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Isolation and Expansion of Mesenchymal Stem/Stromal Cells, Functional Assays and Long-Term Culture Associated Alterations of Cellular Properties

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Abstract

Mesenchymal stem cell/stromal cells (MSCs) can differentiate into a variety of cell types, including osteocytes, adipocytes and chondrocytes. MSCs are present in the multiple types of adult tissue, such as bone marrow, adipose tissue, and various neonatal birth-associated tissues. Given their self-renewal and differentiation potential, immunomodulatory and paracrine properties, and lacking major histocompatibility complex (MHC) class II molecules, MSCs have attracted much attention for stem cell-based translational medicine research. Due to a very low frequency in different types of tissue, MSCs can be isolated and expanded *in vitro* to derive sufficient cell numbers prior to the clinical applications. In this chapter, the methodology to obtain primary bone marrow-derived MSCs as well as their *in vitro* culture expansion will be described. To assess the functional properties, differentiation assays, including osteogenesis, chondrogenesis and adipogenesis, 3-D culture of MSCs and co-culture of MSCs and tumor cells are also provided. Finally, the long-term culture associated alterations of MSCs, such as replicative senescence and spontaneous transformation, will be discussed for better understanding of the use of MSCs at the early stages for safe and effective cell-based therapy.

Keywords: Mesenchymal stem cell/stromal cells, primary culture, co-culture, 3-D culture, replicative senescence, spontaneous transformation

1. Introduction

Mesenchymal stem/stromal cells (MSCs), a multipotent stem/progenitor cell type, were initially described in bone marrow by Friedenstein et al. as rapid adherence to tissue culture vessels and the discrete “fibroblast” colonies approximately 50 years ago [1, 2]. Julius Cohnheim, a German-Jewish pathologist, firstly proposed that a fibroblast-like cell population for nonhematopoietic cells in bone marrow were involved in wound repair over 150 years ago [3]. In the late 1980s, Caplan firstly coined the name “mesenchymal stem cell (MSC)” [4]. Since then, MSCs have gained much attention over the last three decades. Many laboratories focusing on MSCs have developed diverse methods to isolate and expand MSCs from a variety of tissues. While the

assessment of characteristics of MSCs is necessitated in different platforms/laboratories, most researchers come to acknowledge the lack of a universally accepted criteria to define MSCs. To address this question of cell equivalence, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposes three minimal criteria to define MSCs [5]: property of MSC plastic adherence, the expression of specific cellular surface antigen, and capacity for trilineage mesenchymal differentiation (osteogenesis, chondrogenesis and adipogenesis).

Human MSCs from different tissues have the varied phenotypic features, the morphologic inconsistency, and heterogeneous functional behavior [6–8]. Indeed, the properties of stem cell have not been well established yet. Due to the unknown *in vivo* multipotent properties of MSCs, the issue of MSC nomenclature remains actively controversial. In 2019, ISCT MSC committee issued a position statement on nomenclature of MSCs clarifying the functional definition to emphasize the functional distinction of mesenchymal stem versus stromal cells [9].

MSCs have been considered as a promising therapeutic tool in tissue engineering and regenerative medicine. MSCs are well known to be present in almost every type of adult tissues, such as bone marrow [10–12], adipose tissue [10, 13, 14], lung [11, 15], synovial tissue [16, 17], dental pulp and periodontal ligament [18]. Notably, it has become apparent that MSCs are identified in the various human embryonic tissues, such as fetal bone marrow [19], fetal liver [20], aorta gonad-mesonephros and yolk sac [21], as well as multiple neonatal birth-associated tissues, such as placenta [10, 22, 23], amniotic and chorionic membrane [23, 24], umbilical cord tissue [10, 23–25], and umbilical cord blood [26, 27]. Therefore, different platforms/laboratories may use different type of tissue sources and methodologies for isolation and expansion of MSCs. This chapter firstly outlines protocols for standardized isolation and expansion of human bone marrow-derived MSCs (BM-MSCs), a major source of human MSCs, as well as BM-MSCs' characteristics, cryopreservation and thawing. Protocols for the preparation of MSCs derived from the other tissue types are similar to that of BM-MSCs, except tissue sample processing differentially. Human BM-MSCs are estimated at a very low frequency at approximately 0.001–0.01% of total nucleated cells [28, 29], and, therefore, human BM-MSCs are likely to be kind of difficult to isolate and harvest. This chapter will then focuses on optimal functional assays and application on the basis of our previous studies, which would be useful for researchers working with MSCs in basic research and translational and clinical applications, such as osteogenesis, chondrogenesis, adipogenesis, colony forming unit-fibroblast (CFU-F) assay, 3-D cellular co-culture, MSC homing and migration. Last but not least, the long-term culture associated alterations of MSCs' properties will be also discussed in this chapter.

2. Isolation, culture expansion, phenotypic analysis, freezing and thawing of human BM-MSCs

2.1 Materials

1. Growth medium (pH 7.1–7.5): Dulbecco's modified Eagle's medium (DMEM)-low glucose (Sigma-Aldrich, St Louis, MO), 10% fetal bovine serum (FBS, heat-inactivated) (Atlanta Biologicals, Lawrenceville, GA), 0.37 g% sodium bicarbonate, and 1% penicillin–streptomycin. Adjust growth medium to pH 7.1–7.5.
2. Ficoll-Paque (Density gradient medium) (Stem Cell Technologies, Cambridge, MA).

