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ABSTRACT

THE SYNERGISTIC EFFECT OF PULSED ELECTROMAGNETIC FIELDS AND DEMINERALIZED BONE MATRIX ON THE OSTEOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS

by
John Manocchio

Orthopaedic bone grafting continues to be a mainstay in the treatment of non unions and other difficult to heal bone defects. Tissue engineering strategies have focused on providing bone graft materials that are osteoconductive, osteoinductive and osteogenic in order to achieve optimal bone healing. Furthermore, electrical stimulation technologies such as pulsed electromagnetic fields (PEMF) have been used to enhance the effectiveness of bone graft materials. PEMF has been shown to have a synergistic effect with BMP-2 and it was hypothesized that PEMF would have the same effect with demineralized bone matrix (DBM). In the present study human mesenchymal stem cells (hMSCs) were seeded on DBM scaffolds in the presence of PEMF to determine if an increased osteogenic response could be induced. hMSCs were harvested from scaffolds at 5, 7, 14, and 21 days and osteogenic differentiation was assessed by testing for expression of alkaline phosphatase (ALP) and osteocalcin. Results demonstrated that PEMF was able to induce an increased osteogenic response throughout the 21 day culture. ALP and osteocalcin were significantly increased for PEMF treated groups at the earlier day 7 timepoint. DBM contains several osteoinductive growth factors and BMPs including BMP-2. The synergistic response seen between PEMF and DBM was most likely attributed to the osteoinductive factors found in DBM.

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To my parents for their continued support.

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CHAPTER 1

INTRODUCTION

1.1 Orthopaedic Bone Grafting

The enhancement of bone formation through the use of different bone graft materials is an area of active research. Bone grafts have been used for various orthopaedic applications including the reconstruction or replacement of skeletal defects, augmenting fracture repairs, and the stimulation of arthrodeses following oncology surgery, trauma, growth defects, and arthritis.[1] Ideally, bone graft materials should fulfill three areas necessary for optimal bone formation: osteoconduction, osteoinduction, and osteogenecity. Osteoconduction is the ability of a bone graft to act as a scaffold for new bone growth. These grafts should provide a three-dimensional structure that functions as a trellis for osteoprogenitor cells to form new bone as well as support the ingrowth of blood vessels and capillaries through angiogenesis.[2,3] Osteoinduction involves a cascade of biological steps centering around chemotaxis, mitosis, and differentiation of osteoprogenitor and mesenchymal stem cells (MSCs) in order to induce bone formation.[4] Bone grafts that are osteoinductive contain proteins and growth factors that induce bone formation through the recruitment of osteoprogenitors and MSCs that undergo osteoblastic differentiation.[3,5-7] Several factors and proteins have been investigated for their osteoinductive potential, most notably bone morphogenic proteins (BMPs) and transforming growth factor-beta (TGF-ß).[3,8] BMPs are low molecular weight proteins capable of initiating endochondral bone formation by stimulating osteoprogenitor cells and by enhancing collagen synthesis. TGF- ß is growth factor closely related to BMPs, that is synthesized in many tissues including bone and is capable of stimulating bone

formation.[1,8] Osteogenic grafts contain living bone forming cells such as osteoblasts or cells capable of becoming bone forming cells such as osteoprogenitors and MSCs. An ideal bone graft will be a suitable osteoconductive scaffold and also contain the necessary osteoinductive growth factors and proteins to induce bone formation when coupled with living osteogenic cells.

Autogenous cancellous bone graft contains all three of the aforementioned characteristics and therefore has long been considered the gold standard for orthopaedic bone grafting.[1,3,8,9] The organic collagen matrix and inorganic hydroxyapatite of autogenous bone serves as an excellent osteoconductive scaffold for bone formation.[1] Numerous osteoprogenitor and MSCs are contained within the periosteum lining as well as bone marrow taken along with the graft making it osteogenic.[9] Finally, the autogenous bone and adjacent clot contain a host of growth factors and proteins, including BMPs and TGF-ß making it osteoinductive.[8] It should be noted that cancellous bone is more commonly used, but autogenous cortical bone is used on occasion for its superior mechanical properties.[1] Additionally, autogenous grafts have the advantage of being both immunocompatible and histocompatible because they are being harvested and implanted within the same donor.

Although autograft is considered the gold standard, it does have several disadvantages opening the door for the use of alternative bone graft materials. Disadvantages of autograft include limited quantity and significant donor-site morbidity. Donor-site morbidity rates related to autograft harvesting have been reported as high as 25% and include complications such as infections, pain, increased anesthesia time, and increased operative blood loss.[10,11] Recent efforts to reduce the morbidity associated with

autogenous bone grafting have led to the development of several alternatives such as allograft and synthetic ceramic grafts (Table 1.1). Additionally, certain orthopaedic procedures may require a large volume of bone graft. In some cases the amount of autograft that can be safely harvested is not sufficient, requiring the use of alternative grafts as bone graft extenders.

Approximately 800,000 allografts are used in the USA each year.[12] Allograft bone is typically harvested from a cadaveric donor, which is then frozen or processed to be later implanted in a patient as a bone graft. Once harvested, allografts are either frozen at -60°C or lyophilized to reduce degradation by enzymes, decrease immunogenecity and preserve mechanical integrity.[1,8] Several different forms of allograft bone are currently used in orthopaedics, including various sizes of chips, granules, struts, segments, shafts, corticocancellous grafts and massive structural bone allografts.[13] Allografts are osteoconductive and depending on the format and application, can sometimes be used as a structural grafts. Processing of allogeneic bone is done aseptically and is designed to remove all cellular components, including any osteogenic cells. Although BMPs and growth factors naturally reside in bone, they are bound within the mineral portion, significantly reducing the osteoinductive ability of allograft.[8] A major concern with the use of allogeneic bone is the risk of disease transmission. Although the risk is low, transmission of the hepatitis C virus and human immunodeficiency virus (HIV) through allograft transplantation are both well documented.[14,15] Recent trends with allograft bone have focused on donor screening procedures and tissue processing standards to cut down on the risk of disease transmission.[16]

Demineralized bone matrix (DBM) is a form of allograft in which the mineral portion of bone is removed in order to expose osteoinductive proteins and growth factors. Demineralization is achieved by treating bone with an acid in order to remove the mineral hydroxyapatite portion, leaving non-collagenous proteins, growth factors and collagen. DBM was first developed in the 1960's by Urist[17], who later went on to isolate BMPs in DBM. The amount of BMPs and growth factors found in DBM varies among donors[18], which has led to the development of several different assays used to quantify the osteoinductive potential of DBM. Preliminary assays for osteoinductivity have focused on the intramuscular implantation of DBM into athymic rats in order to test for ectopic bone formation.[19] While this model has proven to be an accurate test for assessing DBM osteoinductvity, additional assays have been sought out due to the relatively long implantation time required. Recent work has focused on the development of in-vitro assays, which can be correlated to the athymic rat model. Han et. al.[20] has developed an in vitro assay looking at alkaline phosphatase expression of pluripotent myoblast C2C12 cells in the presence of DBM. The assay was correlated to the in vivo athymic rat model and proved to be a less cumbersome model for assessing DBM osteoinductivity. DBM has no cellular component and therefore is not osteogenic. Removal of the hydroxyapatite mineral portion eliminates any structural properties of the allograft, but the collagen that is left over is somewhat osteoconductive[21].

Ceramic bone graft materials provide users with a synthetic alternative to autogenous and allogeneic grafts. Ceramic bone grafts are generally comprised of calcium sulfates, calcium phosphates (hydroxyapatite), tricalcium phosphates (TCP), calcium carbonates or combinations of different ceramics.[22-27] The grafts are

manufactured commercially with no biological components and therefore cannot be osteogenic or osteoinductive, but are osteoconductive.[1,8,28] Ceramics are biocompatible and experience no adverse effects such as inflammation or foreign-body responses when they are in a structural arrangement. [8,26,29,30] Ceramics can be sterilized via traditional means[31] and because they are synthetic avoid the risk of disease transmission seen with allograft. Ceramics can vary by crystallinity, porosity, mechanical strength and by their resorption rates in the body.[28]

Bone Graft	Features / Properties		
	Osteoconductive	**Osteoinductive**	**Osteogenic**
Autogenous Bone	**YES** *Autogenous bone serves as scaffold for new bone growth*[1]	**YES** *Bone and adjacent blood clot contains growth factors including TGF-β & BMPs*[8]	**YES** *Periosteum lining and entrapped bone marrow contain MSCs and osteoprogenitors*[9]
Allogenic Bone	**YES** *Allogenic bone serves as a scaffold for new bone growth*[13]	**NO** *Native BMPs and growth factors are trapped within mineral portion*[8]	**NO** *Processing of allogenic bone destroys all cellular components*[1,8]
DBM	**YES** *Collagen portion may act as a scaffold for new bone growth*[21]	**YES** *Demineralization allows native BMPs and growth factors to diffuse out*[17]	**NO** *Processing of allogenic bone destroys all cellular components*[1,8]
Ceramics	**YES** *Ceramics serve as a scaffold for new bone growth*[1,8,28]	**NO** *Ceramics are synthetic materials, which do not contain any native biological components*	**NO** *Ceramics are synthetic materials, which do not contain any native biological components*

Table 1.1 Current bone grafting options and their respective features / properties.

1.3 Electrical Stimulation and Bone Healing

Electrical stimulation has been utilized in orthopaedics for several decades. Dating back to the 1960s Bassett et. al. investigated the electrical response in living bone when subjected to mechanical strain.[32-34] The investigation revealed that when hydrated and deformed under mechanical strain, living bone became piezoelectrically charged.[32] Experiments also demonstrated that the polarity of the electric potential correlated to the underlying physiological response in bone. Specifically, bone growth is associated with negative electric potentials and bone resorption to positive electric potentials.[34] Based on this initial work it was hypothesized that there was a link between the electrical fields generated in bone and Wolf's Law.[32-36] Wolff's Law states that bone structure responds to mechanical strain by remodeling to accommodate the applied forces.[37] Essentially bone under stress will undergo bone formation and bone not under stress will experience bone resorption. Subsequently, bone under stress exhibits a positive electric potential and bone under no stress has a negative potential. These same principals have also been shown to play a role physiologically during injury and bone growth.[37,38] Injury potentials are electric fields generated by both soft and hard tissue in response to an injury. Growth potentials are electric potentials produced by bone at sites of rapid bone growth such as the growth plates during development.

Once a link between electric potentials and bone remodeling was established, researchers sought out to investigate the response when electric potentials were induced in bone. Initial pre clinical studies established that direct negative electric currents induced from a cathode could increase the rate of bone formation and healing.[34,39-41] This

work led to future clinical studies to see if the same concept could produce efficacious and safe results in a clinical setting. One of the first clinical studies looking at electrical stimulation for bone repair consisted of a multicenter study evaluating the use of direct current to treat non-unions.[42] In total 175 patients were enrolled in the study and it was determined that direct current achieved a healing rate (83.7%) that was comparable to the current standard of autogenous bone grafting. The initial success of electrical stimulation in bone healing has spawned continued research in the field, which has led to the development of several different technologies that are used today. They include inductive coupling devices such as pulsed electromagnetic fields and combined magnetic fields, direct current, and capacitive coupling.

1.4 Pulsed Electromagnetic Fields

Inductive coupling stimulation consists of one or two external coils connected to a signal generator and designed to deliver a magnetic field (Fig. 1.a). Inductive coupling signals vary in types of pulse (single pulse or pulsed burst), frequency, amplitudes, and time varying electromagnetic fields (EMFs) of 0.1 to 20G.[37] Pulsed electromagnetic field (PEMF) technologies produce magnetic fields consisting of repetitive pulsed burst or single pulsed signals (Fig. 1.b). Unlike DC technologies, which require implantation, PEMF can be applied non-invasively over a fracture site in order to elicit a bone healing response. Several pre-clinical animal studies have been performed in order to substantiate the ability of PEMF to stimulate bone healing. PEMF has been shown to stimulate healing rates of fractures in canines[43,44] and rats[45,46], and non unions in canines.[47,48] In a recent rabbit tibial distraction model PEMF was shown to accelerate

the rate of bone formation.[49] Biomechanical analysis demonstrated that by 16 days post-distraction, the PEMF group had achieved a torsional strength equivalent to intact bone. Similarly, PEMF resulted in a statistically significant increase in torsional stiffness when compared to a control in a canine tibial osteotomy model.[50] Histomorphometric analyses from the study revealed greater bone formation, increased mineral apposition rate, and decreased porosity in the cortex adjacent to the osteotomy line for PEMF treated groups. Finally, PEMF has shown increased benefit in terms of healing and graft encorporation when used in combination with ceramic implants and scaffolds.[51-53] Histological and biomechanical analysis of hydroxyapatite implants implanted into femoral cortical bone of rabbits demonstrated that PEMF increased the direct contact of bone to the implant as well as mechanical fixation of the implant.[52]

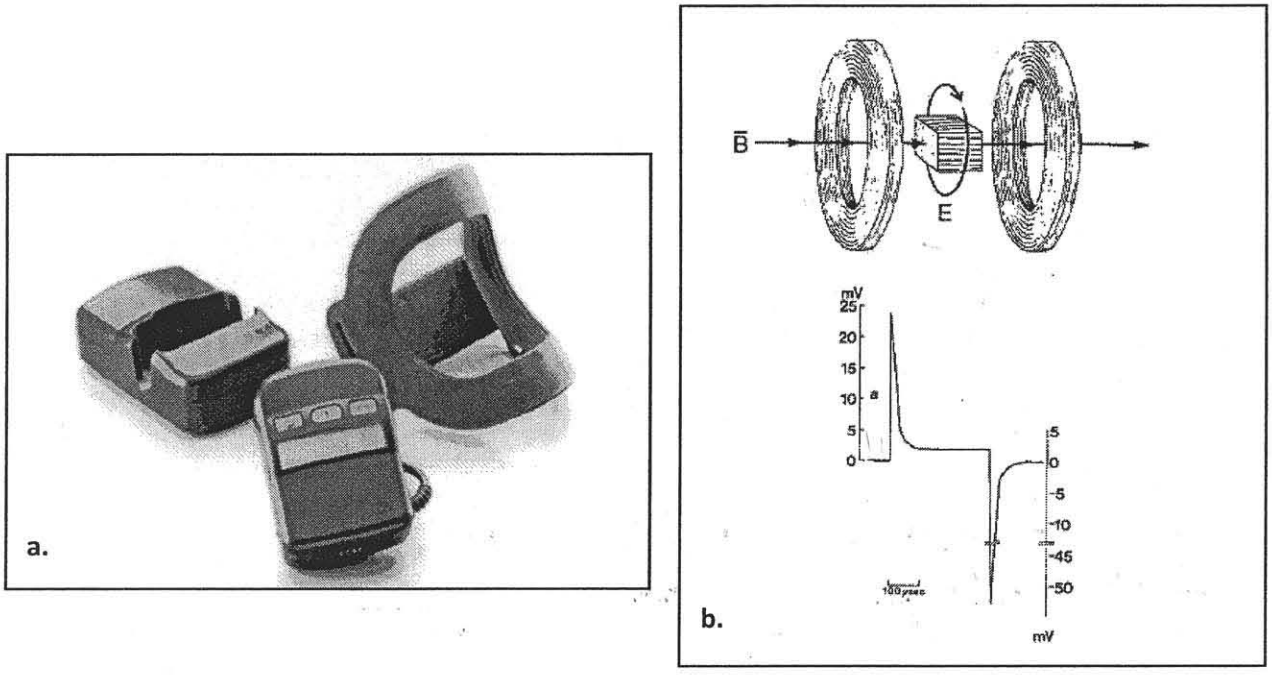

Figure 1.1 (a) Example of a commercial PEMF bone healing unit[54], (b) Diagram of a voltage waveform induced in cortical bone by single pulse PEMFs oriented along the long axis of the bone.[55]

In addition to animal studies, many surgeons have performed clinical studies aimed at evaluating PEMF in several different bone healing applications. PEMF has been shown to be effective in treating scaphoid nonunions[56-58], knee arthrodesis[59], congenital pseudarthrosis of the tibia[60,61], Jones fractures[62], and osteonecrosis of the femoral head.[63-65] The bulk of the published clinical studies with PEMF center on the treatment of non unions. Basset et. al.[66] evaluated the use of PEMF and autogenous bone grafts to treat 83 patients with ununited fractures. Fractures included both lower and upper extremities with the PEMF / autograft treatment resulting in a 90% fusion rate. In 1990 a multi-center double-blind clinical study was published investigating PEMF for the treatment of tibial fracture delayed unions.[67] A total of 45 patients with fractures were chosen for the study for their liability to delayed union due to the presence of moderate or severe displacement, angulation or comminution or a compound lesion with moderate or severe injury to skin and soft tissues. Patients were treated with a hard cast and either an active or inactive PEMF unit. Radiographic assessment of the fractures at 12 weeks revealed a statistically significant increase in the rate of fusion for patients treated with PEMF. Patients that were actively treated with PEMF had a 45% fusion rate, while those who did not receive active treatment had only a 12% healing rate. More recently, a similar double-blind study evaluating PEMF for the treatment of tibial non-unions was performed by Simonis et. al.[68] A total of 34 patients with tibial non-unions were randomly assigned to an active and non active PEMF group. Patients were assessed clinically and radiographically for healing at 6 months post surgery. The PEMF group had an overall fusion rate of 89% or 39% higher than that of the non active group.

