2

Mesenchymal Stem Cells for Tissue Engineering

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1. BACKGROUND AND LOGIC

1.1. Introduction

Tissue engineering, a multidiscipline approach to reconstructing biological tissues, is generally considered to include three main components: cells that fabricate the lost or damaged tissue, materials intended to serve as delivery vehicles and scaffolds for the cells, and cytokines and other bioactive factors consistent with appropriate cell proliferation and differentiation [Ringe et al., 2002]. Although differentiated cells have been used successfully in both experimental and clinical protocols [Bell et al., 1983; Aigner et al., 1998; Brittberg et al., 2001], numerous advantages attend the use of autologous stem cells as the source of donor cells in tissue engineering: These include low donor site morbidity, diminished or absent immune response, high proliferative potential, and relative ease of access to the cell repository [Bruder et al., 1994; Ringe et al., 2002]. Thus, in the past decade, stem cells, generally defined as cells that have the capacity both to self-renew and to generate differentiated progeny [Morrison et al., 1997; Jackson et al., 2002], have generated substantial interest and have been the object of intensive research efforts.

A wide array of studies supports the contention that various tissues may be repositories of mesenchymal stem or progenitor cells. These tissues include bone marrow [Caplan, 1991; Haynesworth et al., 1992a,b; Prockop, 1997; Bianco et al., 1999], periosteum [Nakahara et al., 1991], adipose tissue [Halvorsen et al., 2000; Erickson et al., 2002; Safford et al., 2002], dermis [Toma et al., 2001], muscle [Lee et al., 2000; Jankowski et al., 2002], and vasculature (pericytes) [Brighton et al., 1992].

1.2. History

The notion that stem cells are present in bone marrow was suggested by Friedenstein [1976], based on characterization of clonal populations of marrow stromal cells isolated from mouse, guinea pig, and rabbit bone marrow and on implantation of the cells in diffusion chambers in syngeneic host animals [Friedenstein et al., 1970; Friedenstein, 1976; See Phinney, 2002 for review]. Owen [1985, 1988], and Owen and Friedenstein [1988] proposed a model of differentiation for progenitor cells in the stromal system analogous to that of the hematopoietic system. According to this hypothesis, marrow stromal stem cells give rise to committed

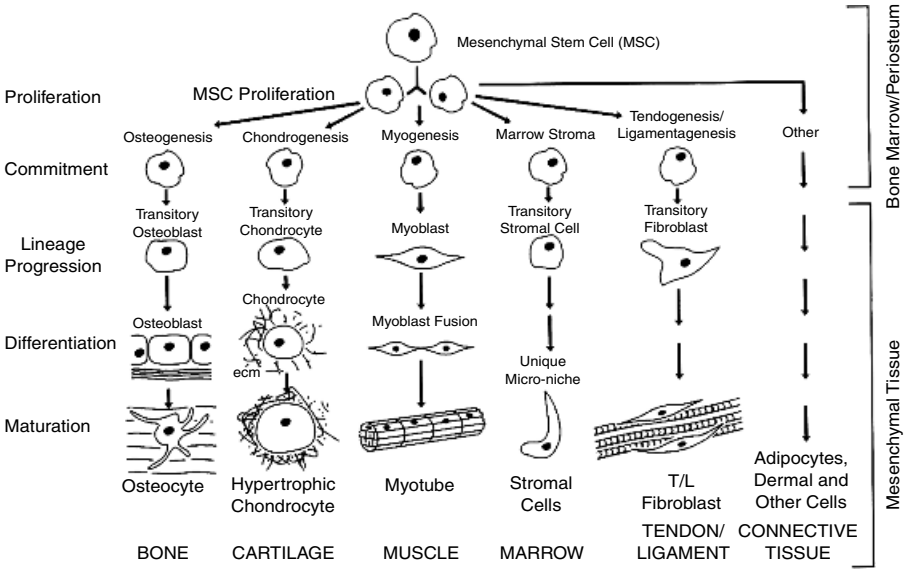

Figure 2.1. The mesengenic process. A schematic representation of some of the differentiation capabilities of mesenchymal stem cells and a simplified illustration of the cellular transitions from stem cell to well-differentiated cells. Reprinted from Clinics in Plastic Surgery, volume 21, number 3, Arnold I. Caplan, "The Mesengenic Process", pp. 429–435, copyright 1994, with permission from Elsevier.

progenitors of fibroblastic, reticular, adipogenic, osteogenic, and possibly other cells. Our vision of the lineage pathways available to mesenchymal stem cells was originally depicted in the mesengenic process diagram seen in Fig. 2.1. In this scheme, the pathways are arranged so that the best-understood are on the left, and the least established are on the right [Caplan, 1994].

Our laboratory's interest in marrow-derived mesenchymal stem cells (MSCs) can be viewed as a logical consequence of our earlier in vitro studies involving mesenchymal cells of the embryonic chick limb bud system [Caplan, 1977, 1984]. Although there are obvious differences between the two systems, there are also certain parallels between the limb bud and marrow stromal systems. Most importantly, both bone and cartilage development in the embryonic limb bud and bone turnover and repair in the adult limb result from the division of small numbers of progenitor cells (MSCs) and the ultimate commitment and differentiation of the progeny of these cells [Caplan, 1991; Bruder et al., 1994]. Thus Ohgushi et al. [1989, 1990] demonstrated that intact or disaggregated rat marrow forms bone and cartilage when loaded into porous ceramic (hydroxyapatite-tricalcium phosphate) cubes and implanted into subcutaneous sites in syngeneic hosts. Goshima et al. [1991a] achieved similar results when they loaded the same type of ceramics with cultured rat marrow cells, referred to as mesenchymal stem cells.

An important development occurred when Haynesworth et al. [1992b] demonstrated that cultured human marrow MSCs also generate bone, but rarely cartilage, when introduced into the same assay system. Interestingly, companion human cells loaded into diffusion chambers did not form bone; accordingly, we consider the

ceramic cube assay to be the standard for testing MSCs. In these experiments, Haynesworth and co-workers (1992b) used marrow cells isolated by two different techniques. In the first, cancellous bone marrow from femoral heads was mechanically disrupted and a single-cell suspension was isolated and seeded into tissue culture dishes in serum-containing medium. In the second method, bone marrow aspirated from the iliac crest was rinsed, resuspended in serum-containing medium, loaded onto a Percoll density gradient, and centrifuged. In preliminary experiments, it was determined that the majority of the adherent cells were restricted to the top 25% of the gradient. Cells recovered from this fraction were rinsed in serum-containing medium, counted, and seeded into tissue culture dishes. Ultimately, the second method was found to be superior to the isolation of cells by direct plating and has been used extensively by our laboratory and many others; this method is described in detail in Protocol 2.3.

Although the isolated cell fraction is seeded at a rather high density (1×10^7 nucleated cells per 10-cm dish), the total cell inoculum includes, along with a small fraction of erythrocytes, many nucleated cells, probably from the hematopoietic lineage, that are not capable of attaching to the culture substrate. The unattached cells are eventually removed during the course of routine changes of medium, and a smaller subset of cells becomes anchored to the substrate. These primarily fibroblast-like cells begin to proliferate and form colonies, which can first be seen around days 4–6 of culture. The ability of these cells to attach to the culture substrate represents the first and most important step in the selection of MSCs from the total nucleated cell population. Because of variability among individual marrow donors, the number of colonies per dish may vary; in our experience a typical 10-cm dish may contain 100 to 200 colonies. Assuming, as suggested by Friedenstein [Friedenstein et al., 1970; Friedenstein, 1976], that a colony of these cells arises from a single attached cell, we would conclude that these cells are rare indeed (1–2 per 10^5 nucleated cells from the marrow of young donors).

1.3. Assays for Phenotypic Potencies

Having isolated cells from an aspirate of human bone marrow, how does an investigator verify the "stem" nature of the cells? That is to ask, what assays and cell markers are relevant to identifying mesenchymal stem cells? As indicated above, we believe that the in vivo ceramic cube assay, described in Section 6, is the standard for identifying MSCs. Human MSC-ceramic composites implanted subcutaneously in immunocompromised host animals almost always produce bone, whereas bone and cartilage are never present when dermal fibroblasts are used in the same manner [Lennon et al., 2000]. Moreover, empty cubes used as a negative control do not contain bone in the central portion of the cubes, although long-term implants rarely include small portions of bone in the periphery of the cube. It should be noted that marrow-derived MSCs from different species produce different proportions of bone and cartilage. Cartilage, almost never seen in human MSC-ceramic composites, is frequently observed with rat MSCs, but much less so than

bone [Dennis and Caplan, 1993; Lennon et al., 2001]. On the other hand, cartilage is more commonly observed than bone for rabbit MSCs.

The in vivo ceramic cube assay system is complemented by the use of a number of in vitro assays, each of which is specific for different differentiated cells. Elevated alkaline phosphatase activity, although not unique to osteogenic cells, is a useful early marker for osteogenic differentiation. Accordingly, biochemical and cytochemical assays for alkaline phosphatase are used early in the culture of MSCs in osteoinductive conditions. On the other hand, calcium biochemistry and von Kossa staining to detect mineralization are used later in the culture period. These assays and the conditions that promote osteogenic differentiation are described in Section 7.

Aggregate or pellet cultures can be established in a defined medium to promote chondrogenic differentiation of MSCs [Johnstone et al., 1998]. Such cultures will not be covered in this section as they are treated thoroughly in Chapter 4 of this book.

