Chapter 4
Characterization of MSCs: From Early Studies to the Present

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Abstract Studies on mesenchymal stem cells/mesenchymal stromal cells (MSCs) have increased dramatically in the last 10 years, and many clinical trials are underway to take advantage of their properties. Early studies on MSC-like cells were performed in laboratories studying either bone repair or hematopoiesis, but the overlap in these studies was not broadly appreciated. The relationship between MSCs, osteoblastic progenitor cells, and the bone marrow stromal cells that provide support for hematopoietic stem cells has emerged. A variety of assays, in vitro and in vivo, allowed for a broader understanding of the MSCs and their characteristics. The MSCs from different animal species have properties similar to those from man, and this has allowed for many animal studies that provided preclinical support for human clinical trials with MSCs. While there are many established characteristics, new understanding of the MSC and the interaction of MSCs with other cell types, including HSCs and those of the immune system, will continue to reveal new and useful understanding of MSC properties.

Introduction

The early, underlying research behind mesenchymal stem cells, also commonly referred to as mesenchymal stromal cells, (MSCs) was slow to develop and can be traced to the fields of bone and blood research. However, by 2012 over 14,000 documents are found when one searches "mesenchymal stem cells" on PubMed. Bone marrow is commonly harvested for MSC isolation because it is considered renewable and does not require the sacrifice of healthy, nonrenewable tissue, although a variety of different tissues can be used to isolate MSCs. This is likely related to the

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existence of microvascular pericytes found along the capillaries in all tissues that have cells with the properties of MSCs. Isolation and propagation procedures for MSCs should be optimized and followed carefully for reproducibility as the cells are sensitive to culture conditions. Procedures used to isolate and propagate stem cells determine many of the properties of the resulting cells, and this is true for MSCs. Like all multipotent progenitor cells, MSCs are poised to respond to environmental conditions, including growth factors and cytokines, basal nutrients, cell-cell contact, as well as two and three dimension formats. The development of a combination of in vitro and in vivo assays greatly aided the characterization of MSCs and progress in this field. Whether for research purposes or clinical therapy, the time in particular culture conditions plays a role in the properties and characterization of MSCs, as well as their differentiation. In many ways, the MSC is an ideal, model adult stem cell; in the proper media, it does not require feeder cell layers, and it expands a millionfold or more and differentiates to desired or specified lineages in a reproducible fashion. Our early studies to characterize human MSCs from bone marrow provided necessary framework for many subsequent studies and clinical trials that continue today.

Early MSC Characterization: Born of Bone and Blood

Although the field of MSC research is expanding exponentially, the early work in this area was slow to develop. There is a modern body of literature that goes back 50 years from both the fields of blood and bone research that has many elements of research on cells we now call mesenchymal stem cells, multipotential stromal cells, or simply MSCs. Hematologists sought blood stem cells and the feeder cells that could support them in vitro in order to develop therapies for hematological malignancies. In the early 1960s, Till and McCulloch published their seminal work on hematopoietic spleen colony-forming units (CFU-Sp) in several papers where they were able to transplant the blood-forming ability of marrow into lethally irradiated mice [1, 2]. Although the term “stem cell” was already in use, this in vivo assay provided an experimental avenue for isolation of the responsible progenitor cells. The field of bone research long sought the progenitor cells for new bone formation to understand osteobiology and to use for repair strategies for bone and cartilage. Drs. Marshall Urist and Frank McLean transplanted decellularized fragments of bone to ectopic sites in 1953 and described new bone formation, but the responding tissue resident cells were unknown [3]. Dr. Urist’s research led many years later to the isolation and cloning of the bone-inducing molecules termed bone morphogenetic proteins (BMPs). In the 1960s, Alexander Friedenstein was researching interactions between bone and hematopoietic tissues at the Gamaleya Institute, USSR Academy of Medical Science in Moscow. He was culturing and characterizing the fibroblastic colony-forming units (CFU-F) from guinea pig bone marrow. The cells were placed in chambers with dialysis membranes to prevent the ingress of host cells, and the chambers were implanted under the skin of same specie host animals.

After several weeks, the chambers were excised, and some chambers provided evidence of new bone and cartilage. In a series of papers beginning from 1966, Friedenstein characterized the CFU-F from guinea pigs and rabbits [4–6]. These studies suggested the CFU-F was quiescent in vivo (in G_0) as it was resistant to radiation and slow to begin dividing. Dr. Maureen Owen and colleagues, working at Oxford University, investigated similar marrow-derived cells using athymic mice as hosts for cell chambers for better reproducibility [7–9]. Dr. Owen was the first to propose that mesenchymal tissues arose from a common progenitor cell similar to the hierarchal diagrams developed at the time for hematopoietic cells. Dr. Arnold Caplan at Case Western University was investigating bone and cartilage formation and repair in animal models in the 1980s. He championed the mesenchymal lineage hierarchy hypothesis and coined the term "mesenchymal stem cell" to focus attention on these powerful cells [10]. In pursuing the logical goal of isolation and therapeutic use of human MSCs, the Caplan lab developed a reliable in vivo bone-forming assay, which has been used to demonstrate their multilineage capabilities and identify fetal bovine serum lots that can maintain the multilineage potential of MSCs during ex vivo expansion [11]. The Caplan group also developed several monoclonal antibodies against surface antigens on the human MSC that proved useful for identification and further cell characterization [12, 13]. These are the SH-2 and SH-3 antibodies, now known to detect the surface molecules CD105 (endoglin) and CD73 (5′exonuleotidase, a salvage pathway enzyme), respectively [14, 15].

To develop the Caplan MSC methods for clinical use, Osiris Therapeutics, Inc. was founded. My cell biology group was tasked with developing in vitro assays that could be utilized to characterize human MSCs and test their differentiation to mesenchymal lineages. The osteogenic differentiation assay was in use in the Caplan lab and further developed for human MSCs by Scott Bruder [16, 17]. We developed an in vitro adipogenic differentiation assay based on the early work of Dr. Howard Green whereby the cells acquire all the attributes of adult adipocytes [18–20]. The chondrogenic differentiation assay was developed by Drs. Brian Johnstone and Jun Yoo at Case Western, and my group at Osiris, largely based on the in vitro study of rat chondrocytes by Drs. Tracy Ballock and Hari Reddi [21–23]. We presented the results from human MSC differentiation to these three lineages at the American Society for Cell Biology annual meeting in 1996 [24]. We added further gene expression studies, chromosome cytology, telomerase assays, and clonal studies and submitted those results in 1998, which were published in 1999, laying the groundwork for many subsequent studies [20]. Over several years, we performed differentiation assays on over 100 unique donors, providing substantiation for the mesenchymal stem cell paradigm.