3. $1 \times$ Phosphate buffered saline (PBS), pH 7.2.
4. $1 \times$ PBS + 2% + 1 mM ethylenediaminetetraacetic acid (EDTA).
5. Trypan blue, 0.4%.
6. Freezing medium: 90% FBS and 10% (v/v) dimethyl sulphoxide (DMSO).
7. Trypsin–EDTA solution 0.25% (Sigma, St. Louis, MO).
8. RosetteSep™ Human Mesenchymal Stem Cell Enrichment Cocktail (Stem Cell Technologies, Cambridge, MA).
9. Nalgene® Cryo 1°C Freezing Container.
10. Cryogenic storage vials.
11. Suitable box for storage in liquid nitrogen.
12. Tissue culture ware: T25 flasks, T75 flasks, 100-mm tissue culture dishes, 6-well plates.
13. Hemocytometer.
14. Water bath at 37°C.
15. Biological safety cabinet class II.
16. Bench centrifuge with swinging bucket rotor.
17. Inverted phase microscope.
18. Sterile conical centrifuge tubes (15 and 50 mL).
19. Sterile cell culture plastic pipettes (2, 5, 10, 25 mL).
20. Automatic pipettor.
21. 1.5 mL eppendorf tubes.

2.2 Isolation and culture expansion of human BM-MSCs

1. Make sure BM sample, $1 \times$ PBS + 2% FBS + 1 mM EDTA, Ficoll-Paque and centrifuge are all at room temperature.
2. Spray the sample tube with 70% ethanol and conduct the isolation of BM-MSCs in the Biological Safety cabinet.
3. For 10 mL size of BM, divide BM sample into two 50 mL conical tubes at room temperature. Add 50 μ L RosetteSep Human Mesenchymal Stem Cell Enrichment Cocktail per mL of bone marrow and mix well.
4. Incubate for 20 minutes at room temperature.

5. Dilute sample with about same volume of $1 \times \text{PBS} + 2\% \text{FBS} + 1 \text{ mM EDTA}$ solution; mix gently.
6. Prepare two 50 mL conical tubes with 10 mL Ficoll-Paque each tube. Layer the diluted sample on top of Ficoll-Paque. Be careful to minimize mixing of Ficoll-Paque and sample. Tilt the tube to 45 degree angle and slowly add the sample drop by drop to form a layer on top.
7. Centrifuge at $1200 \times g$ for 10 minutes at room temperature in a swinging bucket rotor and set the centrifuge to brake on.
8. Remove the enriched cells from the Ficoll-Paque. Collect the interphase containing the mononuclear cells. Be sure not to touch the red pellet at the bottom. It is advisable to remove some of the Ficoll-Paque and a bit of upper plasma layer in order to endure complete recovery of the desired cells.
9. Wash enriched cells with the $5 \times$ volume of $1 \times \text{PBS} + 2\% \text{FBS} + 1 \text{ mM EDTA}$ solution. Spin $300 \times g$ for 10 minutes.
10. Carefully discard the supernatant and resuspend cells in 1 mL of MSC growth medium per tube. Perform a viable cell count with a hemocytometer using Trypan blue.
11. Seed cells into one T-75 flask finally for 10 mL size marrow (a final cell concentration of $0.5\text{--}1.5 \times 10^6 \text{ cells/cm}^2$). 12 mL of MSC growth medium is supplemented.
12. Put the flask in incubator at 37°C with 5% humidified CO_2 for 48 hours to allow cells to attach.
13. After 48 hours, observe with phase contrast microscopy and then remove growth medium and non-adherent cells.
14. Wash cells twice with pre-warmed medium and add 13 mL of fresh MSC growth medium. Return the flask to the incubator.
15. Change growth medium every 3 days and observe the cellular colony forming.
16. CFU-F become in the next 3–5 days. Continue to culture until the cells reach 80% confluence in the 2 weeks.
17. Remove the medium and wash with PBS 2–3 times.
18. Add 3–4 mL pre-warmed trypsin–EDTA solution to cover cells in the flask. Return the flask to the incubator for 5 minutes.
19. Check with phase contrast microscopy. When most cells become detached, gently tap the side of the flask.
20. Add 5 mL growth medium to the flask to stop Trypsin–EDTA action. Resuspend cells by pipetting and transfer the entire cell suspension into a 15 mL conical tube.