Differentiation of MSCs to adipocytes can be induced through the use of a unique medium in cultures seeded at a slightly higher density than that used for osteogenic differentiation. Flow cytometric analysis after Nile Red staining is used to assess expression of the adipocytic phenotype after induction. This methodology is described in Section 8.

The ability of MSCs to differentiate along these various phenotypic lines is strongly suggestive of their stem cell nature. MSCs, although sometimes referred to with different terminology and derived from a number of species, have also been shown to differentiate into other mesenchymal lineages, including skeletal muscle [Wakitani et al., 1995], cardiac muscle [Tomita et al., 1999], and hematopoietic supportive tissue [Koç et al., 2000], in addition to nonmesenchymal tissues including neurons [Woodbury et al., 2000; Black and Woodbury, 2001] and retinal cells [Tomita et al., 2002]. Importantly, differentiation of clonal populations of human [Pittenger et al., 1999] and murine [Dennis et al., 1999] MSCs along multiple lineages has been demonstrated.

Although no cell surface marker unique to MSCs has been identified to date, an extensive expression profile of cytokines and their receptors and adhesion and extracellular matrix molecules shared by these cells has been described [Mosca et al., 1997; Majumdar et al., 1998; Minguell et al., 2001; Shur et al., 2002]. Moreover, a number of monoclonal antibodies reactive with MSCs have been developed; antibodies SH-2, SH-3, and SH-4 are reactive with MSCs, but not with hematopoietic or differentiated bone cells [Haynesworth et al., 1992a].

1.4. Key Technical Details

Selection of the proper lot of fetal bovine serum (FBS) for use in culturing MSCs is perhaps the most important parameter in MSC technology [Lennon et al., 1996] and is described briefly in Section 9. It has been our experience that it is not good practice to purchase serum "off the shelf" (that is, without testing). We have also found that a batch of serum optimal for MSCs from one species will

probably not support proliferation of MSCs from another. Although the selection of the proper batch of FBS is extremely important, all of the technical details involved in the isolation, expansion, and analysis of MSCs are also important. We believe that these details influence the outcome of our experimentation. With this in mind, we have organized the remainder of this chapter to provide all of these technical details.

2. PREPARATION OF MEDIA AND REAGENTS

2.1. Density Gradients

Protocol 2.1. Preparation of Percoll Density Gradient

Reagents and Materials

Sterile

- ❑ Percoll
- ❑ Sodium chloride, 1.5 M
- ❑ Tyrode's or Hanks' balanced salt solution (TBSS or HBSS)
- ❑ Polycarbonate high-speed centrifuge tubes, 50 ml

Nonsterile

- ❑ High-speed centrifuge (20,000 *g*)
- ❑ Fixed-angle rotor for 50-ml tubes

Protocol

(a) Combine the following sterile solutions (or any convenient multiples thereof) in a sterile container:
   i) 22.05 ml Percoll
   ii) 2.45 ml 1.5 M sodium chloride
   iii) 10.5 ml TBSS or HBSS

(b) Mix the solutions thoroughly, and then add 35 ml per sterile 50-ml polycarbonate centrifuge tube. Place caps on tubes.

(c) Centrifuge at 20,000 *g* for 15 min at room temperature in an appropriate centrifuge (preferably in a fixed-angle rotor).

(d) Remove tubes and store at 4 °C until they are needed (See Protocol 2.3).

2.2. Solutions for In Vitro Osteogenic Induction

2.2.1. Dexamethasone (dex)

1. Prepare a stock solution of 1×10^{-3} M dex in 100% ethanol (3.92 mg dexamethasone in 10 ml 100% ethanol).
2. Prepare a solution of 1×10^{-5} M dex by making a 1:100 dilution of the 10^{-3} M dex in serum-free medium (the medium appropriate for the cells being cultured).
3. Filter the solution through a 0.22-μm filter and store at −20 °C

2.2.2. β-Glycerophosphate (BGP)

(i) Prepare a 200 mM solution by dissolving 0.216 *g* BGP in 5 ml TBSS or other balanced salt solution (BSS).

(ii) Sterilize by passing solution through a 0.22-μm filter and store at 4 °C.

2.2.3. Ascorbic Acid 2-Phosphate

(i) Prepare a 5 mM solution of ascorbic acid 2-phosphate by dissolving 0.0347 g ascorbic acid 2-phosphate in 10 ml TBSS or other BSS.

(ii) Sterilize with a 0.22-μm filter and store at 4 °C.

2.3. Solutions for Quantitative Biochemical Alkaline Phosphatase Assay

2.3.1. Substrate Buffer

Glycine, 50 mM, $MgCl_2$, 1 mM, pH 10.5.: Dissolve 1.88 g of glycine and 0.1017 g of $MgCl_2 \cdot 6H_2O$ in 500 ml of water. Adjust to pH 10.5 with 1 N NaOH.

2.3.2. Substrate

Dissolve 1 tablet (5 mg) of phosphatase substrate (*p*-nitrophenyl phosphate) per 5 ml of substrate buffer.

2.3.3. *p*-Nitrophenol for Standard Curve

Prepare a 50 nmol/ml solution of *p*-nitrophenol by combining 50 μl of 10 μmol/ml *p*-nitrophenol standard solution with 9.95 ml of 0.02N NaOH. Prepare further dilutions as illustrated in the following table:

p-Nitrophenol Concentration (nmol/ml)	Volume (ml) of Diluted *p*-Nitrophenol Solution (50 nmol/ml)	Volume (ml) of 0.02 M NaOH
4.5	0.1	1.0
9.0	0.2	0.9
18	0.4	0.7
27	0.6	0.5
36	0.8	0.3
45	1.0	0.1

2.4. Solutions for Qualitative Cytochemical Alkaline Phosphatase Assay

2.4.1. Fast Violet Stain

Dissolve 1 fast violet capsule in 48 ml of water. This solution can be stored as 12-ml aliquots at 4 °C.

2.4.2. Citrate Working Solution

Add 2 ml of citrate concentrated solution to 98 ml of water.

2.4.3. Citrate Buffered Acetone

Combine 60% citrate working solution and 40% acetone.

2.5. Solutions for Adipogenic Induction and Flow Cytometry

2.5.1. Adipogenic Induction Medium

3-Isobutyl-1-methylxanthine (IBMX) and indomethacin:

(i) Combine 0.1789 g indomethacin and 0.555 g IBMX in a 15- or 50-ml centrifuge tube.

(ii) Add dimethyl sulfoxide (DMSO) to a volume of 5 ml. This gives a concentration of 0.1 M indomethacin and 0.5 M IBMX.

(iii) Add 0.5 ml to a 500-ml bottle of serum-supplemented DMEM-HG to give final concentrations of 100 μM indomethacin and 500 μM IBMX.

(iv) Store unused stock solution at −20 °C.

2.5.2. Insulin

(i) Prepare a 10 mg/ml solution of bovine insulin in 0.01 N HCl.

(ii) Add 0.6 ml to 11.4 ml DMEM-HG (0.5 mg/ml).

(iii) Add 10 ml per 500 ml DMEM-HG for a final concentration of 10 μg/ml.

(iv) Sterilize by passing solution through a 0.22-μm filter and store at 4 °C.

2.5.3. Dexamethasone

(i) Prepare a stock solution of 10^{-3} M dex in 100% ethanol (0.00392 g dexamethasone in 10 ml 100% ethanol).

(ii) Add 0.5 ml per 500 ml of Dulbecco's modification of Eagle's medium with high glucose, 4.5.g/l (DMEM) to give a final concentration of 10^{-6} M dex.

(iii) Sterilize by passing solution through a 0.22-μm filter and store at 4 °C.

2.5.4. Nile Red Working Solution

(i) Prepare a 1 mg/ml solution of Nile Red in DMSO.

(ii) Add 250 μl of this solution per final volume of 10 ml in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBSA)

3. ISOLATION OF HUMAN MARROW-DERIVED MESENCHYMAL STEM CELLS (HMSCS)

3.1. Aspiration of Human Bone Marrow

Aspiration of bone marrow from the posterior superior iliac crest is carried out by physicians of the Department of Hematology-Oncology at University Hospitals of

Cleveland, which is affiliated with Case Western Reserve University. Normally, we receive the bone marrow sample in a 20-ml syringe and proceed to the steps listed in Protocol 2.3. The details of Protocol 2.2 were provided by Dr. Omer Koç of the Department of Hematology-Oncology; although this protocol is outside the scope of cell culture, it is provided here for the reader's information. The Institutional Review Board of the hospital must approve research protocols involving human marrow, and donors must give informed consent.

Protocol 2.2. Collection of Human Bone Marrow

Reagents and Materials

Sterile

- ❑ Betadine
- ❑ Lidocaine (1%)
- ❑ Scalpel with # 15 blade
- ❑ Jamshidi needle, 11-gauge
- ❑ Syringe, 20 ml, containing 2 ml heparin (preservative-free, 400 units per ml)

Protocol

(a) Marrow donors who have given informed consent lie in a lateral decubitus position.
(b) The donor's skin is wiped with Betadine.
(c) The posterior superior iliac crest is located.
(d) Lidocaine (1%) is used to anesthetize the skin and subcutaneous tissue superficial to the iliac crest.
(e) A small cut (the width of a scalpel blade) is made though the skin and subcutaneous tissue with a scalpel blade.
(f) An 11-gauge Jamshidi needle is inserted though the cut and is anchored into the posterior superior iliac crest.
(g) Once the needle is anchored, the hub is removed.
(h) A 20-ml syringe containing 2 ml heparin (preservative-free, 400 units per ml) is attached to the needle.
(i) The marrow is aspirated by pulling the syringe plunger back briskly.
(j) The needle is rotated 90 degrees clockwise several times, and marrow is aspirated at each new position.
(k) After the needle is removed, pressure is applied to the skin until bleeding stops.

