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Original Article

Mesenchymal Stem Cells

Lineage, Plasticity, and Skeletal Therapeutic Potential

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Abstract

The tremendous capacity of bone to regenerate is indicative of the presence of stem cells with the capability, by definition, to self-renew as well as to give rise to daughter cells. These primitive progenitors, termed mesenchymal stem cells or bone marrow stromal stem cells, exist postnatally, and are multipotent with the ability to generate cartilage, bone, muscle, tendon, ligament, and fat. Given the demographic challenge of an ageing population, the development of strategies to exploit the potential of stem cells to augment bone formation to replace or restore the function of traumatized, diseased, or degenerated bone is a major clinical and socioeconomic need. Owing to the developmental plasticity of mesenchymal stem cells, there is great interest in their application to replace damaged tissues. Combined with modern advances in gene therapy and tissue engineering, they have the potential to improve the quality of life for many. Critical in the development of this field will be an understanding of the phenotype, plasticity, and potentiality of these cells and the tempering of patients' expectations driven by commercial and media hype to match current laboratory and clinical observations.

Index Entries: Osteoprogenitor; bone regeneration; cell therapy; mesenchymal stem cell; tissue engineering; osteoblast; marrow stromal cells; regenerative medicine.

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Introduction

In the adult, the osteoblast, the cell responsible for bone formation, is derived from a multipotent marrow stromal fibroblastic stem cell termed mesenchymal stem cell (MSC). These MSCs can give rise to cells of the adipogenic, reticular, osteoblastic, myoblastic, and fibroblastic lineages and generate progenitors committed to one or more cell lines with an apparent degree of plasticity or interconversion. With an increasing aging population, clinical imperatives to augment and facilitate skeletal tissue lost as a consequence of trauma or degeneration have led to increased interest in these progenitor cells. Currently, multiple approaches exist for the treatment of tissue or organ failure or loss, although, in general, none of these options cures the patient, rather disease management (reduced symptom or progression) is the outcome. In contrast, regenerative medicine proposes three main approaches to restore human tissues and organs. Although small-molecule pharmaceutical compounds will undoubtedly have a role to play *(1,2)*, the initial and simplest approach to regenerative medicine is to employ genes or proteins as therapies *(3,4)*. If this initial approach is inappropriate then cell therapies and tissue engineering come to the fore. Cell therapies involve the direct introduction of cells into appropriate structures within the patient, for example, stem cells are injected into the heart

muscle to restore cardiac function (5,6). In skeletal trauma, where there is concomitant loss of tissue architecture, the direct addition of cells alone may not be sufficient to repair damage and therefore the potential regenerative medicine option is to grow a replacement organ or tissue—tissue engineering. This can be done either outside or inside the body, or by a combination of the two approaches. Regenerative medicine thus offers a continuum of overlapping treatment options for a broad range of complex pathologies.

This review concentrates on the characterization and properties of MSCs and their potential application using regenerative medicine strategies to the currently unmet needs of bone regeneration. Current orthopedic surgery procedures to restore failing or damaged skeletal bones require some form of replacement components. Conventionally, these components include totally artificial prostheses (e.g., metal hip joints), nonliving processed tissue (e.g., cadaveric bone), and autologous transplant (e.g., harvested fibula). Unfortunately, all these approaches, although routinely deployed in routine clinical practice for pragmatic reasons, are far from ideal. Given the demographic challenges of an ageing population combined with rising patient expectation and the growing emphasis placed on cost containment by healthcare providers, economic regenerative medicine approaches for skeletal regeneration is a major clinical and socioeconomic need. This rising clinical demand has brought adult MSC and osteoprogenitor cell biology to center stage. To date, a number of evidences has accumulated detailing the factors that regulate and modulate osteoprogenitor differentiation into the mature osteoblast phenotype using a variety of in vitro models, with different species. This has served to provide conflicting observations and interpretations concerning the fate, function, and physiology of the MSC and osteogenic progenitor populations. However, relatively little is known concerning the phenotypic characteristics, whether from a morphological or biochemical standpoint, whereas direct in vivo confirmation of the lineage potential and plasticity or interconversion potential existing for MSCs and osteogenic progenitor cells remains unclear. This review outlines the concepts, properties, and current understanding in MSC biology and their much heralded potential for clinical application in bone regenerative medicine.

MSCs

The capacity for bone to regenerate has long been recognized and yet, to date, there is only limited evidence for the existence of a multipotent MSC. This is, in large part, owing to their low incidence, indeterminate morphology and, despite nearly four decades of research, undefined biochemical phenotype. The bone marrow serves as a reservoir for a variety of stem cells including the nonadherent, circulating hemopoietic stem cells and, in close contact and supporting the haemopoietic compartment, marrow stromal cells. Evidence for a population of cells with multilineage mesodermal differentiation capacity was first demonstrated by Friedenstein and colleagues in seminal studies that demonstrated the ability of mesenchymal populations to generate cartilage, bone, myelosupportive stroma, adipocytes, and fibrous connective tissue (7–9). Since then various names have been ascribed to this population of cells leading to a certain degree of confusion in the literature. These include osteogenic stem cells, marrow stromal fibroblastic cells (10), bone marrow stromal stem cells (11), MSCs (12), stromal precursor cells and, more recently, skeletal stem cells (13). Currently, MSCs (undifferentiated multipotent cells of the mesenchyme) appear to be the favored term adopted by many workers within the field.

