Preface

The discovery of mesenchymal stem cells is credited with Alexander Friedenstein and associates, who over 40 years ago demonstrated that pieces of bone marrow transplanted under the renal capsule of mice formed a heterotopic osseous tissue that was self-maintaining, self-renewing, and capable of supporting host cell hematopoiesis. Furthermore, Friedenstein showed that the osseous-forming activity of bone marrow was contained within the fibroblastoid cell fraction isolated by preferential attachment to tissue culture plastic. These findings confirmed that bone marrow contained separable stem cell populations capable of generating hematopoietic and connective tissue cell lineages. These studies also demonstrated that marrow-derived, plastic adherent fibroblastic (stromal) cells were capable of supporting the growth and differentiation of various hematopoietic cell types. These cells were then used as feeder layers to establish long-term bone marrow cultures in vitro, which fostered a wealth of new knowledge regarding the molecular mechanisms regulating hematopoiesis.

In the decades following Friedenstein's seminal publications, various groups labored to delineate the biological nature and differentiation potential of plastic adherent cells from bone marrow. These efforts revealed much information about their cell surface phenotype, proliferative and differentiation potential and culminated in the demonstration that clonally derived murine and human populations were multipotent, capable of differentiating into adipocytes, chondrocytes, osteoblasts, and hematopoiesis-supporting stromal cells. The latter findings confirmed the existence of a stem cell in marrow capable of generating most connective tissue cell types. Consequently, the marrow-derived, plastic adherent cells first referred to as colony-forming unit fibroblast (CFU-F) by Friedenstein, then in the hematological literature as marrow stromal cells, subsequently became known as mesenchymal stem cells. Recently, a committee from the International Society of Cell Therapy has adopted the term multipotent mesenchymal stromal cells (MSCs) to define these cells owing to the fact that a definitive description of the bona fide mesenchymal stem cell and the molecular mechanisms that regulate its self-renewal versus differentiation remain forthcoming. The literature has been confused by the frequency with which the different names for essentially the same cells have been used (*see* Fig. 1). In this compendium, the terms CFU-F, marrow stromal cell, mesenchymal stem cell, and multipotent mesenchymal stromal cell

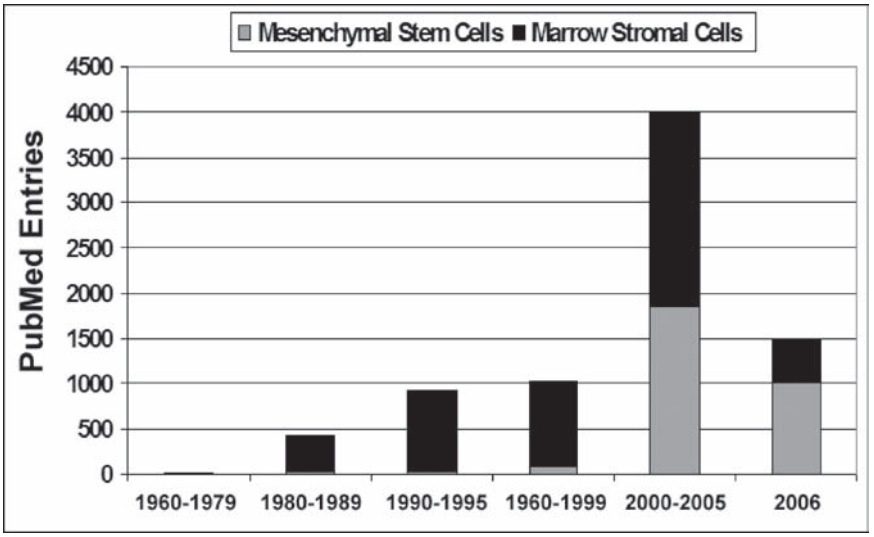

Fig. 1 Illustrated is the number of citations found in the PubMed database that contain the phrase "marrow stromal cells" or "mesenchymal stem cells" in their title or abstract over various time periods.

are deemed equivalent and herein will refer to the plastic adherent, fibroblastoid cells from marrow that are defined functionally by their capacity to undergo multi-lineage differentiation into connective tissue cell lineages.