21. Centrifuge at $400 \times g$ for 5 minutes.
22. Remove the supernatant and resuspend the cells in 2–3 mL pre-warmed PBS. Centrifuge at $400 \times g$ for 5 minutes.
23. Repeat step 22.
24. Harvest cells. This culture is considered as passage 0.
25. Count cells and reseed cells at an optimum density of 5000 cells/cm² in the appropriate tissue culture ware.
26. After 24 hours, remove the growth medium and wash the cells attached to the plate once with PBS.
27. Add the appropriate volume of fresh culture medium and incubate the cells for 2–3 days.
28. When the culture reaches 80–90% confluence, remove the culture medium and wash with PBS 2–3 times.
29. For trypsinization, add an appropriate volume of pre-warmed trypsin–EDTA solution to cover the entire cellular surface. Return the flask to the incubator for 5 minutes.
30. Observe under a phase contrast microscope. When most cells become detached, gently tap the side of the flask.
31. Add the appropriate volume of growth medium to stop Trypsin–EDTA action. Mix and collect the mixture of the entire cell suspension into a 15 mL conical tube.
32. Centrifuge at $400 \times g$ for 5 minutes at room temperature.
33. Remove the supernatant without disturbing the cell pellet and resuspend the cells with an appropriate volume of pre-warmed PBS.
34. Wash and centrifuge at $400 \times g$ for 5 minutes again.
35. Harvest cells. This culture is passage 1. MSCs at passage 1 can be frozen in liquid nitrogen (see the next) or continue to serially passage.

2.3 Characteristics of the expanded human BM-MSCs

The following antibodies are used in flow cytometry analysis of MSC characterization, CD29-PE, CD34-PE, CD44-PE, CD73-PE, CD90-PE, CD45-FITC, CD147-FITC, HLA DR-FITC, IgG1-PE, and IgG1-FITC (BD Biosciences). IgG1 immunoglobulin is used as isotype negative controls and passage 3 MSCs are characterized using PE or FITC conjugated antibodies against the cellular surface markers.

1. Remove the growth medium and wash cells with pre-warmed PBS twice.
2. Add the pre-warmed trypsin–EDTA solution to the flask. Return the flask to the incubator for 5 minutes.

3. When most cells become detached, gently tap the side of the flask.
4. Add growth medium to the flask. Mix and transfer the entire cell suspension into a 15 mL conical tube.
5. Centrifuge at $400 \times g$ for 5 minutes.
6. Remove the supernatant and resuspend the cells in the pre-warmed PBS.
7. Centrifuge at $400 \times g$ for 5 minutes.
8. Carefully discard the supernatant and resuspend cells with pre-warmed PBS.
9. Centrifuge at $400 \times g$ for 5 minutes.
10. Harvest cells.
11. Count cells and make it a single cell suspension at concentration of 1×10^6 cells/mL in PBS.
12. Add 250 μ L cellular suspension to the appropriate number of FACS tubes.
13. Add antibodies to the FACS tubes – 10 μ L per antibody per tube.
14. Incubate the FACS tubes for 20–25 minutes at room temperature on a shaker to prevent aggregation.
15. Then spin down FACS tubes at 150 rpm for 5 minutes.
16. Wash twice with PBS.
17. Add 2 mL MSC growth media.
18. Centrifuge the tubes at 150 rpm for 5 minutes.
19. Pipette off the supernatant.
20. Add 250 μ L MSC growth media.
21. Run samples through the FACS machine.

2.4 Freezing of BM-MSCs

Of note, perform all following steps under sterile conditions.

1. Place freezing medium on ice.
2. Harvest the cultivated MSCs, as described above (steps 1–10 in Section 2.3).
3. Resuspend MSCs in freezing medium at a concentration of $5\text{--}10 \times 10^5$ cells/mL.
4. Transfer cells into appropriate cryogenic storage vials or tubes and close the lid.

5. Place the vials in the pre-cooled Cryo 1°C Freezing Container quickly and store at -80°C directly.
6. After 24 hours, transfer the cryogenic storage vials to the suitable boxes to liquid nitrogen for long-term storage.

2.5 Thawing of BM-MSCs

Perform all following steps under sterile conditions.

1. Place the frozen vial of MSCs rapidly into a 37°C water bath.
2. Gently swirl the cryovial until the ice in the vial has melted.
3. After thawing, transfer the entire content of the cryovial into a sterile 15 mL conical tube containing 5 mL of pre-warmed PBS.
4. Gently swirl and centrifuge at $400 \times g$ for 5 minutes.
5. Aspirate supernatant completely.
6. Wash again.
7. Count and reseed cells at a density of $5000 \text{ cells}/\text{cm}^2$ in the appropriate tissue culture ware.

3. Functional analysis of expanded human MSCs

3.1 *In vitro* osteogenesis of human BM-MSCs

Osteogenic medium: MSC growth medium supplemented with $50 \mu\text{M}$ ascorbic acid phosphate (AsAP) (Wako Chemicals USA, Richmond, VA), $0.1 \mu\text{M}$ dexamethasone (Sigma-Aldrich, St Louis, MO), and 10 mM β -glycerol phosphate (Sigma-Aldrich, St Louis, MO).

Several osteogenic markers, such as alkaline phosphatase (ALP), leptin receptor, and cathepsin K, are used as the indicator of early osteogenesis [28], calcium deposition as the indicator of late-stage osteogenesis [28, 29]. ALP activity and calcium deposition are exemplified to assess osteogenic differentiation of human MSCs from passage 4 (**Figure 1**).

3.1.1 Colorimetric quantitative ALP activity

1. Collect cells from passage 4.
2. Prepare cellular suspension in growth medium.
3. Plate cellular suspension in 6-well plates at 1×10^5 cells/well in triplicate.
4. After 24 hours, remove the growth medium and wash cells with osteogenic medium once.
5. Add 2 mL osteogenic medium in each well.

Figure 1. Osteogenic culture of human BM-MSCs. Representative images (10×) of human BM-MSC osteogenesis on day 12 (A), day 17 (B), and day 26 during osteogenic culture.

