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Isolation of Multipotent Mesenchymal Stem Cells from Human Extraocular Muscle Tissue

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[Abstract] Mesenchymal stem cells (MSCs) have attracted significant attention as potential therapeutic cells to treat various diseases ranging from tissue injuries, graft versus host disease, degenerative diseases and cancer. Since the initial discovery of MSCs in the bone marrow cells, MSCs have been successfully isolated from various adult and neo-natal tissues, albeit the procedures are often coupled with difficulties in harvesting tissue and produce low yield of cells, requiring extensive expansion *in vitro*. Here, we explored extra-ocular muscle tissues obtained from patients as a novel source of MSCs which express characteristic cell surface markers of MSCs and show multilineage differentiation potential with high proliferation capacity.

Keywords: Mesenchymal stem cells, Stem cells, Osteogenesis, Adipogenesis, Chondrogenesis, Extraocular muscle

[Background] Mesenchymal stem cells (MSCs) were originally identified as plastic adherent, fibroblastic cells derived from bone marrow and showed multi-lineage differentiation potential (Friedenstein *et al.*, 1974a and 1974b). Over the years, MSCs have been implicated to play a role in a wide range of biological processes such as hematopoiesis (Friedenstein *et al.*, 1974a), immune-modulation (Klyushnenkova *et al.*, 2005; Crop *et al.*, 2010), tissue repair, angiogenesis, tumorigenesis (Yagi and Kitagawa, 2013) and chemoresistance (Kumar *et al.*, 2017). MSCs possess a vast secretome and have been heralded as factories of extracellular vesicles and “injury drug store” (Caplan and Correa, 2011) for their ability to produce a myriad number of growth factors and cytokines. The secreted factors of MSCs were found to have roles in tissue repair of renal (Grange *et al.,* 2014; Zhang *et al.,* 2014), neural (Koc *et al.*, 2002; Zappia *et al.*, 2005), liver (Li *et al.*, 2013), lung (Lee *et al.*, 2012), myocardial (Timmers *et al.*, 2008; Bian *et al.*, 2014) as well as ischemic injuries (Zhang *et al.*, 2012). Therefore, MSCs are being explored as a therapeutic option for ameliorating various diseases such as myocardial infarction, respiratory disorders, Crohn’s disease, graft versus host disease, diabetes, bone disorders, as well as liver cirrhosis (Uccelli *et al.*, 2008; Battiwalla and Hematti, 2009; Luk *et al.*, 2015).

Cells with similar properties of bone marrow derived MSCs (BM-MSCs) have since been isolated from placenta, amniotic fluid (Tsai *et al.*, 2004), umbilical cord blood (Bieback *et al.*, 2004), mobilized peripheral blood, adipose tissue (Kim *et al.*, 2013), connective tissue, skeletal muscle (Young *et al.*, 2001), dental (Gronthos *et al.*, 2000), fetal tissue (Shin *et al.*, 2009) and extra-ocular muscle tissue (Mawrie *et al.*, 2016). This protocol explores extraocular muscle as a novel source of MSCs which is generally excised and discarded during strabismus correction surgery. Strabismus surgery is the third

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most common eye surgery in US with up to 1.2 million cases per year. The incised tissue is discarded after procedure, which can be used as a source of MSCs. Extraocular muscles are unaffected during Duchenne's muscular dystrophy (Kaminski *et al.*, 1992; Khurana *et al.*, 1995) and contain 15 times more side population stem cells than skeletal muscle (Pacheco-Pinedo *et al.*, 2009). The extraocular muscle derived MSCs (EOM-MSCs) expressed CD73, CD90 and CD105 (Mawrie *et al.*, 2016) which are characteristic cell surface markers for MSCs (Dominici *et al.*, 2006) and could differentiate into all the three meseodermal lineages (Mawrie *et al.*, 2016). Moreover, EOM-MSCs are relatively easy to isolate, have high proliferation capacity, neuroectodermal differentiation potential and might be a good candidate for stem cell based therapy for treating neurodegenerative disorders (Mawrie *et al.*, 2016).

