Chapter 30

Methodology, Biology and Clinical Applications of Human Mesenchymal Stem Cells

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Abstract

Stem cells are known by their capacity of self-renewal and differentiation into at least one specialized cell type. Mesenchymal stem cells (MSCs) were isolated initially from bone marrow but are now known to exist in any vascularized organ or tissue in adults. MSCs have a great therapeutic potential, due to their ability to migrate to sites of tissue injury and secrete trophic factors that hasten endogenous repair. They have also been shown to present immunosuppressive properties that may be used in the treatment of autoimmune or graft-versus-host diseases. Clinical trials employing MSCs show that the therapy is safe, but the efficiency needs to be in tested in phase III and IV studies. We describe here protocols for the isolation of human MSCs from human bone marrow and adipose tissue. The safe use of these cells demand a thorough in vitro characterization, as described in protocols of immunophenotyping by flow cytometry and analysis of their capacity to differentiate into adipogenic, osteogenic, and chondrogenic lineages.

Key words: Mesenchymal stem cells, Clinical applications, Bone marrow, Adipose-derived mesenchymal stem cells, Differentiation

1. Introduction

The study of stem cells has received considerable attention during the past 15 years. Although the therapeutic potential of embryonic stem cells remains undefined, hematopoietic stem cells have been in clinical use for over 40 years, in bone marrow transplantation, and a large number of clinical trials have shown that adult stem cell therapy is safe and potentially effective for the treatment of many non-hematological diseases (1). Adult stem cell compartments have been extensively investigated, and are well known for many tissues and organs. The bone marrow contains at least three stem/progenitor compartments, including hematopoietic, endothelial, and mesenchymal progenitor cells (2).

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Mesenchymal stem cells (MSCs) are plastic-adherent adult stem cells, operationally defined based on their ability to proliferate and differentiate into cells such as osteoblasts, adipocytes, and chondrocytes (3). Cultured MSCs secrete various bioactive molecules that display anti-apoptotic, immunomodulatory, angiogenic, anti-scarring, and chemoattractant properties, providing a basis for their therapeutic use in clinical practice (4). MSCs may be isolated from virtually any tissue (5), and it has been shown that the adipose tissue is one of the richest sources of these cells (6). Adipose-derived stem cells (ADSC) have attracted increasing interest and these cells have already been used in a number of clinical trials, but the determination of their true potential still depends on further research on their fundamental biology (7).

The number of clinical trials that uses MSCs increases every year. A search for "mesenchymal stem cells" at the Clinical Trials website (ClinicalTrials.gov) sponsored by the National Institute of Health returns more than 140 trials for different diseases. Many of them do not clearly describe the cell type or the procedure for isolation of MSCs and in several cases, they use bone-marrow mononuclear cells instead of MSCs. This lack of standardization in isolation, ex vivo expansion, and characterization of MSCs for clinical studies is an issue that needs special attention. Most of the studies using MSCs are phase I and II demonstrating the safety and feasibility with minimal side effects. Few studies are at phase III and no study at phase IV. Osiris Therapeutics Inc. developed a MSC-based product called Prochymal™ and tested in phase III studies to treat Crohn's disease and graft-versus-host disease (GvHD). They are also recruiting patients for two phase II studies for type 1 diabetes and chronic obstructive pulmonary disease. Other examples of diseases targeted in clinical trials using MSCs are critical limb ischemia, kidney transplantation, spinal cord injury, osteodysplasia, and cartilage defects (Table 1).

In this chapter, we present methods for the isolation, cultivation, and characterization of MSCs from human bone marrow and adipose tissue, for use in basic studies or clinical trials.

2. Materials

All reagents should be prepared with sterile, ultrapure water. Solutions prepared must be sterile filtered. All reagents are from Sigma Chemical Co., unless otherwise stated. Plasticware is from TPP (Trasadingen). Tissue culture supplies, including centrifuge, laminar flow hood, Neubauer chamber, inverted phase contrast microscope, culture plates, and flasks, 10-mL pipettes, 15- and 50-mL plastic tubes and a motorized pipettor, will not be described in detail. All surgical instruments and glassware should be sterile.

