells (ASC) using an in vitro three-dimensional micromass culture system. *Biochem Biophys Res Commun* **359**, 311–6.
23. Rangappa, S., Entwistle, J. W., Wechsler, A. S., and Kresh, J. Y. (2003) Cardiomyocyte-mediated contact programs human mesenchymal stem cells to express cardiogenic phenotype. *J Thorac Cardiovasc Surg* **126**, 124–32.
24. Rodríguez, L. V., Alfonso, Z., Zhang, R., Leung, J., Wu, B., and Ignarro, L. J. (2006) Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cells. *Proc Natl Acad Sci U S A* **103**, 12167–72.
25. Kokai, L. E., Rubin, J. P., and Marra, K. G. (2005) The potential of adipose-derived adult

stem cells as a source of neuronal progenitor cells. *Plast Reconstr Surg* **116**, 1453–60.

26. Safford, K. M., Hicok, K. C., Safford, S. D., et al. (2002) Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochem Biophys Res Commun* **294**, 371.
27. Casteilla, L., Planat-Bénard, V., Cousin, B., Silvestre, J. S., Laharrague, P., Charrière, G., Carrière, A., and Pénicaud, L. (2005) Plasticity of adipose tissue: a promising therapeutic avenue in the treatment of cardiovascular and blood diseases? *Arch Mal Coeur Vaiss* **98**, 922–26.
28. Planat-Bénard, V., Menard, C., André, M., Puceat, M., Perez, A., Garcia-Verdugo, J. M., Pénicaud, L., and Casteilla, L. (2004) Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. *Circ Res* **94**, 223–29.
29. Cao, Y., Sun, Z., Liao, L., Meng, Y., Han, Q., and Zhao, R. C. (2005) Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization in vivo. *Biochem Biophys Res Commun* **332**, 370–9.
30. Rehman, J., Traktuev, D., Li, J., Merfeld-Clauss, S., Temm-Grove, C. J., Bovenkerk, J. E., Pell, C. L., Johnstone, B. H., Considine, R. V., and March, K. L. (2004) Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells *Circulation* **109**, 1292–98.
31. Seo, M. J., Suh, S. Y., Bae, Y. C., and Jung, J. S. (2005) Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. *Biochem Biophys Res Commun* **328**, 258–64.
32. Taléns-Visconti, R., Bonora, A., Jover, R., Mirabet, V., Carbonell, F., Castell, J. V., and Gómez-Lechón, M. J. (2006) Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells. *World J Gastroenterol* **12**, 5834–45.
33. Taléns-Visconti, R., Bonora, A., Jover, R., Mirabet, V., Carbonell, F., Castell, J. V., and Gómez-Lechón, M. (2007) Human mesenchymal stem cells from adipose tissue: differentiation into hepatic lineage. *Toxicol In Vitro* **21**, 324–29.
34. Lu, F., Gao, J. H., Ogawa, R., Mizuro, H., and Hykusoku, H. (2006) Adipose tissues differentiated by adipose-derived stem cells harvested from transgenic mice. *Chin J Traumatol* **9**, 359–64.
35. Choi, Y. S., Cha, S. M., Lee, Y. Y., Kwon, S. W., Park, C. J., and Kim, M. (2006) Adipogenic differentiation of adipose tissue derived adult stem cells in nude mouse. *Biochem Biophys Res Commun* **345**, 631–7.
36. Halvorsen, Y. D., Bond, A., Sen, A., Franklin, D. M., Lea-Currie, Y. R., Sujkowski, D., Ellis, P. N., Wilkison, W. O., and Gimble, J. M. (2001) Thiazolidinediones and glucocorticoids synergistically induce differentiation of human adipose tissue stromal cells: biochemical, cellular, and molecular analysis. *Metabolism* **50**, 407–413.
37. Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P., and Hedrick, M. H. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* **7**, 211–22.
38. Lee, J. A., Parrett, B. M., Conejero, J. A., Laser, J., Chen, J., Kogon, A. J., Nanda, D., Grant, R. T., and Breitbart, A. S. (2003) Biological alchemy: engineering bone and fat from fat-derived stem cells. *Ann Plast Surg* **50**, 610–17.
39. Wall, M. E., Bernacki, S. H., and Loboa, E. G. (2007) Effects of serial passaging on the adipogenic and osteogenic differentiation potential of adipose-derived human mesenchymal stem cells. *Tissue Eng* **13**, 1291–98.
40. Aksu, A. E., Rubin, J. P., Dudas, J. R., and Marra, K. G. (2008) Role of gender and anatomical region on induction of osteogenic differentiation of human adipose-derived stem cells. *Ann Plast Surg* **60**, 306–22.
41. Schipper, B. M., Marra, K. G., Zhang, W., Donnenberg, A. D., and Rubin, J. P. (2008) Regional anatomic and age effects on cell function of human adipose-derived stem cells. *Ann Plast Surg* **60**, 538–44.

Chapter 29

Methods for Analyzing MicroRNA Expression and Function During Osteogenic Differentiation of Human Adipose Tissue-Derived Mesenchymal Stem Cells

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Abstract

MicroRNAs (miRNA) are single-stranded RNA molecules of 21–23 nucleotides in length that regulate gene expression at the posttranscriptional level. They may play important roles during osteogenic differentiation of adipose tissue-derived mesenchymal stem cells (hASC). In this chapter, we focus on the methods and strategies for elucidating miRNA function during osteogenic differentiation. We describe a miRNA expression analysis protocol, and a lentiviral vector strategy for the ectopic expression of miRNA in hASC to determine the role of miRNA during osteogenic differentiation. We also describe miRNA inhibition to further determine the role of miRNA during osteogenic differentiation, and a luciferase assay to demonstrate direct binding between a specific miRNA and its putative target.

Key words: MicroRNA, Noncoding RNA, Osteogenic differentiation, Lentiviral expression, Adipose tissue-derived mesenchymal stem cells

1. Introduction

MicroRNAs (miRNA) are endogenous 22-nucleotide RNA, some of which play important regulatory roles in animals by posttranscriptionally regulating gene expression. The first miRNA was identified in *Caenorhabditis elegans* as a gene important for timing larval development (1, 2); since then, nearly 1% of all predicted mammalian genes have been found to encode miRNA (1–3). They are first transcribed as primary miRNA (pri-miRNA) by RNA polymerase II, after which they are cut by an RNase III enzyme (Drosha) into ~70-nucleotide precursors (pre-miRNA), which are transported to the cytoplasm. Another enzyme, Dicer,

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processes the pre-miRNA to mature miRNA, which are recruited into the RNA-induced silencing complex (RISC). Finally, this complex interferes with the translation and stability of target mRNA: these RISC complexes either bind mRNA with exact complementarity, leading to RISC-mediated cleavage of the mRNA, or else they bind with partial complementarity, leading to translational repression. miRNA have been implicated in many processes in invertebrates, including cell proliferation and apoptosis (4, 5), fat metabolism (4), neuronal patterning (6), and tumorigenesis (7).

Cell differentiation involves complex pathways that are regulated at both the transcriptional and posttranscriptional levels. Recent evidence has shown that small, noncoding miRNA influence the complexity of the "stemness" of the cell by negatively regulating gene expression at the posttranscriptional level (8). Only a few mammalian miRNA have been assigned a functional role in developmental processes: miR-181 promotes B cell development in mice (9), and it targets the homeobox protein Hox-A11 during mammalian myoblast differentiation (10). miR-196a regulates several Hox genes that encode a family of developmentally relevant transcription factors in animals (11). The brain-specific miR-134 regulates dendritic spine development (12). miR-1, miR-133, and miR-206 are specifically induced during myogenesis (13, 14). miR-143 increases adipocytic differentiation in preadipocytes (15), and Luzi et al. (16) reported that miR-26a inhibited osteogenic differentiation in human adipose tissue stromal cells by targeting SMAD1.

Like bone marrow, adipose tissue is a mesodermally derived organ with a stromal cell population encompassing microvascular endothelial, smooth muscle, and stem cells (17). These cells can be enzymatically digested out of adipose tissue and separated from buoyant adipocytes by centrifugation. This population, termed adipose tissue-derived mesenchymal stem cells (ASC), shares many of the characteristics of its counterparts in bone marrow, including extensive proliferative potential and the ability to differentiate toward adipogenic, osteogenic, chondrogenic, and myogenic lineages (18–20). Previous studies have demonstrated the osteogenic potential of hASC (17–21) by showing that they possess an in vivo bone-forming capacity, but the molecular mechanisms that underlie hASC differentiation toward the osteoblastic phenotype are still unknown. In an earlier study, we reported a systematic approach to examine the role of miRNA 196a during osteogenic differentiation processes (22).

In this chapter, we describe detailed protocols for analyzing the expression of miRNA in hASC and for generating viral constructs expressing miRNA. We also explain in vitro assay systems to assess the roles of miRNA during osteogenic differentiation of hASC. The methods and principles to be described in this chapter

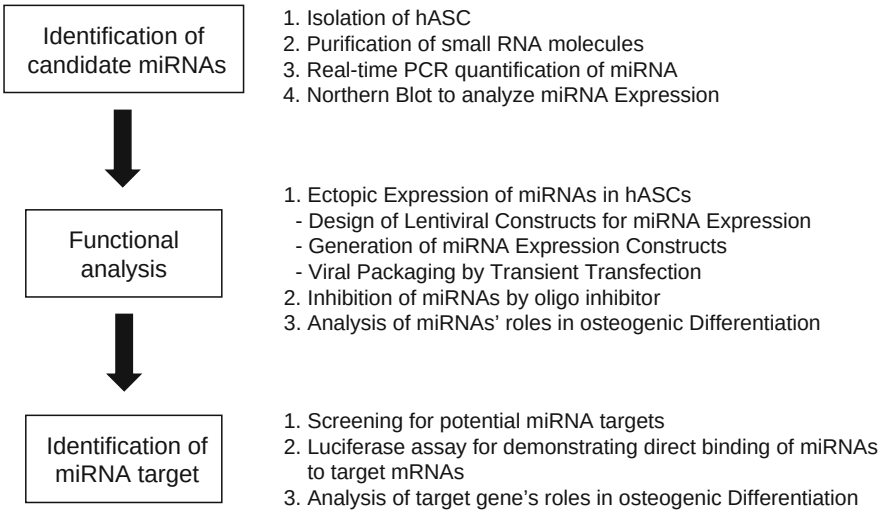

Fig. 1. Basic scheme for microRNA study.

can be used for investigating the functions of miRNA in human mesenchymal stem cells (hMSC) isolated from other tissues (Fig. 1).

2. Materials

2.1. Preparation of hASC for RNA Isolation

1. 0.075% collagenase type 1A (Sigma, St. Louis, MD).
2. α-modified Eagle's medium (α-MEM), 10% fetal bovine serum (FBS), Hank's Buffered Salt Solution (HBSS).
3. 100 U/ml Penicillin, 100 μg/ml streptomycin (Pen/Strep).

2.2. Purification of Small RNA Molecules

1. Trizol reagent (Invitrogen, Carlsbad, CA).
2. mirVana miRNA isolation kit (Ambion, Austin, TX).
3. Acid-phenol:chloroform (Ambion, Austin, TX).
4. RQ1 RNase-Free DNase (Promega, Madison, WI).
5. Glycogen.
6. Chloroform.
7. 100% Isopropyl alcohol.
8. 100% Ethanol.
9. 75% Ethanol.
10. DEPC-treated water.
11. Eppendorf tubes, pipet tips (see Note 1).

2.3. Real-Time PCR

1. NCode miRNA First-Strand cDNA Synthesis and qRT-PCR kits (Invitrogen, Carlsbad, CA).

2. LightCycler FastStart DNA Master SYBR Green I (Roche Molecular Systems, Pleasanton, CA).

2.4. Labeling of Oligonucelotide Probes

1. DNA annealing buffer (100 mM potassium acetate, 30 mM HEPES pH 7.4, 2 mM magnesium acetate).
2. T7 promoter primer: 5′-d(TAATACGACTCACTATAGGGA GACAGG)-3′.
3. T7 polymerase (Promega, Madison, WI).
4. Exo-Klenow enzyme (Ambion, Austin, TX).
5. RNase inhibitor (Promega, Madison, WI).
6. DIG RNA Labeling Mix (10×): 10 mM ATP, 10 mM CTP, 10 mM GTP, 10 mM UTP, 3.5 mM DIG-11-UTP, pH 7.5 (Roche Molecular Systems, Pleasanton, CA).
7. 10× Transcription buffer: 0.4 M Tris–HCl (pH 7.5), 0.1 M NaCl, 60 mM $MgCl_2$, 20 mM spermidine, store in aliquots at –20°C.
8. 10 mM dNTP, 0.2 M EDTA (pH 8.0).

2.5. Northern Blot (See Note 2)

1. Vertical slab gel electrophoresis apparatus, glass plates, gel spacers, binder clips, combs, and aluminum plates. Aluminum plates are used to evenly distribute the heat generated during electrophoresis and eliminate the “smile” effects.
2. 15% Acrylamide gel (8 M urea, 1.5 ml of 10× TBE, 5.6 ml of 40% acrylamide [acryl:bis acryl = 19:1] and add nuclease-free water to 15 ml. Stir to mix, then add 75 µl of 10% ammonium persulfate, 15 µl TEMED).
3. 10× TBE buffer: dissolve 0.9 M of Tris-base and 0.9 M of boric acid in H_2O. Add 20 ml of 0.5 M EDTA, pH 8.0. Adjust final volume to 1 L (see Note 3).
4. 4 µg/ml Ethidium bromide (EtBr) in 0.5× TBE.
5. 2× Gel-loading buffer: 8 M urea, 20 mM EDTA, pH 8.0, 1 mg/ml xylene cyanole FF, and 1 mg/ml bromophenol blue. To make 1 L of 2× gel-loading buffer, mix 480 g urea, 40 ml of 0.5 M EDTA, 2 ml of 1 M Tris–HCl, 1 g xylene cyanole FF, and 1 g bromophenol blue.
6. Nylon membrane (Hybond N+, Amersham-Pharmacia GeneScreen).
7. 20% Sodium dodecyl sulfate (SDS).
8. Anti-digoxigenin-AP (Roche Molecular Systems, Pleasanton, CA).
9. Blocking reagent (Roche Molecular Systems, Pleasanton, CA).
10. 20× Standard sodium citrate (SSC): 3 M NaCl and 0.3 M sodium citrate, pH 7.0. Dissolve 175.3 g NaCl and 88.2 g

sodium citrate in 800 ml H_2O, adjust pH to 7.0 with 1 M HCl, and adjust the volume to 1 L with H_2O.

11. Maleic acid buffer: 0.1 M Maleic acid, 0.15 M NaCl (pH 7.5).
12. Washing buffer: 0.1 M Maleic acid, 0.15 M NaCl (pH 7.5), 0.3% (v/v) Tween 20.
13. Rapid hybridization buffer (Amersham-Pharmacia GeneScreen).
14. CDP-Star, ready-to-use (Roche Molecular Systems, Pleasanton, CA).
15. Hybridization bags (Roche Molecular Systems, Pleasanton, CA).

2.6. miRNA Cloning (See Note 2)

1. hsa-miR-196a mature sequence: 5′-uagguaguuucauguuguuggg

   The engineered pre-miRNA sequence:

   (a) Top 5′-TGCTG-TAGGTAGTTTCATGTTGTTGG-GTTTTTGGCCACTGACTGAC-CCAACAAC GAAACTACCTA.

   (b) Bottom 5′-5′-CCTG-TAGGTAGTTTCGTTGTTGG-GTCAGTCAGTGGCCAAAAC-CCAACAACAT GAAACTACCTA-C.
2. BLOCK-iT Pol II miR RNAi Expression Vector kit with EmGFP (Invitrogen, Carlsbad, CA).
3. pLenti6/V5-DEST, pDONR 221 Vector (Invitrogen, Carlsbad, CA).
4. One Shot® Stbl3 Chemically Competent *Escherichia coli* (Invitrogen, Carlsbad, CA).
5. Gateway® BP Clonase II Enzyme Mix (Invitrogen, Carlsbad, CA).
6. Gateway® LR Clonase II Enzyme Mix (Invitrogen, Carlsbad, CA).
7. 293FT Cell Line.

2.7. Generation of miRNA Expression Constructs

1. pLP1:pLP2:pVSVG:lentiviral vector (Invitrogen, Carlsbad, CA).
2. Lipofectamine Transfection Reagent (Invitrogen, Carlsbad, CA).
3. PLUS Reagent (Invitrogen, Carlsbad, CA).
4. Opti-MEM I Reduced-Serum Medium (1×), liquid (Invitrogen, Carlsbad, CA).
5. 1,000× polybrene: Bring 50 mg polybrene (Sigma, St. Louis, MD) up to 10 ml with H_2O, filter sterilize, and store at –20°C.
6. Millex-HV 25-mm Durapore PVDF 45-μm nonsterile (Millipore, Billerica, MA).

2.8. Luciferase Assay

1. pMIR-REPORT luciferase, pMIR-REPORT β-gal control plasmid (Ambion, Austin, TX).
2. Restriction enzymes from Promega: HindIII and SpeI.
3. Quick Ligation kit, ligases, NEB (New England Biolabs, Ipswich, MA).
4. Cell Culture Lysis Reagent (CCLR buffer: 100 mM potassium phosphate [pH 7.8], 1 mM ethylenediamine tetraacetic acid, 7 mM 2-mercaptoethanol, 1% [v/v] Triton X-100, 10% [v/v] glycerol with H_2O).
5. Luciferase Assay System (Promega, Madison, WI).
6. BETA-GLO® ASSAY SYSTEM (Promega, Madison, WI).

2.9. Osteogenic Differentiation Assays

1. α-MEM supplemented with 10% FBS, Pen/Strep.
2. Osteogenic Induction Medium (OM): 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μM L-ascorbic acid-2-phosphate in α-MEM.
3. 4% Paraformaldehyde (in PBS).
4. 2% Alizarin Red S (Sigma, St. Louis, MD).

3. Methods

The spatial and temporal patterns of miRNA expression provide important clues regarding their biological functions. miRNA cloning (23–25), Northern blot analysis (2), miRNA microarray analysis (26–32), the invader assay (33), and the ribonuclease protection assay have been developed to examine miRNA expression. In this chapter, we only provide detailed protocols for real-time analysis PCR, Northern blot, and miRNA cloning.

3.1. Real-Time PCR Quantification of miRNA

miRNA are challenging molecules to amplify by PCR because the miRNA precursor consists of a stable hairpin and the mature miRNA is roughly the size of a standard PCR primer. Despite these difficulties, successful real-time RT-PCR technologies have been developed to amplify and quantify miRNA. An overview of real-time PCR technologies developed by us to detect precursor and mature miRNA is presented here.

In this chapter, we describe only the modifications we made to the protocol. A detailed description of the original protocol can be found in the manufacturer's instructions.

3.1.1. Isolation of hASC

All protocols involving human subjects were approved by the Institutional Review Board of the Pusan National University. Leftover materials were obtained from individuals undergoing elective abdominoplasty after obtaining informed consent from

each individual. The hASC were isolated according to the methods described in the previous studies (34). Briefly, adipose tissues were digested at 37°C for 30 min with 0.075% type I collagenase. The enzyme activity was neutralized with α-modified Eagle's medium (α-MEM), containing 10% fetal bovine serum (FBS), and the mixture was centrifuged at 2,000 rpm (740 × *g*) for 10 min to obtain a pellet. The pellet was incubated overnight at 37°C/5% CO_2 in a control medium (α-MEM, 10% FBS, Pen/Strep). Following incubation, the tissue culture plates were washed to remove any residual nonadherent cells and were maintained at 37°C/5% CO_2 in the control medium. When the monolayer of adherent cells reached a level of confluence, the cells were trypsinized and subcultured at a concentration of 2,000 cells/cm^2 in α-MEM containing 10% FBS. We used the third to fifth passages of hASC for this experiment.

