IN VITRO ANALYSIS OF HUMAN FEMORAL CANCELLOUS BONE AS A VIABLE SOURCE OF OSTEOPROGENITOR CELLS AND THE EFFECTS OF BONE MORPHOGENETIC PROTEIN 2 AND DOXYCYCLINE ON THEIR DIFFERENTIATION INTO BONE FORMING COLONIES

BY

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OSTEOPROGENITOR ISOLATION & EFFECTS OF BMP-2 & DOXYCYCLINE

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ABSTRACT

The purpose of this investigation was 1) to establish an optimal technique for isolating osteoprogenitor cells in vitro using human femoral cancellous bone as a donor site, and 2) to evaluate the effects of various factors on differentiation of osteoprogenitor cultures. Two isolation techniques evaluated were enzyme digestion and primary explant technique. Furthermore, bone morphogenetic protein 2 (BMP-2) and doxycycline were supplemented in a dose dependent manner into various osteoprogenitor cell containing culture dishes. To compare isolation techniques and the effect of supplemented factors, we isolated cell populations from cancellous bone of the femoral neck from 7 and 6 patients, respectively, with osteoarthritis undergoing total hip replacement surgery. Bone derived osteoblasts and their bone nodules were identified using Von Kossa stain. The cell yield of the two isolation techniques was quantified by hemocytometer counts. The ability of the cells to differentiate into bone forming osteoblasts was evaluated by comparing numbers of Von Kossa positive nodule counts. Using alkaline phosphatase (ALP) staining, ALP quantitative assay and osteocalcin polymerase chain reaction, our study is the first attempt to determine the effects of doxycycline, along with the more commonly used BMP-2, on the differentiation of osteoblasts into bone forming colonies.

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LIST OF ABBREVIATIONS

α-MEM	Alpha minimal essential medium
ALP	Alkaline phosphatase
ATF4	Activating transcription factor 4
BMP	Bone morphogenetic protein
BMP-2	Bone morphogenetic protein 2
bp	Base pair
BRC	Bone remodeling compartment
BSA	Bovine serum albumin
Co-Smad	Common mediated Smad
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FZD	Frizzled
HH	Hedgehog
HSC	Hematopoietic stem cell
IGF	Insulin-like growth factor
JNK	C-Jun N-terminal kinase
LRP	Lipoprotein receptor related protein
MAP	Mitogen activated protein
M-CSF	Macrophage colony stimulating factor
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NO	Nitric Oxide
OCN	Osteocalcin
OPG	Osteoprotegerin
PBS	Phosphate buffered saline
PTH	Parathyroid hormone
RANK	Receptor activator of NF-kappaB
RANKL	Receptor activator of NF-kappaB ligand
rhBMP-2	Recombinant human BMP-2
R-Smad	Receptor regulated Smad
RT-PCR	Reverse transcriptase polymerase chain reaction
Runx2	Runt related factor 2
SDD	Subantimicrobial dose doxycycline
Smad	Mothers against decapentaplegic
TGF-β	Tumour growth factor β
TRANCE	TNF-related activation-induced cytokine
VEGF	Vascular endothelial growth factor
VK	Von Kossa

CHAPTER ONE

INTRODUCTION

1.1 An Overview of Bone

Bone is a highly organized organ composed of different types of cells, interacting in an organic matrix of mineralized hydroxyapatite and amorphous calcium phosphate crystals (Kartsogiannis and Ng, 2004). Bone has an organic component and an inorganic component. The organic component is composed mainly of type I collagen which gives bone its tensile strength. On the other hand, the inorganic component of bone is composed mainly of hydroxyapatite and imparts bone with stiffness to compression. It is in this way that bone provides protection, while acting as a storehouse for calcium and phosphorus (Buckwalter and Cooper, 1987).

1.1.1 Predominant Cells in Bone

The cells most prominent in bone are osteoclasts, osteocytes and osteoblasts. Osteoclasts are multinucleated cells from the hematopoietic lineage, primarily involved in bone resorption. The principle bone forming cell is the osteoblast, which secretes unmineralized matrix, called osteoid, that will eventually be calcified into bone. Once the osteoblast has become trapped by the surrounding mineralized osteoid, it differentiates into an osteocyte, the most abundant cell type in bone (Elabd et al., 2007). The function of the osteocyte is not well understood. However, it is believed that these cells are responsible for maintaining calcium homeostasis and sensing mechanosensory fluctuations in order to relay a chemical message for bone resorption or deposition (Kartsogiannis and Ng, 2004).

1.1.2 Types of Bone

There are two different types of bone: cortical and cancellous bone. Cortical bone or compact bone is found on the surface or cortex and contributes to 80% of the weight of the human skeleton. The unit microarchitecture of this type of bone is the osteon. At the centre of the osteon is the Haversian canal which surrounds blood vessels, nerves and connects with osteocytes via canaliculi which contain osteocyte cytoplasmic processes. The cytoplasmic processes allow for osteocytes to communicate with the presence of gap junctions. Furthermore, osteons are connected to each other and the periosteum by Volkmann's canals. The periosteum covers the bone exterior and is composed of two layers, the innermost of which aids in bone formation because it consists of progenitors which develop into osteoblasts (Buckwalter and Cooper, 1987).

Cancellous bone or trabecular bone, on the other hand, makes up 20% of the human skeleton but has greater surface area per unit volume and a greater metabolic rate compared to compact bone. The interior of cancellous bone is filled with red marrow which is the site of hematopoiesis. Due to its high surface volume and rapid turnover rate, the quantity and quality of cancellous bone is important in maintaining the mechanical integrity of bone and in maintaining calcium homeostasis (Hordon and Peacock, 1990).

1.1.3 Interaction Between Osteoblasts and Osteoclasts

Modern developments in bone cell biology have changed our conceptions of the interaction between osteoblasts and osteoclasts in resorption and bone formation, respectively. Bone morphogenetic proteins (BMPs) play critical roles in osteoblast

differentiation. BMPs act to differentiate early osteoblastic cells to the non-dividing terminally differentiated phenotype via a mothers against decapentaplegic (Smad) mediated pathway. Transcription factors, runt related factor 2 (Runx2) and Osterix, are essential molecules in this pathway, experimentally demonstrated by Runx2-null mice and Osterix-null mice having neither mineralized bone nor osteoblasts. Smad transcriptional factors are shown to interact with these tissue-specific transcription regulators, including Runx2. Furthermore, the fairly recent discovery of the receptor activator of NF-kappaB ligand (RANKL)-RANK interaction substantiates the hypothesis that osteoblasts play a key role in osteoclast differentiation. Osteoblasts express RANKL as a membrane-associated factor which is recognized by RANK ligand found on osteoclast precursors via cell-cell interactions (Figure 1). This recognition results in osteoclast differentiation into mature bone resorbing cells. Therefore, the proper interaction of both the bone formers and resorbers is vital for maintenance of bone, which is continually remodeling (Katagiri and Takahashi, 2002).

1.2 Bone Remodeling

Bone is a metabolically active organ which undergoes continuous remodeling throughout life. In adults, ten percent of the skeletal bone mass is replaced every year (Alliston and Derynck, 2002). This remodeling is essential for bone to maintain its integrity and regulate metabolic levels of calcium and phosphate ions. Skeletal remodeling can be initiated by mechanical stress, damage to the microarchitecture or by hormonal signals to calcium and phosphorus serum levels (Raisz, 1999).

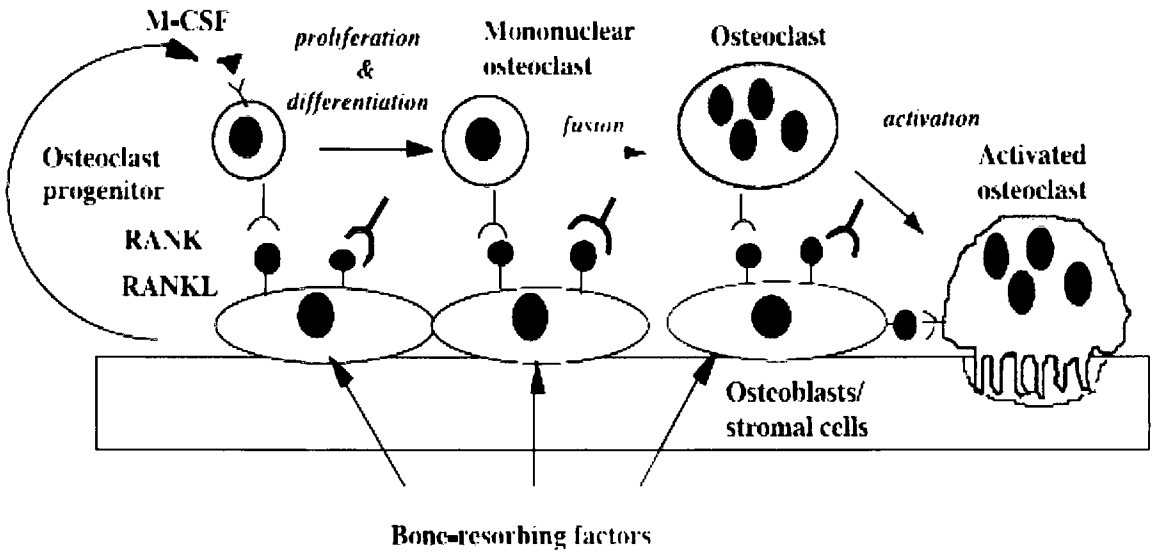

Figure 1. Schematic representation of osteoclast differeentation via interaction with osteoblasts. Osteoclast progenitors recognize the RANKL expressed by osteoblasts via cell-cell interaction. RANKL is also essential for the proper functioning of osteoclasts. M-CSF, produced by osteoblasts, is also essential for osteoclast differentiation (adapted from Katagiri and Takahashi, 2002).

1.2.1 Bone Remodeling Compartments

Five years ago, Hauge et al. demonstrated that cancellous bone in contrast to cortical bone undergoes remodeling via specialized vascular spaces referred to as bone remodeling compartments (BRC; Figure 2). These compartments were found to be externally lined with cells staining positive for osteoblastic characteristics. The inner wall of the compartment on the other hand was composed of cells with either resorptive or formative characteristics, depending on the remodeling phase the BRC was undergoing. This study concluded that the outer wall lining cells were osteoblasts which stained positive for a variety of proteins, including osteocalcin, alkaline phosphatase and type I collagen. The study also concluded that the number of BRCs found on the bone was proportional to that bone's metabolic activity. Higher turnover rates were accompanied with the observation of a greater number of remodeling compartments (Eriksen et al., 2007).

1.2.2 Systemic regulation

Bone remodeling has to be tightly regulated because a disturbance in the balance between resorption and formation can have detrimental consequences, whether it involves accelerated bone loss or bone gain (Eriksen et al., 2007). Systemic management of skeleton metabolism involves calcium-regulating hormones: parathyroid hormone (PTH), calcitonin and the active form of vitamin D, 1, 25-dihydroxy vitamin D. PTH regulates serum levels of calcium by acting as a potent stimulator of bone resorption and

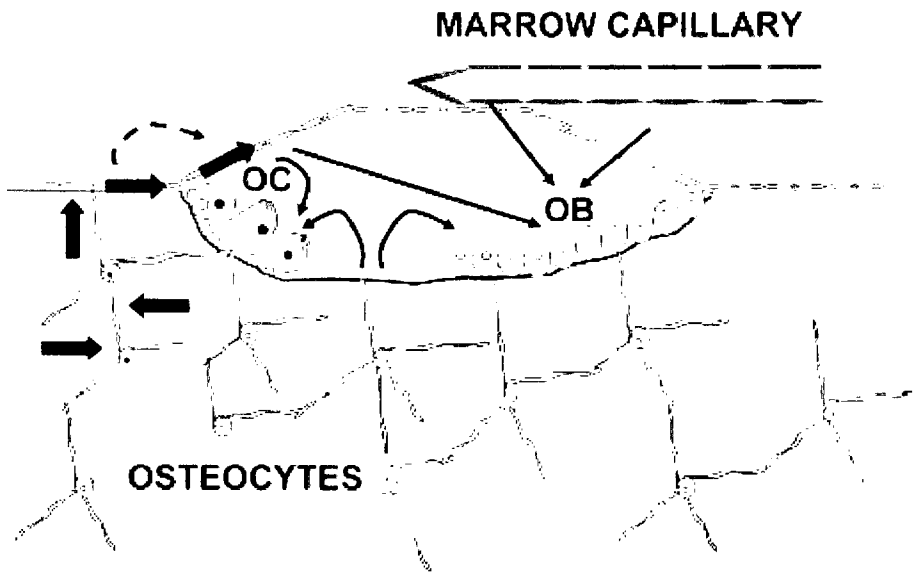

Figure 2: Schematic of the proposed connections between the osteocytes, lining cells and the BRC. All cells in the network are connected with gap junctions, which may provide a pathway (black arrows) through which signals generated deep in bone can reach the surface and elicit remodeling. Factors liberated from marrow capillaries can also play a role in remodeling. The broken arrow represents paracrine factors liberated from lining cells which can also influence remodeling events (Eriksen et al., 2007).

has been shown to have biphasic effects on bone formation. The effects of PTH are counteracted with calcitonin which promotes mineralization of bone. Active vitamin D affects intestinal absorption of calcium and phosphate and can have direct effects on bone. This form of vitamin D has also been shown to have a role in osteoblast and osteoclast differentiation. Other hormones which effect bone turnover are growth hormone which stimulates bone formation via insulin-like growth factor (IGF) production and glucocorticoids which can inhibit bone formation. Perhaps the most important regulator of bone turnover is estrogen. Decreasing levels of estrogen result in an increased skeletal turnover rate with the characteristic imbalance favouring bone resorption over bone formation, causing net bone loss (Raisz, 1999).

1.2.3 Local Regulation

Maintenance of the remodeling cycle is also determined locally by cytokines, prostaglandins and growth factors, which have been identified in the last thirty years. As previously mentioned, osteoblast precursors express a protein which is an osteoclast differentiation factor called RANKL, also known as TNF-related activation-induced cytokine (TRANCE). This protein activates cells in the osteoclastic lineage by associating with the receptor RANK. Osteoprotegerin (OPG) can also be produced by cells in the osteoblastic lineage and acts as a decoy receptor for RANKL, thereby inhibiting osteoclast differentiation by inhibiting the RANKL/RANK interaction. Bone also contains a number of growth factors which are important modulators in the remodeling process. These include IGFs and BMPs. Other growth factors, such as PTH–

related protein and fibroblast growth factor play a more important role with remodeling associated with skeletal repair (Raisz, 1999).

