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Skeletal and Adipose Tissue Engineering with Adipose-Derived Stromal Cells

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1. Introduction

There are wide ranges of pathologies leading to critical adipose and skeletal tissue loss including trauma, cancer resection and congenital anomalies. With half of the adult population in the United States estimated to be affected by a chronic disease according to the World Health Organization, the biomedical burden for replacement tissue continues to rise. By 2030, the number of adults over the age of 65 is expected to double to over 70 million in the United States (Institute of Medicine, 2008). The use of autologous tissue grafts and allografts for multiple organ systems have been successfully employed clinically, however many inherent disadvantages to these strategies exist including graft failure, infection, donor site morbidity etc. (Becker et al. 2011). While synthetic materials are not limited by availability, they are prone to infection, rejection, and breakdown over time (Keefe, 2009). Due to these inherent limitations, true regenerative medicine remains the ultimate goal for tissue replacement.

In 2001, researchers isolated a new population of adult multipotent cells in lipoaspirate (Zuk, et al. 2001, 2002). Since the discovery of adipose-derived stromal cells, a tremendous amount of work has been done in characterizing this population of cells and defining their capacity for multipotent differentiation. Adipose-derived stromal cells have potential advantages over other types of stem cells such as mesenchymal stem cells in that they are widely available and easily harvested through a simple liposuction procedure without altering their viability. Adipose-derived stromal cells can differentiate into mesodermal cell lines and offer a broad range of possibilities for application within the field of tissue engineering. These cells have already been put through several clinical trials in the treatment of a diverse array of pathologies including ST-elevated myocardial infarctions, Crohn's fistulas, and spinal cord injuries (Clinical Trials.gov, 2011).

Bone regeneration is an area of great interest in the field of tissue engineering. Over sixty million Americans are expected to be diagnosed with osteoporosis or low bone mass by the year 2020 (Samelson & Hannon, 2006). This can generate critical pathology as 1 in 2 women and 1 in 4 men over the age of fifty experiences an osteoporotic fracture in their lifetime (National Osteoporosis Foundation, 2011). With limited availability of autologous bone grafts, a bioengineered tissue replacement would be an ideal clinical alternative. Adipose-derived stromal cells were shown early on to have the capability for osteogenic

differentiation *in vitro* (Gimble & Gullak, 2003) and the ability differentiate into osteogenic tissue *in vivo* (Cowan & Longaker, 2004, Cowan & Longaker, 2005). With adipose-derived stromal cells osteogenic capability defined, research has focused on identifying specific methods and pathways that enhance the osteogenic capability of adipose-derived stromal cells. In this chapter, we will focus on the major pathways involved in osteogenic differentiation and their modulation to allow for greater osteogenic capability for skeletal tissue engineering.

A critical aspect in skeletal tissue engineering is the ability for the implanted cells to function within the three-dimensional structure of the surrounding skeleton. The implanted cells must have the proper biomechanical characteristics of bone to allow it to have the form and function necessary for successful skeletal regenerative tissue. The development of scaffolds with osteoinductive properties has allowed for cells to be placed within a three-dimensional structural environment mimicking the skeletal system. These scaffolds provide an environment or niche for cell differentiation and integration within the surrounding tissue and promote the proper healing of the skeletal defect. In this chapter, we will discuss several of the major synthetic scaffolds being used in skeletal tissue engineering and how certain scaffold properties can enhance osteogenic healing within the skeletal defect.

Another potential clinical application of adipose-derived stromal cells with tissue engineering is their use for soft tissue replacement. Of particular importance is the use of adipose-derived stromal cells for reconstruction in post-mastectomy patients. According to the National Cancer Institute, 12.15% of woman will develop breast cancer in their lifetime. The safety of adipose-derived stromal cells for reconstruction was shown within the setting of breast cancer once active disease was eliminated (Zimmerlan et al. 2011). In 2007, Cytori Therapeutics, Inc. received FDA approval for Celution® System apparatus which automates sorting of lipoaspirate to isolate adipose-derived stromal cells for the application of breast reconstruction. This device has already been used in Europe and Japan in the clinical setting and the application to breast reconstruction could be an avenue for a widespread clinical use of adipose-derived stromal cells meeting FDA approval.

It is important for both clinicians and scientists to understand the current research with adipose-derived stromal cells given the great potential these cells have for future therapeutics. Therefore, we will cover the current research with adipose-derived stromal cells and their potential for osteogenic and adipogenic differentiation along with some of the major relevant pathways leading to their differentiation. In addition, we will cover different scaffolds that have been used to place these cells within the three-dimensional network of the surrounding tissue. Finally, we will discuss possible directions for future research.

2. Adipose-derived stromal cells and osteogenesis

The generation of skeletal tissue remains an elusive clinical goal of regenerative medicine. The relative paucity of available donor sites for autogenous bone grafts limits their use in clinical application. The need for bone replacement is increasing with an aging population, leading to an increase in the number of patients diagnosed with osteoporosis with subsequent fractures. In addition, the treatment of non-union fractures across all ages and populations remains a high biomedical burden. Since the isolation and characterization of adipose-derived stromal cells, significant work has been done defining protocols for inducing osteogenic differentiation within this population of multipotent cells.

2.1 *In vitro* methods of human and mouse adipose-derived stromal cell harvest

Adipose derived stromal cells can be harvested following a liposuction procedure or an adipose tissue resection. While surgeons initially believed ultrasonic techniques would have a negative effect on adipose-derived stromal cell viability, it has been shown that they retain their osteogenic capacity following ultrasonic assisted liposuction (Panetta, et al, 2009). The other major difference between harvesting adipose-derived stromal cells after a liposuction procedure rather than an adipose tissue resection is the use of tumescent or saline fluid that is injected prior to liposuction procedures. This injection decreases surgical bleeding and makes the procedure technically easier to perform. However, the additional fluid injected during lipoaspiration dilutes the adipose-derived stromal cells, and thus the total number of adipose-derived stromal cells able to be isolated in liposuction tissue. Therefore, adipose-derived stromal cells harvest yields following a lipoaspiration procedure are often less than following an adipose tissue resection.

