Cell-based therapies for skeletal regenerative medicine

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Skeletal deficits represent a substantial biomedical burden on the US healthcare system. Current strategies for reconstructing bony defects are fraught with inadequacies. Cell-based therapies for skeletal regeneration offer a paradigm shift that may provide alternative solutions. Substantial work has identified a host of cellular sources that possess the potential for osteogenic differentiation. Significant efforts have been devoted toward characterizing the role of postnatal cellular sources that are relatively abundant and easily accessible. Among these, the potential of using adipose-derived stromal cells for skeletal regeneration has garnered much interest. Integral to these efforts directed at characterizing cellular sources are studies that seek to understand the factors that initiate and regulate osteogenic differentiation of progenitor cells. Specifically, focus has been directed on elucidating the role of bone morphogenetic protein and fibroblast growth factor signaling in regulating osteogenic differentiation of osteoprogenitor cells. Concurrent studies in the field of scaffold design have also helped to advance the potential for cell-based therapies.

INTRODUCTION

The presence of a skeleton in vertebrates most saliently distinguishes this subphylum from lower phylogenetic groups. In humans, the skeletal system, composed of over 200 bones, affords unique shape, erect posture and mobility. This bony framework also protects our vital organs, such as the brain, spinal cord, heart and lungs, from injury. Furthermore, it plays critical roles in hematopoiesis and calcium homeostasis. Injury or disease afflicting the skeleton can adversely affect any or all of these functions.

With the US population demographics shifting toward an older profile, injury and disease of the skeletal system have become an increasingly relevant biomedical issue. Over the last half century, the US population has nearly doubled, growing from 151 million to 296 million between 1950 and 2005 (1). During this same period, Americans who are 65 years of age and older increased from 12 to 37 million, growing at an average rate of 2.0% per year. The population 75 years of age and older has increased from 4 to 18 million at an even higher average rate of 2.8% per year. Projections from the US Census Bureau predict that the proportion of older age groups will continue to expand. Within these groups of older Americans, the US Department of Health and Human Services reports that musculoskeletal conditions are the leading cause of chronic health problems that limit activity. These trends highlight the tremendous and ever-growing biomedical burden of skeletal deficits exacerbated by an aging population. One of the most challenging problems faced by clinicians is the reconstruction of skeletal deficits that are incapable of healing because of defect size or patient co-morbidities.

Before considering current therapies for skeletal deficits and the role of regenerative medicine in this arena, it will be helpful to briefly review the composition of bone. In broad terms, bone tissue consists of specialized cells and the extracellular matrix that these cells secrete and remodel (2). Osteoblasts, which mature into osteocytes, are responsible for depositing the proteinaceous and calcified matrix and secreting the necessary growth factors for osteogenesis. Osteoclasts, derived from the monocyte–macrophage lineage, participate in the critical function of bone remodeling. The maintenance of bone mass is dependant upon a delicate balance between the actions of these two cell types. The extracellular matrix...
is composed of collagenous proteins (predominantly collagen type I), non-collagenous proteins (osteocalcin, matrix gla protein, osteopontin and bone sialoprotein) and mineralized matrix (hydroxyapatite).

In cases of large skeletal defects, the gold standard for reconstructing these deficits is the use of autogenous bone grafts (3). However, the use of autogenous bone is limited by concerns for donor site morbidity and its finite supply. When autogenous bone grafts cannot be used, clinicians have at their avail allogenic bone substitutes. A variety of such materials is demineralized bone matrix, which is composed of extracellular matrix proteins and growth factors but devoid of cells and minerals (4). A host of inorganic/alloplastic materials such as metal alloys, glass, polymethylmethacrylate, plaster of Paris and a variety of polymers are also employed to reconstruct bony defects (5–7). The use of these materials is tempered by concerns for infection, contour irregularities and structural failure among others. More recently, surgeons have begun employing bone morphogenetic protein (BMP)-2 and BMP-7 absorbed on to a collagen sponge for spinal fusion, tibial fracture non-union and alveolar reconstruction. (8–10) While studies have demonstrated the efficacy of recombinant human BMP application, these are often expensive treatments because of the supra-physiological doses of BMP required.