3.2. Enrichment of Mesenchymal Stem Cells from Human Marrow

A cell fraction enriched for mesenchymal stem cells is isolated by density gradient centrifugation.

Protocol 2.3. Isolation and Seeding of Human Mesenchymal Stem Cells (hMSCs)

Reagents and Materials

Sterile

- ❑ DMEM-LG-10FB: Dulbecco's modified Eagle's medium with 1 g/l glucose (DMEM-LG) supplemented with 10% FBS. The FBS is preelected [Lennon et al., 1996], as described in Section 9, to support proliferation and differentiation (given the appropriate conditions) of hMSCs.
- ❑ Polypropylene centrifuge tubes, 50 ml
- ❑ Centrifuge tube, 15 ml
- ❑ Tissue culture flasks, 75 cm^2, or Petri dishes, 10 cm

△ *Safety note.* Personnel wearing the proper personal protective equipment, including a lab coat, goggles or a face shield, gloves, and a surgical mask, process the marrow sample in a Class II biological safety cabinet.

The marrow sample and all cells derived from it are treated with standard biohazard precautions. That is, it is assumed that the sample is contaminated with hepatitis B or HIV. Use appropriate waste containers for all sharp and nonsharp disposable supplies that come into contact with human tissue or cells, or with medium that has contacted these cells. All liquid waste generated must be treated with bleach before it is disposed of in a sink; bleach is added to produce a 20% concentration, and the solution may be disposed of after 30 min. These safety precautions apply to all procedures involving unfixed human cells described in this chapter.

Protocol

(a) The marrow sample is usually delivered in a 20-ml syringe. Eject the contents of the syringe into a 50-ml polypropylene centrifuge tube.

(b) Add 20 to 30 ml DMEM-LG-10FB to the tube.

(c) Pipette up and down thoroughly to mix the medium and the marrow sample, and then transfer a small aliquot (about 200 μl) of the suspension to a 15-ml centrifuge tube.

(d) Centrifuge the suspension in the 50-ml tube at 450 *g* for 5 min in a bench-top centrifuge.

(e) While the sample is being centrifuged, conduct a preliminary count of the cells with the suspension in the small tube.
   i) Transfer 50 μl of this suspension to another suitable small tube.
   ii) Add 50 μl DMEM-LG-10FB.
   iii) Add 100 μl 4% acetic acid (to lyse the red blood cells).
   iv) Count the nucleated cells with a hemocytometer, and determine the total number of such cells in the 50-ml tube.

(f) After the sample has been centrifuged, remove the supernate.
(g) Determine the number of tubes of preformed Percoll (density 1.03–1.12 g/ml) (See Protocol 2.1) that will be required to fractionate the nucleated cells. The number of tubes of Percoll to be used depends on the number of nucleated cells determined in step (e) and on the volume of the cell pellet. The maximum number of nucleated cells per tube of Percoll is 2×10^8. However, if the cell number is lower than this figure, but the pellet volume is greater than 5 ml, more than one tube of Percoll will also be required.
(h) Adjust the volume of the pellet with DMEM-LG-10FB to allow 5 ml cell suspension per tube of Percoll.
(i) Carefully load 5 ml of the cell suspension per tube of Percoll with a pipette. Transfer the suspension slowly, so that it remains at the top of the gradient.
(j) Carefully transfer the tubes to a centrifuge, and spin at 480 *g* for 15 min with the brake off, preferably in a fixed-angle rotor.
(k) Return the sample to the biological safety cabinet and transfer the top 10 to 14 ml of each Percoll tube to a sterile 50-ml polypropylene centrifuge tube.
(l) Increase the volume in the tube to 50 ml with DMEM-LG-10FB and mix completely by pipetting up and down.
(m) Centrifuge the tube at 450 *g* in a bench-top centrifuge.
(n) Remove the supernatant and resuspend the cell pellet in 10 ml DMEM-LG-10FB.
(o) Determine the final number of nucleated cells in the same manner as indicated in step (e)
(p) Adjust the volume as necessary with DMEM-LG-10FB and seed the cells at a density of 1.8×10^5 per cm^2 in tissue culture dishes or flasks.
(q) Place dishes in a humidified tissue culture incubator at 37 °C/5% CO_2.
(r) Change the medium after 3 days, and every 3 to 4 days thereafter.

3.3. Primary Culture

Primary cultures of MSCs are seeded at 1×10^7 cells per 10-cm culture dish. This cell inoculum contains a mixture of cells, including red blood cells, unidentified nucleated cells of the hematopoietic lineage, monocytes, macrophages, and fibroblast-like cells. Erythrocytes and leukocytes are not capable of attaching to the culture substrate and are eventually removed during the course of routine changes of culture medium. Nonadherent cells are not rigorously removed by rinsing. Instead, medium is simply pipetted or aspirated from the dish without vigorous swirling or rinsing, as it is assumed that the nonadherent cells provide paracrine factors needed for the optimal growth of the attached cells.

Attachment of cells to the negatively charged culture dish is, in fact, considered a method of selection for fibroblastoid cells [Phinney, 2002]. Only a relatively small number of cells attach to the dish. The fibroblast-like cells begin to proliferate and form loose colonies of spindle-shaped cells that can usually be identified between days 4 and 6 of culture (Fig. 2.2). The colonies greatly increase in size

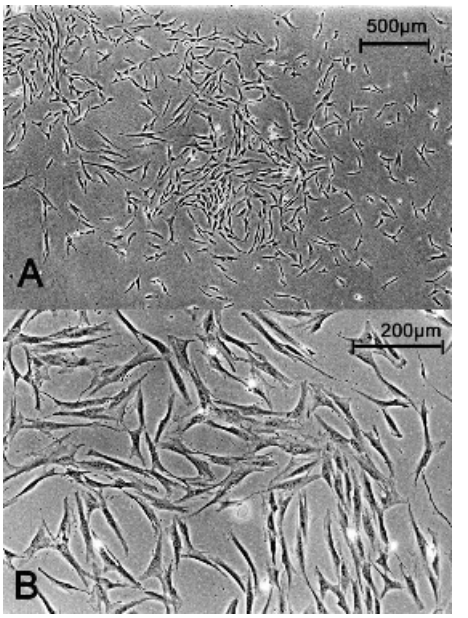

Figure 2.2. Phase-contrast photomicrographs of human mesenchymal stem cells (MSCs) on day 6 of primary culture. A) A low-magnification view of two adjacent colonies of MSCs. B) MSCs from another colony of the same preparation of cells at higher magnification.

over the next 7 days and should be subcultured before the cells become dense and multilayered (proliferation of the cells is not contact inhibited).

4. PROPAGATION OF MESENCHYMAL STEM CELLS

The density of the colonies is the primary consideration in determining when the cells should be passaged. Thus, if the cells in the colonies are becoming densely associated, the cultures should be passaged, even though the cells throughout the dish may not be confluent.

Protocol 2.4. Subculture of Human Mesenchymal Stem Cells (hMSCs)

Reagents and Materials

Sterile

- ❑ BSS: balanced salt solution, e.g., Tyrode's or Hanks' BSS
- ❑ Trypsin-EDTA: 0.25% trypsin, 1 mM EDTA in Hanks' BSS lacking Ca^{2+} and Mg^{2+}
- ❑ BCS: bovine calf serum
- ❑ DMEM-LG-10FB

Protocol

(a) Primary isolates of hMSCs should be subcultured before individual cell colonies become overly dense. The cultures are usually trypsinized around day 14 of culture ($\pm$3 days).

(b) Remove the culture medium.
(c) Rinse the cell layer with 5 ml BSS (for 10-cm tissue culture dishes).
(d) Add 4 ml trypsin-EDTA and return the vessel to the incubator for 5–10 min. Keep the time of exposure as brief as possible. (See (e) and (f) below.)
(e) When the majority of cells have become well rounded or have detached from the culture dish, stop the reaction by adding a volume of BCS equal to 1/2 the volume of the trypsin.
(f) Draw up the cell suspension with a pipette and, with the same pipette, use the suspension to gently wash the remaining cells from the dish. It is not necessary to remove all of the cells from the dish, as most of the nonfibroblastoid cells (which are not likely to be MSCs) in these cultures are more trypsin resistant than the spindle-shaped cells. Thus trypsinization represents, along with separation of nucleated cells by density centrifugation and attachment of fibroblastic cells to plastic, an important component of the process of the selection of MSCs from the total marrow cell population.
(g) Transfer the cell suspension from all of the cultures to an appropriate-size centrifuge tube or tubes. In our laboratory, we prefer to keep groups of culture dishes from a single preparation segregated from one another throughout the subculture process as a form of insurance against contamination of the entire group, because we have found that low levels of contamination are not always easily detectable. For example, a preparation consisting of 12 culture dishes might be divided into 4 groups of 3 dishes; we would attempt to ensure that there was no cross-contamination among the groups by using separate pipettes and tubes for each group.
(h) Centrifuge the tubes at 400 *g*.
(i) Remove the supernatant with a pipette or other suitable device.
(j) Resuspend the cell pellet in a suitable volume of DMEM-LG-10FB (usually 5 or 10 ml).
(k) Remove a sample of the suspension with a Pasteur pipette or micropipettor and count the cells with a hemocytometer.
(l) Adjust the volume of medium as necessary, and seed the cells at 3500 to 4000 cells per cm^2.
(m) Change the culture medium every 3 to 4 days.
(n) Further subculture of hMSCs is conducted in essentially the same manner except for the following considerations:
    i) Subcultured hMSCs are evenly distributed on the tissue culture vessel surface and are not in colonies as for primary cultures. Therefore, the key criterion for determining when the cells should be trypsinized is the degree of confluence; basically, hMSCs should be trypsinized before they become confluent.
    ii) Passaged hMSCs are more easily trypsinized than primary cultures, so exposure of these cells to trypsin is usually limited to 5 min.