Once the lipoaspirate is collected, the stromal cells within the heterogeneous population of cells within the lipoaspirate must be isolated. The adipose tissue settles into two layers consisting of the supernatant, which contains suctioned adipocytes along with stroma and the bottom layer of liposuction aspirate fluid containing larger pieces of lipoaspirate (Levi & Longaker, 2011). Adipose-derived stromal cells can be isolated from either layer, but there is a higher yield of stromal cells from the adipocyte supernatant (Yoshimura, et al. 2006). From there, adipose-derived stromal cells are isolated though a series of digestion, washing, straining, and neutralizing steps (Yu, et al. 2011) through readily available protocols. Once the progenitor cells are isolated, they can be plated for *in vitro* expansion and manipulation in culture or direct injection into a defect site.

Adipose-derived stromal cells have been shown to be similar in profile to bone marrow and umbilical cord blood derived stem cells (Kern, et al. 2006). Adipose-derived stromal cells were first characterized by their ability to differentiate into certain mesenchymal cell lineages. Using flow cytometry analysis, researchers have attempted to define these stromal cells by specific cell surface markers. However, generating a defined list of concrete markers has been a challenge. This is likely due to variability in patient profile and number of *in vitro* passages along with variability in stages of differentiation. Despite these difficulties, the International Society for Cellular Therapy released a position paper in 2006 stating that the minimal criteria for being classified as a mesenchymal stromal cell according to cell surface markers to include the expression of CD105 (Endoglin), CD73, and CD90 while lacking expression of hematopoietic markers CD45, CD34, CD14, CD11b, CD79α, CD19, and HLA-DR (Dominici M, et al. 2006).

2.2 Osteogenic *in vitro* differentiation protocols

The cocktails used for osteogenic differentiation do not vary as much as those for adipogenic differentiation. In general, all osteogenic media protocols utilize β-glycerol phosphate and ascorbic acid, albeit in slightly different concentrations (**Table 1)**. Interestingly, mouse adipose-derived stromal cells have been shown to be less osteogenic than human adipose-derived stromal cells when using these two components alone. Studies have shown that mouse adipose-derived stromal cells require an additional osteogenic stimulus, such as retinoic acid (Wan, et al. 2006). Moreover, growth factors such as fibroblast growth factor (FGF)-2 inhibit the osteogenic differentiation of mouse adipose-derived stromal cells, while human adipose-derived stromal cells osteogenic differentiation proceeds relatively

unabated in the presence or absence of FGF-2 (Quarto & Longaker 2006; Quarto & Longaker 2008). Interestingly, preliminary data from our group has demonstrated that TGF-β acts to inhibit both osteogenesis and adipogenesis in mouse adipose-derived stromal cells. Instead, it seems that TGF-β acts to drive mouse adipose-derived stromal cells toward a chondrogenic fate (James, et al, 2009; Xu, et al. 2007). TGF-β has also been shown to inhibit osteogenic human adipose-derived stromal cell differentiation in large doses. Additionally, while retinoic acid can be utilized to augment mouse adipose-derived stromal cells differentiation, it has little effect on the ability of human adipose-derived stromal cells to undergo osteogenic differentiation (Levi, et al, 2010a; Wan, et al, 2007a) Human adipose-derived stromal cells osteogenic differentiation can, however, be enhanced by supplementation with several cytokines, such as Insulin Like Growth Factor (IGF) (Levi & Longaker 2010a), Platelet Derived Growth Factor Alpha (PDGF-α)(Levi & Longaker 2010a), Sonic Hedgehog (SHH) (James, et al. 2010), or Bone Morphogenetic Protein-2 (Knippenberg M, et al. 2006).

Cytokine	Stimulatory or Inhibitory	Target Osteogenic Concentration
Retinoic Acid	Stimulatory (in mouse ASCs)	1-10 uM
Sonic Hedgehog	Stimulatory	100-750 ng/ml
BMP-2	Stimulatory	50-200 ng/ml
BMP-4	Stimulatory	10-50 ng/ml
IGF-1	Stimulatory	25-50 ng/ml
PDGF-α	Stimulatory	10-20 ng/ml
Noggin	Inhibitory	100-400 ng/ml
BMPR-IB/ALK-6/Fc Chimera	Inhibitory	0.5-2.0 ug/ml

Table 1. Stimulatory and Inhibitory Cytokines for ASCs for use in Osteogenic Differentiation Media

For osteogenic differentiation, cells should be plated in a 6-well plate (80,000-100,000 cells per well), a 12-well plate, (35,000-45,000 cells per well), or a 24-well plate (15,000-25,000 cells per well). After attachment, adipose-derived stromal cells can be cultured in osteogenic differentiation medium.

Early and late osteogenic differentiation has been defined to occur at specific time points, and these time points vary between species. While mouse adipose-derived stromal cells undergo early osteogenesis after 7 days in culture and late osteogenesis after 14 days in

culture. In contrast, human adipose-derived stromal cells begin to differentiate much earlier than mouse adipose-derived stromal cells and show evidence of early and late osteogenesis following 3 and 7 days in culture, respectively. RNA analysis of adipose-derived stromal cells for specific osteogenic gene markers is a commonly used method to assess osteogenic differentiation in a quantitative manner. Specific gene markers for early osteogenic differentiation include *Alkaline Phosphotase (ALP), Runt Related Protein-2 (RUNX-2)*, and *Collagen Ia1 (COL1A1)*. Gene markers for intermediate and late osteogenesis include *Osteopontin (OPN)* and *Osteocalcin (OCN),* respectively. Alkaline phosphatase staining and quantification is another method used to assess early osteogenic differentiation of adipose-derived stromal cells (day 3 for human adipose-derived stromal cells, day 7 for mouse adipose-derived stromal cells). Late osteogenic activity can be assessed by Alizarin red or Von Kossa staining, which are assays for extracellular mineralization (day 7 in human adipose-derived stromal cells, day 14 in mouse adipose-derived stromal cells).

