

Chapter 2

Isolation and Expansion of Mesenchymal Stem Cells/Multipotential Stromal Cells from Human Bone Marrow

Patrice Penfornis and Radhika Pochampally

Abstract

In recent years, human mesenchymal stem cells (multipotential stromal cells) from bone marrow (hMSCs) have attracted enormous attention owing to their broad therapeutic potential. One of the problems in the overall therapeutic use of hMSCs has been the significant variability in the culture conditions used for their isolation and expansion. Since the seminal publications by Friedenstein and colleagues, the isolation and expansion of mesenchymal stromal cells (MSCs) from bone marrow have been of interest to several laboratories. As a result, numerous isolation protocols have been published. This chapter provides a simple protocol whereby a total of 80–100 million human MSCs, with an average viability greater than 90%, can be produced from a relatively small (1–3 mL) bone marrow aspirate in 14–20 days using double stacks culture chambers. MSCs were originally referred to as fibroblastoid colony forming cells because one of their characteristic features is adherence to tissue culture plastic and generation of colonies when plated at low densities. The efficiency with which they form colonies still remains an important assay for the quality of cell preparations. To assess the quality of cell preparations, two different colony forming unit (CFU) assays are also provided.

Key words: MSCs, Isolation, Expansion, Culture, Colony forming unit assay

1. Introduction

The recent explosion of interest in developing cell and gene therapies using adult stem/progenitors cells from human bone marrow can be partly attributed to the ease of isolation and expansion of cells from this source in vitro. In addition, the possibility of generating genetically manipulated bone marrow-derived stem cells to introduce specific genes of interest makes them attractive vehicles for gene therapy (1–5). In this review, the term human mesenchymal stem cell (hMSC) will be used to describe the plastic adherent cells from human bone marrow, first defined in the

literature as fibroblastoid colony forming units (CFU), then as mesenchymal stem/progenitor cells, and most recently as multipotent mesenchymal stromal cells (MSCs) (6).

Human MSCs are readily isolated from bone marrow by their adherence to tissue culture plastic and can be expanded through multiple passages in medium containing high concentrations of fetal bovine serum (FBS) (4, 7–13). However, the proliferation rates and other properties of the cells gradually change during expansion, and therefore, it is advisable to not expand hMSCs beyond four or five passages (14, 15). As originally indicated by Friedenstein, the most prominent properties of MSCs are their ability to generate colonies after they are plated at a low density, but both the colonies and the cells within a colony are heterogeneous in morphology, rates of proliferation, and efficacy with which they differentiate (8). Also, cultures of expanded cells are heterogeneous in their content of cells possessing an early progenitor phenotype. Human MSCs are highly sensitive to plating density, and early progenitors are rapidly lost if the cultures are grown to confluence (14, 16). Although the most recent definition of MSCs includes the expression of CD105, CD90, and CD73 surface antigens as potential biomarkers for MSCs, they alone are not sufficient to isolate cells directly from human bone marrow (6). Therefore, it is important to devise standardized assays for isolating and characterizing MSCs.

For the primary isolation of bone marrow-derived MSCs, the critical steps include the isolation of mononucleated cells from a marrow aspirate by centrifugation on a density gradient followed by recovery and expansion of cells that adhere to tissue culture plastic in standard serum-containing medium (passage zero cells). Passage zero cells are subsequently expanded by plating at a low density, which enhances the percentage of rapidly proliferating spindle-shaped cells. These cells would be replaced by large, flat, and thereby more mature hMSCs if the passage zero cells were plated at higher density or continually passaged for more than four to six times (Fig. 1a, b). Mature hMSCs will expand more slowly and have less multilineage differentiation potential, but still retain the ability to differentiate into mineralizing osteoblasts and secrete factors that enhance the growth of hematopoietic stem cells and perhaps other cells (13).