Until recently electrical stimulation technologies such as PEMF were considered a "black box". Animal studies and clinical studies have demonstrated that PEMF can increase the rate of bone healing, but recent in vitro studies have begun to look at the mechanism of action behind PEMF. In vitro cultures of hMSCs on calcium phosphate scaffolds have shown increased osteoblastic differentiation in response to PEMF.[69] Increased osteoblastic differentiation was only seen in cultures treated with BMP-2, suggesting that PEMF induced surface-dependent changes making cells more responsive to BMP-2 induced differentiation. In addition to increasing cell responsiveness to BMP-2, PEMF has been shown to increase mRNA expression of several BMPs, including BMP-2, 4, 5 and 7.[70-72] In a calvaria chick embryo model, PEMF was shown to enhance BMP-2 mRNA levels by a 2.7 and 1.6 fold increase on days 15 and 17, respectively.[70] Similarly, PEMF resulted in a 1.6 fold increase of BMP-4 on day 15 and 1.5 fold increase on day 17. In the same study PEMF was shown to enhance in vivo bone formation in young embryos. The increased bone formation was correlated to the upregulated expression of BMP-2 and 4 mRNA, suggesting that the upregulation of BMP-2 and 4 may mediate the bone inductive effect of PEMF. Yajima et. al.[71] used RTPCR to examine PEMF treated human osteoblasts and their mRNA expression for various BMPs. PEMF was shown to significantly enhance levels of BMP-2, 4, 5 and 7 in a time dependent manner, with a maximum increase after 24 hours of treatment. Similar experiments using RTPCR have indicated that PEMF exposure to rat calvarial osteoblasts can lead to increased mRNA transcription of BMP-2 and 4.[73] PEMF has shown similar effects in eliciting increased mRNA expression of TGF-ß.[74] PEMF was able to

moderately increase levels of TGF-ß for a sustained period of time suggesting the ability to generate temporal concentration gradients of growth factors to stimulate bone repair.

In vitro experiments with PEMF have also demonstrated changes at the bone cell surface, which in turn affect mRNA and DNA synthesis of proteins and growth factors.[37,75-82] Exposure to PEMF leads to increased calcium uptake of bone and makes it insensitive to parathyroid hormone (PTH)[81,82] by inhibiting cyclic-Adenosine Monophosphate (cAMP)[77] and PTH cell surface receptors.[79] Similarly, in vitro studies have demonstrated increased calcium uptake of osteoblasts with PEMF.[78] Calcium influences interactions between cell surface receptors, antibodies, hormones and neurotransmitters.[75,80] Bone cell surface interactions modulate bone healing through amplification of signal transduction pathways[76] that eventually effect mRNA and DNA synthesis.

1.5 MSCs and Osteogenesis

Adult stem cells are resting cells present in small numbers in adult tissue with the capacity for asymmetric cell division and self renewal.[83] Biological signals and factors capable of stimulating resting stem cells are responsible for inducing mitotic cell divisions and morphogenic cell differentiations. MSCs are multipotent adult stem cells capable of differentiating into cartilage, bone, tendon, ligament, marrow stroma and other connective tissues within the mesodermic lineage(Fig. 1.2).[84] MSCs can be harvested from several sources including, muscle, adipose, and placental tissue, but bone marrow remains to be the most readably available source.[85-88] MSCs are present in concentrations of approximately 36 per 1 million nucleated cells or approximately 2000 MSCs per 2 ml

of bone marrow aspirate (BMA).[86] Characterization and quantification of MSCs is often performed using in vitro colony forming unit (CFU) assays specifically looking for the expression of fibroblasts[89] or alkaline phosphatase[90]. Several different antibodies, including CD105, CD44, CD166, CD29, CD90, and CD73 have also been identified as markers that can be used to characterize MSCs.[91]

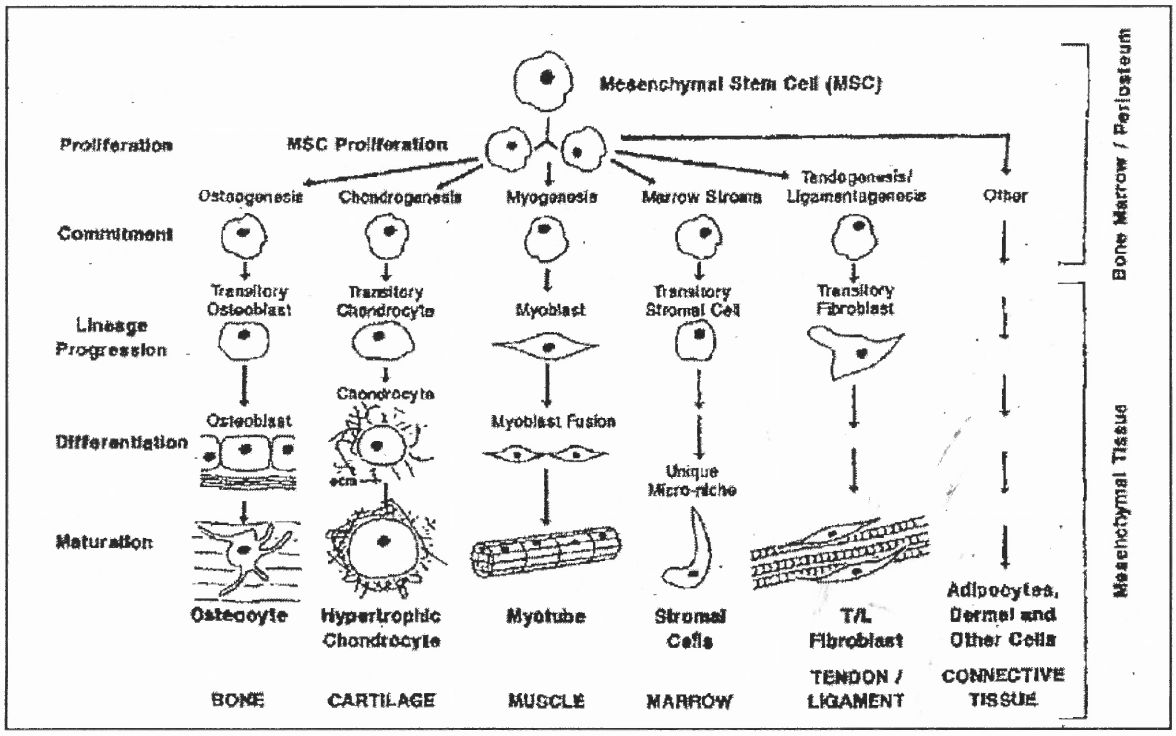

Figure 1.2 MSCs are multipotent adult stem cells capable of differentiating into cartilage, bone, tendon, ligament, marrow stroma and other connective tissues.[84]

The progression from MSCs to a final phenotype such as bone is multistep transition dependent upon both autocrine and paracrine regulation.[92] Bruder and Caplan et. al. [93-97] have looked at the monoclonal antibodies generated against specific surface antigens on differentiating osteogenic cells in order to define the various stages of osteoblastic differentiation. Once committed to the osteogenic pathway, MSCs go

through a series of cell differentiations marked by different surface antigens (Fig 1.3).[92] In vitro and in vivo phenotypes differ slightly, but MSCs generally start out as early osteoprogenitors, which differentiate into pre then transitory osteoblasts and eventually into dormant osteocytes.

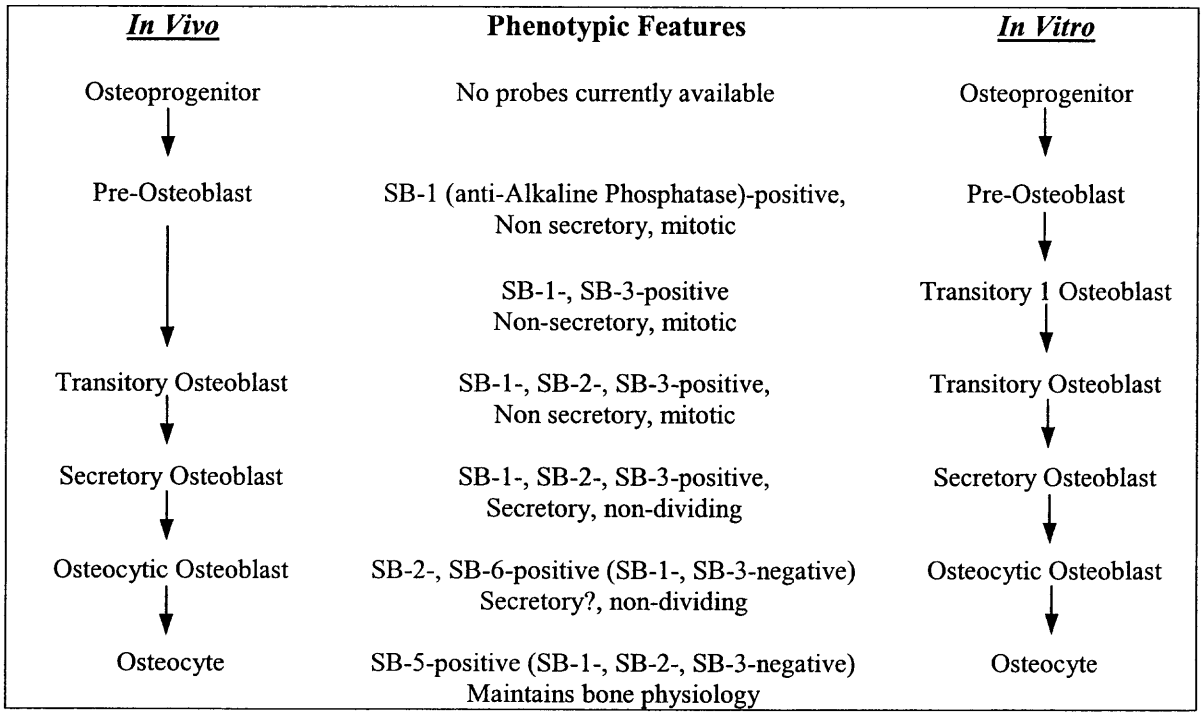

Figure 1.3 MSCs express several different phenotypic features as they undergo osteoblastic differentiation in vivo and in vitro.

Researchers have also looked into defining the mechanism of action for the biological signals and factors responsible for inducing MSCs down the osteogenic lineage. Members of the TGF-ß super-family, which includes TGF-ß1, BMPs, and other growth and differentiation factors, are believed to play significant roles in stimulating the osteogenic differentiation of MSCs.[98,99] TGF-ß proteins, specifically BMPs and TGF-ß1, have been studied extensively to determine how they interact with MSCs during osteogenic differentiation.[99-106] TGF- ß family members signal by binding activin

receptor-like kinases (ALKs), which initiate intracellular signaling through the phosphorylation of specific Smad proteins. Once phorphorylated, Smad proteins move from the cytoplasm to the nucleus where they go on to control transcription of certain genes.[99] BMPs and TGF-ßs have been shown to upregulate Runx2 mRNA expression levels when inducing osteoblastic differentiation of C2C12 cells (Fig. 1.4).[99,102] Runx2 has been shown to interact with BMP specific R-Smads, which are believed to be important in inducing osteoblastic differentiation.[99,106] In humans, mutations in the Runx2 gene have been linked to cleidocranial dysplasia (CCD), a disorder causing delayed closure of sutures, small or absent clavicles, and tooth anomalies.[101,103] Similarly, Runx2 deficiencies in mice have been linked to a decrease in osteoblastic proliferation.[100] Finally, recent studies have demonstrated that BMPs induce the expression of homeobox containing transcription factors Distal-less (Dlx-5), Msx-1, and Msx-2, which have been shown to be important in osteogenesis.[104,105]

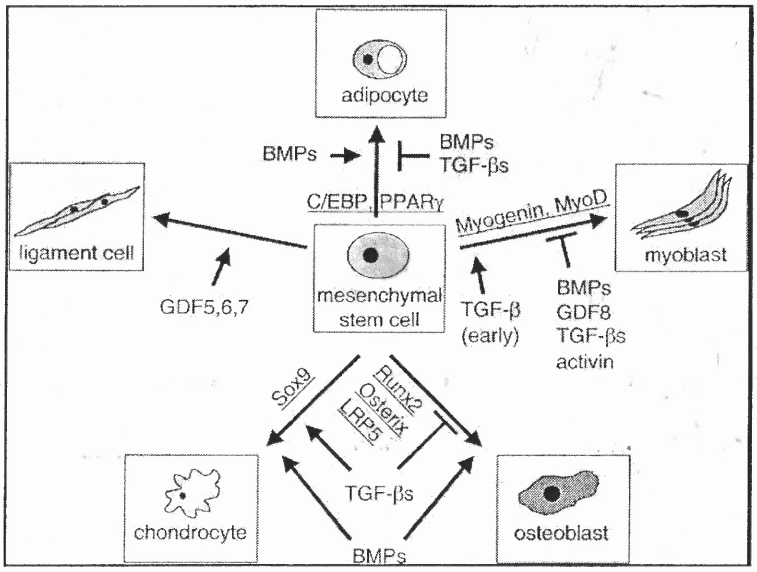

Figure 1.4 Stimulatory or inhibitory effects of TGF-ß super-family members on differentiation of MSCs toward different lineages.[99]

1.6 Bone Marrow Derived MSCs in Orthopaedics

Recent research in the field of orthopaedics has examined the use of bone marrow derived MSCs for different bone grafting applications. Pre clinical research has centered on evaluating the osteogenic potential of MSCs when combined with osteoconductive scaffolds.[107-113] Bruder et. al.[107] performed a canine segmental defect model with autologous MSCs loaded onto porous ceramic (β-TCP/hydroxyapatite) cylinders. Cylinders with and without cells were implanted into critical sized femoral defects and evaluated radiographically and histologically at 16 weeks post implantation. Cylinders loaded with MSCs had a radiographic union rate of 100% compared to 83% without cells. Histomorphometry confirmed what was seen in the radiographs. Porous cylinders loaded with MSCs were shown to have 39.9% new bone ingrowth, which was statistically greater than the 24.0% ingrowth seen in cylinders with out MSCs. A sheep tibial diaphyseal defect model was recently performed in order to evaluate isolated MSCs combined with porous hydroxyapatite cylindrical scaffolds.[108] Scaffolds with and without cells were implanted into the osseous defect and evaluated for healing 2 months post implantation. Biomechanical analysis revealed higher stiffness values for the MSC group compared to scaffolds without MSCs. Histomorphometry performed on explants demonstrated a significantly higher volume of new bone formation for MSC loaded implants (54.2%) compared to the scaffold without MSCs group (8.6%).

Although the amount of published clinical data for bone marrow derived MSCs is low, there are a few key studies that highlight the efficacy of using MSCs and BMA in orthopaedics. Connolly et. al.[114,115] performed one of the earliest studies looking at the use of BMA as a substitute for autograft in the treatment of tibial nonunions. In the study

a total of 20 patients were treated percutaneously with injections of BMA, resulting in a 90% healing rate. Healing time was reported at 7 months and was essentially equivalent to healing times for autograft.[115-117] In a retrospective study for posterior spinal fusion in adolescent idiopathic scoliosis BMA was combined with DBM and fusion was assessed radiographically.[118] The BMA/DBM composite graft was able to achieve an 88.9% fusion rate, which was equivalent to the fusion rate for autograft (87.5%). BMA has also been shown to improve the effectiveness of xenogeneic bone for different bone grafting procedures in pediatrics.[119] More recently Hernigou et. al.[120-123] has published data on the use of concentrated BMA (cBMA) for the treatment of nonunions and avascular necrosis (AVN). BMA was processed using a centrifugation device in order to concentrate the number of MSCs available for treatment. Nonunion patients treated with cBMA had a healing rate of 88% by an average of 12 weeks.[121] cBMA was shown to have approximately a 4.2 times mean increase in MSCs over baseline BMA. The investigator also reported that a minimum of 1500 MSCs/cc (3 X 10^5 MSCs total) was delivered to those patients that healed; speculating that there is a minimum number of MSCs required for the treatment of nonunions. Studies by the same author were performed evaluating the use of cBMA to treat AVN of the femoral head[123]. When patients were treated at stages I or II AVN, cBMA was able to achieve a success rate of 94%. Similarly, better outcomes were seen in patients treated with a higher number of MSCs.

