Adipose-derived Mesenchymal Cells (AMCs): A Promising Future for Skeletal Tissue Engineering

YUE XU, PREETI MALLADI, DIANE R. WAGNER, MONIKA TATARIA, MICHAEL CHIOU, KARL G. SYLVESTER AND MICHAEL T. LONGAKER*

Department of Surgery, Stanford University School of Medicine, 257 Campus Drive, Stanford University, Stanford, CA 94305-5148, USA

Introduction

The reconstruction of bony defects due to congenital deficiencies, degenerative skeletal disease, complex post-surgical deficits, osteomyelitis, and fracture non-union represents a substantial biomedical burden. These repairs often require harvesting autogenous bone from other anatomic sites, potentially causing donor site morbidity (Saito et al., 2001; Boo et al., 2002; Ikeuchi et al., 2002). Alternatively, the implantation of a prosthetic bone substitute is less than optimal given its lack of physiologic attributes, which can lead to infection, unpredictable graft resorption, structural failure, or unacceptable aesthetic outcomes (Mulliken et al., 1980; Fong et al., 2003). We propose that, in the future, cell-based therapies that exploit the regenerative potential of adult multipotent stromal cells (MSCs) will be a substantial improvement over currently available treatment modalities for skeletal defects.

MSCs are a heterogeneous population of cells defined by an ability to differentiate to various mesodermal-derived structural tissues such as the osteogenic, chondrogenic, myogenic, and adipogenic lineages. These cells have thus far been isolated from several adult tissues such as bone marrow (BM), peristeum, trabecular bone, synovium, skeletal muscle, deciduous teeth, and adipose tissue (Barry and Murphy, 2004). Recent studies have suggested that adipose tissue contains multipotent cells that are similar to those derived from other tissues, such as bone

*To whom correspondence may be addressed (Longaker@stanford.edu)

Abbreviations: ABCG2, ATP-binding cassette sub-family G member 2; Cbfal/Runt2, core binding factor alpha-1/runt-related gene-2; ERK1/2, extracellular signal-related kinase 1/2; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; HLA-DR, human leukocyte antigen-D-related; HSC, haematopoietic stem cell; hTERT, human telomerase reverse transcriptase; SCID, severe combined immunodeficiency.
marrow. Mesenchymal cells isolated from adipose tissue have, to date, demonstrated differentiation capacity along osteogenic, chondrogenic, myogenic, and adipogenic lineages. In particular, researchers have demonstrated that adipose-derived mesenchymal cells (AMCs) share similar characteristics as MSCs derived from adult bone marrow through their differentiation capacity in vitro (Zuk et al., 2001, 2002; Gimble and Guilak, 2003a,b; Gimble and Nuttall, 2004). AMCs have generated a large amount of interest for clinicians and stem cell biologists because they are a relatively abundant and easily accessible source of multipotent cells.

Characterization of AMCs and their differentiation pathways is critical to further development and optimization of these cell-based therapies. In this context, recent studies relating to the osteogenic and chondrogenic capacity of AMCs are discussed. This article reviews the current knowledge of AMCs, with a particular focus on efforts to direct these cells towards skeletogenesis. We anticipate that cell-based therapies using AMCs will be of great utility for skeletal tissue engineering.