Table 1
Clinical trials for several diseases using mesenchymal stem cells (from ClinicalTrials.gov)

Study	Disease	Source/via	Phase/status
Extended evaluation of Prochymal™ adult human stem cells for treatment-resistant moderate-to-severe Crohn's disease	Crohn's disease	Bone-marrow/intravenous	III/completed
Adult human mesenchymal stem cells for treatment of moderate-to-severe Crohn's disease (Prochymal™)	Crohn's disease	Bone-marrow/intravenous	II/completed
Safety and efficacy of prochymal for the salvage of treatment-refractory acute GvHD patients	GvHD	Bone-marrow/intravenous	II/completed
Efficacy and safety of adult human mesenchymal stem cells to treat patients who have failed to respond to steroid treatment for acute graft-versus-host disease (GvHD)	Acute GvHD	Bone-marrow/intravenous	III/completed
Stem cell therapy for vasculogenesis in patients with severe myocardial ischemia	Myocardial ischemia	Bone-marrow/intramyocardial injections	I/II/completed
Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery (PROMETHEUS)	Myocardial infarction	Bone-marrow/intramyocardial injections	II/active not recruiting
Comparison of autologous mesenchymal stem cells and mononuclear cells on diabetic critical limb ischemia and foot ulcer	Critical limb ischemia and foot ulcer in type 2 diabetic patients	Bone-marrow/intramuscular injection	I/completed
Induced wound healing by application of expanded bone marrow stem cells in diabetic patients with critical limb ischemia	Critical limb ischemia (diabetic foot)	Bone marrow/intraarterial and intramuscular	II/completed

(continued)

Table 1
(continued)

Study	Disease	Source/via	Phase/status
Human adult stem cells for the treatment of moderate-to-severe chronic obstructive pulmonary disease (COPD) Prochymal™	Moderate to severe chronic obstructive pulmonary disease	Bone-marrow/intravenous	II/active not recruiting
Induction therapy with autologous mesenchymal stem cells for kidney allografts	Kidney transplantation	Bone-marrow/intravenous	Completed
Autologous adipose derived MSCs transplantation in patient with spinal cord injury	Spinal cord injury	Adipose tissue/intravenous	I/completed
Induction therapy with autologous mesenchymal stem cells for kidney allografts	Meniscectomy	Bone-marrow + hyaluronic acid/intrarticular injection	I/II/completed
The effect of human adipose tissue-derived MSCs in Romberg's disease	Romberg's disease	Adipose tissue/intramuscular injection	II/completed
Stromal therapy of osteodysplasia after allogeneic bone marrow transplantation	Osteodysplasia	Bone-marrow/intravenous	II/completed
The use of autologous bone marrow mesenchymal stem cells in the treatment of articular cartilage defects	Cartilage defects	Bone-marrow/in situ	II/III/recruiting
Autologous transplantation of mesenchymal stem cells for treatment of patients with onset of type 1 diabetes	Type I diabetes mellitus	Bone-marrow/intravenous	II/III/recruiting
Using mesenchymal stem cells to fill bone void defects in patients with benign bone lesions	Benign bone defects	Bone-marrow/in situ	II/III/active not recruiting

2.1. Isolation of Mesenchymal Stem Cells from Human Bone Marrow

Adult bone marrow is the most common source of MSCs, whether for basic studies or for clinical trials. Bone marrow is harvested by puncture of the iliac crests, and safety guidelines should be closely observed.

1. Complete medium, composed of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 10 mM HEPES free acid.
2. HB-CMF-HBSS (Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution containing 10 mM sodium HEPES).
3. Ficoll-Hypaque 1.077 g/L.
4. Bone marrow harvest needle.
5. Heparinized 20-mL syringe (BD Bioscience).

2.2. Isolation of Mesenchymal Stem Cells from Adipose Tissue

ADSC are easier to collect than bone marrow derived cells. The method was originally described by Zuk et al. (6).