6. Culture cells in incubator at 37°C with 5% humidified CO₂.
7. Change osteogenic medium every 3 days.
8. Measure ALP activity about 1–2 weeks after MSC osteogenetic culture [29, 30]. Aspirate medium and gently wash cells twice with PBS.
9. Add 600 µL of lysis buffer (0.5% Triton-X 100 in molecular grade ddH₂O) to each well and then scrape the cells off the surface using the end of a pipette tip.
10. Transfer lysates to centrifuge tubes. Prepare 15 mL conical tubes equal to the number of tubes maintaining lysates.
11. Dissolve the contents of a 40 mg capsule of phosphatase substrate (Sigma) in 10 mL ddH₂O. Scale up if necessary.
12. Add 500 µL of 1.5 M alkaline buffer solution (Sigma) to each tube. Add 500 µL of phosphatase substrate solution into each tube. Keep tubes in 37°C water bath.
13. Vortex samples and add 100 µL of each lysate to 15 mL tubes within 30 seconds. Fifteen minutes after the first sample is added, add 1 mL of 1 N NaOH to each tube in 30 second intervals removing tubes from water bath. The reaction will take place for 15 minutes at 37°C for each tube.
14. Prepare standard curve (Sigma) (Note: prepare 100 nmol/mL solution of p-nitrophenol by combining 100 µL of 10 mM p-nitrophenol standard solution with 9.9 mL 0.02 N NaOH).
15. Add 300 µL of standards and samples in triplicate to a 96-well plate.
16. Measure the absorbance using excitation filter of 405 nm. One enzyme unit of ALP is defined as the quantity of enzyme which produces 1 nmol p-nitrophenol per 15 minutes [29].

3.1.2 Colorimetric quantitative calcium assay

1. Aspirate or pipette out all culture medium from each well of the 6-well culture plate that contains induced or control cells to be tested.

2. Rinse the cells twice with PBS. Add 200 μL of PBS to the side of each well, not to dislodge the cells. Aspirate off the PBS and re-rinse.
3. Add 125 μL of 0.5 N HCl to each well.
4. Scrape the cells off of the surface using a cell scraper and transfer the cells and HCl to a 1.5 mL polypropylene microcentrifuge tube with a tight fitting cap.
5. Add an additional 125 μL of 0.5 N HCl to each cell to recover any cells remaining in the well, and transfer this to the appropriate tube.
6. Samples may be capped tightly and stored at -20°C if they are not to be tested immediately.
7. Extract the calcium from the cells by shaking the tubes on an orbital shaker for 4 hours at 4°C . If using frozen samples, allow extra time for samples to thaw.
8. Centrifuge the sample tubes at 500 g for 2 minutes.
9. Carefully collect the supernatant with extracted calcium, without disrupting the cell pellets, and transfer these to a new tube.
10. Prepare a standard curve with the calcium standard and determine the amount of calcium in each control and osteogenesis-induced samples. Follow the instructions provided in the Stanbio Total Calcium LiquiColor® Procedure No. 0150 (Stanbio Laboratory).
11. Three μL of sample vs. 297 μL of assay reagent (1:100 ratio for sample to reagent) for 96-well plate is used for each calcium determination. Assay reagent is mixed by equal volume of two solutions (Color Reagent and Base Reagent) provided in the kit. Distribute the assay reagent by multipipettor after adding samples. Absorbance is read at 550 nm.
12. Unused sample extract may be re-frozen for future re-assay. If the reading was out of range, the sample can be diluted with ddH₂O in a total volume of 3 μL (e.g. 1 μL of sample + 2 μL of ddH₂O) and re-assayed again.

3.1.3 3-D culture of human BM-MSCs and calcium deposition measured by von Kossa staining

PuraMatrix™ hydrogel (BD Biosciences) is a 16-amino acid synthetic peptide hydrogel composed of a repeating sequence of arginine, alanine, aspartate, and alanine (RADA16) [31], which is widely used for 3-D culture.

1. 3-D culture of human BM-MSCs on PuraMatrix hydrogel as follows.
2. The stock of 1% peptide solution can be sonicated for 30 minutes to decrease its viscosity and then diluted with sterile ddH₂O to a final concentration of 0.25% (w/v).
3. 300 μL of 0.25% gel solution is loaded into each well of the 24-well plate until it is uniformly spread.

4. The gelation is initiated by slowly dripping the medium along the wall of the well. 300 μ L of medium is again added carefully on top and the plate is incubated at room temperature for one hour equilibration.
5. After the peptide has assembled into hydrogel, the medium is changed two times over one hour to equilibrate the growth environment to physiological pH.
6. The equilibrated samples are stored overnight at 37°C incubator and the cells are seeded the next day.
7. Prepare the total of 4×10^4 cells suspended in MSC growth medium and 4×10^4 cells are seeded onto the hydrogel. The following day (Day 0), the medium will be replaced by osteogenic medium.
8. Von Kossa staining can be conducted at day 24 after differentiation [30]. MSCs are rinsed with the Tyrode's balanced salt solution and fixed with 10% buffered formalin (Fisher Scientific) for 30 minutes.
9. Incubated with 2% silver nitrate solution for 10 minutes in the dark.
10. Rinse with ddH₂O and expose to light for 15 minutes.
11. Bright-field images of stained samples are captured with an inverted microscope.