**Phenotypic studies of adipose-derived mesenchymal cells**

In order to biologically characterize AMCs and their similarities to bone marrow (BM)-derived MSCs (BMSCs), numerous early studies focused on an array of various cell surface markers (Otto and Rao, 2004). Zuk carefully studied the phenotype of human AMCs and found that they share many of the previously described mesenchymal cell surface markers as BMSCs, including CD29, CD44, CD71, CD90, CD105, and Stro-1 (human only). Combinations of these same surface antigens have been used to enrich MSCs from bone marrow and adipose tissue (Baksh et al., 2004). Most reports on BMSCs have concluded that various hematopoietic markers, including CD31, CD34, and CD45, are not represented in the MSC population of cells in BM. Direct comparisons of mesenchymal stem cells from human bone marrow and adipose tissue have been reported by Lee (Lee et al., 2004). These studies demonstrate that human-derived AMCs have similar expression profiles to BMSCs. Studies utilizing flow cytometry have revealed several differences in the surface antigen expression profiles of BMSC and AMCs. Most notably, several markers originally described in the hematopoietic system and for histocompatibility are more highly represented in human BMSCs, including c-Kit, CD34, HLA-DR, and CD14 (Grontos et al., 2001, 2003; Lee et al., 2004). Preliminary microarray studies comparing undifferentiated AMCs and BMSCs detected no significant differences in an expression panel of 28 genes (Winter et al., 2003). Most recent characterizations of human AMCs using microarray and FACS approaches have revealed many similarities to the profile of BMSCs. Results from these and other studies have shown that AMCs do not possess other well-described characteristics of stem cells from other systems, including increased telomerase expression, and cannot be enriched as a side population of cells through the activity of the membrane transporter ABCG2 (Katz et al., 2005). Future efforts to isolate and characterize the subset of MSCs, from both bone marrow and adipose tissue, which sustains the plasticity of progenitor cells, will enhance the clinical utility of MSCs and potentially advance our basic understanding of them.

**Self-renewal potential and maintenance of AMC plasticity**

As a potential cell source, bone marrow-derived mesenchymal cells (BMSCs) have
demonstrated significant, but highly variable, self-renewal capacity in vitro. Higher population doublings (PD) have been achieved by the addition of specific growth factors such as fibroblast growth factor (FGF)-2 (Bianchi et al., 2003). A comparison of the growth kinetics of AMCs and BMSCs demonstrated that the population doubling times were not significantly different (de Ugarte et al., 2003). An increasing number of studies have focused on extending the life span of the AMCs. One approach (Lin et al., 2005) was to modify the culture conditions by reducing the calcium concentration of the media, which accelerated the growth rate and extended the life span of AMCs. That study demonstrated that cumulative population doubling level (cpdL) of human AMCs cultured in low calcium supplemented with N-acetyl-L-cysteine and L-ascorbic acid-2-phosphate reached a total of 35 cpdL in 62 days, respectively, compared to the reported AMC cultivation of 21 cpdL in 165 days in standard (non-low calcium conditions). Furthermore, the cells were characterized and retained their ability to differentiate into osteoblasts, chondrocytes, and adipocytes. A genetic approach introduced a viral vector expressing telomerase (TERT) into the AMCs; this extended longevity (AMCs underwent more than 100 PDs) and maintained the osteogenic potential of these cells in vivo (Jun et al., 2004; Kang et al., 2004). As expected, the transduced AMCs had an increased level of telomerase activity and increased mean telomere length. In contrast, the non-transduced cells only underwent 12 passages of self-renewal in vitro. In our laboratory, we have observed that juvenile AMCs have a more robust potential for extensive proliferation than mature AMCs in mice (Shi et al., 2005).

Cytokines and growth factors can modulate stem cell growth through their physiological signalling pathways. A recent report has shown that treatment by oncostatin (OSM), a member of the interleukin-6 (IL-6) family, stimulated the proliferation of human AMCs (Song et al., 2005). Platelet-derived growth factor (PDGF) was reported to induce the proliferation and regulate the migration of human AMCs (Kang et al., 2005). One study using modulation by FGF-2 in BMSC cultures demonstrated that low dose FGF-2 supplementation significantly increased cell proliferation and markers such as collagen I, collagen III, fibronectin, and α-smooth muscle actin (α-SMA). This result implicated the potential application of using FGF-2 in tissue regeneration of tendons and ligaments (Hankemeier et al., 2005). Given that AMCs are a promising source for skeletal regeneration, we have aggressively attempted to maintain the plasticity and self-renewal capability of these cells. The growth factors, FGF-2, Noggin, transforming growth factor (TGF)-β1, were applied to AMCs and growth rates were assessed (unpublished data). Low concentrations of FGF-2 significantly increased the proliferation of AMCs. However, the effect of FGF-2 alone appears incapable of enhancing self-renewal in a clonal population.