3.1.2. Purification of Small RNA Molecules

We extracted the small RNA molecules from hASC during osteogenic differentiation hASC using the mirVana miRNA isolation kit and following the manufacturer's instructions (Ambion).

To remove the genomic DNA contamination, treat DNase I in small RNA and incubate at 37°C for 10 min. For precipitation of small RNA, adjust the monovalent cation concentration of the solution (for example to 0.5 M ammonium acetate). Add glycogen to a final concentration of 10–150 μg/ml, mix well, and then mix with 1 volume of isopropanol or 2 volumes of ethanol. Chill the mixture for at least 15 min at –20°C or below, then centrifuge for at least 15 min at ≥10,000 × *g*. Carefully remove the supernatant fluid, and resuspend the small RNA in DEPC-treated water.

The best way to quickly analyze small RNA is to run an aliquot of the preparation on a denaturing 15% polyacrylamide gel. To prepare RNA samples for gel electrophoresis, dissolve RNA samples (1–2 μg of small RNA molecules) in 10 μl of DEPC-treated water (or less) and add an equal volume of 2× gel-loading dye. Heat samples at 95°C for 2–5 min.

3.1.3. Reverse Transcription

miRNA was reverse transcribed by using Ncode miRNA first-strand cDNA synthesis kits (Invitrogen), according to the manufacturer's specification.

miRNA and other noncoding RNA are polyadenylated by poly(A) polymerase and subsequently converted into cDNA by reverse transcriptase with oligo-dT priming. mRNA are converted into cDNA by reverse transcriptase using both oligo-dT and random priming.

3.1.4. Real-Time PCR

The cDNA serves as the template for real-time PCR analysis using the miScript Primer (Invitrogen) in combination with LightCycler® DNA Master SYBR Green I (Roche). A single cDNA preparation is sufficient to interrogate multiple miRNA by real-time PCR

using different miRNA-specific primers. The forward primer sequences are designed to the corresponding mature miRNA sequences. These values are then assessed relative to 5 S rRNA as a normalizing control. The oligonucleotides we used were: hsa-miR-196a primer: 5′-TAGGTAGTTTCATGTTGTTGG-3′, and 5 S primer: 5′-ATACCACCCTGAACGCGCCCGATC-3′.

PCR was performed using a LightCycler Instrument (Roche) at 95°C for 10 min, followed by 50 cycles of 95°C for 10 s, 60°C (primer dependent) for 5 s, and 72°C for 5 s. LightCycler software version 3.3 (Roche Diagnostics) was used to analyze PCR kinetics and calculate quantitative data. A standard curve generated in a separate run was loaded into runs of each sample (without standard curves). For each sample, copy numbers of target gene mRNA were divided by those of 5 S RNA to normalize for target miRNA expression and thus avoid sample-to-sample differences in RNA quantity (see Note 4).

3.2. Northern Blot to Analyze miRNA Expression

Northern blot is a well-established laboratory method for analyzing the presence and level of miRNA genes.

3.2.1. Total RNA Preparation

We use Trizol reagent (Invitrogen) to isolate total RNA from hASC. Compared with other commercial column- or resin-based RNA purification kits, the Trizol protocol gives a better yield and is more reliable in isolating small RNA.

3.2.2. Preparation of Labeled miRNA Probes

Radioactively or nonisotopically labeled RNA, DNA, or oligodeoxynucleotide probes can be used for Northern blots. RNA oligonucleotide probes are used for Northern blots of miRNA. Conventional approaches rely on a radioisotope-labeled probe for Northern blot analysis. However, the usage of isotope-labeled probes is not convenient, especially for routine screening experiments, because of strict safety regulations. As an alternative, the digoxigenin (DIG)-labeling system has several advantages compared with radioactive labeling techniques: high sensitivity, short exposure times, longer shelf life, and increased safety. RNA probes labeled with DIG are generally produced by two methods: an in vitro transcription method using a linearized plasmid DNA or a PCR product as a template that has had a suitable promoter added during amplification; or direct chemical addition of DIG to RNA using total RNA and poly(A)C mRNA (35).

1. Design and synthesize the antisense oligonucleotide probes against the mature miRNA targets and add the T7 promoter sequence (8nt sequence 5′-CCTGTCTC-3′) to the end of the mature miRNA sequence (see Note 5).
2. Resuspend the oligonucleotides template to a concentration of 100 μM in TE buffer.

3. Hybridize the oligonucleotides template to the T7 promoter primer.
   (a) Mix 1 µl T7 promoter primer, 3 µl DNA annealing buffer, and 1 µl oligonucleotides template (100 µM).
   (b) Heat the mixture to 70°C for 5 min.
4. Fill in with Klenow DNA polymerase.
   (a) Add 1 µl Exo Klenow, 1 µl 10× Klenow buffer, 1 µl 10 mM dNTP, and 2 µl nuclease-free water to the hybridized oligonucleotides.
   (b) Incubate at 37°C for 30 min and store at –20°C.
5. DIG labeling.
   (a) Prepare 4 µl dsDNA template by mixing 2 µl DIG RNA labeling mix, 2 µl 10× transcription buffer, 0.5 µl RNase inhibitor, 2 µl T7 RNA polymerase, and 9.5 µl nuclease-free water. Incubate at 37°C for 2 h.
   (b) Add 2 µl DNase I, RNase-free to remove template DNA. Incubate at 37°C for 10 min.
   (c) Stop the reaction by adding 2 µl of 0.2 M EDTA (pH 8.0).
   (d) Removal of unincorporated nucleotides: we recommend a protocol for ammonium acetate/ethanol precipitation. Resuspend the pellet in 20–50 µl of nuclease-free water or TE (see Note 6).

3.2.3. Electrophoresis of RNA Samples

Prepare a 15% acrylamide/8 M urea gel. Prerun the gel at 30 mA for 15 min in 1× TBE. Mix 1–3 µg of the small RNA molecules with an equal volume 2× gel loading buffer and heat sample for 3 min at 95–100°C. Load the RNA samples and run the gel at 30 mA for ~1 h, until the bromophenol blue band reaches the end of the gel. Separate the gel onto plastic wrap and stain the gel with 4 µg/ml EtBr in 1× TBE for 5 min. Examine the EtBr-stained image to evaluate the RNA quality and loading consistency. Discrete transfer RNA (78-nt) and 5 S ribosomal RNA (120-nt) bands indicate a good quality of RNA preparation.

3.2.4. Gel Transfer

Reduce the gel size by cutting off the loading wells and empty lanes. Cut the nylon membranes and six sheets of 3 M Whatman filter paper to the size of the gel. Soak the nylon membrane and filter papers in 0.25× TBE. Assemble the transfer sandwich on the surface of a semidry transfer unit in the following order: three sheets of soaked filter paper, nylon membrane, gel, and three sheets of soaked filter paper. Make marks on the RNA side of the nylon membrane with a pencil. Remove possible air bubbles within the transfer sandwich by gently rolling a plastic serological pipette over the sandwich. Place the top on the semidry transfer unit, and

run the transfer at 200 mA for 1 h. Extending this time does not result in loss of sample. After blotting, keep the membrane damp. Finally, UV crosslink the RNA to the membrane using a commercial crosslinking device (120 mJ burst over 30 s). Store it at –20°C or proceed to the prehybridization step.

3.2.5. Hybridization

Wet the membrane with water, place it in a hybridization tube, and add 10 ml warm Rapid Hybridization Buffer (Amersham Pharmacia Biotech). Rotate at 65°C for 1 h and incubate with 3′-DIG-labeled RNA probe (100 ng/ml) for 15 h at 65°C. Recover probes and store at –20°C (probes can be reused two to three times within a week). Wash one time with 25 ml of 6× SSC/0.2%SDS wash solution in the hybridization oven at 42°C for 10 min. Repeat the wash two more times with 25 ml of washing buffer at room temperature. Finally, wash with washing buffer in the hybridization oven at 42°C for 5 min (see Note 7).

3.2.6. Detection

CDP-Star (Roche), a chemiluminescent substrate for alkaline phosphatase, can be used to detect hybridized probe. In brief, the blot is incubated in 1% Blocking Reagent (Roche) in maleic acid buffer containing 1:20,000 anti-DIG-AP (Roche), then incubated in CDP-Star working solution for 5 min before exposure to X-ray film.

3.3. Ectopic Expression of miRNA in hASC

miRNA are endogenously expressed small ssRNA sequences of ~22 nucleotides in length that naturally direct gene silencing through components shared with the RNAi pathway (36). Unlike shRNA, however, the miRNA Unlike shRNA, however, the miRNA are found embedded, sometimes in clusters, in long primary transcripts of several kilobases in length containing a hairpin structure and driven by RNA polymerase II (37).

Gain-of-function analysis has been widely used for unveiling many of the key protein players in biological processes. This strategy can be a fruitful way for revealing miRNA functions as well (29, 31, 33). To ectopically express miRNA in hASC, the miRNA-encoding oligonucleotides downstream of a polII promoter are inserted into a lentiviral expression vector. The pcDNA 6.2-GW/EmGFP-miR vector (Invitrogen) offers a rapid and efficient way to clone ds oligo duplexes encoding a desired miRNA target sequence into a vector containing a Pol II promoter for use in RNAi analysis. To transfer your pre-miRNA expression cassette into the destination vector, first generate an entry clone by performing a BP recombination reaction between the pcDNA 6.2-GW/EmGFP-miR expression clone and a suitable donor vector (pDONR 221, Invitrogen), then perform an LR recombination reaction between the resulting entry clone and a destination vector (LentiV5/6, Invitrogen). As a marker for infection, emerald green fluorescent protein (EmGFP) is incorporated into the

vector such that the pre-miRNA insertion site is in the 3′ untranslated (3′ UTR) region of the fluorescent protein mRNA. Addition of EmGFP allows tracking of the miRNA expression and provides strong correlation of EmGFP expression with the knockdown of the target gene by your miRNA.

The pre-miRNA that is transcribed from the overexpression construct is then actively transported into the cytoplasm by Exportin 5 in a Ran guanosine triphosphate-dependent manner (38, 39) and further processed into a approximately 21-nt duplex by Dicer in the cytoplasm (40–42).

3.3.1. Design Pre-miRNA Primers

1. Obtain mature miRNA sequences from Rfam – "the miRNA Registry" (http://www.sanger.ac.uk/cgi-bin/Rfam/mirna/browse.pl).
2. To generate the top oligo sequence, combine these elements (from 5′ end to 3′ end):
   (a) 5′-TGCTG.
   (b) Reverse complement of the 21-nucleotide sense target sequence. This is the mature miRNA sequence.
   (c) GTTTTGGCCACTGACTGAC (terminal loop).
   (d) Nucleotides 1–8 (5′–3′) of sense target sequence.
   (e) Nucleotides 11–21 (5′–3′) of sense target sequence.
3. To generate the bottom oligo sequence, perform the following steps:
   (a) Remove 5′ TGCT from top oligo sequence (new sequence starts with G).
   (b) Take the reverse complement of the sequence from step 2.
   (c) Add CCTG to the 5′ end of the sequence from step 3.
4. Annealing oligo.
   (a) Mix 1 µl top strand DNA oligo (10 µM), 1 µl bottom strand DNA oligo (10 µM), 1 µl 10× DNA Annealing Buffer, and 7 µl DNase/RNase-Free Water. Incubate at 95°C for 4 min and 70°C for 10 min.
   (b) Dilute the 1 µM ds oligo mixture 50-fold into DNase/RNase-free water to obtain a final concentration of 20 nM. Store the diluted ds oligo stocks at –20°C (see Note 8).

3.3.2. Generation of miRNA Expression Construct

In this chapter, we describe only the modifications we made to the protocol. A detailed description of the original protocol can be found in the manufacturer's instructions.

The engineered pre-miRNA sequence is cloned into the cloning site of BLOCK-iT Pol II miR RNAi Expression vectors (Invitrogen), which exhibits a co-cistronic expression of EmGFP,

thus allowing the transfection efficiency to be determined by fluorescence microscopy. The construct pcDNATM6.2-GW/EmGFP-miLacZ (targeting β-galactosidase) is used as a control for the effects of any nonspecific RNAi. The pcDNATM6.2-GW/EmGFP-miR vector is recombined into pLenti6/V5 using LR clonase (Invitrogen), resulting in the generation of a pLenti6/V5-miR-196a plasmid. The plasmids are sequenced with the primer to confirm the correctness of the constructed plasmid.

3.3.3. Viral Packaging by Transient Transfection

Replication-defective lentiviruses were produced via the transient transfection of 293FT cells using Lipofectamine Plus (Invitrogen), lentivirus vectors, and a packaging mix (pLP1, pLP2, and pVSVG [Invitrogen]).

1. 293FT cells are 80–90% confluent the day of transfection. Prewarm Opti-MEM (stored in cold room at 4°C) for ~10 min.
2. Add DNA (pLP1:pLP2:pVSVG:lentiviral vector = 1:1:1:2 [5 μg:5 μg:5 μg:10 μg in 10-cm^2 dish]) to an Eppendorf tube in the hood.
3. Add 750 μl of Opti-MEM to the Eppendorf tube to dilute the DNA and 20 μl of Lipofectamine Plus. Incubate for 15 min at room temperature.
4. Add 30 μl of Lipofectamine and 750 μl of Opti-MEM to another Eppendorf tube.
5. Combine tubes generated in steps 3 and 4.
6. Mix well by tapping or vortexing slowly and incubate for 15 min at room temperature.
7. Remove the Lipofectamine–DNA mix by aspiration. Add prewarmed normal media (5 ml).

Two samples of viruses are harvested at 48 and 72 h after transfection, filtered through a Millex-HV 0.45-μm PVDF filter (Millipore); keep at −80°C until use. The transduction of the hASC is performed by exposing the cells to dilutions of the viral supernatant in the presence of polybrene (5 μg/ml) for 6 h.

3.4. Inhibition of miRNA by Oligo Inhibitor

miRNA inhibitors are chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNA molecules. These inhibitors can be introduced into cells using transfection or electroporation parameters similar to those used for siRNA, and enable detailed study of miRNA biological effects. Use of the miRNA inhibitors enables miRNA functional analysis by downregulation of miRNA activity. Specific experimental designs include.

Anti-miR™ miRNA inhibitors (Anti-miRs) and scrambled RNA oligomer (can be purchased from Ambion). These can be

transfected into hASC at a final concentration of 50 nM using DharmaFECT Transfection Reagent as per the manufacturer's instructions.

3.5. Luciferase Assay for Demonstrating Direct Binding of miRNA to Target mRNA

miRNA target the 3′-untranslated region of messenger RNA sequences, affect RNA stability, and prevent translation. HOXC8 is one of the known targets of miR-196a. To assess whether a specific miRNA can target putative targets, we validated the binding of the miRNA with HOXC8 mRNA using a luciferase reporter bearing miRNA target sequences cloned into its 3′-UTR.

3.5.1. Reporter Vectors and DNA Constructs

To construct reporter vectors bearing miRNA-binding sites, a putative miRNA196a-recognition element (as single copy) from the *HOXC8* gene can be cloned in the 3′-untranslated region (UTR) of the firefly luciferase reporter vector. The oligonucleotide sequences are designed to carry the HindIII and SpeI sites. The oligonucleotides are: pMIR-WT HOXC8, 5′-CTAGTCCCAACAACTGAG ACTGCCTA A-3′ (SpeI recognition sequence underlined), 5′-AGCTT TAGGCAGTCTCAGTTGTTGGG A-3′; (HindIII recognition sequence underlined), pMIR-Mut HOXC8, 5′-CTAGT ACCGAAAGTTTAGCCCGCTTA A-3′, 5′-AGCTT TAAGCGGG CTAAACTTTCGGT A-3′.

1. The sense and antisense strands of the oligonucleotides are annealed by adding 2 μg of each oligonucleotide to 46 μl of annealing solution (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, and 2 mM magnesium acetate) and incubating at 90°C for 5 min and then at 37°C for 1 h.
2. pMIR-Report luciferase vector (Ambion, Inc.) is linearized by HindIII/SpeI restriction. We typically retrieve the vector from the restriction digestion by agarose gel electrophoresis and purification of the band corresponding to digested pMIR-REPORT Luciferase.
3. Ligate the insert into pMIR-REPORT Luciferase using T4 ligase. We used a three- to tenfold molar excess of insert into prepared pMIR-REPORT Luciferase. It is also a good idea to include a no-insert negative control ligation in the experiment.
4. Transform *E. coli* with the ligation products.

3.5.2. Reporter Gene Assay

All transient transfections are conducted using Lipofectamine Plus Reagent (Invitrogen). The pMIR-report, pMIR-*HOXC8*, and pMIR-Mut HOXC8 plasmids are transfected with a β-galactosidase construct (pMIR-REPORT-β-gal control plasmid). The transfections are performed in duplicate, and all experiments are repeated several times. The luciferase assays are normalized according to their β-galactosidase activity. Note, in all cases, the total amount of DNA used for the transfection is 2.0 μg.

3.5.2.1. Luciferase Assay

1. Remove the growth medium from the cells to be assayed 48 h after transfection. Rinse the cells twice in PBS buffer, being careful not to dislodge any of the cells.
2. Add a minimal volume of 1× CCLR to cover the cells (100 µl for a 60-mm dish) and incubate for a short period at room temperature.
3. Scrape the attached cells free from the culture dish and transfer the cells and solution to a microcentrifuge tube and vortex mix for 30 s. Centrifuge for 5 s at 12,000 rpm (8,000 × *g*) to pellet the cell debris. Transfer the supernatant (cell extract) to a new tube and discard the pelleted cell debris.
4. Mix 50 µg of room temperature cell extract with 100 µl room temperature Luciferase Assay Reagent (Promega). Place the reaction in a luminometer.
5. Measure the light produced for 10 s.
6. Record data.

3.5.2.2. Measurement of β-Galactosidase Activity

1. Add a volume of reagent equal to the volume of culture medium in each well. For 96-well plates, typically 100 µl of reagent is added to 100 µl cell lysate (10 µg).
2. Mix the sample contents for 30 s using a plate shaker.
3. Incubate the samples for at least 30 min at room.
4. Place the reaction in a luminometer. Measure the light produced for 2 s.
5. Record data.

3.6. Analysis of miRNA Roles in Osteogenic Differentiation

hASC contain a population of cells capable of differentiating along multiple mesenchymal cell lineages. Optimal osteogenic differentiation can be determined by osteoblastic morphology, expression of alkaline phosphatase (APase), reactivity with anti-osteogenic cell surface monoclonal antibodies, modulation of osteocalcin mRNA production, and the formation of a mineralized extracellular matrix. Cultured hMSC provide a useful model for evaluating the multiple factors responsible for the stepwise progression of cells from undifferentiated precursors to osteoblasts, and eventually terminally differentiated osteocytes (43).

3.6.1. Induction of Osteogenic Differentiation

1. Plate the cell suspension in 10% α-MEM at a density of 50,000 cells/well in a 12-well culture dish with 1-ml volume per well.
2. Incubate the cells at 37°C in a 5% CO_2 humidified incubator overnight. *Note*: Cells should be attached and 90% confluent after overnight incubation. If they are not confluent, replace medium every 3–4 days until the cells are confluent. It is important that the cells be 90% confluent before initiating osteogenesis differentiation.

3. When the cells are 90% confluent, carefully aspirate the medium from each well and add 1 ml Osteogenesis Induction Medium. This medium change corresponds to differentiation day 1.
4. Replace with fresh Osteogenesis Induction Medium every 2–3 days for 10–14 days.