Apart from cytokines and growth factors, simple molecules, hypoxia and acidosis have been shown to exert regulatory effects on bone remodeling. Nitric oxide (NO) has been shown to have biphasic effects on osteoclast activity, with higher concentrations inhibiting resorption. Low concentrations of NO have been shown to regulate normal osteoblast growth and the role of estrogens on bone formation. Acidosis and hypoxia almost always inhibit bone formation and increase resorption. These two conditions may also work synergistically and can alter the release of pro-angiogenic factors such as vascular endothelial growth factor (VEGF; Eriksen et al., 2007).

Angiogenesis is closely intertwined with bone resorption and formation. Angiogenic factors such as VEGF and endothelin regulate the activity of osteoblasts and osteoclasts. Also, the formation of blood vessels serves as a transport system which delivers circulating osteoblast and osteoclast precursor cells to sites undergoing active remodeling (Eriksen et al., 2007).

1.3 Osteoblasts

Osteoblasts are of particular interest when considering bone healing because of their principle bone forming role. Osteoblasts originate from mesenchymal stem cells (MSCs). Osteoblasts form bone by secreting type I collagen which is the main component of osteoid. Other proteins osteoblasts secrete in smaller but significant amounts are osteocalcin and various growth factors including BMPs. Terminally differentiated

osteoblasts are active in that they deposit osteoid only on pre-existing mineralized matrix. They contain the genes that are necessary and sufficient to induce mineralization of the secreted osteoid. Ligation of existing matrix occurs via β1 integrins, forming a monolayer linked by cadherins. Integrins convey information about the extracellular matrix by functioning as a physical link between the extracellular matrix and actin cytoskeleton and by functioning as signal transduction molecules (Franceschi, 1999).

As aforementioned, osteoblasts also play a key role in the differentiation of the hematopoietic stem cell (HSC) derived osteoclast by secreting macrophage colony-stimulating factor (M-CSF) and RANKL. M-CSF acts as a survival factor for osteoclasts, while also exhibiting effects on the formation and chemotaxis of mature osteoclasts. In fact, it has been demonstrated that osteoclasts undergo apoptosis in the absence of M-CSF, even in the presence of RANKL (Huang et al., 2007; Woo et al., 2002).

Osteoblasts have also been linked with HSCs. Recent studies have identified osteoblasts as playing a crucial role in the HSC microenvironment. These genetic studies have provided insight into the mechanism of osteoblast-mediated HSC expansion (Huang et al., 2007). Furthermore, imaging studies have demonstrated the close proximity of subsets of HSCs with osteoblast lining cells and blood vessels, suggesting a supportive niche for HSCs (kiel et al, 2005).

Overall, osteoblasts are necessary for the formation of unmineralized bone and for its subsequent mineralization.

1.4 Introduction to Osteoprogenitors

MSCs can differentiate into chondrocytes or osteoblasts depending on the presence of various transcriptional factors (Huang et al, 2007). MSCs which are committed to the osteoblastic lineage are named osteoprogenitors. In differentiation towards the osteoblastic lineage, high levels of transcription factors Runx2 and β-catenin are necessary to suppress the chondrogenic potential of uncommitted MSCs (Phimphilai et al., 2006).These findings were determined in vivo using a mouse model to analyze the effects of the inactivation of β-catenin on skeletal progenitors. It was demonstrated that osteoprogenitors differentiated into chondrocytes in the absence of β-catenin. In vitro deletion studies further substantiated the role that β-catenin plays in differentiating the bipotential precursor, termed osteochondroprogenitor, to a committed osteoprogenitor. Furthermore, the final commitment of osteoprogenitors to preosteoblasts requires the osteoblast commitment transcription factor Osterix. Perichondrial or periosteal cells which failed to express Osterix have been shown to acquire a chondrogenic fate. A few upstream factors to Runx2 are Satb2 and Hoxa2. Hoxa2 inhibits bone formation by inhibiting osteoblast differentiation. The nuclear matrix protein Satb2 represses Hoxa2 expression and allows for the Runx-2 dependent differentiation of osteoblasts (Figure 3). Therefore, one can appreciate the complexity in the differentiation of MSCs to committeed osteoprogenitors destined to progress in the osteoblastic lineage (Huang et al., 2007).

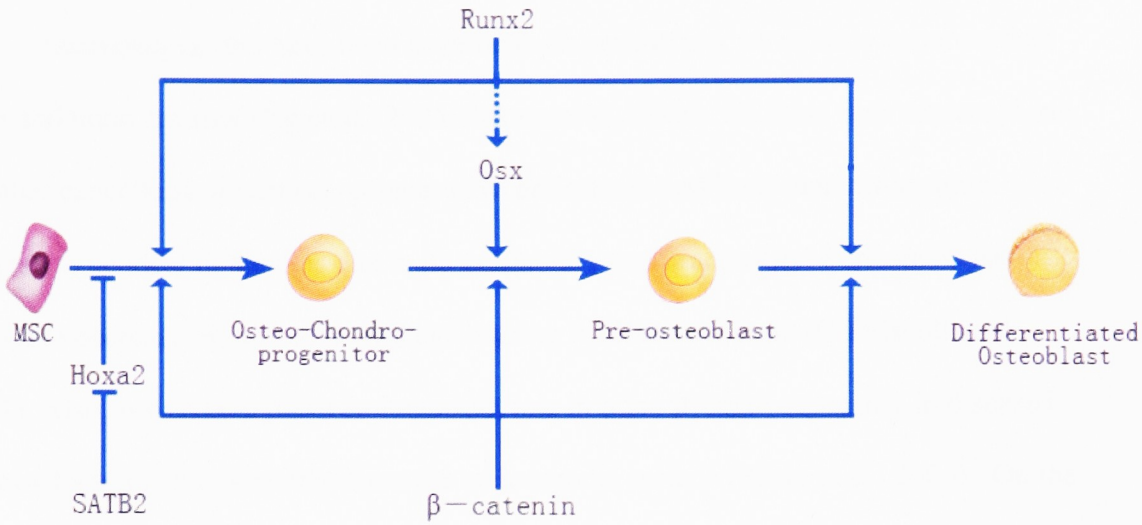

Figure 3: Regulation of osteoblast differentiation by transcription factors. In osteoblast differentiation, high levels of Runx2 and β-catenin are necessary to suppress the chondrogenic potential of uncommitted progenitors, such as the depicted osteochondroprogenitor. Osterix is required for the final commitment of progenitors to preosteoblasts (adapted from Huang et al., 2007).

1.5 Source of Osteoprogenitors

Osteoprogenitors have been isolated from periosteum, cancellous bone, cortical bone and bone marrow (Ng et al., 2005; Sakata et al., 2006). Of these four sources, bone, whether cancellous or cortical, compared to periosteum and bone marrow aspirate appears to be ideal in humans due to its availability and practicality. Periosteum has shown to decrease in thickness with age making it harder to obtain (O'Driscoll et al., 2001). Also, periosteum plays an important role in bone strength especially in diseased states which involve bone loss from the endocortical surface (Szulc et al., 2006). On the other hand, bone marrow osteoprogenitors exhibit slower growth and might require more extensive osteoinduction with various factors thus making it less ideal for proliferation and differentiation purposes when compared to bone and periosteum (Ng et al., 2005). In our study, cancellous bone from the femoral neck was used as a source of osteoprogenitors due to its availability, practicality and clinical relevance.

There have been reports on skeletal site differences in trabecular bone. Anatomical sites investigated in studies are based on clinical relevance and include the iliac crest, lumbar spine, femoral neck and calcaneus. The iliac crest is the most common and convenient site for bone biopsies, the lumbar spine and femur are the most common sites of osteoporotic fractures, and the calcaneus is used to determine the quality of the femoral neck and lumbar spine via ultrasound imaging measurements. Despite the differences in anatomical areas, all sites showed a trend in age-related decrease of collagen production and an increase in mineral parameters (Aerssens et al., 1997).

As previously mentioned, our study utilized cancellous bone from the femoral neck. It is thus of particular interest that the aforementioned studies found the femoral neck to have the highest mineralization accompanied by the lowest concentration of collagen and non-collagenous proteins. This was significantly different from other sites and thus should be taken into account when attempting to make generalizations on cancellous bone characteristics (Aerssens et al., 1997; Currey et al., 1996).

1.6 Osteoblastogenesis

There are three stages in osteoblastogenesis, differentiation of preosteoblasts to the terminally differentiated osteoblastic phenotype: proliferation, matrix maturation and mineralization. Early and advanced stages can be characterized by specific markers which will be discussed in further detail in the next section (Huang et al., 2007; Siggelkow et al., 1999).

The differentiation of osteoblasts is not completely understood, however recent advances have enabled for a better understanding of the signaling pathways and factors that control the process. There are 6 important signaling pathways of osteoblast differentiation: 1) Wnt signaling, 2) Tumour growth factor β (TGF-β) signaling, 3) Hedgehog (HH) signaling, 4) Fibroblast growth factor (FGF) signaling, 5) Ephrin signaling and 6) Sympathetic signaling (Figure 4; Huang et al., 2007). Wnt signaling and TGF-β signaling will be elaborated upon in this introduction because of the relevance of these pathways to osteoblast commitment and maturity and BMP signaling, respectively.

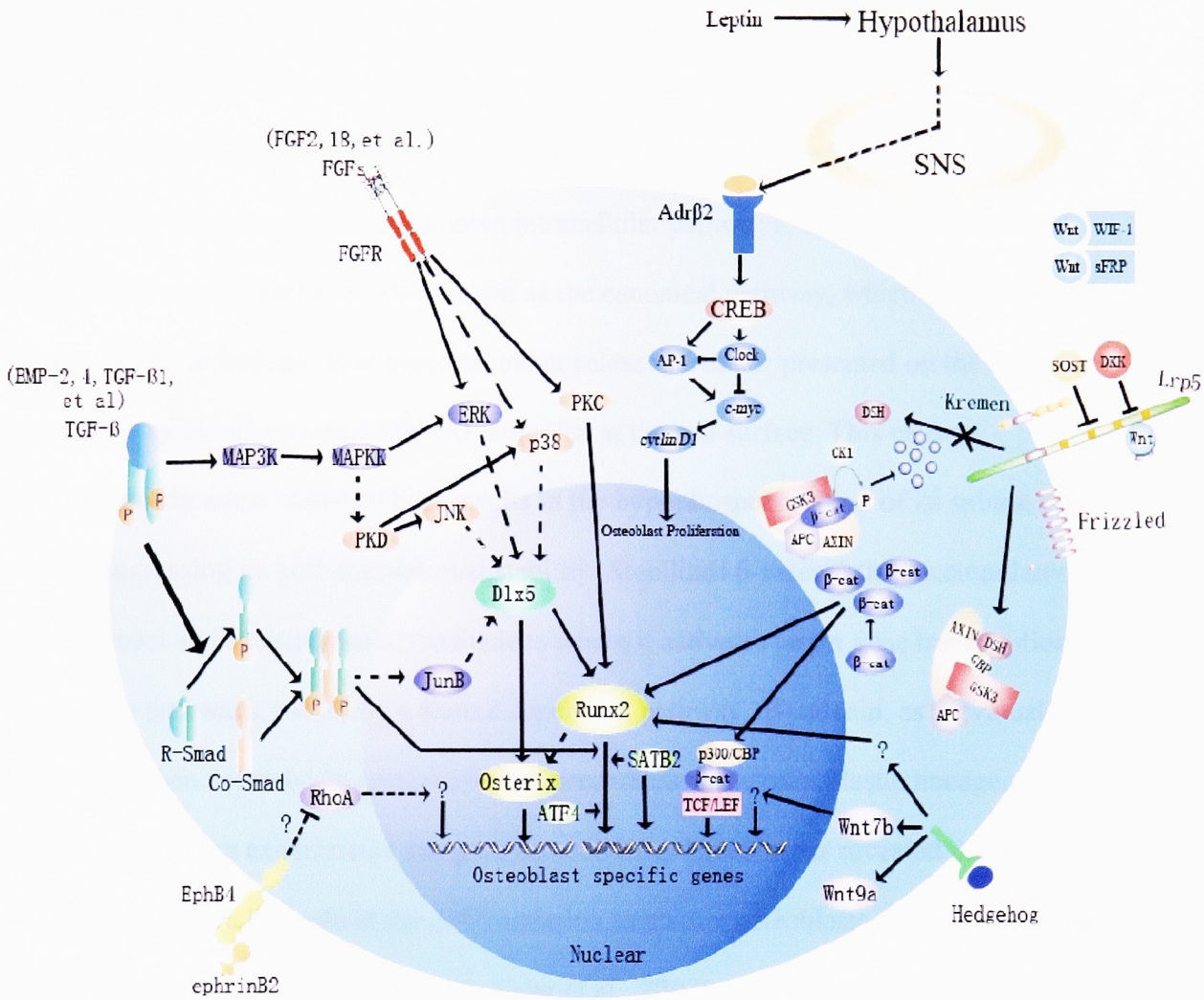

Figure 4: The six important signaling networks of osteoblast differentiation. Several transcription factors have been found to be critical for osteoblast differentiation downstream of this signaling pathway, such as Runx2, Osterix, and ATF4. These factors are essential for the differentiation of MSCs into differentiated osteoblasts. Dotted lines indicate that the physiological function or the stage at which the factor works remains to be proven (adapted from Huang et al., 2007).

1.6.1 Signaling

Wnts are a family of 19 secreted proteins that mediate critical biological processes. There are two classes of Wnt proteins that bind to either Frizzled (FZD) G-protein coupled receptors or low-density lipoprotein receptor related proteins (LRPs). This binding activates one of four known intracellular pathways. The best characterized is the Wnt/β-catenin pathway, also known as the canonical pathway, which is also the one involved in bone biology. Wnt proteins, either released from or presented on the cell, target cells by binding to the FZD/LRP complex at the cell surface. This causes downstream chemical signals which results in the hypophosphorylation of its subtrate, β-catenin, increasing its post-translational stability. Stabilized β-catenin then accumulates in the cytosol and translocates to the nucleus where it activates target gene transcription via various pathways, including a Runx2 dependent pathway. β–catenin, as previously mentioned, then controls the commitment of precursors to the osteoblastic lineage. Recent inactivation experiments using certain cell lines have further revealed that β-catenin also plays a key role in the differentiation to mature osteoblasts and not just at the preosteoblast stage (Huang et al., 2007; Kang et al., 2007).