In recent years MSCs have garnered much attention owing to their broad therapeutic efficacy. Initially, MSC administration to children afflicted with osteogenesis imperfecta was found to have a significant positive impact by reducing the severity of the disease. Promising results were subsequently reported using MSCs or related cells from bone marrow in the treatment of Hurler's syndrome, metachromatic leukodystrophy, graft versus/host disease and to enhance engraftment of heterologous bone marrow transplants. Most recently, MSCs have been shown to afford a therapeutic benefit in the treatment of myocardial infarction, stroke, lung diseases, spinal cord injury, and other neurological disorders. These results, together with the fact that MSCs can be readily isolated from small volume bone marrow aspirates, expanded to large numbers ex vivo and engineered genetically have made them extremely attractive as therapeutic cellular vectors.

Despite these advances, it has been difficult to assess the overall therapeutic use of MSCs owing to conflicting reports in the literature regarding their engraftment levels in tissues in vivo, their overall differentiation potential in vitro and in vivo, as well as their therapeutic efficacy in disease models. Although some of these discrepancies are related to limitations associated with experimental methodologies, critical differences in the preparation and expansion of donor cells used for the experiments certainly contribute to this problem, as well. Consequently, the

necessity of developing standardized methods to isolate, phenotype, and evaluate the quality of MSCs is ever increasing. Accordingly, the following compendium provides detailed methodologies for the isolation and characterization of human and rodent MSCs contributed by a group of assembled leaders in the field.

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Chapter 2
Mesenchymal Stem Cells from Adult Bone Marrow

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Abstract Mesenchymal stem cells (MSCs), sometimes referred to as marrow stromal cells or multipotential stromal cells, represent a class of adult progenitor cells capable of differentiation to several mesenchymal lineages. They can be isolated from many tissues although bone marrow has been used most often. The MSCs may prove useful for repair and regeneration of a variety of mesenchymal tissues such as bone, cartilage, muscle, marrow stroma, and the cells produce useful growth factors and cytokines that may help repair additional tissues. There is also evidence for their differentiation to nonmesenchymal lineages, but that work will not be considered here. This chapter will provide the researcher with some background, and then provide details on MSC isolation, expansion and multilineage differentiation. These are the beginning steps toward formulating tissue repair strategies. The methods provided here have been used in many laboratories around the world and the reader can begin by following the methods presented here, and then test other methods if these prove unsatisfactory for your intended purpose.

Keywords Mesenchymal stem cells (MSCs); direct plating isolation; density gradient isolation, lineage differentiation protocols; chondrogenic; adipogenic; osteogenic.

1 Introduction

Tissue healing takes place more rapidly in children than in adults and this is likely owing to a number of factors. One of those factors appears to be the abundance of stem and progenitor cells in the developing tissues of the child. As we reach adulthood, these cells are not so necessary for tissue growth and appear to diminish over time, perhaps some differentiating to adult cell types whereas some are retained as resident tissue stem cells. Over the years, the number of tissue resident stem cells further diminishes as they are called on for normal tissue repair and maintenance,

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and normal cellular senescence. That said, there appear to be small numbers of progenitor or stem cells that can be isolated from many tissues at all stages of life. These cells appear to afford a wonderful opportunity, indeed, a responsibility, to understand important aspects of human biology involving tissue repair and regeneration.

One of these adult stem cells that can be found in several tissues throughout life and that can be isolated and propagated in culture was termed the mesenchymal stem cell or MSC *(1,2)* by Arnold Caplan of Case Western University. A key element in the acceptance of MSCs as a potential cellular therapeutic was the early demonstration of safety in humans by Drs. Hillard Lazarus and Stan Gerson, hematological oncologists, who first tested MSCs as support cells during hematopoietic stem cell (HSC) transplantations *(3)*. Work began on the isolation of MSCs and examination of their multipotential nature in 1994, and the multilineage in vitro differentiation of these cells was demonstrated at the 1996 annual meeting of the American Society for Cell Biology. This work led to a key paper in the stem cell field published in Science in 1999 demonstrating multilineage differentiation of clonal populations of human MSCs that has now been cited in over 2,500 publications *(4)*.

The first descriptions of fibroblastic cells that could be isolated and grown from bone marrow samples, that retained the ability to differentiate to bone tissue was presented by Dr. Alexander Friedenstein of the Gamalaya Institute in Moscow in the 1960s, using guinea pig bone marrow as the source *(5–7)*. When these cells were culture expanded ex vivo, and then placed in capsules under the skin of a recipient syngeneic animal, new bone and cartilage tissue was identified when histology was performed. Although the same type of cell, or a close homologue of it, can be found in many tissues, including adipose *(8–11)*, the endosteal surface of bone and bone itself, bone marrow has proven to be a reproducible and convenient source of these cells from all species tested. MSCs have been isolated from mouse *(12–16)*, rat *(17–21)*, guinea pig *(5,6)* rabbit *(22–24)*, dog *(25,26)*, goat *(27)*, pig *(28–31)*, nonhuman primates *(32–35)*, and man *(1–4,36–40)*.