3.6.2. Confirmation of Osteogenic Differentiation

Extracellular matrix calcification is estimated using Alizarin Red S stain.

1. Carefully aspirate the medium from each well. Be careful to not aspirate the cells.
2. Fix osteocytes by incubating in 4% paraformaldehyde for 10 min at room temperature.
3. Carefully aspirate fixing solution and rinse twice (5–10 min each) with water.
4. Aspirate the water and add enough Alizarin Red Solution to cover the wells.
5. Incubate at room temperature for 30 min.
6. After 30 min, remove the Alizarin Red Solution and wash the wells four times with 1 ml water. Aspirate after each wash.

Note: Osteocytes containing calcium deposits will be stained orange-red by the Alizarin Red Solution.

4. Notes

1. Wear laboratory gloves at all times during this procedure and change them frequently. They will protect you from getting the reagent on your skin. Use RNase-free pipette tips to handle the wash solutions and the Elution Solution.
2. Prepare all reagents used in this protocol with DEPC-treated H_2O.
3. Do not treat TBE with DEPC-treated H_2O.
4. Since PCR is a powerful technique capable of amplifying trace amounts of DNA, all appropriate precautions should be taken to avoid cross-contamination.
5. miRNA probe sequences were selected based on published sequences listed in the miRNA Registry (http://www.sanger.ac.uk/Software/Rfam/mirna/).
6. We recommended gel purification of the probes. This can be done directly after the DNase I treatment. Do not phenol/chloroform extract your DIG-labeled probe because it will partition into the organic phase. Labeled probes are stable for

a minimum of 1 year when stored at –15 to –25°C. Avoid repeated freezing and thawing of the labeled probe.

7. Do not allow the membrane to dry out at any stage during the washing procedure. Hybridization solutions containing labeled RNA can be stored frozen and reused, provided that all solutions are RNase free.
8. When performing the dilutions, be careful not to cross-contaminate the different ds oligo stocks. Remember to wear gloves and change pipette tips after every manipulation. When using the diluted ds oligo stock solutions, thaw the solutions on ice. When using the 20 nM ds oligo stock solution for cloning, thaw the solution on ice. Do not thaw the ds oligo by heating or the ds oligo duplexes may melt and form intramolecular hairpin structures. After use, return the tube to –20°C storage.

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References

1. Nelson P., Kiriakidou M., Sharma A., Maniataki E., Mourelatos Z. (2003) The microRNA world: Small is mighty. *Trends Biochem Sci* **28**, 534–540.
2. Lee R.C., Feinbaum R.L., Ambros V. (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843–854.
3. Rodriguez A., Griffiths-Jones S., Ashurst J.L., Bradley A. (2004) Identification of mammalian microRNA host genes and transcription units. *Genome Res* **14**, 1902–1910.
4. Xu P., Vernooy S.Y., Guo M., Hay B.A. (2003) The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. *Curr Biol* **13**, 790–795.
5. Brennecke J., Hipfner D.R., Stark A., Russell R.B., Cohen S.M. (2003) Bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell* **113**, 25–36.
6. Johnston R.J., Hobert O. (2003) A microRNA controlling left/right neuronal asymmetry in *Caenorhabditis elegans. Nature* **426**, 845–849.
7. Esquela-Kerscher A., Slack F. (2006) Oncomirs-microRNAs with a role in cancer. *Nat Rev Cancer* **6**, 259–269.
8. Yi R., Poy M.N., Stoffel M., Fuchs E.A. (2008) Skin microRNA promotes differentiation by repressing "stemness." *Nature* **452**, 225–229.
9. Chen C.Z., Li L., Lodish H.F., Bartel D.P. (2004) MicroRNAs modulate hematopoietic lineage differentiation. *Science* **303**, 83–86.
10. Naguibneva I., Ameyar-Zazoua M., Polesskaya A., Ait-Si-Ali S., Groisman R., Souidi M., Cuvellier S., Harel-Bellan A. (2006) The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation. *Nat Cell Biol* **8**, 278–284.
11. Yekta S., Shih I.H., Bartel D.P. (2004) MicroRNA-directed cleavage of HOXB8 mRNA. *Science* **304**, 594–596.
12. Schratt G.M., Tuebing F., Nigh E.A., Kane C.G., Sabatini M.E., Kiebler M., Greenberg M.E. (2006) A brain-specific microRNA regulates dendritic spine development. *Nature* **19**, 283–289.

13. Rao P.K., Kumar R.M., Farkhondeh M., Baskerville S., Lodish H.F. (2006) Myogenic factors that regulate expression of muscle specific microRNAs. *Proc Natl Acad Sci U S A* **6**, 8721–8726.
14. Kim H.K., Lee Y.S., Sivaprasad U., Malhotra A., Dutta A. (2006) Muscle-specific microRNA miR-206 promotes muscle differentiation. *J Cell Biol* **28**, 677–687.
15. Esau C., Kang X., Peralta E., Hanson E., Marcusson E.G., Ravichandran L.V., Sun Y., Koo S., Perera R.J., Jain R., Dean N.M., Freier S.M., Bennett C.F., Lollo B., Griffey R. (2004) MicroRNA- 143 regulates adipocyte differentiation. *J Biol Chem* **279**, 52361–52365.
16. Luzi E., Marini F., Sala S.C., Tognarini I., Galli G., Brandi M.L. (2008) Osteogenic differentiation of human adipose tissue–derived stem cells is modulated by the miR-26a targeting of the SMAD1 transcription factor. *J Bone Miner Res* **23**, 287–295.
17. Zuk P.A., Zhu M., Mizuno H., Huang J., Futrell J.W., Katz A.J., Benhaim P., Lorenz H.P., Hedrick M.H. (2001) Multilineage cells from human adipose tissue: Implications for cell-based therapies. *Tissue Eng* **7**, 211–228.
18. Zuk P.A., Zhu M., Ashjian P., De Ugarte D.A., Huang J.I., Mizuno H., Alfonso Z.C., Fraser J.K., Benhaim P., Hedrick M.H. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279–4295.
19. Rodriguez A.M., Elabd C., Amri E.Z., Ailhaud G., Dani C. (2005) The human adipose tissue is a source of multipotent stem cells. *Biochimie* **87**, 125–128.
20. Gimble J.M., Guilak F. (2003) Differentiation potential of adipose derived adult stem (ADAS) cells. *Curr Top Dev Biol* **58**, 137–160.
21. Hicok K.C., Du Laney T.V., Zhou Y.S., Halvorsen Y.D., Hitt D.C., Cooper L.F., Gimble J.M. (2004) Human adipose-derived adult stem cells produce osteoid in vivo. *Tissue Eng* **10**, 371–380.
22. Kim Y.J., Bae S.W., Yu S.S., Bae Y.C., Jung J.S. (2010) miR-196a regulates proliferation and osteogenic differentiation in mesenchymal stem cells derived from human adipose tissue. *J Bone Miner Res* **24**, 816–825.
23. Lee R.C., Ambros V. (2001) An extensive class of small RNAs in *Caenorhabditis elegans. Science* **294**, 862–864.
24. Lau N.C., Lim L.P., Weinstein E.G., Bartel D.P. (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans. Science* **294**, 858–862.
25. Lagos-Quintana M., Rauhut R., Lendeckel W., Tuschl T. (2001) Identification of novel genes coding for small expressed RNAs. *Science* **294**, 853–858.
26. Liang R.Q., Li W., Li Y., et al. (2005) An oligonucleotide microarray for microRNA expression analysis based on labeling RNA with quantum dot and nanogold probe. *Nucleic Acids Res* **33**, e17.
27. Baskerville S., Bartel D.P. (2005) Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA* **11**, 241–247.
28. Sioud M., Rosok O. (2004) Profiling microRNA expression using sensitive cDNA probes and filter arrays. *Biotechniques* **37 (574–576)**, 578–580.
29. Miska E.A., Alvarez-Saavedra E., Townsend M., et al. (2004) Microarray analysis of microRNA expression in the developing mammalian brain. *Genome Biol* **5**, R68.
30. Liu C.G., Calin G.A., Meloon B., et al. (2004) An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc Natl Acad Sci U S A* **101**, 9740–9744.
31. Barad O., Meiri E., Avniel A., et al. (2004) MicroRNA expression detected by oligonucleotides microarrays: system establishment and expression profiling in human tissues. *Genome Res* **14**, 2486–2494.
32. Krichevsky A.M., King K.S., Donahue C.P., Khrapko K., Kosik K.S. (2003) A microRNA array reveals extensive regulation of microRNAs during brain development. *RNA* **9**, 1274–1281.
33. Allawi H.T., Dahlberg J.E., Olson S., et al. (2004) Quantitation of microRNAs using a modified Invader assay. *RNA* **10**, 1153–1161.
34. Kim Y.J., Kim H.K., Cho H.H., Bae Y.C., Suh K.S., Jung J.S. (2007) Direct comparison of human mesenchymal stem cells derived from adipose tissues and bone marrow in mediating neovascularization in response to vascular ischemia. *Cell Physiol Biochem* **20**, 867–876.
35. Ramkissoon S.H., Mainwaring L.A., Sloand E.M., Young N.S., Kajigaya S. (2006) Nonisotopic detection of microRNA using digoxigenin labeled RNA probes. *Mol Cell Probes* **20(1)**, 1–4.
36. Bartel D.P., Chen C.Z. (2004) Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat Rev Genet* **5**(5), 396–400.
37. Lee Y., Kim M., Han J., Yeom K.H., Lee S., Baek S.H., Kim V.N. (2004) MicroRNA genes are transcribed by RNA polymerase II. *Embo J* **23**, 4051–4060.

38. Yi R., Qin Y., Macara I.G., Cullen B.R. (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* **17**, 3011–3016.
39. Lund E., Guttinger S., Calado A., Dahlberg J.E., Kutay U. (2004) Nuclear export of microRNA precursors. *Science* **303**, 95–98.
40. Grishok A., Pasquinelli A.E., Conte D., et al. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* **106**, 23–24.
41. Ketting R.F., Fischer S.E., Bernstein E., Sijen T., Hannon G.J., Plasterk R.H. (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans. Genes Dev* **15**, 2654–2659.
42. Hutvagner G., McLachlan J., Pasquinelli A.E., Balint E., Tuschl T., Zamore P.D. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the *let-7* small temporal RNA. *Science* **293**, 834–838.
43. Jaiswal N., Haynesworth S.E., Caplan A.I., Bruder S.P. (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem* **64(2)**, 295–312.

Chapter 30

The Generation and the Manipulation of Human Multipotent Adipose-Derived Stem Cells

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Abstract

In this chapter, we describe a method to isolate and to expand multipotent adipose-derived stem (hMADS) cells from human adipose tissue. We also describe culture conditions to differentiate them into adipocytes at a high rate. This culture system provides a powerful means for studying the first steps of human adipose cell development and a route for investigating effects of drugs on the biology of adipocytes. Finally, we provide a protocol to investigate gene function during proliferation and differentiation of hMADS cells by means of siRNA-mediated gene silencing approaches or forced expression by transducing hMADS cells permissive to infection with murine retrovirus vectors.

Key words: Human mesenchymal stem cells, Adipose tissue, Human adipogenesis, hMADS cells

1. Introduction

Up to now, preadipocyte clonal lines from rodents have been mainly used to gain insight into cellular and molecular mechanisms of adipogenesis (1). Much less is known about the molecular regulation of human adipogenesis, partly due to the absence of appropriate human cellular models. Primary cultures of preadipocytes derived from the stromal-vascular fraction (SVF) of adipose tissue, although able to differentiate into adipocytes in vitro, undergo a dramatic decrease in their ability to differentiate before growth arrest and replicative senescence with serial subculturing, making it difficult to investigate molecular mechanisms in a fully reproducible manner. Recently, we have isolated multipotent stem cells from the SVF of infant adipose tissues, cells called

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human multipotent adipose-derived stem (hMADS) cells (2). Diverse terms, from adipose mesenchymal stem cells to preadipocytes, have been used to name stem cell populations isolated from adipose tissue (for discussion see (3, 4)). The International Federation of Adipose Therapeutics and Science (IFATS) reached a consensus to adopt the term "adipose-derived stem cells (ASCs)." The name hMADS cells has been utilized as these cells display additional and specific characteristics. hMADS cells are isolated from infant adipose tissues and hMADS cell lines can be established. Cells exhibit the characteristics of mesenchymal stem cells, i.e., the capacity for self-renewal, as cells can be expanded in vitro for more than 160 population doublings (i.e., around 30 passages) while maintaining a normal diploid karyotype and the potential to undergo differentiation into adipocytes, osteoblasts, and chondrocytes at the single-cell level (2, 5). When transplanted into animal models for muscular dystrophies, hMADS cells participate in muscle regeneration, emphasizing their therapeutic potential (2, 6). Several other papers describe the therapeutic potential of stem cells derived from human adipose tissue. In vitro, these cells enter the adipose lineage at a high rate, the differentiation yield is estimated at more than 80%, and they differentiate into cells that display a unique combination of properties similar, if not identical, to those of native human adipocytes (7, 8). Therefore, the hMADS cell line is an appropriate model to study human fat cell metabolism. Cells can be genetically modified, making them a powerful model to investigate function of genes during the earliest step of human adipogenesis.

2. Materials

2.1. Isolation of MADS Cells from Human Adipose Tissue

1. Growth medium: DMEM low glucose, supplemented with 1× glutamine, 10 mM HEPES buffer, penicillin–streptomycin 5,000 IU/ml–5,000 µg/ml (Pen/Strep), and 10% fetal bovine serum (FBS).
2. Collagenase A: 2 mg collagenase A/ml supplemented with 20 mg BSA (Fraction V). The solution is freshly prepared in PBS and filtered (0.2 µm).

2.2. Expansion of hMADS Cells and Thawing Cells from Freezing Tubes in Liquid Nitrogen

1. Growth medium (see Subheading 2.1, item 1).
2. Solution of 5 µg/ml of human FGF2 (see Note 1).
3. Solution of trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA) (1 mM).
4. Solution of DMSO/FBS (10/10%) in growth medium.

2.3. Adipocyte Differentiation of hMADS Cells

1. Differentiation medium: DMEM low glucose/Ham's F12 (50/50) supplemented with glutamine, HEPES buffer 1 M, and Pen/Strep and an adipogenic cocktail consisting of (final concentrations) insulin (5 µg/ml), transferrin (10 µg/ml), T3 (0.2 nM), rosiglitazone (1 µM), IBMX (100 µM), and dexamethasone (1 µM) (see Notes 2–5).

2.4. siRNA-Mediated Gene Silencing in hMADS Cells

1. siRNA transfection medium: 60% DMEM low glucose, 40% MCDB-201, 1× ITS, dexamethasone (10^{-9} M), ascorbic sodium acid (100 mM). This medium is supplemented with 2% FBS or 0.5% FBS with or without 2.5 ng/ml FGF2.
2. HiPerfect reagent.

2.5. Retroviral Infection of hMADS-EcoRec Cells

1. Ecotropic retrovirus vector.
2. Plat-E cells.
3. Growth medium.
4. Fungene 6 transfection reagent.
5. Polybrene 5 mg/ml.
6. Blasticidin 5 mg/ml.

3. Methods

3.1. Isolation of hMADS Cells from Adipose Tissue

hMADS cells were obtained from the SVF of human adipose tissue of young donors as described previously (2). Discarded adipose tissue was collected during surgery with the informed consent of the parents, as approved by the Centre Hospitalier Universitaire de Nice Review Board.

1. Adipose tissue (from 0.2 to 2 mg) is transported from the hospital to the laboratory in 15 ml of growth medium immediately after the biopsy (see Note 6).
2. At the laboratory, biopsies are cut into small fragments with forceps in PBS in a culture dish, under a flow laminar hood. Tissues with no adipose tissue (white, soft and easy to shred) appearance are removed and discard (some biopsies contain a majority of nonadipose tissue). Then, small fragments are digested with 1 ml of collagenase A/BSA solution.
3. Collagenase digestion is performed at 37°C in a water bath. Shake the tube vigorously every 5 min until dissociation of the fragments. It takes between 10 and 20 min depending on the size of the adipose tissue fragments. We prefer to stop collagenase digestion after 20 min even if a few fragments are not totally dissolved.

4. Stop collagenase digestion by adding 10 ml growth medium. The crude SVF is separated from the adipocyte fraction by centrifugation at 200×*g*, 10 min at room temperature.
5. The adipocyte fraction (upper fraction) is discarded and cells from the SVF are seeded onto one (or two if starting with 2 g of tissue) 100-mm culture dish in 10 ml of growth medium.
6. The day after, culture medium is eliminated and adherent cells are maintained for expansion.

3.2. Expansion of hMADS Cells and Thawing Cells from Freezing Tubes in Liquid Nitrogen

Adherent cells are dissociated when they reach 80% confluence. hMADS cells are shown to be able to proliferate in culture for more than 30 passages without reaching senescence. However, during propagation in vitro, we noticed that the cell morphology changed dramatically, from spindle-shaped cells to large and flat cells (see Fig. 1). This morphological change is accompanied by a change in cell proliferation ability. Two major stages of cell proliferation could be identified: a fast-cycling stage, which extended until passage 15–20, followed by a slow-cycling stage. Fast-cycling cells exhibited an average population doubling time of 36 h, whereas slow-cycling cells only divided every 72–96 h. At that stage, addition of FGF2 is required both to maintain hMADS cells in a fast-cycling state and to retain their differentiation potential (for details, see (5)).

1. For a 100-mm dish, aspirate medium off and wash twice with 10 ml of PBS. Aspirate off the PBS and add 1 ml of trypsin solution. Ensure the trypsin covers the cell monolayer and incubate at 37°C, 5% CO_2, for 2–3 min. Check under an inverted microscope that cells are detached from the bottom of the dish.
2. Add 10 ml of growth medium to stop trypsinization and suspend the cells by pipetting. Transfer the cells to a sterile tube and centrifuge at 250×*g* for 5 min at room temperature.

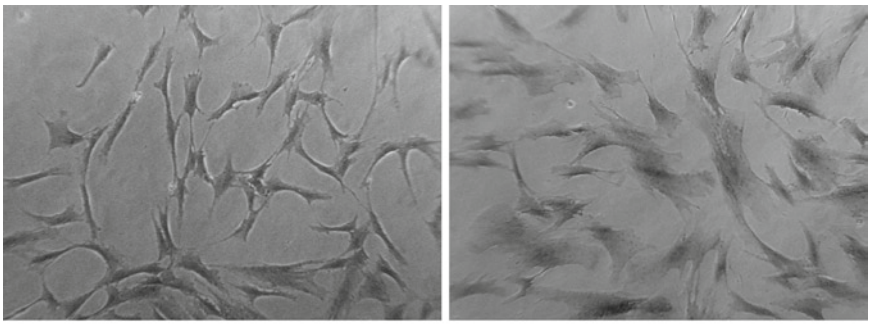

Fig. 1. Change in morphology of hMADS cells as the number of passage increases. Light microscopy photographs of hMADS cells stained with crystal violet.