All members of the TGF-β superfamily signal through a dual receptor system of type I and type II transmembrane kinases. Transmission of signals is carried out by Smad-mediated signaling in all receptors activated by members of this superfamily. One of the members of TGF-β is the BMPs, which have potent osteogenic effects. BMP-1 to -7 are expressed in skeletal tissue, and the most detectable BMPs in osteoblast cultures are

BMP-2, -4 and -6. BMPs involved in bone formation are BMP-2, -6, -7, and -9 (Cheng et al., 2003; Huang et al., 2007). BMP specific signaling will be discussed in section 1.9.

1.6.2 Transcription factors

Various transcription factors regulate osteoblast differentiation. The master genes are Runx2, Osterix and activating transcription factor 4 (ATF4). As previously mentioned, Runx2 is expressed in early osteoprogenitors in order to induce gene expression required for lineage differentiation of MSCs to the osteoblastic phenotype. Runx2 however is also necessary for osteoblast function beyond differentiation. Various DNA binding proteins interact with Runx2 to facilitate the recruitment of coactivators and transactivation complexes of osteoblast specific genes, which include the BMP-responsive Smads (Smad1 and 5; Xiao et al., 2005).

Osterix, a BMP-2 inducible gene, is a transcriptional regulator of the final stages of osteoblast differentiation. This was demonstrated in studies using the murine model in which bone deposition failed to occur in Osterix null mutant mice. Knockout studies have shown that Osterix null mice cannot form bone in spite of Runx2 expression, suggesting that Osterix acts downstream to Runx2 (Katagiri and Takahashi, 2002). Currently, it is not known whether Runx2 and Osterix interact either functionally or physically (Huang et al., 2007). On the other hand, it is well known that Runx2 interacts with ATF4 to stimulate osteoblastic specific osteocalcin gene expression, an important marker for terminal osteoblastic differentiation (Xiao et al., 2005).

1.7 Markers of Differentiation

Markers most frequently used to determine the differentiation process of osteoblasts include alkaline phosphatase (ALP), type I collagen and osteocalcin (OCN). In general, markers such as ALP and type I collagen are considered early markers of osteoblastogenesis, while markers such as OCN, associated with mineralization, are considered advanced markers because they appear later in the differentiation process (Huang et al., 2007).

1.7.1 Alkaline Phosphatase

Another marker denoting cells destined towards the osteblastic lineage is ALP. There are various isoforms of ALP which are divided into tissue specific and tissue non specific subgroups. ALP derived from bone falls under the tissue non specific category; however, it can be determined that it is bone specific via carbohydrate side chain analysis: bone ALP is the only isoenzyme which undergoes O-glycosylation. ALP is attached on the extracellular surface of the cell membrane via phosphotidylinositol and enhances mineralization in various ways, including by cleaving inorganic pyrophosphate which acts as an inhibitor to mineralization at high concentrations. ALP is an early marker of osteoprogenitor cells and increases as these cells progress towards the mature osteoblastic phenotype (Figure 5). This makes ALP a good marker in assessing osteoprogenitor lineage by staining to detect presence of the enzyme and in quantifying activity to detect any increase which would indicate differentiation towards the desired phenotype (Bilezikian et al., 2003).

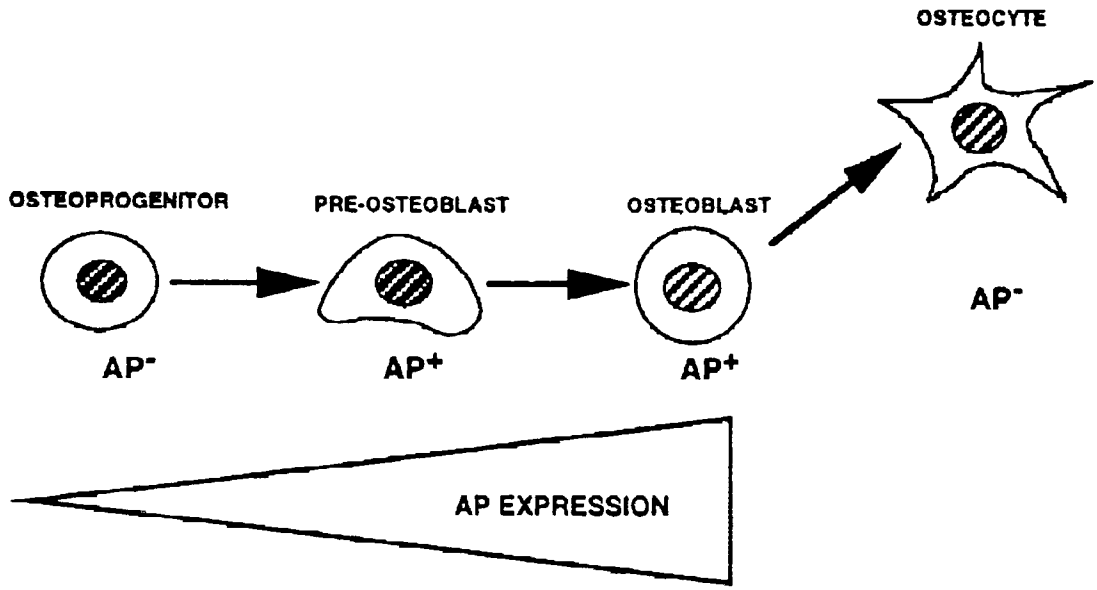

Figure 5: ALP expression during osteoblast differentiation in normal human trabecular bone samples. ALP (AP in this figure) expression begins in the mature osteoprogenitors and preosteoblast and further increases as differentiation progresses (adapted from Gronthos et al., 1999).

1.7.2 Osteocalcin

The best indicator of the differentiation of osteoprogenitors to the terminal osteoblastic phenotype is mineralization (Declercq et al, 2005). A marker closely associated with mineralization and specific to bone is the osteoblast related protein, OCN (Seibel, 2005). OCN is a non-collagenous protein deposited in the extracellular matrix of bone; however, some escapes into the bloodstream and can be found in serum, where it has a half life of 20 minutes. Serum OCN level is a specific and sensitive marker for osteoblastic activity and reflects the bone formation rate (Bilezikian et al, 2002). It is involved in binding the hydroxyapatite crystals, found in between collagen fibers, making it is essential for bone formation (Rho et al., 1998). It is for this reason that OCN assays and reverse transcriptase polymerase chain reaction (RT-PCR) are widely used in literature surrounding osteoblasts and bone mineralization. A nonspecific method that also tests for mineralization is the Von Kossa (VK) staining procedures, which can be used along with more specific markers as aforementioned. VK staining should not be used on its own as it stains the anion that binds calcium and not the calcium cation itself (Bonewald et al., 2003).

1.8 Bone Morphogenetic Protein 2

For differentiation, BMP-2 and doxycycline were supplemented into osteoprogenitor cell containing cultures in order to determine any osteoinductive effect. BMP-2, which belongs in the TGF-β superfamily, has been shown to stimulate proliferation, expression of bone matrix proteins, mineralization and ALP activity (Cheng

et al., 2003; Gueorguieva et al., 2006; Yamaguchi et al., 2000; Koch et al., 2005). Common doses used on human cells range from 25 to 100 ng/ml, resulting in a dose dependent response (Kuo et al., 2005). However, doses as low as 1 ng/ml have been used and have been shown to elicit an increase in osteoblast differentiation compared to controls, which is comparable to the findings in our study (Chaudhari et al. 1997). Furthermore, BMP 2 has been shown to act via Runx2 dependent and independent pathways (Wang et al., 2007; Jeon et al., 2006; Liu et al., 2007).

1.9 Bone Morphogenetic Protein Signaling

BMP signaling components are essential in the healing of human fracture callus. BMPs transmit signals through Smad-dependent pathways as previously mentioned. Receptor regulated Smads (R-Smads), Smads 1, 5 and 8, are receptor-regulators activated by BMPs. Common mediator Smads (Co-Smads), Smad4, are mediators which allow the transduction of the signaling by R-Smads. R-Smads in the BMP-2 signaling pathway are Smad1, 5 and 8. There are also inhibitor Smads, such as Smad6. Smad6 is a specific inhibitor of BMP signaling (Kloen et al., 2003).

Upon ligand stimulation and activation of BMPs by type II receptors, type I receptors phosphorylate R-Smads (Smad1 and 5), which then form complexes with Co-Smad, Smad4. The R-Smad/Co-Smad complex translocates to the nucleus and interacts with various transcription factors to regulate target gene transcription (Figure 6). R-Smads and Runx2 physically interact with each other in BMP signaling: BMP induces Runx2 expression in MSCs through activation of R-Smads which in turn interact with

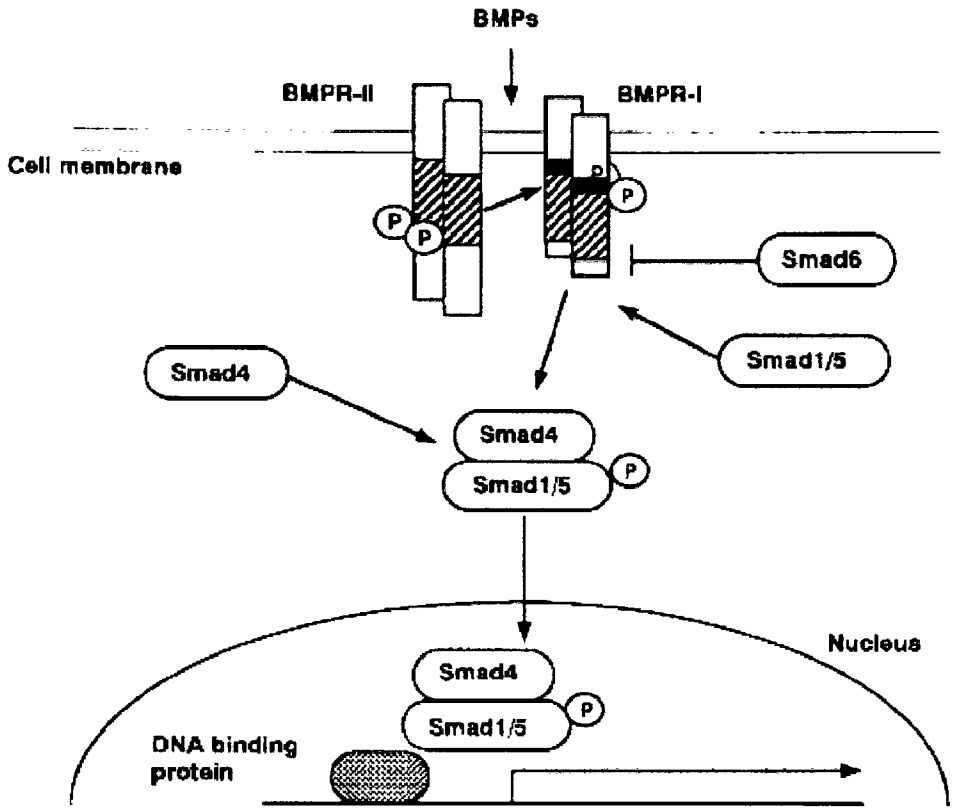

Figure 6: Schematic illustrating possible mechanism of BMP signaling through BMP receptors and Smads. BMPs bind to BMP receptors in target cells. Smad 1 and 5 transduce the signals interacting with Smad4. Smad6 inhibits BMP activity by binding to BMP receptor I. Thus, Smads1, 4 and 5 act positively and Smad6 acts negatively in BMP signaling (adapted from Yamaguchi et al., 2000).

Runx2 to further stimulate osteoblast differentiation (Huang et al., 2007). Thus, Runx2 interacts closely with BMP signaling through Smads in osteoblastogenesis (Katagiri and Takahashi, 2002).

There is a substantial body of evidence demonstrating the existence of Smad-independent pathways, including JNK and p38, upon BMP-2 activation. BMP-2 has been shown to activate MAP kinases and further regulate the expression of ALP and OCN. BMP-2 has been shown to activate the mentioned JNK and p38 pathways via protein kinase D (Lemonnier et al., 2004). It has been further demonstrated that Smad and MAP kinase pathways converge at the Runx2 gene to control MSC differentiation. Thus, Runx2 plays a pivotal role in BMP-2 induced osteoblast differentiation (Huang et al., 2007).

However, BMP-2 has also been shown to have Runx2-independent effects. Specifically, BMP-2 has been reported to induce Osterix independent of Runx2 (Huang et al., 2007). Furthermore, Liu and colleagues demonstrated that Runx2 deficient mice were able to express markers related to osteoblasts upon BMP-2 stimulation but were unable to induce precursor cells to differentiation to mature bone forming osteoblasts, which suggests a pathway independent to Runx2 (Liu et al., 2007).

1.10 Doxycycline

BMP-2 has been approved for use in the clinic however it is used when all other therapeutic options have been exhausted, as it is very expensive. It is for this reason that an alternative osteoinductive agent is desirable. Doxycycline is the most potent matrix

metalloproteinase (MMP) inhibitor of the tetracycline family (Gomes et al., 1984; Duivenvoorden et al., 2002). MMPs degrade extracellular matrix, composed primarily of collagen type I in bone. Since doxycycline inhibits MMP function, it is also described as exhibiting anticollagenase activity (Pytlik et al., 2004). In fact, doxycycline has been studied on several occasions with respect to its effect of decreasing tumour burdon in bone metastatic models. In this model, it has been demonstrated that doxycycline can compensate for the increased resorption sometimes seen in bone metastases (Duivenvoorden et al., 2002). This phenomenon was further studied and it was found that this tetracycline actually increased several parameters of bone formation (Duivenvoorden et al., 2007). The ability of this drug to stimulate bone formation is most likely due to its inhibitory effect on collagenases and certain MMPs by a mechanism which has been shown to be independent of its antimicrobial ability (Golub et al., 1987; Golub et al., 1991).

1.11 Doxycycline Mechanistic Action

Several mechanisms have been proposed for the demonstrated in vivo and in vitro bone parameter increase of doxycycline (Gomes et al., 1984; Pytlik et al., 2004). The most studied mechanism has been its role as a MMP inhibitor (Gomes et al., 1984). In this suggested mechanism, doxcycyline, a calcium chelator, diminishes calcium thereby inhibiting MMPs which are functionally dependent on the calcium cation. Thus, doxycycline may indirectly increase bone formation by inhibiting bone resorption via decreased MMP activity. Furthermore, osteoblastic differentiation has been documented

as being dependent on the stability of the collagenase environment. Thus, doxycycline may also increase bone formation by increasing osteoblastic differentiation due to the instability in the extracellular matrix caused by its primary anticollagenase activity. It has also been proposed that doxycycline may have direct effects at the transcriptional level by decreasing collagenase mRNA. Regardless of its mechanism however, doxycycline has been shown in studies, including our study, to inhibit collagen breakdown and to be conducive to osteoblastic differentiation between 10 to 50 μM concentrations, above which there is an inhibition of proliferation and growth (Gomes et al., 1984; Pytlik et al., 2004).