Moreover, manipulation of the micro-environment in which multipotent cells are cultivated also has been examined as a possible means of altering their proliferative and differentiation capabilities (Gimble et al., 2003b). Among a variety of non-genetic factors, perhaps the most robust example is oxygenation (Salim et al., 2004). BMSCs demonstrated a pronounced growth rate enhancement in a relatively low oxygen environment (Danet et al., 2003). Given the natural, relatively low oxygen milieu of adipose tissue, we found that oxygen reduction to 2–5% with humidified incubation significantly promoted proliferation of AMCs, which has implications
for the physiological growing conditions of progenitor cells (unpublished data). Furthermore, expansion of AMCs in a reduced oxygen environment preserved the capability of differentiation towards chondrocytes, osteocytes, and adipocytes.

Hedgehog (Hh) and Wnt signalling have been implicated as critical signalling pathways for maintaining plasticity, promoting self-renewal, and regulating the differentiation capabilities of the mesenchymal progenitor cells (Edwards et al., 2005). Studies of Wnt protein effects on haematopoietic stem cells have implied the critical role of Wnts in MSC differentiation (Boland et al., 2004; de Boer et al., 2004a,b). In BMSCs, blocking Wnt signalling by using one of the Wnt inhibitors, Dkkopf (DKK)-1, stimulated the proliferation of human BMSCs (Gregory et al., 2003, 2005). Data demonstrating that DKK-1 promotes re-entry into the cell cycle in MSCs and suggests the critical involvement of this signalling pathway in regulating mesenchymal cell biology. Furthermore, a recent study has shown that over-expressing Sonic hedgehog (Shh) in bone marrow-derived progenitors and adipose-derived cells leads to the successful regeneration of cranial bone defects in an in vivo rabbit model (Edwards et al., 2005). Moreover, conditional deletion of β-catenin in limb and head mesenchyme has demonstrated the critical role of Wnt/β-catenin signalling in control of lineage branch points between osteoblast and chondrocyte precursors (Kolpakova and Olsen, 2005). Therefore, we hypothesized that Wnt/β-catenin contributes to osteoconductive precursor cells in their lineage commitments. We anticipate that additional studies unravelling the role of Wnt signalling in AMC plasticity will be forthcoming and provide further impetus toward the development of gene enhancement strategies for the tissue engineering of bone.

**Osteogenic differentiation capacity of AMCs**

**SUCCESSFUL BONE FORMATION USING AMCS IN VIVO**

As an example of the potential for AMCs to be applied to tissue engineering applications in vivo, recent studies have reported that AMCs are capable of healing calvarial defects in both mice and humans. Our group has demonstrated that AMCs can regenerate critical size calvarial defects in mice by using a novel apatite-coated poly lactic-co-glycolic acid (PLGA) scaffold (Cowan et al., 2004). Our results provide evidence that AMCs have an osteogenic potential that is similar to the BMSCs. An exciting recent case report from Germany described the use of autologous AMCs, bone chips, and fibrin glue to treat a large traumatic calvarial defect in a seven-year-old child (Lendeckel et al., 2004). In that study, autologous cells derived from fat were processed and applied to the skull defect. Clinical follow-up showed uneventful skull healing with no neurological deficits. These in vivo experiments and clinical data highlight AMCs’ therapeutic potential in skeletal tissue engineering and regenerative medicine (Strem and Hedrick, 2005).

In addition to mouse and human in vivo studies, several studies have utilized AMCs as the osteogenic cell source with which to investigate various combinations of augmented or altered scaffolds to heal bony defects. Most recently, Peterson et al. applied lipoaspirated human AMCs on a collagen-ceramic carrier into rats with femoral defects. In that study, in order to enhance osteoconductivity, AMCs were virally transduced with BMP-2. Histological analysis and biomechanical testing
revealed that the femurs healed with no significant difference compared to non-operated animals (Peterson et al., 2005). Lee et al. demonstrated that in vivo bone formation can occur when pre-differentiated rat AMCs are seeded subcutaneously in rat (Lee et al., 2003). Hicok et al. reported that human AMCs produced osteoid matrix when implanted on hydroxyapatite/tricalcium phosphate (HA-TCP) cubes that had been implanted in SCID mice (Hicok et al., 2004). Taken together, these results demonstrate that multipotent stromal cells isolated from adipose tissue are an attractive source of autologous cell-based therapy. These cells may have the added advantage over BMSCs as they are easily obtainable and, thus far, are readily propagated in vitro.