3. Aspirate the medium off and resuspend the cell pellet with 5 ml of growth medium by pipetting up and down two or three times. Count the cells.
4. Add 2.5×10^5 cells into 10 ml of prewarmed growth medium then transfer to a 100-mm dish.
5. Change the medium every other day.
6. Trypsinize the cultures 3–4 days later, as in step 1. Cultures should be subcultured before cells have reached confluence (*s*ee Note 7).
7. Cell harvesting for freezing tubes in liquid nitrogen: Centrifuge 5×10^5 cells at $250 \times g$ for 5 min at room temperature. Aspirate the supernatant off and resuspend cells in 1 ml of growth medium containing 10% DMSO. Fill the cell suspension in one freezing tube and put the tube at −80°C for one night and then in liquid nitrogen for storage.
8. Thawing cells from freezing tubes: Thaw the tubes by holding them in a 37°C water bath. Immediately after thawing, transfer cells into a 15-ml tube containing 5 ml of prewarmed growth medium. Centrifuge at $250 \times g$ for 5 min at room temperature. Discard the supernatant and resuspend the cell pellet in 10 ml growth medium and plate onto a 100-mm dish. Incubate the dish in the incubator (37°C, 5% CO_2).

3.3. Adipocyte Differentiation of hMADS Cells

1. Plate cells at a high density, i.e., 4×10^4 cells/cm^2 in growth medium containing 2.5 ng/ml FGF2.
2. Two days after, switch cells into growth medium with no FGF2. The day after cells have reached confluence, change growth medium for differentiation medium (see Note 8).
3. Three days later, switch cells into differentiation medium but with no IBMX and no dexamethasone.
4. Adipocytes should appear 5–7 days after induction of the differentiation. Adipose cells with lipid droplets are easily visualized microscopically, especially under bright field illumination, or after Oil Red staining (Fig. 2).

3.4. siRNA-Mediated Gene Silencing in hMADS Cells

Cells are maintained in siRNA transfection medium and are transfected with small interfering RNA (siRNA) duplexes using HiPerfect reagent either during the exponential growth cell phase to investigate gene function in cell proliferation, or a day before confluence to investigate gene function on differentiation.

1. Cells are plated at the 28×10^3 cells/12-well plate or at 7×10^4 cells/24-well plate for studies in proliferation or differentiation, respectively, in the siRNA transfection medium supplemented with 2% FBS and 2.5 ng/ml FGF2 the day before transfection.

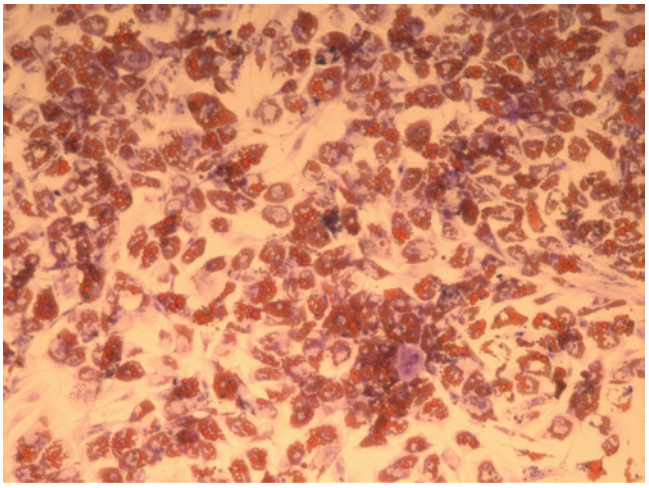

Fig. 2. Adipocytes derived from hMADS cells. Cells were maintained for 10 days in adipogenic medium as described. Then, cells were fixed and stained with Oil Red O for lipid droplets and with crystal violet.

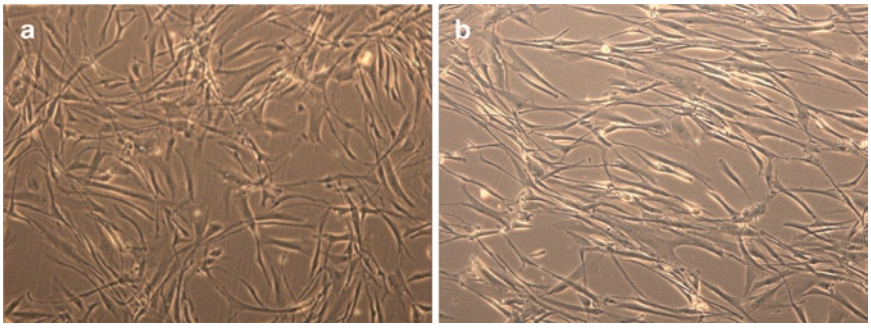

Fig. 3. hMADS cells transfected with siRNAs. Cells were plated at 28×10^3 cells/12-well plate and maintained for 5 days in the absence (**a**) or the presence (**b**) of a HiPerfect-scrambled siRNA mixture.

2. Change the medium to siRNA transfection medium supplemented with 0.5% FBS with no FGF2 1 h before transfection (1 ml/24 well plate).
3. Under the hood, prepare the siRNA transfection mixture in a 15-ml tube. For each transfected well of a 12-well plate, add: 3 µl siRNA 2 µM + 97 µl siRNA transfection medium with no serum + 6 µl HiPerfect. Mix gently and let sit for 10 min. Then, add drop by drop the 106 µl of the HiPerfect–siRNA mixture into the well.
4. The HiPerfect–siRNA mixture can be maintained throughout the experiment for proliferation studies, or can be maintained present for the first 3 days of differentiation, as no toxic effect of a HiPerfect-scrambled siRNA mixture has been observed (see Fig. 3).

3.5. Retroviral Infection of hMADS-EcoRec Cells

hMADS cells permissive to infection with murine retrovirus have been established. For that purpose, cells have been transduced by a lentivirus vector expressing the ecotropic receptor (EcoRec), according to standard procedures. This hMADS-EcoRec cell line allows the infection of human cells at a high efficiency with virus expressing genes having potential oncogenic properties and without safety issues for the operators. The hMADS-Ecorec cell line is resistant to blasticidin (see Note 9).

1. Transfection of Plat-E cells and retrovirus production: Plat-E cells are transfected with retrovirus vectors using the Fungene 6 transfection reagent. Plat-E cells (4×10^6 cells/10-cm dish) are plated the day before transfection (see Note 10).
2. The day of transfection: Mix 300 µl DMEM (with no antibiotics and no serum) with 27 µl Fungene 6 transfection reagent in a 15-ml tube (see Note 11). Mix gently by finger tapping. Leave for 5 min at room temperature.
3. Add 9 µg of each retrovirus DNA into the DMEM/Fungene mix. Mix gently by finger tapping and leave at room temperature for around 20 min.
4. Add the DNA/Fungene complex drop by drop into the Plat-E plate. Incubate overnight at 37°C.
5. The day after transfection, aspirate the transfection reagent-containing medium. Add new medium containing Pen/Strep and return cells to the incubator.
6. At 24 h, collect the medium from the Plat-E dish by using a sterile disposable syringe. Filtering the medium through a 0.22-µm-pore size cellulose acetate filter, and transfer it into a Falcon tube. Add 10 ml of medium containing antibiotics to the Plat-E dish for the second collection the day after. Important: Plat-E cells attach poorly to the dish. Add medium very gently, on one "corner" of the plate and return plate to 37°C for 24 h.
7. Transduction of hMADS-EcoRec cells. hMADS-EcoRec cells are maintained in growth medium in the presence of 2.5 µg/ml blasticidin. The day before transduction, cells are plated at 3×10^5 cells/10-cm plate with FGF2 (2.5 ng/ml).
8. The day of transduction: change the growth medium for 10 ml of the first supernatant containing retrovirus completed with 5 µg/ml Polybrene and 2.5 ng/ml FGF2.
9. The day after, change the medium by adding 10 ml of the second supernatant containing retrovirus complete with 5 µg/ml Polybrene and 2.5 ng/ml FGF2. This step is optional (see Note 12).
10. The day after transduction, remove the medium containing virus and change for growth medium containing FGF2 for 24 h.

11. Split the cells and plate them at low density to investigate the effects on cell proliferation, or at cell confluence to investigate the effects on the adipocyte differentiation.

4. Notes

1. Proliferation medium for expansion of hMADS cells is supplemented with 2.5 ng/ml FGF2 when cells reach passage 15–20, as previously reported (5).
2. Transferrin is prepared at 1 mg/ml in PBS and sterilized by filtration. Aliquot (1 ml) and store at –20°C.
3. IBMX is prepared at 100 mM in H_2O. For this, dissolve 250 mg in 9 ml H_2O and add NaOH 10 N (around 5–10 µl) until the pH is around 12. Bring up to 11 ml H_2O, sterilize by filtration, and store aliquots at –20°C.
4. Dexamethasone is prepared at 10^{-3} M in ethanol. Dissolve 3.9 mg in 10 ml ethanol.
5. Rosiglitazone (BRL4953) was a gift from Dr. J.F. Dole (GlaxoSMithKline, King of Prussia, PA, USA).
6. The efficiency of hMADS cell isolation seems to decrease when biopsies are stored compared with immediate use. However, no comparative studies have been carried out to confirm this issue.
7. The plate should contain between 1 and 1.5×10^6 cells.
8. The addition of rosiglitazone is required to differentiate cells into adipocytes.
9. Generation of hMADS-EcoRec cells. Briefly, 293 FT cells were transfected with pLenti6/Ubc-Slc7a1 (Addgene plasmid 17224) along with packaging mix using Lipofectamine 2000, according to the supplier's instructions. Forty-eight hours after transfection, the supernatant was collected, filtered through a 0.45-mm-pore size filter, and transferred to dishes containing proliferating hMADS3 cells for 24 h in the presence of 5 µg/ml polybrene. Cells expressing the mouse receptor for retrovirus were then selected in the presence of 2.5 µg/ml blasticidin. hMADS cells and hMADS-EcoRec cells displayed a similar proliferation rate and the same capacity to undergo differentiation into adipocytes. These data indicated that infection of hMADS cells with a lentivirus vector expressing the mouse receptor did not modify their proliferation and differentiation potential.
10. Do not add antibiotics (Pen/Strep) into the culture medium when cells are transfected. Plat-E cells attach poorly to the plate.

11. Important: do not touch the wall of the tube when adding Fungene. Add the reagent directly into the DMEM medium.
12. The second round of transduction is optional. Using retrovirus vector expressing GFP, around 80% of hMADS-EcoRec cells can be transduced after the first round. Depending on the efficiency of the transfection of Plat-E cells, on the titer of the supernatant, and the level of transgene wished, a second round could be performed. However, keep in mind that a high copy number of retrovirus integrated in the hMADS genome could have side effects on the proliferation and differentiation. GFP-retrovirus-transduced hMADS cells is the appropriate control to run in parallel with hMADS cells transduced with the gene of interest.

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References

1. Rosen ED, Spiegelman BM. (2000) Molecular regulation of adipogenesis. Annu Rev Cell Dev Biol;16:145–71.
2. Rodriguez A-M, Pisani D, Dechesne CA, et al. (2005) Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. J Exp Med;201(9):1397–405.
3. Mitchell JB, McIntosh K, Zvonic S, et al. (2006) Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells;24(2):376–85.
4. Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C. (2008) Adipose-derived stem cells: isolation, expansion and differentiation. Methods;45(2):115–20.
5. Zaragosi LE, Ailhaud G, Dani C. (2006) Autocrine fibroblast growth factor 2 signaling is critical for self-renewal of human multipotent adipose-derived stem cells. Stem Cells;24(11):2412–9.
6. Goudenege S, Pisani DF, Wdziekonski B, et al. (2009) Enhancement of myogenic and muscle repair capacities of human adipose-derived stem cells with forced expression of MyoD. Mol Ther;17(6):1064–72.
7. Poitou C, Divoux A, Faty A, et al. (2009) Role of serum amyloid a in adipocyte-macrophage cross talk and adipocyte cholesterol efflux. J Clin Endocrinol Metab;94(5):1810–7.
8. Bezaire V, Mairal A, Ribet C, Lefort C, Girousse A, Jocken J, Laurencikiene J, Anesia R, Rodriguez A-M, Ryden M, Stenson BM, Dani C, Ailhaud G, Arner P, Langin D. (2009) Contribution of adipose triglyceride lipase and hormone-sensitive lipase to lypolysis in human hMADS adipocytes. J Biol Chem;284(27):18282–91.

Chapter 31

Cell Sheet Technology for Tissue Engineering: The Self-Assembly Approach Using Adipose-Derived Stromal Cells

Benoît Labbé, Guillaume Marceau-Fortier, and Julie Fradette

Abstract

In the past years, adipose tissue has spurred a wide interest, not only as a source of adult multipotent stem cells but also as a highly eligible tissue for reconstructive surgery procedures. Tissue engineering is one field of regenerative medicine progressing at great strides in part due to its important use of adipose-derived stem/stromal cells (ASCs). The development of diversified technologies combining ASCs with various biomaterials has lead to the reconstruction of numerous types of tissue-engineered substitutes such as bone, cartilage, and adipose tissues from rodent, porcine, or human ASCs. We have recently achieved the reconstruction of connective and adipose tissues composed entirely of cultured human ASCs and their secreted endogenous extracellular matrix components by a methodology known as the self-assembly approach of tissue engineering. The latter is based on the stimulation of ASCs to secrete and assemble matrix components in culture, leading to the production of cell sheets that can be manipulated and further assembled into thicker multilayer tissues. In this chapter, protocols to generate both reconstructed connective and adipocyte-containing tissues using the self-assembly approach are described in detail. The methods include amplification and cell banking of human ASCs, as well as culture protocols for the production of individual stromal and adipose sheets, which are the building blocks for the reconstruction of multilayered human connective and adipose tissues, respectively.

Key words: Mesenchymal stem cells, Adipose tissue, Adipose substitutes, Reconstructed tissues, Tissue engineering, Self-assembly, Cell sheet, Connective tissue, Extracellular matrix

1. Introduction

Regenerative medicine and tissue engineering both harness the potential of human cells to repair, regenerate or even recreate a tissue/organ with the goal of restoring the architecture and functionality of the tissue of origin. Mesenchymal cell populations are prime candidates for such purposes since they harbour subpopulations of cells exhibiting multipotentiality as well as beneficial

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secretory functions stimulating repair. Such mesenchymal stem cells have now been isolated from a variety of connective tissues and stroma including bone marrow (1–4), dermis (5, 6), umbilical cord Wharton's jelly (7, 8) and adipose tissue (9). It is also the hallmark of mesenchymal cell types to produce and reorganize extracellular matrix (ECM) elements to ensure homeostasis and repair of connective tissues throughout adult life. As such, dermal fibroblasts have been widely used for tissue-engineering applications and have proven their ability to reconstitute in vitro or repair stromal compartments such as dermis after grafting (10–14). The use of adipose-derived stromal/stem cells (ASCs) has rapidly expanded over the last years and their potential for regenerative therapies are now described in an increasing number of studies. This chapter highlights how we harnessed their natural propensity to produce and efficiently organize ECM elements in a tissue-engineering context. In particular, the self-assembly approach of tissue engineering will be described. This strategy relies on the stimulation of mesenchymal cells with serum and ascorbic acid (vitamin C) during long-term culture resulting in the formation of a manipulatable cellular sheet made of cells and ECM components produced from these cells. Then, multiple connective tissue sheets can be assembled to form multilayered reconstructed tissues of increased thickness.

The self-assembly approach has been intensively developed by the research group LOEX from Université Laval in Québec City, Canada (11). The first tissues reconstructed by the LOEX group were human blood vessels tissue-engineered from smooth muscle cells, dermal fibroblasts and endothelial cells (15), as well as reconstructed human skin produced using dermal fibroblasts in combination with keratinocytes (13, 14, 16). The use of the self-assembly approach of tissue engineering rapidly spread to the reconstruction of other tissues such as corneas (17) and urological structures (18). My team recently produced tissues based on this cell sheet technology using human ASCs as a cell source (19, 20). The results indicate that ASCs are very well suited for the self-assembly approach: they feature a high capacity for ECM production and organization, leading to the production of a dense reconstructed connective tissue (Fig. 1a and b). These can be used as stromal compartments for various epithelial cell types such as keratinocytes, resulting in reconstructed skin with features very similar to native human skin or to skin reconstructed using dermal fibroblasts (21).

Next, we took advantage of the differentiation potential of ASCs to create a specialized connective tissue containing in vitro terminally differentiated cells: adipose tissue. We therefore adapted the method to include an adipogenic differentiation step concomitant with ECM deposition stimulated by ascorbic acid. This led to the production of adipocyte-containing cell sheets, which, although more fragile than their corresponding connective cell sheets, can

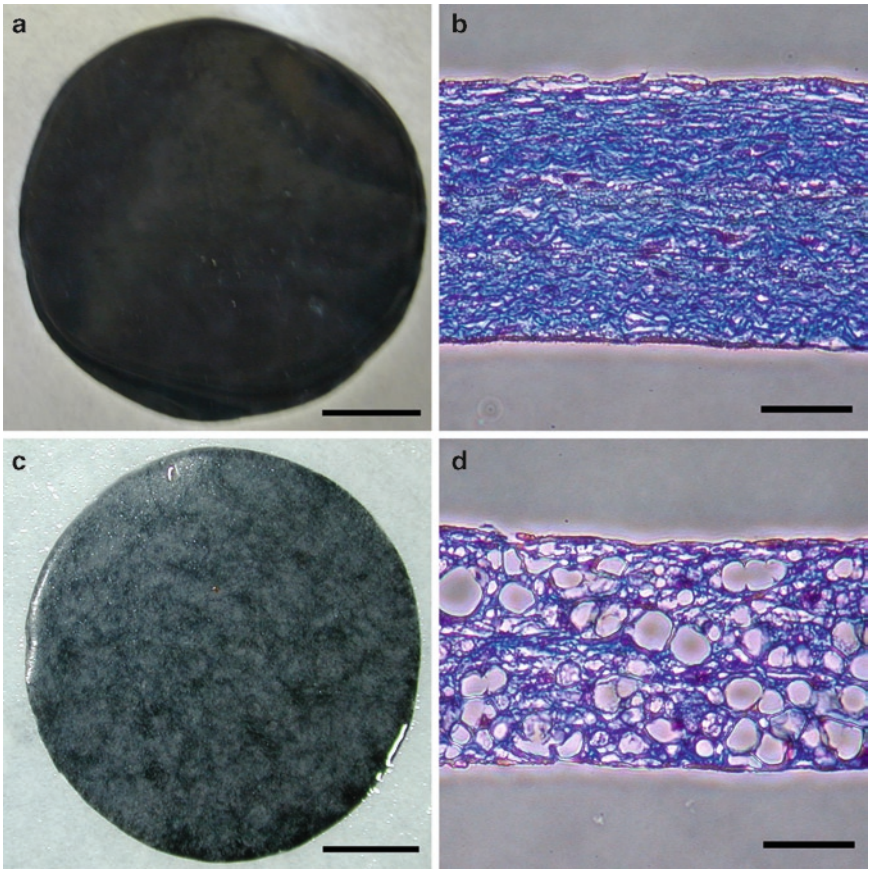

Fig. 1. Reconstructed connective (**a**, **b**) and adipose (**c**, **d**) tissues produced using the self-assembly approach. Macroscopic images (**a**, **c**) and histological appearance (cross-sections) after Masson's trichrome staining (**b**, **d**) reveal the presence of an abundant human extracellular matrix and the presence of numerous adipocytes only when adipogenic differentiation is induced (**d**). Tissues (**b**, **d**) were reconstructed by layering three cell sheets. Bars: (**a**, **c**) 0.5 cm; (**b**, **d**) 50 μm.

also be lifted from the culture plates, manipulated and assembled into multilayered tissues (Fig. 1c and d). These reconstructed adipose tissues feature adequate structural (19, 20) as well as functional properties such as adipokine secretion and β-adrenergic receptor-mediated lipolysis (19).

Other researchers have also developed a cell layer technology without the use of traditional scaffold-based methods. For example, mesenchymal cells such as ASCs and fibroblasts have been used to generate cell sheets using temperature-responsive culture surfaces. One of the first applications described has been the production of stromal sheets used as cardiac patches in a rat model of tissue repair after chronic heart failure (22). Now, a variety of differentiated cell types have also been used for the production of cell sheets using that technology, including chondrocyte (23), islet cell (24) and corneal cell sheets (reviewed in ref. (25)).