Materials and Reagents

1. Bio-hazard waste container (Tarsons, catalog number: 583254)
2. Cell culture dish, 35 x 10 mm (Eppendorf, catalog number: 0030700112)
3. Cryo-vials (Tarsons, catalog number: 523182)
4. Cryo-tags (Tarsons, Cryo-babies, catalog number: 526070)
5. FACS tubes (Corning, catalog number: 352063)
6. Graduated centrifuge tubes, 15 ml (Tarsons, catalog number: 546021)
7. Graduated centrifuge tubes, 20 ml (Tarsons, catalog number: 546041)
8. Graduated 20 µl micro-tips (Tarsons, catalog number: 521000)
9. Graduated 200 µl, 1,000 µl micro-tips (Thermo Fisher Scientific, catalog numbers: 90030100, 90030210-P)
10. Tissue culture treated, flat bottom 96-well plate (Eppendorf, catalog number: 6030730119)
11. Whatman filter paper (Whatman, catalog number: 1001 125)
12. 10 ml serological glass pipettes (Himedia, catalog number: CG316-1x10NO)
13. Aseptic Petri dish (Tarsons, catalog number: 460051)
14. Sterile filtration unit (0.22 µm) (Thermo Fisher Scientific, catalog number: 450-0020)
15. Antibodies (Table 1)

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Table 1. Antibody details

S. No.	Marker	Antibody	Manufacturer	Dilution	Amount added per 50 µl of FACS buffer
1.	CD29	Anti-human CD29 PE	BD Biosciences, catalog number: 555443	1:100	0.5 µl
2.	CD34	Anti-human CD34 FITC	Thermo Fisher Scientific, catalog number: CD3458101	1:100	0.5 µl
3.	CD44	Anti-human CD44 FITC	BD Biosciences, catalog number: 555478	1:100	0.5 µl
4.	CD49e	Anti-human CD49e PE	BD Biosciences, catalog number: 555617	1:100	0.5 µl
5.	CD73	Anti-human CD73 PE	BD Biosciences, catalog number: 550257	1:100	0.5 µl
6.	CD90	Anti-human CD90 FITC	BD Biosciences, catalog number: 555595	1:100	0.5 µl
7.	CD105	Anti-human CD105 PE	BD Biosciences, catalog number: 560839	1:100	0.5 µl
8.	HLA1	Anti-human HLA1 FITC	BD Biosciences, catalog number: 555552	1:100	0.5 µl
9.	Isotype control	Anti-mouse IgG1 PE	BD, catalog number: 556561	1:100	0.5 µl
10.	Isotype control	Anti-mouse IgG1 FITC	BD, catalog number: 554109	1:100	0.5 µl

16. 10x Trypsin (2.5%) (Thermo Fisher Scientific, catalog number: 15090-046)
17. Alizarin Red S (Sigma-Aldrich, catalog number: 199962)
18. Ascorbic acid-2-phosphate (Sigma-Aldrich, catalog number: A8960)
19. β-Glycerophosphate (Sigma-Aldrich, catalog number: G9891)
20. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D8418-250ML)
21. Dexamethasone (Sigma-Aldrich, catalog number: D2915)
22. Fibronectin solution (Sigma-Aldrich, Fibronectin human plasma, catalog number: F0895)
23. Formaldehyde solution 37-41% (w/v) (Merck, catalog number: 61780805001730)
24. Hank's balanced salt solution (HBSS) (Sigma-Aldrich, catalog number: 55021C)
25. IBMX (Sigma-Aldrich, catalog number: I-5879)
26. Indomethacin (Sigma-Aldrich, catalog number: I-7378)
27. Insulin (Sigma-Aldrich, catalog number: 91077C)
28. Isopropanol (Merck, catalog number: 1.07022.2521)
29. Oil Red O (Sigma-Aldrich, catalog number: O-0625)
30. Potassium chloride (KCl) (Merck, catalog number: 61779205001730)
31. Potassium dihydrogen phosphate (KH_2PO_4) (Merck, catalog number: 60487305001730)
32. Propidium Iodide (Sigma-Aldrich, catalog number: P4170)
33. Safranin O (Sigma-Aldrich, catalog number: S8884)
34. Sodium bicarbonate (Sigma-Aldrich, catalog number: S5761)