In the early 1990s, the therapeutic potential of MSCs was already under study at University Hospital in Cleveland as an autologous treatment to support peripheral blood stem cell or bone marrow transplant for hematological malignancies [25, 26]. At this time, immunology studies on MSCs at Osiris Therapeutics demonstrated that human MSCs did not stimulate allogeneic T cell proliferation, and this was reported at international meetings and later published Klyushnenkova et al. [27]. These data correlated well with the lower than expected graft-versus-host disease incidence in

cancer patients undergoing matched unrelated bone marrow or mobilized peripheral blood transplants. That is, patients under treatment for hematological malignancies were found to have poor production of MSCs from their bone marrow, and matched donor MSCs were investigated as an improved therapy [28]. Allo-MSCs were also tested in patients with metachromatic leukodystrophy and Hurler syndrome [29]. (The hMSC immunology studies will be covered in other chapters.) It became apparent that allogeneic MSCs may be just as potent as autologous MSCs in preventing GVHD and stimulating beneficial responses from host cells and tissues and since then many studies have utilized allogeneic bone marrow MSCs. The autologous versus allogeneic MSC debate remains lively, and each may see therapeutic use in the future.

It should be emphasized that clinical use of MSCs has required careful characterization of the identity, purity, viability, potency, and stability of the therapeutic "product," and that the supporting preclinical studies performed in several mammalian species required a similarly rigorous if not quite as thorough characterization of the species' MSCs. In this regard, MSCs from rat [30–34], guinea pig [4], rabbit [35–37], dog [38, 39], goat [40], pig [41–46], and nonhuman primates [47–51] have very similar characteristics to their human counterparts. Therefore, many of these species have been useful for developing the necessary preclinical studies that allowed clinical development of MSC therapies. It is worth remembering that the rat has long been the preferred animal model for understanding aspects of human physiology/biology prior to gene knockout techniques that catapulted the mouse to the head of the line for understanding questions of gene functions and development.

Studies with mouse MSCs are plentiful, and many efforts have gone toward isolating mouse MSCs by similar methods as the above species [52–55]. However, the inherent co-purification/co-proliferation of mouse MSCs and cells derived from the hematopoietic lineages during ex vivo culture, something not seen in the other species listed above, has brought up the question of which cells in the cultures may be responding in experiments. This issue of mouse MSCs containing HSC progeny that continue to coculture throughout the in vitro culture process remains a problem today for the study of mouse MSCs. A method to eliminate HSCs progeny in mouse MSC preparations requires a final immunoselection step to negatively select and eliminate the HSCs before experimenting with the mouse MSCs [56]. This can limit the number of mouse MSCs available for study as they may no longer propagate without the presence of the hematopoietic cells.

"Mesenchymal stem cell" captures the potential of these cells to do more than differentiate to one or two lineages in vitro and in vivo, although to date there are no methods to differentiate these cells to *all* mesenchymal lineages. Other names including mesenchymal progenitor cell, multipotential stromal cell, and multipotential mesenchymal stromal cell are used by different investigators, yet the abbreviations MSC and MSCs, for the plural, are universally common. While some researchers continue to refer to MSCs as mesenchymal stromal cells, the reader should recognize that not all stromal cells are MSCs – actually very few. We suspect that MSCs were probably part of the bone marrow stromal cell preparations used in past years to propagate hematopoietic progenitor cells, but this is not assured

because MSCs are very rare in the bone marrow and their in vitro expansion while retaining multilineage potential is dependent on *optimized* in vitro *culture conditions*. The reliance of hematopoietic stem cell research on irradiated feeder layers led to the isolation of a number of characterized stromal cell lines, both mouse and human, but these generally were not tested for differentiation to any other lineages as such methods were not developed. Early cultured populations of stromal cells may have supported hematopoietic expansion, but they were only partially characterized, and what differentiation potential to other lineages or immunemodulatory capacity these cells may have had was not tested. Therefore, the percentage of early cultured stromal cells with the properties of MSCs cannot be known. Hence, given the rarity of MSCs in adult bone marrow and the need for careful culture methods for their propagation, a general claim of stromal cells as MSCs must be thoughtfully examined.

Source Tissues for MSCs

Isolation of MSCs (BM-MSCs) from human adult bone marrow drawn from the iliac crest is common, and this marrow or the isolated and cultured cells can be ordered from vendors. Adipose tissue has been used as a MSC source (AT-MSCs), the MSCs likely deriving from the adipose vascular pericytes [57–59]. The discarded placenta and umbilical cord *tissues* appear to be good sources of MSCs, although cord blood has very few MSCs [60–65]. Even the pulp of shed teeth has been used as a source of MSCs [66, 67]. From all species, bone marrow is most commonly used and adipose the next likely source as it is easily harvested in small quantities, or in larger amounts through liposuction procedures (for veterinary uses, see www.VetStem.com). Despite the origin of MSCs, they need to be characterized before use for in vitro or in vivo experiments. Clinical use requires highly characterized MSCs and full compliance with current Good Manufacturing Practices (cGMP). Many of the methods presented here have been adapted for Good Laboratory Practice (GLP) and cGMP use.