The multitude of reconstructive options for skeletal defects belies the inadequacies of any one approach for reconstructing missing bone. Many of these approaches involve the application of only one specific component of bone. What is most saliently absent in these approaches is the delivery of osteoprogenitor cells that will be capable of responding to the local environment and facilitate new bone formation in a robust and rapid fashion. Regenerative medicine offers a potential solution to this conundrum.

**CELL SOURCES**

Research on cellular-based, skeletal tissue engineering applications has remained focused on identifying an ideal cellular source. When considering potential cells, options include osteoblasts, embryonic stem cells and multipotent postnatal cells. Despite their lineage commitment to bone formation, osteoblasts derived from autologous bone represent a relatively limited source and result in additional loss of bone. Furthermore, studies of mouse and human osteoblasts have described an attenuation of osteogenic differentiation and the proliferative response to mitogenic stimuli with aging (11,12).

Embryonic stem cells, which are harvested from the inner cell mass of the blastocyst, are touted for their pluripotency and unlimited capacity for self-renewal. (13) In vitro and in vivo experiments have clearly demonstrated the ability of embryonic stem cells for osteogenic differentiation (14,15). While the pluripotency of embryonic stem cells makes them an attractive cellular source, the predisposition of these cells for teratoma formation calls for a more thorough understanding of the mechanisms controlling their tumorigenic properties (16). Furthermore, the political and ethical debate currently surrounding the use of embryonic stem cells also poses substantial challenges for forward progress on these cells (17,18).

As a result, significant efforts have been directed at identifying postnatal sources for multipotent cells. Multipotent cells have been identified in bone marrow, adipose tissue, placenta, umbilical cord, human amniotic fluid, dental pulp and skeletal muscle among others (19–26). Bone marrow-derived mesenchymal stem cells (MSCs) have received the most attention. MSCs were first described by Friedenstein as what he termed colony forming units of fibroblasts (19,20). MSCs are isolated based on their adherence to plastic culture surfaces and possess the potential for differentiation into bone, cartilage, adipose and muscle.

Our laboratory is particularly interested in the presence of osteoprogenitor cells within the stroma of subcutaneous adipose tissue. Previous work has demonstrated that clonal populations within adipose-derived stromal cells (ASCs) are multipotent and possess the potential for differentiation along adipose, chondrogenic and osteogenic lineages (22,27). ASCs represent a potentially desirable cellular source not only because of their multipotency, but also because of their ease of harvest and relative abundance. Our laboratory has previously demonstrated the ability of mouse ASCs, without any perturbation, to regenerate bone in a critical-sized, calvarial defect model. (28) ASCs were seeded on to apatite-coated poly(t-lactic-co-glycolic acid) (PLGA) scaffolds and implanted into 4 mm parietal bone defects of adult mice. Radiographical analysis of calvarial healing revealed that mice treated with ASCs had greater bone regeneration than mice treated with osteoblasts at the 4 week time point, with comparable healing between these two groups at the 8 and 12 week time points (Fig. 1). Defects treated with scaffold alone demonstrated minimal osseous healing over a 12 week course. These findings were corroborated by histology. Furthermore, using sex-mismatched donors, we have demonstrated that the cellular contribution to the osseous regenerate derived from implanted ASCs was at least 92%.

The recent demonstration of induced pluripotency in postnatal somatic cells by two independent groups led by Yamanaka and Thompson, respectively, opens yet another avenue for cellular-based, skeletal regenerative therapies (29,30). Specifically, these groups demonstrated that the ectopic expression of a select group of genes can enable postnatal, human fibroblasts and other somatic cells to exhibit many of the hallmarks of human embryonic stem cells. These findings are especially important as they demonstrate the potential for reprogramming postnatal somatic cells to a pluripotent state. Given the abundance and ease with which skin fibroblasts can be harvested autogenously, it is foreseeable that such an approach could one day be tailored to provide patients with specific cell types that may be needed for tissue/organ regeneration, including bone. These studies remain a proof of principle and much remains to be worked out before contemplation of this approach in the clinical arena, including the use of viral vectors and the potential tumorigenicity of these cells. Nevertheless, these results provide a promising direction for generating patient-, tissue- and disease-specific stem cells, presumably without immunological rejection concerns.