The efficiency with which hMSCs form colonies still remains an important assay for the quality of cell preparations. This chapter also describes two methods used to assay the colony forming ability of MSCs: (a) a traditional assay for colony forming units – fibroblast assay (CFU-F, Fig. 1c, d) and (b) single-cell colony forming unit assay (sc-CFU, Figs. 2 and 3). In the traditional CFU assay, cells are plated at low density in large plates and discrete colonies counted after 2 or 3 weeks. When used for assay of human MSCs, a single cell generates each colony. Noted when

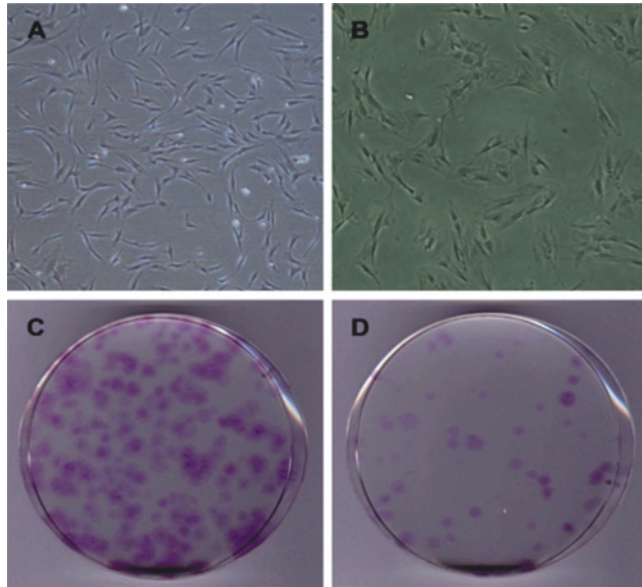


Fig. 1. Representative phase contrast microscopic images of cultures with (a) rapidly proliferating early passage mesenchymal stromal cells (MSCs) and (b) slowly proliferating late passage MSCs. Crystal violet stained plates of colony forming units – fibroblast assays (CFU-F) performed on (c) rapidly proliferating MSCs (as shown in (a)) and (d) slowly proliferating MSCs (as shown in (b)).

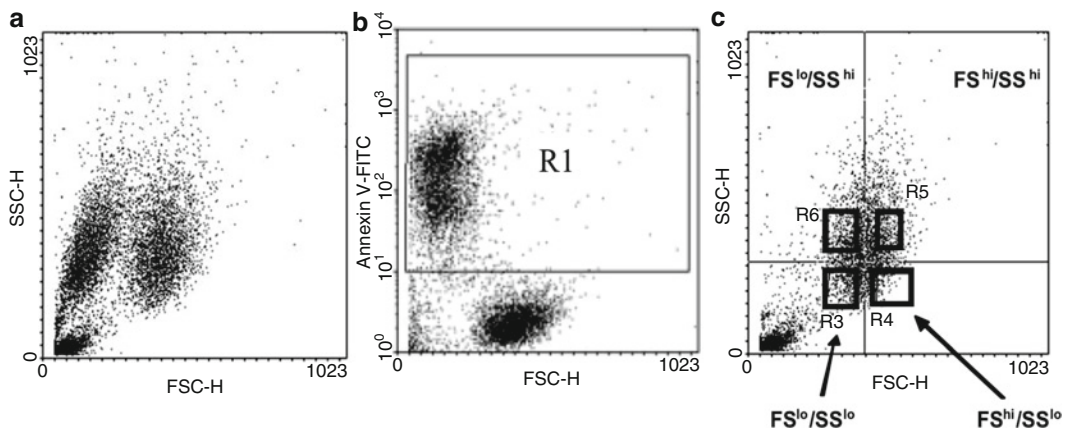


Fig. 2. Representative assay of FS/SS of passage two human mesenchymal stem cells (hMSCs) initially plated at 500 cells/cm² and incubated for 6 days to obtain high-density P3 cells. The cells were lifted with trypsin/EDTA and assayed on the open stream flow cytometer. (a) Uncorrected plot of FS/SS. (b) Same sample stained with Annexin V-FITC (R1). (c) Same sample after gating out Annexin V⁺ events. A small fraction of very low FS/SS events was Annexin V⁻ debris. Cell sorting based on FS/SS results in fractions that differ on subsequent analysis for FS/SS at the same instrument settings. FITC fluorescein isothiocyanate; FS forward scatter; SS side scatter.