A number of criteria need to be fulfilled before a cell can be termed a stem cell, namely, self-renewal, the ability to differentiate into more than one cell type and the capacity for cell division to be maintained throughout life—the definition adopted from the hemopoietic system. Thus a stem cell can be defined as maintaining its own numbers (self-renewal) in spite of physiological or artificial removal of cells from the population by differentiation (14). Stem cells can be divided into two classes based on their capacity to differentiate; pluripotent cells have the capacity to generate every cell type of the organism, whereas multipotent cells are restricted to differentiate into more than one cell type (15). In contrast, progenitor cells derived from stem cells may be multipotential but they lack the capacity for self-renewal and thus are an intermediate between a stem cell and differentiated progeny. It is important to note therefore that osteogenic progenitors are an intermediate between a stem cell and differentiated progeny (i.e., osteoblast).

Friedenstein and colleagues provided evidence for an osteoblast stem cell around four decades ago. Initial studies showed that bone marrow transplanted under the renal capsule of mice or within an enclosed environment (diffusion chamber) generated osteogenic tissue, indicating that marrow contained osteogenic precursors (reviewed in refs. 8 and 16). MSCs give rise to a hierarchy of cell populations within the bone, which can be artificially divided into a number of developmental stages including MSC, determined osteoprogenitor cell, preosteoblast, osteoblast, and ultimately, osteocyte. It is generally accepted the osteoblast is derived from a multipotent MSC, which can give rise to cells of the adipogenic, osteoblastic, chondroblastic, myoblastic, reticular, and fibroblastic lineages (8,16–19) (Figs. 1 and 2). However, it is important to note that distinct boundaries among the populations do not exist; instead, cells form a developmental continuum. Furthermore, homogenous human MSC populations have yet to be isolated.

Isolation of MSCs

The adherent nature of MSCs has been used for over 30 yr to isolate these cells from bone marrow aspirates. The culture of bone marrow mononuclear cells on tissue culture grade plastic enriches for a population of fibroblastic cells which can be expanded in vitro. However, these cells are heterogeneous, containing cells having a range of differentiation potentials (19). In order to understand the biology of the MSC an appropriate homogenous population is a prerequisite. However, such analysis is hampered by the paucity of developmental stage-specific markers for MSCs and osteogenic progenitor cells (20). A few antibodies do exist and have been used to isolate subpopulations of cells from the bone marrow, which after expansion retain their ability to differentiate into bone, cartilage, and fat. Antibodies described to date include monoclonal antibodies SB-10 (21), STRO-1 (22–24), SH-2 (25), and HOP-26 (26). Gronthos and co-workers (27) found the heterogeneity of the stromal cell population could be reduced by isolation using the monoclonal antibody STRO-1, which recognizes

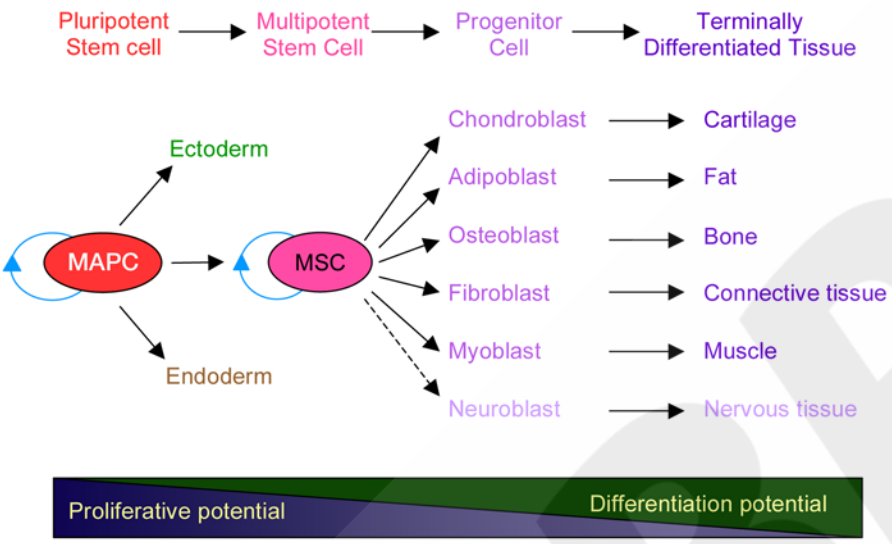

Fig. 1. Differentiation of mesenchymal stem cells from self-renewing stem cells to generate all mesenchymal cell lineages. It should be noted the postulated steps are highly schematic and derived from a wealth of in vitro and in vivo experimentation over the last decade. The potential for neuronal tissue formation remains controversial and is indicated by the dashed line.