STANDARD OSTEOGENIC DIFFERENTIATION IN VITRO

Although the methods for osteogenesis of mesenchymal cells, especially bone marrow-derived, have been well documented, the mechanisms underlying osteogenic induction have not yet been elucidated fully. Zuk and colleagues initially demonstrated that human AMCs have a strong capacity for osteogenic differentiation in vitro (Zuk et al., 2002). In that study, freshly isolated heterogeneous AMCs were exposed to different media treatments and underwent lineage-specific differentiation. Osteogenesis was induced using a standard osteogenic media containing Dulbecco's modified Eagle's medium (DMEM), 50 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate, and 10% fetal bovine serum supplemented with 0.1 μM of dexamethasone (see Figure 15.1). Osteogenesis was assessed using a combination of early and late osteogenic gene expression. In addition, the osteoid phenotype of matrix mineralization was demonstrated by the appropriate staining of cultured cells. For example, at two weeks of differentiation, an increase in alkaline phosphatase activity and staining corresponded to an induction of early genetic markers of osteogenesis: alkaline phosphatase, osteopontin, and the osteogenic transcription factor, Cbfa1/Runt2 (Zuk et al., 2002). Sequentially, at four weeks, terminal differentiation was detected by von Kossa/Alizarin Red S staining specific for calcium deposits, and osteocalcin gene expression was mostly upregulated, corresponding to the extracellular matrix mineralization (de Ugarte et al., 2003). In contrast, for the differentiated AMCs, the gene expression of peroxisome proliferator-activated receptor (PPAR)γ, an adipogenic differentiation marker, significantly decreased while the osteogenic markers were induced. The reduction of adipogenic marker such as PPARγ is consistent with pro-osteogenic differentiation. In summary, the above lineage-specific gene markers were utilized to examine the reciprocal relationships among multi-lineage differentiation.

ENHANCEMENT OF OSTEOGENESIS BY HORMONES AND CYTOKINES

Besides dexamethasone, Zuk et al. has demonstrated that osteogenic differentiation is promoted by adding 0.01 μM 1,25 dihydroxyvitamin D3 (vitamin D) to osteogenic cultures of AMCs (Zuk et al., 2002). Our laboratory has found recently that retinoid acid (RA), which influences cellular differentiation and regulates gene expression in embryonic stem cells, also plays an important role in AMC osteogenic differentiation (Cowan et al., 2005; Shi et al., 2005). This hormone is known to influence
Figure 15.1. Osteogenic and chondrogenic differentiation pathways for AMCs. Differentiation media components are indicated next to the large arrows. The effects of mechanical stimuli, such as tension and compression, on AMC differentiation are still under investigation.
gene expression via two families of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). Although the detailed mechanisms of these pathways are not yet elucidated, hormones that harbour the identical phenomenon in osteoblast differentiation are worth investigating. Published work from our laboratory (Cowan et al., 2005) has shown that in vitro pre-induction of bone by RA can have unwanted consequences in vivo, such as increased rates of bone turnover. In our study, we clearly showed the bone turnover was induced by ex vivo RA treatment of AMCs that subsequently underwent osteoclast differentiation when implanted in vivo. Hence, induced osteoclasts surprisingly reabsorbed the newly formed bone in mouse skull (Cowan et al., 2005). Alternatives such as novel vitamin D analogues, that selectively increase osteoblastic formation, are being developed (Holliday et al., 2000; Shevde et al., 2002), and these compounds may be incorporated into AMC-based strategies for skeletogenesis.

Cytokine signalling via the bone morphogenetic protein (BMPs) members of the transforming growth factor-β superfamily, have been implicated in differentiation of osteo/chondro-progenitor cells (Dragoo et al., 2003; Peterson et al., 2005). BMPs are well established as essential for embryonic skeletogenesis. Studies into the synergistic effects between cytokines such as BMPs, BMP antagonists, and hormones (RA and/or vitamin D) may provide insight into the molecular mechanisms of AMC osteo/chondro-lineage commitments.