used with rat or mouse MSCs, single cells can generate more than one colony because the cells can detach as they expand and reseed the plate (17, 18). In this chapter, we also describe a refined assay in which single MSCs are plated using a fluorescent flow cytometer

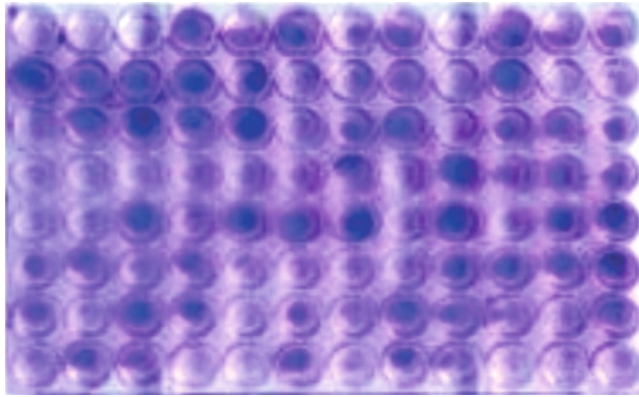


Fig. 3. Representative microtiter plate in which single FS^{lo}/SS^{lo} cells were deposited. The plate shown was stained with crystal violet to count colonies after incubation in standard serum-containing medium for 12–14 days.

with an automated cell sorter (FACSVantage SE with Clonesort accessory; Becton-Dickinson) to plate single cells into individual wells of a 96-well microtiter plate as described in Smith et al. (15). The cells are incubated in complete medium for 10–14 days and assayed visible colonies by staining the plates with Crystal Violet. With the sc-CFU assay, it is possible to distinguish the colony forming potential of two distinct kinds of MSCs present in early passage cultures: (1) spindle-shaped cells that are rapidly self-replicating are predominant in the first few days after plating the cells at low density, and (2) broader, slowly replicating cells that predominate as colonies or cultures become confluent. The proliferative spindle-shaped cells can be distinguished from larger, slower proliferating cells by their lower forward scatter (FS^{lo}) and lower side scatter (SS^{lo}) of light. As the delineation of subpopulations based upon FS/SS is somewhat difficult to standardize (Fig. 2), the sc-CFU assay is more useful in estimating the proportion of early progenitors in different preparations of MSCs.

2. Materials

2.1. Isolation and Culture of Bone Marrow-Derived hMSCs

1. Complete culture medium (CCM): α -MEM (Invitrogen, Carlsbad, CA) containing 16.5% (v/v) FBS (Atlanta Biologicals, Lawrenceville, GA) and 1% (v/v) Penicillin–Streptomycin (Invitrogen).
2. Hank's Balanced Salt Solution (HBSS) without Ca²⁺ and Mg²⁺ (Invitrogen).
3. Ficoll-Paque (GE Healthcare, Piscataway, NJ).

4. Phosphate buffered saline (PBS), pH 7.4 (Invitrogen).
5. Trypsin-EDTA in HBSS (Invitrogen).
6. Trypan blue, 0.4% (Invitrogen).
7. PBS, pH 7.4 (Invitrogen).
8. Cryopreservation medium: α -MEM (Invitrogen) containing 30% FBS (Atlanta Biologicals, Lawrenceville, GA) and 5% (v/v) DMSO (Sigma-Aldrich, St. Louis, MO).
9. T175 flasks or 150-mm tissue culture dish (Nunc, Thermo-Fisher Scientific, Rochester, NY).
10. Cryo 1°C Freezing Container (Nalgene, Thermo-Fisher Scientific).
11. Biological Safety cabinet Class II plugged to a vacuum system.
12. Water bath set at 37°C.
13. Water jacketed CO₂ incubator with HEPA filter system in humidified atmosphere and set at 37°C and 5% CO₂.
14. Bench centrifuge with swinging bucket rotor and brake ON/OFF option.
15. Inverted phase microscope.
16. Hemocytometer.
17. Sterile cell culture plastic pipets individually wrapped (2, 5, 10, 25 mL).
18. Pipet-Aid.
19. Sterile conical centrifuge tubes (15 and 50 mL).
20. 175 cm² Tissue culture flasks (Nunc).
21. Sterile plastic transfer pipets.
22. CellSTACK (2-stack) culture chambers (Corning, Corning, NY).
23. Solid cap, 33 mm threaded cap (Corning).