3.1.4 3-D cellular culture conducted by encapsulation of human MSCs in PuraMatrix™ hydrogel

1. To generate a 0.25% final concentration of PuraMatrix™ hydrogel for cells encapsulation, one part of 1% PuraMatrix™ hydrogel is diluted with same volume of sterile 20% sucrose, to reach 0.5% PuraMatrix™ hydrogel in 10% sucrose, and then mix with one part of 2× concentration of cells resuspended in 10% sucrose.
2. For 24-well plates, 300 μ L of PuraMatrix mixture is loaded into each well and 300 μ L of medium is layered on top of the gel. The gelation of the PuraMatrix is completed in an incubator for 60 minutes.
3. Change medium the next day.
4. Von Kossa staining can also be conducted, as described above, or collect cells as follows for other experiments.
5. Mechanically disrupt BD PureMatrix™ and cells in the well or cell culture insert by pipetting the media and gel up and down.
6. Transfer to a 15 mL conical tube.
7. Rinse out the well or cell culture insert using PBS.
8. Centrifuge at $150 \times g$ for 5 minutes. Discard supernatant. The pellet at the bottom of the tube contains cells and BD PuraMatrix fragments.

9. Resuspend pellet in 2 mL of PBS. Spin and collect pellet again.
10. Resuspend pellet in 1 mL of trypsin–EDTA and incubate at 37°C for 5–10 minutes. This will help separate cells that are still attached to each other.
11. Add 5 mL PBS to spin cell pellet again.
12. Aspirate the supernatant (do not disturb the gel). Resuspend pellet again in 1 mL of trypsin–EDTA and incubate at 37°C for 5–10 minutes.
13. Add 5 mL PBS to spin cell pellet again.
14. Aspirate the supernatant. Carefully take out one third of the gel pellet. Do not disturb the bottom (two-third) part of the gel.
15. Wash with PBS twice.
16. Add appropriate amount of lysis buffer to perform cell lysis and collect cell sample.

3.2 3-D cell pellet culture and chondrogenesis of human BM-MSCs

Chondrogenic medium: 95% DMEM high-glucose medium (Sigma-Aldrich, St Louis, MO), 1% 1 × ITS+1 solution (BD Biosciences, San Jose, CA), 1% Pen-Strep, 100 µg/mL sodium pyruvate (Invitrogen, Carlsbad, CA), 50 µg/mL AsAP, 40 µg/mL L-proline (Sigma-Aldrich, St Louis, MO), 0.1 µM dexamethasone, and 10 ng/mL recombinant human TGF-β3 (Lonza, Walkersville, MD).

Passage 4 human BM-MSCs are used for chondrogenic differentiation. Chondrogenic differentiation is induced by chondrogenic medium (**Figure 2**).

1. In order to form a chondrogenic pellet, approximately 2.5×10^5 human BM-MSCs are centrifuged down in a 15 mL conical tube at $150 \times g$ for 5 minutes at room temperature.
2. Five hundred µL of chondrogenic medium is used to resuspend the 2.5×10^5 cells to a final concentration of 0.5×10^6 cells/mL.

Figure 2. Chondrogenic culture of human BM-MSCs. Representative images (10×) of immunohistochemical staining of collagen II (A) and aggrecan (B) in pellet culture samples at day 17 during chondrogenesis [29].

3. Cells are centrifuged down again. Place the 15 mL conical tube in the incubator at 37°C with 5% humidified CO₂.
4. MSCs are shaped into the pellet after 24 hours incubation without disturbance.
5. The cell pellet is fed every 3 days for about 2–4 weeks and, after that, the chondrogenic pellet is harvested and sample processing is described as follows for immunohistochemistry analysis (e.g. examination of the expression levels of Collagen II, X and Aggrecan) [29, 32].

3.2.1 Sample processing for immunohistochemistry analysis

Day 1: Fixation and dehydration

1. Rinse specimen in PBS.
2. Fix samples for 45 minutes in acid-formalin at 4°C.
3. Rinse specimen in PBS twice.
4. Embed specimen in 2% agarose.
5. Transfer to vial and treat with 50% absolute ethanol for 1 hour at room temperature.
6. Transfer to 70% Absolute ethanol for 1 hour at room temperature.
7. Transfer to 95% Absolute ethanol for 1 hour at room temperature.
8. Repeat the step 7.
9. Transfer to 100% absolute ethanol for 1 hour at room temperature.
10. Leave specimen at 4°C overnight.

Day 2: Clearing and infiltration

1. Transfer to 100% CitriSolv (Fisher Scientific, catlog 22-143-975) for 1 hour at room temperature.
2. Transfer to 100% CitriSolv for 1 hour at 55°C.
3. Transfer to 1:1 mixture of CitriSolv/Micro-cut paraffin for 1 hour at 55°C.
4. Repeat the step 3.
5. Transfer to 100% Micro-cut paraffin for 1 hour at 55°C.
6. Repeat the step 5.