Fetal Calf Serum Qualification and the "In Vivo Cube Assay"

Following their initial studies with cells from rats and rabbits, Arnold Caplan and colleagues specifically sought to isolate human mesenchymal stem cells that could be expanded in culture and used for clinical studies for hematopoietic support and/or bone and cartilage therapies. Steve Haynesworth working with Dr. Caplan sought to isolate and study human MSCs and developed several monoclonal antibodies that identified rare cells in bone marrow that could be isolated and cultured in vitro. These were the SH-2, SH-3, and SH-4 antibodies now known to bind to cell surface

endoglin, or CD73, (SH-2), and the two that bound epitopes on 5′-exonucleotidase or CD105 (SH-3, SH-4). The other aspect was the development of an in vivo assay to test the ability of the isolated cells to differentiate to bone and cartilage and demonstrate endochondral bone development [11]. This entailed choosing a porous osteoconductive matrix material that was not osteoinductive. Specifically, hydroxyapatite/tricalcium phosphate matrix would allow osteo differentiation of cells attached to it, but would not induce osteoid formation when placed into tissue. The cells in question were allowed to attach for several hours and then implanted under the skin of athymic mouse recipients with up to six cubes per mouse. Usually 3 and 6 weeks later, the cubes were removed and examined histologically for the presence of new bone and cartilage. Culture medium and supplements, particularly fetal bovine serum (FBS), have been analyzed by the cube assay to develop a method to reliably grow human MSCs. When the porous cubes with no cells were implanted, no bone or cartilage was seen, and only some host fibrous tissue may be present. Similarly, when fibroblasts were placed in the cubes, no bone or cartilage was seen when the cubes were sectioned and analyzed. However, when a "good" prospective MSC population was placed in the cubes, abundant bone and cartilage tissue could be found in the matrix pores (see Fig. 4.1), whereas poor MSC preparations showed limited bone and little cartilage. The astute reader will recognize that the cube assay analyzes the results of the in vitro culturing of the cells of interest and their in vivo differentiation. Therefore, the cube assay can be used to develop the in vitro culture conditions as well as examine the resultant cultured cells. Importantly, the cube assay can identify and qualify fetal bovine serum that supports the expansion of multipotential MSCs. This iterative "boot strapping" process of both culture conditions and isolated cells was essential to the development of human MSCs, and no hindsight or prospective MSC isolation procedure suffices to replace the process even today because FBS or its replacement(s) must still be optimized and qualified in some manner. Most vendors of reagents for MSC research use some version of the cube assay or other methods to qualify lots of fetal bovine serum for MSC growth. Fetal serum is a complex solution of growth factors, and cytokines and quantities of each factor vary from one calving season to the next, and from lot-to-lot. Simply using more FBS does not seem to work. Although many efforts to use defined growth factors instead of FBS have been published, each growth factor needs to meet its own release criteria following manufacturing, and most are not produced to clinical standards.

Flow Cytometric Analysis of MSCs

Fluorescence-activated cytometry or simply flow cytometry can analyze the presence of known molecules on the cell surface with the use of antibodies, although internal molecules and some other characteristics can be analyzed as well. For MSCs, the characteristic surface molecules do not identify MSC stemness per se, but some have proven useful to routinely assay the cultured cells for homogeneity,

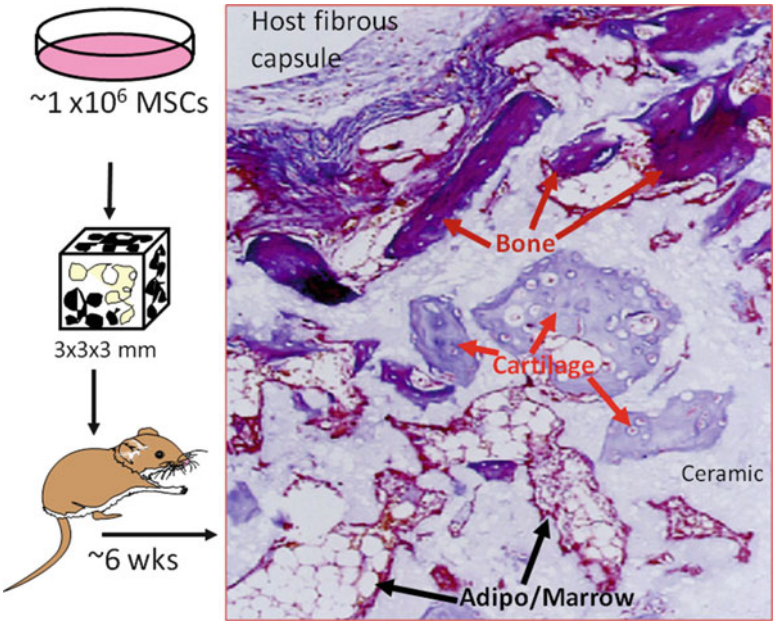

Fig. 4.1 Developing preferred MSC culture conditions. Mononuclear cells are isolated from bone marrow or other tissues sources, propagated in controlled tissue culture conditions which may include different additives such as fetal calf serum, particular growth factors, different basal media, etc. The cultured cells are placed on "inert" carriers and placed under the skin of immune-deficient mice, and the in vivo culture continued for ~6 weeks. The animal is sacrificed; the carriers are recovered and analyzed for the presence of tissues with differentiated cell types such as bone, cartilage, and adipose. This method was used in an iterative fashion to understand and improve the culture conditions for human BM-MSCs

"subpopulations," or any contaminating cells. Isolated MSC populations that performed well in the cube and differentiation assays were found to have consistently high levels of certain surface markers and low or undetectable levels of others. These positive and negative markers can be used as one facet for the characterization of MSCs for research or clinical purposes, and the presence and/or absence of certain surface molecules can help to determine the purity of the sample [68]. The markers are not sufficient to identify stem cells but do indicate the surface molecules available for interaction with other cells, extracellular matrix, etc. Cultured expanded human MSCs are commonly >95% positive for CD29, CD44, CD73, CD105, and CD166 and negative for hematopoietic markers CD11, CD34, and CD45, and a subset of these markers has been used as one aspect to qualify MSCs used in clinical studies. Table 4.1 contains a list of surface markers on human MSCs as analyzed by flow cytometry; this list is not complete, and culture conditions can affect some expression data. Attempts to isolate subpopulations of MSCs based on low and high expression of particular surface molecules has met with limited success, partly due to the limited number of cells isolated, but it is also debatable whether further isolation identifies "new cells" or just reveals temporal variations in expression. It is