This chapter focuses on the self-assembly approach of tissue engineering for the production of entirely autologous reconstructed connective and adipose tissues using human ASCs from subcutaneous lipoaspirated fat. First, we describe the amplification and freezing of ASCs, followed by a detailed description of the production of reconstructed connective as well as adipose sheets, including engineering of multilayer tissue constructs from those sheets. These models allow us to study fundamental aspects of adipogenesis and represent attractive new substitutes that can be produced in an autologous fashion for soft-tissue repair.

2. Materials

2.1. Stromal Cell Culture Media

1. Preparation of stromal cell culture medium components
   (a) *D-MEM-Ham*: Dulbecco's Modified Eagle's medium (D-MEM) Ham's F12 medium 1:1, 1.2 g/L $NaHCO_3$, 3.574 g/L HEPES free acid, 312.5 µL/L 2 N HCl. Dissolve in apyrogenic ultrapure water. Adjust pH to 7.4. Sterilize by filtration through a 0.22-µm low-binding disposable filter. Distribute in 450-mL aliquots and store at 4°C.
   (b) *Fetal calf serum (FCS)*: Thaw in cold water. Inactivate in hot water (56°C) for 30 min. Distribute in 50-mL aliquots and store at –20°C (see Note 1).
   (c) *Penicillin G and gentamicin*: Dissolve 50,000 IU/mL of penicillin G and 12.5 mg/mL of gentamicin sulfate in apyrogenic ultrapure water to make a 500× stock solution. Sterilize by filtration through a 0.22-µm low-binding disposable filter, distribute in single-use aliquots (1 mL) and store at –80°C.
2. Thaw serum and add to D-MEM-Ham for a final concentration of 10% FCS. Thaw antibiotics and add 1 mL for a bottle of 500 mL of media.

2.2. Adipogenic Induction Medium

1. Preparation of induction culture medium (for final concentrations see Table 1).
   (a) *D-MEM-Ham*.
   (b) *FCS*.
   (c) *Isobutylmethylxanthine (IBMX)*: Dissolve 250 mg in 1,125 µL pure sterile DMSO for a 1,000 mM stock solution. Distribute in single-use 28-µL aliquots and store at –20°C.
   (d) *Rosiglitazone*: Dissolve 5 mg in 1,400 µL pure sterile DMSO for a 10-mM stock solution. Distribute in single-use aliquots and store at –20°C.

Table 1
Adipogenic induction medium

Component	Final concentration	Concentrated stock	Quantity for 100 mL total
D-MEM-Ham	96% (v/v)		96.4 mL
Fetal calf serum	3% (v/v)		3 mL
IBMX	0.25 mM	1,000 mM	25 µL
Rosiglitazone	1 µM	10 mM	100 µL of a 1:10 dilution of the stock aliquot
Insulin	100 nM	100 µM	100 µL
Dexamethasone	1 µM	1 mM	100 µL
T3	0.2 nM	200 nM	100 µL
Penicillin G and Gentamicin	Penicillin G 100 IU/mL Gentamicin 25 µg/mL	500×	200 µL

(e) *Insulin*: Dissolve 58 mg in 100 mL of 0.005 N HCl (250 µL 2 N HCl/100 mL apyrogenic ultrapure water) for a 100-µM stock solution. Use a plastic container and dissolve under gentle agitation. Sterilize by filtration through a 0.22-µm low-binding disposable filter, distribute in single-use aliquots and store at –80°C.

(f) *Dexamethasone*: Dissolve 25 mg in 1 mL of methanol (63.7 mM). Add 62 mL of D-MEM-Ham for a 1-mM stock solution. Sterilize by filtration through a 0.22-µm low-binding disposable filter, distribute in single-use aliquots and store at –80°C.

(g) *Thyroxine (T3)*: Dissolve 6.8 mg in 3 mL 0.02 N NaOH (10 µL 10 N NaOH/4.99 mL apyrogenic ultrapure water). Complete to 50 mL with apyrogenic ultrapure water for a 2×10^{-4} M solution stored at 4°C. Prepare a 200-nM stock solution by adding 99.9 mL of apyrogenic ultrapure water to 100 µL of 2×10^{-4} M solution. Sterilize by filtration through a 0.22-µm low-binding disposable filter, distribute in single-use aliquots and store at –80°C.

(h) *Penicillin G and Gentamicin*.

2. Thaw all components. For 100 mL, refer to Table 1 (see Note 2). Warm IBMX aliquots to 37°C and add to prewarmed media to prevent precipitate formation.
3. Complete adipogenic induction medium can be stored at –20°C in single-use aliquots for approximately 3 months.
4. If needed, make induction control media by combining the appropriate amount of DMSO, the vehicle for IBMX and

rosiglitazone, into 3% FCS stromal cell culture media. For 100 mL of control media, add 38 µL of DMSO.

2.3. Adipocyte Maintenance Medium

1. Preparation of adipocyte medium components (for final concentrations see Table 2).
   (a) D-MEM-Ham.
   (b) FCS.
   (c) Insulin.
   (d) Dexamethasone.
   (e) T3.
   (f) Penicillin G and gentamicin.
2. Thaw all components. To make 500 mL, refer to Table 2 (see Note 2).
3. Complete adipogenic maintenance medium can be stored at 4°C for 10 days.

2.4. Freezing Medium

1. Preparation of freezing medium components
   (a) *FCS.*
   (b) *DMSO.* Distribute the stock solution (99.7%) in single-use aliquots and store at –20°C.
2. Thaw all components at 4°C. For 10 mL, combine 1 mL of DMSO with 9 mL of FCS for a final concentration of 10% DMSO.
3. Keep on ice. Can be stored at 4°C for 24 h (see Note 3).

Table 2
Adipocyte maintenance medium

Component	Final concentration	Concentrated stock	Quantity for 500 mL total
D-MEM-Ham	90% (v/v)		447.5 mL
Fetal calf serum	10% (v/v)		50 mL
Insulin	100 nM	100 µM	500 µL
Dexamethasone	1 µM	1 mM	500 µL
T3	0.2 nM	200 nM	500 µL
Penicillin G and Gentamicin	Penicillin G 100 IU/mL Gentamicin 25 µg/mL	500×	1 mL

2.5. Primary Cultures (P0) of Human ASCs

1. Cell suspension of freshly extracted ASCs from human adipose tissue obtained after proper ethical approbations, either from lipoaspirated or resected subcutaneous adipose tissues (see Note 4).
2. Tissue culture flasks (75 cm^2).
3. Stromal cell culture medium.

2.6. Subculture of Human ASCs (Passage)

1. Stromal cell culture medium (see Subheading 2.1).
2. Trypsin/EDTA 2.8 mM D-glucose: 0.05% (w/v) trypsin 1-500, 0.00075% (v/v) phenol red, 100,000 IU/L penicillin G, 25 mg/L gentamicin, 0.01% (w/v) EDTA. Dissolve in 1× PBS. Adjust pH to 7.45. Sterilize by filtration through a 0.22-μm low-binding disposable filter, distribute in 10-mL single-use aliquots and store at –20°C.
3. 15-mL or 50-mL conical tubes.
4. Tissue culture flasks, 75 cm^2.

2.7. Cryopreservation and Thawing of Human ASCs

1. Freezing medium.
2. Sterile cryogenic vials.
3. Stromal cell culture medium.
4. Tissue culture flasks, 75 cm^2.

2.8. Production of Self-Assembled Connective or Adipose Cell Sheets from ASCs

1. For connective sheets, follow Subheading 2.8, steps 1–8 using confluent ASC cultures preferably before their fourth passage.
2. (+)-Sodium L-ascorbate: Dissolve 10 mg/mL of (+)-sodium L-ascorbate in cold D-MEM-Ham for a 200× stock solution of ascorbic acid. Sterilize by filtration through a 0.22-μm low-binding disposable filter (see Note 5).
3. Stromal cell culture medium.
4. Ingots (stainless steel grade # 316).
5. Curved forceps.
6. 6-well cell culture dish (see Note 6).
7. Anchoring paper (Whatman sheet, cat. no. 1004-185, see Note 6).
8. Phosphate-buffered saline (PBS)–penicillin G/gentamicin (PBS-P/G): 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4. Dissolve in apyrogenic ultrapure water for a 10× stock solution. Store at room temperature. To dilute 10× PBS to 1×, add apyrogenic ultrapure water. Verify pH is 7.4. Before use, add penicillin G and gentamicin 500× stock solution by diluting these additives to 1×.
9. For self-assembled adipose tissue sheets, use the same material as for Subheading 2.8, items 1–8, with the exception of

the following additional culture media: adipogenic induction medium and adipocyte maintenance medium.

2.9. Production of Multilayered Connective and Adipose Tissues from Stromal and Adipose Cell Sheets

1. Cell sheets produced as in Subheading 3.5 or 3.6.
2. Ascorbic acid solution.
3. Culture media:
   (a) Stromal cell culture medium.
   (b) Adipocyte maintenance medium.
4. Merocel® sponges (Medtronic) (see Note 6).
5. Anchoring ring (stainless steel grade # 316) (see Note 6).
6. Ingots.
7. Deep Petri dish.
8. Curved forceps.
9. Straight forceps.
10. Ligaclips.
11. Ligaclip applicator (cat. no. LX-105, Ethicon).

3. Methods

3.1. Primary Cultures (P0) of Human ASCs

All further manipulations are performed under a sterile laminar flow hood cabinet.

1. Human ASCs are isolated from subcutaneous adipose tissue of normal adult donors (see Notes 4 and 7).
2. Seed 8×10^4 ASCs/cm^2 in a 75-cm^2 tissue culture flask with 18 mL of stromal cell culture medium. Incubate in 8% CO_2, 100% humidity atmosphere at 37°C. Change the culture medium the following day to eliminate non-adherent cells and thereafter change medium three times a week.
3. When cells nearly reach confluence, subculture (see Subheading 3.2) or freeze (see Subheading 3.3) cells.

3.2. Subculture of Human ASCs (Passage)

For a 75-cm^2 culture flask of ASCs:

1. Remove medium and rinse with 2 mL of warm (37°C) Trypsin/EDTA and discard.
2. Add 3 mL of Trypsin/EDTA. Incubate at 37°C until the cells detach from the flask. Gently tap the flask after 5 min.
3. Add a minimum of 3 mL of warm stromal cell culture medium. Collect the cell suspension into a conical tube. Rinse the flask with stromal cell culture medium, and add it to the tube.

4. Centrifuge cell suspension at $300 \times g$ for 10 min at room temperature.
5. Resuspend cell pellet in a given volume of stromal cell culture medium.
6. Count the cells and measure the viability by Trypan blue staining. The cell viability should be >95%.
7. Seed 5×10^5 to 6×10^5 cells/75-cm^2 tissue culture flask with 18 mL of stromal cell culture medium. Change culture medium three times a week.

3.3. Cryopreservation of Human ASCs

1. Resuspend the trypsinized cells in a given volume of cold (4°C) freezing medium in order to obtain $1–2 \times 10^6$ cells/mL (see Note 8).
2. On ice, aliquot in sterile cryogenic vials.
3. Freeze overnight at –80°C.
4. Store in liquid nitrogen.

3.4. Thawing of Human ASCs

1. Put the cryogenic vial in 37°C water until only a small cluster of ice remains.
2. Using a pipette, quickly transfer the content of the cryogenic vial into a tube containing 9 mL of cold stromal cell culture medium (4°C), then rinse the vial with 1 mL of cold medium and combine for a total of 10 mL.
3. Centrifuge cell suspension at $300 \times g$ for 10 min at room temperature.
4. Resuspend cell pellet in a given volume of warm stromal cell culture medium.
5. Count the cells and assess viability by Trypan blue staining.
6. Seed 6×10^5 cells/75-cm^2 tissue culture flask with 18 mL of stromal cell culture medium. Incubate in 8% CO_2, 100% humidity atmosphere at 37°C. Change culture medium three times a week.

3.5. Production of Connective Tissue Sheets from ASCs Using the Self-Assembly Approach of Tissue Engineering

All further manipulations are performed under a sterile laminar flow hood cabinet.

1. Put the customized peripheral anchoring papers into the desired cell culture dish (see Note 6). Rinse anchoring papers with PBS three times.
2. If using a 6-well culture plate, seed a cell suspension corresponding to 1.5×10^5 ASCs/well in 3 mL of stromal cell culture medium containing 50 µg/mL ascorbic acid. Incubate in an 8% CO_2, 100% humidity atmosphere at 37°C.
3. The following day of culture, place ingots on the peripheral anchoring papers to prevent moving.

4. Two days after cell seeding, add 3 mL of stromal cell culture medium containing 100 µg/mL ascorbic acid into each well. From then on, change culture media three times a week by removing 3 mL of culture media and adding 3 mL of stromal cell culture medium supplemented with 100 µg/mL ascorbic acid into each well.
5. After 21–28 days of culture, individual cell sheets can be detached, superimposed and clipped together in order to engineer multilayered reconstructed tissues (see Subheading 3.7).

3.6. Production of Self-Assembled Adipose Tissue Sheets from Induced ASCs Using the Self-Assembly Approach of Tissue Engineering

1. Follow steps 1–4 of Subheading 3.5.
2. After confluence or typically after 7 days of culture, proceed to induction of adipogenic differentiation by removing all media and adding 3 mL of 50 µg/mL ascorbic acid supplemented induction media per well.
3. After 3 days of induction, add 3 mL of adipogenic media. From then on, change culture media three times a week by removing 3 mL of culture media and adding adipogenic media containing 100 µg/mL ascorbic acid into each well.
4. After 21–28 days of culture, individual cell sheets can be detached and superimposed to engineer multilayered reconstructed adipose tissues (see Subheading 3.7).

3.7. Reconstruction of Multilayered Connective and Adipose Tissues from Stromal and Adipose Cell Sheets

1. After typically 28 days of culture, carefully detach and lift cell sheets by holding the paper anchorage using fine forceps. Transfer into a deep Petri dish containing 25 mL of either stromal or adipogenic medium, depending on the type of reconstruction, containing 50 µg/mL ascorbic acid.
2. Repeat step 1 with the desired number of cell sheets. Transfer each cell sheet on top of the preceding cell sheet. Typically, three cell sheets are superposed to produce thicker tissues for routine experiments (Fig. 1). Cell sheets can be clipped together through the anchoring device using ligaclips.
3. In order to promote cohesion between cell sheets, place a stainless steel ring on top of the construct and add two sterile Merocel® sponges presoaked in the appropriate medium. Keep Merocel® sponges in place with ingots.
4. After 2 days, remove Merocel® sponges and ingots. Incubate in an 8% CO_2, 100% humidity atmosphere at 37°C. Culture for a week after the assembly step to favour cohesion between sheets, changing culture medium three times.
5. Reconstructed tissues are then ready for various types of analysis after removal of the anchoring device.

4. Notes

1. Various lots of serum are screened to select the serum lot ensuring maximal cell performance.
2. Serum must be added first followed by insulin. Insulin must be added with a sterile plastic pipette.
3. DMSO is an oxidative agent toxic for cells, especially at temperatures above 10°C. Thus, it must be used at 4°C.
4. Adipose tissue from donors with a body mass index (BMI) below 25 was used. Either lipoaspirated or *en bloc* resected fat can be used as starting material for ASCs extraction. However, when isolated and cultured according to our protocols, cells extracted from lipoaspirates performed better in terms of cell yield at extraction, adipogenic differentiation and matrix secretion reflected by tissue thickness (19, 20), than cells extracted from resected fat. Thus lipoaspirated ASCs represent better candidates for tissue reconstruction using the self-assembly approach.
5. Ascorbic acid stock solution prepared from (+)-sodium L-ascorbate must be prepared immediately before use and protected from light.
6. The size of the cell culture dish to be used is not limited to 6-well culture plates and is to be chosen according to the desired type of reconstruction and experiment to be performed. Therefore, anchoring devices should be customized for that particular type of cell culture dish. Merocel® and anchoring rings should also be designed to accommodate the specific form and size of the reconstructed tissue.
7. The institution's committee for the protection of human subjects approved protocols.
8. This solution must be prepared the day of its use.

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References

1. Caplan, A. I. (1991) Mesenchymal stem cells. *J Orthop Res* **9**, 641–50.
2. Prockop, D. J. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276**, 71–4.
3. Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–7.
4. Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W. C., Largaespada, D. A., and Verfaillie, C. M. (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41–9.
5. Toma, J. G., McKenzie, I. A., Bagli, D., and Miller, F. D. (2005) Isolation and characterization of multipotent skin-derived precursors from human skin. *Stem Cells* **23**, 727–37.
6. Gingras, M., Champigny, M. F., and Berthod, F. (2007) Differentiation of human adult skin-derived neuronal precursors into mature neurons. *J Cell Physiol* **210**, 498–506.
7. Wang, H. S., Hung, S. C., Peng, S. T., Huang, C. C., Wei, H. M., Guo, Y. J., Fu, Y. S., Lai, M. C., and Chen, C. C. (2004) Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells* **22**, 1330–7.
8. Sarugaser, R., Lickorish, D., Baksh, D., Hosseini, M. M., and Davies, J. E. (2005) Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. *Stem Cells* **23**, 220–9.
9. Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P., and Hedrick, M. H. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* **7**, 211–28.
10. Laplante, A. F., Germain, L., Auger, F. A., and Moulin, V. (2001) Mechanisms of wound reepithelialization: hints from a tissue-engineered reconstructed skin to long-standing questions. *FASEB J* **15**, 2377–89.
11. Auger, F. A., Remy-Zolghadri, M., Grenier, G., and Germain, L. (2000) The self-assembly approach for organ reconstruction by tissue engineering. *e-Biomed: A J Regen Med* **1**, 75–86.
12. Boyce, S. T., Kagan, R. J., Greenhalgh, D. G., Warner, P., Yakuboff, K. P., Palmieri, T., and Warden, G. D. (2006) Cultured skin substitutes reduce requirements for harvesting of skin autograft for closure of excised, full-thickness burns. *J Trauma* **60**, 821–9.
13. Michel, M., L'Heureux, N., Pouliot, R., Xu, W., Auger, F. A., and Germain, L. (1999) Characterization of a new tissue-engineered human skin equivalent with hair. *In Vitro Cell Dev Bio* **35**, 318–26.
14. Pouliot, R., Larouche, D., Auger, F. A., Juhasz, J., Xu, W., Li, H., and Germain, L. (2002) Reconstructed human skin produced in vitro and grafted on athymic mice. *Transplantation* **73**, 1751–7.
15. L'Heureux, N., Pâquet, S., Labbé, R., Germain, L., and Auger, F. A. (1998) A completely biological tissue-engineered blood vessel. *FASEB Journal* **12**, 47–56.
16. Larouche, D., Paquet, C., Fradette, J., Carrier, P., Auger, F. A., and Germain, L. (2009) Regeneration of skin and cornea by tissue engineering. *Methods Mol Biol* **482**, 233–56.
17. Carrier, P., Deschambeault, A., Audet, C., Talbot, M., Gauvin, R., Giasson, C. J., Auger, F. A., Guerin, S. L., and Germain, L. (2009) Impact of cell source on human cornea reconstructed by tissue engineering. *Invest Ophthalmol Vis Sci* **50**, 2645–52.
18. Magnan, M., Levesque, P., Gauvin, R., Dube, J., Barrieras, D., El-Hakim, A., and Bolduc, S. (2009) Tissue engineering of a genitourinary tubular tissue graft resistant to suturing and high internal pressures. *Tissue Eng Part A* **15**, 197–202.
19. Vermette, M., Trottier, V., Menard, V., Saint-Pierre, L., Roy, A., and Fradette, J. (2007) Production of a new tissue-engineered adipose substitute from human adipose-derived stromal cells. *Biomaterials* **28**, 2850–60.
20. Vallee, M., Cote, J. F., and Fradette, J. (2009) Adipose-tissue engineering: taking advantage of the properties of human adipose-derived stem/stromal cells. *Pathol Biol (Paris)* **57**, 309–17.
21. Trottier, V., Marceau-Fortier, G., Germain, L., Vincent, C., and Fradette, J. (2008) IFATS collection: Using human adipose-derived stem/stromal cells for the production of new skin substitutes. *Stem Cells* **26**, 2713–23.
22. Miyahara, Y., Nagaya, N., Kataoka, M., Yanagawa, B., Tanaka, K., Hao, H., Ishino, K., Ishida, H., Shimizu, T., Kangawa, K., Sano, S., Okano, T., Kitamura, S., and Mori, H. (2006) Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nat Med* **12**, 459–65.