1.12 Implantation and Cellular Based Therapy

With an aging population, fractures and various disorders in bone metabolism become prevalent, which makes the development of therapeutic approaches to bone healing so critical. Current bone regeneration methods involve autografts, allografts, metal devices, etc. Each of these methods has its limitations, including donor site morbidity and graft rejection in allografts (Goldberg and Stevenson, 1987). These methods do not sufficiently consider the cells that constitute bone, which are necessary for regeneration to occur.

The idea of cell-based therapy emerged as a way to counteract the disadvantages set forth by traditional methods. Cell based therapy involves providing patients with the cells necessary for optimal bone healing. This innovative method involves harvesting the necessary cells, osteoprogenitors, from a patient and proliferating the cultures in vivo.

These cells are then osteoinduced to differentiate to bone nodule forming osteoblasts and then transplanted percutaneously back into the donor.

Overall, the project focuses on the acquisition and priming of osteogenic cells necessary for bone grafts to be effective in healing bone defects. The desired future outcome of this study would involve the ability to harvest cells from the patient, proliferate and differentiate the cells towards an osteoblastic phenotype, and finally transplant the cells back into the patient along with a scaffold to heal the bone.

1.13 Hypothesis

The hypothesis is that viable osteoprogenitor cells can be isolated from human cancellous bone of the femoral neck and grown in vitro. These osteoprogenitors can then be differentiated into nodule forming osteoblasts with the use of BMP-2 and doxycycline.

1.14 Specific Objectives

1. To determine an optimal cell isolation technique from human cancellous bone of the femoral neck.
2. To examine whether differentiation of these osteoprogenitors into bone forming osteoblasts occurs upon stimulation with either BMP-2 or doxycyline.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Primary Cell Culture

Human cancellous bone (2-4 grams) was harvested from biopsies of the femoral neck of total hip replacement surgery patients, aged 61 or older. Dr. Nigel Colterjohn, the orthopaedic surgeon in this study, was generous to obtain all our biopsy material. Patients were consented and approval for the study was obtained from the Hamilton Research Ethics Board. Cells were isolated from bone chips and placed for 9 days in Standard Media (SM) consisting of alpha minimal essential medium (αMEM) supplemented with fungizone (0.3 μg/mL), penicillin-streptomycin (100 μg/mL), gentamicin (50 μg/mL), L-Glutamine (200 mM) and 10% Fetal Bovine Serum (FBS). All tissue culture media and reagents were obtained from Invitrogen life technologies (Burlington, ON). Media was changed 3 times per week and cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

2.2 Cell Isolation Technique:

Bone samples were cut into 3x3 mm chips, washed twice with 1x phosphate buffer saline (PBS; 13.8 mM NaCl, 2.7 mM KCl, pH 7.4; Sigma-Aldrich, Oakville, ON) and placed in 1x red blood cell lysis buffer (BioLegend, San Diego, CA) for 15 minutes.

2.2.1 Enzyme Digestion

One gram of bone was set aside for enzyme digestion technique. Bone chips were placed in 50 mL Falcon tubes at 1 g per tube and 3 mL of collagenase (2000 units/mL; Invitrogen Life Technologies) was added to the tubes. The chips were incubated for 10

minutes. The collagenase was replaced with 6 mL of fresh collagenase solution and incubated for 2 hours and 30 minutes. The collagenase treatment was stopped by adding 30 % FBS. Bone chips were filtered out of solution with a cell sieve. The cell suspension was centrifuged for 7 minutes at 1500 rpm. The supernatant was aspirated and replaced with 2-4 mL, dependent on the size of the cell pellet, of SM and the number of cells was counted using a hemocytometer.

2.2.2 Explant Culturing

One gram of bone was set aside for explant. Chips were placed into 25 µL droplets of plasma clots which included: αMEM combined with 20 % FBS, 15 % citrated bovine plasma, antibiotics (100 µg/mL penicillin-streptomycin and 50 µg/mL gentamicin) and an antimycotic (0.3 µg/mL fungizone) in 6 well Falcon tissue culture plastic dishes (BD Biosciences, Oakville, ON). After allowing 2 hours for droplets to clot, 2 mL of SM were placed in each well. Primary cultures were set up as 8 chips per well in a 6 well plate in order for the cells to migrate out of the bone. Cultures were grown for 18 days in order to reach 70 % confluence. On day 18, bone chips were taken off the plates with forceps and reweighed to check for 1 g of bone. The cells were detached with 1 % collagenase and 0.12% trypsin (Invitrogen Life Technologies). The enzyme activity was stopped with 30% FBS and counted.

2.3 Treatment Groups

Cells were harvested using enzyme digestion as described above and grown for 9 days in SM to reach 70 % confluence (2.5×10^6 cells per T75 flask). On day 9, cells were detached with 0.05 % trypsin. The enzyme activity was stopped using 30 % FBS. Cells were then plated into subcultures at a seeding density of 2.0×10^5 cells per well in a 6 well plate in OM containing BMP-2 or doxycycline at different concentrations. SM was used as a negative control (no addition of any osteoinductive factors). Osteogenic Media (OM) was used as a positive standard control containing the minimum requirements for differentiation to osteoblastic phenotype (SM with the addition of 10 μM β-glycerophosphate and 50 μg/mL L-ascorbic acid; Sigma Aldrich). Recombinant human BMP-2 (rhBMP-2; R&D systems, Minneapolis, MN) and doxycycline (Sigma Aldrich) solutions were made using OM at three different doses each (rhBMP-2: 1 ng/mL, 10 ng/mL and 100 ng/mL and doxycycline: 10 μM, 50 μM and 100 μM). Day 0.5 of culture occurred 12 hours after cells were subcultured into their groups.

2.3.1 BMP-2 Preparation

A 4 mM hydrochloric acid (HCl) stock solution (molecular weight of 36.46 g/mol) was prepared in distilled water, with the addition of 0.1 g of bovine serum albumin (BSA; Sigma Aldrich). The stock solution was then filter sterilized using a 0.22 μM syringe filter (Millipore, Mississauga, ON). One milliliter of this stock HCl solution was added into the vial of rhBMP-2. The rhBMP-2 and HCl mix was then reconstituted

into 250 µL aliquots and stored in -20°C. Aliquots were taken out of storage as needed and used to prepare 1, 10 and 100 ng/mL solutions of BMP-2 using OM.

2.3.2 Doxycycline Preparation

Doxycycline containing treatment media was prepared fresh per feeding. A 5mM stock was prepared with distilled water and then filter sterilized with a 0.22 µM syringe filter. This stock solution was used to prepare 10, 50 and 100 µM doxycycline solutions using OM.

2.4 Von Kossa Staining

On day 8 of treatment with either BMP-2 or doxycycline, subcultures were processed with Von Kossa (VK) staining to assess mineralized bone nodule production following the protocol used in Bhargava and colleagues (Bhargava et al., 1988). Briefly, media was suctioned off and rinsed once in warm 1x PBS. The wells were then fixed in 10% neutral buffered formalin (Millipore). After 15 minutes, the formalin was suctioned off and disposed of in accordance with regulations. The wells were rinsed once with distilled water and then left in distilled water for 15 minutes. The water was then suctioned off and replaced with prepared 2.5% silver nitrate (BDH Inc., Toronto, ON) for 30 minutes. The silver nitrate was disposed of as required and the wells were rinsed 3 times with distilled water and covered with fresh distilled water for 15 minutes. After this, the wells were rinsed once more with distilled water to ensure the elimination of all

silver nitrate. Sodium carbonate formaldehyde was then added for 5 minutes to allow the reaction to deepen the colour of the silver nitrate stained mineralized nodules. The sodium carbonate and formaldehyde was then discarded and the wells were washed 3 times with distilled water and covered with fresh distilled water for 1 hour. Wells were rinsed once more with fresh distilled water and then analyzed under the microscope for mineralization. Stained wells were able to be stored in the fridge filled with distilled water containing fungizone (0.3 μg/mL) for a month.

2.4.1 Preparation of Solutions in VK protocol

Solutions used in the VK protocol were prepared fresh for each experiment. A 2.5% silver nitrate solution was prepared by dissolving 2.5 g of silver nitrate in 100 mL of distilled water.

Sodium carbonate formaldehyde was prepared by adding 25 mL of formaldehyde (Sigma Aldrich) and 5 g of sodium bicarbonate (Sigma Aldrich) to 100 mL of distilled water.

2.5 Nodule Assay

Nodule counts were done on 100x magnification using a graticule (Ted Pella, Inc., Redding, CA). Nodules stained black were included in the counts when the diameter was equal to or greater than 25 μm.

2.6 Alkaline Phosphatase Staining

On day 8 of treatment with either BMP-2 or doxycycline, subcultures were stained with Alkaline Phosphatase (ALP) staining kit (procedure No. 85, Sigma Aldrich) to assess osteoblast phenotype of primary cell colonies.

2.7 Alkaline Phosphatase and Protein Assays

ALP protein levels were quantified on day 0.5 and 8 of treatment and control subcultures using the EnzyoLyte pNPP ALP Assay from Anaspec, Inc. (San Jose, CA) with some modifications.

Cell isolation from wells began by washing the cells with 1x PBS three times. Five hundred microlitres of 0.5% Triton X-100 was added per well. Adherent cells were scraped off using a cell scraper (Corning Life Sciences, Lowell, MA) and collected in microcentrifuge tubes. The cell suspension in the tubes was frozen in -80°C and thawed in 37°C waterbath three times in order to disrupt the cells and cause lysis. Cell lysates were stored in -80°C until required for ALP analysis using the rest of the Anaspec kit reagents. Once ready to be analyzed, the cell lysate mixtures were thawed and reaction mixtures and standards prepared according to the manufacturer's protocol. Fifty microlitres of sample lysate mixture was added to 50 µL of pNPP reaction mixture in a 96 well plate; samples were prepared in triplicates. The 96 well plate including the samples and standards were incubated for 30 minutes at 37°C. Plates were read at 405 nm using Bio-Rad plate reader (Bio-Rad, Hercules, CA). The step involving the optional

addition of 50 μL stop solution after incubation but before plate reading was unnecessary and not used in our protocol.

ALP levels were normalized per microgram protein using Micro BCA protein assay (Fisher Scientific, Ottawa, ON). The protocol followed was that suggested by the manufacturer. Samples were prepared in triplicate. The BCA mix was prepared and 100 μL of this mix was added to 100 μL of the cell lysate extracted from the pNPP protocol using a 96 well plate. The plate was gently mixed, sealed in parafilm and incubated for 30 minutes at 37°C. The plate was read at 562 nm using Bio-Rad plate reader. The standard curve used in the protein assay was revised for this project: 0, 20, 40, 50, 100, 120, 160, 180, 200 and 400 μg/mL concentrations of BSA in Milli-Q water were used.

2.8 RNA isolation and Reverse Transcriptase Reaction

Total RNA was isolated on day 0.5 and 8 from treated and control cells cultured in a 6 well plate using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions. Complementary DNA (cDNA) was made using a Superscript III RT-PCR kit (Invitrogen) with 500 ng of total RNA. The specific protocol used was that of random primers (5 ng/uL). Briefly, a mixture of random hexamers, 500 ng total sample RNA, and 1 mM dNTP was made and the final volume per reaction was brought to10 μL using DEPC-treated water. This 10 μL mixture was then incubated for 65°C for 5 minutes and then placed on ice for 1 minute. A separate mixture of 1x RT buffer, 2.5 mM $MgCl_2$, 0.01 M DTT, and 1 μL RNaseOUT recombinant ribonuclease inhibitor was prepared. Nine microlitres of this reaction mixture was added to each sample reaction tube and collected

briefly by centrifugation. Following a 2 minute incubation at 25°C, 1 µL (50 units) of Superscript III RT was added to each tube except the no RT control and each sample reaction tube was incubated for an additional 10 minutes at 25°C. The tubes were then transferred to 42°C for 50 minutes. Termination of the reaction was carried out by placing the tubes at 70°C for 15 minutes, followed by chilling on ice. The tubes were then centrifuged briefly to collect the reactions and 1 µL of RNase H was added to each tube and incubated for 20 minutes at 37°C before proceeding to amplification. cDNA which was not used that day was stored in -20°C.

2.9 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

The OCN primers (Sigma Aldrich) used for amplification by PCR have been used in various studies (Diefenderfer et al., 2003; Rifas et al., 1989; Sakaguchi et al., 2004). The sequence of the primers generated for amplification of a 297 base pair (bp) product were;

- sense 5' – ATGAGAGCCCTCACACTCCTC
- antisense 5' – GCCGTAGAAGCGCCGATAGGC

According to Invitrogen PCR protocol, 0.2 µM of the sense and antisense primers were mixed with 1x PCR Buffer, 0.2 mM dNTP mix, 1 mM $MgCl_2$, 2 µL template DNA and 0.04 units Taq DNA polymerase. Milli-Q water was then added to reach a final volume of 50 µL. The reaction was performed in a thermal cycler (PerkinElmer, Waltham, MA) and the PCR conditions repeated for 45 cycles were:

1) Denaturation: 94°C for 15 seconds

2) Annealing: 65°C for 30 seconds

3) Extension: 72°C for 30 seconds

Samples were resolved by electrophoresis in a 1.2% agarose gel containing 0.5 μg/ml ethidium bromide. The gel was run at 110 volts for approximately 50 minutes in 1x TAE buffer. A 1 kb ladder (Invitrogen Life Technologies) was run alongside samples as a size marker. Gels were visualized under UV light using the imager Bio-Rad ChemiDoc XRS system (Bio-Rad).