2.3 Molecular pathways of differentiation

There are several key molecular pathways that lead to the differentiation of a multipotent cell into a mature osteoblast in the process of osteogenesis. Runx-2 is considered the principal osteogenic switch and has a key role in the commitment of stem cells into an osteoblastic lineage. Runx-2 null mice show a complete deficiency in skeletal formation (Komori and Kishimoto, 1998). Runx-2 is transcriptionally upregulated by the bone morphogenetic proteins (BMP) through transduction via Smad 1 and 5 (Chen et al, 2004). Retinoic acid has also been shown to increase Runx-2 expression and stimulate mouse adipose-derived stromal cell osteogenic differentiation by increasing the expression of BMPR-IB protein (Wan, Longaker 2006). While Transforming Growth Factor-Betas (TGF-βs) have been shown to be increased during bone development and growth, their effect on precursor cell differentiation into osteoblasts depends on cell type, stage of differentiation, and levels of expression (Linkart 1996, Komori 2002).

The Wnt signaling pathway is a group of proteins involved in a wide array of developmental and cellular processes. In the canonical Wnt pathway, Wnts bind to cell-surface receptors of the Frizzled family and their co-receptors, low-density lipoprotein receptor related protein (LRP), leading to inactivation of the axin-glycogen synthase kinase 3β complex which stabilizes Beta-catenin. Beta-catenin translocates into the nucleus to promote Wnt gene expression. LRP5 knockout mice show a phenotype with low bone mass (Gong et al, 2001) while overexpression of the same gene is associated with high bone mass (Boyden et al, 2002). Wnt3A has been shown to have differing results on osteogenic differentiation depending on dosage and level of baseline differentiation along with age of the cells. (Quarto, Longaker 2010). Low dose treatment with Wnt3a enhanced bony regeneration in a mouse critical calvarial defect treated with differing doses of Wnt3a in juvenile mice, while higher doses decreased repair (Quarto, Longaker 2010). Conversely, in adult mice, increasing doses of Wnt3a enhanced bone regeneration.

In the non-canonical Wnt pathway, Wnts bind to the receptors Frizzled and Dishevelled but the non-canonical pathway does not mediate its signal through the glycogen synthase 3β kinase complex or the Beta-catenin complex, but rather through G-proteins and GTPases. While the most studied aspect of the non-canonical Wnt pathway is their regulation in cell polarity, a growing body of evidence suggests that this pathway is important in bone development. Wnt5a has been shown to increase osteoblast formation from human bone

mesenchymal stem cells *in vitro* (Baksh & Tuan, 2007). In human adipose-derived stromal cells, the addition of Wnt5a *in vitro* increased the expression of Runx2 and osteocalcin and mineralized nodule formation (Santos, et al, 2010).

2.4 Growth factors and hormones that promote osteogenesis

Growth factors are signaling molecules that have a wide effect on cellular processes. The majority of growth factors are secreted as proteins or hormones and act in an autocrine, paracrine, or endocrine manner to affect cellular proliferation and differentiation by regulating gene expression, protein synthesis, and cell signaling. Through the modulation of specific growth factors, osteogenesis can be enhanced with the result of improved skeletal healing.

Bone morphogenetic proteins are a group of secreted cytokines that belong to the Transforming Growth Factor-β superfamily. Throughout history, skeletal tissue was known for its ability to regenerate and efforts were undertaken to try to isolate an intrinsic property secreted by these cells that led to their regenerative properties. BMPs were first discovered and named in 1965 (Urist, 1965) and the first BMP gene was isolated and cloned by Wozney in 1988 (Wozney et al, 1988). In 2002, the FDA approved the use of recombinant BMP-2 to be used on a three-dimensional scaffold for anterior lumbar fusion (McKay et al, 2007). Today, over twenty different genes belong to the BMP family mainly involved in the regulation of bone and cartilage formation (Bessa C et al. 2008). The BMP proteins signal (**Fig. 1**) through BMP receptor Type I and II, which leads to the phosphorylation of receptor-regulated SMAD 1,5, and 8, which combine with the common-mediator SMAD 4. This complex then migrates into the nucleus to affect gene expression.

A large body of research has validated the importance of BMP-2's role in bone formation and healing. BMP-2 has been shown to induce chondrocyte and osteoblast precursor formation in mesenchymal stem cells (Wall et al. 1994) and specifically shown to induce osteogenic differentiation in adipose-derived stromal cells (Dragoo et al. 2003) *in vitro*. Our group (Cowan, Longaker 2005) showed the use of BMP-2's effect combined with adipose derived stromal cells *in vivo* on bony healing by culturing adipose-derived stromal cells with rhBMP-2 for 4 weeks prior to implantation onto a PLGA scaffold into a critical-sized calvarial defect in a mouse. With the treatment of BMP-2 *ex-vivo*, cells treated with recombinant human BMP-2 showed accelerated healing over unstimulated scaffolds with complete healing in as little as two to four weeks as demonstrated by histology and microCT. Interestingly, prolonged exposure to recombinant human BMP-2 was also shown to increase osteoclast activity and increase bone turnover and resorption. Therefore, to avoid increased bone turnover, our group used direct *in vivo* injection of recombinant human BMP-2 without the prolonged *ex-vivo* treatment (Levi, Longaker, et al 2010b) into a critical calvarial defect with and without adipose-derived stromal cells. Cells treated with recombinant human BMP injected subcutaneously into the skeletal defect on post-op days 1-3 showed increased healing at 8 weeks over unstimulated scaffold alone and untreated cells. The increase in osteogenesis was observed with this method of BMP-2 augmentation, but without the stimulation of osteoclast-induced bone turnover and resportion. The current scope of research is concentrating on how to deliver BMP-2 into the skeletal wound microenvironment to promote osteogenesis and bony healing while minimizing side effects such as ectopic bone formation (Zara, Siu, Soo et al. 2011).