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35. Sodium chloride (NaCl) (Merck, catalog number: 1.93206.0521)
36. Sodium dihydrogen phosphate (NaH_2PO_4) (Merck, catalog number: 61787405001730)
37. DMEM-low glucose with L-Glutamine medium (Sigma-Aldrich, catalog number: D2902-1L)
38. DMEM-high glucose with L-Glutamine medium (Sigma-Aldrich, catalog number: D5648-1L)
39. Fetal Bovine Serum (Thermo Fisher Scientific, catalog number: 10270)
40. 100x Penicillin (10,000 Units/ml)-Streptomycin (10,000 Units/ml) antibiotic (Thermo Fisher Scientific, catalog number: 15140-122)
41. StemPro chondrogenesis differentiation kit (Thermo Fisher Scientific, catalog number: A10071-01)
42. De-ionized water (dH_2O) (Merck, Elix Type 2 pure water)
43. Trypan blue (Sigma-Aldrich, catalog number: T6146)
44. Liquid nitrogen (99.999%)*
45. Sodium hydroxide (NaOH) (Merck, catalog number: 61843805001730)
46. Hydrochloric acid 35% (HCl) (Merck, catalog number: 61762505001730)
47. DMEM medium (see Recipes)
    a. DMEM-LG (low glucose)/DMEM-HG (high glucose) basal medium
    b. MSC growth medium
48. Tissue collection medium (see Recipes)
49. Fibronectin solution (see Recipes)
50. Phosphate buffered saline (PBS) (see Recipes)
    a. 10x PBS
    b. 1x PBS
    c. PBS with 1x antibiotic
51. 1x Trypsin (0.25%) (see Recipes)
52. 0.4% trypan blue (see Recipes)
53. Heat-inactivated FBS (see Recipes)
54. Freezing media (see Recipes)
55. FACS buffer (see Recipes)
56. Propidium iodide staining solution (see Recipes)
57. Osteogenesis induction media (see Recipes)
58. Adipogenesis induction media (see Recipes)
59. 4% formaldehyde solution (see Recipes)
60. Alizarin red staining solution (see Recipes)
61. Oil Red O solution (see Recipes)
    a. 1% Oil Red O stock solution
    b. Oil red O staining solution
62. Chondrogenic differentiation medium (see Recipes)
63. 0.1% Safranin O staining solution (see Recipes)

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Equipment

1. Cryo-cooler (Tarsons, catalog number: 525000)
2. Sterilized sharp-tip forceps and scalpel
3. Analytical balance (Sartorius, Quintix Analytical Balance 60, 120 g x 0.01, 0.1 mg)
4. Cryogenic cell storage container (International Cryogenics, model: D-2000C)
5. Biosafety level 2 cabinet (Thermo Fisher Scientific, model: 1300 series A2)
6. CO_2 incubator (Thermo Fisher Scientific, Hera Cell 150i)
7. Flow cytometer (Becton Dickinson, FACS calibur and BD cell quest software)
8. Hemocytometer (Bright line hemocytometer) (Sigma-Aldrich, catalog number: Z359629)
9. Inverted microscope with camera (Zeiss, Axio Vert. A1)
10. Pipette controller (Socorex, Profiller 446)
11. Single-channel pipettes, 0.5-20 µl, 20-200 µl, and 100-1,000 µl (Gilson, Pipetman classic)
12. Refrigerated centrifuge (Thermo Fisher Scientific, Sorvall legend X1R)
13. Water bath (Thermo Fisher Scientific, Labline water bath)
14. Ultra-low temperature freezer (-80 °C freezer) (Thermo Fisher Scientific, Forma 88000 series)
15. 4 °C refrigerator*
16. -20 °C freezer*
17. Autoclave*

**Note: These items can be ordered from any qualified company.*

Software

1. FlowJo software (FlowJo, LLC)

Procedure

A. Isolation of extra ocular muscle derived MSCs (EOM-MSCs)

1. Collect excised EOM tissue (approximately 3-8 mm^3) from the patient and transfer it to a vial with 3-4 ml of collection media (see Recipes). Use ice packs to maintain the tissue at low temperature during transportation.

Note: The following steps are to be performed inside a biosafety level 2 cabinet following aseptic culture techniques.

2. Before processing the tissue sample, coat the isolation dish with fibronectin at a concentration of 20 ng/cm^2. To a 35 mm cell culture dish, add 500 µl of fibronection solution (400 ng/ml; see Recipes) and incubate the dish at 37 °C in a CO_2 incubator for 1 h. Remove the excess fibronectin and rinse the dish once with 3 ml of 1x PBS (see Recipes).