1. Glass separating funnel.
2. Low-glucose DMEM.
3. Culture medium: Low-glucose DMEM containing 10% FCS (see Note 1).
4. Collagenase type I solution (0.5–1.0 mg/mL in DMEM/10 mM HEPES).
5. Phosphate buffered saline (PBS) solution. Prepare a 10× stock solution: dissolve 80 g of NaCl, 2.0 g of KCl, 14.4 g of Na_2HPO_4 and 2.4 g of KH_2PO_4 in 800 mL distilled water, adjust pH to 7.4, adjust volume to 1 L with additional distilled water.
6. Red blood cell (RBC) lysis buffer: 160 mM NH_4Cl.
7. 70-μm strainer or nylon screen.

2.3. Establishment and Maintenance of MSC Cultures

According to the minimal criteria proposed by the International Society for Cellular Therapy to define human MSC, cells must be plastic adherent when maintained in standard culture conditions (8). Bone marrow- or adipose tissue-derived cells should therefore be cultivated for some weeks.

1. HB-CMF-HBSS.
2. Trypsin–EDTA solution (0.25% Trypsin and 0.01% EDTA in HB-CMFHBSS).
3. DMEM supplemented with 10% FCS and 10 mM HEPES free acid.

2.4. Flow Cytometry

According to the same guidelines mentioned above, human MSC must express CD105, CD73, and CD90, whereas markers such as CD14 or CD11b, CD34, CD45, CD79a or CD19, and HLA-DR,

that can be found in macrophages, endothelial cells, leucocytes, B cells, and antigen-presenting cells, respectively, should be absent.

1. DMEM/10 mM HEPES supplemented with 10% FCS.
2. Solution of trypsin (0.25%) with 1 mM ethylenediamine (trypsin–EDTA), diluted in HB-CMF-HBSS.
3. Solution of penicillin (50 IU/mL)/streptomycin 100 μg/mL.
4. PBS.
5. Primary antibodies: fluorescein isotiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies specific for human CD11b or CD14, CD19, CD34, CD44, CD45, CD73, CD90, CD105, and HLA-DR (BD Bioscience), tested for flow cytometry application.
6. Flow cytometer equipped with 488-nm argon laser (FACSCalibur, BD Bioscience).

2.5. Cellular Differentiation

Finally, the minimal criteria proposed by the International Society for Cellular Therapy to define human MSC state that cells must differentiate to osteoblasts, adipocytes, and chondroblasts in vitro. For that, the cells are cultivated with differentiation-inducing media for some weeks and analyzed by chemical or molecular methods.

2.5.1. Adipogenic Differentiation

1. Iscove's Modified Dulbecco's Medium/10 mM HEPES + 20% FCS, supplemented with the following reagents:
   (a) 2.5 μg/mL insulin from bovine pancreas. Prepare a 1,000× stock solution at 2.5 mg/mL, in water. Store at −20°C in single-use aliquots (see Note 2).
   (b) 3.5 μM rosiglitazone. Prepare a 1,000× stock solution: 12.5 mg of rosiglitazone powder dissolved in 10 mL of 5 mM dimethyl sulfoxide (DMSO). Store at −20°C in single-use aliquots.
   (c) 100 μM indomethacin. Prepare a 1,000× stock solution: 350 mg of indomethacin powder dissolved in 10 mL of 5 mM DMSO. Store at room temperature (RT).
   (d) 10^{-8} M dexamethasone. Prepare a 1,000× stock solution: dissolve 1 mg dexamethasone in 1 mL ethanol (2.5×10^{-3} M). Transfer 10 μL of the above solution to 2.5 mL of DMEM without serum to obtain a 10^{-5} M solution. Both solutions can be stored at −20°C.
2. Sodium heparin.
3. Oil Red O solution: add 3.75 g of Oil Red O to 100 mL isopropanol. Mix three volumes of this solution to two volumes of dH_2O and filter on filter paper. Store at RT.

4. Harris hematoxylin.
5. Glycerin jelly: Add 10 g of gelatin to 60 mL of dH_2O and stir at 60°C to dissolve. Mix to 70 mL of glycerin and 1 mL of phenol. Store at 4°C.