**SIGNALING FACTORS**

A multitude of signaling pathways participate in controlling the progression from undifferentiated progenitor cell to a
lineage committed osteoblast, including the Wnts, transforming growth factor-β superfamily, Notch, Hedgehog and fibroblast growth factors (FGFs) (31). Although many of the major transcription factors regulating osteogenic differentiation, such as Runx2 and Osterix, have been identified, the events leading to activation and regulation of osteogenic differentiation remain to be fully elucidated.

Because of our laboratory’s interest in the use of ASC for skeletal regeneration applications, we have focused our efforts on dissecting some of the pathways regulating osteogenic differentiation of these cells. As BMPs are known to impart potent osteoinductive signals, our laboratory has examined the role of BMP signaling during osteogenic differentiation of mouse ASCs. BMPs, members of the transforming growth factor-β superfamily, are secreted proteins that mediate their effects by binding to dimers of the transmembrane serine–threonine kinase receptors, BMP receptor type-I (BMPR-I) and type-II (BMPR-II) (32). These receptors in turn phosphorylate Smads, a group of transcription factors, which then translocate to the nucleus and activate BMP-responsive genes. Our laboratory, thus, examined the role of BMP signaling in regulating the transition from undifferentiated mouse ASCs to lineage committed osteoblasts (33). When mouse ASCs were exposed to an in vitro cocktail that was capable of inducing osteogenic differentiation, Wan et al. noted a 7-fold increase in transcript levels of BMPR-IB. This pattern of BMPR-IB expression was confirmed at the protein level. This observation suggested the importance of BMP signaling for osteogenic differentiation of ASCs through the receptor isoform type IB. To confirm this, Wan et al. suppressed BMPR-IB expression using RNA interference. Subsequent in vitro osteogenic differentiation of ASC with suppressed BMPR-IB expression resulted in significantly impaired osteogenesis. These results confirmed the
necessity of BMPR-IB for osteogenic differentiation of mouse ASC and identified a promising molecular target for future skeletal tissue engineering applications (Fig. 2).

Fibroblast growth factor signaling is also known to play an important role in regulating osteogenic differentiation. Although FGF-2 is a known mitogen and regulator of bone formation, its effects on osteogenic differentiation remain complex. Our laboratory has directed efforts toward understanding the effect of FGF-2 on osteogenic differentiation of mouse ASCs (34). Quarto et al. found that when mouse ASCs were cultured in the presence of FGF-2, their proliferative capabilities were substantially enhanced. Of interest, however, Quarto et al. demonstrated that exposure of mouse ASCs to FGF-2 significantly inhibited their potential for osteogenic differentiation in a dose-dependent and reversible fashion. Quarto et al. then cultured mouse ASCs for 11 passages, either with or without FGF-2. Upon removal of FGF-2, the capacity of these high passage ASCs for proliferation and osteogenic differentiation was subsequently evaluated. Notably, mouse ASCs cultured for high passages in the presence of FGF-2 exhibited preserved potential for proliferation and osteogenic differentiation, while those cultured in the absence of FGF-2 demonstrated significant diminution of these capabilities. These results suggest the potential role of FGF-2 as an agent for selection and expansion of osteoprogenitor cells.

**BIOMIMETIC SCAFFOLDS**

While the identification of an ideal cellular source and an understanding of the molecular mechanisms controlling osteogenic differentiation remain a central focus, discussion of skeletal tissue engineering would be incomplete without an overview of scaffold design. recent advances in materials science and bioengineering have resulted in a multitude of scaffold options. For skeletal regeneration, qualities that are desired in scaffolds include osteoconduction (the potential for cells to attach, migrate and proliferate), osteoinduction (the potential to initiate osteogenic differentiation), biocompatibility and controlled biodegradability with maintenance of structural integrity. The choices range from natural materials to synthetic polymers, each with its attendant advantages and disadvantages. Natural scaffolds generally involve the use of collagen, hyaluronic acid and chitosan (5). Natural scaffolds often are available in forms that allow for molding to fit irregular defects. Unfortunately, the lack of rigidity of these scaffolds limits their use to non-load-bearing regions. Mineral-based scaffolds, such as hydroxyapatite and tricalcium phosphates, are attractive because of their osteoinductive nature. These scaffolds, however, are also not ideal for load-bearing regions because of their brittle consistency. A host of polymeric options exist for scaffolds. While polymers lack osteoinductive signals, they are attractive because of their durability. Material scientists often combine polymers to allow for fine tuning of physical properties, such as structural integrity or degradation rates, by varying the ratios of the two polymers.