Mesenchymal stem cells have a high proliferative capacity and may be subcultured repeatedly. During the process of cell expansion, MSCs maintained in DMEM-LG supplemented only with FBS remain in an undifferentiated state, as indicated by negative results in in vitro assays for osteogenesis (See Section 7). Bruder et al. [1997] maintained hMSCs through as many as 15 passages. At each passage some cells were continued in serum-supplemented medium, while others were placed into an osteoinductive medium also containing dexamethasone (See Section 7). The conservation of osteogenic potential at each passage was demonstrated by increased alkaline phosphatase activity and the formation of mineralized matrix in cells of the latter group. Careful documentation of cell numbers at each passage revealed that hMSCs could undergo an average of 38 population doublings before reaching senescence (as indicated by arrested cell growth and a broad, flattened cell morphology).

Subcultured MSCs are similar to primary isolates of the cells in that they remain spindle-shaped fibroblastoid cells. As indicated above, however, passaged MSCs are distributed evenly over the culture dish rather than being organized into colonies. They are also slightly wider than for primary cultures, a feature that becomes more pronounced with additional subcultivation.

5. CRYOPRESERVATION AND THAWING OF HMSCS

Another interesting aspect of the report by Bruder et al. [1997] is that a portion of the cells trypsinized at the end of primary culture was cryopreserved in liquid nitrogen and then thawed. These cells were then taken through the same process of extensive subcultivation and exposure to osteoinductive treatment as for the unfrozen cells. Cryopreservation was not found to have an adverse effect on further cell expansion or on osteogenic differentiation at each passage. The procedures involved in freezing and thawing hMSCs are described in Protocol 2.5 and Protocol 2.6, respectively.

Protocol 2.5. Cryopreservation of Human Mesenchymal Stem Cells (hMSCs)

Reagents and Materials

Sterile

- ❑ Trypsin-EDTA: 0.25% trypsin, 1 mM EDTA in Hanks' BSS lacking Ca^{2+} and Mg^{2+}
- ❑ DMSO
- ❑ FBS (from the same lot of serum selected for growth of hMSCs)

Nonsterile

- ❑ Nalgene freezing container, with a 1 °C/min cooling rate when filled to the appropriate level with isopropanol

Protocol

(a) Prepare freezing medium consisting of 10% DMSO and 90% FBS. Use only freshly prepared freezing medium.
(b) The cells must be in log phase (rapidly dividing) for the cryopreservation to be successful.
(c) Trypsinize the cells in the manner described in Protocol 2.4.
(d) Count the resuspended cells with a hemocytometer or electronic cell counter to determine the total cell yield.
(e) Centrifuge the cell suspension at 400 *g* for 5 min at room temperature.
(f) Gently resuspend the cells in freezing medium at a concentration of 1×10^6 cells per ml.
(g) Label cryovials with the appropriate information, including complete cell identification (cell type, donor number, passage number, and cell density), date, and initials of the operator.
(h) Transfer 1 ml of the cell suspension to each labeled cryovial, and then seal the vial with the cap.
(i) Place the cryovial(s) into the Nalgene freezing container.
(j) Place the freezing container in a $-70\,^{\circ}C$ freezer. The Nalgene containers are designed to reduce the temperature of the cell suspension by $1\,^{\circ}C$/min under these conditions.
(k) After 24 h transfer the vials to a liquid nitrogen freezer. (See Safety note.)

△ ***Safety note.*** Wear safety goggles or, preferably, a mask, in addition to gloves and protective clothing when handling liquid nitrogen, and ensure the room is properly ventilated.

Protocol 2.6. Thawing Cryopreserved Human Mesenchymal Stem Cells (hMSCs)

Reagents and Materials

Sterile

- ❑ DMEM-LG-10FB
- ❑ Plastic tissue culture dishes, 10 cm

Nonsterile

- ❑ Trypan Blue viability stain
- ❑ Water bath at $37\,^{\circ}C$

Protocol

(a) Warm DMEM-LG-10FB to room temperature.
(b) Remove cryovials to be thawed from liquid nitrogen (See Safety note above).
(c) Partially thaw in a $37\,^{\circ}C$ water bath (i.e., some ice should still be present).

△ *Safety note.* Wear safety goggles and place a lid on the water bath while thawing the cells. If liquid nitrogen has entered the vial, it may explode on thawing. Ideally, store vials in the vapor phase of liquid nitrogen.

(d) Transfer gently into a tube containing 35 ml DMEM-LG-10FB (the sample will have completed thawing by this time).
(e) Centrifuge at 250 *g* for 5 min.
(f) Remove supernate from cells.
(g) Add 2 to 5 ml DMEM-LG-10FB and resuspend cells gently.
(h) Combine 100 μl of the cell suspension with an equal volume of Trypan Blue and count the unstained cells with a hemocytometer.
(i) Seed cells into a 10-cm tissue culture dish or dishes in 7 ml medium per dish at a density of 2.5×10^5.
(j) Change medium every 3–4 days. Plates should be confluent in 7–10 days.
(k) Cells may be subcultured as described in Protocol 2.4.

Thawed hMSCs are similar in morphology to those that have never been frozen and then thawed. They may have elongated, slender processes or may be somewhat more compact 1 day after being seeded but, after readapting to culture, become identical to unfrozen cells. A variable number of cells fail to attach to the culture dish and remain as floating cells; these are removed in the course of routine medium changes. Some presumably donor-dependent variability in recovery from cryopreservation has been observed.

6. IN VIVO ASSAY FOR OSTEOGENESIS

We consider the in vivo assay, in which ceramic cubes are loaded with isolated and culture-expanded putative stem cells and then implanted subcutaneously into immunocompromised host animals, to be the definitive test of the osteogenic and chondrogenic potential of stem cells in general. However, as indicated in Section 1, cartilage formation is almost never observed when human MSCs are used in this assay. Although bone formation has been observed in diffusion chambers loaded with cultured nonhuman marrow-derived stromal cells, the frequency of bone-positive results and the rate of bone formation are higher when the same cells are implanted in ceramic vehicles [Dennis and Caplan, 1993].

Our laboratory routinely uses biphasic ceramics consisting of 60% tricalcium phosphate and 40% hydroxyapatite, but we have also had success with coral-based ceramics. Pretreatment of the ceramics with fibronectin or laminin facilitates attachment of the cells to the ceramic surface and, in turn, results in the development of bone at earlier time points than for untreated cubes [Dennis and Caplan, 1993].

Subcutaneously implanted cell-loaded ceramic cubes provide a critical control for cells used in experimental tissue engineering designs. For example, Kadiyala

et al. [1997] used ceramic cubes loaded with rat marrow-derived MSCs as a positive control in experiments testing the efficacy of these cells in aiding the repair of a critical-size defect in rat femora. We have also used this assay to test the effect of in vitro factors, such as reduced oxygen tension, on differentiation of MSCs [Lennon et al., 2001] and to attempt to detect the presence of MSCs or osteoprogenitor cells in tissues other than bone marrow, including dental pulp [Mann et al., 1996] and peripheral blood [Lazarus et al., 1997].

Protocol 2.7. Preparation and Cell Loading of Ceramic Cubes

Reagents and Materials

Sterile

- ❑ Fibronectin: Prepare fibronectin as instructed by the manufacturer. Store as 100-μl aliquots at a concentration of 1 mg/ml in 12 × 75-mm tubes at −20 °C
- ❑ Tyrode's or Hanks' BSS
- ❑ Trypsin-EDTA: 0.25% trypsin, 1 mM EDTA in Hanks' BSS lacking Ca^{2+} and Mg^{2+}
- ❑ BCS
- ❑ DMEM-LG, serum-free
- ❑ Additional caps for 12 × 75-mm tube
- ❑ Conical centrifuge tubes, 15 or 50 ml
- ❑ Syringes, 20 ml and 30 ml, with 20-gauge needles
- ❑ Tissue culture Petri dish, 10 cm

Nonsterile

- ❑ Ceramic rods
- ❑ Glass beaker, 250 ml, with ultrapure water (UPW)
- ❑ Glass Petri dish
- ❑ Paper towel

Protocol

1. Cube Preparation and Coating

(a) Cut ceramics into 3-mm cubes. Trim to ideal size with a razor after cutting to approximate size with a hacksaw.