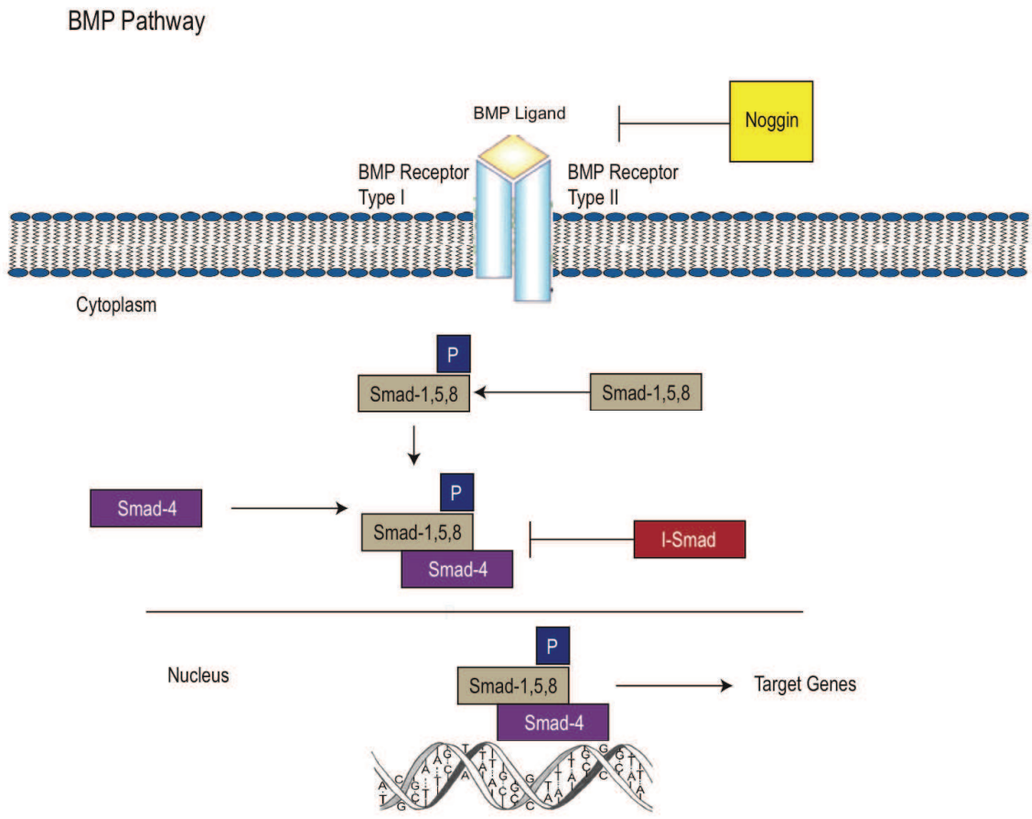

Fig. 1. Bone Morphogenetic Protein Pathway

BMP ligand binds to BMP-R1 or 2, which phosphorylates SMAD 1,5, and 8. Co-smad 4 complexes with Smad 1,5, and 8 and translocates into the nucleus and regulates gene expression

In addition to BMP-2, BMP-7 (Osteogenic Protein-1) has also been shown to be effective in the augmentation of skeletal healing. In a monkey model, recombinant human BMP-7 was injected into a scaffold at the skeletal injury site and demonstrated healing in an ulnar/tibial segmental defect model. (Cook, et al 1995). A randomized clinical trial compared the use of BMP-7 versus autogenous bone graft in the treatment of tibial non-unions (Friedlaender, 2004) and found that using BMP-7 was equivalent in clinical outcomes without the morbidity of using an autogenous bone graft. Specifically with adipose-derived stromal cells, researchers using an adenovirus vector *ex vivo* to induce BMP-7 expression showed increased bone formation both *in vitro* and *in vivo* using rat adipose-derived stromal cells (Yang, et al. 2005) and in human adipose-derived stromal cells (Kang Y, et al. 2007).

The fibroblast growth factor family has over twenty members and four receptors involved in diverse cellular processes from angiogenesis to wound healing. Similar to the BMP family, fibroblast growth factors have been shown to have a significant effect on osteogenesis (Ornitz and Marie, 2002). In particular, FGF-2 is expressed in osteoblastic cells and has been

shown to have important functions in the regulation of bone and cartilage formation. FGF-2 knockout mice show decreased bone mass and bone formation compared to control mice (Montero et al. 2000). Initial work on the effect of FGF-2 on adipose-derived stromal cells showed that FGF-2 expression was critical in maintaining clonogenicity and differentiation potential (Zaragosi et al. 2006) along with the maintenance of adipose-derived stromal cells proliferation *in vitro* (Quarto and Longaker, 2006). Evaluation of FGF expression during osteogenic differentiation of adipose-derived stromal cells showed that FGF-2 is down-regulated during osteogenic differentiation (Quarto and Longaker, 2008) while FGF-18 is upregulated. This was explored further by examining different isoforms of FGF-2. The high molecular weight protein form of FGF-2 increased expression during osteogenesis while the low-molecular weight form decreased, suggesting a regulation at the translational level in the balance between maintaining multipotency and lineage commitment. In order to explore the effects of FGF-2 *in vivo,* we engineered feeder cells capable of secreting the FGF-2 protein when driven by the presence of a synthetic ligand, Shield-1, which stabilizes a protein-destabilizing domain fused to the FGF-2 gene (Kwan, Longaker, 2011). Feeder cells and adipose-derived stromal cells were both seeded on a scaffold and placed into a mouse critical-sized calvarial defect. Shield-1 was delivered intraperitoneally and the mice were followed up to 20 weeks. There was significantly improved healing with FGF-2 versus the non-injected group of control adipose-derived stromal cells and scaffold alone in the defect. A possible explanation for the improved osteogenesis and calvarial defect healing *in vivo* may be linked to the increased proliferation of osteoprogenitor cells due to FGF-2 expression.