Table 4.1 Cell surface molecules on MSCs by flow cytometry

Surface antigen	Pos/Neg
CD11a,b	Neg
CD13	+
CD14	Neg
CD18 integrin β2	Neg
CD29 integin β1	+
CD31 PECAM	Neg
CD34	Neg
CD44	+
CD45	Neg*
CD49b integrin α2	+
CD49d integrin α4	Neg
CD49e integrin α5	+
CD50 ICAM3	Neg
CD51 integrin αV	+
CD54 ICAM1	+
CD56 NCAM	+
CD62E E-selectin	Neg
CD71 transferrin rec	+
CD73 SH-3	+
CD90 thy-1	+
CD105 endoglin, SH-2	+
CD106 VCAM	+
CD117	Neg
CD133	Neg
CD166 ALCAM	+
CD271 p76 LNGFR	+
Trk A, B, C	+
HLA A, B, C	+
HLA-DR	Neg, IFNγ inducible
B2 microglobulin	+
Nestin	+
SSEA-3	+
SSEA-4	+

*Primary MSCs may be poscultured MSCs are ref

important to note that the flow cytometry data are routinely presented on a log scale, and purified populations of cells often have positive expression levels that vary tenfold or more. Another aspect of flow cytometry that has been useful in MSC characterization is the shift in expression levels of some surface molecules when MSCs are treated with certain biologically active molecules. For example, when MSCs are treated with interferon-γ, they now express HLA-DR on their surface. A shift in a single peak is found in the flow cytometry results, further suggesting MSCs are a

single population of cells responding uniformly to a biological stimulus and not a heterogeneous population.

In Vitro Differentiation of MSCs

In vivo assays such as the cube assay involve the added complexity of different host animals and several handlers at different steps and can lead to varying results. We developed a series of in vitro assays to test the differentiation of human MSCs [20, 24, 68] which also perform well for MSCs from other species. The assays were then miniaturized to allow for full testing with a minimum number of cells to compare parental cells and progeny derived from single cell clones. That is, from a single human cell from bone marrow, we expanded the progeny 21–22 population doublings (PD) to yield 500,000–1,000,000 cells that could be analyzed by flow cytometry and in vitro differentiation illustrating that the differentiation of the parental cells to different lineages was due to their multilineage potential rather than the outgrowth and subsequent differentiation of separate subpopulations [20]. If some MSC clones do not differentiate to each lineage, it indicates either that the colony has expanded beyond its capability to differentiate to all lineages or that the original single cell did not have multilineage capacity.

Adipogenic Differentiation of MSCs

The method for adipogenic differentiation of human MSCs is similar to the method developed by Dr. Howard Green for differentiation of 3T3-L1 preadipocytes [19]. With 3T3-L1 cells, the differentiation occurs easily in a few days, but with MSCs, repeating the induction conditions commits more cells to adipocytes, so several treatments were found to be optimal [20]. Briefly, MSCs are cultured as monolayers in dishes with low glucose (1 g/l) DMEM with 10% FBS and allowed to become confluent. The cells are cultured for ~3 days more, and then the medium is changed to adipogenic induction medium (MDI + I medium) containing 0.5-mM methylisobutylxanthine, 1-μM dexamethasone, 100-μM indomethacin, 10-μg/ml insulin, and 10% FBS in low glucose DMEM. The MSCs are then incubated for 48–72 h, and the medium is changed to adipogenic maintenance medium (AM medium) containing 10 μg/ml insulin and 10% FBS in the DMEM for 24 h. Greater commitment to the adipogenic lineage is seen when the cells are retreated with (MDI + I) for a second and third treatment round. The cultures are then maintained in AM medium for about 1 week to allow the lipid vesicles to enlarge and coalesce and then assayed. Nile Red, a fluorescent vital dye, is used to quantify lipid vacuoles using a UV plate reader and counterstaining the cells with DAPI to label DNA content as described

[20]. If desired, the adipogenic MSCs can then be fixed and stained with oil red O for nonquantitative histological presentation [20].

Chondrogenic Differentiation of MSCs

The chondrogenic differentiation of human MSCs utilizes an in vitro culture method described for rat chondrocytes and optimized for human MSCs [21–23]. Although MSCs are usually cultured in low glucose (1 g/l glucose) and 10% FBS, during chondrogenic differentiation in a compact micromass, this leads to cell death so it is important to use high-glucose (4.5 g/l glucose) DMEM but no FBS. In the micromass or "pellet culture," there is little or no cell proliferation, but the abundant amount of extracellular matrix produced leads to enlargement of the pellets. For chondrocytic differentiation of human MSCs, approximately 250,000 cells are placed in a polypropylene conical tube (to prevent easy adhesion) with DMEM, and cells are gently centrifuged to the bottom. Cells will form a cell micromass in 24 h that should be dislodged and free floating. The chondrogenic media consists of high-glucose DMEM supplemented with ITS+ (6.25-μg/ml insulin, 6.25-μg/ml transferrin, 6.25-μg/ml selenous acid, 5.33-μg/ml linoleic acid, 1.25-mg/ml bovine serum albumin), 0.1 μM dexamethasone, 10-ng/ml TGF-β3, 50-μg/ml ascorbate 2-phosphate, 2-mM pyruvate, and antibiotics. This medium is changed every day due to the labile TGF-β. The TGF-β3 is stored at −80 °C in small aliquots. For rat MSCs, BMP-2 is added at 10 ng/ml to improve chondrogenic differentiation. During the first week, little change is observed, but in 2–3 weeks, the extensive extracellular matrix leads to larger hard cell pellets that appear cartilaginous (if not obvious, extend the culturing for another week). The chondrogenic MSCs can undergo further maturation in vitro to become hypertrophic chondrocytes with addition of thyroxine, demonstrating their chondrocyte biology [21, 23]. Gene expression studies, immunofluorescence, and histological examination will reveal extensive differentiation that resembles neo-cartilage during embryonic development, and electron microscopy evaluation will show the glycoproteins are extensive but perhaps less cross-linked than adult cartilage [21].