The housekeeping gene used was GAPDH (Sigma Aldrich). The sequence of the primers generated for amplification of the 375 bp product was:

sense 5' – ATGTTCCAATATGATTCC

- antisense 5' – ACGATACCAAAGTTGTCA

All aspects of the protocol used for the OCN primer was the same as that used for GAPDH. The only difference was in the PCR conditions which were run at 35 cycles:

1) Denaturation: 94°C for 15 seconds

2) Annealing: 55°C for 30 seconds

3) Extenstion: 72°C for 30 seconds

CHAPTER THREE

RESULTS

3.1 Comparison of Cell Isolation Techniques: Enzyme digestion yields a 10 fold higher number of cells compared to explant culturing

Isolation of cells from bone samples using enzyme digestion for two and a half hours with collagenase yielded a 10 fold higher number of cells per gram of bone when compared to the explant culturing technique (Table 1).

The enzyme digestion technique also yielded more cells in a shorter time frame: two and a half hours after sample collection and treatment with red lysis buffer and a 10 minute collagenase conditioning incubation. On the other hand, explant cultures took 18 days to become confluent with cells migrated out from adherent bone chips. Thus, it took a longer time for cells to become available to be used in subculture. The shorter wait period for cell availability when using enzyme digestion makes this technique superior to explant culturing. This is of particular interest when considering harvesting cells in a clinically relevant time frame.

3.2 Nodule Assay: Subcultures treated with BMP-2 at all doses and doxycycline at all doses except at 100 μM displayed an increased number of mineralized nodules relative to control

Mineralized nodules which were included in the counts had a black mineralized core, usually but not always outlined with a yellow-brown perimeter representing unmineralized osteoid (Figure 7).

Treatment with BMP-2 at 1 ng/mL, 10 ng/mL and 100 ng/mL significantly increased the number of mineralized bone nodules when compared to controls (Figure 8). BMP-2 at 10 ng/mL produced the most nodules per well when compared to 1 ng/mL and 100 ng/mL treatment doses.

Doxycycline at 10 μM and 50 μM significantly increased the number of mineralized bone nodules when compared to controls. However, doxycycline at 100 μM decreased the amount of nodule formation when compared to controls (Figure 9).

In regards to statistical analysis, BMP-2 and doxycycline treatment groups were compared to OM controls in a two tailed t-test. SM groups were also compared to OM controls. As previously mentioned, SM contained no differentiation factors, neither β-glycerophosphate nor L-ascorbic acid, and were thus used as negative controls. As expected, SM subculture groups yielded significantly lower number of mineralized nodules when compared to the OM control groups.

3.3 Alkaline Phosphatase Staining: All treatment and control groups stained positive for ALP

ALP staining was used to determine the presence of cells in the osteoblastic lineage. All treatment groups were stained positively for ALP protein when stained on day 8 of treatment. Positive staining was presumed as it demonstrated the well known phenomenon of progressing ALP expression during osteoblastic differentiation. As expected, BMP-2 and doxycycline treated groups stained positively for ALP (Figure 10

and 11). This is also in accordance to the increased mineralization seen on day 8 in the nodule assay.

ALP staining of SM subcultures was used to determine if the cells being extracted and proliferated in vitro exhibited osteoprogenitor phenotype without stimulation with either the minimal factors in OM or either treatment group. As predicted, these cells stained positively for ALP which further confirmed that osteoprogenitors committed to the osteoblastic lineage were being harvested from femoral cancellous bone and expanded in vitro (Figure 12). This further explains why 100 μM doxycycline treated groups still exhibited ALP staining even though this dosage had less mineralized nodules than controls. The reason lies in the fact that the isolated cell populations, irrespective of being effectively stimulated towards the osteoblast phenotype, initially contain a significant amount of committed osteoprogenitors expressing ALP

3.4 Alkaline Phosphatase Assay: BMP-2 at all doses and doxycycyline at all doses except 100 μM increased ALP protein levels relative to control

Alkaline phosphatase assay was used to quantify the levels of ALP in order to determine ALP levels during differentiation. Twelve hours after being placed in subculture (day 0.5), BMP-2 treatment groups showed higher levels of ALP at all doses compared to controls; while, doxycycline at all doses except for 100 μM had higher levels of ALP compared to controls. Doxycycline at 100 μM had lower ALP levels compared to controls. The same trend was observed on day 8 of subculture, however with

smaller differences within groups. These results however were not statistically significant on either day (Figure 13 and 14).

Another trend observed fairly consistently was ALP levels decreasing from day 0.5 to day 8. On day 8, wells stained positively for VK staining which confirmed bone forming cell differentiation into osteoblasts capable of mineralization. This suggests that as the cells become terminally differentiated their ALP levels decrease. Thus, an increased mineralization correlated with a decrease in ALP protein formation in vitro.

3.5 Osteocalcin RT-PCR: BMP-2 at all doses and Doxycycline at all doses except 100 µM increased OCN expression

OCN expression was analyzed to determine terminal differentiation into the mature osteoblastic phenotype. On day 0.5 and 8 in control groups and day 0.5 of treatment, negative to weak OCN expression was observed (Figure 15 and 16). On day 8 of treatment with BMP-2, there was strong expression of OCN at all doses (Figure 17). On day 8 of doxycycline treatment, there was strong expression of OCN at low doses of 10 µM and 50 µM. However, the 100 µM high concentration had a negative expression for OCN (Figure 18). This correlates with the decreased ALP activity and nodule formation on day 8 of the 100 µM doxycycline treatment, compared to controls.

Cell Isolation Technique	Cell Number Per Gram of Bone (N=7)
Enzyme Digestion	$4.99 \pm 0.92 \times 10^6$ *
Explant Culture	$4.10 \pm 0.86 \times 10^5$

Table 1: Cell yield per gram of bone from enzyme digestion technique compared to explant culturing technique.

*significantly higher yield compared to explant culturing technique using a paired t-test ($p<0.05$).

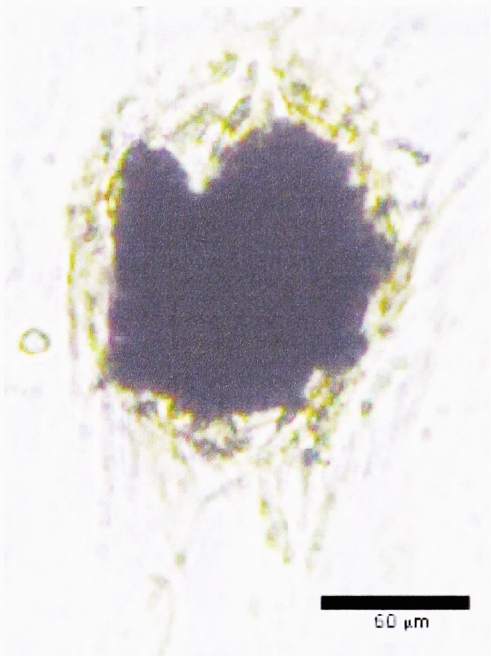

Figure 7: Image of mineralized nodule; Image at 100x magnification; Magnification bar is 60 µM.

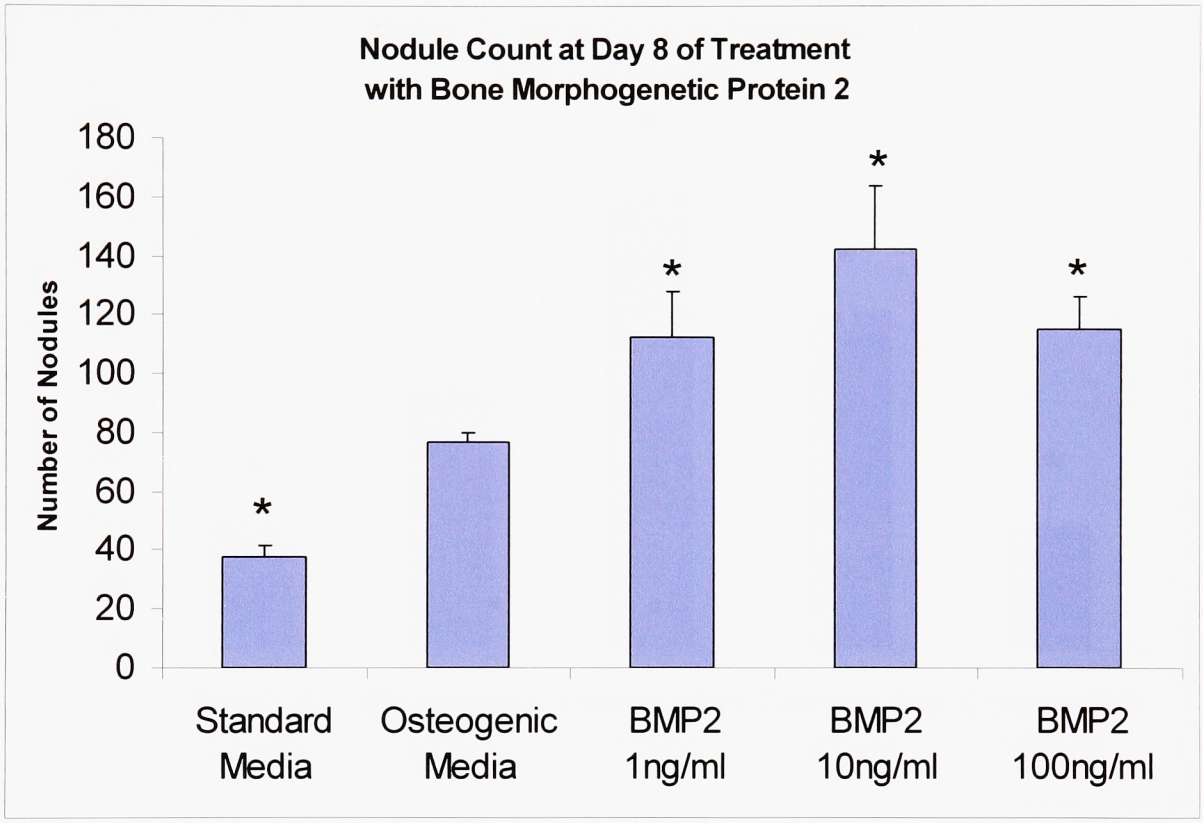

Figure 8: Nodule Count at Day 8 of Treatment with Bone Morphogenetic Protein 2.

Nodule counts in BMP-2 treatment groups. Nodule Assay using VK staining procedure to count mineralized nodules in day 8 subcultures. Nodule count on BMP-2 treated cells at three different doses and non-treated cells (in standard media), all statistically compared to osteogenic media.

*significantly different from osteogenic media control group using t-test ($p<0.05$).

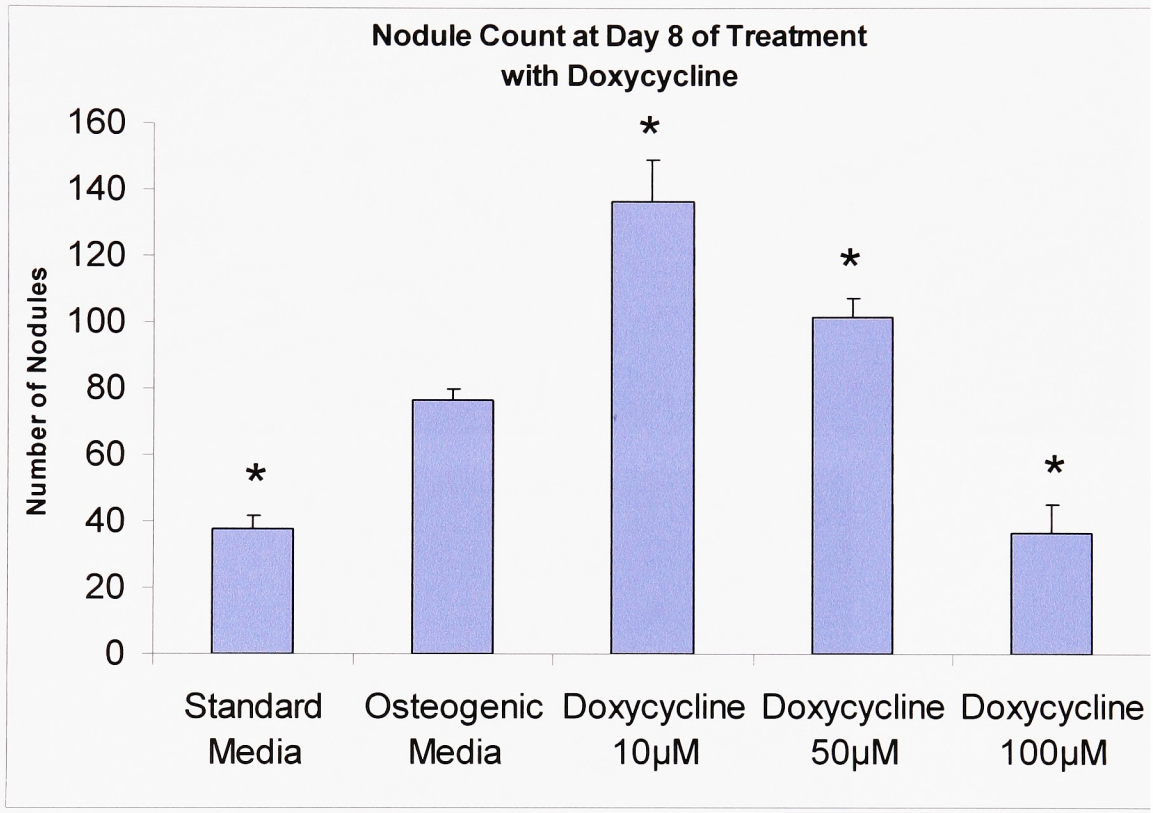

Figure 9: Nodule Count at Day 8 of Treatment with Doxycycline. Nodule counts in doxycycline treatment groups. Nodule Assay using VK staining procedure to count mineralized nodules in day 8 subcultures. Nodule count on doxycycline treated cells at three different doses and non-treated cells (in standard media), all statistically compared to osteogenic media.

*significantly different from osteogenic media control group using t-test ($p<0.05$).