2.5.2. Osteogenic Differentiation

1. DMEM/10 mM HEPES + 10% FCS, supplemented with the following reagents (see Note 3):
   (a) 10 mM β-glycerophosphate. Prepare a 100× stock solution: 2.16 g of β-glycerophosphate powder dissolved in 10 mL DMEM without serum.
   (b) 5 μg/mL ascorbic acid 2-phosphate. Prepare a 1,000× solution: 50 mg of ascorbic acid diluted in 10 mL of DMEM/10 mM HEPES without serum. Store at 4°C.
   (c) 10^{-5} M dexamethasone.
2. Alizarin Red S solution: add 2 g Alizarin Red S in 90 mL of dH_2O. Adjust pH to 4.1 by adding ammonium hydroxide and complete the volume to 100 mL. Filter with filter paper and store at RT.

2.5.3. Chondrogenic Differentiation

1. DMEM/10 mM HEPES + 10% FCS, supplemented with the following reagents:
   (a) Insulin from bovine pancreas, 2.5 μg/mL in water
   (b) 5 μg/mL ascorbic acid
   (c) 1 ng/mL TGF-β1 (Millipore)
2. Alcian Blue solution: add 1 g Alcian Blue to 100 mL of acetic acid 3%. Mix well and adjust pH to 2.5. Filter with filter paper to remove undiluted particles. Store at RT.

3. Methods

MSCs may be used for therapeutic purposes as part of the mononuclear fraction obtained from bone marrow aspirates, or of the stromal fraction obtained from liposuction material, isolated as described below. A short period of cultivation (24–72 h) allows the enrichment of MSCs in the adherent fraction. The preparation of conventional MSCs demands around 2 weeks of culture, with expansion of the adherent layer which progressively becomes more homogeneous and free from contaminant cells.

For clinical use, the isolation and cultivation of stem cells should be done in a certified laboratory under Good Manufacturing Practice (GMP) conditions. Local regulations must be observed. In most countries, the therapeutic use of stem cells in non-hematologic diseases is still considered as an experimental procedure,

and studies must be approved by a Human Research Ethics Committee, with signature of an informed consent form by the patient.

3.1. Isolation of Mesenchymal Stem Cells from Human Bone Marrow

1. Bone marrow should be collected with iliac crest aspiration by an experienced hematologist. Aspirates can be obtained from the sternum.
2. Add to the bone marrow aspirate the same volume of HBSS and homogenate well by pipetting with a 10-mL pipette.
3. Add 15 mL of Ficoll-Hypaque to a 50-mL centrifuge tube and carefully add 10 mL of the bone marrow/HBSS mixture using a 10-mL pipette (see Note 4).
4. Centrifuge tubes for 25 min at 400 × *g* for 30 min at RT, with the brake off.
5. After centrifugation, cells will be separated by density, from bottom to top, in RBCs/granulocytes followed by the Ficoll-Hypaque layer, the mononuclear cells, and the layer of plasma/HBSS.
6. Remove the top plasma/HBSS layer, collect the mononuclear layer by aspiration using a 5-mL pipette or glass Pasteur (see Note 5) and transfer to a new 15-mL tube.
7. Wash cells two or three times by adding 10 mL of fresh HBSS. This will eliminate residual Ficoll-Hypaque.
8. Resuspend cells in 5–10 mL of complete medium and count them in a Neubauer chamber. Cell viability should be higher than 90%.
9. Plate cells at 2×10^6 cells/mL in complete medium on 6-well (3.5 mL/well) plates, and incubate at 37°C in a humidified chamber with 95% air, 5% CO_2.

3.2. Isolation of Mesenchymal Stem Cells from Adipose Tissue

The methodology described below is applied to liposuction tissue. To use the technique on fragments of adipose tissue, it should be cut into small pieces, washed with serum-free medium, and digested with collagenase (0.75–1%).

1. The liposuction procedure is performed by a qualified surgeon, and the material is collected in sterilized tubes (see Note 6).
2. Wash the liposuction material extensively with PBS, with the use of a separating funnel (Fig. 1), to remove erythrocytes and leukocytes.
3. Add 1 mg/mL collagenase type I solution to the material, in a 1:3 proportion (vol:vol).
4. Incubate in a water bath at 37°C for 30 min, with agitation (see Note 7).