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3. Using sterile forceps, transfer the tissue to an aseptic Petri dish containing 10 ml of PBS with 1x antibiotic (see Recipes). Incubate the tissue at room temperature for 5 min to disinfect and remove excess blood.
4. Carefully transfer the tissue to a sterile 35 mm cell culture dish coated with fibronectin and tear it into small pieces using sterile forceps or if required cut the tissue into small pieces with a sterile scalpel.
5. Add 500 µl of warm fresh MSC growth medium (see Recipes) to the dish and allow the tissue pieces to attach by incubating the dish for 2 h in a CO_2 incubator maintaining 5% CO_2 and 37 °C.
6. After 2 h, add additional 2 ml of warm MSC growth medium without disturbing the tissue pieces and return the dish to the incubator (Figure 1).
   Note: Handle the plate gently during this time since the tissue can be dislodged easily by excessive shaking.

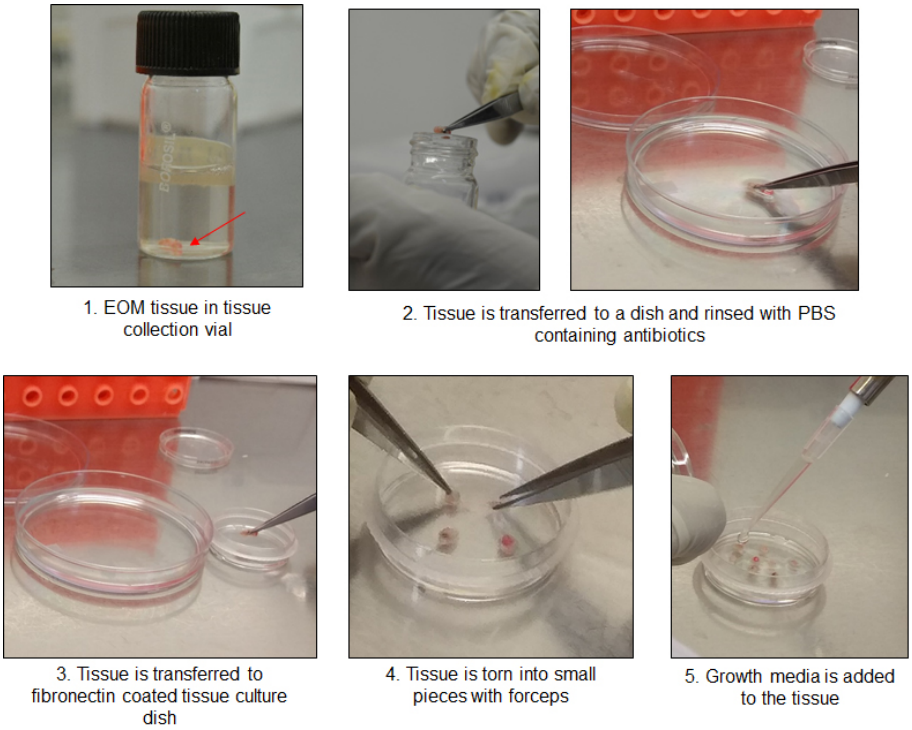

Figure 1. Procedure of processing EOM tissue

7. After 48 h, carefully aspirate the medium and replenish with 1.5-2 ml of fresh MSC growth medium (see Recipes) without disturbing/dislodging the tissue.
   Note: MSC growth medium has to be replaced every 72 h thereafter.
8. After 7-10 days, check for attached cells around the explants using an inverted microscope (Figure 2). Ensure that the culture is free of any contamination at this point.
9. Once a sufficient number of attached cells (200-300 cells) are observed around the tissue pieces, gently remove the tissue explants using a fine tipped forceps without scrubbing the tissue over the dish surface (Figure 2).