MSCs secrete growth factors and cytokines that have autocrine and paracrine activities. The MSCs produce vascular endothelial growth factor (VEGF), stem cell factor (SCF-1), leukemia inhibitory factor (LIF), granulocyte colony stimulatory factor (G-CSF), macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor(GM-CSF), interleukins (IL-1, -6,-7, -8, -11, -14, and -15), stromal cell-derived factor (SDF-1), Flt-3 ligand, and others *(4,36–39)*. The expression of these factors may be modulated through interactions with other cell types *(48–50)*.

Some additional interesting and important aspects of MSCs that have come to light include their homing to sites of tissue injury, particularly ischemic regions of heart *(26,29,43,44)* where the MSCs may prevent deleterious remodeling *(28,30,31)*. MSCs also have the ability to modify immune responses and engraft in allogeneic recipients, and MSC treatment has been used to clinically treat graft-versus-host disease (GVHD) *(46–51)*. MSCs are also under evaluation for clinical use in children with osteogenesis imperfecta, and glycogen storage diseases *(52–54)*. Although methods in these areas are not detailed here, clearly, MSCs represent a new, exciting and potentially powerful paradigm for cellular therapy.

Although a number of research groups investigated MSCs from nonhuman species, Arnold Caplan, Steve Haynesworth, and colleagues at Case Western Reserve

University were the first to systematically pursue the isolation of the MSC from human tissue. The reasoning was that a human source should be sought that could be harvested as a simple procedure in the doctors office under local anesthetic, without sacrificing or harming the tissue that was to be repaired and regenerated. In this case, bone marrow was chosen as it was remarkably renewable, harbored MSCs, was a known source of hematopoietic stem cells (HSCs), and could be isolated from the marrow cavity of the hipbone in a simple procedure under local anesthetic. I can personally attest to the simplicity of the harvest procedure. This will be the method used to obtain the starting material below. The methods presented here work well for most species, but the mouse MSCs are often contaminated with HSCs that seem to be required during propagation of MSCs, perhaps because of production of a growth factor or cytokine.

As a senior scientist at Osiris Therapeutics, Inc. a company formed to develop and commercialize products based on human MSCs, I was able to perform studies on MSCs and evaluate many of their properties. The results have been presented in a number of peer-reviewed papers that form the basis for this chapter and all the information here has been made available previously. The first public presentation of the 3 lineage differentiation of MSCs to chondrocytes, adipocytes, and osteocytes was at the American Society for Cell Biology Annual Meeting in December 1996 in Washington, D.C.

2 Materials

2.1 MSCs from Bone Marrow Aspirates

2.1.1 Biohazard Considerations

Bone marrow may contain blood borne pathogens, and therefore one must take care to avoid exposure, splashing, or spills. The handler should be trained and familiar with "universal precautions" to protect exposed skin, mucus membranes etc, and it is a good idea to walk through the planned steps before obtaining the bone marrow sample. Recommendations can be found in the Code of Federal Regulations, 29 CFR Chapter XVII (7-1-98 Edition), Occupational Safety and Health Administration, US Department of Labor. § 1910.1030. Although the bone marrow donors may be pretested for HIV and hepatitis, such tests are not available for many pathological agents.

2.2 Cell Culture Laboratory

It is understood that culturing MSCs will require typical equipment found in a cell culture laboratory including a laminar flow Biological Safety Cabinet, a 5% CO_2 incubator at 37 °C, inverted microscope with interference phase optics for observing cultured cells, and a hemocytometer (glass) for counting cells with the microscope.