Osteogenic Differentiation of MSCs

The osteogenic differentiation of MSCs is perhaps the easiest assay and has been used for many years to demonstrate the potential of bone-derived osteoblasts as well as MSCs [16]. We refer to the in vitro differentiated cells as osteoblasts and not osteocytes because the cells first proliferate (blasts) but do not encase themselves in mature bone extracellular matrix as osteocytes. However, the in vivo-differentiated MSCs are found as osteocytes with extensive matrix production around each cell. For in vitro osteogenic differentiation of MSCs, approximately 3×10^4 cells (low density) are seeded onto 35-mm dishes or six well plates in low glucose DMEM

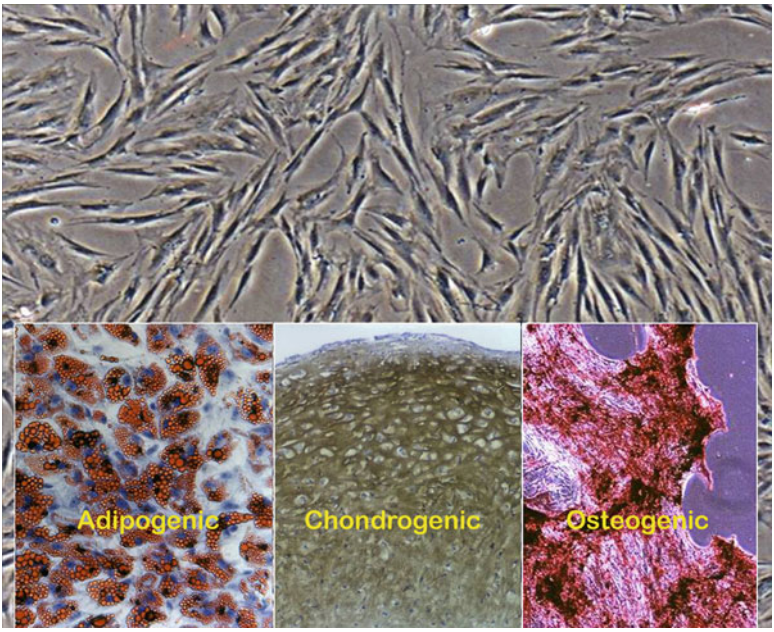

Fig. 4.2 Cultured MSCs can be exponentially propagated in culture and tested for in vitro differentiation. We demonstrated culture conditions that resulted in complete differentiation of human BM-MSCs. Under these protocols, virtually every MSC in the culture progressed to the fully differentiated cell type and exhibited gene expression and properties of the differentiated phenotype of the adult tissue, that is, it was not a mixture of differentiated and undifferentiated cell types. *Left to right*: Adipogenic oil red O stained lipid vesicles; chondrogenic MSCs immunostained for type II collagen shows abundant extracellular matrix in *brown* (DAB staining); osteogenic MSCs stained for alkaline phosphatase (*red*) and calcium deposits by silver staining by the von Kossa method (*black*)

with 10% FBS, glutamine, and antibiotics. In 24 h, the medium is replaced with the same supplemented with 50-μM ascorbate 2-phosphate, 10-mM β-glycerol phosphate, and 100-nM dexamethasone. The medium is changed every ~3 days, and periodically a sample is stained with Alizarin Red and compared to MSCs maintained in their normal culture medium. The differentiation is largely complete in 10 days. The culture wells can otherwise be stained for increased expression of alkaline phosphatase and deposition of mineralization by silver staining by the method of von Kossa [17]. In a separate set of culture wells, mineralization is quantified by measuring calcium deposition using commercially available kits [17] (Fig. 4.2).

Stromal Support Assay

Cultured MSCs produce a large number of cytokines and growth factors that are necessary for support of hematopoietic stem cells or even human embryonic stem cells. MSCs produce macrophage colony-stimulating factor (M-CSF), granulocyte

colony-stimulating factor (G-CSF), and granulo-macrophage colony-stimulating factors (GM-CSF). MSCs also produce interleukins IL-1a, IL-1b, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12, IL-14, and IL-15. MSCs and each of these can be assayed by western blot, ELISA, or Elispot assays [20, 68]. MSCs also express surface molecules including intercellular adhesion molecules and vascular cell adhesion molecules, ICAM and VCAM, respectively, which interact with receptors on HSCs or ES cells. These surface molecules are easily assayed by flow cytometry [20]. Therefore, MSCs can be used to provide stromal support for the expansion of HSCs in culture.

Gene Expression MicroArrays in MSC Characterization

Analyzing gene expression of MSCs by microarray analysis is very promising, and several studies have been completed. The power of microarray analysis is the ability to analyze thousands of transcripts in a single experiment. Phinney and colleagues utilized serial analysis of gene expression (SAGE) to sample 2,300 transcripts from MCSs and found mRNAs from multiple cell lineages [75]. We previously used an array of 8,400 gene tags with highly purified MSCs, and the results demonstrated MSCs-transcribed genes normally associated with many differentiated cell types including astrocytes, neurons, epithelial and endothelial cells, as well as osteocytes, myocytes, tenocytes, adipocytes, chondrocytes, and other mesenchymal lineages (unpublished.) Analyses of the proteomes and transcriptomes of various MSC preparations from lab to lab reveal the transcription and translation of the genes of multiple lineages, but results can be dependent on lab-specific culture conditions. Moreover, microarray analysis is also dependent on initial cell sample isolation and multiple steps isolation of RNA, reverse transcription and amplification of the DNA copy – and performing these steps in an identical manner is essential for reproducibility and validation. From a clinical regulatory perspective, microarray data should only include that which is reliable and necessary, and variations should be within a specified range or be otherwise explicable. In this regard, a downsized custom microarray of ~100 transcripts may be more useful for MSC characterization for clinical purposes. Any “data for information purposes only” can be useful for future cell characterization but should not be confusing or non-reproducible, because they raise concern among regulatory agencies.

MSC Population Properties: Homogeneous or Heterogeneous MSCs?

MSCs constitute a discrete cell population that can be isolated reproducibly from bone marrow and other tissues and become a highly homogeneous population with consistent assayable properties after only a few passages ex vivo. Such properties

are maintained after further expansion through many passages. The expanded cells from different donors are found to have the same flow cytometric profile of positive and negative cell surface molecules; the growth characteristics and morphology are the same; and the results of differentiation assays are remarkably consistent. The flow cytometry scattergrams show a highly reproducible normal distribution with few outliers. Further, the search for the presence of known cells of other lineages is characteristically negative or produces a nominal 0–2% of uncharacterized cells. The contribution of any small population of contaminating cells to assay results is likely to be very small, and any contaminating cells with an uncharacterized phenotype are likely fewer than other clinical therapeutic preparations such as mobilized peripheral blood mononuclear cells. From a clinical perspective, it is necessary to have reproducible methods and a thorough description of the cellular product and its possible contaminants. When procedures are established and consistently followed, laboratories thousands of miles apart using marrow from different donors isolate MSCs that are indistinguishable from one another. Many clinical trials are underway with MSCs characterized similarly to the methods described in this chapter with the understanding that the methods are reproducible. In virtually all respects, cultured MSCs are much more homogeneous than other stem cells such as ESCs, iPSCs, neural stem cells, and others.