2.2. Colony Forming Unit Assay

1. Crystal violet (3%) (Sigma-Aldrich) in methanol. Filter through 25 μ m filter paper and store at room temperature. Before use, dilute to 0.5% in PBS.
2. Annexin V-FITC apoptosis detection kit (Sigma-Aldrich).
3. Flow Cytometry Microbead Standards (Polysciences Inc., Warrington, PA).
4. 96-Well tissue culture plate (Nunc).
5. EPICS FC500 flow cytometer running with CXP software (Beckman-Coulter, Brea, CA).
6. FACSVantage SE with FACSDiva Option (BD Biosciences, San Jose, CA).

3. Methods

3.1. Isolation and Culture of Bone Marrow-Derived hMSCs

1. Source bone marrow aspirates from the iliac crest and placed in 10 mL heparinized tubes prefilled with 3 mL of plain α -MEM. Keep samples on ice until to be processed.
2. Transfer each aspirate into a 50 mL conical tube and dilute to 15 mL with HBSS.
3. Rinse aspirate tubes twice with 5 mL of HBSS and combine with the diluted aspirate (25 mL total volume).
4. For each aspirate, place 10 mL of prewarmed (37°C) Ficoll-Paque into a separate 50 mL conical tube.
5. Gently overlay each aspirate onto the Ficoll. Take care to angle the tube containing Ficoll and very slowly pipet on the diluted aspirate over the border of the Ficoll meniscus. Once done, gently replace the tube in a vertical position (see Note 1).
6. Centrifuge tubes at $1,800 \times g$ for 30 min at room temperature in a swinging bucket rotor with the brake OFF (see Note 2).
7. After centrifugation, carefully collect the buffy coat, located at the Ficoll-HBSS interface, with a sterile Pasteur transfer pipet and place the cells into a clean 50 mL conical tube.
8. Dilute each sample to 25 mL with HBSS and invert the tube three to five times to mix (see Note 3).
9. Centrifuge tubes at $1,000 \times g$ for 10 min in a swinging bucket rotor with the brake ON.
10. Remove the supernatant by vacuum aspiration and resuspend the cells with 30 mL of prewarmed CCM.
11. Count viable cells with a hemocytometer using Trypan blue and plate at a cell density of 50–100 cells/cm² in 175 cm² flasks or 150 mm dishes.
12. Incubate the cells at 37°C with 5% humidified CO₂ for 24 h to allow adherent cells to attach.
13. After 24 h, remove the media and nonadherent cells (see Note 4).
14. Add 10 mL of prewarmed PBS to the culture, rock gently to cover the entire surface area and aspirate. Repeat the wash two additional times (see Note 5).
15. Add 30 mL of fresh CCM to the flask and return flasks to the incubator.
16. Examine cultures daily by phase microscopy.
17. Every 3 days, remove the medium and rinse the flask with 10 mL of prewarmed PBS. Aspirate the wash and feed cultures with 30 mL of fresh CCM. Continue until the cells reach 70–80% confluence (see Note 6).