In vitro cultures have been used extensively to examine osteogenic differentiation of MSCs. These cultures have relied on several different proteins and genes that have been identified as markers of MSC osteoblastic differentiation[124]. The initial proliferative phase of MSCs in vitro is characterized by the expression of nuclear proteins H4 histone,

c-fos, and c-jun. Differentiation follows with expression of Cbfa1 and then upregulation of type 1 collagen, osteonectin and alkaline phosphatase (ALP). The final phase is linked to extracellular mineralized matrix formation by secreting osteoblasts and can be identified by the expression of osteocalcin, bone sialoprotein (BSP), and responsiveness to 1,25-dihyroxy vitamin D and parathyroid hormone.[124-128] These markers are often used to characterize the osteogenic response of MSCs cultured on various scaffolds in order to determine optimal characteristics such as material, surface texture and porosity. MSCs have been cultured in vitro on ceramic[129-132], polymer[133,134], ceramic/polymer composites[135], silk[136,137], DBM[138-140] and collagen[141,142] scaffolds to ascertain their osteoconductive and osteoinductive potential. Coralline hydroxyapatite (HA) scaffolds with pore sizes of 200 and 500 μm were recently evaluated for the expansion and differentiation of hMSCs in vitro.[130] Cells were harvested at days 1, 7, 14 and 21. Scanning electron microscopy (SEM) performed on seeded scaffolds revealed that cells coated both scaffolds on the surface and within the pores (Fig 1.5). ALP activity peaked at day 14 for both scaffolds, but was significantly higher for the 200 μm pore scaffold. mRNA expression of ALP and osteocalcin quantified using RTPCR saw similar results with increased activity for the 200 μm pore size.

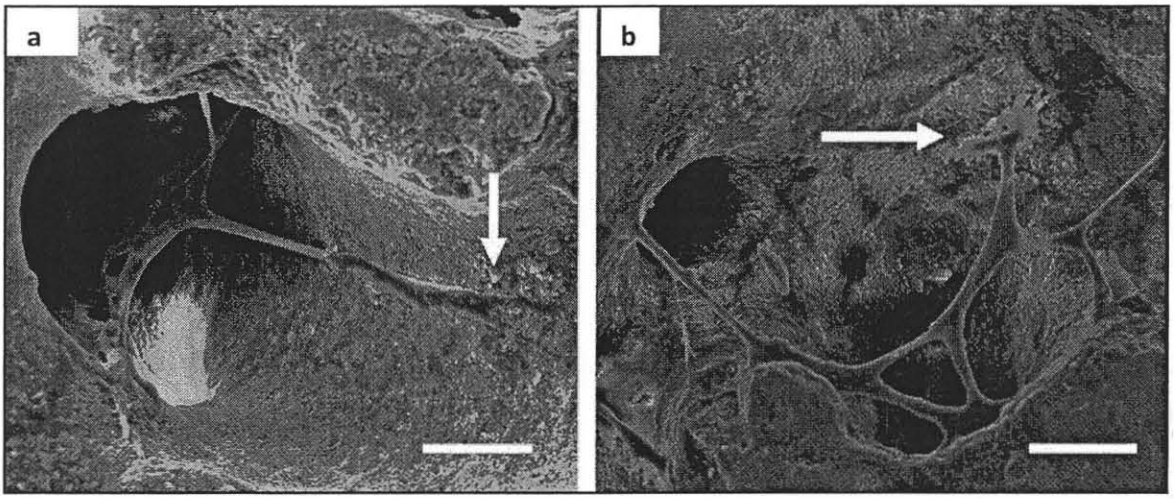

Figure 1.5 Scanning electron micrographs of hMSCs cultured on the top surface of coralline HA scaffolds with (a) 200 μm and (b) 500 μm pores.[130]

An in vitro study performed by Muller et. al.[129] compared scaffolds made of calcium phosphate/silicon oxide (CPS) and hydroxyapatite to standard tissue culture plastic. MSCs were harvested after 14 days and mRNA expression of several osteogenic markers including ALP, BSP, and osteocalcin were measured. mRNA expression was significantly higher for the scaffold groups in standard growth media, while expression was the same for scaffolds and tissue culture plastic in osteoinductive media supplemented with dexamethasone. Dexamethosone is used with in vitro cell cultures to induce osteogenic differentiation of MSCs. Typical osteoinductive media containing small amounts of dexamethasone results in a significant increase in the expression of osteogenic markers in vitro.[143] Increased osteogenic differentiation of MSCs cultured on CPS and hydroxyapatite scaffolds in standard growth media is indicative of an osteoinductive response caused by the scaffolds. DBM scaffolds have also been evaluated with MSCs due to their ability to release naturally occurring growth factors and BMPs. In a recent study, AP levels were assessed for hMSCs cultured on partially

demineralized cancellous bone scaffolds.[138] AP levels were significantly greater the DBM scaffold group at days 7 and 14, with peak levels at day 7. SEM analysis revealed that the majority of cells on the DBM scaffold had a cuboidal shape indicative of mature osteoblasts, which contrasted to the fibrolast cell morphology seen on control scaffolds.

1.7 Bone Marrow Derived MSCs and PEMF

PEMF has been shown to be effective in stimulating bone healing through the upregulation of several growth factors and BMPs. Previous work has investigated PEMF's ability to stimulate osteoblastic differentiation of MSCs in vitro.[69,144-147] hMSCs treated with PEMF and BMP-2 resulted in increased levels of osteocalcin at 24 days post confluence.[69,146] Within the same study hMSCs cultured on calcium phosphate discs in the presence of PEMF and BMP-2 were shown to have increased levels of AP, TGF-β1 and prostaglandin E2 (PGE_2). In both cases PEMF and BMP-2 were shown to have a synergistic effect on the osteoblastic differentiation of MSCs. Similar studies have shown increased gene expression for MSCs cultured with weak, low-frequency PEMF.[145] EMFs have been shown to stimulate osteoblastic differentiation of mouse MSCs (mMSC).[144] Cylic AMP (cAMP) levels were also increased at days 1-3, indicating that cAMP may be involved with EMF induced osteoblastic differentiation at early timepoints. Finally, Moioli et al.[147] recently used PEMF with MSCs seeded on 3D scaffolds in order to evaluate the technology with a more clinically relevant model. HMSCs were cultured on UV-photo polymerized polyehtyleneglycol diacrylate (PEGDA) hydrogels with and without PEMF and analyzed for expression of calcium and

osteocalcin. PEMF was able to induce increased levels of both calcium and osteocalcin at 2 weeks, however PEMF had no affect at 4 weeks.

Studies have shown that PEMF and BMP-2 can have a synergistic effect and induce an increased level of osteogenic differentiation of MSCs. DBM is an osteoconductive scaffold that has been shown to support cellular differentiation in vitro. Furthermore, DBM is also an osteoinductive material that contains several growth factors and BMPs including BMP-2. The present study will look to evaluate whether PEMF can induce an elevated osteogenic response in hMSCs cultured in vitro on DBM scaffolds.

CHAPTER 2

METHODS

2.1 hMSC Isolation and Expansion

Human bone marrow aspirated from the iliac crests of a healthy adult donor (Cambrex Corp, East Rutherford, NJ) was prepared according to previous protocols.[148] Briefly, the 25 mL marrow sample was washed with saline, followed by centrifugation over a density gradient. The interface layer was removed, washed, and nucleated cell counts were performed. Nucleated cells recovered from the density separation were washed and plated in tissue culture flasks in growth medium (GM) consisting of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. Colony formation was monitored for a 14-17 day period. When the tissue culture flasks were near confluent (~80%), the hMSCs were passaged. At the end of the first passage, MSCs were enzymatically removed from the culture flask using trypsin-EDTA and re-plated at a lower density for further expansion. At the end of the second passage, MSCs were cryopreserved for later use.

2.2 Scaffold Preparation

Lyophilized human cortical allograft bone cylinders were obtained from an adult donor who had successfully passed US Food and Drug Administration FDA and American Association of Tissue Banks AATB screening procedures (University of Miami Tissue Bank, Miami, FL).[149] The 5.0 mm (0.197 in) diameter cortical cylinders were cut into 0.50 mm (0.019 in) discs using a rotary diamond coated saw (Buehler, Lake

Bluff, IL). Demineralization was performed by a modification of the technique described by Reddi and Huggins.[150] The cortical discs were demineralized in 0.5 N HCL for 24 hrs (3 hr at room temperature, 21 hr at 4° C) with constant stirring, using 50 mL of HCL per gram of cortical bone. The DBM scaffolds were then washed in purified water, pH 7.4 phosphate buffer, followed by a final water wash. The DBM scaffolds were then frozen at - 20° C and lyophilized (Virtis, Gardiner, NY). DBM scaffolds were stored at -20° C in order to preserve their osteoinductivity.[151] Deproteinized guanidine-extracted inactive DBM scaffolds were generated using a modification of the technique described by Han et al.[20] DBM scaffolds were partially deproteinized and extracted with 10 ml of 4M guanidine-HCL (50 mM Tris, pH 7.4) for 24 hr at room temperature with constant agitation. The inactive DBM (dDBM) scaffolds were rinsed several times with distilled water. Scaffolds were sterilized via an ethanol immersion for 20 minutes followed by 3 successive rinses in PBS totaling 50 minutes.

In vitro cultivation of hMSCs

Sterile DBM and dDBM scaffolds were placed in 96 well polypropylene non-adherent tissue culture plates (BD Biosciences, San Jose, CA). The cryopreserved hMSCs were thawed and seeded at 15.15 x 10^3 cells/cm^2 on the DBM and dDBM scaffolds. hMSCs were also seeded at the same density in 96 well polystyrene tissue culture (PTC) treated plates (BD Biosciences), which served as a control. Cells were grown in GM for 2 days in a humidified 37°C/5% CO_2 incubator to allow the cells to reach confluency. At confluence GM was replaced with Osteogenic Media (OM) consisting of GM supplemented with 1mM Dexamethasone (0.01% v/v), 1M ß-

Glycerophosphate (1.0% v/v) and 10mM ascorbic acid (0.5% v/v). Media was replaced every 2nd or 3rd day until harvest at days 5, 7, 14 and 21 days post confluence.

2.3 Application of PEMF

To determine if pulsed electromagnetic fields (PEMF) could induce an osteogenic response PEMF was applied via a Helmholtz coil (Biomet, Parsippany, NJ) specially configured to two matched incubators.[69,152] Metal shelves inside the incubator were replaced with acrylic shelves to reduce any potential interference of the electromagnetic fields caused by current flow through the surrounding shelves. Prior to starting any experiments with PEMF, background fields in the incubator and surrounding room were measured. Background fields with the incubators off with and without the PEMF coils placed inside were the same at less than 1 mG (rms). Similarly, fields were 4 mG (rms) with incubator heaters and air circulators on. The PEMF signal that was used for this experiment is the same signal used clinically for the treatment of fracture nonunions or delayed fracture healing. The applied field consisted of 5-millisecond bursts of 20pulses, repeating at 15 Hz. During each pulse, the magnetic field increased from 0 to 18 G within 200 microseconds and then decayed back to 0 G in 25 microseconds. The PEMF coils were activated for 8 hours per day to simulate clinical use.

2.4 Cell Harvesting

hMSCs were harvested from the scaffolds and the amount of DNA was quantified in order to determine the total number of cells. Briefly, the scaffolds were rinsed and the cultured MSCs were lysed with 0.1% Triton-X. Scaffolds were immersed in 0.1%

Triton-X for 1 hr and sonicated for 120s. PicoGreen® dsDNA reagent (Invitrogen Corp., Carlsbad, CA) was used to quantify DNA in the cell lysate. PicoGreen dsDNA reagent is an ultrasensitive fluorescent nucleic acid stain for quantifying double-stranded DNA in solution. The amount of reagent bound DNA was quantified by reading absorbance on a FLx800™ Multi-Detection Microplate Reader (BioTek Instruments Inc, Winooski, VT) at 480 nm excitation / 520 nm emission using KC Junior software (BioTek Instruments Inc, Winooski, VT). The amount of DNA was then used to calculate the total number of cells harvested for each scaffold/group from a standard curve, which was created for each timepoint.

2.5 Alkaline Phosphatase Activity

Harvested hMSCs were assayed for the presence of alkaline phosphatase (ALP) at 5, 7, 14 and 21 days post confluence with and without PEMF to assess osteogenic differentiation. Cell lysate was assayed for ALP using *p*-nitrophenyl phosphate as a substrate (Sigma-Aldrich Corp, St. Louis, MO). The presence of ALP was measured by the hydrolysis of p-nitrophenylphosphate to p-nitrophenol using an absorbance plate reader (Molecular Devices, Sunnyvale, CA) at 410 nm with SoftMax Pro Software (Molecular Devices, Sunnyvale, CA).

2.6 Osteocalcin Activity

Harvested hMSCs were assayed for the expression of Osteocalcin at 7, 14 and 21 days post confluence in order to assess osteogenic differentiation. Cell lysate was assayed for intact osteocalcin using an EIA kit (Biomedical Technologies Inc, Stoughton,

MA). Monoclonal antibodies directed toward the amino and carboxy terminal regions of intact osteocalcin were utilized and absorbance was measured at 450 nm.

2.7 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed on scaffolds at 1, 7 and 21 days to assess cell morphology and distribution. Scaffolds were rinsed and fixed in paraformaldehyde – glutaraldehyde solution (Karnovsky fixative) followed by a dehydration using a series of immersions in increasing concentrations of ethanol. Dehydrated fixed scaffolds were then left to dry in Freon for 24 hours. Fixed scaffolds were carbon coated and viewed with an Electro Scan 2020 SEM (Electroscan, Wilmington, MA).

2.8 Statistical Analysis

All assays were performed with an $n = 4$ for each data point. Results in graphs are presented as mean ± standard error of the mean (SE). Results were analyzed using a one-way ANOVA (Fisher's, individual error rate). Statistically significant values were defined as $p \leq 0.05$. All statistical analyses were performed using Microsoft Excel and Minitab version 15.1.0.0 (Minitab Inc., State College, PA).

CHAPTER 3

RESULTS

3.1 Cell Harvest

hMSCs were harvested from dDBM and DBM scaffolds and PTC controls at 5, 7, 14 and 21 days post confluence. An average of 10.84 X 10^3 ± 4.41, 13.32 X 10^3 ± 3.15, and 16.50 X 10^3 ± 1.77 cells were harvested at day 5 from the control, dDBM and DBM scaffolds, respectively (Fig. 3.1). The total number of cells harvested from each scaffold remained relatively consistent throughout the culture, with trends showing a slight increase for all groups on day 14. PEMF had no effect on cell proliferation for any of the scaffold groups (Fig. 3.2). The complete dsDNA assay results for PTC controls and dDBM scaffolds can be found in Appendix A.

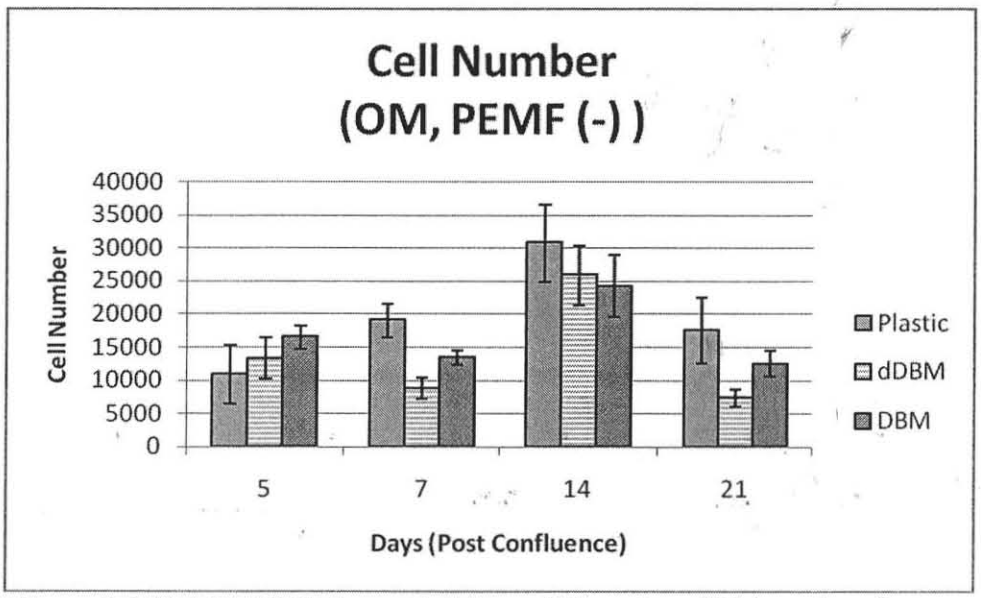

Figure 3.1 Total hMSCs harvested from different scaffolds and surfaces. hMSCs were grown in osteogenic media and were not treated with PEMF. Number of cells was determined using a DNA assay. Values are Mean ± SE.