Immunoselection of cells from fresh bone marrow with different antibodies can obtain a subpopulation of the cells present in bone marrow. We previously selected cells from human bone marrow using a variety of antibodies and expanded them in culture. The resultant cells had similar properties to MSCs isolated by density centrifugation or direct plating. Other researchers have also utilized antibodies to select bone marrow cells. For example, Covas et al. used anti-CD146 to select cells from bone marrow aspirates, and the cultured cells had the desired phenotype of MSCs [69]. Similarly, McGonagle and coworkers used anti-CD271 immunoselection to select a population of primary cells from bone marrow to produce MSCs [70]. Other authors have utilized antibodies whose antigen is unknown and claimed that they have isolated a superior population of MSCs, but the field has been slow to confirm such claims. The antibody selection of a desired subset of bone marrow cells may be advantageous in focusing attention on the population of interest in an immediate fashion but the selected and culture-expanded cells appear to be virtually identical to the MSCs derived from density centrifugation or direct plating methods from other labs, rather than obtaining unique stem cell populations.

Recent studies of MSC heterogeneity usually choose a point in time and analyze differences in gene expression in isolated subpopulations and assume no or limited interconvertibility. The question is whether these are unique stable phenotypes or a phenotype that is time- and culture condition-dependent. For HSCs, it is known that subpopulations can be interchangeable, and that phenotype can depend on cell cycle, injury proximity, cytokines, and interactions among homologous and heterologous neighboring cells [71]. For MSCs, it seems apparent that their plasticity is at least as complex as HSCs, yet it is quite possible to use the “MSC population properties” to design revealing studies and clinical trials. Nevertheless, MSCs can exhibit microheterogeneity within the isolated cell population, and this may be a common and useful property of all stem cells. MSCs express a variety of surface

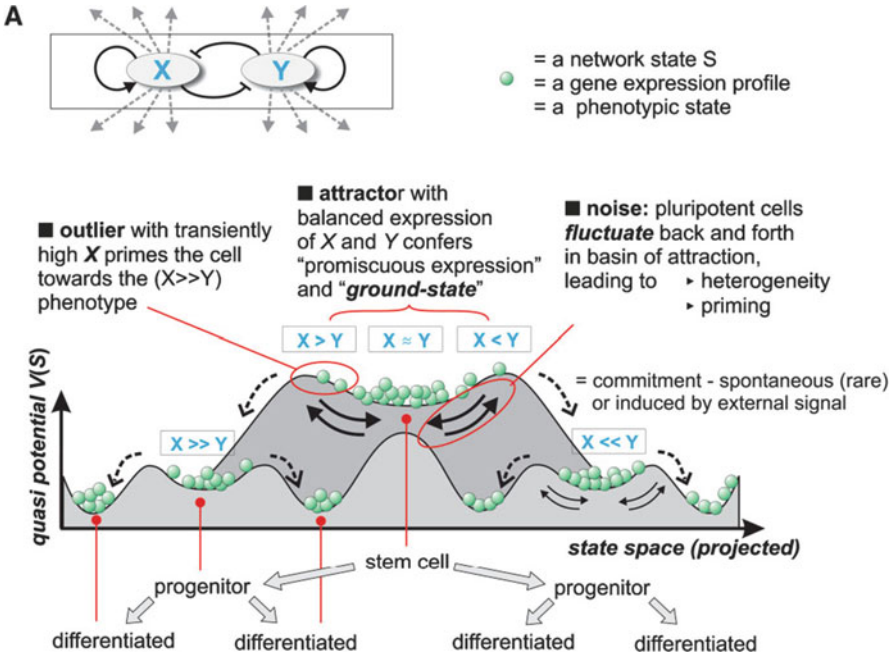

Fig. 4.3 Stem cells can express a range of genes without losing identity or differentiating. A hypothetical epigenetic landscape with a field of multipotential cells. A population of MSCs, even if clonally derived, can express a range of genes, and the levels of gene and protein expression can oscillate, although some states are more common or "preferred." X and Y may represent master genes leading to different pathways such as X = PPARγ (adipocyte pathway) and Y = BMP2 (osteocyte pathway) (Drawing from Figure 3A in Huang [74]. With permission from *Bioessays*)

receptors and internal signaling pathways that allow them to respond to neighboring cells and different external signals. It should be recognized that dispersed single MSCs growing in the culture dish at low confluency have different and measurable properties from MSCs that have contacts with their neighbors, or those that are confluent, or contact non-MSCs such as endothelial cells, lymphocytes, or HSCs. Thus, culture conditions partly determine the properties of any stem cell populations; hence, closely following protocols results in greater reproducibility.

Mesenchymal stem cell microheterogeneity may be an adaptation to the needs of the cells in the embryo, expanding fetal tissues, or repairing adult tissues where the stem cell is constantly modifying its response to environmental input. Several papers in system dynamics have modeled stem cell biology by describing preferred states within a continuum that allows the interchange between states, some more likely than others, based on transcription factors energy levels and other factors that must be overcome to pass from one state to the other [72, 73, 74] (see Fig. 4.3). Thus, describing MSC gene expression with its stochastic fluctuations yet constrained by interacting gene regulatory pathways, the availability of ATP, and input from the environment and neighboring cells, gives a dynamic yet stable phenotype. Such

approaches are powerful and useful alternatives to the stem cell heterogeneity paradigm. Moreover, such a view explains well the notion that multipotential stem and progenitor cell states are not so rigid and can sometimes move back and forth.