Chondrogenic differentiation capacity of AMCs

CARTILAGE PATHOLOGY AND DEFECTS

Pathologic states of cartilage repair and homeostasis from trauma and degenerative joint disease in an expanding elderly, yet active, population is projected to produce the largest increase in the number of new patients of any disease in the US (CDC, 1994, 1999). Because of the poor regenerative capability of cartilage tissue, current therapies are limited. The only cell-based therapy being utilized is autologous chondrocyte transplantation (Brittberg et al., 2001). This technique requires invasion of the joint space to harvest donor chondrocytes from cartilage at the periphery of the joint, expansion of the cells in vitro, and then a second operative procedure for implantation at the site of the cartilaginous defect. In addition to donor site morbidity and a low cell yield from cartilage, a frustration for clinicians is that chondrocytes, especially from aged donors, have a poor proliferative capacity, thus limiting their expansion capabilities (Barbero et al., 2004). Thus, there is increasing need for an alternative cell source for cell-based cartilage repair.

DEVELOPMENT OF CHONDROGENIC DIFFERENTIATION TECHNIQUES

The in vitro model of chondrogenesis in AMCs is still in its early stages, with only a few studies published in the literature. Since chondrocytes are known to maintain their phenotype only in a three-dimensional culture and de-differentiate in monolayer, embryonic stem cells and bone marrow stem cells have been cultured in a variety of three-dimensional culture systems using either a micromass technique (Ahrens et al., 1977), pellet culture, or by seeding into a scaffold to induce chondrogenesis. A comparative study of chondrogenic phenotypical changes between
'alginate bead' and 'pellet' culture systems has proven that the 'alginate bead' culture is the ideal model for articular cartilage as it induced a greater relative expression of collagen II (Yang et al., 2004). Consistent with these culture methods, AMCs have been studied using three-dimensional culture systems under the influence of chondrogenic media including TGF-β (Zuk et al., 2001; Lisignoli et al., 2005). These AMCs expressed chondrocyte markers such as aggrecan, collagen II, chondroitin-4-sulfate, and keratin-sulfate (Huang et al., 2004b,c). Awad and colleagues have examined AMCs in alginate, agarose, and gelatin scaffolds, finding that all support chondrogenesis (Awad et al., 2004). Other pro-chondrogenic cytokines, such as members of the TGF-β superfamily, have been used in studies of BMSC chondrogenesis, but yet have to be studied in AMC differentiation culture (see Figure 15.1). An exciting in vivo study by Nathan demonstrated that osteochondral defects in rabbits repaired with implantation of AMCs were superior biomechanically compared to osteochondral plugs or implantation of cells from periosteum (Nathan et al., 2003).

REGULATION OF CHONDROGENIC DIFFERENTIATION

Of all the effectors that have been noted during chondrogenesis, ambient oxygen in the micro-environment has a known dominant effect during chondrogenic differentiation in vivo (Wilson, 1986; Malda et al., 2003). Cartilage is an avascular tissue and its formation is one of the early morphogenetic processes during embryonic development involving a series of coordinated events (Olsen et al., 2000). Cultivation of rat BMSCs in reduced oxygen tension (5% oxygen) demonstrated increased alkaline phosphatase activity, calcium content, and von Kossa staining at first passage (Lennon et al., 2001). Subsequently, these cells sustained an increased proliferation compared to cells grown in the control oxygen environment (20% oxygen) and differentiated toward cartilage and bone nodules in vivo under reduced oxygen tension. These data encourage the development of an in vitro system for chondrogenesis in AMCs that has the ability to alter oxygen tension during culture. Our laboratory has established a well-defined, three-dimensional micromass culture system using alcian blue staining to evaluate proteoglycan content. We have identified that hypoxia inducible factor (Hif)1–alpha plays a critical role in chondrogenic differentiation in AMCs (unpublished data).