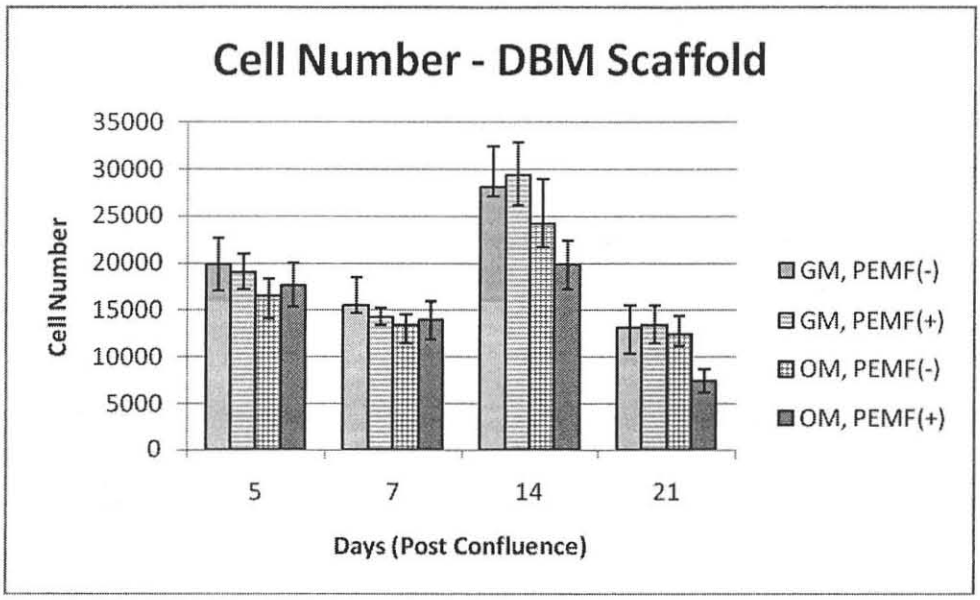

Figure 3.2 Total hMSCs harvested from dDBM & DBM scaffolds in OM with (+) or without (-) PEMF. Number of cells was determined using a DNA assay. Values are Mean ± SE.

3.2 Alkaline Phosphatase Activity

hMSCs harvested from scaffolds were assessed for ALP activity at 5, 7, 14, and 21 days post confluence. ALP is often used as a marker to assess osteogenic differentiation of MSCs in vitro. ALP expression increased significantly for hMSCs on both dDBM and DBM scaffolds with and without PEMF during the 21 day culture (Fig. 3.3: dDBM, PEMF(-) $p = 0.003$; dDBM PEMF(+) $p = 0.004$; DBM, PEMF(-) $p = 0.009$; DBM PEMF(+) $p = 0.009$). Cells harvested from PTC controls had no significant increase in ALP expression. No significant differences in ALP expression were revealed upon comparing performance of the different scaffolds. For both scaffold groups ALP expression increased steadily for the first 14 days and then significantly increased between days 14 and 21 days when exposed to PEMF (dDBM $p = 0.004$; DBM $p = 0.050$). Results indicated that there was a general trend where PEMF resulted in higher

mean ALP expression for hMSCs on dDBM (53%) and DBM (95%) scaffolds throughout the culture, but the increase over baseline groups without PEMF was only shown to be significant earlier timepoints. For hMSCs cultured on dDBM scaffolds PEMF resulted in a significant increase of ALP initially at day 5 (78%, $p = 0.004$) and then peaked at day 14 (110%, $p = 0.005$) (Fig. 3.4). hMSCs cultured on DBM scaffolds with PEMF saw a slight increase of ALP expression at day 5 (65%) and then peaked at day 7 (%257), although the increase was only significant at the latter timepoint ($p = 0.050$). Complete ALP results for both scaffolds and the PTC control can be found in Appendix B.

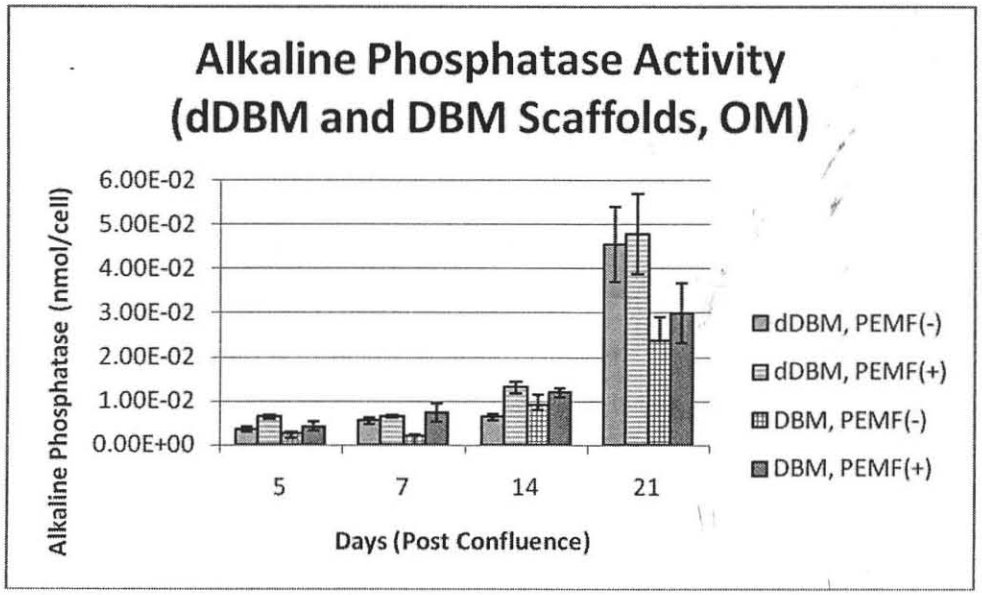

Figure 3.3 Alkaline phosphatase activity for hMSCs harvested from dDBM and DBM scaffolds in OM with (+) and without (-) PEMF.
Values are Mean ± SE.

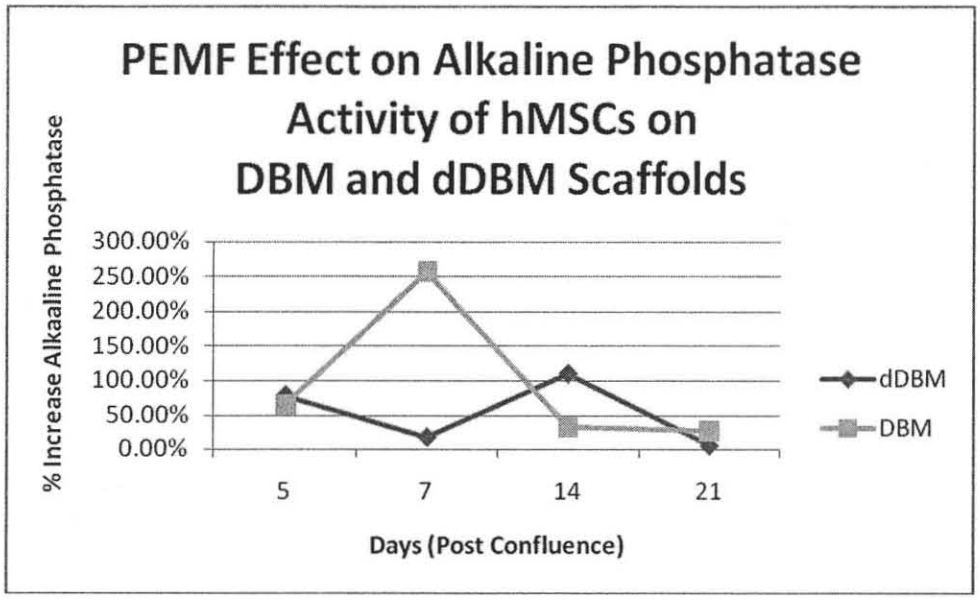

Figure 3.4 PEMF effect on alkaline phosphatase activity for hMSCs harvested from dDBM and DBM scaffolds in OM. Values are Mean.

3.3 Osteocalcin Activity

hMSCs harvested from scaffolds were assessed for osteocalcin activity at 5, 7, 14, and 21 days post confluence. Osteocalcin is used as an osteogenic marker in vitro and is typically expressed by MSCs during the latter stages of osteogenic differentiation. Osteocalcin levels remained relatively level for hMSCs on both dDBM and DBM scaffolds with and without PEMF during the 21 day culture (Fig. 3.5). Cells harvested from PTC controls had no significant increase in osteocalcin expression. No significant differences in osteocalcin expression were revealed upon comparing performance of the different scaffolds. PEMF did result in a general trend of increased mean osteocalcin expression for hMSCs seeded on dDBM (%44) and DBM (34%) scaffolds throughout the 21 day culture, but this increase was not significant. hMSCs on dDBM scaffolds had no PEMF induced osteocalcin increase initially at day 7, but then their was a slight increase

for days 14 and 21 (%75) (Fig. 3.6). However, the PEMF induced response was only significant for day 7 when hMSCs were cultured in GM. For hMSCs on DBM scaffolds PEMF resulted in a sporadic increase of osteocalcin that dropped slightly at day 14 (9%) and was higher at days 7 and 21 (~45%). Similarly, the PEMF induced response was only significant for day 7 when hMSCs were cultured on DBM scaffolds in GM. Complete osteocalcin results for both scaffolds and the PTC control can be found in Appendix C.

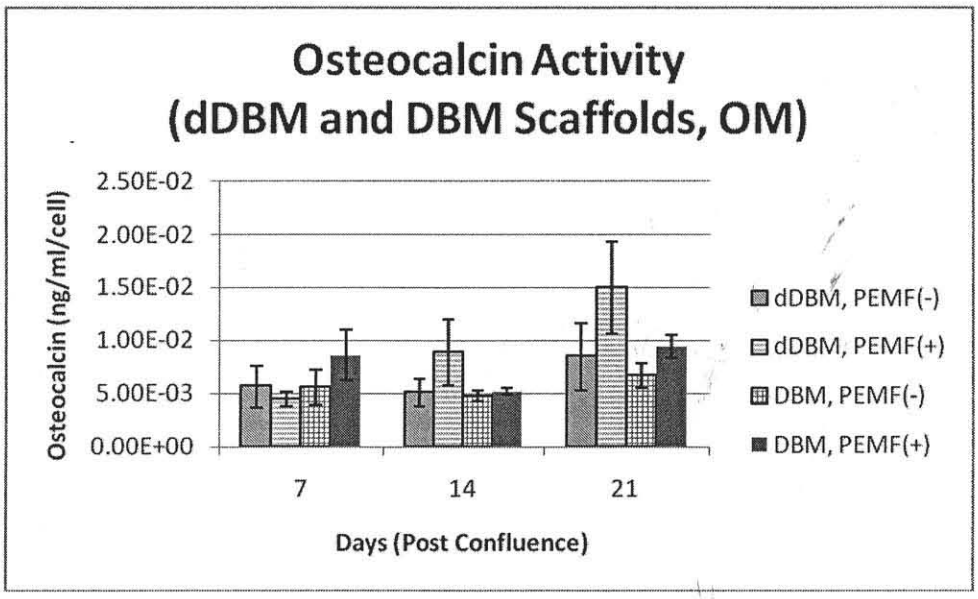

Figure 3.5 Osteocalcin activity for hMSCs harvested from dDBM and DBM scaffolds in OM with (+) and without (-) PEMF. Values are Mean ± SE

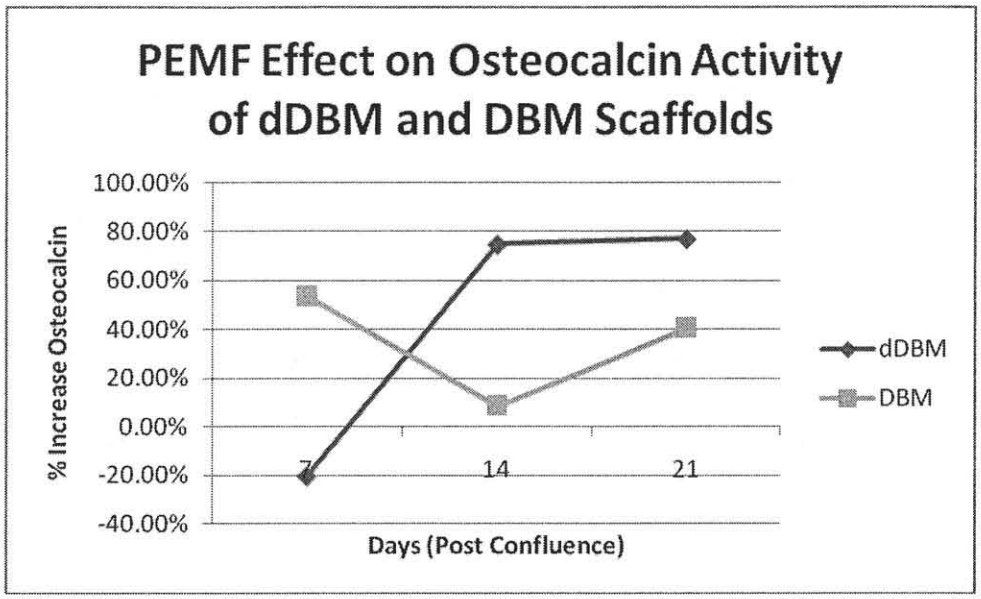

Figure 3.6 PEMF effect on osteocalcin activity for hMSCs harvested from dDBM and DBM scaffolds in OM. Values are Mean.

3.4 SEM Analysis

Qualitative SEM analysis was performed on scaffolds to assess cell morphology and extracellular matrix (ECM) deposition at 0, 7, and 21 days post confluence. Both DBM and dDBM scaffold SEM images had visible lacunae ranging from 20 – 70 μm in diameter (Fig. 3.7). No differences in cell morphology, distribution, or ECM deposition were seen between scaffolds with and without exposure to PEMF at any timepoint. At day 1 hMSCs were present on the surface of both scaffolds (Fig. 3.8.a - b). At day 7 ECM deposition had initiated and it was difficult to identify cells on the surface of the scaffolds, but cells could be seen both inside and spanning individual lacunae (Fig. 3.8.c - d). No visible cells were located on day 21 scaffolds. Considerable ECM deposition had taken place by day 21 with extensive matrix formation both on the surface and covering the lacunae of the scaffolds.

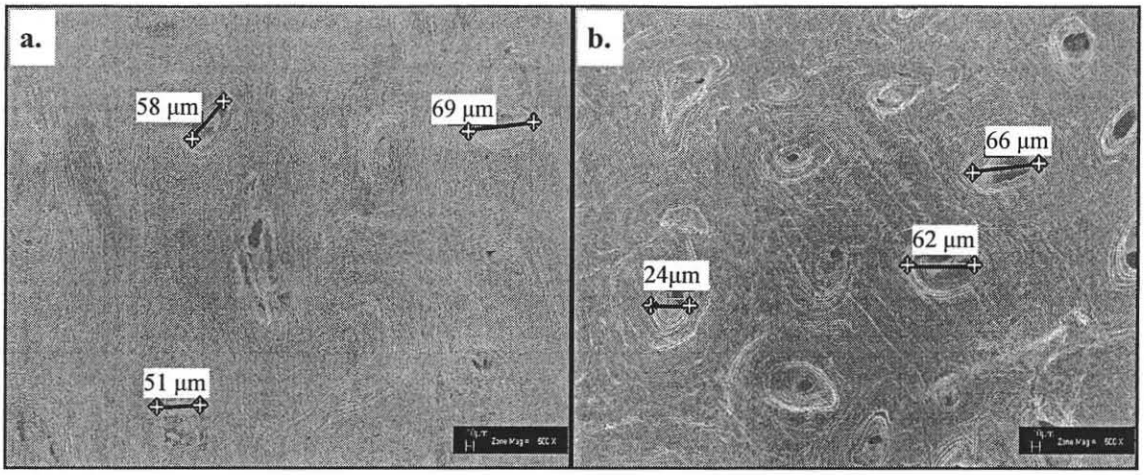

Figure 3.7 SEM micrographs of (a) DBM and (b) dDBM scaffolds with visible lacunae on the surfaces.