Conclusions

Current methods can produce highly reproducible populations of mesenchymal stem cells for research purposes or clinical therapies. The characterization of MSCs will continue to improve until highly successful or preferred methods become more obvious, and agreement among investigators is achieved. New assays will always be needed to further characterize stem cells, including RNA microarrays, glycoprotein arrays, transcription factors, DNA methylation sites, and differentiation assays for new lineages. Some such work has been published, but more is needed. Due to the inherent microheterogeneity of stem cells, it may be necessary to constrain some parameters in the assays to limit the cells' degrees of freedom. This can be accomplished by controlling one or more dominant parameters such as using defined medium containing a single growth factor or a culture surface that signals through a particular cell adhesion molecule. Well-characterized animal models and in vivo assays are also further needed to develop particular clinical therapies with MSCs. It is just as important to define the limitations of MSCs as well as their diverse potential. Overall, it seems it is not the MSCs that are limiting; it is the ingenuity and creativity of the investigators.

References

1. McCulloch EA, Till JE (1960) The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Radiat Res 13:115–125
2. Till JE, McCulloch EA, Siminovitch L (1964) A stochastic model of stem cell proliferation based on the growth of spleen colony-forming cells. Proc Natl Acad Sci USA 51:29–36
3. Urist MR, McLean FC (1953) The local physiology of bone repair with particular reference to the process of new bone formation by induction. Am J Surg 85:444–449
4. Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV (1966) Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 16:381–390
5. Friedenstein AJ, Gorskaja JF, Kulagina NN (1974) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267–274
6. Luria EA, Owen ME, Friedenstein AJ, Morris JF, Kuznetsow SA (1987) Bone formation in organ cultures of bone marrow. Cell Tissue Res 248:449–454
7. Bab I, Ashton BA, Gazit D, Marx G, Williamson MC, Owen ME (1986) Kinetics and differentiation of marrow stromal cells in diffusion chambers in vivo. J Cell Sci 84:139–151
8. Owen ME, Cave J, Joyner CJ (1987) Clonal analysis in vitro of osteogenic differentiation of marrow CFU-F. J Cell Sci 87:731–738
9. Owen ME, Friedenstein AJ (1988) Stromal stem cells: marrow derived osteogenic precursors. Ciba Found Symp 136:42–60

10. Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641–650
11. Lennon DP, Haynesworth SE, Bruder SP, Jaiswal N, Caplan AI (1996) Human and animal mesenchymal progenitor cells from bone marrow: identification of serum for optimal selection and proliferation. In Vitro Cell Dev Biol 32:602–611
12. Haynesworth SE, Goshima J, Goldberg VM, Caplan AI (1992) Characterization of cells with osteogenic potential from human marrow. Bone 13:81–88
13. Haynesworth SE, Baber MA, Caplan AI (1992) Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. Bone 13:69–80
14. Barry FP, Boynton RE, Haynesworth S, Murphy JM, Zaia J (1999) The monoclonal antibody SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105). Biochem Biophys Res Commun 265:134–139
15. Barry F, Boynton R, Murphy M, Haynesworth S, Zaia J (2001) The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human mesenchymal stem cells. Biochem Biophys Res Commun 289:519–524
16. Bruder SP, Jaiswal N, Haynesworth SE (1997) Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem 64:278–294
17. Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 6:295–312
18. Pittenger MF (1998) Adipogenic differentiation of human mesenchymal stem cells. US Patent #5,827,740. US Patent Office
19. Green H, Kehinde O (1975) An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5:19–27
20. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147
21. Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF (1998) Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 4:415–428
22. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU (1998) In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 238:265–272
23. Ballock RT, Reddi AH (1994) Thyroxine is the serum factor that regulates morphogenesis of columnar cartilage from isolated chondrocytes in chemically defined medium. J Cell Biol 126:1311–1318
24. Pittenger MF, Mackay AM and Beck SC (1996) Human mesenchymal stem cells can be directed into chondrocytes, adipocytes and osteoblasts. Mol Bio Cell Dec 7:305a 1172
25. Lazarus HM, Haynesworth SE, Gerson SL, Rosenthal NS, Caplan AI (1995) Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. Bone Marrow Transplant 16:557–564
26. Koç ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI et al (2000) Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol 18:307–316
27. Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, Deans RJ, MCintosh KR. T cell responses to allogeneic human mesenchymel stem cell: immunogenicity, tolerance, and suppression. J Biomed Sci. 2005;12(1):47–57
28. Lazarus HM, Koc ON, Devine SM, Curtin P, Maziarz RT, Holland HK et al (2005) Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant 11:389–398
29. Koç ON, Day J, Nieder M, Gerson SL, Lazarus HM, Krivit W (2003) Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH). Bone Marrow Transplant 30:215–222

30. Grigoriadis AE, Heersche J, Aubin JE (1990) Continuously growing bipotential and monopotential myogenic, adipogenic and chondrogenic subclones isolated from the multipotential RCJ3.1 clonal cell line. Dev Biol 142:313–318
31. LeBoy PS, Beresford J, Devlin C, Owen M (1991) Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J Cell Physiol 146:370–378
32. Beresford JN, Bennett JH, Devlin C, Leboy PS, Owen ME (1992) Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. J Cell Sci 102:341–351
33. Kadiyala S, Jaiswal N, Bruder SP (1997) Culture-expanded bone marrow-derived mesenchymal stem cells can regenerate a critical-sized segmental bone defect. Tissue Eng 3:173–185
34. Neuhuber B, Gallo G, Howard L, Kostura L, Mackay A, Fischer I (2004) Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. J Neurosci Res 77:192–204
35. Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI et al (1994) Mesenchymal cell based repair of large, full thickness defects of articular cartilage. J Bone Joint Surg Am 76:579–592
36. Grande DA, Southerland SS, Manji R, Pate DW, Schwartz RE, Lucas PA (1995) Repair of articular defects using mesenchymal stem cells. Tissue Eng 1:345–353
37. Young RG, Butler DL, Weber W, Caplan AI, Gordon SL, Fink DJ (1998) Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J Orthop Res 16:406–413
38. Kadiyala S, Young RG, Thiede MA, Bruder SP (1997) Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro. Cell Transplant 6:125–134
39. Kraitchman DL, Tatsumi M, Gilson WD, Ishimori T, Kedziorek D, Walczak P et al (2005) Dynamic imaging of allogeneic mesenchymal stem cells trafficking to myocardial infarction. Circulation 112:1451–1461
40. Murphy JM, Fink DJ, Hunziker EB, Barry FP (2003) Stem cell therapy in a caprine model of osteoarthritis. Arthritis Rheum 48:3464–3474
41. Shake JG, Gruber PJ, Baumgartner WA, Senechal G, Meyers J, Redmond JM, Pittenger MF, Martin BJ (2002) Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. Ann Thorac Surg 73:1919–1925
42. Kraitchman DL, Heldman AW, Atalar E, Amado LC, Martin BJ, Pittenger MF, Hare JM, Bulte JW (2003) In vivo magnetic resonance imaging of mesenchymal stem cells in myocardial infarction. Circulation 107:2290–2293
43. Amado LC, Saliaris AP, Schuleri KH, St John M, Xie JS, Cattaneo S et al (2005) Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc Natl Acad Sci USA 102:11474–11479
44. Freyman T, Polin G, Osman H, Crary J, Lu M, Cheng L et al (2006) A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction. Eur Heart J 27:1114–1122
45. Schuleri KH, Amado LC, Boyle AJ, Centola M, Saliaris AP, Gutman MR et al (2008) Early improvement in cardiac tissue perfusion due to mesenchymal stem cells. Am J Physiol Heart Circ Physiol 294:H2002–H2011
46. Quevedo HC, Hatzistergos KE, Oskouei BN, Feigenbaum GS, Rodriguez JE, Valdes D et al (2009) Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. Proc Natl Acad Sci USA 106:14022–14027
47. Mahmud N, Pang W, Cobbs C, Alur P, Borneman J, Dodds R et al (2004) Studies of the route of administration and role of conditioning with radiation on unrelated allogeneic mismatched mesenchymal stem cell engraftment in a nonhuman primate model. Exp Hematol 32:494–501
48. Chapel A, Bertho JM, Bensidhoum M, Fouillard L, Young RG, Frick J et al (2003) Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome. J Gene Med 5:1028–1038