Day 3: Embedding

1. Transfer to 100% Micro-cut paraffin for 1–2 hours at 55°C.

2. Position specimen in Peel-Away mold with 100% paraffin.
3. Allow specimen to harden overnight.
4. Specimen may be sectioned the following day.
5. Conduct immunohistochemistry.

3.3 *In vitro* adipogenesis of human MSCs

Adipogenic medium: DMEM (1 g/L glucose), 10% FBS, 1% penicillin/streptomycin, 10 mg/mL insulin, 1 mM dexamethasone, 0.5 mM methylxanthine, and 200 mM indomethacin.

Passage 4 human BM-MSCs are used for adipogenic differentiation. Adipogenic differentiation is induced by adipogenic medium.

1. Harvest cells from passage 4, as described in the previous section.
2. Resuspend cells in adipogenic medium carefully.
3. Transfer the single cell suspension in triplicate to 6-well plates (1×10^5 cells/well).
4. Culture cells in the incubator at 37°C with 5% humidified CO₂ for 3 weeks.
5. Change medium every 2–3 days.
6. After 3 weeks, aspirate adipogenic medium and wash cells twice with PBS.
7. Cells are fixed in ice-cold methanol for 10 minutes.
8. Aspirate methanol completely and wash cells twice with ddH₂O.
9. Add Oil Red O staining reagent to the wells to stain lipid vacuoles at room temperature and mix slowly about 20 minutes on a shaker plate.
10. Aspirate Oil Red O staining reagent and wash cells twice with ddH₂O.
11. Observe and check the stained cells with phase contrast microscopy.

3.4 CFU-F assay

CFU-F assay can be used *in vitro* to evaluate the proliferation potential of MSCs. CFU-F assay is a well-established method for the quality control of MSCs' preparation. This section describes a traditional assay for CFU-F to evaluate the colony forming ability of human MSCs.

1. Collect cells from passage 4.
2. Prepare the single cell suspension in growth medium and seed cells in the 6-well plates in triplicate at three different densities, 1.5×10^5 , 2.5×10^5 , and 5×10^5 cells/well, in 2 mL growth medium, respectively.
3. Culture cells in the incubator at 37°C with 5% humidified CO₂ for two weeks.

4. Change medium twice each week and check with phase contrast microscopy daily to prevent overgrowth. Stop cell culture as soon as colonies are forming visibly and proceed with the Giemsa staining of CFU-F colonies on a benchtop as follows.
5. Wash the culture dishes twice with PBS.
6. Fix cells by adding 2 mL methanol to each well for 5 minutes at room temperature.
7. Gently remove the methanol and discard into the bio-hazardous waste.
8. Air dry the culture vessels and add the diluted Giemsa staining solution for 5–10 minute at room temperature.
9. Remove Giemsa staining solution and wash twice with ddH₂O.
10. Count visible colonies manually with a diameter greater than 5 mm.

3.5 Co-culture of MSCs with cancer cell line

There are various 2-D or 3-D, dyeing or not dyeing, co-culture models of human MSCs and other cell sources to study cell–cell interaction, cell proliferation, MSCs' immunomodulatory capacity, and the cellular contribution of each cell type. These methods making co-cultures of MSCs and other cells are well-established, such as co-cultures of MSCs and human peripheral blood mononuclear cells [33, 34], MSCs and T cells [35], MSCs and human hematopoietic stem cells [36], MSCs and umbilical vein endothelial cells [37]. MSC-cancer cell (PC-9) co-culture will be described in this section (**Figure 3**).

3.5.1 Preparation of MSCs with CellTracker™ Red dye (CellTracker™ CM-DiI, C7000)

1. Culture MSCs in an appropriate tissue culture ware (e.g. a 6-well plate)
2. Remove culture medium and wash with PBS twice.

Figure 3. Human BM-MSCs-PC-9 co-culture. Representative images ((A) 5×; (B) 20×) of co-cultures of human BM-MSCs labeled with CellTracker™ red dye and PC-9 cells transfected with copGFP, nuclei counterstained with DAPI.

3. Prepare CellTracker™ working solution in MSC growth medium. Make stock solution of lipophilic tracers in DMSO at 2 mM. Dilute the stock solution in MSC growth medium at 2 µM.
4. Add the working solution in the tissue culture vessel to cover the entire cell surface.
5. Incubate for 5 minutes or less in incubator at 37°C with 5% humidified CO₂.
6. Remove the CellTracker™ working solution.
7. Wash with MSC growth medium once.
8. Add MSC growth medium and return the tissue culture vessel to the incubator.

3.5.2 Preparation of PC-9 cells labeling with GFP

1. PC-9 cells labeling with copGFP (Santa Cruz, sc-108083) is performed using the lentiviral technique. Lentivirus is produced by using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's instructions. After transfection, PC-9 cells expressing copGFP may be isolated via puromycin (2 µg/mL) selection.
2. Continue to serially passage.
3. Collect PC-9 cells for cell co-culture.

3.5.3 MSC-PC-9 cell co-culture

1. Harvest MSCs with CellTracker™ red dye and wash with PBS once.
2. Prepare the single cell suspension in growth medium at an appropriate cell concentration and transfer the cell suspension in a 6-well plate or a special chamber.
3. Culture cells in the incubator at 37°C with 5% humidified CO₂ for about 3 hours to allow cells to attach.
4. Change fresh growth medium slowly.
5. Add PC-9 cells in the 6-well plate (the same cell number of MSCs). Gently tap the side of the flask.
6. Return the 6-well plate to the incubator and culture cell overnight.
7. Check the cell co-culture under the contrast microscope and image under microscopy.