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Notes:

a. *Since most of the MSCs attach below the explants, care must be taken not to disturb the cells while removing the tissue.*
b. *Discard the tissue in a bio-hazard waste container. All the liquid waste should be appropriately disposed of after treatment with sodium hypochlorite.*

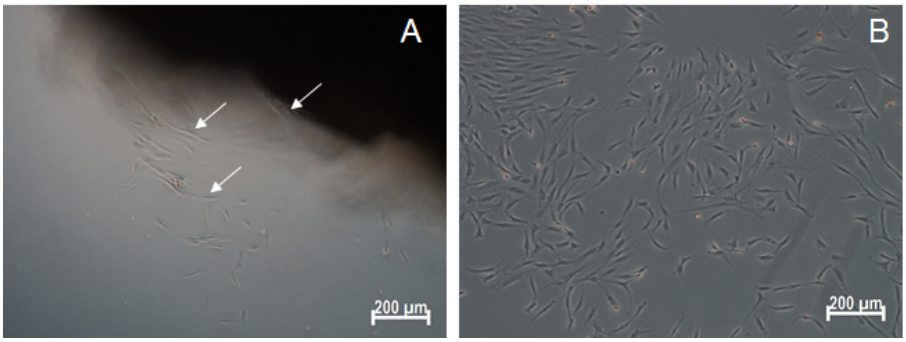

Figure 2. Microscopic image of isolated EOM-MSCs. A. Phase-contrast image showing the emergence of MSCs from the tissue explant within one week of isolation. White arrows represent the cells that have migrated out of the tissue. B. Phase contrast image of EOM-MSCs at passage 0. Scale bars: 200 µm.

B. Maintenance of culture

Once the cell colonies reach 50%-80% confluence, sub-culture them as follows.

1. Aspirate the medium (spent medium) and collect it in a sterile 15 ml centrifuge tube. Wash the cells twice with PBS.
2. Add 500 µl of 1x trypsin (see Recipes) to the dish and incubate at 37 °C for 5-7 min.

   Notes:

   a. *Keep trypsin at room temperature for 5-10 min after retrieving from 4 °C before use.*
   b. *Monitor the cell detachment intermittently under the microscope. Gentle taps on the side of the dish might be required to dislodge the rounded cells. Do not leave the cells in trypsin for more than 10 min.*
3. Add 1-2 ml of spent medium to neutralize trypsin and transfer the cells to a labeled 15 ml centrifuge tube.
4. Centrifuge at 300 *x g* for 5 min at 4 °C to obtain the cell pellet. Discard the supernatant and re-suspend the pellet in 1 ml of fresh MSC growth medium.
5. Aliquot a small volume (~50 µl) of cells and add an equal volume of 0.4% trypan blue. Mix properly and count the viable cells using a hemocytometer.
6. Seed cells at 1-2 x 10^3 cells/cm^2 in a tissue culture dish and add 2 ml of fresh MSC growth medium. Incubate the dish in 5% CO_2 incubator at 37 °C.
7. Change the MSC growth medium every 72 h until the cells attain 70%-80% confluence. Cells can be passaged up to 10-12 times by repeating the Steps B1-B6.

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C. Freezing and thawing of EOM-MSCs

1. After Step B5, re-suspend 1 x 10^6 cells in 0.5 ml of pre-chilled sterile FBS (see Recipes).
2. Transfer the cell suspension to a labeled cryo-vial and add an equal volume of pre-chilled freezing media (see Recipes). Gently mix and transfer the vial immediately to a cryo-cooler.
3. Keep the cryo-cooler in a -80 °C freezer for 24 h before transferring the vial to a cryogenic cell storage container with liquid nitrogen for long-term storage.
4. For reviving the cells, thaw by placing the cryo-vial immediately into a water bath at 37 °C for 45-60 s.
5. Add 0.5 ml of fresh warm MSC growth medium to the vial and transfer the cell suspension to a sterile 15 ml centrifuge tube. Add additional 4 ml of MSC growth medium drop-wise.
6. Centrifuge the cells at 300 *x g* for 5 min at 4 °C. Re-suspend the cell pellet in 1 ml of fresh MSC growth medium to remove any remaining DMSO.
7. Count the viable cells as described in Step B5 and seed 2-3 x 10^5 cells in a 35 mm cell culture dish with 2 ml fresh MSC growth medium.
8. Replace the growth medium after 12 h and maintain the cells according to Step B7 thereafter.

