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

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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), periosteum, 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

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Abbreviations: ABCG2, ATP-binding cassette sub-family G member 2; Cbfa1/Runx2, 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.

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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 (Baksh *et al.*, 2004). Most reports on BMSCs have concluded that various haematopoietic 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 haematopoietic system and for histocompatability are more highly represented in human BMSCs, including c-Kit, CD34, HLA-DR, and CD14 (Gronthos *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, Dickkopf (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/Runx2* (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-actived 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 D_3 (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

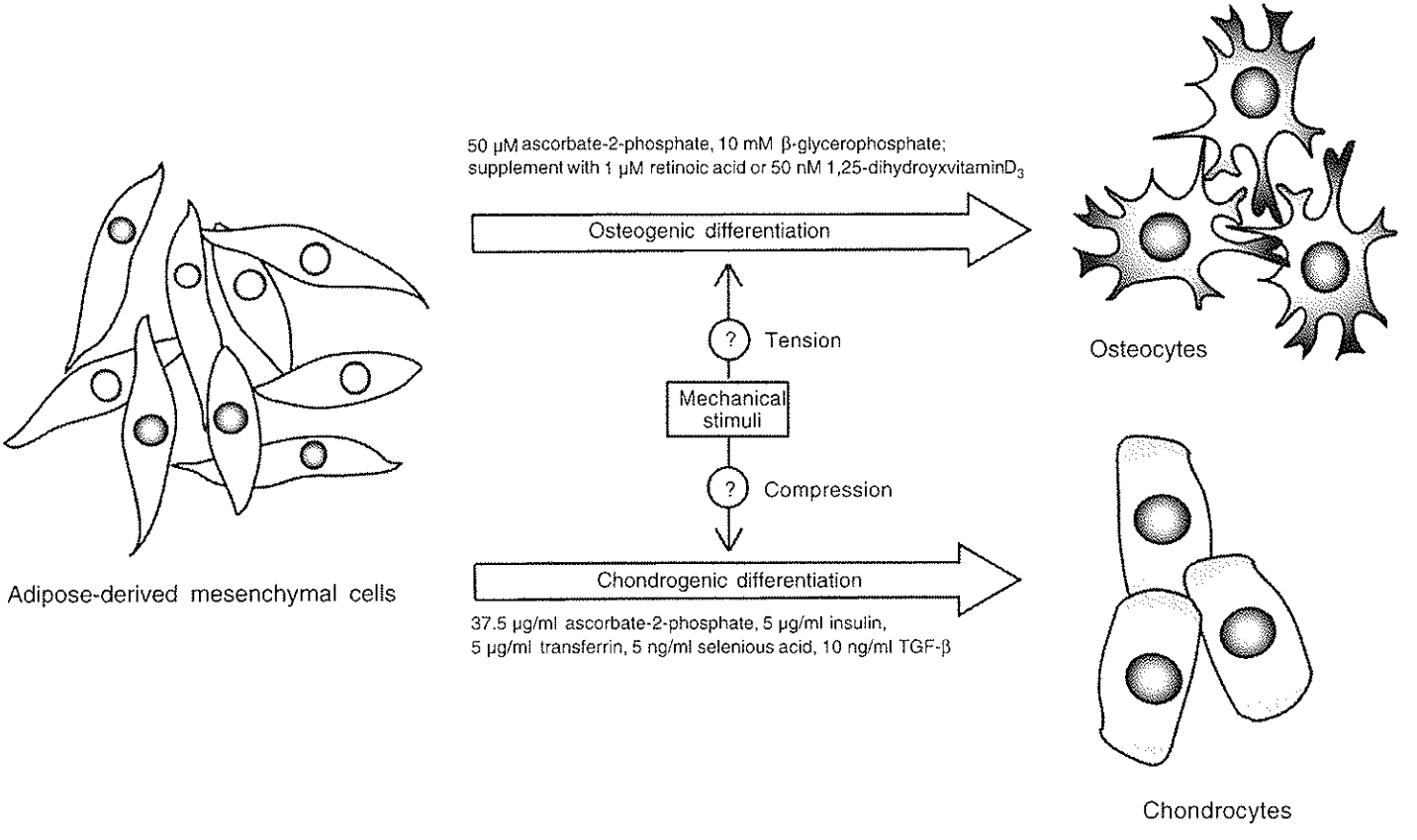

Figure 15.1. Osteogenic and chondrogenic differentiation pathways for AMCs. Differentiation media components are indicated next to the large arrows. The effects of mechanical stimuli, such as tension and compression, on AMC differentiation are still under investigation.

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 skeletalgenesis.

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 straining 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 expession. 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 *cbfa1/Runx2*. 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 capabiltities (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.

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