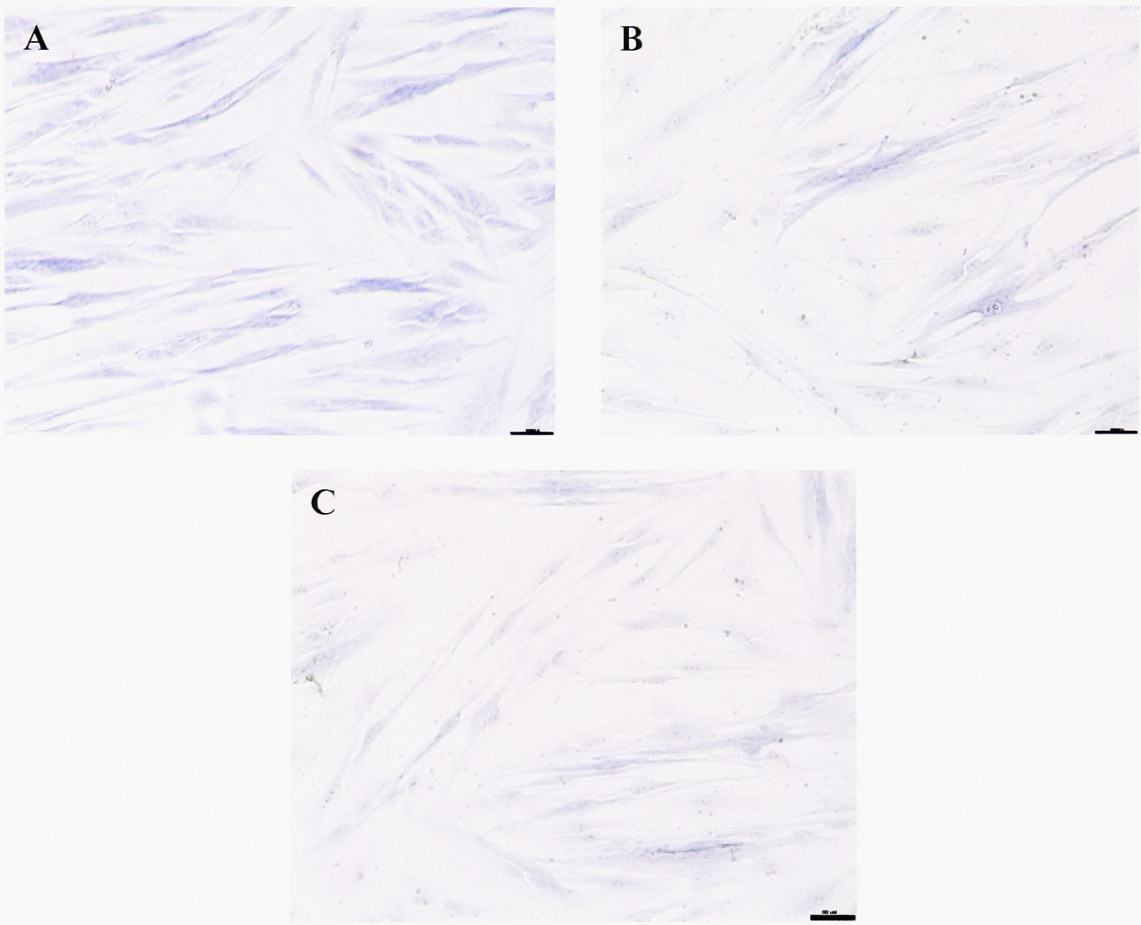

Figure 10: ALP staining to determine presence of early marker of osteoblastic phenotype. Staining done on day 8 of subculture. (A) BMP-2 1 ng/mL; (B) BMP-2 10 ng/mL; (C) BMP-2 100 ng/mL; 100x magnification with magnification bar of 60 μM. Control group images in figure 12.

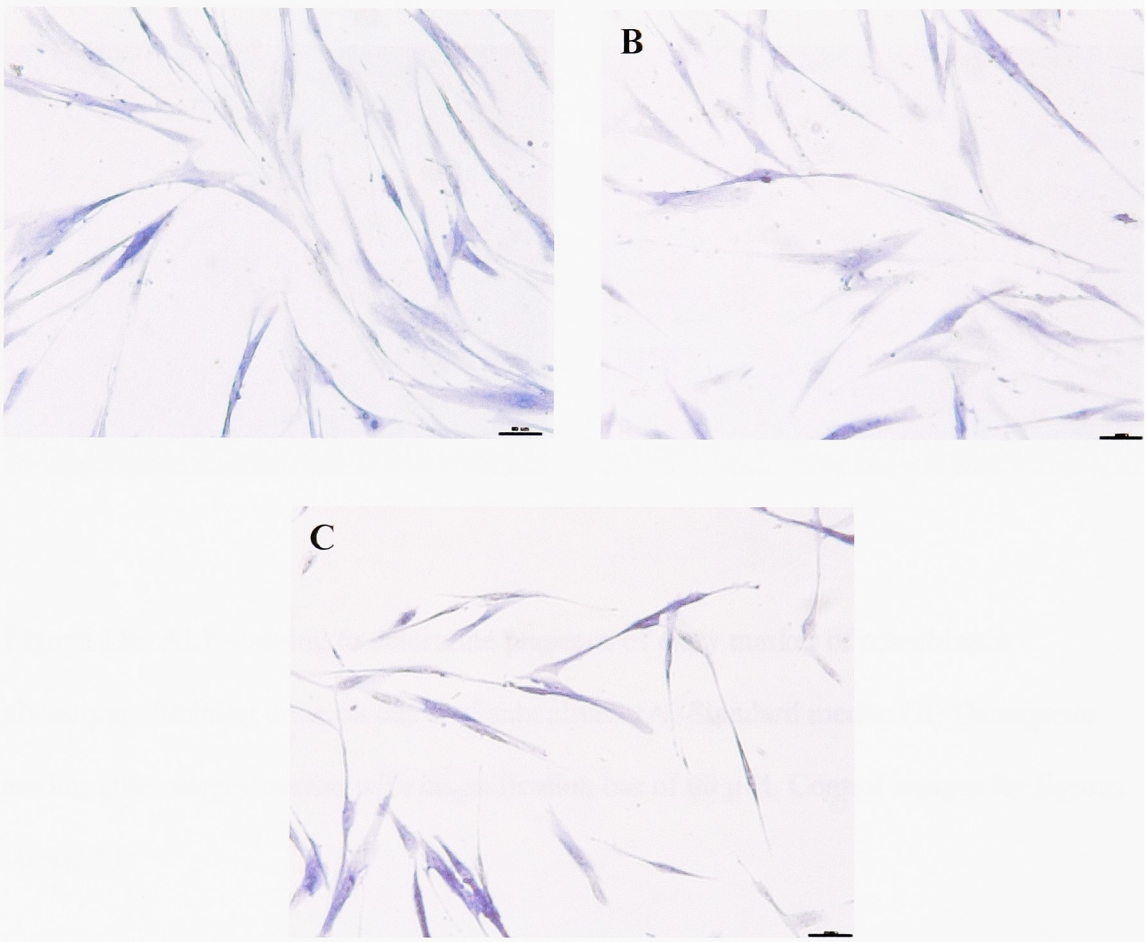

Figure 11: ALP staining to determine presence of early marker of osteoblastic phenotype. Staining done on day 8 of subculture. (A) Doxycycline 10 μM; (B) Doxycycline 50 μM; (C) Doxycycline 100 μM; 100x magnification with magnification bar of 60 μM. Control group images in figure 12.

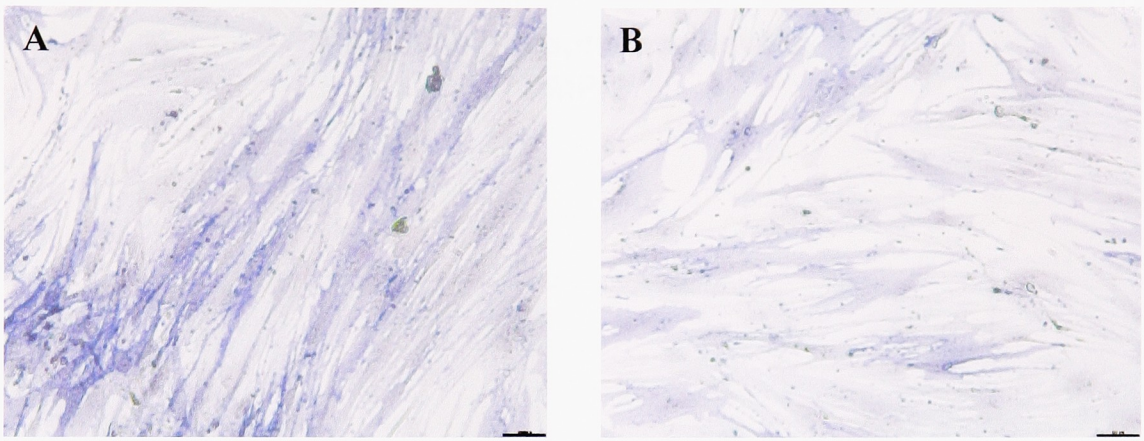

Figure 12: ALP staining to determine presence of early marker of osteoblastic phenotype. Staining done on day 8 of subculture. (A) Standard media; (B) Osteogenic media; 100x magnification with magnification bar of 60 μM. Control images for figures 10 and 11.

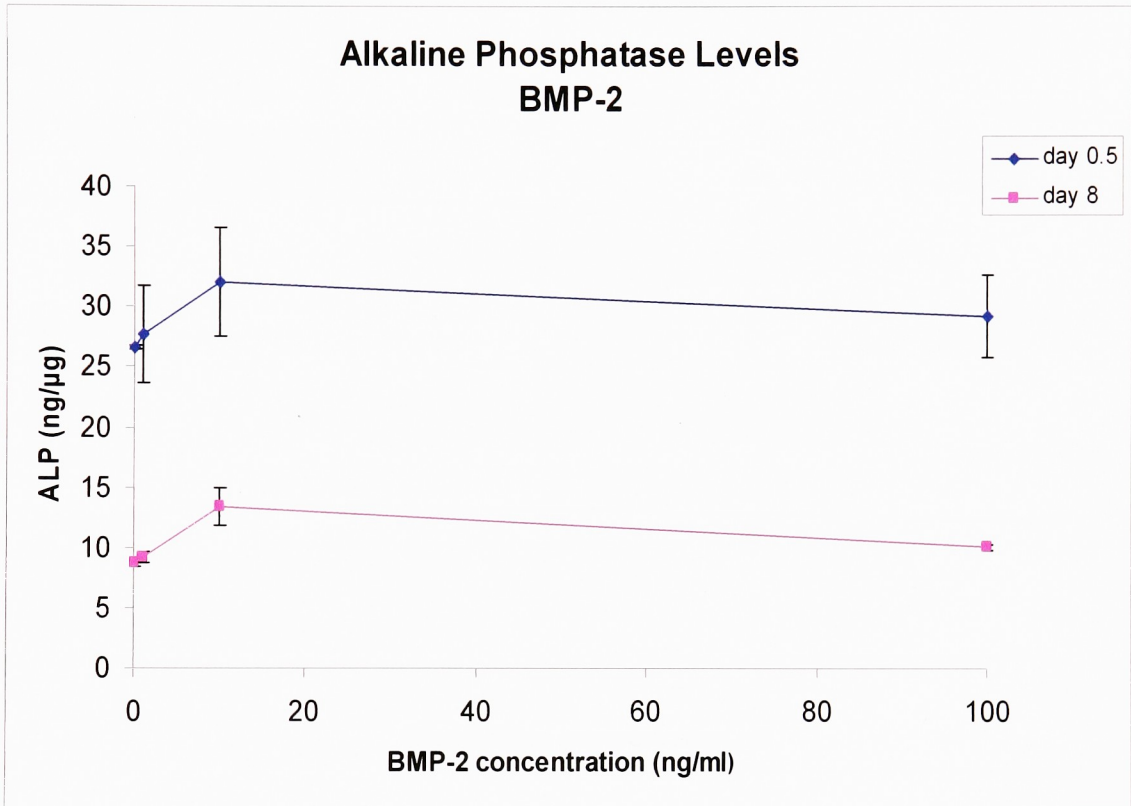

Figure 13: Alkaline Phosphatase Levels BMP-2. ALP assay results showing ALP activity in ng ALP per µg protein on day 0.5 and day 8 of BMP-2 treated subcultures.

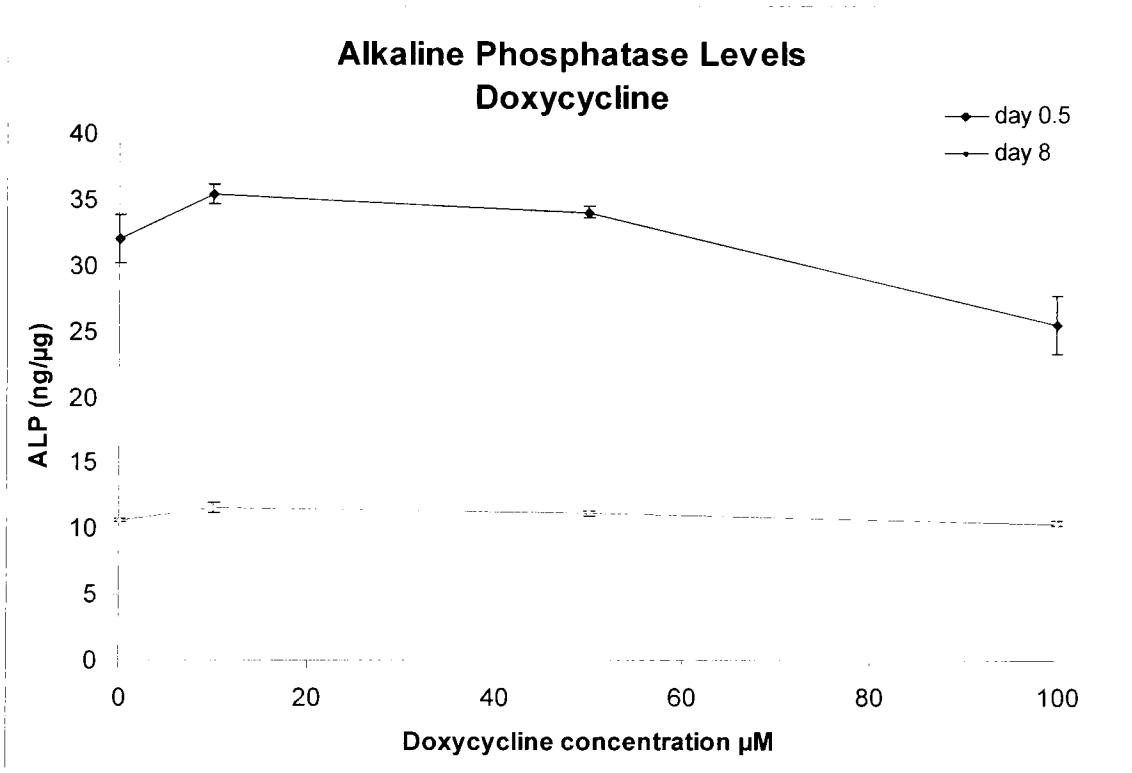

Figure 14: Alkaline Phosphatase Levels Doxycycline. ALP assay results showing ALP activity in ng ALP per µg protein on day 0.5 and day 8 of doxycycline treated subcultures.