18. To harvest cultures, remove the media and rinse the flask with 30 mL PBS and aspirate.
19. Add 10 mL of prewarmed trypsin-EDTA solution to the flask. Distribute the trypsin across the surface area of the flask. Incubate the flask for 2–5 min at 37°C. Examine the cells by phase microscopy.
20. After 80–90% of the cells have rounded up or become detached, gently tap the sides of the flask to dislodge any remaining attached cells.
21. Add 10 mL CCM to the flask. Rock the flask back and forth to swirl the media around the flask and transfer the entire cell suspension into a clean 50-mL conical tube.
22. Rinse the flask with 30 mL of 1× PBS and combine with the cell suspension.
23. Centrifuge at $1,000 \times g$ for 10 min in a swinging bucket rotor with the brake ON.
24. Remove the supernatant and resuspend the cells in 1–2 mL of prewarmed PBS.
25. Count the cells with a hemocytometer and trypan blue or preferred method (see Note 7).
26. Reseed harvested cells at a density of 50–100 viable cells/cm² in an appropriate culture vessel. The resultant hMSC cultures can usually be successfully expanded through passage three or four without significant loss of the stem cell phenotype.

The remainder of this procedure will describe the expansion of hMSCs in a Corning CellSTACK (2-stack) culture chambers (total surface = 1,272 cm²).
27. In order to obtain between 0.8 and 1×10^8 cells, we recommend using five 2-stack culture chambers. Add 300 mL of CCM/double stack culture chamber and place each chambers in incubator for at least 2 h before seeding (see Note 8).
28. Plate 6×10^4 cells/stack and carefully distribute cells evenly by gentle agitation using solid caps.
29. Grow cells for 2–3 weeks with complete medium changes made every 3–4 days. To remove medium, use a vacuum aspirator and a Pasteur pipet. Gently angle the culture chambers to avoid bubbling. The bottom stack can be monitored by using a regular inverted microscope.
30. To harvest cells, wash stacks with 100 mL PBS/stack and aspirate. Add 15 mL trypsin-EDTA/stack and incubate for 5 min. Follow cells lifting with the microscope, stop trypsinization when almost all cells are detached, reincubate an additional minute if needed but no more than 7 min.

31. Use the harvested cells for experimental purposes or reseed additional flasks or stacks at a density of 50–100 cells/cm². It is recommended to determine expanded MSC quality by using the CFU assays, see Subheadings 3.2.1 and 3.2.2.
32. Cryopreserve unused expanded hMSCs in Cryopreservation Medium (see Subheading 2.1) at 1×10^6 cells/mL (see Note 9).

3.2. Colony Forming Unit Assays

The efficiency with which MSCs form colonies still remains an important assay for the quality control of MSCs preparations. This section describes two methods to assay the colony forming ability of MSCs including (1) a traditional assay for CFU-F and (2) a sc-CFU.

3.2.1. Colony Forming Unit: Fibroblast Assay

1. Expand hMSC cultures to 70–80% confluence and harvest with trypsin-EDTA (see Subheading 3.1).
2. To ensure cell separation, a glass Pasteur pipet can be flamed to create a narrowed tip. Draw cells through the narrowed pipet several times (see Note 10).
3. Count the number of cells using a hemocytometer.
4. Dilute cells in CCM and plate at 100 cells/100-mm tissue culture dish or 10 cells/well in a 6-well plate.
5. Incubate for 10–14 days at 37°C in a humidified 5% CO₂ incubator.
6. Wash plates with PBS and stain with 0.5% (v/v) Crystal Violet solution for 5–10 min at room temperature.
7. Wash thoroughly with water and count visible colonies with a diameter greater than 5 mm. (Fig. 1).

3.2.2. Colony Forming Unit: Single Cell Assay

Rapidly self-renewing MSCs are characterized by low forward scatter (FS^{lo}) and low side scatter (SS^{lo}) of light. The following protocol describes the isolation of FS^{lo}/SS^{lo} MSCs that are rapidly self-renewing. It is also a rapid, standardized assay for FS/SS, a useful protocol to identify preparations of MSCs enriched for proliferative cells that will expand rapidly during subsequent passage in culture. The use of the assay should help to resolve discrepancies in data obtained by different laboratories with presumably similar preparations of hMSCs.