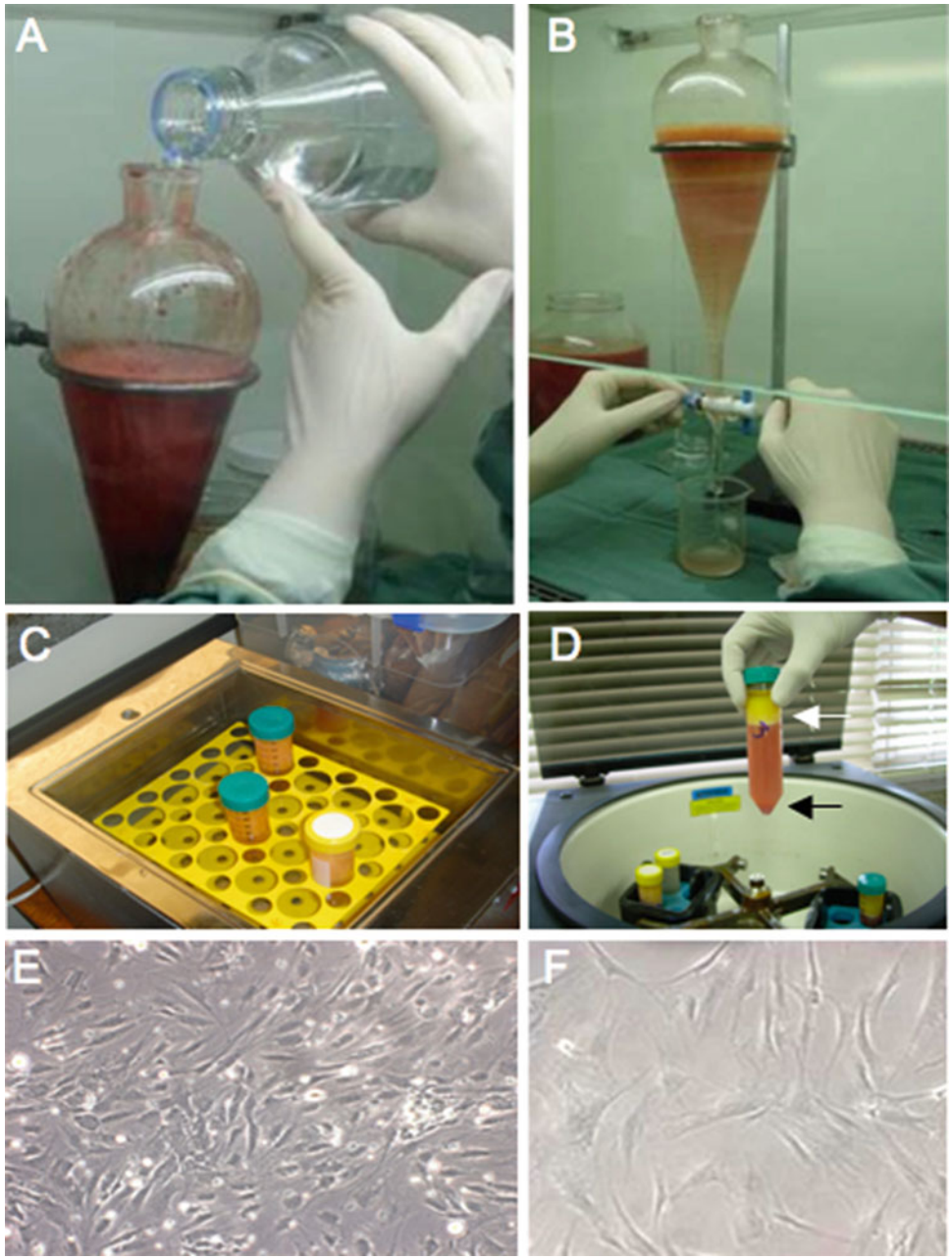

Fig. 1. Isolation of the stromal fraction from liposuctions material. The sample is extensively washed with PBS (**a**, **b**), incubated with collagenase at 37°C (**c**), and centrifuged (**d**) to separate mature adipocytes from the stromal fraction (*white* and *black arrows*, respectively). ADSC cultures are initially heterogeneous, with different types of contaminating cell (**e**), but become progressively more homogeneous (**f**). Original magnification ×200.