Insulin-like growth factors are proteins with sequence homology similar to insulin under the control of growth hormone. There are two types, IGF-1 and IGF-2, which play a wide variety of cellular roles from the regulation of proliferation to apoptosis. Both IGF-1 and IGF-2 have been shown to be associated with increase in bone formation and density (Adami et al. 2010, Chen et al, 2010) while their reduction has been shown to be associated with decreased bone density (Bennett, 1984). In order to specifically identify genes upregulated during osteogenesis, our group isolated RNA from human adipose-derived stromal cells during i*n-vitro* osteogenesis and performed a microarray analysis (Lee, Longaker, 2010). Gene expression of IGF-1 was found to be elevated 3.5 fold from baseline levels at day three of osteogenic differentiation with the microarray. IGF-1's ability to increase osteogenesis was then tested by augmenting osteogenic differentiation media with IGF-1 *in vitro* (Levi, Longaker 2010a). Alkaline phosphatase activity was elevated with the addition of IGF-1 and there was increased matrix mineralization evident with the treated group stained with alizarin red. Quantitative real-time polymerase chain reaction showed elevated levels of Runx2 and Osteocalcin when adipose-derived stromal cells were treated with IGF-1.

The effect of estrogen on bone formation is well known. Decreasing levels of estrogen are one of the main culprits in the pathogenesis of osteoporosis with estrogen withdrawal causing an increase in bone remodeling (Seeman, 2003). While men do not have a comparable deceleration of hormone production, circulating levels of free estrogen also declines with men with aging leading to comparable long term loss of trabecular bone (Seeman, 2004). The addition of estrogen through 17-β estradiol increased levels of osteogenesis in human bone marrow mesenchymal stem cells through modulation of estrogen receptor α and β (Hong, et al 2006). Human adipose-derived stromal cells

augmented with 17-β estradiol *in vitro* showed increased evidence of osteogenesis through increased expression of alkaline phosphatase and osteocalcin as compared to control cells without estrogen augmentation (Hong et al, 2007).

The effect of androgens on bone formation is less clear. It is recognized that patients with androgen deficiency have lower bone mass and higher bone turnover compared to patients with normal levels of androgen (Hofbauer & Khosia, 1999). The majority of *in vitro* studies indicate that testosterone and dihydrotestosterone have proliferative effects on osteoblast progenitors, but there is no consensus as to whether androgens promote or inhibit osteogenic differentiation (Vanderschueren, et al, 2004). Androgen replacement therapy *in vivo* has shown to be effective in increasing bone mass in hydogonadal patients, the effect is mitigated in patients with more modest decreases in testosterone (Vanderschueren, et al, 2004). The full effects of estrogen and androgen and differences in sex hormones still must be worked out as well as the effect of sex of the donor adipose-derived stromal cells in differences on osteogenic differentiation.

2.5 Gene therapy

Another strategy for improving osteogenic differentiation is gene therapy to either express or knockdown specific proteins for the desired effect altering cellular function. Strategies in genetic therapy include *in vivo* direct gene delivery using a vector for transfection or *ex vivo* techniques, which involves treating the cell of interest with modification of the genome outside of the host prior to implantation.

Gene therapy requires a vector to transfer the genetic material into the target cell. Vectors can be divided into viral and non-viral and also whether the strategy integrates the vector into the host-genome or is based on a transient effect. Viral vectors have the advantage of being efficient in their ability to infect host cells. Common viral vectors are adenovirus, lentivirus, and retrovirus. While the integrating viral vectors are efficient and express the DNA of question for the life of the cell, there is no way to completely control the site of integration leading to concern regarding mutations and oncogenic activity. Viral vectors can also elicit a host immune response causing rejection. Non-integrating strategies such as liposome based particles and electroporation transiently transfect the cells and have a decreased immune response, but are not as efficient and have variable expression.

Zhang et al. (2006) used an adenovirus vector to overexpress Runx-2 in mouse adipose-derived stromal cells to increase osteogenesis *in vitro* and *in vivo* by implanting seeded scaffolds on the back of nude mice. Several strategies have used BMP-2 gene therapy to promote bone healing and osteogenic differentiation. A segmental defect created in New Zealand white rabbits was treated with adenoviral vectors expressing BMP-2 resulting in increased healing in the treatment group (Baltzar, 2000). Lee SJ et al (2010) created a bicistronic vector for co-delivery of both Runx-2 and BMP-2 into human adipose-derived stromal cells. Using microporation for their transfection strategy, they showed increases in osteogenesis *in vitro* and implanted transfected human adipose-derived stromal cells on a PLGA scaffold subcutaneously *in vivo* showing increase in ectopic bone formation compared to control.

Osterix is a zinc finger transcription factor that is expressed in osteoblasts whose role is critical during osteoblast differentiation. Nakashima et al. (2002) first identified osterix through a screen of expressed genes during osteogenic differentiation. Through homologous recombination, they produced osterix-null mice, which showed no bone formation. Osterix

appears to be downstream of Runx-2 as osterix-null mice had normal Runx-2 expression and normal cartilage formation. However, Runx-2 negative mice show impaired chondrogenesis and decreased osterix expression. Overexpression of osterix has been shown to increase osteogenic differentiation in embryonic stem cells by increased in levels of Osteocalcin and Runx-2 (Tai, et al. 2004) and in adipose-derived stromal cells (Wu L, et al 2007). Lee SJ et al. (2011) used electroporation to co-transfect Runx-2 with osterix into adipose-derived stromal cells. Transfected cells with osterix alone and osterix with Runx-2 both showed significant increase in osteogenesis over control cells.

Msx-2 (Hox-8) is a homeodomain transcription factor that functions as a transcriptional regulator of the osteocalcin promoter (Towler DA, et al 1994). Msx-2 has been found to be vital in craniofacial development, as mutations within the homeodomain of Msx-2 have been shown to lead to craniosynostosis, the premature fusion of calvarial sutures (Jabs, et al. 1993; Liu et al. 1995). Msx2 null mice have deficient endochondral bone formation, defects in skull ossification and a persistent calvarial foramen (Satokata, et al, 2000). Using *CMV-Msx2* transgenic mice, Cheng et al, (2008) showed that overexpression of Msx2 leads mesenchymal progenitor cells into an osteogenic lineage enhancing osteoblast formation along with trabecular bone formation by activation of the canonical Wnt pathway.