23. Mitani, G., Sato, M., Lee, J. I., Kaneshiro, N., Ishihara, M., Ota, N., Kokubo, M., Sakai, H., Kikuchi, T., and Mochida, J. (2009) The properties of bioengineered chondrocyte sheets for cartilage regeneration. *BMC Biotechnol* **9**, 17.
24. Shimizu, H., Ohashi, K., Utoh, R., Ise, K., Gotoh, M., Yamato, M., and Okano, T. (2009) Bioengineering of a functional sheet of islet cells for the treatment of diabetes mellitus. *Biomaterials* **30**, 5943–9.
25. Yang, J., Yamato, M., Shimizu, T., Sekine, H., Ohashi, K., Kanzaki, M., Ohki, T., Nishida, K., and Okano, T. (2007) Reconstruction of functional tissues with cell sheet engineering. *Biomaterials* **28**, 5033–43.

Chapter 32

Differentiation of Adipose-Derived Stem Cells for Tendon Repair

A. Cagri Uysal and Hiroshi Mizuno

Abstract

The goal of primary tendon repair is to increase tensile strength at the time of mobilization. Tendon repair and regeneration using mesenchymal stem cells have been described in several studies; however, the use of adipose derived stem cells (ASCs) for tendon repair has only recently been considered. In order to establish a suitable experimental model for the primary tendon repair using ASCs, this chapter describes the detailed methods for: (1) isolating stem cells from adipose tissue, (2) generation of a primary tendon injury and repair model, (3) evaluating functional restoration by measuring tensile strength, and (4) investigating the mechanisms involved in ASC-mediated tendon healing by histological and immunohistochemical analyses. Topical administration of ASCs to the site of injury accelerates tendon repair, as exhibited by a significant increase in tensile strength, direct differentiation of ASCs toward tenocytes and endothelial cells, and increases in angiogenic growth factors. These findings suggest that ASCs may have a positive effect on primary tendon repair and may be useful for future cell-based therapy.

Key words: Wound healing, Adipose-derived stem cells, Tendon, Wound healing, Tensile strength, Angiogenesis, Growth factors

1. Introduction

A damaged tendon never completely regains the biological and biomechanical properties of a normal tendon. The collagen fibrils remain thinner, and the tendon's biomechanical strength is reduced (1). Tendons heal through both extrinsic and intrinsic mechanisms. Previous studies have implicated basic fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor beta subfamily (TGF-β) in the tendon healing process (2, 3).

The goal of tendon rehabilitation after injury and operative repair is to achieve optimal function and gliding while preventing

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tendon rupture (4). Thus, different studies have examined ways to increase the strength of the repaired tendon mainly by altering the suture type and repair techniques (5). However, regenerative medicine has established a new, promising experimental and clinical epoch (6). The differentiation of postnatal somatic stem cells or mesenchymal stem cells (MSCs) into different cell lineages and tissues, including tendon, has been described. In addition, the MSCs (mainly bone marrow derived stem cells [BMSCs]) were proven to enhance tendon healing (7–9). Adipose-derived stem cells (ASCs) have been shown to be as robust as other MSCs in their multipotency and proliferative efficiency (10–14). In addition to tissue regeneration, ASCs have a positive effect on wound healing in a murine skin ulcer model (15, 16). Moreover, our previous study demonstrated that topical application of ASCs is beneficial in primary tendon healing. This chapter highlights the details of our model for primary tendon healing and methods to evaluate tendon repair.

2. Materials

2.1. ASC Harvesting from Rabbit Adipose Tissue

1. Ten-week-old white Japanese rabbits (see Note 1).
2. Sodium pentobarbital.
3. Phosphate-buffered saline (PBS).
4. Collagenase dissolved at 0.10% in PBS, freshly prepared prior to use.
5. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic as a control medium. Store at 4°C.
6. 100-mm culture dish.
7. 50-ml centrifuge tube.
8. Trypan blue.
9. 100-μm nylon mesh.

2.2. Cell Culture

1. DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic as a control medium. Store at 4°C.
2. 100-mm culture dish.
3. Trypsin–EDTA.

2.3. Cell Labeling

1. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) (see Note 2).
2. PBS.
3. 15-ml centrifuge tube.

2.4. Platelet-Rich Plasma Preparation

1. Ten-week-old white Japanese rabbits.
2. 23-Gauge needle and 10-ml disposable syringe.
3. 10-ml tubes containing 3.8% sodium citrate.
4. Standard laboratory centrifuge.
5. 10% Calcium chloride solution.

2.5. Tendon Incision and Repair

1. Ten-week-old white Japanese rabbits.
2. Scalpel.
3. Polypropylene 3/0.

2.6. Biomechanical Testing

1. Uniaxial compression testing machine.
2. NTS load cell.

2.7. Histological Analysis

1. 10% Formaldehyde.
2. Hematoxylin.
3. Eosin.

2.8. Evaluation of Dil-Labeled ASCs

1. 10% Formaldehyde.
2. Hematoxylin.
3. Software (LuminaVison).

2.9. Immunohistochemical Analysis

1. 10% Paraformaldehyde.
2. Antibody dilution buffer.
3. Anti-collagen type I antibody (Collagen I ab6308).
4. Fluorescein isothiocyanate (FITC) (Fluorescein IgG, FI-5000).
5. Antibodies to TGF-β1, 2, and 3 (TGF-β1 [sc-146]; TGF-β2 [sc-90]; TGF-β3 [sc-82]).
6. Anti-FGF antibody (sc-1365).
7. Anti-VEGF antibody (VEGF ab28775).

3. Methods

3.1. Harvesting ASCs from Rabbit Adipose Tissue

1. Anesthetize the white Japanese rabbits by intravenous injection of sodium pentobarbital (35 mg/kg), and shave the surgical area.
2. Make an incision in the inguinal region, harvest the fat pads, place them in the hood, and wash extensively with PBS.
3. Mince the fat pads for 10 min with fine scissors in culture dishes and transfer the material to 50-ml centrifuge tubes.

Add 0.12% collagenase and enzymatically digest at 37°C for 40 min (see Note 3).

4. Add an equal volume of control medium to neutralize the collagenase.
5. Centrifuge the cell suspension at 1,300 rpm (260×*g*) for 5 min to obtain a high-density ASC pellet.
6. Aspirate the supernatant, being careful not to disturb the cell pellets. Resuspend the cell pellets in control medium and incubate at room temperature for 10 min.
7. Filter the cell suspension through 100-μm nylon mesh to remove cellular debris.
8. Count the cells using trypan blue and plate at a concentration of 5×10^5 cells/100 mm in tissue culture dishes.

3.2. Cell Culture

1. Maintain the primary ASCs in control medium at 37°C in 5% carbon dioxide. Change the culture medium every 3 days.
2. Once adherent cells become subconfluent, aspirate the culture medium and wash the cells with 5 ml of PBS three times. Add 3–5 ml of trypsin–EDTA at 37°C for 5 min to detach the cells.
3. Resuspend the ASCs with an equal volume of control medium.
4. Centrifuge the cell suspension again and resuspend in a small volume of control medium.
5. Count and calculate the cell concentration using trypan blue and adjust the volume to obtain 1.5×10^7 in 20 μl of control medium for the subsequent transplantation.

3.3. Cell Labeling

1. Make a 1:100 dilution of DiI with PBS.
2. Centrifuge the solution at 650 xg for 5 min and collect the supernatant.
3. Apply 200 μl of the supernatant to the cell pellet so that pellet floats. The number of the cells labeled is not important.
4. Incubate the solution for 5 min at 4°C.
5. Add 7 ml of PBS to the tube and centrifuge at 260 xg for 5 min to collect the cells (see Note 4).

3.4. Platelet Rich Plasma Preparation

1. Draw 10 ml of whole blood from each 10-week-old white male Japanese rabbit with a 23-gauge needle into tubes containing 3.8% sodium citrate.
2. Centrifuge the blood in a standard laboratory centrifuge for 10 min at 2,400 rpm (450×*g*).
3. Collect the supernatant plasma and the buffy coat, which contains platelets and leukocytes, with a long cannula and transfer into a 15-ml centrifuge tube.

4. Centrifuge at 3,600 rpm (850 × *g*) for 15 min to concentrate the platelets.
5. Resuspend the infranatant containing the buffy coat with 1.3 ml of the residual plasma to prepare the final platelet rich plasma (PRP) mixture.
6. Activate the PRP gelatin with a 10% calcium chloride solution immediately before administration in vivo (see Note 5).

3.5. Tendon Incision, Repair, and Cell Transplantation

1. Expose both Achilles tendons with a lazy S incision.
2. Incise the tendons with scalpel, and repair them using the modified Kessler method with 3/0 polypropylene.
3. Mix the labeled cells with the prepared PRP solution and then add 10% calcium chloride to activate the PRP gelatin.
4. Place the PRP-ASC gel mixture around the circumference of the repaired tendon to cover the tendon completely.
5. Use PRP gel lacking ASCs for the control group (Fig. 1).
6. Immobilize the legs of the rabbits with a cast for 2 weeks.

3.6. Biomechanical Testing

1. Harvest the tendons 4 weeks after the surgery between the muscle and the insertion to the bone.
2. Clamp the free ends of the tendon in the vice of the uniaxial compression testing machine.
3. Set the head speed to 10 mm/min and test the tendons until they reach mechanical failure. Record the maximum load for each sample (Fig. 2). Gap formation is not assessed during this dynamic testing procedure.

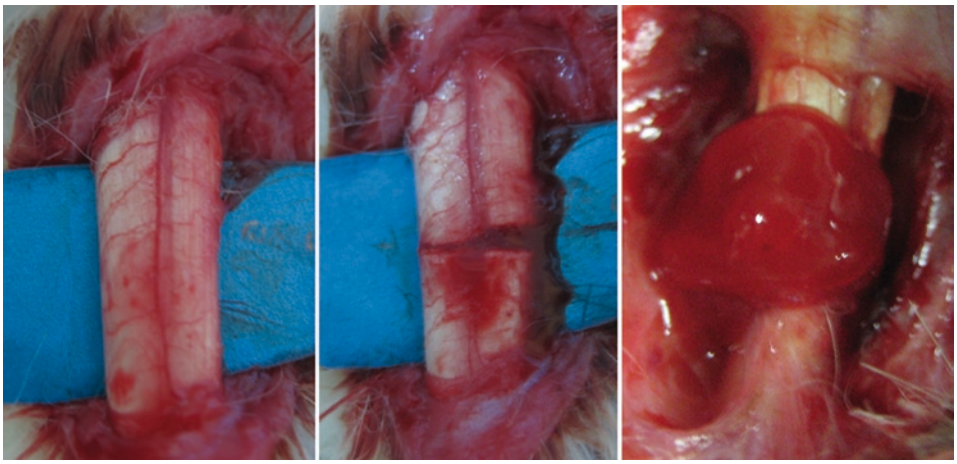

Fig. 1. Representative images of the tendon injury and repair model. The Achilles tendon was exposed (*left*) and a sharp incision was performed with a scalpel (*center*). Primary tendon repair was performed using a modified Kessler technique. The repaired tendon was exposed to platelet-rich plasma (PRP) mixed with 1×10^7 autologous adipose-derived stem cells (ACSs), or PRP alone as a control (*right*).

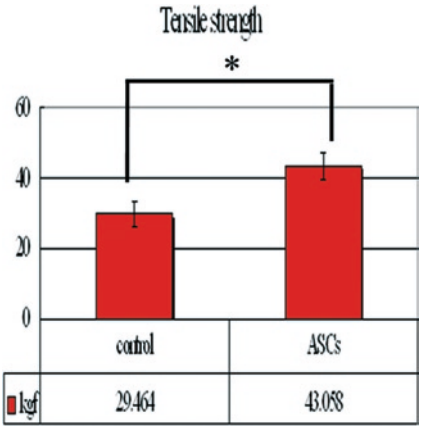

Fig. 2. Graph of the mean tendon tensile strength as measured by biomechanical testing. There was a statistical difference between the groups (*$p < 0.05$).

3.7. Histological Analysis

1. Fix the tendon specimens in 10% formaldehyde at room temperature overnight.
2. Embedded the samples in paraffin.
3. Section the samples in 4-μm increments using a microtome and place on glass slides.
4. Deparaffinize sections in xylene I for at least 10 min.
5. Immerse the slides in xylene II and then xylene III.
6. Immerse the slides in 100% ethanol three times and then in 90, 80, 70, and 50% ethanol.
7. Rinse the sections with tap water.
8. Incubate the sections in Carazzi's Hematoxylin for 8 min.
9. Rinse the slides with tap water for 3–5 min.
10. Incubate the sections in eosin solution for 3 min.
11. Dehydrate slides in a graded ethanol series (70%, 90%, 100% × 3), followed by successive immersion in xylene I to III.
12. Mount slides using mounting medium and observe at 40× magnification to visualize the collagen pattern and scar tissue (Fig. 3).

3.8. Evaluation of DiI-Labeled ASCs

1. Section consecutive tissue samples in 4-μm increments using a microtome and place on glass slides.
2. Immerse the slides in a series of xylenes, ethanol, and water followed by Hematoxylin and eosin staining as described above in steps 6–11, Subheading 3.7. Mount slides using mounting medium, and visualize DiI at 565 nm.
3. Visualize the DiI-positive cells (565 nm) and the total tenocyte population (transmitted light) of the same area at a 40× magnification using a fluorescence microscope.

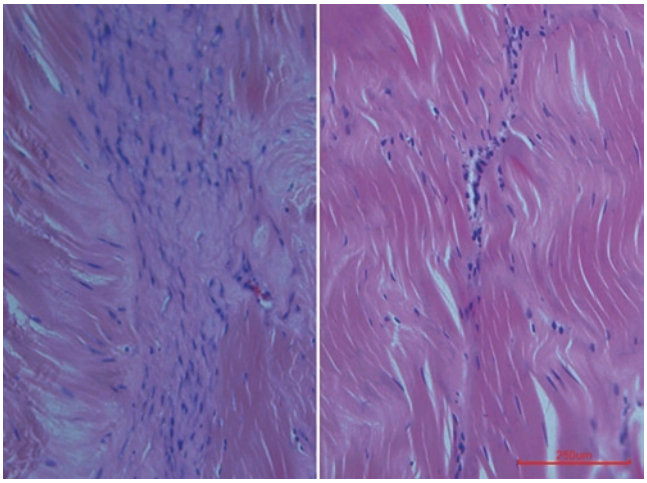

Fig. 3. Hematoxylin and eosin staining of tendon samples. In the control group, the alignment of collagen bundles was irregular, and connective tissue and inflammatory cells were evident at the incision site (*left*). In contrast, alignment of collagen bundles in the experimental group was partially regular and longitudinal, and the border between the incision line and the tendon itself was less distinct (*right*). *Bar*, 100 μm.

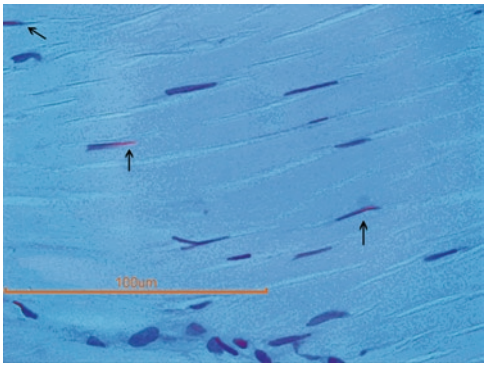

Fig. 4. Representative images of DiI and hematoxylin staining. Merged images show DiI-positive tenocytes (fibroblast-like elongated cells) in between collagen bundles (*arrows*). *Bar*, 100 μm.

4. Merge the two photographs using Lumina Vision software and quantitate the number of DiI-positive and -negative tenocytes (Fig. 4).

3.9. Immunohistochemical Evaluation

1. Section consecutive tissue samples in 4-μm increments using a microtome and place on glass slides.
2. Deparaffinize sections in xylene I for at least 10 min.
3. Immerse the slides in xylene II and then xylene III.
4. Rinse the sections with PBS.

5. Fix with the sections with 10% paraformaldehyde and then rinse with PBS.
6. Incubate the tissue for 30 min with 5% bovine serum albumin to block nonspecific binding of immunoglobulin.
7. Incubate the tissues with primary antibody (1:200 in antibody dilution buffer for 1 h at room temperature in a humidified chamber).
8. Rinse the sections with PBS three times for 5 min each.
9. Incubate the tissues with secondary antibody (1:100 in antibody dilution buffer) for 30 min in a humidified chamber.
10. Rinse the sections with PBS three times for 5 min each.
11. Dehydrate the tissue by immersing the slides in a graded ethanol series (70%, 90%, and 100%) three times each, followed by immersion in xylenes I to III.
12. Mount the slides using mounting medium.
13. Image 20 distinct fields at 40× magnification using the appropriate fluorescent filters.
14. Evaluate the florescence ratio using the histogram tool (mean intensity value in pixels) in Adobe Photoshop7.0®.

4. Notes

1. Chickens are the ideal animal model for tendon experiments. However, their use depends on ethics committee rules regarding bird flu.
2. Other labeling methods, such as transfecting cells with GFP and DNA labeling with Hoescht dye, are also available and should be taken into consideration depending on the animal model.
3. Vascular structures in the adipose tissue should be excised to obtain a clean cell population. The adipose tissue should be minced as finely as possible to harvest the maximum number of cells.
4. The cells should be checked visually using a fluorescence microscope to confirm proper labeling.
5. The amount of calcium chloride may be increased if gel fails to form or if the total PRP volume is greater than expected. The amount of PRP depends on the platelet concentration, which can vary from animal to animal.