Recent work has emphasized how the physical properties of the extracellular matrix and, by default, the material qualities of scaffolds may play a role in directing the lineage differentiation of stem cells. Data presented by Engler et al. suggest that human MSCs cultured on soft, collagen-coated gels, with elasticity similar to that of brain, were induced to commit along a neurogenic lineage (35). More rigid matrices specified MSCs along a myogenic lineage, whereas the most rigid matrices upregulated markers of osteogenesis. This idea has since been further elaborated upon by other groups. Khatiwala et al. demonstrated that the degree of osteogenic differentiation of MC3T3 preosteoblasts could be upregulated by culturing these cells on rigid substrates instead of soft hydrogels (36,37). Furthermore, Khatiwala et al. implicated mitogen-activated kinase (MAPK) as a mediator of this effect by their observation of decreased osteogenic differentiation when MC3T3 cells were cultured on rigid surfaces in the presence of the MAPK inhibitor, PD98059. These provocative data serve to further emphasize the potential influence of the scaffold’s physical properties on specification of cell fate.

Given the abundance of work identifying regulators of osteogenesis, a natural extension of such research is the incorporation of pro-osteogenic, molecular factors onto scaffolds. The bulk of these studies involve adsorbing growth factors
on a scaffold (38–40). However, local concentrations of adsorbed growth factors often decrease dramatically soon after implantation due to rapid and uncontrolled diffusion. Two innovations in scaffold technology have significantly bolstered the armamentarium of tissue engineers for controlled release of growth factors. Recently, Hubbell et al. demonstrated that encapsulation of BMP-2 with a unique polyethylene glycol (PEG) hydrogel resulted in its release over a greater period of time and a significantly reduced dose of BMP-2 required for in vivo bone formation (41). Hubbell’s group accomplished this effect by designing a PEG hydrogel that incorporated RGD motifs to enhance cell attachment and metalloproteinase (MMP) recognition sites, which facilitated the release of the encapsulated BMP-2. As cells migrate onto the scaffold and secrete MMPs, these proteinases in turn specifically degrade the hydrogel to release the encapsulated BMP-2 in a cell-responsive manner.

Another recent strategy for regulating scaffold-mediated growth factor delivery involves tethering the bioactive molecule to the scaffold. Most recently, Liu et al. described tethering BMP-2 to a PLGA scaffold using a PEG linker (42). This approach immobilizes the growth factor to maintain its local influence. Release kinetics comparing this strategy to adsorbed BMP-2 revealed a more constant and prolonged profile of BMP-2 release. When Liu et al. examined this scaffold in the setting of leporine calvarial defects, they demonstrated significantly greater bone formation in the BMP-2 tethered group relative to control groups.

CONCLUSION

The ability to harvest progenitor cells and coax them along an osteogenic lineage has propelled the field of cellular-based, skeletal tissue engineering as a viable solution for many of the shortcomings of current, clinical approaches. An understanding of the molecular mechanisms regulating this process will undoubtedly be critical for optimizing this strategy. Paralleling these advances are efforts to develop biomimetic scaffolds that provide the necessary molecular and physical cues for osteogenic differentiation. The ultimate goal is to deliver competent osteoprogenitor cells on a compatible scaffold along with the necessary signals to facilitate robust and rapid regeneration of the missing bone.

As the options of cellular sources grow, consideration for ease of harvest, frequency of osteoprogenitors and patient safety continues to play an important role in deciding what the ideal cellular source should be. However, two major questions remain to be answered. First, it will be important to evaluate whether progenitor cells derived from these varied sources are equivalent in their biology, specifically their potential for osteogenic differentiation. Second, it will be critical to define distinguishing characteristics of osteoprogenitor cells, whether by cell surface markers or by identification of a unique cluster of gene expression. The ability to define and thus identify an osteoprogenitor cell will not only facilitate comparison of osteoprogenitors derived from different sources, but will also have a profound impact on optimizing cell-based skeletal tissue engineering applications.

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REFERENCES