3.6 *In vitro* migration assay

The intercellular communication can be executed through a direct cell–cell interaction or through paracrine signaling mediated by a combination of active molecules. The major signaling molecules include cytokines, growth factors, chemokines, which can be generated and expressed in a wide variety of cell types including tumor cells and MSCs in response to multiple signals such as

inflammatory or tumor microenvironment. Circulating MSCs are driven by such signaling molecules to home and subsequently migrate into the sites of tissue injury or disease. It is critical for the ability of MSCs to migrate and identify the injury sites for tissue repair and regeneration. Clinical data are still lacking for MSCs' homing and distribution of transplanted MSCs in the body, albeit a large number of *in vivo* studies are conducted on homing and migration pathways of MSCs for targeted stem cell-based therapies.

There are different approaches for improvement of MSC homing and migration. In this section, *in vitro* migration capacity of human BM-MSCs is evaluated by using an 8 µm-pore transwell chamber inserts (Corning).

1. Harvest MSCs and prepare cell suspension in the serum-free medium.
2. Transfer the cell suspension to the upper layer of a transwell insert at a density of 4×10^4 cells/cm² and allow cells to migrate to the lower compartment containing MSC growth medium overnight in the incubator at 37°C with 5% humidifies CO₂.
3. Cells from the upper chamber of transwell are migrated. Gently scrape the MSCs using the cotton swab at the upper layer of the membrane.
4. The migrated MSCs at the lower layer are stained with 0.1% crystal violet.
5. Check the cells and image under a light microscope.
6. Count the number of stained MSCs manually.

4. *In vitro* long-term culture associated alterations of MSCs

4.1 Morphological and immunophenotypic alterations of MSCs

It is well known that MSCs demonstrate biological alterations in the course of *in vitro* long-term culture. Different tissue derived MSCs may present different morphological and immunophenotypic characteristics in the expansion culture. At present, there is lack of a unifying definition for the “passage” of MSCs. Morphological changes are continuous during the long-term culture and expansion of BM-MSCs [38–40], which display a fibroblast-like appearance at early passages while the flattened and larger morphology as well as a visible increase of cellular granularity in late passages. For example, one previous study showed that human BM-MSCs were consistent with a morphological appearance from passage 1 to passage 6–8 and beyond that period such cells became large and flat [40].

During further cultivation, MSCs demonstrate the altered common immunological surface markers. Comparison of the early and late passages of BM-MSCs reveals that no differences are observed between passage 2 and 6 MSCs in expression of CD44, CD90, CD105, HLA-ABC, and HLA-DR, while CD106 is downregulated in MSCs of passage 6 [41]. Research has also reported that the expression pattern of the common surface markers maintains consistently with consecutive passaging up to passage 8 of BM-MSCs [40]. In contrast, the positive expression of the common surface markers such as CD73, CD90 and CD105 presents at the passage 30 of human adipose-derived MSCs (AD-MSCs) [42] and human umbilical cord MSCs (UC-MSCs) [43].

4.2 Alterations of proliferation and differentiation of MSCs

MSCs exhibit a high proliferation rate at lower passage and, however, the rapid growth kinetics decrease gradually with consecutive cell passaging. A linear correlation is observed between cumulative population doubling and days in culture up to passage 6–8 of human BM-MSCs and the passage-dependent decrease in the proliferation rate is also observed beyond that period [40]. A reduction in the proliferation in the course of long-term cultivation has been reported in human dental pulp tissue-derived MSCs [44] and human tonsil-derived MSCs [45].

The differentiation ability of human BM-MSCs varies in long-term culture manifested by the significant reduction in expression levels of the osteogenic markers, such as ALP and osteocalcin, and adipogenic markers, such as fatty acid binding protein-4 and lipoprotein lipase at the late passages [45]. It has been reported that 25% samples of BM-MSCs from different donors in the 8th passage and the 20% in the 10th passage lost their osteogenic differentiation potential [46]. Similarly, 10% BM-MSC samples in the 6th passage, the 50% in the 8th passage, and the 60% in the 10th passage also lost their adipogenic differentiation [46]. In contrast, an *in vitro* differentiation study has also reported that the potential of adipogenesis decreases in higher passages (from the 5th passage) whereas the propensity for osteogenesis increases in the long-term culture [39].

4.3 Replicative senescence during long-term culture expansion of MSCs

Replicative senescence is known as the irreversible growth arrest of the mitotic cells and is induced by telomere shortening. The expression of senescence markers such as senescence-associated β -galactosidase, heterochromatin protein-1, and p16INK4a increase during aging [47, 48]. Molecular damage and epigenetic alterations occur in aging stem cells [49], which can result in the impairment of stem cell function.