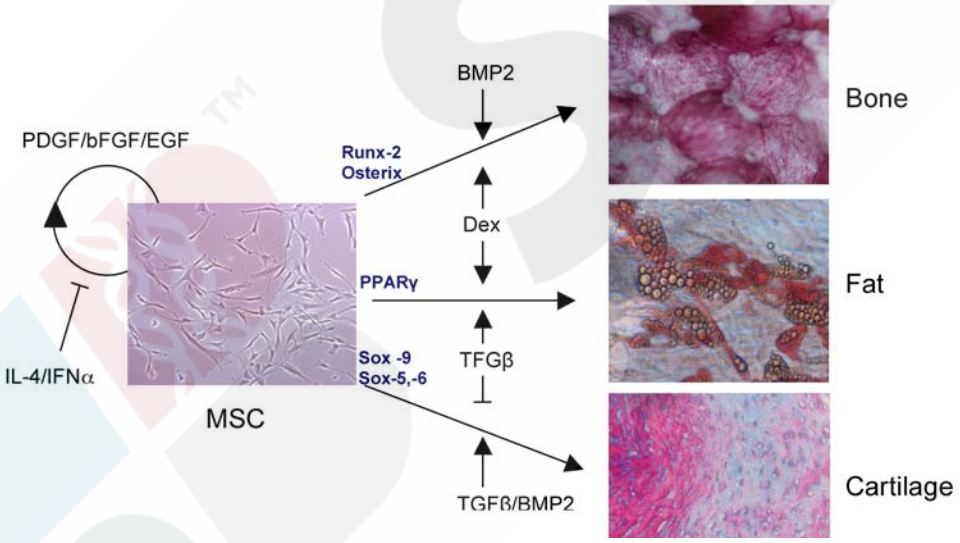

Fig. 2. Isolated mesenchymal stem cells from human bone marrow have been shown to differentiate to mesenchymal lineages. Known regulatory transcription factors are indicated and a selection, although far from exhaustive, of growth factors, hormones and cytokines implicated in commitment, differentiation and proliferation along the selected lineages. Osteogenesis can be identified, typically by increased alkaline phosphatase, collagen type I and osteocalcin expression. Adipogenesis can be identified by neutral lipid vacuoles that stain with oil red O and with adipocyte specific markers such as aP2. Chondrogenesis can be identified by type II collagen, proteoglycan and matrix synthesis typically visualized using alcian blue Sirius red staining.

a trypsin-resistant cell surface antigen present on a subpopulation of bone marrow cells. The cells expressing STRO-1 have been found to include predominantly the adherent, high growth potential, colony-forming units (fibroblastic) (CFU-F) *(27)*. However, the epitope for STRO-1 remains to be characterized although the antibody does not bind to myeloid cells, megakaryocytes, or macrophages but is present on erythroid progenitors *(27)*. The STRO-1-selected populations have been shown to give rise to fibroblastic, adipogenic, and smooth muscle cells as well as cells of the osteoblastic lineage. The cells of the osteoblastic lineage, under osteogenic conditions, become alkaline phosphatase-positive, respond to 1,25(OH)2D3, form mineralized tissue in vitro and in vivo, and form osteogenic tissue *(27–29)*. Beresford and co-workers *(28)* have deployed cell sorting to show that there is an inverse association between the expression of STRO-1 and alkaline phosphatase. Preosteoblasts or progenitor populations were observed to be STRO-1-positive and alkaline phosphatase-negative, whereas osteoblasts were observed to be STRO-1-negative and alkaline phosphatase-positive. Epitopes for a number of monoclonal antibodies shown to be reactive with early osteoprogenitor populations have been identified; thus SB-10 which has been