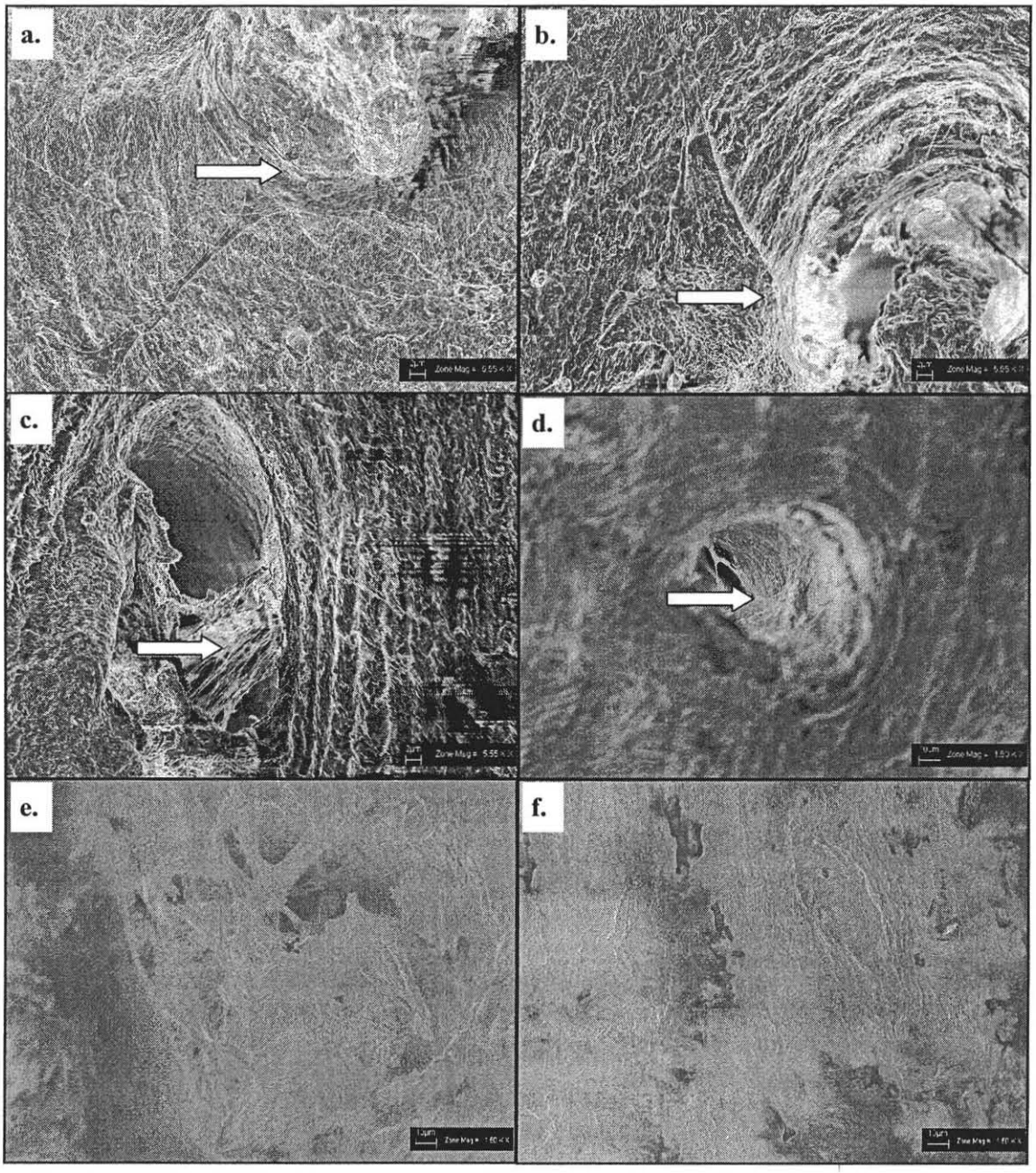

Figure 3.8 SEM micrographs of scaffolds cultivated with hMSCs: (a) DBM, Day 0, (b) dDBM, Day 0, (c) DBM, Day 7, (d) dDBM, Day 7, (e) DBM, Day 21, (f) dDBM, Day 21. White arrows in (a – d) indicate cellular focal adhesions. (e – f) ECM deposition covering surface and spanning lacunae of scaffolds.

CHAPTER 4

DISCUSSION

Orthopaedic bone grafting continues to be a mainstay in the treatment of non unions and other difficult to heal bone defects. Tissue engineering strategies for bone formation have focused on the use of bone graft materials that have osteoconductive, osteoinductive and osteogenic properties. Traditional autogenous cancellous bone grafts remain to be the gold standard in orthopaedics, but synthetic and allogenic grafts have garnered attention due to the increased morbidity associated with harvesting autograft. To date, none of the current alternative grafts collectively possess osteoconductive, osteoinductive and osteogenic properties (Table 1.1). Recent trends have shifted towards the use of composite grafts made up of two or more different bone grafts in order to provide all three of the necessary components for optimal bone formation. Additionally, the use of electrical stimulation technologies such as PEMF have been used to further stimulate the effectiveness of bone graft materials. In the present study, MSCs were cultured on DBM scaffolds in the presence of PEMF to determine if an increased osteogenic response could be induced in vitro.

Studies have demonstrated that DBM scaffolds can be used to support osteogenic differentiation of MSCs in vitro.[138-140,153] SEM micrographs of the dDBM and DBM scaffolds used in this study revealed that cells could be detected on the surface of scaffolds starting at day 0. ECM deposition had taken place by day 7, and by day 21 extensive ECM had formed covering the entire surface of the scaffolds including the lacunae.

In the present study, ALP expression for MSCs cultured on DBM scaffolds increased slightly through days 5 and 7 and then rapidly increased on day 14. Exposure to PEMF resulted in a similar response where there was a slight elevation in ALP expression early on, but then a rapid increase on day 21. ALP expression for MSCs on dDBM scaffolds with and without PEMF also saw a slight rise in ALP early on and then a rapid increase at later timepoints. For all groups, ALP activity was greater in cultures with OM compared to GM. ALP is expressed by MSCs at early to intermediate stages (days 7 – 10) of osteoblastic differentiation in vitro.[130,154,155] This was seen for hMSCs cultured on TCP controls where ALP peaked at day 7. Interestingly, ALP activity for MSCs harvested from both scaffolds in this study were highest at day 21, suggesting that differentiation was occurring later than what is typically seen in vitro. Osteocalcin expression of MSCs cultured on DBM scaffolds remained relatively level through day 21, but saw a slight increase on day 21 in the presence of PEMF. Osteocalcin levels associated with dDBM scaffolds both with and without PEMF also remained consistent throughout the 21 day culture. Osteocalcin is a bone-specific gene that is often expressed by cells in the latter stages of differentiation (days 14 – 21).[130,154] In general, osteocalcin expression did not peak for MSCs cultivated on scaffolds in the 21 day culture, indicating that the cells were still in the early to intermediate stages of differentiation at day 21. ALP results suggested the same, in that ALP levels typically peaked at later timepoints. Previous work by Mauney et. al.[139] has looked at the osteogenic differentiation of MSCs cultured on DBM scaffolds in vitro. Although differentiation was only assessed at days 7 and 14, ALP levels were relatively the same for both days. Based on the results from the present study one can speculate that differentiation was still in the earliest stages and

that an increase in ALP activity would have occurred at later timepoints such as day 21. Similarly, in vitro cultures with BMP-2 conditioned media have also demonstrated increasing ALP for MSCs through 21 days.[69,146] BMPs and growth factors may therefore have a delayed effect on MSCs in vitro, where osteogenic differentiation is induced at later timepoints such as 21 days. Hence, ALP activity was highest at day 21 and osteocalcin remained the same for cells harvested from scaffolds in the present study.

Although no quantitative analysis was performed to determine cellular distribution on the scaffolds used in this study, cells were observed both on the surface and inside the lacunae of the scaffolds. The DBM and dDBM scaffolds were derived from allograft cortical bone because of its inherent low porosity, as opposed to trabecullar bone, which is known for its larger and more prevalent porosity. It was originally thought that the cells would remain on the surface of the scaffolds, making this a 2-D in vitro model. This was not the case and it was evident early on that cells were able to locate the lacunae and make there way in, indicating that the scaffolds were in fact supporting 3-D differentiation of cells. Cultivation of cells in a 3-D versus 2-D in vitro environment have been shown to have differences in cellular proliferation and differentiation[139], and thus may explain the differences in ALP and osteocalcin activity seen between the 3-D scaffold groups and 2-D TCP controls in this study.

The use of in vitro cultures to evaluate both DBM scaffolds and PEMF for osteogenic differentiation of MSCs is limited. To date much of the data on the mechanism of action behind PEMF suggests a strong relation between BMPs and PEMF induced osteogenesis. Recent work has shown that PEMF can elicit an increased osteogenic response when BMP-2 conditioned media is used.[69,146] PEMF has also been

shown to upregulate BMP mRNA expression in vitro.[71,73,74] DBM is a known osteoinductive material that contains BMP-2 among other growth factors and it was hypothesized that MSCs cultured on DBM scaffolds in the presence of PEMF would elicit an increased osteogenic response. MSCs cultivated from DBM scaffolds in the presence of PEMF exhibited increased levels of ALP throughout the 21 day culture. ALP levels for MSCs seeded on DBM scaffolds in the presence of PEMF had an overall mean increase of 95%. The greatest response was seen at day 7 where PEMF resulted in a 257% mean increase of ALP activity. Similar results were seen with osteocalcin expression, although not as pronounced. PEMF resulted in an overall mean increase of 34% in osteocalcin expression, with the highest increase also seen on day 7 (54%). Despite the consistent elevation in ALP and osteocalcin, the increase was only shown to be significant at day 7 for both markers (ALP/OM, osteocalcin/GM). These results differ from other studies examining the effect of PEMF on MSC osteogenic differentiation. Specifically, PEMF was not able to induce such a robust response as previously reported by Schwartz et. al.[146] who saw significant PEMF induced increases in ALP expression on days 12 through 24. The elevated response due to PEMF was only seen in MSCs cultured in OM supplemented with BMP-2 (40 ng/ml). While DBM is known to contain several growth factors including BMP-2, the amount of growth factors is relatively low. Specifically, DBM has been shown to contain the following: BMP-2 = 21.4 +/- 12.0 ng/g DBM, BMP-4 = 5.45 +/- 2.04 ng/g DBM, and BMP-7 = 84.1 +/- 34.4 ng/g DBM.[18] Assuming a 100% diffusion of proteins from the DBM scaffolds (0.075 g DBM), the amount of BMP-2 released would still be significantly lower than a continuous treatment of BMP-2 (4.0 ng BMP-2/100 μl media) with every media change. Within the same

study, calcium phosphate (CP) scaffolds were also used. CP comprises the mineral portion of bone (hydroxyapatite) and is an excellent osteoconductive scaffold. Studies looking at osteogenic differentiation on different allograft scaffolds have seen significantly higher ALP expression with fully mineralized bone than DBM in certain donor lines of MSCs.[139] Based on those results, CP may serve as a better scaffold for osteogenic differentiation in vitro. This is not entirely surprising because PEMF has been shown to increase calcium uptake of cells in vitro. [81,82] Furthermore, PEMF may have a synergistic effect with BMP-2 in combination with CP. Recent experiments have also used 3D PEGDA hydrogels to investigate the effect of PEMF on MSC osteogenic differentiation.[147] Results revealed elevated levels of osteocalcin and calcium for MSCs exposed to PEMF at 2 weeks. In this case PEMF was able to induce an increased osteogenic response without BMP-2, suggesting that the PEGDA hydrogels may have interacted with PEMF. Such was not the case in the present study where PEMF had no effect on hMSCs cultivated on tissue culture treated polystyrene. Although the response to PEMF was not as dramatic compared to previous studies, the response was greater for the DBM and dDBM groups, suggesting that the scaffold may play a significant role in PEMF induced osteogenic differentiation.

Deproteinized dDBM scaffolds were used to confirm whether a synergistic effect between DBM derived BMPs and PEMF existed. dDBM scaffolds were created by performing a guanidine extraction in order to remove all the native proteins found in DBM. Interestingly, hMSCs cultivated from dDBM scaffolds saw a mean increase in ALP of 53% in response to PEMF, with a peak increase of 110% at day 14. Osteocalcin expression was elevated by 44% in response to PEMF, with a peak increase at day 21

(77%). MSCs cultured on DBM scaffolds did not express increased osteogenic activity when compared to dDBM scaffolds with or without PEMF. These results were surprising in that despite the deproteinization (1) PEMF had a slight positive effect on ALP and osteocalcin activity for the dDBM scaffolds and (2) there was no significant difference in ALP or osteocalcin expression between the two scaffolds. The guanidine extraction method used for deproteinization in this study has been validated to extract the BMPs and growth factors found in DBM.[20] C2C12 cells were cultured in the presence of DBM and dDBM powder for 2 days and assessed for ALP expression. The study demonstrated an increased response in ALP for cells cultured in the presence of DBM when compared to dDBM powder. The particle size for DBM powder is small in volume (200 –500 μm)[156] and therefore has a relatively high surface to volume (S/V) ratio. Assuming the powder is spherical with an average diameter of 350 μm, DBM powder would have a S/V ratio of 8.56. The scaffolds used in this study had a significantly lower S/V ratio (3.07), which would most likely limit the rate of diffusion and in turn the reaction kinetics for the guanidine extraction. Therefore, it is possible that the dDBM scaffolds used in this study were not completely deproteinized and contained residual BMPs and growth factors. This would explain why a response to PEMF was seen for dDBM groups and why there was no difference in ALP or ostecalcin activity between the two scaffolds. Similarly, the same argument can also be applied to the rate of elution for BMPs and growth factors. Simply, the smaller S/V ratio would limit the elution rate of proteins out of the scaffold when compared to DBM powder. Previous studies have compared ALP activity for fully and partially demineralized bone scaffolds.[139] ALP activity between the two scaffolds remained the same, despite the fully demineralized scaffolds having more DBM and

accessible BMPs and growth factors. Surprisingly, histology performed on the same scaffolds implanted in vivo revealed higher bone formation scores for the DBM groups. In vivo the release of BMPs and growth factors is aided by the enzymatic digestion and osteoclastic resorption of DBM. The in vitro release of growth factors and BMPs is dependent upon diffusion and therefore much less efficient, hence the disparity between the in vivo and in vitro results.

CHAPTER 5

CONCLUSION

DBM scaffolds were used to determine if PEMF could induce an osteogenic response for hMSCs in vitro. While PEMF did result in increased osteogenic activity, the response was only significant for the early day 7 timepoint. Surprisingly, PEMF was also shown to have a positive effect on dDBM scaffolds, but again the response was only seen early on and was not as dramatic as previous studies evaluating PEMF with BMP-2 conditioned media. This can be attributed to several factors including the limited elution of BMPs and growth factors from the scaffolds in vitro. This is not to say that DBM is not as effective as previously thought. In vivo, the release of proteins is aided by enzymatic digestion and osteoclastic resorption of DBM. Furthermore, DBM powder has been shown to be an effective osteoinductive material both in vivo and in vitro, partly because of its high S/V ratio. Scaffolds made of DBM powder held together with a carrier may prove to be more effective in this vitro model. Future in vitro studies evaluating PEMF and DBM scaffolds should look to simulate in vivo conditions more closely, but results from this study were promising. Despite limited levels and elution of BMPs, PEMF was still able to induce an osteogenic response, suggesting that the scaffold and PEMF had a synergistic effect. Additional work with PEMF and DBM should focus on in vivo studies to evaluate MSCs seeded on DBM scaffolds in the presence of PEMF to determine efficacy in a more clinically relevant model.