In looking for specific genetic targets to augment adipose-derived stromal cells for osteogenic differentiation, our group focused on the noggin protein, which is an inhibitor of BMP signaling. We evaluated the effect of noggin suppression on osteoblast differentiation by using siRNA constructs against the noggin protein in primary osteoblasts (Wan, Longaker et al, 2007b). Noggin suppression was confirmed using western blot and QT-PCR. Evaluation of BMP signaling *in vitro* with cells transfected with Noggin siRNA constructs showed an increase in BMP signaling with Smad1/5 along with evidence of osteogenic differentiation of preosteoblasts as compared to control. Noggin knockdown also increased skeletal healing *in vivo* when osteoblasts transfected with Noggin siRNA were seeded on a PLGA scaffold as demonstrated by microCT and histomorphometric analysis.

3. Adipogenesis and adipose-derived stromal cells

The adipogenic differentiation of multipotent stromal cells is of interest to many specialties of medicine. The growing body of research in obesity and adipose biology has opened the field of adipogenesis to numerous specialties in clinical medicine and science. Stem cell scientists, bone biologists, endocrinologists, and tissue engineers all have a vested interest in the study of adipogenesis. From a clinical standpoint, surgeons are faced with challenging reconstructive cases in patients afflicted with soft tissue deficiencies. For example, burn patients often have soft tissue atrophy and severe skin contractures and would greatly benefit from soft tissue augmentation. Similarly, the widespread use of HAART (Highly-active retroviral therapy) medications for HIV has left many HIV patients with facial lipodystrophy, which can be disfiguring and socially troublesome. In the pediatric population, patients with congenital malformations would greatly benefit from new soft tissue engineering techniques. For example, Parry-Romberg disease causes progressive hemifacial soft tissue atrophy in the face. Outcomes of these patients would greatly improve with the use of a tissue engineering approach to reconstruct their inadequate adipose compartment.

3.1 Adipogenic *in vitro* differentiation protocols

The adipogenic differentiation protocols of primary mesenchymal stromal cells vary as reported in the literature. For the purposes of this chapter, two of the most commonly studied mesenchymal stem cells populations were examined: bone marrow mesenchymal stem cells and adipose-derived stromal cells. Generally, a three component cocktail is used to supplement media for bone marrow mesenchymal stem cell adipogenic induction, including Insulin, Dexamethasone, and IBMX (3-isobutyl-1-methylxanthine). There are several differences between mouse and human adipogenic differentiation protocols. For example, Insulin was not used in the majority of publications in human bone marrow mesenchymal stem cell adipogenic differentiation protocols, while insulin is present in the majority of murine adipogenic differentiation protocols. Additionally, human adipogenic protocols used an increase in dexamethasone along with the addition of Indomethacin in comparison to mouse bone marrow mesenchymal stem cell protocols.

Adipose-derived stromal cells are multipotent mesenchymal cells and thus can be differentiated into adipocytes. With regards to adipose-derived stromal cell adipogenic differentiation, there are a wide variety of reports of varied differentiation cocktails with many published articles in the last year (Romo-Yanex, et al 2011; Ghosh, et al, 2010; James, et al. 2010; Valorani, et al, 2010, Lee JE, et al. 2010). Generally, a three or four component cocktail is used for adipose-derived stromal cell adipogenic differentiation, including Indomethacin, Insulin, Dexamethasone and IBMX. Similar to bone marrow mesenchymal stem cell adipogenesis protocols, there are a wide variety of recommended cocktails that become even more heterogeneous when comparing protocols for mouse adipose-derived stromal and human adipose-derived stromal cell adipogenesis. For example, though insulin concentrations in the adipose differentiation media of both mouse adipose-derived stromal cell and human adipose-derived stromal cell are relatively similar, an approximate 10-fold increase in dexamethasone concentration exists in mouse adipose-derived stromal cell adipogenesis protocols when compared to human adipose-derived stromal cell protocols

3.2 Molecular pathways of differentiation

At the molecular level, several key genes are expressed during the differentiation of pre-adipocytes to mature adipocytes including lipoprotein lipase (LPL), peroxisome proliferator-activated receptor (PPARγ), and enhancer binding proteins (EbP) (Kronenberg & Williams, 2008). In addition, several Hox genes have been recently identified that are highly expressed during the adipogenic differentiation of human adipose-derived stromal cell (Cowherd, et al. 1997; Cantile, et al. 2003). Other genes such as TAZ have been shown to activate the Runx-2 transcription factor and stimulate osteogenesis while inhibiting adipogenesis (Hong, et al. 2005).

PPARγ is one of the most widely studied genes involved in adipogenesis, and has been translated for clinical use in the form of the agonist rosiglitazone, as well as the similar agents (troglitazone, etc). These PPARγ agonists have been used in the treatment of diabetes as they have been shown to speed up the differentiation process of pre-adipocytes or adipoprogenitor cells *in vitro*. Rosiglitazone functions by binding to PPARγ, thus 'sensitizing' adipocytes to insulin (Mayerson, et al 2002). One of the major side effects of this class of pharmaceuticals is bone mineral density reduction, which is thought to be due to the diversion of mesenchymal stem cells to adipogenesis rather than osteogenesis *in vivo* (Bodmer, et al, 2009; Rosen, et al. 2006). To the full extent that rosiglitazone is able to induce

mesenchymal stem cell adipogenesis as a single agent is unknown. However, it is believed that this class of drugs is capable of enhancing the differentiation of pre-adipocytes to adipocytes *in vivo* (Ninomiya, et al 2010). Thus, PPARγ agonists may be useful in adipocyte induction cocktails for adipose-derived stromal cells. Furthermore, from a clinical standpoint, targeting the PPARγ pathway may improve the adipogenic potential of transplanted stem cells further improving soft tissue engineering outcomes.