D. Cell surface marker staining and flow cytometry

1. Trypsinize and count the EOM-MSCs as described in Procedure B.
   Note: *Keep the cells on ice during the whole procedure to maintain viability and reduce internalization of surface markers.*
2. For each marker to be analyzed, distribute 1 x 10^5 cells per FACS tube for staining with fluorophore-labeled antibody. For each type of antibody used, keep additional FACS tube to be stained with a non-specific isotype control antibody as control.
3. Centrifuge the cells and wash the pellet twice with 3 ml PBS.
   Note: All centrifugation steps are to be done at 300 x g for 5 min at 4 °C.
4. Resuspend the cells in 50 µl of FACS buffer (see Recipes). Add 2 µl of diluted antibody (refer to antibody details in Table 1) followed by short gentle vortexing at low RPM. Incubate the tubes at 4 °C for 30 min in the dark.
5. Wash the cells with 1 ml of FACS buffer and re-suspend in 300 µl of propidium iodide staining buffer (see Recipes). Analyze the samples in a flow cytometer using proper gain/amp settings for the channels (Figure 3).

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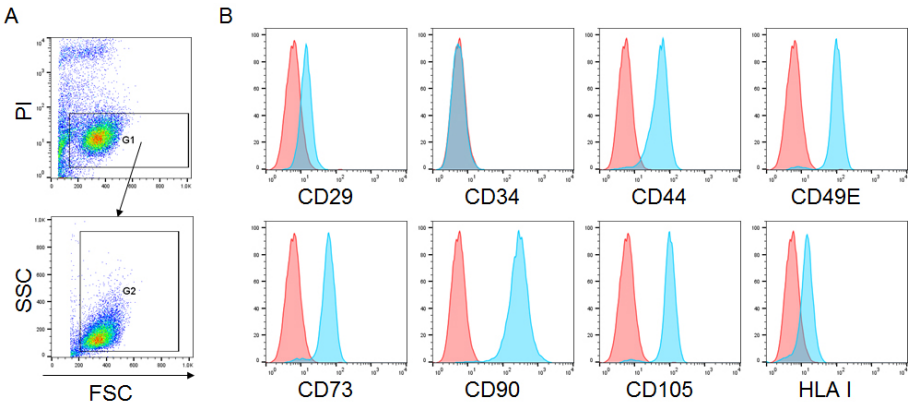

Figure 3. Cell surface marker expression of EOM-MSCs. A. Plots showing the gating for flow cytometry analysis; live cells were gated (G1) on the basis of propidium iodide (PI) negative (FL3 low) population in FL3 vs. FSC plot followed by gating (G2) in FSC vs. SSC plot to remove the cell debris. Cells in gate (G2) were further analyzed for their fluorescence intensity. B. EOM-MSCs were stained with fluorescently conjugated antibodies against CD29, CD34, CD44, CD49E, CD73, CD90, CD105 and HLA class I, and the expression was analyzed by flow cytometry. The histogram in red represents the isotype control and blue represents the stained sample. Y-axis represents the number of events/counts and X-axis represents the fluorescence intensity of the mentioned cell surface marker.

E. Osteogenic and adipogenic differentiation
   1. Trypsinize and count the EOM-MSCs as described in Procedure B.
   2. Seed EOM-MSCs at a density of 5,000 cells/cm^2 for osteogenesis and 20,000 cells/cm^2 for adipogenesis induction in a 96-well plate with adequate MSC growth medium (see Recipes).
   3. After 24 h, add osteogenesis or adipogenesis induction media (see Recipes) to the osteogenic and adipogenic conditions respectively.
   4. Replace the induction media every 3-4 days and allow the cells to differentiate for 14-21 days.

F. Alizarin red staining for assessing calcium deposition during osteogenesis
   1. Aspirate the medium and wash the cells twice with PBS.
   2. Fix the cells in 4% formaldehyde solution (see Recipes) for 1 h at room temperature.
   3. Wash the cells twice with dH_2O.
   4. Stain with freshly prepared Alizarin red staining solution (see Recipes) for 10 min at room temperature.
   5. Wash cells five times with dH_2O followed by a wash with PBS for 15 min to remove excess stain. Add PBS to the cells to prevent them from drying.
   6. Proceed to imaging the cells under an inverted microscope with a camera (Figure 4A).

G. Oil red O staining for assessing lipid accumulation during adipogenesis
   Note: Prepare Oil Red O staining solution (see Recipes) at least 1 h prior to staining.