APPENDIX A - DNA ASSAY RESULTS

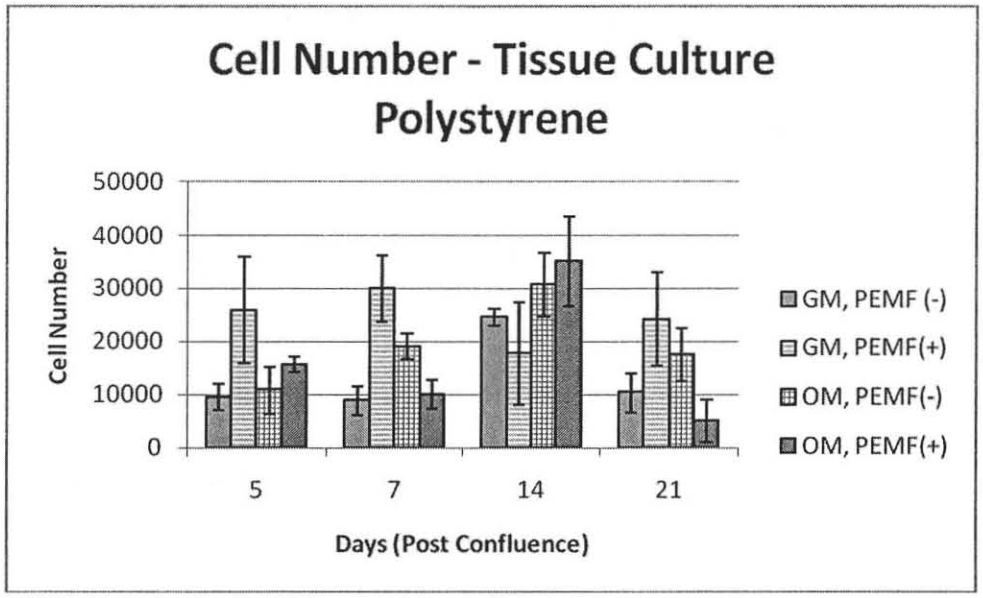

Figure A.1 Total hMSCs harvested from polystyrene tissue culture treated plates in GM/OM with (+) and without (-) PEMF . Values are Mean ± SE.

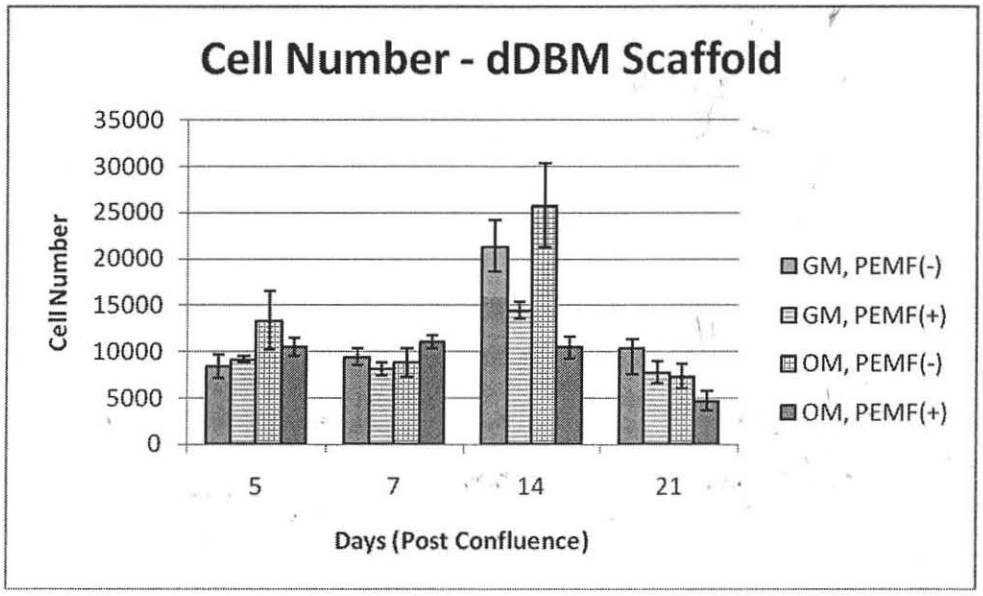

Figure A.2 Total hMSCs harvested from dDBM scaffolds in GM/OM With (+) and without (-) PEMF. Values are Mean ± SE.

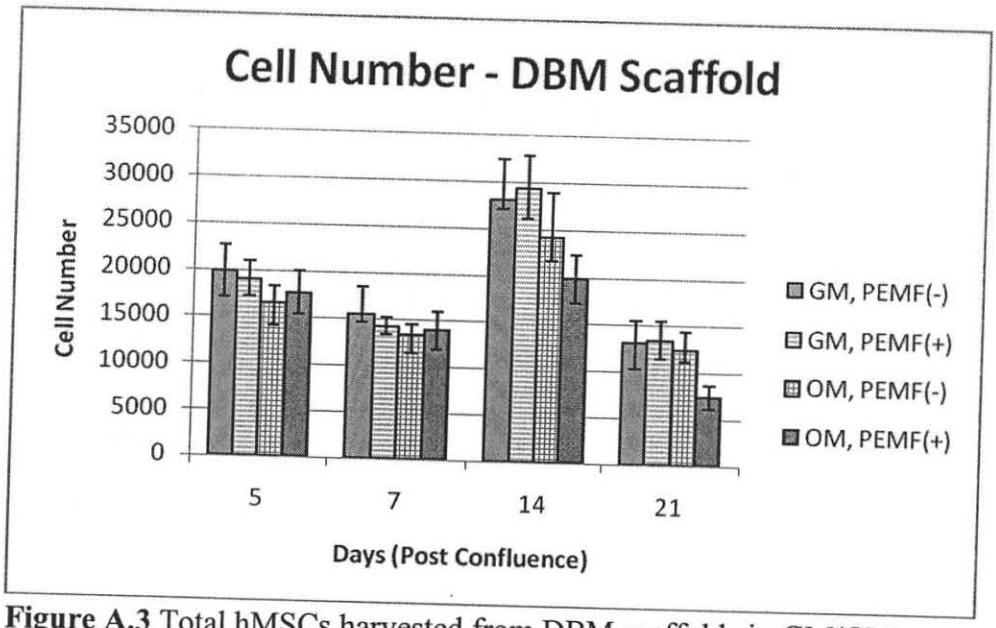

Figure A.3 Total hMSCs harvested from DBM scaffolds in GM/OM With (+) and without (-) PEMF. Values are Mean ± SE.

APPENDIX B - ALKALINE PHOSPHATASE ASSAY RESULTS

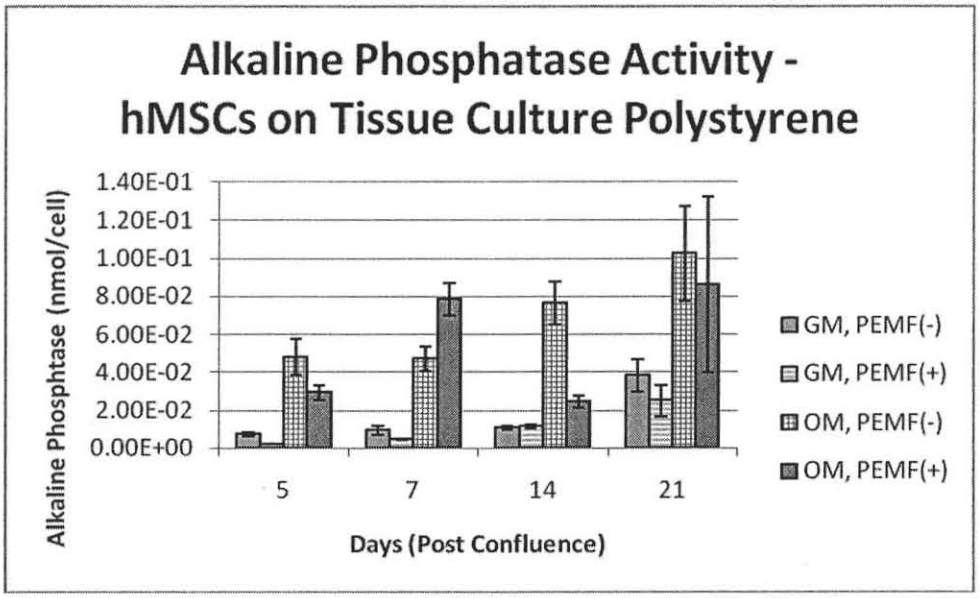

Figure B.1 Alkaline phosphatase activity for hMSCs harvested from polystyrene tissue culture treated plates in GM/OM with (+) and without (-) PEMF. Values are Mean ± SE. $P<0.05$, (#) timepoint v. previous timepoint.

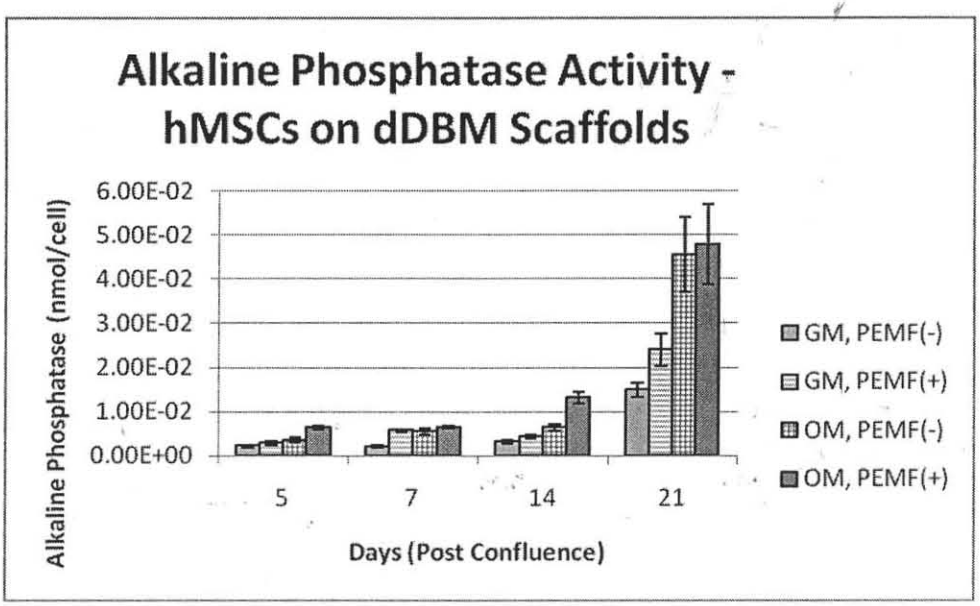

Figure B.2 Alkaline phosphatase activity for hMSCs harvested from dDBM scaffolds in GM/OM with (+) and without (-) PEMF. Values are Mean ± SE. $P<0.05$, (#) timepoint v. previous timepoint; (*) PEMF(+) v. PEMF(-); (◊) dDBM v. DBM.

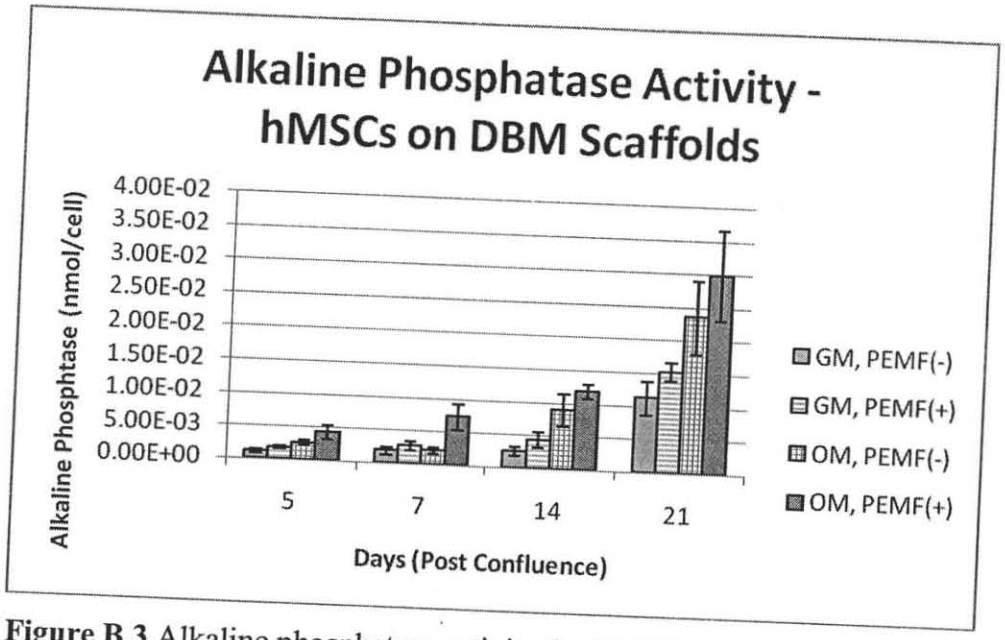

Figure B.3 Alkaline phosphatase activity for hMSCs harvested from DBM scaffolds in GM/OM with (+) and without (-) PEMF. Values are Mean ± SE. $P<0.05$, (#) timepoint v. previous timepoint; (*) PEMF(+) v. PEMF(-).

APPENDIX C - OSTEOCALCIN ACTIVITY

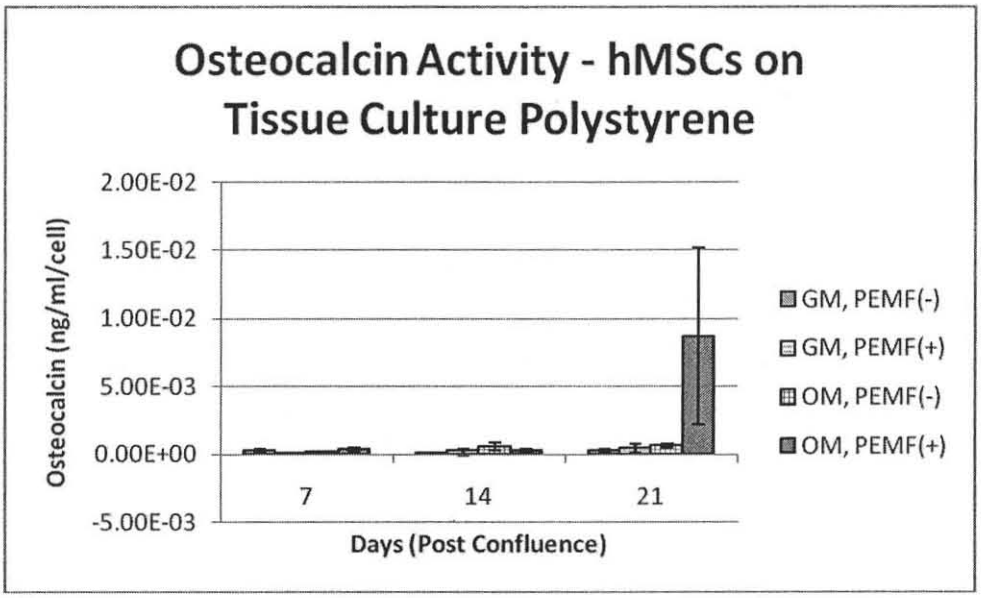

Figure C.1 Osteocalcin activity for hMSCs harvested from polystyrene tissue culture treated plates in GM/OM with (+) and without (-) PEMF. Values are Mean ± SE. $P<0.05$, (#) timepoint v. previous timepoint.

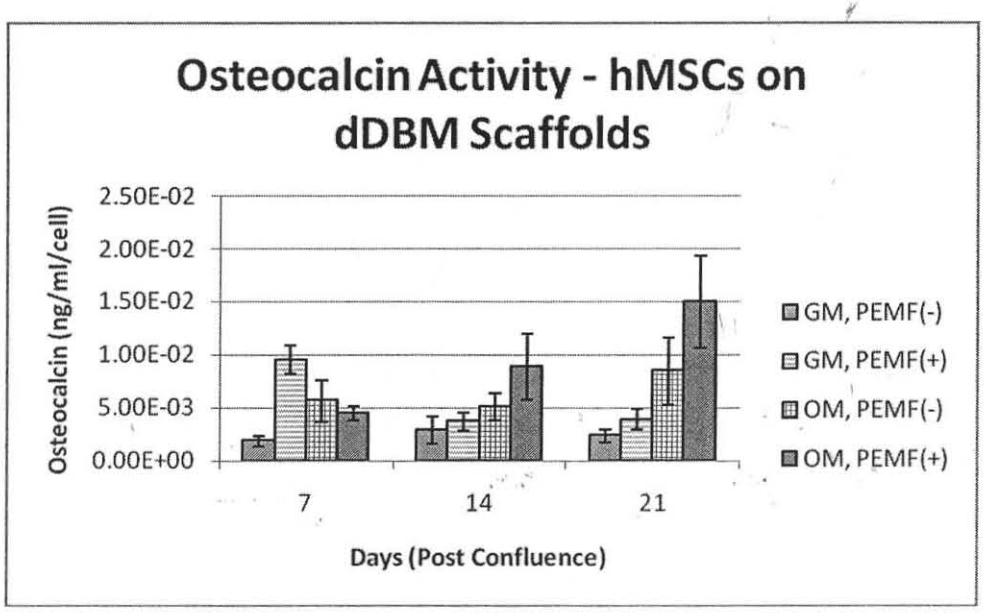

Figure C.2 Osteocalcin activity for hMSCs harvested from dDBM scaffolds in GM/OM with (+) and without (-) PEMF. Values are Mean ± SE. $P<0.05$, (#) timepoint v. previous timepoint; (*) PEMF(+) v. PEMF(-).