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References

1. Frank C, McDonald D, Shrive N. (1997) Collagen fibril diameters in the rabbit medial collateral ligament scar: A longer term assessment. *Connect Tissue Res.* 36:261.
2. Duffy FJ, Jr, Seiler JG, Gelberman RH, Hergrueter CA. (1995) Growth factors and canine flexor tendon healing: Initial studies in uninjured and repair models. *J Hand Surg (Am).* 20:645.
3. Ngo M, Pham H, Longaker MT, Chang J. (2001) Differential expression of transforming growth factor-beta receptors in a rabbit zone II flexor tendon wound healing model. *Plast Reconstr Surg.* 108(5):1260.
4. Lehfeldt M, Ray E, Sherman R. (2008) MOC-PS(SM) CME article: Treatment of flexor tendon laceration. *Plast Reconstr Surg.* 121(4 Suppl):1.
5. Evans RB, Thompson DE. (1993) The application of force to the healing tendon. *J Hand Ther.* 6(4):266.
6. Gurtner GC, Callaghan MJ, Longaker MT. (2007) Progress and potential for regenerative medicine. *Annu Rev Med.* 58:299.
7. Awad HA, Butler DL, Boivin GP, et al. (1999) Autologous mesenchymal stem cell-mediated repair of tendon. *Tissue Eng.* 5:267.
8. Young RG, Butler DL, Weber W, et al. (1998) Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. *J Orthop Res.* 16:406.
9. Juncosa-Melvin N, Boivin GP, Gooch C, et al. (2006) The effect of autologous mesenchymal stem cells on the biomechanics and histology of gel-collagen sponge constructs used for rabbit patellar tendon repair. *Tissue Eng.* 12:369.
10. 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.* 7:211.
11. Mizuno H, Zuk PA, Zhu M, Lorenz HP, Benhaim P, Hedrick MH. (2002) Myogenic differentiation by human processed lipoaspirate cells. *Plast Reconstr Surg.* 109(1):199.
12. Tobita M, Uysal AC, Ogawa R, Hyakusoku H, Mizuno H. (2008) Periodontal tissue regeneration with adipose-derived stem cells. *Tissue Eng Part A.* 14(6):945.
13. Ogawa R, Mizuno H, Watanabe A, Migita M, Shimada T, Hyakusoku H. (2004) Osteogenic and chondrogenic differentiation by adipose-derived stem cells harvested from GFP transgenic mice. *Biochem Biophys Res Commun.* 313(4):871.
14. Lu F, Mizuno H, Uysal CA, Cai X, Ogawa R, Hyakusoku H. (2008) Improved viability of random pattern skin flaps through the use of adipose-derived stem cells. *Plast Reconstr Surg.* 121(1):50.
15. Nambu M, Ishihara M, Nakamura S, Mizuno H, Yanagibayashi S, Kanatani Y, et al. (2007) Enhanced healing of mitomycin C-treated wounds in rats using inbred adipose tissue-derived stromal cells within an atelocollagen matrix *Wound Repair Regen.* 15:505–10.
16. Nambu M, Kishimoto S, Nakamura S, Mizuno H, Yanagibayashi S, Yamamoto N, et al. (2009) Accelerated wound healing in healing-impaired db/db mice by autologous adipose tissue-derived stromal cells combined with atelocollagen matrix. *Ann Plast Surg.* 62:317–21.

Chapter 33

Adipose-Derived Stem Cells for Skin Regeneration

Hiroshi Mizuno and Masaki Nambu

Abstract

Intractable skin ulcers resulting from diabetes, ischemia and collagen diseases represent significant problems with few solutions. Cell-based therapy may hold promise in overcoming such disorders. In order to establish a suitable experimental model for the treatment of such ulcers using stem cells, this chapter describes detailed methods for: (1) isolation of stem cells from adipose tissue, termed adipose-derived stem cells (ASCs), (2) preparing a hybrid-type artificial dermis that consists of a type I collagen sponge and ASCs, (3) preparing intractable ulcers using Mitomycin C, and (4) evaluating the effect of wound healing histologically. ASCs seeded onto a type I collagen sponge are applied to intractable ulcers induced by topical application of Mitomycin C. Histological evaluation after 1 and 2 weeks revealed an increase in capillary density and granulation thickness of the hybrid-type artificial dermis. These findings suggest that ASCs may have a positive effect on wound healing and may be a useful tool for future cell-based therapy.

Key words: Wound healing, Adipose-derived stem cells, Intractable ulcers, Angiogenesis, Granulation, Type I collagen sponge, Mitomycin C

1. Introduction

Recent studies demonstrate that the incidence of peripheral arterial diseases and diabetes mellitus increases with age (1). In association with this increase, there is also a greater incidence of intractable skin ulcers, particularly in the lower leg, which eventually results in severe gangrene and subsequent limb amputation (2, 3). Cell-based therapeutic approaches have been recently introduced to treat intractable skin ulcers with favorable results (4–6). Although dermal fibroblasts derived from either autologous or allogeneic origin are mainly used in clinical situations, stromal/stem cells isolated from various donors are also found to be effective in treating intractable ulcers (7, 8).

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Stromal/stem cells isolated from adipose tissue, termed adipose-derived stromal/stem cells (ASCs), have also shown to be effective in treating intractable skin ulcers (9–11). The mechanism of wound healing by ASCs is generally thought to occur by direct differentiation toward lineage-committed cells or endothelial cells and ASC release of angiogenic growth factors, such as bFGF, PDGF, and VEGF.

Strategies for ulcer treatment via cell-based therapeutics can be divided into two approaches: (1) transplantation in conjunction with the appropriate scaffolds, such as a collagen sponge matrix, and (2) injection of the cells around the wound periphery. This chapter focuses on scaffold-based transplantation and describes detailed methods for this approach.

2. Materials

2.1. Harvest of ASCs from Murine Adipose Tissue

1. Inbred male Fisher 344 rat weighing approximately 250 g (see Note 1).
2. Sodium pentobarbital.
3. Phosphate-buffered saline (PBS).
4. Collagenase dissolved at 0.12% in PBS, freshly prepared prior to use.
5. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic as a control medium. Store at 4°C.
6. 100-mm culture dish.
7. 50-ml centrifuge tube.
8. Trypan blue.
9. 100-μm nylon mesh.

2.2. Cell Culture

1. DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic as a control medium. Store at 4°C.
2. 100-mm culture dish.
3. Trypsin–EDTA.

2.3. Intractable Ulcer Preparation

1. Inbred male Fisher 344 rat weighing approximately 250 g.
2. Mitomycin C dissolved at 1 mg/ml in PBS, freshly prepared prior to use.

2.4. Cell Transplantation onto the Wound

1. Type I collagen sponge (1.5 cm in diameter in size).
2. 24-well culture plate.
3. External cylinder of a 10-ml disposable syringe.

2.5. Histological Analysis

1. 10% Formaldehyde.
2. Hematoxylin.
3. Eosin.

3. Methods

3.1. ASC Harvesting from Murine Adipose Tissue

1. Anesthetize the Fisher 344 rats with an intraperitoneal injection of sodium pentobarbital at (1 mg/100 g) and shave the surgical area.
2. Make an incision in the inguinal region, harvest the fat pads, place them in the hood, and wash extensively with PBS using sterile technique.
3. Mince the fat pads for 10 min with fine scissors in culture dishes, and transfer the material into a 50-ml centrifuge tube. Add 0.12% collagenase and enzymatically digest at 37°C for 40 min with 0.12% collagenase.
4. Add an equal volume of control medium to neutralize the collagenase.
5. Centrifuge the cell suspension at 1,300 rpm ($260 \times g$) for 5 min to obtain a high-density ASC pellet.
6. Aspirate the supernatant, being careful not to disturb the cell pellets. Resuspend the cell pellets in control medium and incubate at room temperature for 10 min.
7. Filter the cell suspension through a 100-μm nylon mesh to remove cellular debris.
8. Count the cells using Trypan blue and plate at a concentration of 5×10^5 cells/100 mm in tissue culture dishes.

3.2. Cell Culture

1. Maintain the primary cultures of ASCs in control medium at 37°C in 5% carbon dioxide. Change the culture medium every 3 days.
2. Once the adherent cells become subconfluent, aspirate the culture medium and wash the cells with 5 ml of PBS three times. Add 3–5 ml of trypsin–EDTA at 37°C for 5 min to detach the cells.
3. Resuspend the cells with an equal volume of the control medium.
4. Centrifuge the cell suspension to pellet the cells and resuspend the pellet in a small volume of control medium.
5. Count and calculate the cell concentration using Trypan blue and adjust the volume to obtain 1×10^6 cells/1.5 ml for subsequent transplantation.

3.3. Intractable Ulcer Generation

1. Anesthetize the Wistar rats with an intraperitoneal injection of sodium pentobarbital (40 mg/kg) and shave the surgical area.
2. Depilate the dorsal area of the skin.
3. Make two round wounds (approximately 1.5 cm in diameter) through the full thickness of the skin bilaterally on the back of each rat using a pair of sharp scissors and a scalpel.
4. Apply the Mitomycin C solution onto both of the wounds with a sterile paintbrush (see Note 2).
5. After 10 min, thoroughly wash out the Mitomycin C from the wounds with distilled water.

3.4. Cell Transplantation

1. Place a sterile type I collagen sponge with a silicone membrane (1.5 cm in diameter) into a well of a 24-well culture plate such that the membrane is on the bottom of the dish.
2. Gently apply 1.5 ml of the ASC suspension (1×10^6 cells) onto the type I collagen sponge (see Note 3).
3. Incubate for 2 h at 37°C in 5% carbon dioxide to allow the cells to incorporate into the collagen matrix.
4. Apply the type I collagen sponge incorporated with ASCs onto one of the wounds so that the silicone membrane faces up.
5. Place a silicon ring made from a circular piece of the external cylinder of a 10-ml disposable syringe (approximately 1.5 cm in diameter) on the ulcers to prevent epithelialization from the surrounding wounds. Suture the silicone ring together with the hybrid-type artificial dermis using 5-0 nylon suture. A representative image is shown in Fig. 1 (see Note 4).

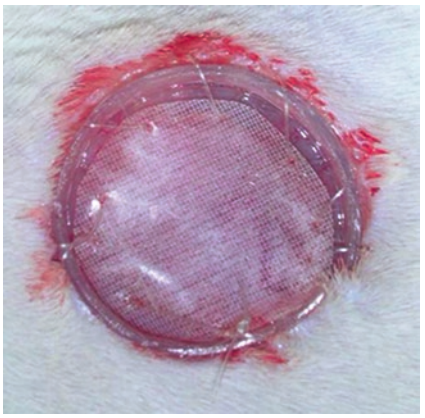

Fig. 1. Representative image of an intractable ulcer covered by a type I collagen sponge incorporated with ASCs. Once the hybrid-type artificial dermis is placed on the Mitomycin C-induced ulcer, a silicon ring is applied along the wound margin and sutured to the hybrid-type artificial dermis with 5-0 nylon.

6. As a control, apply a type I collagen sponge without ASCs to the other wound in the same manner and suture with 5-0 nylon.
7. Keep each rat in a separate cage to maintain the stability of the wound.

3.5. Histological Analysis

1. Euthanize the rats with an overdose of sodium pentobarbital injected intraperitoneally 1 and 2 weeks after surgery. Macroscopic appearance of the wound at 1 week is shown in Fig. 2.
2. Dissect out the dorsal skin, including the wound tissue, with fine scissors.
3. Fix the specimen in 10% formaldehyde at room temperature overnight.
4. Embed the sample in paraffin.
5. Section the tissue in 4-μm increments using a microtome (see Note 5) and place the sections on a glass slide.
6. Deparaffinize sections in xylene I for at least 10 min.
7. Immerse the sections briefly in xylene II and III.
8. Immerse the sections in 100% ethanol three times, and then in 90%, 80%, 70%, and 50% ethanol.
9. Rinse sections with tap water.
10. Incubate the sections in Carazzi's Hematoxylin for 8 min (see Note 6).
11. Rinse slides with tap water for 3–5 min.
12. Incubate the sections in eosin solution for 3 min.
13. Dehydrate the slides in a graded ethanol series (70%, 90%, 100%×3) and then immerse them successively in xylene I to III.

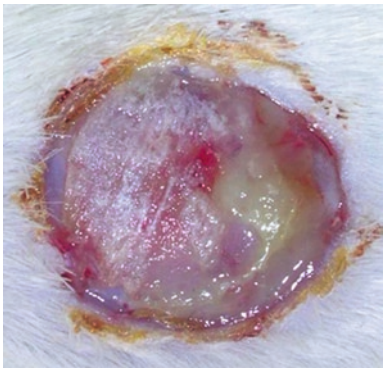

Fig. 2. Macroscopic appearance of an intractable ulcer covered by a type I collagen sponge incorporated with ASCs. Abundant granulation occurs within the type I collagen sponge.

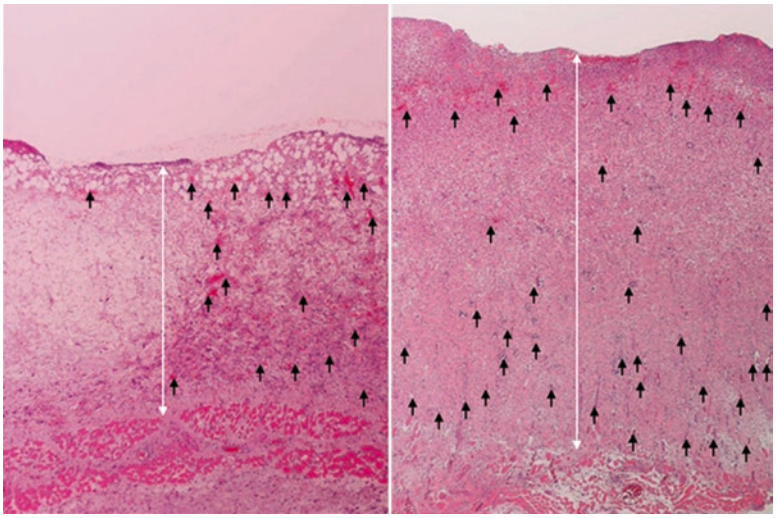

Fig. 3. Hematoxylin and eosin-stained cross section of an intractable ulcer 2 weeks after treatment by a type I collagen sponge with or without ASC incorporation. More abundant capillary formation (*black arrows*) and thicker granulation (*white arrow*) are found with ASC incorporation into the type I collagen sponge (*left*) than without (*right*).

14. Mount slides using mounting medium.
15. Choose several areas at random and measure the granulation thickness and count the capillary number. Representative results from 2 weeks posttransplantation are shown in Fig. 3.

4. Notes

1. Male mutant diabetic mice, C57BL/ksJ *db/db*, are also available as a delayed wound-healing model (10).
2. The series of procedures for inducing an intractable ulcer using Mitomycin C can be omitted if C57BL/ksJ *db/db* mice are used in the study.
3. The cell suspension is incorporated into the type I collagen sponge spontaneously within several minutes. Do not compress the type I collagen sponge to incorporate the cell suspension or the porous structure of the sponge will be destroyed.
4. The purpose of the silicon ring is to exclude granulation and subsequent epithelialization from surrounding tissue. However, epithelialization can be also evaluated if a silicon ring is not applied. In this case, digital photographs can be taken 1 and 2 weeks after the silicon membrane is removed, and the area of raw surface measured using image-processing software. Epithelialization rate is calculated by the following formula: $(1 - \text{raw surface area}/\text{original wound area}) \times 100\%$.

Our previous study demonstrated that within 2 weeks the epithelialization rate is faster in the ASC-treated group than the control (10).

5. Sections should be generated perpendicular to the anterior–posterior axis and perpendicular to the surface of the wound.
6. Application time is subject to be changed depending on temperature of the room.

Acknowledgments

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References

1. Hiatt, W. R. (2001) Medical treatment of peripheral arterial disease and claudication. *N Engl J Med* **344**, 1608–21.
2. Aulivola, B., Hile, C. N., Hamdan, A. D., Sheahan, M. G., Veraldi, J. R., Skillman, J. J., et al. (2004) Major lower extremity amputation: outcome of a modern series. *Arch Surg* **139**, 395–9; discussion 9.
3. Faglia, E., Clerici, G., Clerissi, J., Gabrielli, L., Losa, S., Mantero, M., et al. (2009) Long-term prognosis of diabetic patients with critical limb ischemia: a population-based cohort study. *Diabetes Care* **32**, 822–7.
4. Brem, H., Young, J., Tomic-Canic, M., Isaacs, C., and Ehrlich, H. P. (2003) Clinical efficacy and mechanism of bilayered living human skin equivalent (HSE) in treatment of diabetic foot ulcers. *Surg Technol Int* **11**, 23–31.
5. Gentzkow, G. D., Iwasaki, S. D., Hershon, K. S., Mengel, M., Prendergast, J. J., Ricotta, J. J., et al. (1996) Use of dermagraft, a cultured human dermis, to treat diabetic foot ulcers. *Diabetes Care* **19**, 350–4.
6. Hanft, J. R. and Surprenant, M. S. (2002) Healing of chronic foot ulcers in diabetic patients treated with a human fibroblast-derived dermis. *J Foot Ankle Surg* **41**, 291–9.
7. Ichioka, S., Kouraba, S., Sekiya, N., Ohura, N., and Nakatsuka, T. (2005) Bone marrow-impregnated collagen matrix for wound healing: experimental evaluation in a microcirculatory model of angiogenesis, and clinical experience. *Br J Plast Surg* **58**, 1124–30.
8. Rogers, L. C., Bevilacqua, N. J., and Armstrong, D. G. (2008) The use of marrow-derived stem cells to accelerate healing in chronic wounds. *Int Wound J* **5**, 20–5.
9. Nambu, M., Ishihara, M., Nakamura, S., Mizuno, H., Yanagibayashi, S., Kanatani, Y., et al. (2007) Enhanced healing of mitomycin C-treated wounds in rats using inbred adipose tissue-derived stromal cells within an atelocollagen matrix. *Wound Repair Regen* **15**, 505–10.
10. Nambu, M., Kishimoto, S., Nakamura, S., Mizuno, H., Yanagibayashi, S., Yamamoto, N., et al. (2009) Accelerated wound healing in healing-impaired db/db mice by autologous adipose tissue-derived stromal cells combined with atelocollagen matrix. *Ann Plast Surg* **62**, 317–21.
11. Rigotti, G., Marchi, A., Galie, M., Baroni, G., Benati, D., Krampera, M., et al. (2007) Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: a healing process mediated by adipose-derived adult stem cells. *Plast Reconstr Surg* **119**, 1409–22; discussion 23–4.

Chapter 34

Adipose-Derived Stem Cells for Periodontal Tissue Regeneration

Morikuni Tobita and Hiroshi Mizuno

Abstract

Mesenchymal stem cells can effectively regenerate destroyed periodontal tissue. Because periodontal tissues are complex, mesenchymal stem cells that can differentiate into many tissue types would aid periodontal tissue regeneration. Indeed, periodontal tissue regeneration using mesenchymal stem cells derived from adipose tissue or bone marrow has been performed in experimental animal models, such as rat, canine, swine, and monkey. We have shown that rat periodontal tissue can be regenerated with adipose-derived stem cells. Adipose tissue contains a large number of stromal cells and is relatively easy to obtain in large quantities, and thus constitutes a very convenient stromal cell source. In this chapter, we introduce a rat periodontal tissue regeneration model using adipose-derived stem cells.

Key words: Periodontal tissue, Adipose-derived stem cells, Rat model, Platelet-rich plasma, Periodontal disease

1. Introduction

The dental attachment apparatus consists of two mineralized tissues: the cementum and the alveolar bone. These tissues are interposed with vascular connective tissue that is fibrous and cellular, termed the periodontal ligament (1). Periodontal disease can lead to destruction of the periodontium, including the alveolar bone, cementum, the periodontal ligament, and the gingiva. Developing effective treatments for periodontal tissue disease is important because only limited regeneration of these tissues occurs naturally. Several methods, such as guided tissue regeneration (2, 3) and enamel matrix protein (4, 5), have been investigated for periodontal tissue regeneration in a clinical setting. However, the effectiveness of these treatments was limited (6, 7) because they

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lacked key factors to recruit cells to the site of damage, a critical step in successful periodontal tissue regeneration. To overcome this limitation, mesenchymal stem cells have been adapted for regeneration of complex structures, such as periodontal tissues, and have yielded promising results (8, 9).

Adipose-derived stem cells (ASCs) can differentiate into several tissues, such as bone, cartilage, adipose, and neurons (10–13). In recent years, it was also suggested that ASCs can promote periodontal tissue regeneration (14). ASCs are a promising source of cells for tissue-engineering strategies since adipose tissue is available in large quantities and is relatively easy to obtain. Moreover, liposuction procedures used to obtain the ASCs have minimal donor site morbidity and induce limited patient discomfort.