One of the surgical limitations of fat transfer is the high rate of adipocyte resorption over time. It is thought that adipocyte resorption during fat transfer is due to the inadequate blood supply and thus, surgeons have set out to improve circulation to transplanted adipocytes. Besides alterations in technique, recently, Yoshimura *et al* has reported enhanced adipocyte viability and vascularization when seeding adipocytes with human adipose-derived stromal cells from the same patient at the time of surgery (Suga et al 2010, Yoshimura, et al. 2008, Yoshimura, et al. 2009, Matsumoto, et al. 2006). This technique offers the use of an autologous scaffold seeded with autologous multipotent stromal cells. Human adipose-derived stromal cells are a known vasculogenic cell type that have been shown to secrete vasculogenic cytokines, thus working in an autocrine and paracrine fashion to allow for improved survival of adipocytes and enabling possible adipogenic differentiation. Therefore, potential benefits of using human adipose-derived stromal cells to differentiate into adipocytes, include their ability to proliferate after transplantation, their release of angiogenic cytokines, and their ability to differentiate into endothelial cells and undergo neovascularization (Planat-Barnard et al., 2004; Miranville, et al. 2004, Moon, et al. 2006)

4. Scaffold technology and adipose-derived stromal cells

Almost as important as the proper engineering of multipotent cells into the desired lineage is their placement within the three-dimensional context of the target organ. This is especially true with skeletal and soft tissue engineering as their structure is critical to their function. Recent advancements in the design and quality of scaffold technology is critical for the advancement of successful *in vivo* application of multipotent cells. With the goal of engineering functional tissue, biomimetic scaffolds are designed to provide 3-dimensional structural support for the engineered tissue, uniform pore size and structure for a matrical distribution of cells, and combinations of substrates and growth factors to promote viability and differentiation along the appropriate cell lineage. With technologies enabling the reliable production of mineralized biopolymers to nanospun fibers, scaffolds engineered from these materials promote healing *in vivo*, (Lew, et al. 1997; Osathanon, et al. 2009) and prove a powerful delivery system for adipose-derived stromal cells in the generation of functional tissue replacement.

The growing incidence of craniofacial bone defects arising from congenital malformation, surgical resection and trauma necessitates the development for skeletal regeneration of bony defects to restore functional movement and protection to the head and its critical structures. Regenerated bone exists in a dynamic environment and must provide adequate structural support and tensile strength. One of the important models for skeletal regeneration in the craniofacial context is the critical size calvarial defect, in which a bony deficit is created in an animal model that is of a size that will not heal within the lifetime of an animal. Thus, any healing observed is due to the experimental contribution. Because of the availability of experimental animals and analytical tools, the laboratory mouse is a common model for

bone regeneration in calvarial critical-sized defects, which are between 3-4mm in diameter (Mooney & Siegel, 2005).

Critical to the engineering of an ideal scaffold for bone regeneration are the biocompatibility, osteoconductive, and osteoinductive properties of the materials. A biocompatible scaffold should be manufactured under sterile conditions, have an even and consistent pore size and distribution, and be non-toxic to the host tissues as it breaks down over time. Osteoconductive properties refer to the characteristics of the scaffold framework and its impact on the resulting structure of newly formed bone, while osteoinduction is the promotion and induction of host or donor cells toward mature osseous formation. A variety of inorganic polymers and synthetic nanofibers are being used as the basis for manufacturing scaffolds and vary in their capability for osteoinduction, rate of resorption, and moldability.

Polylactide (PLA) and poly lactic-co-glycolic acid (PLGA) polymers have been shown to be safe in human tissue (Frazza & Schmitt, 1971) and are resilient to fracture but lack significant osteoconduction. Calcium phosphate and calcium sulfate salts, on the other hand, tend to be brittle and difficult to mold but contain natural bone elements of crystal salts found in bone matrix and tend to be more osteoconductive scaffolds than their polymer counterparts. Of particular interest in this group is hydroxyapatite, a mineral salt composing a large proportion of naturally-occurring bone matrix with strong osteoconductivity (Chang, et al 2000).

In order to combine the tensile strength and load-bearing capabilities of a polymer scaffold with the osteoconduction and cellular substrate of mineral salts, Cowan et al employed a strategy combining osteogenic mouse adipose-derived stromal cells seeded on a PLGA scaffold coated with hydroxyapatite for bony regeneration of a 4mm critical-sized defect in mouse. (Cowan, et al, 2004) The combined osteogenic potential of adipose-derived stromal cells and osteoconduction of apatite-coated PLGA promoted complete healing within 12 weeks with major contribution from the transplanted adipose-derived stromal cells as shown by chromosomal analysis. Levi et al have recapitulated these results with apatite-coated PLGA scaffolds in mouse calvarial defect using human adipose-derived stromal cells in immunodeficient nude mice, demonstrating the potential of human cellular therapy delivered on hybrid polymer mineral scaffold for translational development. (Levi, et al. 2010b)

Although significant bone regeneration has been achieved using osteoconductive scaffolds and adipose-derived stromal cells without the use of additional growth factors (Cowan, et al 2004; Levi, et al. 2010b), the stimulation of well-characterized osteogenic pathways may enhance efficient new bone deposition in conjunction with biomimetic scaffolds and cell therapy. Bone morphogenetic proteins (BMPs) along with valproic acid and other factors have demonstrated abilities to accelerate bone formation *in vivo* (Cowan, et al. 2005) and may be candidates for delivery in combination with ASCs and apatite-coated PLGA scaffolds for increased efficiency and reliability of osteogenesis.

Similar to current clinical treatment modalities for bone defects, soft tissue deficits due to trauma, tumor, and congenital etiologies are treated with surgical grafts from autologous, allogenic, or alloplastic sources. The successes of vascularized microvascular flap transfer, the gold standard modality, are often accompanied with limitations in source material and patient morbidity. Because adipose tissue has numerous functions, including energy regulation, secretion of important paracrine and endocrine factors, and mechanical

protection, it is important to develop new strategies for replacement of functional soft tissue deficits. Of major consideration for clinical translation is the regeneration of adipose tissue following mastectomy, requiring adipose stroma and a biomimetic matrix for the replacement of breast tissue.

Cellular transplants for adipogenic regeneration hinge on scaffolds that support cell adhesion and the differentiation and uptake of lipids while providing appropriate structural support. Porosity is a critical factor in the scaffold structure to allow the development of angiogenic support necessary for functional tissue formation. A number of synthetic and biological scaffolds have been studied as delivery systems of adipocyte precursor cells for effective adipose tissue generation with varying degrees of success and limitations. Inorganic synthetics such as PLGA and polyglycolic acid (PGA) offer moldable, highly regular structures and non-toxic breakdown. Gel-based delivery systems promote cell survival but lack the organizational regularity of inorganic scaffolds. Natural proteins found in connective tissue such as collagen can be engineered into scaffold design with homogeneous structure and porosity, and mimic biological matrix found commonly in adipose tissue.