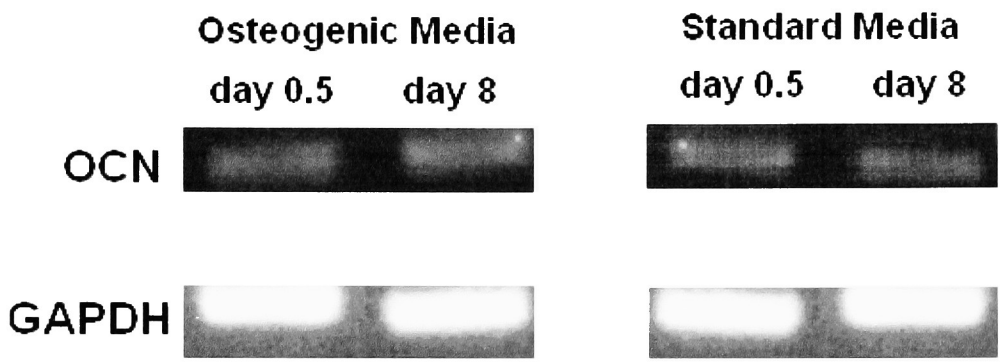

Figure 15: OCN RT-PCR on day 0.5 and 8 of SM and OM subcultures. Control group images for figures 16 and 17. OCN, 45 cycles; GAPDH, 35 cycles.

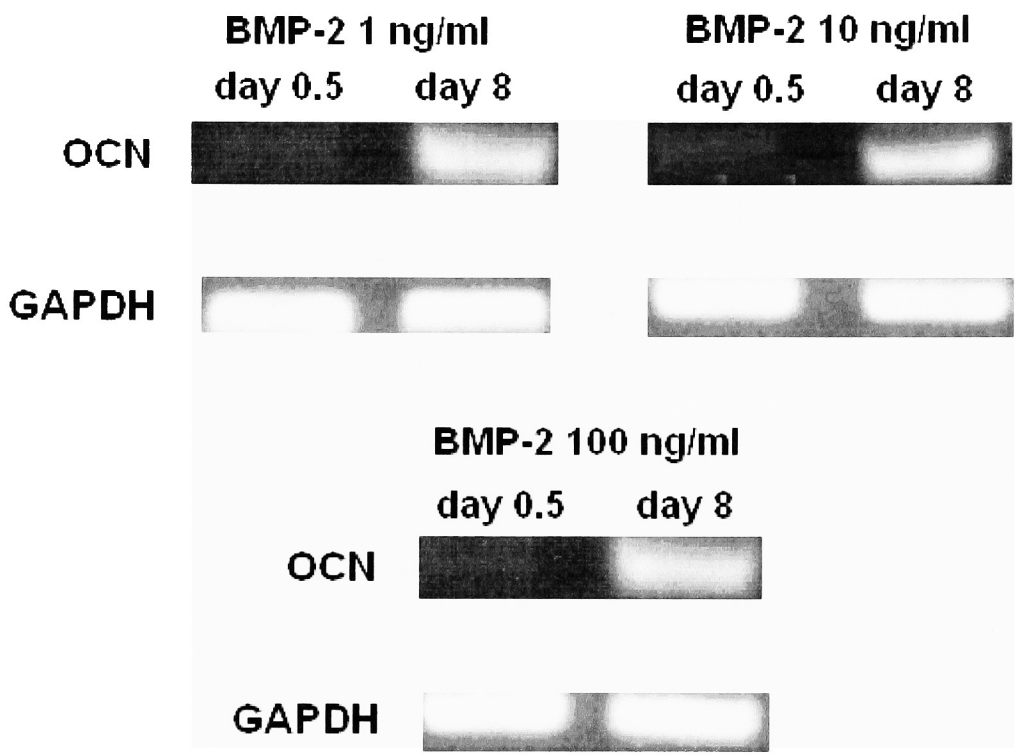

Figure 16: OCN RT-PCR on day 0.5 and 8 of BMP-2 treated subcultures. Control group images in figure 15. OCN, 45 cycles; GAPDH, 35 cycles.

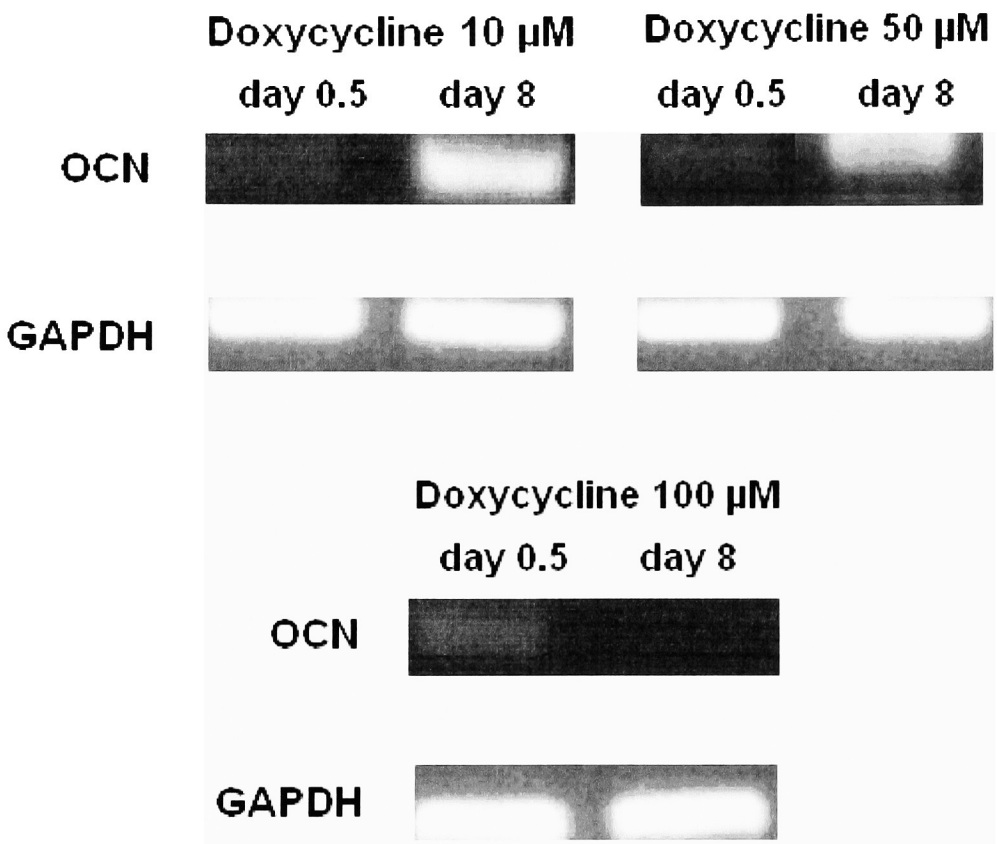

Figure 17: OCN RT-PCR on day 0.5 and 8 of doxycycline treated subcultures. Control group images in figure 15. OCN, 45 cycles; GAPDH, 35 cycles.

CHAPTER FOUR

DISCUSSION

4.1 Use of Enzyme Digestion as isolation technique for harvesting cells from cancellous bone

Enzyme digestion proved to be optimal to explant culturing in producing greater cell yields in a shorter time frame. Furthermore, explant culturing was more difficult to work with due to the tight adherence of bone cells to wells, resulting from the use of bovine citrated plasma as an adhesive. As previously mentioned, cells outgrown from bone chips in the explant technique require a mixture of trypsin and collagenase in order to effectively lift cells (Pei et al., 2003; McDuffee et al., 2006). Even this potent mixture at times did not lift all the cells without having an impact on viability (data not shown). Explant culturing however is still used by some as a preferred isolation technique and does yield sufficient cell numbers for experimentation (Pei et al., 2003; McDuffee et al., 2006).

The lengthy wait time of 18 days for cells to migrate out of bone chips in explant culturing as seen in this thesis project has also been observed by other research groups. One such group lead by Ng, observed a period of 21 to 35 days for explant wells to become confluent (Ng et al., 2005). A better comparison however is research done by Pei and colleagues, who demonstrated that cells took an average of 12 to 15 days to migrate out of bone chips and make wells 70% confluent, using a similar 8 bone chips per 35 mm well strategy (Pei et al., 2003). The cell yield in this research article ranged from $5x10^4$ to $2x10^5$ cells per well. This falls within the range of cell yield per well that was demonstrated in this paper (data is shown per gram of bone in Table 1 which was found to correspond to per 6 wells). Also, cell proliferation is first evident close to the explant

chips and confluence is evident only after a minimum of two weeks time (Robey et al., 1995; Robey and Termine, 1985). This is similar to our findings, as cells were first evident around the bone chips and slowly proliferated outward to reach confluence at 18 days.

Furthermore, Sakaguchi and colleagues compared their usage of enzyme digestion technique with a 28 day explant culturing method performed by Tuli et al. and found that enzyme digestion yielded 50 times more cells that had a 100 fold higher proliferative potential in as little as 14 days (Tuli et al., 2003). Thus, explant culturing can still be used to harvest a high number of cells. However, enzyme digestion should be primarily considered when an optimally efficient isolation and time frame is required as in most clinical settings.

Another important point to consider is the homogeneity of isolated cells which will be discussed in the next section. Enzyme digestion of trabecular bone has shown to yield more homogeneous osteoblastic cell populations compared to explant culturing. This phenomenon can be attributed to the fact that collagenase digestion removes a substantial amount of the connective tissue components, leaving cells mostly derived from within the osteoid matrix (Nöth et al., 2002).

4.2 Potential and practicality of proximal femoral cancellous bone as a source of osteoprogenitor cells

The possibility of isolating osteoprogenitors from cancellous bone is widely accepted, including from the proximal femur (McDuffee et al., 2006; Pei et al., 2003; Ng

et al., 2005). In fact, cancellous bone has been shown to have great osteoprogenitor potential. Recent studies on the properties of the cells isolated from cancellous bone, whether by explant culturing or enzyme digestion, have demonstrated the mesenchymal stem cell characteristics of these osteoprogenitors as having the potential to differentiate into osteoblasts, chondrocytes and adipocytes (Sakaguchi et al., 2004; Schliephake et al., 2000).

Despite the amount of studies on and great interest in osteoprogenitors, these cells are still poorly characterized. The difficulty partly lies in the subtle changes the cells undergo in culture. Clonal colonies and homogeneous populations can be collected however the cells quickly become heterogeneous as they expand so that they contain subpopulations of progenitors and more differentiated cells (Sakaguchi et al., 2004). This nonetheless seems to only be a classification problem in that despite heterogeneity, these harvested cells are still able to differentiate into bone forming osteoblasts, as demonstrated in this and previously mentioned studies. Thus, the heterogeneity of cell populations is not clinically relevant in this instance, and only becomes relevant in studies concerned with cell subpopulations.

Despite its use in this study, using the femoral neck as a harvesting site is not a justifiable source. It is not practical nor proper protocol for surgeons to obtain bone from the neck of the femur since it is in the hip joint which is inaccessible in regular conditions and very invasive, resulting in an increased risk of morbidity. The femoral neck however was used in this study because of the regularity with which the surgeon working on the project conducted total hip replacement surgeries, making the femoral neck readily

accessible. This is the reason why the proximal femur was practical and convenient to work with in this study. However, a more sensible site must be used if harvesting bone from a patient not undergoing total hip replacement surgery, as to minimize invasiveness. This problem can be overcome with what is commonly used currently for bone biopsies: the iliac crest. Cancellous bone from the iliac crest has been characterized and shown to be a source of osteoprogenitors. However, one must be careful in generalizing any of the data from this project to iliac crest bone due to confirmed differences between different anatomical sites. In particular, femoral cancellous bone differs from iliac crest bone with a higher degree of mineralization and a higher OCN expression (Aerssens et al., 1997).

4.3 Similarities in BMP-2 and doxycycline induced differentiation

Both BMP-2 and doxycycline stimulated osteoblast differentiation with regards to mineralization, ALP levels and OCN expression.

4.3.1 Nodule assay

The nodule assay in both treatment groups yielded quite similar results. Both BMP-2 at 10 ng/mL and doxycycline at 10 μM produced close to 140 mineralized bone nodules per well. Also, doxycycline produced close to 100 nodules per well which was similar to nodule counts seen in both BMP-2 1 ng/mL and 10 ng/mL concentrations.

Furthermore, there was no distinguishable difference in nodule colony morphology between both treatment groups, which suggests that doxycycline treatment may trigger similar end-point mineralization patterns to BMP-2 in vitro irrespective of its

actual mechanistic action. More studies need to be done in order to determine the actual signaling pathway with which doxycycline causes mineralization.

4.3.2 ALP activity

ALP levels of doxycycline were similar to those of BMP-2 treated groups. Specifically, doxycycline at 10 μM was close to 35 ng ALP per microgram protein which is similar to BMP-2 at 10 ng/mL which was also close to 35 ng ALP per microgram protein. This resemblance further suggests the similarity of end point between the two treatments.

It must be noted however that doxycycline at its highest dose of 100 μM proved to be toxic to the cells, as is demonstrated by its low ALP levels compared to controls. This suggests that only low doses of doxycycline should be used for osteoblastic differentiation. This is consistent with the literature surrounding doxycycline usage in animal models and in culture. Using a murine model, ovariectomy-induced bone loss has been shown to be ameliorated with the use of low doses of doxycycline, suggesting the possible function of doxycycline as a MMP and cysteine proteinase inhibitor (Pytlik et al., 2004). Also, research done by Gomes and Fernandes on cells extracted from human bone marrow demonstrated that the optimal concentration for differentiation of precursors to osteoblasts was 10 μM, the same concentration used in our study (Gomes and Fernandes, 2007). Thus, low doses have been shown in our study and related studies to be efficient in osteoblastic differentiation.

The effective low dose of doxycycline is of clinical importance since the US Food and Drug Administration only approved the use of doxycycline in subantimicrobial amounts for the use of adult periodontitis in the late nineties (Golub et al., 1991). Thus, it is advantageous that low doses are effective in that it makes the clinical use of doxycycline as a bone formation enhancing therapeutic factor a future possibility, as it abides by current standards of subantimicrobial dose.