1. Standardize the closed stream flow cytometer (EPICS FC500 running CXP software) using microbeads with known uniform diameters (i.e., 6, 10, 15, and 20 μ m).
2. Adjust the gains and voltages on the photomultiplier tubes so that the mean value of the FS peak for the 20 μ m bead is about 650 and the peak of the SS for the 6 μ m bead is about 450. With these settings, the standard deviation for FS of the largest bead should be less than $\pm 0.4\%$ ($n=3$) of the mean and the slope of FS on a linear scale of 0–1,023 at least 41 (see Note 11).

3. For the assay, lift cells expanded as described in Subheading 3.1 with trypsin/EDTA and centrifuge in CCM at $450\times g$ for 10 min (see Note 12).
4. Count cells on a hemocytometer and resuspend in cold PBS (4°C) at a concentration of about 5×10^5 cells/mL. The assay should be run shortly thereafter.
5. Stain cells with the Annexin V-FITC (using manufacturer recommended protocol) and maintain at 4°C to prevent aggregation due to the presence of calcium and reagent-induced toxicity. Staining with Annexin V-FITC demonstrates that the events in the upper left of the plot are cell debris and dead cells (R1 in Fig. 2b). To obtain subfractions of cells, the Annexin V⁺ events are gated out and four subpopulations are defined on the basis of FS and SS (Fig. 2c).
6. Analyze cells using the above method and sort single FS^{lo}/SS^{lo} cells per well of a 96-well plate using the FACSVantage instrument. Divide the AnnexinV⁻ events into four quadrants on the basis of FS and SS (Fig. 2c). Offset the sort gates from the boundaries (see Note 13).
7. Incubate the microtiter plates with one hMSC per well in 0.15 mL CCM at 37°C and 5% CO_2 .
8. Every 4–5 days, aspirate CCM from each well and replace with 0.15 mL fresh medium.
9. After 2 weeks in culture, remove the medium and wash the wells with PBS. Incubate samples with 0.5% crystal violet solution for 5–10 min wash with water and count colonies with diameters greater than 1 mm using an inverted phase contrast microscope with a $4\times$ objective (Fig. 3).

4. Notes

1. If the Ficoll and HBSS-cell suspension layers are mixed, the mononuclear cells will not completely and efficiently separate during centrifugation.
2. The brake is left off to allow a slow deceleration that helps to avoid disturbance of the Ficoll-HBSS cell suspension interface.
3. It is recommended diluting the collected buffy coat with HBSS at a 3:1 volume ratio of diluent to sample.
4. If the nonadherent cells are not removed, hematopoietic cells may become attached and contaminate the hMSC culture.
5. There may not be many adherent cells seen at this point.

6. To preserve progenitor cell phenotype, do not allow the cells to become confluent. Because hMSCs are not evenly distributed in the marrow, some aspirates do not have enough hMSCs to obtain large cultures. If a sample does not grow well or do not have a good morphology by the eighth day, discard it.
7. A typical yield from a 175 cm² primary culture flask is between 1 and 3 × 10⁶ total cells, with an average viability usually greater than 90%.
8. It is important to allow the chambers containing CCM to equilibrate to 37°C and 5% CO₂ before use.
9. Freeze at a rate of −1°C/min using a Nalgene Cryo 1°C Freezing container placed at −80°C. After 24 h, transfer vials to liquid nitrogen for long-term storage.
10. It is critical that the cells are well dissociated.
11. The variation in values for log (%G/%T) should be established against samples containing 0.5 or 1 million MSCs/mL when the following parameters are varied: (a) the flow rate was 250, 500 or 900 cells/s; (b) the FS was assayed with 67 or 122 V and a gain of 2 or with 353 V and a gain of 1; and (c) the peak for FS of the 20 μm bead was set at 550, 650, or 750; and (d) the peak for SS for the 7 μm bead was set at 350, 450, or 550.
12. Cell culture confluence is important, and cells should be harvested when they are less than 80% confluent.
13. The accuracy of sorting single cells into each well of a microtiter plate should be verified routinely by sorting fluorescent beads (i.e., Flowchek; Beckman-Coulter) into a test plate and examining the wells with an epifluorescence microscope.