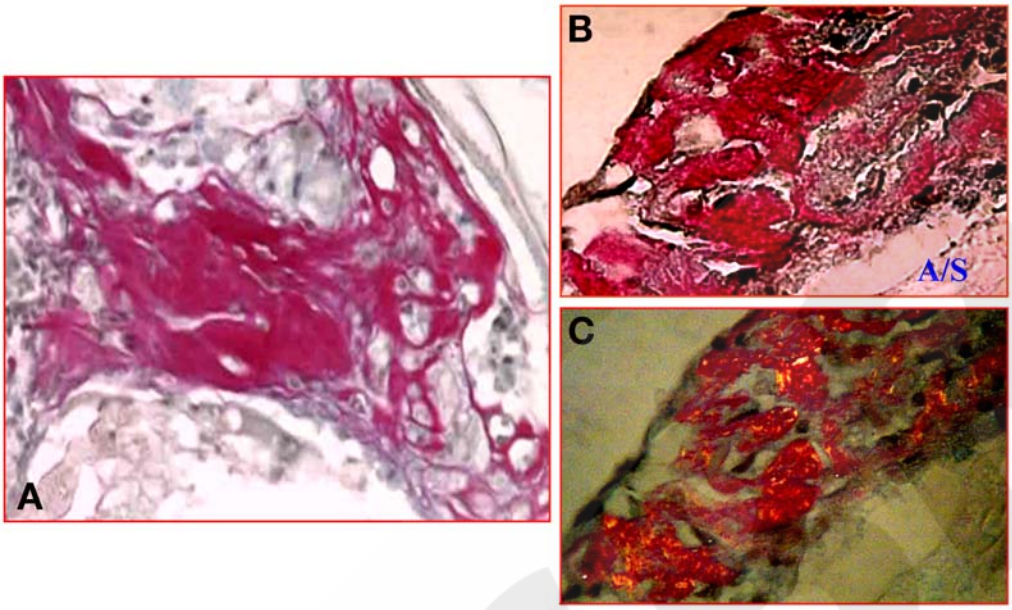

Fig. 3. Bone formation in vivo **(A)** and in vitro **(B,C)** using human osteoprogenitor cells on PLA scaffolds. **(A)** Bone formation observed following subcutaneous implantation of human osteoprogenitors with pleiotrophin on PLA scaffolds in mF1 Nu/Nu mice after 6 wk. **(B,C)** In vitro mineralization on PLA scaffolds using human osteoprogenitor cells and pleiotrophin on PLA scaffolds stained using alcian blue and sirius red with birefringence microscopy **(C)** (x100).

shown to react with osteoprogenitors at the early stages of differentiation has been found to react with activated leukocyte cell adhesion molecule 99 (ALCAM) *(30)*, and SH-2 with CD105 (endoglin) *(31)*. The epitope for HOP-26, derived using human bone marrow fibroblast cultures, has been shown to be the cell surface and lysosomal enzyme CD63 or melanoma-associated antigen, a member of the tetraspan glycoproteins *(32)*. In an attempt to circumvent the limitations in purification of homogenous populations of MSCs, Gronthos and colleagues *(33)* have reported the ability to isolate an enriched population of adult human MSCs using STRO-1 in combination with an antibody directed against vascular cell adhesion molecule 1 or CD106 capable of differentiation along the osteogenic, adipogenic, and chondrogenic lineages. Fluorescence-activated cell sorting analysis by Waller and colleagues *(34)* indicated human fetal bone marrow stromal progenitor cells were present in the $CD34^+$, $CD38^-$, $HLA\text{-}DR^-$, and $CD50^-$ fractions. In an extension of such approaches, Jones and co-workers *(35)* reported that mesenchymal progenitor cells could be isolated from human bone marrow using positive selection with D7-FIB (antifibroblast) conjugated microbeads followed by FACS for $CD45^{low}$ cells to give a $CD105^+$, $LNGFR^+$, $HLA\text{-}DR^+$, $CD1^+$, $CD13^+$, $CD90^+$, $STRO\text{-}1^+$, and $BMPR1A^+$. A surprising observation from this study was that these cells were positive for major histocompatibility complex (MHC) class II when isolated from the bone marrow, but became MHC class-II-negative on culture. All of the above studies highlight the need for the identification of additional markers to allow the definitive identification of MSCs in vivo and in vitro. Progress in this area may soon come from the use of gene expression microarrays allowing the identification of new cell surface-expressed markers.

Plasticity and Interconversion Potentials

The widely accepted key criterion to define a MSC is its ability to form bone cartilage, fat, tendon, and myogenic tissue. Studies from a number of groups have shown that bone marrow cells can be plated onto tissue culture plastic and the initial adherent bone marrow-derived stromal colonies are derived from a single MSC (reviewed in ref. *16*). These colonies are multipotent and can be induced to form bone, cartilage, and fat by simple manipulation of culture conditions *(19,36,37)*. As detailed above, committed progenitors (i.e., restricted in lineage development such as bone under defined conditions) can be identified by functional in vitro CFU-F assays *(38)*. Thus, these stromal colonies retain their multipotentiality in vitro after expansion. Culture in the presence of dexamethasone, methyl isobutylxanthine, insulin, and indomethacin has been found to favor adipogenesis of human bone marrow cells, whereas culture in the absence or presence of serum with transforming growth factor-β (TGF-β) favors chondrogenesis *(36,19)*. In contrast, it is well established that marrow cells cultured in serum with dexamethasone and ascorbic acid favor osteoprogenitor differentiation with enhanced alkaline phosphatase expression, matrix production, nodule formation, and deposition of calcium, confirming the presence of osteoprogenitor cells *(36)* (reviewed in ref. *39*).

With the understanding of defined conditions required to modulate cell phenotype, the potential for cells to "switch" or differentiate among different phenotypes after considerable differentiation (e.g., along the osteogenic lineage to give a mature osteoblast population) has led to the concept of plasticity of phenotype. It has long been known that an association exists between an increase in marrow adipose tissue and osteopenia (reduction in skeletal bone mass) with increasing

age and in a variety of experimental and pathological conditions, such as disuse osteoporosis and glucocorticoid-induced osteoporosis *(40)*. This suggests plasticity or interconversion potential among the lineages and confirms that the adipocytic and osteogenic cells share a common lineage. Park et al. *(36)* have shown the ability to isolate clonal adipogenic cells and to redifferentiate the cells into osteogenic and adipogenic lineages in vitro, whereas Nuttall and co-workers *(41)* have shown the potential of human bone cells to transdifferentiate along the adipogenic lineage. However, osteoadipogenic manipulations in vitro have not yet been conclusively demonstrated in vivo.