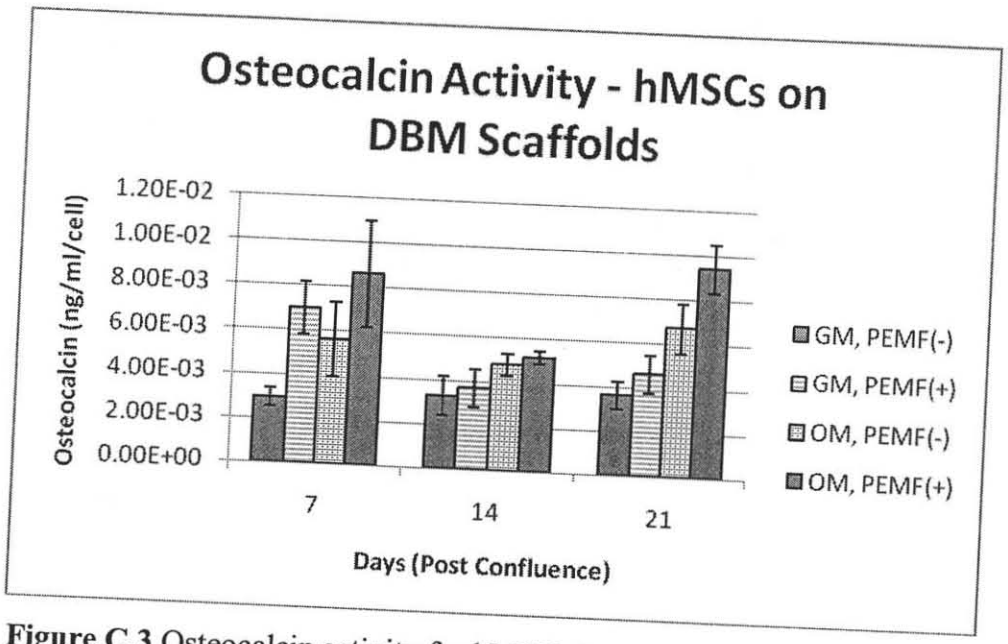

Figure C.3 Osteocalcin activity for hMSCs harvested from DBM scaffolds in GM/OM with (+) and without (-) PEMF. Values are Mean ± SE. $P<0.05$, (#) timepoint v. previous timepoint; (*) PEMF(+) v. PEMF(-).

REFERENCES

1. Lane, J. M. & Bostrom, M. P. Bone grafting and new composite biosynthetic graft materials. *Instr Course Lect* **47**, 525-34 (1998).

2. Goldberg, V. M. & Stevenson, S. Natural history of autografts and allografts. *Clin Orthop Relat Res*, 7-16 (1987).

3. Goldberg, V. M. & Stevenson, S. The biology of bone grafts. *Semin Arthroplasty* **4**, 58-63 (1993).

4. Reddi, A. H. Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nat Biotechnol* **16**, 247-52 (1998).

5. Centrella, M., McCarthy, T. L. & Canalis, E. Transforming growth factor beta is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone. *J Biol Chem* **262**, 2869-74 (1987).

6. Reddi, A. H., Wientroub, S. & Muthukumaran, N. Biologic principles of bone induction. *Orthop Clin North Am* **18**, 207-12 (1987).

7. Triffitt, J. T. Initiation and enhancement of bone formation. A review. *Acta Orthop Scand* **58**, 673-84 (1987).

8. Gazdag, A. R., Lane, J. M., Glaser, D. & Forster, R. A. Alternatives to Autogenous Bone Graft: Efficacy and Indications. *J Am Acad Orthop Surg* **3**, 1-8 (1995).

9. Salgado, A. J., Oliveira, J. T., Pedro, A. J. & Reis, R. L. Adult stem cells in bone and cartilage tissue engineering. *Curr Stem Cell Res Ther* **1**, 345-64 (2006).

10. Summers, B. N. & Eisenstein, S. M. Donor site pain from the ilium. A complication of lumbar spine fusion. *J Bone Joint Surg Br* **71**, 677-80 (1989).

11. Younger, E. M. & Chapman, M. W. Morbidity at bone graft donor sites. *J Orthop Trauma* **3**, 192-5 (1989).

12. Greenwald, A. S. et al. Bone-graft substitutes: facts, fictions, and applications. *J Bone Joint Surg Am* **83-A Suppl 2 Pt 2**, 98-103 (2001).

13. Delloye, C., Cornu, O., Druez, V. & Barbier, O. Bone allografts: What they can offer and what they cannot. *J Bone Joint Surg Br* **89**, 574-9 (2007).

14. Carlson, E. R., Marx, R. E. & Buck, B. E. The potential for HIV transmission through allogeneic bone. A review of risks and safety. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **80**, 17-23 (1995).

15. Tomford, W. W. Transmission of disease through transplantation of musculoskeletal allografts. *J Bone Joint Surg Am* **77**, 1742-54 (1995).

16. Delloye, C. Tissue allografts and health risks. *Acta Orthop Belg* **60 Suppl 1**, 62-7 (1994).

17. Urist, M. R. Bone: formation by autoinduction. *Science* **150**, 893-9 (1965).

18. Pietrzak, W. S., Woodell-May, J. & McDonald, N. Assay of bone morphogenetic protein-2, -4, and -7 in human demineralized bone matrix. *J Craniofac Surg* **17**, 84-90 (2006).

19. Huggins, C. B. & Urist, M. R. Dentin matrix transformation: rapid induction of alkaline phosphatase and cartilage. *Science* **167**, 896-8 (1970).

20. Han, B., Tang, B. & Nimni, M. E. Quantitative and sensitive in vitro assay for osteoinductive activity of demineralized bone matrix. *J Orthop Res* **21**, 648-54 (2003).

21. Oakes, D. A., Lee, C. C. & Lieberman, J. R. An evaluation of human demineralized bone matrices in a rat femoral defect model. *Clin Orthop Relat Res*, 281-90 (2003).

22. Bucholz, R. W. Nonallograft osteoconductive bone graft substitutes. *Clin Orthop Relat Res*, 44-52 (2002).

23. Burger, E. L. & Patel, V. Calcium phosphates as bone graft extenders. *Orthopedics* **30**, 939-42 (2007).

24. Erbe, E. M., Marx, J. G., Clineff, T. D. & Bellincampi, L. D. Potential of an ultraporous beta-tricalcium phosphate synthetic cancellous bone void filler and bone marrow aspirate composite graft. *Eur Spine J* **10 Suppl 2**, S141-6 (2001).

25. Martin, R. B. et al. Bone ingrowth and mechanical properties of coralline hydroxyapatite 1 yr after implantation. *Biomaterials* **14**, 341-8 (1993).

26. Moore, D. C., Chapman, M. W. & Manske, D. The evaluation of a biphasic calcium phosphate ceramic for use in grafting long-bone diaphyseal defects. *J Orthop Res* **5**, 356-65 (1987).

27. Sidqui, M., Collin, P., Vitte, C. & Forest, N. Osteoblast adherence and resorption activity of isolated osteoclasts on calcium sulphate hemihydrate. *Biomaterials* **16**, 1327-32 (1995).

28. LeGeros, R. Z. Properties of osteoconductive biomaterials: calcium phosphates. *Clin Orthop Relat Res*, 81-98 (2002).

29. Altermatt, S., Schwobel, M. & Pochon, J. P. Operative treatment of solitary bone cysts with tricalcium phosphate ceramic. A 1 to 7 year follow-up. *Eur J Pediatr Surg* **2**, 180-2 (1992).

30. Bucholz, R. W., Carlton, A. & Holmes, R. E. Hydroxyapatite and tricalcium phosphate bone graft substitutes. *Orthop Clin North Am* **18**, 323-34 (1987).

31. Tsai, C. H., Lin, J. H. & Ju, C. P. Gamma-radiation-induced changes in structure and properties of tetracalcium phosphate and its derived calcium phosphate cement. *J Biomed Mater Res B Appl Biomater* **80**, 244-52 (2007).

32. Bassett, C. A. Electrical effects in bone. *Sci Am* **213**, 18-25 (1965).

33. Bassett, C. A. & Becker, R. O. Generation of electric potentials by bone in response to mechanical stress. *Science* **137**, 1063-4 (1962).

34. Bassett, C. A., Pawluk, R. J. & Becker, R. O. Effects of Electric Currents on Bone in Vivo. *Nature* **204**, 652-4 (1964).

35. Bassett, C. A. Biologic significance of piezoelectricity. *Calcif Tissue Res* **1**, 252-72 (1968).

36. Becker, R. O. & Bassett, C. A. Bioelectric factors controlling bone structure. *In Bone Biodynamics*, 209-232 (1964).

37. Pietrzak, W. S., Simon, J. & Simon, B. Orthopedic biology and medicine: Musculoskeletal tissue regeneration, biological materials and methods. *Humana Press*, 260-261 (2008).

38. Rubinacci, A. et al. Bicarbonate dependence of ion current in damaged bone. *Calcif Tissue Int* **58**, 423-8 (1996).

39. Friedenberg, Z. B. & Kohanim, M. The effect of direct current on bone. *Surg Gynecol Obstet* **127**, 97-102 (1968).

40. O'Connor, B. T., Charlton, H. M., Currey, J. D., Kirby, D. R. & Woods, C. Effects of electric current on bone in vivo. *Nature* **222**, 162-3 (1969).

41. Spadaro, J. A. Electrically stimulated bone growth in animals and man. Review of the literature. *Clin Orthop Relat Res*, 325-32 (1977).

42. Brighton, C. T. et al. A multicenter study of the treatment of non-union with constant direct current. *J Bone Joint Surg Am* **63**, 2-13 (1981).

43. Bassett, C. A. & K., H. Synergistic effects of pulsed electromagnetic fields (PEMFs) and fresh canine cancellous bone grafts. *In: Orhtop trans* **1984**, 341 (1984).

44. Olmstead, M., Ost, P. & Hohn, R. The effect of pulsing electromagnetic fields on bone healing in fresh canine tibial fractures. *Parsippany* **EBI** (1984).

45. Leisner, S., Shahar, R., Aizenberg, I., Lichovsky, D. & Levin-Harrus, T. The effect of short-duration, high-intensity electromagnetic pulses on fresh ulnar fractures in rats. *J Vet Med A Physiol Pathol Clin Med* **49**, 33-7 (2002).

46. Sarker, A. B. et al. Effect of PEMF on fresh fracture-healing in rat tibia. *Bangladesh Med Res Counc Bull* **19**, 103-12 (1993).

47. Enzler, M. A., Sumner-Smith, G., Waelchli-Suter, C. & Perren, S. M. Treatment of nonuniting osteotomies with pulsating electromagnetic fields. A controlled animal experiment. *Clin Orthop Relat Res*, 272-6 (1984).

48. Enzler, M. A., Waelchli-Suter, C. & Perren, S. M. [Prevention of pseudarthrosis using magnetic stimulation? Experimental demonstration of Bassett's method on beagles]. *Unfallheilkunde* **83**, 188-94 (1980).

49. Fredericks, D. C., Piehl, D. J., Baker, J. T., Abbott, J. & Nepola, J. V. Effects of pulsed electromagnetic field stimulation on distraction osteogenesis in the rabbit tibial leg lengthening model. *J Pediatr Orthop* **23**, 478-83 (2003).

50. Inoue, N. et al. Effect of pulsed electromagnetic fields (PEMF) on late-phase osteotomy gap healing in a canine tibial model. *J Orthop Res* **20**, 1106-14 (2002).

51. Fini, M. et al. The effect of pulsed electromagnetic fields on the osteointegration of hydroxyapatite implants in cancellous bone: a morphologic and microstructural in vivo study. *J Orthop Res* **20**, 756-63 (2002).

52. Fini, M., Giavaresi, G., Giardino, R., Cavani, F. & Cadossi, R. Histomorphometric and mechanical analysis of the hydroxyapatite-bone interface after electromagnetic stimulation: an experimental study in rabbits. *J Bone Joint Surg Br* **88**, 123-8 (2006).

53. Shimizu, T. et al. Bone ingrowth into porous calcium phosphate ceramics: influence of pulsing electromagnetic field. *J Orthop Res* **6**, 248-58 (1988).

54. EBI Bone Healing System Model 2001 Physician Manual and Package Insert. 2 (2005).

55. Bassett, C. A. Fundamental and practical aspects of therapeutic uses of pulsed electromagnetic fields (PEMFs). *Crit Rev Biomed Eng* **17**, 451-529 (1989).

56. Adams, B. D., Frykman, G. K. & Taleisnik, J. Treatment of scaphoid nonunion with casting and pulsed electromagnetic fields: a study continuation. *J Hand Surg [Am]* **17**, 910-4 (1992).

57. Dunn, A. W. & Rush, G. A., 3rd. Electrical stimulation in treatment of delayed union and nonunion of fractures and osteotomies. *South Med J* **77**, 1530-4 (1984).

58. Frykman, G. K. et al. Treatment of nonunited scaphoid fractures by pulsed electromagnetic field and cast. *J Hand Surg [Am]* **11**, 344-9 (1986).

59. Bigliani, L. U., Rosenwasser, M. P., Caulo, N., Schink, M. M. & Bassett, C. A. The use of pulsing electromagnetic fields to achieve arthrodesis of the knee following failed total knee arthroplasty. A preliminary report. *J Bone Joint Surg Am* **65**, 480-5 (1983).

60. Bassett, C. A., Caulo, N. & Kort, J. Congenital "pseudarthroses" of the tibia: treatment with pulsing electromagnetic fields. *Clin Orthop Relat Res*, 136-48 (1981).

61. Fontanesi, G., Giancecchi, F., Rotini, R. & Cadossi, R. Treatment of delayed union and pseudarthrosis by low frequency pulsing electromagnetic stimulation. Study of 35 cases. *Ital J Orthop Traumatol* **9**, 305-18 (1983).

62. Holmes, G. B., Jr. Treatment of delayed unions and nonunions of the proximal fifth metatarsal with pulsed electromagnetic fields. *Foot Ankle Int* **15**, 552-6 (1994).

63. Aaron, R. K., Lennox, D., Bunce, G. E. & Ebert, T. The conservative treatment of osteonecrosis of the femoral head. A comparison of core decompression and pulsing electromagnetic fields. *Clin Orthop Relat Res*, 209-18 (1989).

64. Bassett, C. A., Schink-Ascani, M. & Lewis, S. M. Effects of pulsed electromagnetic fields on Steinberg ratings of femoral head osteonecrosis. *Clin Orthop Relat Res*, 172-85 (1989).

65. Eftekhar, N. S., Schink-Ascani, M. M., Mitchell, S. N. & Bassett, C. A. Osteonecrosis of the femoral head treated by pulsed electromagnetic fields (PEMFs): a preliminary report. *Hip*, 306-30 (1983).

66. Bassett, C. A., Mitchell, S. N. & Schink, M. M. Treatment of therapeutically resistant non-unions with bone grafts and pulsing electromagnetic fields. *J Bone Joint Surg Am* **64**, 1214-20 (1982).

67. Sharrard, W. J. A double-blind trial of pulsed electromagnetic fields for delayed union of tibial fractures. *J Bone Joint Surg Br* **72**, 347-55 (1990).

68. Simonis, R. B., Parnell, E. J., Ray, P. S. & Peacock, J. L. Electrical treatment of tibial non-union: a prospective, randomised, double-blind trial. *Injury* **34**, 357-62 (2003).

69. Boyan, B., Fisher, M., Simon, B. & Schwartz, Z. Pulsed electromagnetic fields modulate OPG expression during osteogenic differentiation of mesenchymal stem cells. *53rd Annual Meeting of the Orthopaedic Research Society*, 1576 (2007).

70. Nagai, M. & Ota, M. Pulsating electromagnetic field stimulates mRNA expression of bone morphogenetic protein-2 and -4. *J Dent Res* **73**, 1601-5 (1994).

71. Yajima, A., Ochi, M. & Hirose, Y. Effects of pulsing electromagnetic fields on gene expression of bone morphogenetic proteins in human osteoblastic cell line in vitro. *J Bone Miner Res* **11**, 381 (1996).