Various cytokines such as the BMPs, and other TGF-β superfamily proteins, are known to stimulate chondrogenesis. A recent study about the function of BMP-2 in bovine synovium-derived progenitor cells showed enhanced chondrogenic differentiation in 3-D alginate hydrogel (Park et al., 2005). Fibroblast growth factor (FGF)-18 signals through FGF receptor 3 and promotes chondrogenesis in 3-D micromass culture using E11.5 limb bud cells (Davidson et al., 2005). FGF-2 enhances the mitotic effects in human BMSCs and potentiates the chondrogenic differentiation in aggregate culture (Solchaga et al., 2005). Moreover, a one day administration of FGF-2 was sufficient for regeneration of 5 mm full-thickness articular cartilage defects in a rabbit model (Chuma et al., 2004). Most interestingly, studies using human BMSCs in an aggregate culture system showed sustained Wnt protein expression (Wnt4 and Wnt5a), implicating Wnt signalling during chondrogenesis (Nishioka et al., 2005). Further experiments exploring the functional
interaction and potential combinatorial effects of these various pathways may additionally enhance cartilaginous tissue engineering approaches.

**Mechanotransduction and osteogenic/chondrogenic differentiation**

**OSTEOBLASTIC INDUCTION**

Longstanding mechanobiology theories propose that mechanical loading can direct the differentiation of mesenchymal tissues (Pauwels, 1980; Carter et al., 1988, 1998). More recently, MSCs, and BMSCs in particular, have been used to demonstrate the link between mechanical loading and osteo/chondrogenic differentiation in vitro. For example, Thomas and El Haj showed in an early study that equibiaxial tension elevated alkaline phosphatase levels in rat BMSCs, and that treatment with indomethacin, a prostaglandin inhibitor, abolished this load response (Thomas and El Haj, 1996). Simmons et al. demonstrated that cyclic equibiaxial tensile strain enhances bone matrix mineralization in BMSCs through the ERK1/2 pathway (Simmons et al., 2003). Although dexamethasone induces osteogenesis in BMSCs (Beresford et al., 1992; Herbertson and Aubin, 1995), Jagodzinski and colleagues found that cyclic stretching of human BMSCs was even more osteoinductive than exposure to this steroid hormone (Jagodzinski et al., 2004). Koike et al. questioned whether an optimal strain magnitude existed for upregulation of osteoblastic markers and saw that relatively moderate levels of strain induced the highest levels of alkaline phosphatase activity and Runx2 expression in bone marrow stromal cell line ST2 (Koike et al., 2005). Fluid flow also promotes osteogenic differentiation in MSCs; Li and colleagues determined that human BMSCs respond to oscillatory fluid flow with transient increases in intracellular \(Ca^{2+}\) and upregulation of osteoblastic gene expression (Li et al., 2004). Kreke and colleagues showed that steady fluid flow stimulated the expression of osteopontin, osteocalcin, and bone sialoprotein in rat BMSCs (Kreke and Goldstein, 2004; Kreke et al., 2005). The study by Li and colleagues demonstrated an increase in proliferation of BMSCs, while Kreke and colleagues saw no increase; this may be due to differences in the type of fluid flow (oscillatory vs. steady), differences in the species of the cell donors, or the presence of dexamethasone in the Kreke study. Because extracorporeal shockwave treatment promotes the healing of bone fractures (Beutler et al., 1999; Rompe et al., 2001), Wang and colleagues studied the effect of shockwaves on rat and human BMSCs and observed that this treatment increases the osteogenic ability of BMSCs (Wang et al., 2001, 2002). Taken together, these data suggest that mechanotransduction may be a promising in vitro approach to studying the effects of in vivo loading on the induction of bone and cartilage differentiation of mesenchymal progenitor cells, including AMCs.

**OSTEOCLASTIC ACTIVITY**

Cytokines, growth factors, and hormones may accelerate bone formation by stimulating mesenchymal cells to exhibit an osteoblastic phenotype, while at the same time recruiting osteoclasts and promoting bone turnover (Cowan et al., 2005). Therefore, it is crucial to consider osteoclastogenesis while stimulating osteogenesis, including stimulation by mechanical loading. Remarkably, the types of
mechanical loading that induce osteogenesis, such as tensile strain and fluid flow, also have been reported to reduce osteoclastic activity. In a series of studies, Rubin and colleagues have examined the effect of stretch on the osteoclastic activity of mouse BMSCs. In these studies, 1,25-dihydroxyvitamin D$_3$ was used to stimulate mouse BMSCs to form osteoclast-like cells. They showed that equibiaxial straining decreased the expression of receptor activator of NF-κB ligand (RANKL) mRNA via the ERK1/2 pathway, where RANKL is the dominant molecule controlling osteoclastogenesis (Rubin et al., 2000, 2002). This group also showed that endothelial nitric oxide synthase (eNOS) is upregulated by biaxial straining via the ERK 1/2 pathway. eNOS generates nitric oxide (NO), which has an inhibitory effect on both osteoclast formation and activation (Kasten et al., 1994; Collin-Osdoby et al., 2000). Additionally, the response of BMSCs to fluid flow was studied by McAllister and colleagues, who observed that flow increased prostaglandin E$_2$, NO, and prostacyclin (McAllister et al., 2000), all of which are thought to inhibit osteoclastogenesis. The inhibition of osteoclast formation with hydrostatic pressure in BMSCs was first reported by Rubin; they also measured a decrease in the expression of macrophage colony stimulating factor (MCSF), a membrane-bound protein that is required for expansion of the monoblastic cells that supply pre-osteoblasts (Rubin et al., 1997).