(b) Place cubes in the 250-ml glass beaker containing UPW, and swirl to remove small ceramic particles from the cubes.

(c) Let cubes settle to the bottom of the beaker, and then pour off the water containing the small ceramic particles.

(d) Repeat steps (b) and (c) 3 or 4 times, or until most of the debris has stopped coming off the cubes.

(e) Blot cubes on a dry paper towel, and then transfer them to a glass Petri dish and dry under a bright light.

(f) Autoclave for 30 min.

(g) Dilute fibronectin to a concentration of 100 μg/ml with sterile Tyrode's or Hanks' BSS in a 12 × 75-mm sterile tube.

(h) Add ceramics, but only enough so they are totally immersed (usually about 16 cubes per ml).
(i) Attach a 20-gauge needle to a 30-ml syringe. Insert the needle through the cap of the tube, and create a partial vacuum in the tube by pulling the plunger back completely. This helps the fibronectin enter the cube pores. Leave under negative pressure for about 60 s. Flick the tubes with your finger to help free air bubbles trapped in the cubes.
(j) Replace the punctured tube cap with a new one.
(k) Leave cubes in fibronectin at room temperature for 2 h.
(l) Aspirate liquid and transfer the cubes to a 10-cm tissue culture dish. Dry overnight in a laminar flow hood.

2. Cell Preparation and Loading

(a) Rinse cultures with 5 ml of TBSS (volumes indicated here are for 10-cm dishes) and remove liquid.
(b) Add 4 ml trypsin-EDTA. Incubate at 37 °C for 5–6 min.
(c) Add 2 ml BCS. Pipette resulting cell suspension up and down and gently rinse remaining cells off plate. Do not try to get all the cells off, just the easily detachable ones.
(d) Transfer suspension to 15- or 50-ml conical tube and centrifuge at 350 *g* for 5 min.
(e) Remove supernate and resuspend cell pellet in 10 ml serum-free DMEM-LG.
(f) Centrifuge as in (d) above.
(g) Remove supernate and resuspend pellet in 5 ml serum-free medium.
(h) Determine the number of cells with a hemocytometer or electronic cell counter.
(i) Centrifuge and resuspend pellet in serum-free medium at 5×10^6 cell/ml.
(j) Transfer cell suspension to a sterile 12 × 75-mm polystyrene tube.
(k) Add an appropriate number of fibronectin-coated cubes to the cell suspension.
(l) Draw off air from the tube as above (step (1)(i)), but with a 20-ml syringe. Flick tube to eliminate bubbles.
(m) Replace cap with a new one.
(n) Place tube in a 37 °C incubator with cap loose (to allow for CO_2 equilibration) for at least 2 h.

Protocol 2.8. Implantation of Ceramic Cubes into SCID Mice

Reagents and Materials

Sterile

- ❑ Ceramic cubes, prepared and loaded with cells as indicated in Protocol 2.7
- ❑ Anesthetic: "rodent cocktail," 1.5 parts ketamine (100 mg/ml), 1.5 parts xylazine (20 mg/ml), and 0.5 parts acepromazine (10 mg/ml). Diluted 1:4 in sterile water
- ❑ Local anesthetic: 0.5% Marcaine

- ❑ Syringes, 1 ml
- ❑ Syringe needles, 25 and 27 gauge
- ❑ Surgical instruments: fine scissors, blunt forceps and sharp forceps
- ❑ Betadine and alcohol swabs
- ❑ Staple sutures and forceps for applying staples
- ❑ Ear-notching forceps or other marking device

Nonsterile

- ❑ Athymic mice
- ❑ Styrofoam block
- ❑ Paper towels

Protocol

(a) Prepare a diagram of the scheme that you will use for implanting the ceramic cubes. The rest of the procedure is done at a facility designed for housing and working with athymic rodents. Adhere to the rules for the facility, including proper attire, use of laminar flow hoods, use of disinfectant solutions, etc. Many of the steps listed here are those used at the Athymic Animal Facility (AAF) at Case Western Reserve University and are included for reference.

(b) Before entering the interior of the AAF put on shoe covers, mask, cap, and sterile gown and gloves.

(c) Once inside the clean room, turn the laminar flow hood fan to the high setting.

(d) Spray entire inside working area of the hood with Clidox disinfectant.

(e) Leave the spray in hood and get the mouse cages from the room in which they are housed. The general rule is to use the oldest available mice first. Use all of the mice in one cage before getting a second cage (if necessary).

(f) Wipe down hood, and place the cage inside the hood.

(g) Spray hands with Clidox, and take a mouse out of the cage.

(h) Inject mouse with 0.1 to 0.15 ml anesthetic per 25 g body weight. Injections are made intraperitoneally (IP) with a new 25-gauge needle for each mouse.

(i) Return mouse to top of cage. Inject another mouse if needed. If more than 2 mice will be used, inject 1 additional mouse just before you begin the implants on 1 of the previously anesthetized mice.

(j) Fill out cage card with the cube location and identification as indicated on your implant sheet.

(k) Place a Styrofoam block in the center of the hood and cover it with a sterile towel. This will serve as the operating field. Place a second sterile towel adjacent to the block and place the instruments on it.

(l) Spray hands with Clidox.

(m) Remove a mouse from the cage and place on the towel over the Styrofoam block. Wipe the mouse's back with Betadine and alcohol swabs.

(n) Inject 0.1 ml 0.5% Marcaine (a local anesthetic) subcutaneously with a 27-g needle over the area to be incised with scissors. If adequate general anesthesia has not been obtained, you may inject an additional 0.05 ml rodent cocktail.

(o) Open skin with scissors and create subcutaneous pockets with blunt forceps.
(p) Insert cubes, up to 12 per mouse.
(q) Close skin with staples.
(r) Wipe ear with alcohol and make ear notch for identification.
(s) Return mouse to cage, and repeat the process with as many mice as needed.
(t) When finished, return cage to its original location. Wipe hood surface with water. Wipe any carts you used with Clidox, then with water.
(u) Turn off hood.

Host animals are normally harvested at 3 and 6 weeks after implantation, although little bone is usually present at the early harvest time. The cubes are fixed overnight with 10% phosphate-buffered formalin, decalcified in RDO (a low-pH rapid bone decalcifying agent), embedded in paraffin, and cut into sections, which are placed on a glass slide, stained with Toluidine Blue or Mallory–Heidenhain, and coverslipped. When viewed by brightfield microscopy the decalcified ceramic material appears as a gray to white granular, amorphous material. Cells can be seen occupying the pore areas of the cube. In some pores a mineralized material consistent in morphology with that classically ascribed to bone can be seen. The bone present within the cubes takes on a medium blue color with Toluidine Blue staining and a blue or red color when stained with Mallory–Heidenhain. The amount of bone can be quantified by histomorphometric techniques or by simpler visual estimates of the number or percentage of bone-containing pores [Dennis et al., 1998]. Formation of bone begins with the development of a sheet of cuboidal cells on the inner surfaces of the pores (Fig. 2.3). The cells, shown to be osteoblasts, begin to proliferate and fabricate extracellular matrix. Some of the osteoblasts become enmeshed in the matrix so that they elaborate and become osteocytes. These osteogenic cells have been shown to be of donor origin in appropriate marking experiments [Goshima et al., 1991b; Allay et al., 1997]. As this process continues, the developing bone progresses toward the center of the individual pores. In the case of human MSCs, most of the bone-containing pores are situated toward the periphery of the cube, but some are more centrally located. Vasculature from the host animal is always present in these cubes and is always associated with developing bone therein.

7. IN VITRO OSTEOGENIC INDUCTION AND ASSAYS FOR OSTEOGENESIS

7.1. In Vitro Osteogenic Induction

Although the in vivo model has been considered the optimal assay for osteogenesis, the in vitro assay is another valuable tool for evaluating hMSCs. The in vivo ceramic cube assay is expensive, includes technology not needed in the in vitro assay (histologic processing), and requires facilities for housing and working with athymic rodents. In vitro assays are free of these requirements and, furthermore, avoid possible undesirable host-contributed factors.

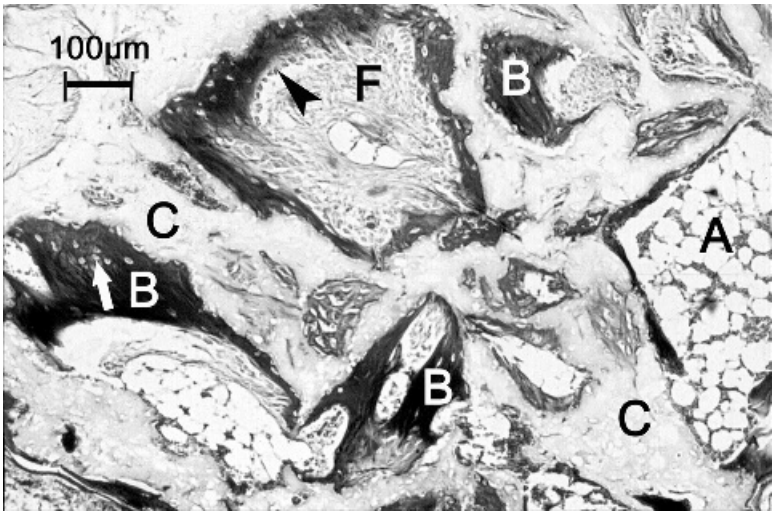

Figure 2.3. Brightfield photomicrograph of a histologic section of an MSC-containing ceramic cube harvested after 6 weeks in vivo and stained with Mallory–Heidenhain. Demineralized ceramic (C) appears as a gray amorphous material. Bone (B), deposited by osteoblasts (arrowhead), can be seen as a dark stained tissue adjacent to the inner surface of a number of pores. A group of osteocytes (arrow) is enmeshed within the osseous matrix. Adipose (A) or fibrous (F) tissues occupy pore space adjacent to bone.