Evidence of skeletal muscle and neuronal plasticity from mesenchymal populations has emerged in the past 5 yr (reviewed in ref. *42*). Ferrari and co-workers *(43)* successfully transplanted genetically-marked mouse bone marrow into immunodeficient mice and showed the migration of the labeled marrow-derived cells into areas of induced muscle degeneration. Limited evidence of myogenic differentiation and the formation of fully differentiated muscle fibers were observed. In addition, Sanchez-Ramos and colleagues *(44)* have shown that human and mouse MSCs, at least under defined conditions in vitro, can differentiate into neuron-like cells (ectodermal tissue) that express neuron-specific nuclear protein and glial fibrillary acidic protein. These are exciting studies; however, caution is required in interpretation of such plasticity—the study from Ferrari et al. *(43)* involved the use of total bone marrow transplantation rather than isolated stromal progenitors; thus a role for hematopoietic cells cannot be excluded, whereas evidence of neural markers is not a definitive evidence of functionality. Thus, the extent and physiological relevance of cell plasticity remains unclear, as does the potential role(s) of MSC plasticity in disease states or variation with ageing. Nevertheless, the ability to exploit MSC plasticity is clinically attractive from a tissue engineering and regenerative medicine perspective.

In 2001, studies from Verfaillie's group identified a population of mesodermal progenitor cells (MPCs) which copurify with the MSC fraction of bone marrow mononuclear cells *(45)*. These cells were enriched from the bone marrow by depleting the mononuclear fraction of $CD45^+/GlyA^+$ cells. The enriched cells were cultured at a low cell density in a low serum media supplemented with a range of growth factors including platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). The expanded progenitor cells were negative for the cell surface markers CD34, CD44, CD45, CD117 (*c*-kit), and MHC classes I and II. MAPCs had a greater expansion capacity than MSCs and were capable of differentiating to mesodermal lineages, but unlike MSCs, had the potential to differentiate in vitro to cells with functional attributes of endothelial cells and hepatocytes *(45–47)*. Verfaillie's group later isolated MPC (later termed multipotent adult progenitor cell [MAPC]) from a range of species including mouse and human. They were able to show that murine MAPCs were pluripotent by the in vitro differentiation of MAPCs into endoderm, ectoderm, and mesoderm lineages as well as by the generation of chimeric mice by the injection of MAPCs into blastocyst-stage embryos *(17)*. However, progress in this field has been hampered by the lack of reproducibility of the original observations outside the lab of Verfaillie. This is due in part to the extreme fastidious culture regime required for expanding these cells. After optimization of the culture conditions described by Verfaillie, the group of one of the authors has successfully isolated MAP-like cells which could be induced in vitro to differentiate into cells with characteristics of mesodermal, endothelial, and neural lineages but not hepatocyte fates (Elliman, Clements, and Boshoff, unpublished data). If MAPCs are identified in vivo and shown to contribute to tissues outside of the mesoderm lineage, then it is possible that these cells represent precursor of MSCs (Fig. 1).

To demonstrate the plasticity of adult bone marrow progenitor cells, many studies have looked at the engraftment of these cells in various animal models. Excitement in the field was provoked when bone marrow-derived cells were found to engraft in a variety of tissues including liver, heart, and brain, giving rise to functionally active cell types. However, excitement has been tempered by a number of issues that remain regarding these results. It has become increasingly evident that many of these reports are not owing to transdifferentiation of the bone marrow cells, but are instead the result of fusion events. It has been found that bone marrow cells can fuse with a variety of tissues generating hybrid cells, expressing characteristics of both lineages *(48,49)*. In studies in which true transdifferentiation of bone marrow cells has been demonstrated, the identity of the progenitor cells has not been established owing to issues of purity of the engrafted material.

In Vitro Differentiation and Expansion of MSCs

A number of growth factors have been shown to affect MSC and osteoprogenitor cell activity although it is important to note culture conditions (e.g., serum-free vs serum), species studied (human marrow vs mouse marrow or rat calvarial-derived populations) play a significant part in the response or interpretation observed. Gronthos and Simmons *(23)* examined the growth factor requirements of selected (STRO-1) human bone marrow stromal precursors in serum-deprived conditions in vitro and found from 25 purified recombinant growth factors examined, PDGF and EGF were able to initiate and support clonogenic growth of CFU-F, whereas interleukin-4 (IL-4) and INF-α inhibited colony formation. In a later study, Kuznetsov and colleagues *(50)* found the initial proliferation of human and mouse marrow stromal fibroblast colony formation required at least four growth factors; PDGF, bFGF, TGF-β, and EGF, with differing requirements between mouse and human marrow stromal fibroblasts. More recently, Oreffo and co-workers *(51)* showed INF-α-inhibited human osteoprogenitor cell proliferation, CFU-F formation, alkaline phosphatase expression, HOP-26 expression as well as modulating bone morphogenetic protein-2 (BMP-2) expression. A number of studies indicate basic FGF (FGF-2) and the glucocorticoids are potent mitogens for marrow stromal fibroblasts. FGF-2 was found to increase both colony number and size markedly for human stromal cells although colony formation was unaffected *(52)*. For human MSCs, FGF-2 was shown to increase the growth rate and life-span of the cells without affecting their ability to differentiate to chondrocytes, osteocytes, or adipocytes *(53)*. Furthermore, it appears that FGF-2 acts on early osteogenic progenitors, possibly maintaining cells in an immature state, since in combination with the synthetic glucocorticoid dexamethasone, further cell maturation was observed *(54)*.