There are various signaling pathways involved in the senescence of MSCs, including oxidative damage [50, 51], age-related defects [52], and senescence associated up-regulation of microRNAs [53]. MSC senescence can be observed with long-term *in vitro* cultivation [54, 55], thus suggesting that a certain proportion of MSCs may undergo senescence during culture expansion. *In vitro* long-term culture of MSCs can induce continuous changes in gene expression [39, 56]. The expression levels of the senescence related genes, such as p16, p21 and p53, increase gradually in MSCs in the course of *in vitro* culture expansion [57]. DNA-methylation changes in MSCs during long-term culture have been investigated as an important epigenomic feature of replicative senescence of MSCs [58–60]. DNA-methylation changes may affect the proliferation and differentiation of MSCs. Differential methylation patterns of gene and miRNAs show between early-passage (passage 5) and late-passage (passage 15) MSCs [60]. Some genes that are hypermethylated at passage 5 present the lower mRNA expression than does these hypermethylated at passage 15 and vice versa [60].

Senescent cells secrete a complex combination of interleukins, chemokines, growth factors, proinflammatory/inflammatory cytokines, which compose the senescence-associated secretory phenotype (SASP) [61, 62]. One previous report has shown that conditioned medium (CM) collected from senescent BM-MSC culture at passage 10 is able to trigger senescence in young cells [63]. The key factors of senescent MSC CM needed for triggering senescence in the young MSCs have been characterized as insulin-like growth factor binding proteins 4 and 7, which are linked to cellular senescence and apoptosis [63]. Similarly, monocyte chemoattractant protein-1 (MCP-1), as a dominant component of the SASP, is markedly

increased in the conditioned medium of the late-phase MSCs and MCP-1 treatment significantly increase the senescence phenotypes of umbilical cord blood-derived MSCs via its cognate receptor chemokine receptor 2 signaling cascade [64]. Senescence-associated changes are observed in the metabolome of MSCs during replicative senescence, including down-regulation of nicotinamide ribonucleotide and up-regulation of orotic acid, which may be used to monitor the cellular senescent state during culture expansion of MSCs [65].

4.4 *In vitro* long-term culture associated spontaneous transformation of MSCs

Sarcoma represents a very heterogeneous group of relatively rare tumors and a variety of different studies have investigated to support the MSC origin of sarcoma. There are a number of cellular and molecular mechanisms of MSC transformation for better understanding of MSCs' contribution to sarcomagenesis [66]. The majority of published research articles indicate that various sarcoma types have been shown MSCs' origin. Several group have reported spontaneous transformation in human and murine MSCs after long term culture [67–70]. For example, one study has reported that murine BM-MSCs are spontaneously transformed at passage 29 under standard conditions and that these transformed MSCs are able to generate fibrosarcoma in immunocompromised mice [70]. Accumulated chromosomal abnormalities, such as chromosome instability, chromosomal imbalances and aneuploidy, are suggested to be associated with the transformation of BM MSCs [70]. Indeed, chromosomal aberrations (chromosomal level) in *in vitro* cultures of human MSCs have been reported in previous studies, including human BM-MSCs after passage 4 [71], human AD-MSCs from passage 5 [72], and UC-MSCs from passage 5 [73].

There are also studies that have not detected the transformation of MSCs in long-term culture [74–76]. One previous study reports that human BM-MSCs do not undergo malignant transformation after long-term *in vitro* culture for up to 44 weeks and these cells maintain a normal karyotype [75]. In agree with the previous report [75], another study has described the occurrence of aneuploidy in cultivated human MSCs without evidence of transformation either *in vitro* or *in vivo* [76]. In addition, Røsland G.V. *et al* have reported that human BM-MSC spontaneous transformation phenomenon occurred in consequence of the cross-contamination between the transformed human MSCs and human cancer cells [77]. To date, there is no solid evidence for the transformation of different sarcoma subtypes from MSCs and it leaves an uncertainty for MSCs with the ability to spontaneously transform.

5. Conclusion and perspective

MSCs provide huge opportunities in translational medicine for treatment of a range of diseases or medical conditions. MSCs are multipotent stem cells and such cells can be isolated from various tissues including bone marrow, a major source of human MSCs. Given that a large number of MSCs are required for the clinical application, *in vitro* expansion of MSCs is critical. However, MSCs at higher passage could lead to the culture-associated alterations, such as cellular morphology, immunological surface markers, proliferation, differentiation, and cell genetics. Due to *in vitro* long-term culture associated spontaneous transformation of MSCs, the safety of MSC therapy remains the major concerns. Human MSCs from various tissues present the varies biological properties. At present, consensus is lacking regarding materials and culture protocols, culture conditions, supplement of growth factors,

freezing and thawing (e.g. media), and functional assays. Therefore, standardizing the procedures for preparation of MSCs would be needed for pre-clinical studies and clinical application of MSCs.

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Conflict of interest

No competing interests for this work.

Abbreviations

AD-MSCs	Adipose-derived mesenchymal stem cell/stromal cells
ALP	Alkaline phosphatase
AsAP	Ascorbic acid phosphate
BM-MSCs	Bone marrow-derived MSCs
CM	Conditioned medium
CFU-F	Colony forming unit-fibroblast
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
ISCT	International Society for Cellular Therapy
MCP-1	Monocyte chemoattractant proten-1
MHC	Major histocompatibility complex
MSCs	Mesenchymal stem cell/stromal cells
PBS	Phosphate buffered saline
SASP	Senescence-associated secretory phenotype
UC-MSCs	Umbilical cord mesenchymal stem cell/stromal cells

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