CHONDROGENIC EFFECTS

The chondrogenic effect of mechanical loading on BMSCs was reported in 1994 in an in vivo study: Wakitani and colleagues transplanted BMSCs into large articular cartilage defects of rabbit knees. They observed that different local mechanical environments resulted in differences in the mechanical properties of the regenerative tissue (Wakitani et al., 1994). In vitro studies have shown that compressive loading significantly enhances chondrogenesis of BMSCs (Angele et al., 2003, 2004; Huang et al., 2004a; Scherer et al., 2004). While one study reported that mechanical loading alone was sufficient to induce upregulation of chondrogenic genetic markers (Huang et al., 2004a), another study reported that it was not sufficient (Scherer et al., 2004). This discrepancy may be explained by differences in the culture conditions (cells embedded in agarose or not) and differences in loading (compressive loading vs. hydrostatic pressure). In summary, the mechanisms of load-induced differentiation on multipotent cells have yet to be fully elucidated. In the future, AMCs may be used to explore the effects of loading on osteo/chondroinduction and the pathways of mechanotransduction.

McBeath and colleagues have described another interesting mechanism of osteogenic differentiation in human BMSCs (McBeath et al., 2004). This study elegantly demonstrated that cytoskeletal tension, mediated by RhoA and Rho kinase, directs lineage-specific differentiation. Our laboratory has observed a similar association between cell tension and osteogenic differentiation in AMCs (unpublished data).

Future perspectives

CHARACTERIZATION OF AMC SUBPOPULATIONS

Research regarding the application of AMCs for regenerative medicine is still in its
infancy. One caveat regarding the study of AMCs and the associated differentiation signalling pathways is that primary culture AMCs are a heterogeneous population of cells. We and others are beginning to show that primary culture AMCs likely exist as an equilibrium of multipotent cells and more committed progenitors. The several pro-/pre-lineage-specific cells within this population create variables that will likely confound high throughput attempts at profiling gene expression. One potential strategy that has been pursued in other adult stem cell populations is enrichment by specific cell surface markers and characterization of the function of these populations after prospective sorting to increase homogeneity. This strategy can be pursued in AMCs by utilizing the described BMSC markers to identify the multipotent cells or more committed lineage-specific progenitors in AMCs. This approach may allow for enrichment of cells primed to differentiate along a specific lineage, such as bone and cartilage.

An alternative approach to identify subpopulations within AMCs is to sort genetically modified cells based on the functional modification. For example, AMCs can be transfected with vectors containing reporters (such as GFP) attached to lineage-specific gene promoters such as chfl1/Rumx2. The cells initiating bone differentiation can thus be sorted by their GFP activation. It has been shown that AMCs can be transduced by adenoviral, retroviral, and lentiviral vectors, and thus achieve long-term transgene expression (Morizono et al., 2003). Genetic modification of AMCs can be used not only for furthering our understanding of AMC biology, but also as a method for cell-based gene therapy (Peterson et al., 2005). However, given the problems associated with gene therapy, an extensive examination of the possible side effects of gene delivery would need to be carried out in experimental studies before these methods can be considered in the clinical setting.