TGF-β and other members of the TGF-β superfamily, including the BMPs, play a significant role in the induction, differentiation, and modulation of osteoprogenitor cells (reviewed in refs. *55* and *56*). TGF-β has complex biphasic actions on progenitors and on their differentiation. TGF-β has been shown to act on committed or determined osteogenic precursors to stimulate proliferation and chemotaxis to generate populations of committed osteoblast cells *(57)*. Locklin and co-workers *(58)* showed TGF-β in the absence of serum-promoted cell growth and alkaline phosphatase activity, whereas in the presence of serum, matrix proteoglycan and collagen synthesis were increased in human bone marrow stromal fibroblasts. In the same model system, TGF-β inhibited dexamethasone-induced adipogenesis. More recently, Walsh and co-workers *(59)* found that TGF-β1 limited the expansion of the osteoprogenitor fraction of human bone marrow cells and thus significantly reduced subsequent alkaline phosphatase expression with implications for the clinical potential of these cells on expansion.

The BMPs, which like the TGF-βs are sequestered within bone matrix, play a pivotal role in the initiation of MSCs or inducible osteogenic precursors into the osteoblast lineage. BMP-2, BMP-4, and BMP-7 have been shown to induce the mouse pluripotent fibroblast cell line C3H10T1/2, and mouse 3T3 cells to differentiate into osteoblasts, chondroblasts, and adipocytes *(60)*. Similarly, Rickard et al. *(61)* have shown that BMP-2 can induce osteoblast differentiation in rat bone marrow cell cultures. In addition, differential roles for BMP receptor types IB and IA in the differentiation and specification of mesenchymal progenitor cells to either osteoblasts or adipocytes have been demonstrated by Chen and co-workers *(56)*.

In vitro, dexamethasone has been shown in rat, mouse, and rabbit as well as human marrow stromal cultures to stimulate osteoprogenitor cell differentiation although the known effects of glucocorticoid-induced osteoporosis and impaired fracture repair remain confounding issues *(58,62–67)*. Other stimulatory factors of note include (1) PGE_2, which may act to promote osteogenesis by the recruitment of osteoprogenitor cells from the nonadherent MPCs in bone marrow acting possibly via EP2 receptors *(68–71)*, (2) pleiotrophin (PTN), an extracellular matrix associated protein, which has been found to induce human osteoprogenitor chemotaxis, proliferation, differentiation, and bone formation *(72,73)*, and (3) osteoclastotrophic or bone remodeling factors IL-1, IL-6, granulocyte/macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF), which have all been implicated in the modulation of osteoprogenitor proliferation and differentiation *(74–76)*. Ultimately, delineation of the growth factors and hormones required for human MSCs and osteoprogenitors will be needed to generate protocols for the rapid expansion, ex vivo, of these cells (using autologous serum), whereas retaining their differentiation potential for tissue engineering or regeneration applications.

Aging and MSCs

Ageing is associated with decreased fracture repair and reduced skeletal bone mass as a consequence of a net reduction in bone formation. This can result in osteoporosis with devastating socioeconomic consequences. As indicated, bone formation depends on MSCs present within bone marrow which in vitro give rise to CFU-F that differentiate into the osteogenic, adipogenic, fibroblastic, and reticular cell lineages *(8,16,17,39)*. Studies on human osteoprogenitor number and age have been limited and contradictory. Nishida et al. *(77)* found that the ability to form alkaline phosphatase-positive (AP^+) CFU-F was significantly reduced between the ages of 10 and 20 yr and then only gradually reduced after the age of 20. Similarly, D'Ippolito et al. *(78)* found a significant decrease with age in AP^+-CFU-F in bone progenitors isolated from human vertebrae. Other workers have recorded decreases with age in CFU-F colonies or AP^+-CFU-F in human *(79)*, rat *(80)*, and mouse *(81)* marrow. However, Oreffo et al. *(82,83)* in a study of 99 patients who were osteoarthritic, osteoporotic, or without evidence of metabolic bone disease, found no differences in CFU-F or AP^+CFU-F with age, disease state, or gender, although a significant decrease in CFU-F colony size was observed. The decreased CFU-F colony size may be owing to replicative senescence (growth arrest) caused by reduced telomere length associated with age. Telomeres are repetitive DNA sequences that protect the end of chromosome during replication *(84)*. During replication telomeres become shorter, eventually leading to chromosome instability resulting in replicative senescence *(85)*. This acts as a countdown mechanism to limiting the proliferative capacity of cells. Telomere length decreases with age and hence could explain the limited proliferative capacity of mesenchymal cells in older patients *(86)*. However, this process can be reversed by the overexpression of telomerase in MSCs by genetic manipulation *(87–89)*. Overexpression of telomerase restores telomere length extending the replicative capacity of the cells indefinitely with the cells retaining their ability to differentiate into osteocytes, chondrocytes, and adipocytes. However, researchers are still uncertain for the safety of such immortalized stem cells. For example, in vitro, Serakinci et al. *(90)* have demonstrated that some populations of immortalized human MSCs lack contact inhibition, anchorage dependence, and also formed tumors in mice. However, others have demonstrated that when transplanted into mice, such cells were able to form bone with no evidence of tumor formation *(88,89)*. These results suggest that the limited proliferative capacity of MSCs can be overcome and it may be a useful strategy in bone regeneration and repair but that caution must be taken to better understand any potential for neoplastic change.