5. Neutralize the collagenase by adding the same volume of ice-cold DMEM supplemented with 10% FCS.
6. Centrifuge at 400 × *g* for 7 min at RT.

7. To eliminate contaminating erythrocytes, add to precipitate the appropriate volume (~10 to 50 mL) of RBC lysis buffer, shake, and incubate for 10 min at RT, with a new agitation after 5 min.
8. Centrifuge for 10 min at 400 × *g* and discard the supernatant.
9. Resuspend the stromal fraction in 10 mL of the culture medium.
10. Filter cell suspension through a 70-μm strainer or a nylon screen to get cleaner preparations.
11. Collect a small sample for determination of yield and viability by counting in a Neubauer chamber (see Note 8).
12. Centrifuge cells for 10 min at 400 × *g*, RT.
13. Discard the supernatant and resuspend in 1 mL of complete medium.

3.3. Establishment and Maintenance of MSC Cultures

After the procedures of isolation and initial plating of MSC from bone marrow or adipose tissue, cells must be expanded in vitro for basic studies or therapeutic application. As MSC adhere to plastic surfaces, they can be easily separated from the non-adherent hematopoietic cells by repeated washing and expanded in culture. Primary cultures generally become confluent within around 6–7 days, when they should be passaged to new tissue culture flasks for further expansion. For that, the cells are detached by incubation with trypsin, collected and split into new flasks. Subsequent passages are performed when cultures reach around 80–90% confluence. The number of cells collected in each passage may be registered to establish the growth kinetics of cultures.

1. Remove the medium and wash twice the cell monolayer with HB-CMF-HBSS.
2. Add the trypsin–EDTA solution in a volume which covers the cell layer and incubates the dish or flask at 37°C for 5–10 min.
3. Resuspend the cells in complete medium, with twice the volume of trypsin–EDTA used, to inactivate the enzyme.
4. Wash cell by centrifugation at 400 × *g* for 10 min at RT.
5. Discard the supernatant and resuspend the cells in appropriate volume of complete medium for plating. The volume will depend on the culture flask or plate to be used. In this step, the cells can be counted and plated as needed by the experiment.
6. Repeat steps 1–5 when the cells reach 80–90% confluence (see Note 9).

3.4. Immunophenotyping by Flow Cytometry

MSC are immunophenotyped by flow cytometry. For most types of procedures, the cells should be analyzed and used in early passages, typically between passages 3 and 5.

1. Prepare MSC cultures for flow cytometry analysis by plating cells in a 75-cm^2 flask and analyze when the culture reaches 80–90% of confluence. In general, the cell yield of one flask is enough for 10–15 immunophenotyping reactions.
2. Trypsinize and collect cells. Count viable cells and adjust concentration for 2×10^6 cell/mL in PBS.
3. Distribute in flow cytometry tubes (50 μL/tube).
4. Add antibodies in the appropriate combinations. Refer to the manufacturer's recommendation for the optimal volume of antibody to be used. For most of them, 5 μL is adequate for 50 μL of cell suspension.
5. Incubate for 30 min at 4°C, protected from light.
6. Add 1 mL of PBS, centrifuge the cells at 1,200 rpm for 10 min and remove supernatant to eliminate unbound antibodies.
7. Resuspend the cells in 0.5 mL PBS.
8. Analyze the cells in a flow cytometer (see Note 10).

3.5. Differentiation of MSC

To determine the differentiation potential of MSC, cultures in early (3–5) passages should be used.