72. Aaron, R. K., Boyan, B. D., Ciombor, D. M., Schwartz, Z. & Simon, B. J. Stimulation of growth factor synthesis by electric and electromagnetic fields. *Clin Orthop Relat Res*, 30-7 (2004).

73. Bodamyali, T. et al. Pulsed electromagnetic fields simultaneously induce osteogenesis and upregulate transcription of bone morphogenetic proteins 2 and 4 in rat osteoblasts in vitro. *Biochem Biophys Res Commun* **250**, 458-61 (1998).

74. Aaron, R. K., Wang, S. & Ciombor, D. M. Upregulation of basal TGFbeta1 levels by EMF coincident with chondrogenesis--implications for skeletal repair and tissue engineering. *J Orthop Res* **20**, 233-40 (2002).

75. Adey, W. R. Biological effects of electromagnetic fields. *J Cell Biochem* **51**, 410-6 (1993).

76. Brighton, C. T., Wang, W., Seldes, R., Zhang, G. & Pollack, S. R. Signal transduction in electrically stimulated bone cells. *J Bone Joint Surg Am* **83-A**, 1514-23 (2001).

77. Cain, C. D., Adey, W. R. & Luben, R. A. Evidence that pulsed electromagnetic fields inhibit coupling of adenylate cyclase by parathyroid hormone in bone cells. *J Bone Miner Res* **2**, 437-41 (1987).

78. Fitzsimmons, R. J., Ryaby, J. T., Magee, F. P. & Baylink, D. J. Combined magnetic fields increased net calcium flux in bone cells. *Calcif Tissue Int* **55**, 376-80 (1994).

79. Luben, R. A. Gene expression of parathyroid hormone (PTH) receptors and functionally related antigens in bone cells: desensitization with hormones and electromagnetic fields (EF). *Transactions of the Bioelectrical Repair and Growth Society* **Washington, DC**, 20 (1988).

80. Luben, R. A. Effects of low-energy electromagnetic fields (pulsed and DC) on membrane signal transduction processes in biological systems. *Health Phys* **61**, 15-28 (1991).

81. Luben, R. A., Cain, C. D., Chen, M. C., Rosen, D. M. & Adey, W. R. Effects of electromagnetic stimuli on bone and bone cells in vitro: inhibition of responses to parathyroid hormone by low-energy low-frequency fields. *Proc Natl Acad Sci U S A* **79**, 4180-4 (1982).

82. Spadaro, J. A. & Bergstrom, W. H. In vivo and in vitro effects of a pulsed electromagnetic field on net calcium flux in rat calvarial bone. *Calcif Tissue Int* **70**, 496-502 (2002).

83. Muschler, G. F. & Midura, R. J. Connective tissue progenitors: practical concepts for clinical applications. *Clin Orthop Relat Res*, 66-80 (2002).

84. Caplan, A. I. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. *Tissue Eng* **11**, 1198-211 (2005).

85. Fraser, J. K., Zhu, M., Wulur, I. & Alfonso, Z. Adipose-derived stem cells. *Methods Mol Biol* **449**, 59-67 (2008).

86. Muschler, G. F., Boehm, C. & Easley, K. Aspiration to obtain osteoblast progenitor cells from human bone marrow: the influence of aspiration volume. *J Bone Joint Surg Am* **79**, 1699-709 (1997).

87. Soncini, M. et al. Isolation and characterization of mesenchymal cells from human fetal membranes. *J Tissue Eng Regen Med* **1**, 296-305 (2007).

88. Bosch, P. et al. Osteoprogenitor cells within skeletal muscle. *J Orthop Res* **18**, 933-44 (2000).

89. Friedenstein, A. J., Chailakhyan, R. K. & Gerasimov, U. V. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet* **20**, 263-72 (1987).

90. Majors, A. K., Boehm, C. A., Nitto, H., Midura, R. J. & Muschler, G. F. Characterization of human bone marrow stromal cells with respect to osteoblastic differentiation. *J Orthop Res* **15**, 546-57 (1997).

91. Kilic, E., Ceyhan, T. & Cetinkaya, D. U. [Evaluation of differentiation potential of human bone marrow-derived mesenchymal stromal cells to cartilage and bone cells]. *Acta Orthop Traumatol Turc* **41**, 295-301 (2007).

92. Caplan, A. I. Mesenchymal stem cells. *J Orthop Res* **9**, 641-50 (1991).

93. Bruder, S. P. & Caplan, A. I. First bone formation and the dissection of an osteogenic lineage in the embryonic chick tibia is revealed by monoclonal antibodies against osteoblasts. *Bone* **10**, 359-75 (1989).

94. Bruder, S. P. & Caplan, A. I. A monoclonal antibody against the surface of osteoblasts recognizes alkaline phosphatase isoenzymes in bone, liver, kidney, and intestine. *Bone* **11**, 133-9 (1990).

95. Bruder, S. P. & Caplan, A. I. Terminal differentiation of osteogenic cells in the embryonic chick tibia is revealed by a monoclonal antibody against osteocytes. *Bone* **11**, 189-98 (1990).

96. Bruder, S. P. & Caplan, A. I. Osteogenic cell lineage analysis is facilitated by organ cultures of embryonic chick periosteum. *Dev Biol* **141**, 319-29 (1990).

97. Bruder, S. P., Fink, D. J. & Caplan, A. I. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. *J Cell Biochem* **56**, 283-94 (1994).

98. Kingsley, D. M. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev* **8**, 133-46 (1994).

99. Roelen, B. A. & Dijke, P. Controlling mesenchymal stem cell differentiation by TGFBeta family members. *J Orthop Sci* **8**, 740-8 (2003).

100. Kato, M. et al. Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J Cell Biol* **157**, 303-14 (2002).

101. Komori, T. et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89**, 755-64 (1997).

102. Lee, K. S., Hong, S. H. & Bae, S. C. Both the Smad and p38 MAPK pathways play a crucial role in Runx2 expression following induction by transforming growth factor-beta and bone morphogenetic protein. *Oncogene* **21**, 7156-63 (2002).

103. Otto, F. et al. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* **89**, 765-71 (1997).

104. Satokata, I. et al. Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. *Nat Genet* **24**, 391-5 (2000).

105. Satokata, I. & Maas, R. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nat Genet* **6**, 348-56 (1994).

106. Zhang, Y. W., Bae, S. C., Takahashi, E. & Ito, Y. The cDNA cloning of the transcripts of human PEBP2alphaA/CBFA1 mapped to 6p12.3-p21.1, the locus for cleidocranial dysplasia. *Oncogene* **15**, 367-71 (1997).

107. Bruder, S. P., Kraus, K. H., Goldberg, V. M. & Kadiyala, S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J Bone Joint Surg Am* **80**, 985-96 (1998).

108. Kon, E. et al. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. *J Biomed Mater Res* **49**, 328-37 (2000).

109. Becker, S. et al. Osteopromotion by a beta-tricalcium phosphate/bone marrow hybrid implant for use in spine surgery. *Spine* **31**, 11-7 (2006).

110. Arinzeh, T. L. et al. Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect. *J Bone Joint Surg Am* **85-A**, 1927-35 (2003).

111. Brodke, D. et al. Bone grafts prepared with selective cell retention technology heal canine segmental defects as effectively as autograft. *J Orthop Res* **24**, 857-66 (2006).

112. Gupta, M. C. et al. Efficacy of mesenchymal stem cell enriched grafts in an ovine posterolateral lumbar spine model. *Spine* **32**, 720-6; discussion 727 (2007).

113. Muschler, G. F. et al. Spine fusion using cell matrix composites enriched in bone marrow-derived cells. *Clin Orthop Relat Res*, 102-18 (2003).

114. Connolly, J. F., Guse, R., Tiedeman, J. & Dehne, R. Autologous marrow injection for delayed unions of the tibia: a preliminary report. *J Orthop Trauma* **3**, 276-82 (1989).

115. Connolly, J. F., Guse, R., Tiedeman, J. & Dehne, R. Autologous marrow injection as a substitute for operative grafting of tibial nonunions. *Clin Orthop Relat Res*, 259-70 (1991).

116. Gershuni, D. H. & Pinsker, R. Bone grafting for nonunion of fractures of the tibia: a critical review. *J Trauma* **22**, 43-9 (1982).

117. Green, S. A., Moore, T. A. & Spohn, P. J. Nonunion of the tibial shaft. *Orthopedics* **11**, 1149-57 (1988).

118. Price, C. T., Connolly, J. F., Carantzas, A. C. & Ilyas, I. Comparison of bone grafts for posterior spinal fusion in adolescent idiopathic scoliosis. *Spine* **28**, 793-8 (2003).

119. Wientroub, S., Goodwin, D., Khermosh, O. & Salama, R. The clinical use of autologous marrow to improve osteogenic potential of bone grafts in pediatric orthopedics. *J Pediatr Orthop* **9**, 186-90 (1989).

120. Hernigou, P. et al. Percutaneous autologous bone-marrow grafting for nonunions. Surgical technique. *J Bone Joint Surg Am* **88 Suppl 1 Pt 2**, 322-7 (2006).

121. Hernigou, P., Poignard, A., Beaujean, F. & Rouard, H. Percutaneous autologous bone-marrow grafting for nonunions. Influence of the number and concentration of progenitor cells. *J Bone Joint Surg Am* **87**, 1430-7 (2005).

122. Hernigou, P., Poignard, A., Manicom, O., Mathieu, G. & Rouard, H. The use of percutaneous autologous bone marrow transplantation in nonunion and avascular necrosis of bone. *J Bone Joint Surg Br* **87**, 896-902 (2005).

123. Hernigou, P. & Beaujean, F. Treatment of osteonecrosis with autologous bone marrow grafting. *Clin Orthop Relat Res*, 14-23 (2002).

124. Fleming, J. E., Jr., Cornell, C. N. & Muschler, G. F. Bone cells and matrices in orthopedic tissue engineering. *Orthop Clin North Am* **31**, 357-74 (2000).

125. Lian, J. B. & Stein, G. S. Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation. *Crit Rev Oral Biol Med* **3**, 269-305 (1992).

126. Lian, J. B., Stein, G. S., Bortell, R. & Owen, T. A. Phenotype suppression: a postulated molecular mechanism for mediating the relationship of proliferation and differentiation by Fos/Jun interactions at AP-1 sites in steroid responsive promoter elements of tissue-specific genes. *J Cell Biochem* **45**, 9-14 (1991).

127. Maniatopoulos, C., Sodek, J. & Melcher, A. H. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res* **254**, 317-30 (1988).

128. Owen, T. A. et al. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J Cell Physiol* **143**, 420-30 (1990).

129. Muller, P. et al. Calcium phosphate surfaces promote osteogenic differentiation of mesenchymal stem cells. *J Cell Mol Med* **12**, 281-91 (2008).

130. Mygind, T. et al. Mesenchymal stem cell ingrowth and differentiation on coralline hydroxyapatite scaffolds. *Biomaterials* **28**, 1036-47 (2007).

131. Niemeyer, P. et al. Evaluation of mineralized collagen and alpha-tricalcium phosphate as scaffolds for tissue engineering of bone using human mesenchymal stem cells. *Cells Tissues Organs* **177**, 68-78 (2004).

132. Takahashi, Y., Yamamoto, M. & Tabata, Y. Osteogenic differentiation of mesenchymal stem cells in biodegradable sponges composed of gelatin and beta-tricalcium phosphate. *Biomaterials* **26**, 3587-96 (2005).

133. Jager, M., Feser, T., Denck, H. & Krauspe, R. Proliferation and osteogenic differentiation of mesenchymal stem cells cultured onto three different polymers in vitro. *Ann Biomed Eng* **33**, 1319-32 (2005).

134. Guo, L. et al. Osteogenic differentiation of human mesenchymal stem cells on chargeable polymer-modified surfaces. *J Biomed Mater Res A* (2008).

135. Leong, N. L., Jiang, J. & Lu, H. H. Polymer-ceramic composite scaffold induces osteogenic differentiation of human mesenchymal stem cells. *Conf Proc IEEE Eng Med Biol Soc* **1**, 2651-4 (2006).

136. Meinel, L. et al. Osteogenesis by human mesenchymal stem cells cultured on silk biomaterials: comparison of adenovirus mediated gene transfer and protein delivery of BMP-2. *Biomaterials* **27**, 4993-5002 (2006).

137. Meinel, L. et al. Bone tissue engineering using human mesenchymal stem cells: effects of scaffold material and medium flow. *Ann Biomed Eng* **32**, 112-22 (2004).

138. Mauney, J. R. et al. Osteogenic differentiation of human bone marrow stromal cells on partially demineralized bone scaffolds in vitro. *Tissue Eng* **10**, 81-92 (2004).

139. Mauney, J. R. et al. In vitro and in vivo evaluation of differentially demineralized cancellous bone scaffolds combined with human bone marrow stromal cells for tissue engineering. *Biomaterials* **26**, 3173-85 (2005).

140. Mauney, J. R. et al. Mechanical stimulation promotes osteogenic differentiation of human bone marrow stromal cells on 3-D partially demineralized bone scaffolds in vitro. *Calcif Tissue Int* **74**, 458-68 (2004).

141. Sumanasinghe, R. D., Osborne, J. A. & Loboa, E. G. Mesenchymal stem cell-seeded collagen matrices for bone repair: Effects of cyclic tensile strain, cell density, and media conditions on matrix contraction in vitro. *J Biomed Mater Res A* (2008).

142. Donzelli, E. et al. Mesenchymal stem cells cultured on a collagen scaffold: In vitro osteogenic differentiation. *Arch Oral Biol* **52**, 64-73 (2007).

143. de Girolamo, L., Sartori, M. F., Albisetti, W. & Brini, A. T. Osteogenic differentiation of human adipose-derived stem cells: comparison of two different inductive media. *J Tissue Eng Regen Med* **1**, 154-7 (2007).

144. Wu, H., Ren, K., Zhao, W., Baojian, G. E. & Peng, S. Effect of electromagnetic fields on proliferation and differentiation of cultured mouse bone marrow mesenchymal stem cells. *J Huazhong Univ Sci Technolog Med Sci* **25**, 185-7 (2005).

145. Walther, M., Mayer, F., Kafka, W. & Schutze, N. Effects of weak, low-frequency pulsed electromagnetic fields (BEMER type) on gene expression of human mesenchymal stem cells and chondrocytes: an in vitro study. *Electromagn Biol Med* **26**, 179-90 (2007).

146. Schwartz, Z. et al. Pulsed electromagnetic fields enhance BMP-2 dependent osteoblastic differentiation of human mesenchymal stem cells. *J Orthop Res* (2008).

147. Moioli, E. K., Kennedy, B. S., Simon, B. J., Woo, C. & Mao, J. J. Pulsed electromagnetic fields influence osteogenic differentiation of mesenchymal stem cells in 3D biomatrix. *Society for Physical Regulation in Biology and Medicine 26th Scientific Conference* (2008).

148. Jaiswal, N., Haynesworth, S. E., Caplan, A. I. & Bruder, S. P. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem* **64**, 295-312 (1997).

149. Hornicek, F., Woll, J. & Kasprisin, D. Standardes for Tissue Banking, 10th ed. *American Association of Tissue Banks* **McLean, VA** (2002).

150. Reddi, A. H. & Huggins, C. Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. *Proc Natl Acad Sci U S A* **69**, 1601-5 (1972).

151. Hosny, M., Arcidi, C. & Sharawy, M. Effects of preservation on the osteoinductive capacity of demineralized bone powder allografts. *J Oral Maxillofac Surg* **45**, 1051-4 (1987).

152. Lohmann, C. H. et al. Pulsed electromagnetic field stimulation of MG63 osteoblast-like cells affects differentiation and local factor production. *J Orthop Res* **18**, 637-46 (2000).

153. Kasten, P. et al. Comparison of human bone marrow stromal cells seeded on calcium-deficient hydroxyapatite, beta-tricalcium phosphate and demineralized bone matrix. *Biomaterials* **24**, 2593-603 (2003).

154. Zhu, J. X., Sasano, Y., Takahashi, I., Mizoguchi, I. & Kagayama, M. Temporal and spatial gene expression of major bone extracellular matrix molecules during embryonic mandibular osteogenesis in rats. *Histochem J* **33**, 25-35 (2001).

155. Young, M. F. Bone matrix proteins: their function, regulation, and relationship to osteoporosis. *Osteoporos Int* **14 Suppl 3**, S35-42 (2003).

156. Schwartz, Z. et al. Ability of commercial demineralized freeze-dried bone allograft to induce new bone formation. *J Periodontol* **67**, 918-26 (1996).