Stenderup et al. *(91)* found the number and proliferative capacity of osteogenic stem cells was maintained during aging and in patients with osteoporosis. The bone loss associated with aging may reflect altered proliferative capacity of progenitor cells or altered responsiveness of CFU-F to systemic or locally released growth factors *(92,93)*, leading to alteration in subsequent differentiation. The complex picture presented from these studies and the discrepancies therein may be attributable to different sample population sizes, populations selected, and laboratory protocols used, but indicate substantial modulation of MSCs with ageing.

Stem Cells, Genes, and Tissue Engineering: Restoring Aging Bones

Osteoporosis is currently defined as a systemic skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone

fragility and susceptibility to fracture (94). Clinically, osteoporosis is recognized by the occurrence of characteristic low-trauma fractures, which typically arise at the hip, spine, and distal forearm. It is estimated that around 40% of US white women and 13% of US white men of 50 yr of age will experience at least one clinically apparent fragility fracture at these sites during their lifetimes (95). However, taking into account sites other than the hip, spine, and distal forearm, the lifetime risk among women aged 50 yr might be as high as 70% (96). The medical costs of osteoporosis and its attendant fractures have been estimated in the US to be \$ (17.9×10^9)/yr, with hip fractures accounting for one-third of this total. In England and Wales, hip-fracture patients alone take up 20% of orthopedic beds, with an estimated cost for all osteoporotic fractures of £ (1×10^9)/yr (97). Furthermore, the overall burden on the public health is set to increase dramatically over the next 60 yr because of the steep predicted increase in the proportion of elderly people in the population. Thus, worldwide, there were an estimated 1.66×10^6 hip fractures in 1990, a figure which is predicted to increase to 6.26×10^6 in 2050 if adequate preventative measures are not taken (96). Around 30–50% of the hip operations will require subsequent revision surgery and, in a significant proportion, bone augmentation will be necessary. With an increasing ageing population, overall health costs are set to rise. In addition, the observation that artificial prostheses, which are subjected to wear owing to lack of integration resulting in aseptic loosening, ultimately fail (reviewed in ref. 98), has further driven research activity to exploit the potential of MSCs in bone repair and regeneration (13,16,99). At present, regimes that encourage bone formation or delivery strategies for osteotropic agents such as the BMPs, which hold the promise of significantly increasing bone density, have proved elusive. Tissue engineering seeks to resolve these issues through a combination of stem or progenitor cells with appropriate growth factors and tailored three-dimensional scaffolds. Thus tissue engineering has been defined as the application of scientific principles to the design, construction, modification, and growth of living tissues (100). As a source of progenitor cells, it has long been known that bone has a vast capacity for regeneration from cells with stem cell characteristics. Kadiyala and co-workers have shown that culture-expanded bone marrow cells will heal a segmental bone defect following reimplantation (101).

Several groups have shown that MSCs and osteoprogenitor populations from a variety of species, including human MSCs, do give rise to osteogenic tissue within diffusion chambers (102–104). As detailed above, human bone marrow osteoprogenitors can be isolated and enriched using selective markers, such as STRO-1, from a $CD34^+$ fraction (105,106) and readily expanded, indicating their potential for marrow repopulation (16,26,107,108). A desirable extension in the use of MSCs for tissue regeneration would be the potential for the use of allogeneic populations allowing the deployment of cells from one or more donors, their preparation, and cryopreservation until required. Studies by Bartholomew et al. (109) and Di Nicola et al. (110) suggest that MSCs may be immune-privileged cells failing to elicit an immune response when combined with allogeneic lymphocytic cells. In support of such an approach, Arinzeh and co-workers (111) have shown that, at least at 4 and 8 wk, allogeneic MSCs aid regeneration in a critical-sized canine segmental defect. No systemic alloantibody production was detected over time (although a few allogeneic cells could be detected at 8 wk); these results add further support to the potential of allogeneic MSCs in cartilage and bone repair. Clinical data from Horwitz and colleagues (112,113) using human bone marrow-derived osteoprogenitors transplanted into children with osteogenesis imperfecta suggest some therapeutic effect of such an approach. Donor-derived MSCs (approx 2%) were capable of homing to the bone marrow and differentiating into the osteoblasts. In follow-up studies, the same group reported an increase in bone mineral content compared with age-matched controls, although the precise contribution of the donor cells to the clinical improvements recorded remains to be determined (114). Connolly (115) as well as Quarto and co-workers (116) have indicated the efficacy of autologous bone marrow stromal cells in the treatment of large bone defects. However, true engraftment of human MSCs, long-term biological effects on the stem cells at the implant site, as well as issues of cell plasticity remains to be defined.