Periodontal tissue regeneration has been performed in several experimental animal models, such as rat, canine, monkey, and swine (8, 14–17). The rat model is especially well suited to investigate the ability of different implanted material to regenerate periodontal tissue. In this chapter, we introduce protocols to regenerate rat periodontal tissue using ASCs.

2. Materials

2.1. Harvesting ASCs from Rat Adipose Tissue

1. Inbred male Wistar rat weighing approximately 350 g.
2. Sodium pentobarbital.
3. Phosphate-buffered saline (PBS).
4. 0.12% Collagenase dissolved in PBS, freshly prepared prior to use.
5. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic as a control medium. Store at 4°C.
6. 100-mm Culture dish.
7. 50-ml Centrifuge tube.
8. 40-μm Nylon mesh.
9. Trypan blue.

2.2. ASC Culture

1. DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic as a control medium. Store at 4°C.
2. Trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA) (1 mM).

2.3. Generation of Periodontal Tissue Defect

1. Dental burs (Round, #2, Shank type).
2. Micro motor (Rotex 780).
3. Surgical blade.

2.4. Preparation of Platelet-Rich Plasma for Cell Vehicle

1. 100-mm Culture dish.
2. Blood collection tubes: 5 ml EDTA–2Na or 3.8% citric acid–Na blood collection tube.
3. Calcium chloride:calcium chloride injection 2%.
4. 15-ml Centrifuge tube.

2.5. Jaw Dissection

1. Micro motor (Rotex 780).
2. Dental burs (Straight Fissure Crosscut, #557, Shank type; HP).

2.6. Fixation and Decalcification

1. 4% Paraformaldehyde PBS.
2. Decalcification solution.
3. 50-ml Centrifuge tube.

2.7. Hematoxylin and Eosin Staining

1. 10% Formaldehyde.
2. Hematoxylin.
3. Eosin.

2.8. Immunohistochemistry

1. Mouse anti-osteocalcin monoclonal antibody (Clone No. OCG3).
2. Goat anti-rabbit IgG polyclonal antibody.
3. 0.03 mol/l 3,3′-diaminobenzidine 4HCl.
4. Tris-buffered saline (TBS).

3. Methods

In rat in vivo experiments, it is important to establish procedures for generating uniform periodontal tissue defect models. During surgery, the researcher should maintain the airway, and minimize surgical stress and the operating time, because oral tissue is delicate.

Periodontal tissue includes the alveolar bone, periodontal ligament, cementum, and gingival tissue. It is important to completely remove the periodontal ligament and cementum in periodontal tissue defect models. Repair of this damage requires regeneration of diverse tissues and thus tests the ability of undifferentiated ASCs to effectively regenerate complex tissue structures (see Fig. 4 below).

3.1. Harvesting ASCs from Rat Adipose Tissue

1. Anesthetize the Wistar rats with an intraperitoneal injection of sodium pentobarbital at 40 mg/kg and shave the surgical area (see Note 1).
2. After a skin incision of the inguinal region, harvest the inguinal fat pads, place them in the hood, and wash extensively with PBS.

3. Mince the fat pads for 10 min with fine scissors in culture dishes, and transfer the material into a 50-ml centrifuge tube. Add 0.12% collagenase and enzymatically digest with 0.12% collagenase at 37°C for 40 min.
4. Add an equal volume of control medium to neutralize the collagenase.
5. Centrifuge the cell suspension at 1,300 rpm ($260 \times g$) for 5 min to obtain a high-density ASC pellet.
6. Aspirate the supernatant, being careful not to disturb the cell pellets. Resuspend the cells pellets in control medium and incubate at room temperature for 10 min.
7. Filter the cell suspension through a 40-μm nylon mesh to remove cellular debris.
8. Count the cells using Trypan blue and plate at a concentration of 5×10^5 cells/100 mm in tissue culture dishes.

3.2. ASC Culture

1. Maintain the primary ASCs in control medium at 37°C in 5% carbon dioxide. Change the culture medium every 3 days.
2. Once adherent cells become subconfluent, aspirate the culture medium and wash the cells with 5 ml of PBS three times. Add 3–5 ml of trypsin–EDTA at 37°C for 5 min to detached the cells.
3. Resuspended the ASCs with an equal volume of control medium.
4. Centrifuge the cell suspension again and resuspend in a small volume of control medium.
5. Calculate the cell concentration and adjust the volume to obtain 1.5×10^7 cells/ml for the subsequent transplantation.

3.3. Generation of the Periodontal Tissue Defect

1. Anesthetize the Wistar rats with an intraperitoneal injection of sodium pentobarbital (40 mg/kg).
2. Place the rat in the supine position and make a mucosal incision from the gingival sulcus of the second molar mesial palatal side to the first molar mesial palatal side. Make an additional incision of approximately 5 mm in the mesial direction from the first molar mesial side. An illustration of the rat palatal region is shown in Fig. 1 (see Note 2).
3. Elevate the mucosal flap and excise the periodontal tissue (the cementum, alveolar bone, and periodontal ligament) bilaterally at the first molar palatal side under irrigation using a dental round bur. The dimensions of the defect should be approximately 1 (width) × 1 (height) × 1–1.5 (width) mm. The rat periodontal tissue defect is shown in Fig. 2.

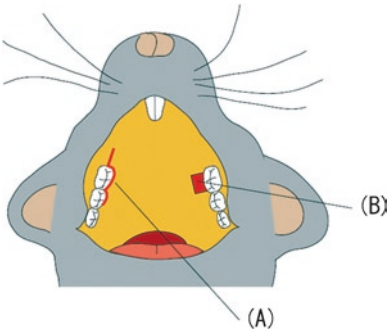

Fig. 1. Illustration of the sulcular incision and the periodontal tissue defect site. (A) Sulcular incision. A sulcular incision was performed using a microscope at the palatal side of the first and second molar, followed by a continuous vertical incision at the mesial side of the first molar. (B) The site at which the periodontal tissue defect was generated.

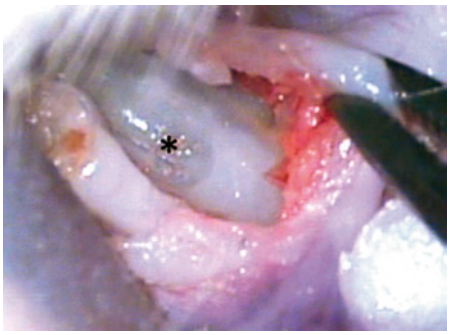

Fig. 2. Representative image of a rat periodontal tissue defect. A periodontal tissue defect (*) of approximately 1 × 1 × 1–1.5 mm was created in the palatal side of the first molar. After removing the alveolar bone, the periodontal ligament and cementum were scaled using a dental explorer and dental scaler.

3.4. Preparation of Platelet-Rich Plasma as a Cell Vehicle

1. Anesthetize 10-week-old male Wistar rats (330–380 g) with diethyl ether and an intraperitoneal injection of 40 mg/kg of sodium pentobarbital.
2. Draw 10 ml of whole blood preoperatively via cardiac puncture with an 18-gauge needle into the blood collection tubes.
3. Centrifuge the blood in a standard laboratory centrifuge for 10 min at 2,400 rpm (450 × *g*).
4. Collect the supernatant plasma and the buffy coat, which contains platelets and leukocytes, using an 18-gauge long cannula, and transfer into a 15-ml centrifuge tube.
5. Centrifuge at 3,600 rpm (850 × *g*) for 15 min to concentrate the platelets.
6. Guide the top of an 18-gauge long cannula to the bottom of the plasma and gently aspirate 1 ml of the infranatant plasma containing the buffy coat. Discard the supernatant plasma (see Note 3).

3.5. Implantation of the ASCs/PRP Admixture

1. Mix 1 ml of the PRP with 1.5×10^7 ASCs using an 18-gauge long cannula and a 1-ml syringe.
2. After mixing the ASCs and PRP well, add 1 ml to a 100-mm dish.
3. Add 10% calcium chloride solution to the ASCs/PRP admixture.
4. After 5–10 min, implant approximately 1 μl of the ASCs/PRP admixture gels onto the generated periodontal tissue defect with tweezers.
5. Finally, place the mucosal flaps back using 6-0 absorbable surgical suture.

3.6. Jaw Dissection

1. Euthanize the rats by intraperitoneal injection of an overdose of sodium pentobarbital.
2. Place the animal in the supine position, and make an incision in the mucosa and muscle in the angle of the mouth toward the Gonial angle in order to expand the view.
3. Incise the buccal side of the mucosa, the backside mucosa of the third molar, and the mucosa between incisor teeth and the first molar. An illustration of the incision line is shown in Fig. 3.
4. Remove the jaw from the rat after separating it from the zygomatic arches and cutting the hard palate. Remove the zygomatic arch using a straight fissure bur with a micro motor.
5. Separate the jaw into two pieces, the right half and the left half.

3.7. Fixation, Decalcification, and Histological Analysis

1. After harvesting the specimens, fix in 4% paraformaldehyde solution for 7 days at room temperature.
2. After fixation, decalcify the specimens using decalcification solution for 2–3 days at 4°C. Change the decalcification solution every day.
3. Cut the specimens at the center of the teeth in the buccal–palatal plane using a #15 surgical blade.

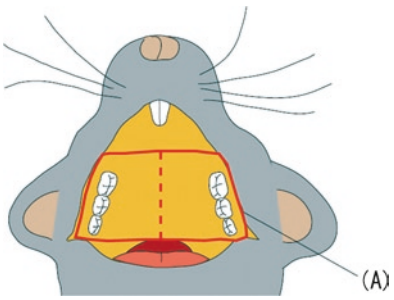

Fig. 3. Illustration of the gingival incision used to harvest tissue samples. (A) The *hand-drawn trapezoid* indicates the incision line on the gingival tissue. After harvesting, the sample was divided into two sections as indicated by the *dotted line.*

4. Wash the specimens with water and then embed them in paraffin (see Note 4).
5. Section the tissue at 5-μm increments using a microtome and place on glass slides.
6. Immerse the slides in xylene I for at least 10 min.
7. Bathe the slides in xylene II and then xylene III for 2–3 min each.
8. Immerse the slides in 100% ethanol three times for 2–3 min each, followed by immersion in 90, 80, 70, and 50% ethanol for 2–3 min each.
9. Wash the slides in tapped water.
10. Immerse the slides in Carazzi's Hematoxylin for 20 min (see Note 4).
11. Wash the slides two times in tapped water for 2–3 min.
12. Immerse the slides in eosin solution for 15 min.
13. Dehydrate by immersing in a graded ethanol series three times (70, 90, and 100%) for 2–3 min each, followed by immersion in xylenes I–III three times for 2–3 min each.
14. Mount slides using mounting medium.
15. Observe the stained slides under a light microscope. Expected histological results for hematoxylin and eosin staining are shown in Fig. 4.

3.8. Immunohistochemistry for Osteocalcin

1. Section the tissue specimens in 4-μm increments using a microtome and place onto a glass slide.
2. Immerse the slides in a series of xylenes, ethanol, and water as described above in Subheading 3.7, steps 6–9.
3. Wipe the area around the sections to carefully to remove excess solution.
4. Immerse sections in 0.1% trypsin for 20 min at 37°C.

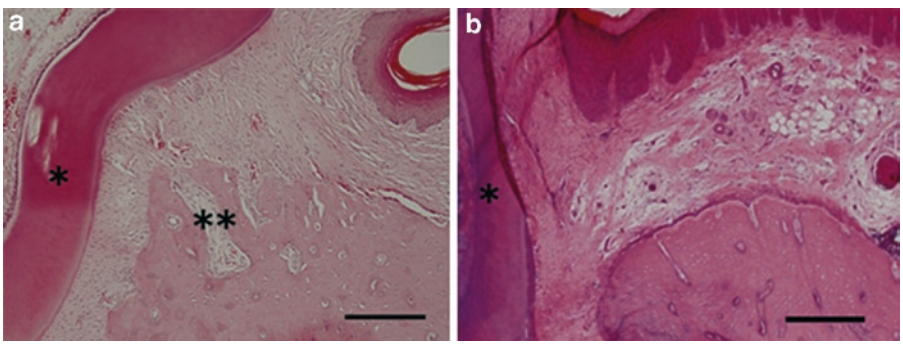

Fig. 4. Histological analysis 8 weeks after surgery. (**a**) Hematoxylin and eosin (H&E) staining of a periodontal tissue defect following implantation of ASCs and PRP. (**b**) H&E staining of a periodontal defect in the absence of implantation. The dental root (*) and regenerated alveolar bone (**) are noted. Bar, 250 μm.

5. Wash the slides in tap water for 2–3 min.
6. Immerse sections in 3% H_2O_2 for 10 min at room temperature to block endogenous peroxidase activity.
7. Wash the slides three times in 50 ml TBS for 5 min each.
8. Remove the slides from the TBS, wipe gently around each section, and cover tissues with blocking reagent (normal swine serum) for 30 min to block nonspecific staining.
9. Remove the blocking buffer, wipe gently around each section, and cover tissues with primary antibody (bovine osteocalcin, mouse monoclonal [1:200 dilution]) or negative control reagent. Incubate sections at room temperature for 60 min.
10. Wash the slides three times in 50 ml TBS for 5 min each.
11. Wipe gently around each section and cover tissues with biotin-conjugated goat anti-rabbit IgG secondary antibody (1:200 dilution). Incubate for 30 min at room temperature.
12. Wash the slides three times in 50 ml TBS for 5 min each.
13. Visualize by incubating with substrate solution containing 0.03 mol/l 3,3′-diaminobenzidine 4HCl for 10 min.
14. Wash the slides in distilled water for 5 min.
15. Immerse the slides in Carazzi's Hematoxylin for 5 s.
16. Wash the slides in tap water.
17. Dehydrate by immersing in a graded ethanol series three times (70%, 90%, and 100%) for 2–3 min each, followed by immersion in xylenes I–III three times for 2–3 min each.
18. Mount slides using mounting medium.
19. Observe the stained slides under a light microscope. A representative image for osteocalcin staining is shown in Fig. 5 (see Note 5).

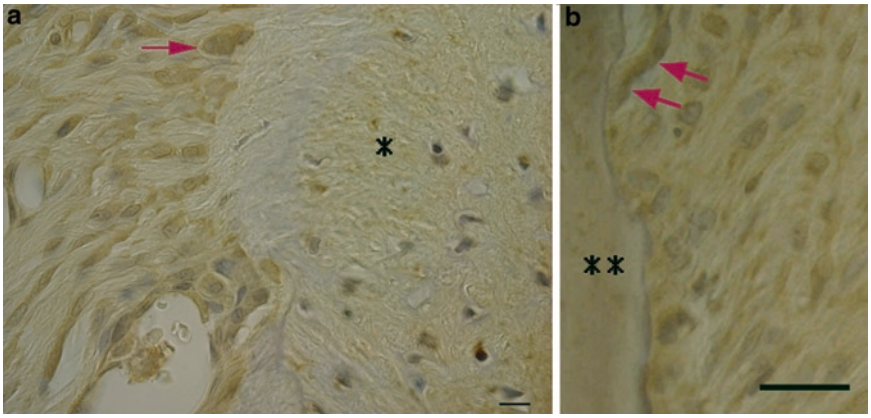

Fig. 5. Immunohistochemical staining for ostecalcin. High-magnification images of the regenerating alveolar bone (**a**; bar, 10 μm) and dental root surfaces (**b**; bar, 25 μm) shown in Fig. 4. *Regenerated alveolar bone, **dental root, *arrow* osteocalcin-positive cells.

4. Notes

1. Green fluorescent protein (GFP) transgenic rats (Crj:Wistar-Tgn[CAG/GFP]) may also be used to harvest ASCs and are helpful in investigating the biologic role of implanted ASCs.
2. Use of a microscope when generating the rat periodontal tissue defect is preferable.
3. Generally, six to seven times of concentrated platelet can be collected from the generated 1 ml of PRP.
4. Application time is subject to change depending on temperature of the room.
5. Osteocalcin is detected in the cementum and on the surface of bone.

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References

1. Glickman, I., and Carranza, F. A. (1984) Glickman's clinical periodontology. Phila delphia: Saunders **6th ed**, 1–61.
2. Nyman, S., Lindhe, J., Karring, T., and Rylander, H. (1982) New attachment following surgical treatment of human periodontal disease. *J Clin Periodontol* **9**, 290–6.
3. Lindhe, J., Pontoriero, R., Berglundh, T., and Araujo, M. (1995) The effect of flap management and bioresorbable occlusive devices in GTR treatment of degree III furcation defects. An experimental study in dogs. *J Clin Periodontol* **22**, 276–83.
4. Hammarstrom, L. (1997) Enamel matrix, cementum development and regeneration. *J Clin Periodontol* **24**, 658–68.
5. Sakallioglu, U., Acikgoz, G., Ayas, B., Kirtiloglu, T., and Sakallioglu, E. (2004) Healing of periodontal defects treated with enamel matrix proteins and root surface conditioning – an experimental study in dogs. *Biomaterials* **25**, 1831–40.
6. Baelum, V., and Lopez, R. (2003) Weak evidence for a benefit of Emdogain in the treatment of intrabony defects. *Evid Based Dent* **4**, 66.
7. Lopez, R., and Baelum, V. (2003) Is grafting biomaterials or biological agents more effective than open-flap debridement in treating deep intraosseous defects? *Evid Based Dent* **4**, 64–5.
8. Kawaguchi, H., Hirachi, A., Hasegawa, N., Iwata, T., Hamaguchi, H., Shiba, H., et al. (2004) Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. *J Periodontol* **75**, 1281–7.
9. Yamada, Y., Ueda, M., Hibi, H., and Baba, S. (2006) A novel approach to periodontal tissue regeneration with mesenchymal stem cells and platelet-rich plasma using tissue engineering technology: a clinical case report. *Int J Periodontics Restorative Dent* **26**, 363–9.

10. Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., et al. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 7, 211–28.
11. Zuk, P. A., Zhu, M., Ashjian, P., De Ugarte, D. A., Huang, J. I., Mizuno, H., et al. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279–95.
12. Ogawa, R., Mizuno, H., Watanabe, A., Migita, M., Hyakusoku, H., and Shimada, T. (2004) Adipogenic differentiation by adipose-derived stem cells harvested from GFP transgenic mice-including relationship of sex differences. *Biochem Biophys Res Commun* **319**, 511–7.
13. Safford, K. M., Hicok, K. C., Safford, S. D., Halvorsen, Y. D., Wilkison, W. O., Gimble, J. M., et al. (2002) Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochem Biophys Res Commun* **294**, 371–9.
14. Tobita, M., Uysal, A. C., Ogawa, R., Hyakusoku, H., and Mizuno, H. (2008) Periodontal tissue regeneration with adipose-derived stem cells. *Tissue Eng Part A* **14**, 945–53.
15. Seo, B. M., Miura, M., Gronthos, S., Bartold, P. M., Batouli, S., Brahim, J., et al. (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* **364**, 149–55.
16. Buser, D., Hoffmann, B., Bernard, J. P., Lussi, A., Mettler, D., and Schenk, R. K. (1998) Evaluation of filling materials in membrane-protected bone defects. A comparative histomorphometric study in the mandible of miniature pigs. *Clin Oral Implants Res* **9**, 137–50.
17. Hammarstrom, L., Heijl, L., and Gestrelius, S. (1997) Periodontal regeneration in a buccal dehiscence model in monkeys after application of enamel matrix proteins. *J Clin Periodontol* **24**, 669–77.

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