As indicated in the Introduction, hMSCs remain in an undifferentiated state through repeated subculture. They can, however, be induced to differentiate along osteogenic lines by exposure to dexamethasone and ascorbic acid, and to mineralize by the later addition of β-glycerophosphate.

Protocol 2.9. Osteogenic Differentiation in Human Mesenchymal Stem Cells (hMSCs)

Reagents and Materials

Sterile or Aseptically Prepared

- ❑ DMEM-LG-10FB
- ❑ Trypsin-EDTA: 0.25% trypsin, 1 mM EDTA in Hanks' BSS lacking Ca^{2+} and Mg^{2+}
- ❑ Dexamethasone (dex) (See Section 2.2.1)
- ❑ β-Glycerophosphate (BGP) (See Section 2.2.2)
- ❑ Ascorbic acid 2-phosphate, (See Section 2.2.3)
- ❑ OS medium: supplement DMEM-LG-FB with 1% (v/v) each of 10 µM dexamethasone and 5 mM ascorbic acid 2-phosphate. Thus the final concentrations are 0.1 µM dexamethasone and 50 µM ascorbic acid 2-phosphate.
- ❑ Control medium: supplement DMEM-LG-FB with 1% (v/v) 5 mM ascorbic acid 2-phosphate
- ❑ Culture vessels: 3.5-cm tissue culture dishes or multiwell plates, 6-, 12-, or 24 well

Protocol

(a) Calculate the number of cultures and the volume of medium needed for the experiment. Osteogenic induction involves treating cells with or without dexamethasone. The former are referred to as OS (osteogenic supplemented) cultures, and the latter are referred to as control cultures. Equal numbers of culture dishes are established for each condition.
(b) Prepare OS and control media.
(c) Subculture hMSCs as described in Protocol 2.4. Second-passage cells are typically used, but later-passage hMSCs can be used.
(d) Count the cells and seed them at 3×10^3 per cm^2 in serum-containing medium, usually into 35-mm dishes or 12- or 24-well plates.
(e) On day 1 of culture (approximately 24 h after cells are seeded), the medium in which the cells were seeded is removed and immediately replaced with an appropriate volume of either control or OS medium.
(f) Media are changed every 3–4 days.
(g) On day 10 and thereafter, the medium for both groups of cultures should be further augmented with 1% 200 mM β-glycerophosphate (for a final concentration of 2 mM).
(h) Cells are maintained in culture up to 21 days.

Human MSCs respond to exposure to dexamethasone by becoming more compact; typically, these cells change from a spindle-shaped morphology to one that is more cuboidal or polygonal, although some fibroblast-like cells remain. After the addition of β-glycerophosphate, granular mineral-like deposits can be seen on the surface of the OS cultures. Unlike rat MSCs, which form discrete multilayered nodules in the presence of dexamethasone, presumptive osteoblasts differentiated from human MSCs are distributed rather evenly throughout the culture dish, although irregularly shaped multilayered areas are seen.

Through at least day 9 of culture, cell proliferation, as determined by quantification of DNA, is lower in OS than in control cultures, although by day 28 the values for the two groups are equivalent [Lennon et al., 2000]. Techniques used to examine osteoinduced MSCs are described in Sections 7.2 through 7.4.

7.2. Determination of Alkaline Phosphatase Activity

Alkaline phosphatase activity, on a per cell basis, reaches its peak between days 9 and 12 of culture for passaged cells. Accordingly, this time period is optimal for assays of the enzyme, although earlier and later determinations of alkaline phosphatase activity may be useful. The biochemical and cytochemical assays are described below. Both assays may be conducted on individual culture dishes as long as the sequence below is followed.

In the biochemical assay, alkaline phosphatase produced by the cells cleaves the phosphate ion from the substrate, *p*-nitrophenyl phosphate. The resulting

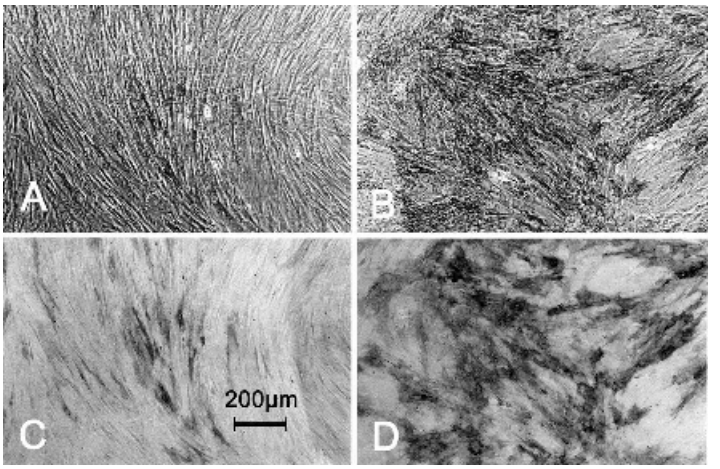

Figure 2.4. Human mesenchymal stem cells stained with Fast Violet B for cytochemical detection of alkaline phosphatase activity on day 10 of second passage. Cells in control medium are shown via phase-contrast microscopy (A) and brightfield optics (C). More intense staining is apparent in cells cultured in the presence of dexamethasone, as seen in phase-contrast (B) and brightfield (D) micrographs. Scale (bar in panel C) is the same for all panels.

p-nitrophenol can be measured colorimetrically by the addition of an alkaline solution. The quantity of *p*-nitrophenol liberated from the substrate can be determined by comparison to a curve generated from known concentrations of *p*-nitrophenol standards. A 5- to 10-fold increase in the specific activity of the enzyme is typical for OS cultures (compared with control cultures).

The substrate used in the cytochemical assay is naphthol AS-MX phosphate. Again, the phosphate group is removed, and the resulting naphthol AS-MX combines with fast violet or fast blue salts to produce violet or blue color at cellular sites of alkaline phosphatase activity. Dark to medium red stain develops in OS cultures stained with fast violet. Most of the stain can be seen in the multilayered areas. Control cultures stain much less intensely (Fig. 2.4).

7.2.1. Alkaline Phosphatase Assays (Biochemical and Cytochemical)

Protocol 2.10A. Biochemical Assay of Alkaline Phosphatase Activity

Reagents and Materials

Nonsterile

- ❑ TBSS
- ❑ Substrate buffer (See Section 2.3.1)
- ❑ Substrate (See Section 2.3.2): Calculate the volume of substrate required, and add substrate tablets to the buffer solution to yield this volume
- ❑ 1 M NaOH
- ❑ *p*-Nitrophenol for standard curve (See Section 2.3.3)

Protocol

(a) Turn on spectrophotometric plate reader in time to have it properly warmed up when samples are ready to read.
(b) Label a 12 × 75-mm tube for each tissue culture dish or well of multiwell plates to be analyzed. Add a volume of 1 M NaOH equal to the volume of substrate used (See step (d)) to each tube.
(c) Rinse cells twice with TBSS.
(d) Add an appropriate volume of substrate solution to tissue culture dishes or wells (1 ml per 3.5-cm dish, 0.5 ml per well of 12-well plate, etc.) Time the addition of substrate so that cells in each well or dish are in contact with the solution for the same length of time.
(e) Incubate for 5 to 15 min, depending on the rate of the reaction (as seen by the density of yellow developing in the substrate; the ultimate OD when measured must fit within the linear range of the standard curve).
(f) Transfer the substrate solution to the NaOH-containing tubes set up earlier. Do this in the same sequence in which substrate was added to the dishes or wells, and remove substrate so that each dish or well is exposed to the substrate for the same length of time.
(g) If cultures are to be used for additional assays (alkaline phosphatase cytochemistry, DNA, etc.) add TBSS to the cells immediately, and do additional rinses and fixation as soon as time permits.
(h) Set up and label a 96-well plate.
    i) To the first row add 200 μl of a mixture of equal parts of 1 M NaOH and substrate buffer to serve as blanks.
    ii) Transfer 200 μl of appropriately diluted (or undiluted) experimental samples to corresponding labeled wells (usually 4 replicate wells per sample). As a rough guide for determining whether samples require dilution, compare the intensity of color of the samples to that of the highest concentration of *p*-nitrophenol. Dilutions are made with the same solution used for blanks.
    iii) Set up a standard curve by transferring 200 μl diluted *p*-nitrophenol standards to wells (See Section 2.3).
(i) Read the absorbance at 405 nm on a microplate reader. Do further dilutions for samples that give readings beyond the linear range of the standard curve and read samples again. Note any dilutions made.