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1. Aspirate the medium and wash the cells twice with PBS.
2. Fix the cells in 4% formaldehyde solution (see Recipes) for 1 h at room temperature.
3. Remove the fixing solution and rinse cells with 60% isopropanol in dH_2O.
4. Stain the cells with Oil Red O staining solution for 10 min.
5. Wash cells five times with dH_2O. Add PBS to the cells to prevent them from drying.
6. Proceed to imaging the cells under an inverted microscope with a camera (Figure 4B).

H. Chondrogenic differentiation
1. Trypsinize and count the EOM-MSCs as described in Procedure B.
2. Re-suspend the pellet in an appropriate volume to generate a cell suspension of 1.6 x 10^7 cells/ml. Add a 5 µl droplet of this cell suspension to the center of each well in a 96-well plate and allow cells to attach by incubating the plate for 2 h in a 5% CO_2 incubator at 37 °C.
3. After 2 h, add adequate (100 µl) chondrogenic differentiation media (see Recipes) to the wells without disturbing the cell micromass and return the plate to the incubator.
4. Replace the chondrogenic media every 2 days and allow cells to differentiate for > 14 days.

I. Safranin O staining for detection of cartilage, mucin and mast cell granules
1. Aspirate the medium and wash the cells twice with PBS.
2. Fix the cells with 4% formaldehyde solution (see Recipes) for 1 h at room temperature.
3. Wash the cells twice with dH_2O.
4. Stain with 0.1% safranin O solution (see Recipes) for 5 min at room temperature.
5. Wash cells with dH_2O. Add PBS to the cells to prevent them from drying.
6. Proceed to imaging the cells under an inverted microscope with a camera (Figure 4C).

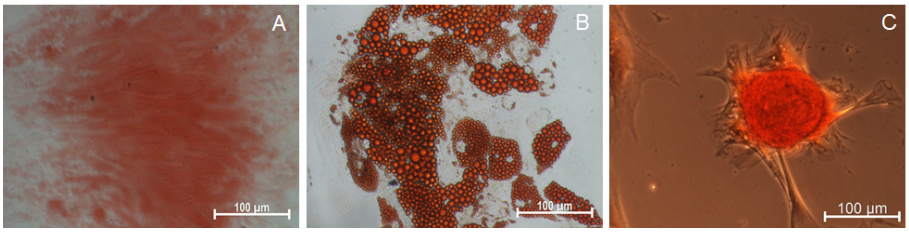

Figure 4. Differentiation of EOM-MSCs into osteogenic, adipogenic and chondrogenic cells. EOM-MSCs were seeded in the respective cell density and induction media for differentiation into osteogenic, adipogenic and chondrogenic lineage cells. The differentiated cells were stained with (A) alizarin red, (B) oil red o (C) safranin o for determining osteogenic, adipogenic and chondrogenic differentiation respectively. Scale bars: 100 µm.

Data analysis

The flow cytometry data for the expression of various surface markers on EOM-MSCs was analyzed using FlowJo software. Live cells were gated on the basis of propidium iodide negative (FL3 low)

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population in FL3 vs. FSC plot followed by gating in FSC vs. SSC plot to remove the cell debris. Histograms of signal in FL1 (for FITC-conjugated antibodies) and FL2 (for PE-conjugated antibodies) were plotted to define negative (unstained) and positive (stained) population using appropriate isotype-matched control antibody. This protocol shows representative data for only one of the EOM-MSCs samples and does not include any statistical analysis.