2.3 Bone Marrow Acquisition

Human bone marrow can be obtained by needle from the iliac crest by a physician in a brief procedure, using local anesthetic. For many laboratories, this means finding a medical doctor, a hematologist/oncologist, who is willing to collaborate. For harvest of bone marrow from nonhuman species the procedure is similar but the subject is appropriately anesthetized. For rodents, they are humanely sacrificed by IACUC approved procedures. In general, for the iliac crest biopsy, the skin area is carefully cleaned, lidocaine is injected locally to numb the area, and a fine trocar is used to gain access to the marrow space. The aspiration syringe is loaded with 3,000 units of heparin to prevent clotting of the marrow sample. A large gauge needle is inserted and marrow is aspirated with rapid pulls on the syringe plunger, moving the needle to gain access to new marrow areas without removing the needle. Slow steady pulling on the plunger is not desirable as this will likely aspirate less marrow, and more blood as it flows into the space. Successful marrow aspiration is usually limited to 15–20 mLs per side, left and right iliac crest. More volume usually yields more blood, not marrow. This step is perhaps the most important in determining the MSC yield at first passage. This procedure requires some practice, but has given good yield in large animals (dog, goat, monkey, and pig) as well as man. The bone marrow sample can be processed immediately or stored at room temperature for up to 36 h without detrimental effects. For human marrow samples, a commercial source is Poietics, Inc. (a Cambrex company), which handles all of the informed consent issues, obtains the marrow sample, and delivers it to the investigator for under $1,000.

For small mammals such as rabbits, access to the bone marrow in the long bones is possible by a surgical cutdown, and using relatively stiff tubing placed over the end of the aspiration needle to gain access to marrow further down the bone shaft. For rodents, it is necessary to sacrifice the animal(s), dissect the long bones, remove attached fascia, tendons, and muscles, and move the bones to the sterile tissue culture hood. The bones of up to 10 rats or 20 mice can be processed as a single preparation. The bones are wiped with 70% ethanol to reduce any contamination. The ends of the femur or tibia are removed with sterile wire-cutter-type nippers and the bone marrow is extruded into a collection dish using a needle and saline-filled syringe. The needle should not be too small but should fit easily into the shaft of the femur or tibia bone. The marrow is expressed into a sterile disposable dish, such as a 10-cm tissue culture dish.

2.4 Choices of Growth Media for MSCs

Many different formulations of growth media can be used to isolate and grow MSCs. We have followed the recommendations of Caplan as found in Haynesworth et al. 1992 and use Dulbecco's Modified Eagles media (DMEM) containing 1.0 g/L glucose as opposed to the formulation with 4.5 g/L glucose, which is used in the

chondrogenic differentiation media below. Other media that have been used successfully to propagate human MSCs include BGJb, Alpha MEM, DMEM:F12, McCoy's 5A, and RPMI 1640. Although some of these media are richer in certain components than DMEM, they have not proven superior to the original formulation reports and many other works using DMEM.

The other major component of MSC isolation and growth media is fetal bovine serum (FBS). Most media formulations usually use 10% fetal calf serum to provide a mixture of undefined growth factors, cytokines, and attachment factors. It is known that FBS contains platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF or FGF-2), and epidermal growth factor (EGF) as well as small amounts of other growth factors. Numerous studies have tested media formulations that included defined growth factors instead of FBS to culture MSCs, but there is not sufficient evidence that these methods produce larger populations of superior MSCs, usually quite the opposite. It is clear that serum-free defined media lack attachment factors to aid MSC attachment and cell yields tend to be low. Some authors have suggested that all media formulations are rendered similar when fetal calf serum is added to the 20% v/v level. In the past, we tested many lots of FBS before selecting one (or two) for purchase. Fetal bovine serum is a by-product of the commodities market, and its price and availability is subject to fluctuations. For that reason it is not always possible to test several lots of serum from different vendors before purchase. For most uses, probably the best solution is to purchase FBS from vendors that market it for use with MSCs or MSC differentiation kits (Cambrex, Inc., Stem Cell Technologies, Inc., Gibco/Invitrogen, Inc.)

2.5 MSC Growth Medium with 10% FBS and Antibiotics

1. 445 mL DMEM low glucose (Gibco/Invitrogen) or alternatives Mesencult (Stem Cell Technologies, Inc.) or MSCGM (Cambrex, Inc).
2. 50 mL FCS selected for MSCs (Gibco/ Invtirogen) or alternatives Mesenchymal Stem Cell Stimulatory Supplement (Stem Cell Technologies) or Bullet Kit for MSCs (from Cambrex, Inc).
3. 5 mL Antibiotic/Antimycotic mixture (Gibco/Invitrogen).
4. Mix solutions, filter sterilize and store at 4 °C in the dark for up to 2 wk of use.

2.5.1 Additional Solutions and Items

A number of additional solutions common in tissue culture studies will also be used.

1. Trypsin/EDTA: 0.05% trypsin/0.23 mM EDTA (Gibco/Invitrogen).
2. Dulbecco's phosphate buffered saline (D-PBS) (Gibco/Invitrogen).
3. Ficoll or Percoll Density Gradient solution of 1.073 g/mL (Gibco/Invitrogen).