Protocol 2.10B. Cytochemical Assay for Alkaline Phosphatase

Reagents and Materials

Nonsterile

- ❑ Fast violet stain (See Section 2.4.1)
- ❑ Citrate-buffered acetone (See Section 2.4.3)
- ❑ Naphthol AS-MX Alkaline Solution (Sigma 85-5)
- ❑ TBSS

Protocol

(a) Rinse cultures twice with TBSS.
(b) Fix for 30 s with citrate-buffered acetone.
(c) Rinse cultures twice with distilled water. Leave water from the second rinse on the cultures until you are ready to proceed with the next step.
(d) Add 0.5 ml Naphthol AS-MX Alkaline Solution per 12 ml of fast violet solution. Cover with aluminum foil to protect solution from the light.
(e) Remove water from cultures and add an appropriate volume of fast violet-naphthol solution to each dish or well.
(f) Incubate at room temperature in the dark for 45 min.
(g) Remove solution and rinse twice with distilled water. Keep the cultures covered with water and store in the dark. The cultures can be further stained according to other protocols (von Kossa or other stain).

7.3. Von Kossa Staining

Staining with the von Kossa method is a qualitative assay for mineralization. In the case of human MSCs, mineralization is usually not readily detectable until day 21. A positive reaction is manifested by brown or black staining, which, in the case of hMSCs, is diffuse and is usually distributed fairly evenly throughout the culture (Fig. 2.5). This contrasts with mineralization in cultures of rat MSCs, in which intense staining can be identified in discrete bone nodules.

Protocol 2.11. Staining for Mineralization in Cultured Mesenchymal Stem Cells (MSCs)

Reagents and Materials

Nonsterile

- ❑ Silver nitrate, 2% in distilled water. Make up only what you will need because the shelf life of the solution is approximately 1 week
- ❑ Tyrode's or Hanks' BSS
- ❑ Phosphate-buffered formalin, 10%
- ❑ Distilled water
- ❑ Ethanol, 100%

Protocol

(a) Rinse cultures twice with cold TBSS (or HBSS).
(b) Fix cultures with 10% phosphate-buffered formalin for 30 min. All steps involving formalin must be carried out in a fume hood.
(c) Rinse cultures twice with distilled water.
(d) Add 2% silver nitrate to cover the cells; place the dishes or flasks in a dark environment for 10 min.

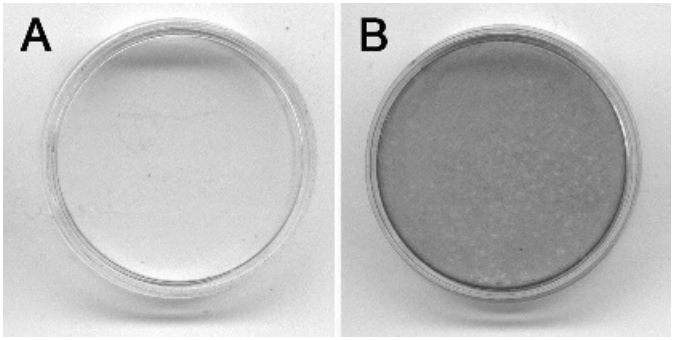

Figure 2.5. Human mesenchymal stem cells stained by the von Kossa method on day 21 of second passage. Second-passage human mesenchymal stem cells were cultivated in 35-mm dishes in control medium (A) or medium supplemented with dexamethasone (B). Media for cultures in both conditions were further augmented with 2 mM β-glycerophosphate, beginning on day 10 of culture. Cultures were fixed on day 21 and stained by the von Kossa method to reveal mineralization. Positive staining (gray to black) is apparent only in the dexamethasone-treated cultures.

(e) Rinse three times with distilled water. Leave the water from the final rinse on the culture dish for step (f).
(f) With water still covering the cells, expose the cultures to bright light for 15 min. Use a white background beneath the dishes to help reflect the light.
(g) Remove the water covering the cells, then rinse two more times with distilled water.
(h) Dehydrate with 100% ethanol; remove the ethanol after 1 min, and then allow the cultures to air dry.

7.4. Calcium Assay

Protocol 2.12. Calcium Assay in Cultured Mesenchymal Stem Cells (MSCs)

Reagents and Materials

Nonsterile

- ❑ TBSS
- ❑ Formalin, 10%, phosphate buffered
- ❑ Distilled water
- ❑ HCl, 0.6 N
- ❑ $CaCl_2$ standards: 6.25, 12.5, 25, 50, 100, and 200 μg/ml
- ❑ Calcium binding reagent and calcium buffer: Sigma calcium diagnostic kit (#587-A)
- ❑ Polystyrene tubes, 12 $\times$ 75 mm
- ❑ Rotary shaker
- ❑ Microtitration plate reader

Protocol

(a) Rinse cultures twice with TBSS.
(b) Fix with 10% phosphate-buffered formalin for 30 min (use fume hood for all steps involving the use of formalin).
(c) Remove formalin by aspiration and save for proper disposal.
(d) Rinse three times with distilled water, and then air dry. The culture dishes may be stored at 4°C so that the assay of all plates from an experiment may be completed at the same time.
(e) After all cultures have been collected, begin the assay by adding 1 ml 0.6 N HCl per 3.5-cm dish (0.5 ml per well of a 24-well plate).
(f) Place dishes on a rotary shaker overnight at about 50 rpm to extract calcium.
(g) Collect the HCl and transfer it to labeled 12 × 75-mm polystyrene tubes.
(h) Set up and label a 96-well plate as follows:
    i) To the first row on the left add 20 μl 0.6 N HCl per well to serve as blanks.
    ii) Add 20 μl per well of dilutions of $CaCl_2$ in 0.6 N HCl to establish a standard curve. The standard curve should include the following concentrations of $CaCl_2$: 6.25, 12.5, 25, 50, 100, and 200 μg/ml. Use 4 replicates for each concentration.
    iii) Add 20 μl of the experimental samples to each of 4 wells. If necessary, first dilute the samples with 0.6 N HCl in separate tubes.
(i) Prepare the required volume of reagents from the Sigma calcium diagnostic kit by combining equal volumes of the calcium binding reagent and calcium buffer. The required volume equals the number of wells × 180 μl, plus about 10%.
(j) Turn on the plate reader to have it properly warmed up.
(k) Add 180 μl of the calcium binding reagent-calcium buffer to each well.
(l) Read the samples on a microtitration plate reader at 575-nm absorbance. Record the results, and calculate a standard curve and the concentration of calcium in the experimental samples by linear regression analysis. Do further dilutions for samples that give readings beyond the linear range of the standard curve, and then read the samples again.

8. IN VITRO ADIPOGENIC INDUCTION AND ASSAYS FOR ADIPOGENESIS

Adipogenic induction involves culturing cells (starting 24 h after they are seeded) with either control or adipogenic induction medium (AIM). The method described below is a variation of that described by Smyth and Wharton [1992, 1993] and by Pittenger et al. [1999]. Accumulation of lipid droplets can be seen in the cytoplasm of induced cells. Approximately 30% to 40% of the cells may include lipid droplets by day 12. After that time the amount of lipid may increase, but few additional cells will differentiate into adipocytes.

8.1. Adipogenic Induction

Protocol 2.13. Induction of Adipogenesis in Mesenchymal Stem Cells (MSCs)

Reagents and Materials

Sterile

- ❑ hMSC cultures, first or second passage
- ❑ DMEM-LG-10FB
- ❑ Adipogenic induction medium (AIM), consisting of DMEM-HG-10FB, 1 μM dexamethasone, 100 μM indomethacin, 500 μM 3-isobutyl-1-methylxanthine (IBMX), and 10 μg/ml insulin (See Section 2.5)
- ❑ Adipogenic maintenance medium (AMM): DMEM-HG-10FB with 10 μg/ml insulin
- ❑ Trypsin-EDTA (See Protocol 2.4)
- ❑ Tissue culture dishes, 3.5 cm, or 6-well plates

Protocol

(a) Calculate the number of cultures needed for the experiment.
(b) Trypsinize first- or second-passage hMSCs as described in Protocol 2.4.
(c) Seed cells into 3.5-cm dishes (or 6-well plates) at a density of 2×10^5 cells in DMEM-LG-10FB.
(d) Incubate at 37 °C overnight.
(e) On day 1, switch medium for cultures to be induced to AIM, and replace medium in control cultures with fresh DMEM-LG-10FB. Set up the desired number of cultures for each condition.
(f) Media are changed twice a week.
(g) On day 10, replace AIM with AMM.

Cells are usually fixed for Nile Red staining on days 0, 2, 12, and 21.

Protocol 2.14. Fixing Mesenchymal Stem Cells (MSCs) for Flow Cytometric Assay of Adipogenesis

Reagents and Materials

Sterile

- ❑ PBSA: phosphate-buffered saline without Ca^{2+} and Mg^{2+}
- ❑ EDTA, 0.1 mM
- ❑ Trypsin-EDTA: 0.25% trypsin, 1 mM EDTA
- ❑ FBS
- ❑ Trypsin inhibitor, Type II-O: chicken egg white, 100 mg/ml in DMEM
- ❑ Pasteur pipettes
- ❑ Polypropylene tubes, 12 × 75 mm

Nonsterile

- ❑ Paraformaldehyde, 4%

Protocol

(a) Remove medium and rinse 3 times with phosphate-buffered saline (PBSA). Leave the third addition of PBSA on the cultures for 3–5 min.
(b) Remove PBSA and add 1 ml 0.1 mM EDTA per well or dish. Incubate at 37 °C for 10–20 min, checking cells at 5-min intervals to see whether they are ready for the addition of trypsin-EDTA, as indicated by retraction of the cells (cells should not be completely round). If the cells are being released on days 1 or 2 of culture, this step can be skipped.
(c) Remove EDTA and add 475 μl trypsin-EDTA per well and incubate at 37 °C for 5 to 7 min.
(d) Return plate to room temperature. Agitate by hand if any cells remain adherent.
(e) Add 25 μl FBS and 25 μl trypsin inhibitor. Swirl or rock plate to mix.
(f) With a Pasteur pipette, combine suspensions from 3 wells (of a 6-well plate) into 1 well. Gently flush empty wells once or twice with the cell suspension.
(g) Transfer cell suspension to a 12 × 75-mm polypropylene tube.
(h) Rinse empty wells with a total volume of 500 ml PBSA and add the rinse to the polypropylene tube.
(i) Cells may be counted at this point.
(j) Bring suspension to a concentration of 0.5% paraformaldehyde with the addition of a suitable volume of 4% paraformaldehyde.
(k) Invert the tube several times to mix contents, and store at 4 °C until the day of staining.