Recipes

1. DMEM medium
   a. DMEM-LG (low glucose)/DMEM-HG (high glucose) basal medium
      10 g DMEM-LG or DMEM-HG media powder
      3.7 g sodium bicarbonate
      10 ml 100x Penicillin-Streptomycin antibiotic solution
      Make up the volume to 1 L with autoclaved deionized H_2O
      Adjust pH to ~7.0 as pH tends to increase after filtration
      Sterilize by filtration using a sterile filtration unit (0.22 µm)
      Can be stored at 4 °C for up to 2 months
   b. MSC growth medium
      10% FBS in DMEM-LG basal medium
      Sterilize by filtration using a sterile filtration unit (0.22 µm)
      Store at 4 °C and use within two weeks
2. Tissue collection medium
   2x Penicillin-Streptomycin antibiotic in DMEM-LG basal medium or HBSS solution
3. Fibronectin solution (400 ng/ml)
   Prepare working stock of fibronectin by diluting 40 µl of 1 mg/ml stock solution in 100 ml dH_2O
   Sterilize by filtration using a sterile filtration unit (0.22 µm) and store at 4 °C
4. Phosphate buffered saline (PBS)
   a. 10x PBS
      80 g NaCl
      2 g KCl
      14.4 g Na_2HPO_4
      2.4 g KH_2PO_4
      Make up the volume to 1 L with autoclaved dH_2O
      Sterilize by filtration using a sterile filtration unit (0.22 µm)
      Store at room temperature
   b. 1x PBS
      Dilute 10x PBS ten times (1 ml in 10 ml) in H_2O
      Adjust pH to 7.4 and autoclave at 121 °C for 40 min

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c. PBS with 1x antibiotic

100 µl of 100x Penicillin-Streptomycin antibiotic in 10 ml of 1x PBS

5. 1x Trypsin (0.25%)

Dilute 10x trypsin ten times in sterile cold 1x PBS (1 ml of 10x trypsin in 9 ml of PBS) and prepare 5 ml aliquots for use

Store aliquots at -20 °C for long term storage. Thaw at room temperature and store at 4 °C after use for up to 1 week

6. 0.4% trypan blue

Dissolve 50 mg of Trypan blue (dye composition 40%) in 5 ml of 1x PBS

Sterilize by filtration using a sterile filtration unit (0.22 µm)

Store as aliquots at 4 °C

7. Heat inactivated FBS

Heat inactivate FBS by incubating at 56 °C for 1 h in a water bath and store at -20 °C as 50 ml aliquots

8. Freezing media

20% DMSO in heat-inactivated FBS (1 ml DMSO in 4 ml of FBS)

9. FACS buffer

2% heat-inactivated FBS in 1x PBS (200 µl FBS in 9.8 ml of PBS)

10. Propidium iodide staining solution

2 µg/ml of propidium iodide in FACS buffer

11. Osteogenesis induction media

DMEM-high glucose basal medium

1x Penicillin-Streptomycin antibiotic

10% FBS

10 mM β-Glycerophosphate

0.1 µM Dexamethasone

0.05 mM Ascorbic acid-2-phosphate

Note: Osteogenesis induction media can be stored for up to 4 weeks at 4 °C.

12. Adipogenesis induction media

DMEM-high glucose basal medium

1x Penicillin-Streptomycin antibiotic

10% FBS

1 µM Dexamethasone

0.2 mM Indomethacin (add 2 drops of 5 M NaOH to dissolve)

0.5 mM IBMX

0.01 M Insulin (add 2 drops of 5 M HCl to dissolve)

Note: Adipogenesis induction media can be stored for up to 4 weeks at 4 °C.

13. 4% formaldehyde solution

Dilute 37%-41% Formaldehyde solution 10 times in 1x PBS

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1 ml of 37%-41% Formaldehyde solution

9 ml of 1x PBS

14. Alizarin red staining solution

    0.19 g Alizarin Red S

    10 ml dH_2O

    Adjust pH to 4.2

15. Oil Red O solution

    a. 1% Oil Red O stock solution

       1 g Oil Red O

       100 ml isopropanol

       Warm in a water bath at 56 °C for 30-60 min. Not all of Oil Red O will dissolve

       Can be stored at room temperature for up to 6 months

    b. Oil red O staining solution

       3 parts of 1% Oil Red O stock solution

       2 parts of dH_2O

       Mix and let stand for 1 h

       Filter the solution with Whatman filter paper

16. Chondrogenic differentiation medium

    10 ml 10x Stem Pro Chondrogenesis supplement

    90 ml Stem Pro Osteocyte/Chondrocyte Differentiation Basal Medium

    1x Penicillin-Streptomycin antibiotic

    Note: Chondrogenic differentiation media can be stored for up to 4 weeks at 4 °C.

17. 0.1% Safranin O staining solution

    0.1 g Safranin O

    100 ml dH_2O

    Stir to help dissolve

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Competing interests

The authors declare no competing interests.

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Ethics

The study was approved by ethics committee of IIT Guwahati and samples were collected after written informed consent from the patients.

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