DEVELOPMENT AND UTILIZATION OF ANIMAL MODELS

The development of robust animal models is critical to advance the understanding of AMC biology and the process of differentiation. Studies in mice are advantageous to study signalling pathways, given the currently available murine reagents and transgenic/knockout animals (Ogawa et al., 2004a,b,c). Larger animal models, such as rabbit and sheep, have been used to study the efficacy of tissue engineered constructs, given the physiologic modelling available in those species. One significant shortcoming yet to be resolved in studies of MSC biology is the lack of a robust in vivo model that allows investigators to determine the stem cell-like properties of MSC, such as re-population of a physiological niche. Early studies aimed at establishing either an ectopic BM niche capable of supporting HSC in vivo or re-constitution of the BM niche by donor MSC during whole bone marrow transplantation have met with little success.

USE OF AMCs AS GENE DELIVERY VEHICLES

Gene therapy represents a promising potential treatment for devastating disorders. MSCs are thought to be a potential target for gene transfer and allowing genetic and phenotypic correction as an application of cell-based therapy. In an attempt to study the mechanisms of self-renewal in MSCs, BMSCs either retrovirally transduced with
human telomerase reverse transcriptase (hTERT) or infected with human papillomavirus showed prolonged cell growth, while maintaining their multipotent differentiation capabilities (Okamoto et al., 2002). However, excessive expansion of stromal cells may lead to a change in their multi-potentials (Pittenger et al., 1999; Reyes et al., 2001); AMCs are ideal candidate cells for gene therapy in this regard because they are abundant and easily obtainable through standard lipoaspiration procedures.

To date, two methods of gene transfer into MSCs have been reported on: non-viral and viral delivery. Non-viral methods of gene delivery, such as electroporation, have been found to be less efficient than viral methods. Keating and Toneguzzo (1990) showed that MSCs from human bone marrow have a lower efficiency of stable integration, thus leading to transient expression of the transgene. Additionally, non-viral transfection requires selection with a marker, such as neomycin resistance, which may result in excessive expansion in vitro. Therefore, utilizing viral vectors such as retroviruses, adenoviruses, and lentiviruses may be the preferred alternative for gene delivery. In the past, human BMSCs have been transduced with adenovirus carrying BMP-2; these cells showed enhanced osteogenic differentiation in vitro (Turgeman et al., 2001; Hamada et al., 2005). When implanted subcutaneously in nude mice, BMP-2 transduced MSCs resulted in ectopic bone and cartilage formation (Turgeman et al., 2001). Similarly, human AMCs transduced with adenovirus carrying BMP-2 have led to in vivo ectopic osteogenesis when seeded onto collagen matrices and implanted into SCID mice (Dragoo et al., 2005). Peterson showed that human AMCs that over-expressed BMP-2 through adenoviral transductions are capable of healing critical femoral defects in a rat model (Peterson et al., 2005). In vivo studies of gene transfer have been performed in BMSCs through adenoviral vectors and may be used alone or in combination with scaffolds for bone regeneration (Franceschi et al., 2004).

Among all these viral delivery methods, in vitro studies have shown that both the lentivirus and adenovirus led to greater than 90% efficiency in AMCs, and were capable of transducing non-dividing cells. Adenovirus, however, led to high levels of cytotoxicity; therefore, lentiviral transduction was found to be preferable (Morizono et al., 2003). After lentiviral transduction, the transgene continued to be expressed after in vitro differentiation (Morizono et al., 2003). The ex vivo expansion and priming by lentiviral transduced genes like BMP-2 is a promising approach for gene therapy using AMCs.

Conclusion

The identification of multipotent cells within adipose tissue has opened a new and exciting chapter in skeletal tissue engineering. AMCs offer great promise for bone reconstruction and cartilage repair based on their capacity for multilineage differentiation. The first steps toward this goal of bedside skeletal tissue engineering are a characterization of AMC subpopulations, a detailed examination of their in vitro and in vivo behaviour, and a better understanding of the signalling pathways active in skeletogenesis.
Acknowledgements

We acknowledge that this work was supported by NIH grants R01 DE-14526 and R01 DE-13194, the Oak Foundation to M.T.L., and NIH grant R21 DE-15368 to K.G.S.

References


GROTHOS, S., FRANKLIN, D.M., LEDDY, H.A., ROBEY, P.G., STORMS, R.W. AND GIMBLE,