1. Collect the cells by trypsinization, wash, resuspend in complete medium, and plate in 6-well culture plates, at the concentration usually used when splitting the cultures.
2. When the cells reach 80% confluence, replace the complete medium with each of the differentiation-inducing media, prepared as described above.
3. Keep the cultures for 1 month, with two medium changes per week (see Note 11).
4. Adipogenic, osteogenic, or chondrogenic differentiation of bone marrow- or adipose tissue-derived MSCs may be analyzed by staining with specific reagents (see Note 12). For that, remove medium, wash the monolayer with PBS, fix cells with 4% paraformaldehyde in PBS for 15–30 min at RT, and wash once with dH_2O. Stain as below.

3.5.1. Adipogenic Differentiation

1. Cover the monolayer with Oil Red O solution and incubate for 5 min, at RT.
2. Remove Oil Red O and wash at least three times with dH_2O.
3. Counterstain with Harris hematoxylin for 10 min and wash with dH_2O.
4. Cover cells with melted glycerin jelly for long-term storage.
5. Lipid drops will stain red and nucleus in purple/blue. Analyze using an inverted microscope (see Note 13).

3.5.2. Osteogenic Differentiation

1. Cover the monolayer with Alizarin Red S solution and incubate for 5 min, at RT.
2. Remove the Alizarin Red S solution and wash at least three times with dH_2O to remove excess of dye.
3. The calcium rich extracellular matrix will stain red and can be seen macroscopically (see Note 13).

3.5.3. Chondrogenic Differentiation

1. Cover the monolayer with the Alcian Blue solution and incubate for 5 min, at RT.
2. Remove the Alcian Blue solution and wash at least three times with dH_2O to remove excess dye.
3. Glycosaminoglycans rich extracellular matrix will stain blue and can often be seen macroscopically (see Note 13).

4. Notes

1. An alternative for avoiding the use of fetal calf serum in clinical protocols, for example, is the use of 5% autologous plasma.
2. You may need to lower the pH of the solution to solubilize insulin.
3. This medium can be stored at 4°C and used several times. Add fresh dexamethasone at final concentration of 1×10^{-8} M to the osteogenic differentiation medium at each medium change (3 mL per each well of a 6-well plate).
4. This step is critical for an efficient separation of mononuclear cells. Change the mode of pipettor to gravity and use a 10-mL pipette slowly to deliver the suspension of cells over the Ficoll-Hypaque layer. This will avoid mixing of the two phases.
5. Take care when collecting the mononuclear layer. Avoid liquid reflux from the pipette or glass Pasteur to the tube when collecting cells. It will disrupt the mononuclear layer leading to cell loss.
6. Liposuction material can be stored at 4°C overnight, but the yield is better when isolation is done no more than 3 h after the tissue is collected.
7. Collagenase type I may be used in concentrations between 0.5 and 1.0 mg/mL. We have a higher cell yield with 1.0 mg/mL. For dense adipose tissue specimens, a concentration of 1.5 mg/mL should be used. In our experience, agitating the sample during incubation with collagenase does not result in better yields.
8. The cell yield obtained in our laboratory ranges from 2×10^5 to 10×10^5 cells/mL of adipose tissue collected from different

sites. Yield depends on the donor site and gender of the patient.

9. Split ratios are defined empirically so that subcultures are performed twice a week, and should be modified as needed. Split ratios may generally be set to 1:6 at passage 5 or 6, 1:9 at around passage 11 and, if necessary, ratios of up to 1:24 may be used, especially for cell populations subjected to extensive subcultivation (more than 20 passages). Culture medium is changed every 3–4 days.
10. Isotypic controls should be systematically included for all analyses. The best way to present data relative to MSC cultures is using histograms.
11. Some MSC cultures, depending on the source, are less prone to differentiate in some of the three lineages described above. In this case, the cells can be maintained for more the 1 month in the differentiation-inducing medium, increasing the probability of differentiation.
12. It is also of great interest to establish molecular methods, such as real-time RT-PCR, for analyzing differentiation. These methods are beyond the scope of the present chapter, but usually involve the use of primers for pluripotency genes (such as Sox2, Nanog, or Oct4) and of at least two genes characterizing each of the differentiation pathways investigated.
13. Differentiated cells can be best seen in inverted microscope with no phase contrast.

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