Protocol 2.15. Staining Mesenchymal Stem Cells (MSCs) with Nile Red for Flow Cytometric Assay of Adipogenesis

Reagents and Materials

Nonsterile

- ❑ Nile Red working solution. (See Section 2.5)
- ❑ Pasteur pipette
- ❑ Nitex nylon filter, 100 μm
- ❑ Polypropylene tubes, 12 × 75 mm
- ❑ Aluminum foil
- ❑ Ice tray
- ❑ Flow cytometer

Protocol

(a) Resuspend each sample with a Pasteur pipette.
(b) Collect 300 μl of each sample and filter through a 100-μm Nitex nylon filter into a fresh polypropylene tube.

(c) Add an equal volume of Nile Red working solution.
(d) Cover tube with foil and store on ice until time of assay.
(e) Cells are analyzed with a flow cytometer. Gold fluorescence is emitted when Nile Red is dissolved in neutral lipids, and can be collected between 560 and 590 nm with a band-pass filter. Ten thousand cells are analyzed from each sample [Smyth and Wharton, 1992, 1993].

9. SELECTION OF FETAL BOVINE SERUM

The process of selecting the best lot of FBS from the lots available has been described previously [Lennon et al., 1996] and will not be reviewed in detail here. It should, however, be emphasized that we regard the process of serum selection as an extremely important aspect of the culture of MSCs. Our experience with testing FBS dates to the time when we were working primarily with stage 24 embryonic chick limb bud mesenchymal cells. We were fortunate that the lot selected for these cells also supported the proliferation of marrow-derived MSCs, although it may be argued that the ability of a single lot of serum to support both cell types is related to the concept that both sets of cells are MSCs (See Section 1). Be that as it may, we do not use stage 24 limb bud cells as part of our current assay for FBS.

We consider the ability of a lot of FBS to promote the initial adherence of MSCs to the culture dish, to support cell proliferation, and to maintain the multipotentiality of the cells, as indicated by their ability to promote osteogenesis in the ceramic cube assay, to be the most important criteria for selection of serum. Theoretically, an ideal lot of serum would meet these standards, and would also promote in vitro chondrogenesis, osteogenesis, and adipogenesis in serum-supplemented media further augmented with the appropriate inductive agents (or in the defined medium appropriate for chondrogenic differentiation). In practice, we have found that it is not always possible to select such an ideal serum. For example, one lot of FBS that supported proliferation and gave good results in the in vivo assay was far from ideal for in vitro osteogenesis. Thus, although we have considered the in vivo assay to be our "gold standard" for serum selection, it may be prudent for investigators to adopt the assay or inductive method that will be most widely utilized with MSCs in their laboratory as their most stringent criterion in the selection process.

Moreover, it should be noted that although in vitro chondrogenic induction for MSCs takes place in a medium devoid of serum (See Chapter 4), exposure to the proper lot of FBS in monolayer culture before the cells are introduced into aggregate or pellet culture is very important.

A brief description of the process of selecting FBS for use in culturing and inducing differentiation in MSCs is outlined below. We usually screen 8 to 10 lots of serum (including the control lot) in a given serum screen.

Protocol 2.16. Selecting FBS for Mesenchymal Stem Cells (MSCs)

Reagents and Materials

Sterile

- ❑ Materials and reagents for Protocols 2.3 and 2.4
- ❑ MSCs as isolated in Protocol 2.3
- ❑ Tissue culture dishes, 10 cm

Protocol

(a) Isolate MSCs as described in Protocol 2.3, but using a concentration of 5% FBS.
(b) Determine the final cell concentration.
(c) Adjust the cell suspension (still in 5% of the FBS currently in use, or the control serum) to a volume sufficient to seed 1 or 2 ml per culture dish.
(d) Before the cells are seeded, pipette 5 or 6 ml of medium supplemented with 10% of the sera being tested into 10-cm dishes
(e) Add the cell suspensions and mix to distribute the cells evenly. In this way, the cells are exposed to the test sera from the beginning of their time in culture.
(f) Maintain the cells in culture as described in Protocol 2.3 and in Section 4. Examine cultures frequently by phase microscopy to evaluate cell morphology, to gain a qualitative assessment of cell proliferation, and to examine cell attachment, as indicated by a comparison of the number of colonies.
(g) Subculture cells as described in Protocol 2.4. Cell yields are determined individually for cultures in the various test sera, with at least two cell counts being taken for each sample.
(h) Seed the cells into 10-cm dishes (all at the same density and again in control or test serum).
(i) Repeat the process of trypsinizing and counting the cells when the culture is just preconfluent. Detailed records of cell yields are kept to assess cell proliferation.

Cells harvested at the end of the first passage (or later passages if an insufficient number of cells is available at the end of the first passage) are used for the assays that are deemed appropriate. In our laboratory, highest priority is given to loading ceramic cubes for implantation. We do this for two reasons. First, as indicated above, we regard this as the definitive assay for stem cells. The second reason is a practical matter relating to the length of the in vivo assay (6 weeks in vivo plus time for histologic processing and evaluation) and the time that companies that provide serum are willing to hold the product on reserve. Because we are usually pushing the limit of the reserve time, we try to get the cubes implanted as quickly as possible.

Additional cell preparations may be needed to provide enough cells to complete all of the required assays, and because duplicate or triplicate data sets are desirable. Among the other assays that we routinely include in the testing of FBS for human MSCs are the in vitro assays for osteogenesis and chondrogenesis. For the former,

we use von Kossa staining to assess mineralization, but do not routinely examine alkaline phosphatase activity. For in vitro chondrogenesis, sera are tested for their ability to support pellet formation as indicated by toluidine blue staining of fixed and embedded pellets.

Data reflecting the ability of the various lots of serum to support proliferation and to promote differentiation in the induction assays are compiled and evaluated. The results are compared with the lot of serum currently in use. If no serum is currently in use for MSCs, the lot providing the best results is selected (if acceptable).

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SOURCES OF MATERIALS

Materials	Supplier	Catalog Number
Acepromazine	Henry Schein	356–7290
Alkaline phosphatase assay materials	Sigma	85L-3R or individual components
Alkaline phosphatase substrate	Sigma	104–105
Ascorbic acid 2-PO_4	Wako	013–12061
Biphasic ceramics consisting of 60% tricalcium phosphate and 40% hydroxyapatite	Zimmer	97-1109-531-00
Buffered formalin	Fisher	SF 100–4
Calcium assay kit	Sigma	587-A
Calf serum	Hyclone	SH30073-03
Citrate concentrated solution	Sigma	85-4C
Clidox disinfectant	Pharmacal Research Laboratories	95120 (Activator) 96120 (Base)
Coral-based ceramics	Interpore	
Cryofreezer	Nalgene	5100-0001
Cryovials	Nalgene	5000-0020
Dexamethasone	Sigma	D 4902
Dimethyl sulfoxide	Sigma	D 2560
Dulbecco's modified Eagle's medium	Sigma	D 5523
	GIBCO	31600-083
Electronic cell counter	Beckman Coulter	
Fast Violet B Salt	Sigma	85-1
Fetal bovine serum	Best available	
Fibronectin	Becton Dickinson	354008
β-Glycerophosphate	Sigma	G 9891

Materials	Supplier	Catalog Number
Halothane	Henry Schein	982-0753
IBMX	Sigma	I 5879
Indomethacin	Sigma	I 7378
Insulin	Sigma	I 1882
Ketamine	Henry Schein	995–5770
Marcaine	NLS Animal Health	108435
Microtitration plate reader	Bio-Rad	2550
Naphthol AS-MX Alkaline Solution	Sigma	85-5
Nile Red	Sigma	N 3013
Nitex nylon filter, 100 μm	TETKO, Inc.	3-100/47
p-Nitrophenol standard solution	Sigma	104-1
p-Nitrophenyl phosphate:	Sigma	104–105
Percoll	Sigma	P 1644
Polycarbonate tubes	Nalgene/Oakridge	3118-0050
RDO decalcifying agent	Apex Engineering Products Corp.	RDO-04
Rotary shaker	New Brunswick	
SCID mice	Charles River	CB17
Silver nitrate	Sigma	S 0139
Toluidine Blue	Sigma	T 3260
Trypan blue	Gibco	15250-061
Trypsin inhibitor	Sigma	T 9253
Trypsin-EDTA: 0.25% trypsin, 1 mM EDTA	Gibco	25200-072
Tyrode's salt solution (TBSS)	Sigma	T 2145
Xylazine	NLS Animal Health	105650

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