MSC or osteoprogenitor delivery, whether by injection or with a matrix, is attractive and carries a reduced risk of morbidity. However, for large skeletal defects these cells will need an appropriate, designed, or "smart" vehicle/scaffold/matrix tailored to the shape and size of the defect and one which will provide mechanical competence. To aid this process, advances in scaffold technology and gene delivery offer the possibility of genetic modification of isolated and expanded cells in constructs to produce populations of progenitor cells overexpressing selected signaling molecules for bone regeneration (13,114,117–119). Lieberman and co-workers have shown regional cell and gene therapy using BMP-2-producing bone marrow cells on the repair of segmental bone defects in rats (120). Similarly, Breitbart and colleagues (121) have cultured periosteal cells retrovirally transduced with BMP-7 in a poly(glycolic acid) (PGA) scaffold in a critical sized calvarial defect model in rabbits. Several studies indicate human bone marrow stromal cells expressing BMP-2 by adenoviral infection may prove efficacious in bone regeneration (29,122–124) (Fig. 2). Musgrave and co-workers (125) reported on the use of direct adenovirus-mediated gene therapy to deliver active BMP-2 and produce bone in skeletal muscle, thus circumventing problems associated with delivery of BMP-2 and the requirements for cell expansion. However, limitations of associated immunogenicity in vivo, fate of adenoviral cells, long-term safety, and requirement for cell expansion in culture before viral infection and reimplantation remain significant hurdles before clinical use.

A variety of materials have been used for bone regeneration together with MSCs and osteoprogenitors, these include ceramics or materials based on hydroxyapatite, ceramic forms of β-tricalcium phosphate and composites of both hydroxyapatite and β-tricalcium phosphate (119). In recent years workers have sought to exploit biological cues that are necessary to mimic the cell–bone matrix interactions with the generation of biomimetic scaffolds based on poly(lactic acid), poly (-lactic-*co*-glycolic acid), as well as PGA (126,127). Healy and co-workers (128) and Dee and co-workers (129) have reported on the ability to modulate the adhesion of osteoblasts selectively and significantly by immobilized –Arg–Gly–Asp– (–RGD–) and –Phe–His–Arg–Arg–Ile–Lys–Ala– (–FHRRIKA), and Lys–Arg–Ser–Arg peptides (KRSR). In addition, Yang et al.

(130) have shown the potential to promote human osteoprogenitor differentiation on RGD-coupled biodegradable scaffolds. Continued advances in gene delivery combined with development of smart designer scaffolds and materials in combination with selection of MSC and osteoprogenitor populations are needed to deliver on the promise of skeletal tissue engineering using MSC populations.

Conclusions and Future Perspectives

With an increasing ageing population with ever expanding medical needs and expectations, the potential to grow human tissues from small populations of stem cells is one of the most exciting medical developments of the current era. The biological understanding of stem cells is improving at an extraordinary rate but much greater developments in our understanding of MSC and osteoprogenitor biology are of paramount importance if stem cell therapies are ever to become routine clinical practice. In particular, from an orthopedic patient's perspective, the ultimate goal will remain the utilization of MSCs and progenitors in simple, safe, and reproducible strategies for the augmentation of bone, cartilage, and tendon repair to address the pressing, clinical, unmet need of many. Tissue engineering could provide suitable, efficacious, alternative therapies for orthopedic applications such as nonunion fracture, healing of critical-sized segmental defects, and regeneration of articular cartilage in degenerative joint disease. It is attractive on a number of fronts.

1. The ability to engineer tissue in vitro for transplantation would reduce the requirement for donor tissue as the number of skeletal cells required could be expanded ex vivo prior to implantation.
2. Treatment of diseased tissue at an early stage with MSCs could alleviate or even cure the disease, reducing the need for life-long treatment and improving the quality of life of the patient and potentially reducing overall costs to society.

Thus, cell delivery vehicles, composite tissue engineering protocols, and smart materials which can orchestrate the bone formation cascade will all undoubtedly have their place. However, it is clear that for many strategies to reach clinical trials, structural, biological, and safety issues will need to be resolved. Nevertheless, the ability to select, expand, and differentiate these cells and the potential for ex vivo gene transfer suggests the potential future use of human osteoprogenitors for gene therapy of metabolic bone diseases. Clearly, harnessing the homing potentials of such cells and channeling their development into the required human tissues for regeneration is a significant challenge. It will be necessary to gain a greater understanding of the continuum of cell development of MSCs to osteoprogenitor to preosteoblast, and ultimately, osteocyte and the role of the matrix microenvironment and factors that control their fate and plasticity. Whereas development pathways are clearly delineated from in vitro observations, their relevance in vivo and the extent of applications to physiological population modulations (e.g., adipocyte to osteoblast) are unclear. Ultimately, the need to define the phenotype of the MSC, to generate a phenotypic fingerprint, will enable isolation and expansion of populations for clinical use. These ultimately may improve the quality of life for many as a result of strategies to augment skeletal regeneration.

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