Matrigel™ is a gel-like substance derived from animal connective tissue proteins and growth factors, and is readily available commercially. Despite the translational barrier of its sarcoma derivation, Kawaguchi et al and others have reported successful angiogenesis and adipogenesis following injection of Matrigel™ in mouse fat pad (Kawaguchi, et al. 1998). The mixture of components in matrigel including collagen, laminin, and growth factors demonstrated sufficiency for the generation of new vascularized adipose tissue, but because of the stochastic organization of proteins within the gel, the distribution of cells in the *de novo* tissue reflect a scattered and uneven pattern of growth. Adipogenic tissue regeneration with eventual clinical translation in mind should promote predictable growth of new soft tissue. An ideal scaffold would define the structure of the newly formed tissue to be populated by transplanted donor cells and stimulated endogenous pre-adipocytes and allow for angiogenic growth and support into newly forming tissue.

One study performed a direct *in vivo* comparison of three disparate scaffold technologies for the delivery of mouse ASCs as an assay for adipogenic ability using PGA, collagen, and hyaluronic acid gel (Itoi, et al. 2010). PGA represents the biodegradable synthetic group of scaffolds, extracellular matrix-derived collagen from tendon is a natural product that is prepped into porous sponges, and hyaluronic acid (HA) is a derivative of extracellular matrix of soft tissue involved in cell proliferation. ASCs were harvested from GFP mice to allow visualization of transplanted cell proliferation and differentiation, grown in adipogenic supplemented media, and seeded into the respective scaffolds and transplanted into athymic mice. In all groups tested, fluorescent cells were present at four weeks and a slight increase in angiogenesis was seen. In contrast, Oil Red O staining for triglycerides was positive only in PGA and collagen groups and negative in HA. At 8 weeks, the most robust GFP positive adipose-like tissue was detected in the collagen scaffold construct indicating that collagen served as the best adipogenic scaffold in this comparison.

Promising data from Davidenko et al (2010) combines the naturally-derived extracellular matrix components collagen and hyaluronic acid into a hybrid cross-linked scaffold designed to provide an array of matrix protein components with the structural advantage of collagen. Thus, this design allows for precise engineering of scaffold structure and porosity, even distribution of transplanted adipogenic cells, and physiochemical safety. Pre-

adipocyte cells were shown to proliferate and differentiate readily on the scaffold during *in vitro* adipogenic induction and gene analysis showed increased expression of Adipsin following differentiation.

5. Future directions

Given the abundant availability of lipoaspirate combined with a relatively simple procedure to harvest, adipose-derived stromal cells have a potential to be critically important in the generation of tissue for clinical use in a wide range of pathologies. While there is great promise for the use of these cells, significant hurdles remain before they become available for wide spread clinical application. The ultimate translational goal is the ability to harvest the lipoaspirate and be able to process and implant the cells into the area of need in the same patient during a single surgical procedure. Research in several key areas must happen prior to this scenario being realized.

Currently, the process for sorting and isolating stem cells from the lipoaspirate is a manual process. The total number of cells typically obtained is around 300,000 cells per milliliter of lipoaspirate (Levi & Longaker, 2011). While this yield is higher then other adult-stem cell populations, the process can still be time consuming and labor intensive. For widespread clinical use to be possible, the ability to have an automated processing unit isolate the stromal cells in the lipoaspirate would be critical to decreasing the time and cost of this procedure. Cytori's Celution® System was developed to automate the sorting process and takes around 1 hour to sort 250 ml of lipoaspirate. Using Flow Cytometry, the cells sorted using the Celution® system were found to be similar to cells isolated on a manual sort with the ability to differentiate into osteogenic and adipogenic cells (Lin, et al. 2008). As more research is done characterizing specific populations within adipose-derived stromal cells, the technology should improve to better match and sort for specific populations of cells more likely to differentiate into the desired cell lineage.

The importance of scaffolds and their ability to deliver cells into the proper niche environment has been stated. As the technology improves with scaffold development, they have become much more than inert materials where cells are placed, but rather can provide a highly inductive environment for cells to be "coached" towards a certain lineage. The ability to augment scaffolds with various growth factors and cytokines has been shown to improve healing within specific wound environments (Brown, et al. 2011). These scaffolds can have the advantage of release kinetics allowing for a controlled delivery of the growth factor of interest into the wound environment (Kwan, et al, 2011). The future of scaffold technology is moving towards the ability to induce genetic therapy within its three-dimensional structure. The delivery of BMP-2 and BMP-7 through vectors within the scaffold structure into host cells has already proven to be effective (Nie, et al. 2007; Zhang, et al. 2011).

6. Conclusion

With the large clinical need for replacement tissue, adipose-derived stromal cells are easily obtained in large numbers and offer a viable option for future therapeutic application given their ability to differentiate into multiple cell lines. Through continued research into pathways of differentiation, the ability to maximize the yield of these multipotent cells will continue to improve. With the increased effectiveness of technology for the sorting and the

application of adipose-derived stromal cells, they could soon become an important clinical tool for the treatment of a wide range of clinical pathologies.

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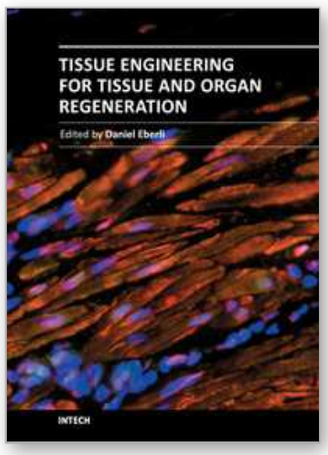

Tissue Engineering for Tissue and Organ Regeneration

Edited by Prof. Daniel Eberli

ISBN 978-953-307-688-1
Hard cover, 454 pages
Publisher InTech
Published online 17, August, 2011
Published in print edition August, 2011

Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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