References

1. Azizi, S. A., Stokes, D., Augelli, B. J., Digirolamo, C., and Prockop, D. J. (1998) Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats – similarities to astrocyte grafts, *Proc. Natl. Acad. Sci. U S A* 95, 3908–3913.
2. Chopp, M., Zhang, X. H., Li, Y., Wang, L., Chen, J., Lu, D., Lu, M., and Rosenblum, M. (2000) Spinal cord injury in rat: treatment with bone marrow stromal cell transplantation, *Neuroreport* 11, 3001–3005.
3. Kopen, G. C., Prockop, D. J., and Phinney, D. G. (1999) Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains, *Proc. Natl. Acad. Sci. U S A* 96, 10711–10716.
4. Liechty, K. W., MacKenzie, T. C., Shaaban, A. F., Radu, A., Moseley, A. M., Deans, R., Marshak, D. R., and Flake, A. W. (2000) Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep, *Nat. Med.* 6, 1282–1286.
5. Pereira, R. F., Halford, K. W., O'Hara, M. D., Leeper, D. B., Sokolov, B. P., Pollard, M. D., Bagasra, O., and Prockop, D. J. (1995) Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice, *Proc. Natl. Acad. Sci. U S A* 92, 4857–4861.
6. Dominici, M., Le, B. K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E.

- (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement, *Cytotherapy* 8, 315–317.
7. Caplan, A. I. (1990) Stem cell delivery vehicle, *Biomaterials* 11, 44–46.
 8. Friedenstein, A. J., Petrakova, K. V., Kurolesova, A. I., and Frolova, G. P. (1968) Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues, *Transplantation* 6, 230–247.
 9. Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S., and Sharkis, S. J. (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell, *Cell* 105, 369–377.
 10. LaBarge, M. A. and Blau, H. M. (2002) Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury, *Cell* 111, 589–601.
 11. Owen, M. and Friedenstein, A. J. (1988) Stromal stem cells: marrow-derived osteogenic precursors, *Ciba Found. Symp.* 136, 42–60.
 12. Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999) Multilineage potential of adult human mesenchymal stem cells, *Science* 284, 143–147.
 13. Prockop, D. J. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues, *Science* 276, 71–74.
 14. Colter, D. C., Class, R., DiGirolamo, C. M., and Prockop, D. J. (2000) Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow, *Proc. Natl. Acad. Sci. U S A* 97, 3213–3218.
 15. Smith, J. R., Pochampally, R., Perry, A., Hsu, S. C., and Prockop, D. J. (2004) Isolation of a highly clonogenic and multipotential subfraction of adult stem cells from bone marrow stroma, *Stem Cells* 22, 823–831.
 16. Sekiya, I., Larson, B. L., Smith, J. R., Pochampally, R., Cui, J. G., and Prockop, D. J. (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality, *Stem Cells* 20, 530–541.
 17. Javazon, E. H., Colter, D. C., Schwarz, E. J., and Prockop, D. J. (2001) Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells, *Stem Cells* 19, 219–225.
 18. Peister, A., Zeitouni, S., Pfankuch, T., Reger, R. L., Prockop, D. J., and Raber, J. (2006) Novel object recognition in ApoE(–/–) mice improved by neonatal implantation of wild-type multipotential stromal cells, *Exp. Neurol.* 201, 266–269.



<http://www.springer.com/978-1-60761-998-7>

Mesenchymal Stem Cell Assays and Applications

Vemuri, M.C.; Chase, L.G.; Lipnick, S. (Eds.)

2011, XVI, 534 p. 114 illus. With online files/update.,

Hardcover

ISBN: 978-1-60761-998-7

A product of Humana Press