49. Francois S, Bensidhoum M, Mouiseddine M, Mazurier C, Allenet B, Semont A et al (2006) Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promotes their widespread engraftment to multiple organs: a study of their quantitative distribution after irradiation damage. Stem Cells 24:1020–1029
50. Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R (2003) Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood 101:2999–3001
51. Bartholomew A, Patil S, Mackay A, Nelson M, Buyaner D, Hardy W et al (2001) Baboon mesenchymal stem cells can be genetically modified to secrete human erythropoietin in vivo. Hum Gene Ther 12:1527–1541
52. Saito T, Dennis JE, Lennon DP, Young RG, Caplan AI (1995) Myogenic expression of mesenchymal stem cells within myotubes of mdx mice in vitro and in vivo. Tissue Eng 1:327–343
53. Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD et al (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 USA 92:4857–4861
54. Dennis JE, Merriam A, Awadalla A, Yoo JU, Johnstone B, Caplan AI (1999) A quadripotent mesenchymal progenitor cell isolated from the marrow of an adult mouse. J Bone Miner Res 14:700–709
55. Short B, Brouard N, Driessen R, Simmons PJ (2001) Prospective isolation of stromal progenitor cells from mouse BM. Cytotherapy 3:407–408
56. Phinney DG, Kopen G, Isaacson RL, Prockop D (1999) Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth and differentiation. J Cell Biochem 72:570–585
57. Carvalho PP, Wu X, Yu G, Dias IR, Gomes ME, Reis RL et al (2011) The effect of storage time on adipose-derived stem cell recovery from human lipoaspirates. Cells Tissues Organs 194(6):494–500
58. Halvorsen YC, Wilkison WO, Gimble JM (2000) Adipose-derived stromal cells–their utility and potential in bone formation. Int J Obes Relat Metab Disord 24(Suppl 4):S41–S44
58. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ et al (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228
60. Fukuchi Y, Nakajima H, Sugiyama D, Hirose I, Kitamura T, Tsuji K (2004) Human placenta-derived cells have mesenchymal stem/progenitor cell potential. Stem Cells 22:649–658
61. In't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH et al (2004) Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells 22:1338–1345
62. Romanov YA, Svintsitskaya VA, Smirnov VN (2003) Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. Stem Cells 21:105–110
63. Campagnoli C, Roberts IA, Kumar S, Choolani M, Bennett PR, Letsky E et al (2001) Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 98:2396–2402
64. Panepucci RA, Siufi JL, Silva WA, Proto-Siquiera R, Neder L, Orellana M et al (2004) Comparison of gene expression of umbilical cord vein and bone marrow-derived mesenchymal stem cells. Stem Cells 22:1263–1278
65. Kern S, Eichler H, Stoeve J, Klüter H, Bieback K (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 24:1294–1301
66. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA 97:13625–13630
67. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG et al (2003) SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci USA 100:5807–5812
68. Pittenger MP, Mbalaviele G, Black M, Mosca JD, Marshak DR (2001) Mesenchymal stem cells. In: Koller MR, Palsson BO, Masters JRW (eds) Human cell culture, vol 5, Primary mesenchymal cells. Kluwer Academic Publishers, Dordrecht

69. Covas DT, Panepucci RA, Fontes AM, Silva WA Jr, Orellana MD, Freitas MC et al (2008) Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. Exp Hematol 36:642–654
70. Jones E, English A, Churchman SM, Kouroupis D, Boxall SA, Kinsey S et al (2010) Large-scale extraction and characterization of CD271+ multipotential stromal cells from trabecular bone in health and osteoarthritis: implications for bone regeneration strategies based on uncultured or minimally cultured multipotential stromal cells. Arthritis Rheum 62:1944–1954
71. Quesenberry PJ, Colvin G, Dooner G, Dooner M, Aliotta JM, Johnson K (2007) The stem cell continuum: cell cycle, injury, and phenotype lability. Ann N Y Acad Sci 1106:20–29
72. Huang S (2010) Cell lineage determination in state space: a systems view brings flexibility to dogmatic canonical rules. PLoS Biol 8:e1000380
73. Wang J, Xu L, Wang E, Huang S (2010) The potential landscape of genetic circuits imposes the arrow of time in stem cell differentiation. Biophys J 7(99):29–39
74. Huang S (2009) Reprogramming cell fates: reconciling rarity with robustness. Bioessays 31:546–560
75. Trimain N, Korkko J, Ibberson D, Kopan GC, DiGirolamo C, Phiney DG. MicroSAGE analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple cell lineages. Stems Cells. 2001;19(5):408–18