4.3.3 OCN expression

Osteocalcin plays a major role during the development of MSCs to mature osteoblasts that can secrete and mineralize osteoid. The expression of OCN is regulated by the aforementioned osteoblast specific transcription factor Runx2. It is believed that during the early stages of osteoblast differentiation, low concentrations of OCN promote maturation to the terminal phenotype. At the stage where osteoblasts are mature and capable of mineralization, high OCN levels inhibit prolonged ossification and maturation to osteocytes, which are no longer able to participate in osteoid secretion and calcification (Handschin et al., 2006).

In this study, OCN expression correlated with mineralization as seen in the literature, discussed above. Both treatment groups had an increase in OCN expression from day 0.5 to day 8 of subculture. The only treatment group that did not have an increase in OCN expression was doxycycline at 100 μM. This lack of expression in the high dose doxycycline group is expected as it is associated with the low mineralization and ALP levels compared to controls on day 8 of subculture. Thus, this study

demonstrates the increased OCN associated with the end stage differentiation of osteoblasts to the bone mineralizing stage.

4.4 OCN as a differentiation marker

Since OCN is associated with the terminal osteoblastic phenotype, it is commonly used to determine the stage of osteoblast differentiation. This however should not be used to determine the normal functioning of bone, as normal OCN expression can not always rule out bone disease such as osteoarthritis.

A number of osteoblast specific markers, including OCN, are differentially expressed in osteoarthritis serum. Osteoblast cells from osteoarthritic bone are biosynthetically active, producing greater amounts of protein such as OCN. This however does not afford the bone more stability. In fact, osteoarthritic bone is more brittle and prone to fracture. The instability in osteoarthritis is believed to be attributed to the increased bone remodeling and collagen fibre arrangement: collagen fibres are narrower and more disorganized compared to normal bone (Aerssens et al., 1997; Shimizu et al., 1983).

In this study, some controls (SM) demonstrated weak OCN expression without any stimulation which confirms the increased OCN expression associated with osteoarthritis. Furthermore, SM controls also exhibited mineralized nodule formation, although significantly lower than OM controls. Another reason for OCN expression and mineralization seen in SM controls may be due to the possibility that some cells extracted from primary cultures were already more differentiated towards the osteoblastic lineage.

This possible explanation arises from the occurrence of heterogeneous cell subpopulations in cancellous bone, which was discussed in section 4.2. Therefore, it is important to be aware of OCN expression in various disease states compared to that found in normal bone, especially when using OCN as a marker for osteoblast differentiation.

4.5 Observable differences in cell morphology using BMP-2 and doxycycline

Osteoprogenitors have been shown in the literature to exhibit a stellar, fibroblast-like morphology in vitro (Ng et al., 2005; Nöth et al., 2002). This is similar to what was observed in this study (data not shown). The osteoprogenitors isolated from femoral neck cancellous bone were spindle shaped and resembled fibroblast cells.

Once placed in subculture, cells in OM and certain treatment groups began to resemble osteoblast morphology which is cuboidal in shape (Kim et al., 2005). Specifically, cells in OM and all BMP-2 treatment groups resembled the normal osteoblast. This can be seen in Figures 10 and 12B which illustrate the cells with ALP staining in BMP-2 subcultures and OM media, respectively. Furthermore, it can be noted that osteoprogenitors cultured in SM media, maintained their spindle shaped. This indicates their undifferentiated state and is expected considering SM contains no differentiation factors (Figure 12A).

In contrast, cells treated with doxycycline displayed vastly different morphology compared to those found in the other treatment groups and controls. The architecture of these cells was elongated, thinner and farther apart. There are no present studies that have

highlighted the in vitro appearance of cancellous bone cultures upon exposure to doxycycline for comparison purposes with our study. However, a study by Bettany and Wolowacz has shown that doxycycline causes morphological changes in murine macrophages. It is also important to note that the mentioned study also found that doxycycline was able to cause apoptosis of macrophages but did not stimulate apoptosis in MSCs (Bettany and Wolowacz, 1998). MSCs being spared from doxycycline induced apoptosis is critical in that the aim of this study is to proliferate and differentiate these MSCs with minimal loss in cell numbers.

Further study is needed to assess the reason for the morphological changes. However, this change is probably not due to toxicity because the cells still differentiate into osteoblasts which show nodule counts comparable to the BMP-2 treatment groups. ALP activity is also comparable to that found in the BMP-2 treated groups. Therefore, the change in morphology is indifferent to the maximal differentiation of osteoblasts with optimal nodule yields. This makes doxycycline a promising alternative to BMP-2.

4.6 Doxycycline as an alternative to BMP-2

The bone enhancing effects of doxycycline have been described in vivo and in vitro using both animal and human models. Duivenvoorden and colleagues have shown that doxycycline decreases tumor burden and compensates for increased resorption by increasing bone forming parameters. Specifically mouse femora was analyzed for bone formation enhancement and a significant increase of osteoid volume, osteoid surface and number of osteoblasts per bone surface was found in mice femora which had been treated

with doxycycline (Duivenvoorden et al., 2002; Duivenvoorden et al., 2007). Doxycycline has also been shown to reverse the effects of ovariectomy-induced bone loss in mice as already mentioned. In more detail, fracture load at the femoral neck was decreased in rats by daily oral gavage (Pytlik et al., 2004). Furthermore, Chang and Ramada observed that doxycycline loaded biodegradable tissue regenerating membranes inserted into bone defects in a canine model increased bone height and bone area significantly when compared to non-treated biodegradable tissue regenerating membranes (Chang and Yamada, 2000).

In vitro studies have also displayed the bone formation increasing properties of doxycycline (Duivenvoorden et al., 2002; Gomes and Fernandes, 2007). Also, Gomes and Fernandes used human bone marrow cells and treated them with doxycycline. They found that at low doses 10 to 50 μM doxycycline enhanced mineralization of cell cultures (Gomes and Fernandes, 2007). This is in line with our study's findings, which included the analysis of these two concentrations of doxycycline on subcultures.

Gomes and Fernandes further found that higher doses of doxycycline inhibited bone formation, which was also shown in the results of this study. Therefore, the findings in the literature surrounding doxycycline are similar to what has been observed in the outlined experiments and shows promise in regards to reproducibility and reliability of results.

Doxycycline has also been used on patients at subantimicrobial levels. Golub and colleagues reported in 1998 that doxycycline inhibited collagenase activity in bone. They went on to further confirm this in different types of arthritic disease conditions (Golub et

al., 1991; Golub et al., 1998). This study is what led the US Food and Drug Administration to approve the aforementioned use of subantimicrobial levels of doxycycline in adult periodontitis patients. This demonstrates the promise of doxycycline as a potential osteoinductive stimulatory factor for human use.

The significance of the need for antimicrobial amounts stems from the idea of eliminating the risk of antibiotic resistant bacteria, as doxycycline is an antibiotic. This led Golub and colleagues to manufacture a non-antimicrobial tetracycline, by separating the bone enhancing portion from the antimicrobial portion of the molecule. Thus, this led to the formation of a modified doxycycline molecule without its antibiotic properties (Golub et al., 1987). The antibiotic aspect of doxycycline however does not seem to be a problem for this project in that the concentration of doxycycline used in the outline expeiments is well below subantimicrobial levels (SDD 20 mg, twice daily; Walker C et al., 2007). Furthermore, this study's aim is to only treat patient cells with doxycycline in vitro and once differentiated implant the cells back into the patient without any treatment factors. This eliminates some of the uncontrolled interaction of the host and its natural flora. Also, if doxycycline was ever to be administered to a fracture patient in order to enhance bone formation parameters, the patient most likely would also need antibiotics, which is usually the case with fracture patients. Thus, the use of an antibiotic is usually warranted in fracture treatment, making the use of doxycycline, with its antibiotic properties, a good candidate for such cases.

4.7 Comparison of in vitro differentiation to in vivo

The textbook's rendition of the progressive increase in osteoblast specific markers associated with differentiation is somewhat different compared to results from in vitro studies. As already discussed, OCN expression is substantially increased as osteoblasts differentiate to occupy their final niche which primarily involves mineralization of matrix. This is consistent with the surrounding literature, as discussed in section 4.3.3.

When referring to ALP enzymatic activity however, there seems to be a contradiction. ALP levels are believed to increase to its highest levels in terminally differentiated osteoblasts (Bilezikian et al., 2002). However, the opposite seems to be true, shown in the results section of this thesis. Other studies have also described similar results.

In a study using human cancellous bone from femoral heads, Nöth and colleagues found that ALP expression and activity progressively increased and then decreased during progression towards the osteoblastic mineralization stage. This is a comparable study to this thesis in that it involves human cancellous bone from the femora and treatment groups of 30 ng/mL BMP-2 were used containing 10 μM β-glycerophosphate, 50 ng/mL L-ascorbic acid, which is the same formulation as found in our treatment mixtures. The only difference is in the concentration of BMP-2 used. We used 1, 10 and 100 ng/mL, with the optimal concentration determined as 10 ng/mL. However, the recently discussed project by Nöth et al. found that the optimal concentration of BMP-2 on the differentiation of osteoblasts was 21.5 ng/mL (Nöth et al., 2002). This is in

between 10 and 30 ng/ml and thus this data can be used to allow for comparisons between our study and Nöth's study, while keeping the concentrations in mind.

Another study already mentioned, using human bone marrow cells treated with a similar concentration of 10 μM as used in our experiments, found that ALP levels increased during differentiation. However, this was followed by a subsequent decrease after the onset of mineralization. Therefore, our study most likely did not use a time point which allowed for the detection of the peak ALP levels. Specifically, they found that ALP activity increased during the first 14 days of culture with doxycycline and this was then followed by a significant decrease (Gomes and Fernandes, 2007). In our cultures, ALP activity had decreased as early as 8 days after treatment. At first it was postulated that perhaps the ALP in our experiments decreased earlier due to the fact that our cells were in culture for 9 days before being placed in treatments for a further 8 days. However, Gomes and Fernandes' study also had a period of cell proliferation for 10 to 15 days before placing cells in subculture (Gomes and Fernandes, 2007). Thus, another, more probable explanation had to be proposed. One such explanation is that our study harvested cancellous bone; whereas, the study mentioned was using bone marrow cells. Bone marrow cells have been shown to require more osteoinduction when compared to cancellous bone, cortical bone and periosteum (Ng et al., 2005). This suggests that bone marrow cells might demonstrate a later increase and thus decrease in ALP levels due to delayed differentiation. Cancellous bone, on the other hand, may comparatively experience a quicker maximal ALP level, followed by a quicker decrease in ALP levels. This would explain why we saw ALP levels decrease as early as day 8 of subculture.

Gomes and Fernandes also found that cultures treated with 100 μM of doxycycline or higher showed impaired production of phosphate ions. Thus possibly a reason why there were decreased ALP levels in the higher dose doxycycline treatment groups relative to controls (Gomes and Fernandes, 2007). Our study also found a decrease in ALP levels relative to controls when analyzing doxycycline at 100 μM, the highest concentration used in this thesis.

It is possible thus that what was thought of as a contradiction is not one at all; there just may be a discrepancy in what stage the osteoblast is considered to be in. Based on the evidence, it can be agreed upon that ALP levels increase as osteoblasts differentiate to the mineralization stage. However, it is hard to distinguish when the osteoblast is at its most active mineralizing stage versus when it is near the end of mineralization. Once the osteoblast is in the end stage of mineralization and completely surrounded by bone, it progresses to differentiate into an osteocyte. Osteocytes are associated with low to negative ALP expression (Gu et al., 2006). Therefore, diminishing ALP expression that were observed from day 0.5 to day 8 in our subcultures suggests that the osteoblasts have already reached end stage mineralization and have began to exhibit characteristics indicative of osteocytes.

4.8 Potential for implantation of isolated and differentiated osteoprogenitor cells

In this present study, osteoprogenitors were shown to have osteoinductive potential into bone forming colonies. This is in accordance with the overall aim of this study which is the autotransplantation of these cells back into the donor for optimal bone

healing in fracture scenarios. Although this has not been done specifically in our experiments, it is the next step of the research. Furthermore, studies on animal models have been successful in demonstrating the idea of directly delivering the machinery (cells) responsible for synthesizing new bone as a more efficient way of promoting the healing at bone defect sites.

Two studies have utilized human cells to induce bone formation in rats (Bruder et al., 1998; Sakata et al., 2006). Bruder and colleagues implanted human bone marrow derived MSCs with a ceramic carrier into femoral defects in rats. They found that by 8 weeks, the defect site contained bone which had integrated with the host bone. By 12 weeks, they reported complete union by the formation of woven bone which was in intimate contact with the cut edge of the host cortex (Bruder et al., 1998). In addition, Sakata and colleagues used human periosteal MSCs and implanted them into rat calvarial defects using a collagen sponge. Although there was significant calcification and bone formation by day 35, they reported that the defect had not been completely filled (Sakata et al., 2006). Although the results are optimistic, this difference in the degree of filling of the defect site suggests that the osteoinductive material required for cell based bone healing strategies must be further researched if it is to be approved for use in humans.

A further two studies have a similar idea to that of this thesis in that they focus on the autoimplantation of host derived MSCs back into the donor for bone defect healing. Both these studies have been conducted using a canine model. In Kawaguchi H et al., MSCs were harvested from the bone marrow of beagles in order to regenerate periodontal bone. Significant tissue regeneration described in percent bone area was found in the

osseous defect after 1 month of autoimplantation (Kawaguchi et al., 2004). The other study is more similar to the type of cells harvested for future implantation. Kraus and his colleagues harvested cancellous bone from the iliac wing of hounds and proliferated them in what was referred to as a special cell culture facility. They then implanted these cells into a synthetically produced defect site in the diaphyseal bone of the dogs' femurs using a ceramic carrier. New bone was found in the pores of the scaffold after 4 weeks post transplantation. By 12 weeks, there was bridging of the defect site. Ceramic carriers which had been implanted without MSCs as controls displayed minimal bone formation, with a high amount of fibrous tissue infiltration in the pores. The study then had a similar suggestion as proposed in this discussion which involves the potential of this process to be used in humans: MSCs to be harvested from a site such as the ilium of the patient, sent to a cell culture facility to be expanded and subsequently delivered to the defect site with the use of osteoconductive material as a scaffold in order to aid in bone healing (Kraus and Kirker-Head, 2006).

There is somewhat of a discrepancy in the actual degree of bone defect filling with the use of MSCs implanted at the defect site, as can be seen by the aforementioned studies. It is important to note however that in all these experiments, the harvested MSCs were not stimulated to differentiated into osteoprogenitors and preosteoblasts. As already mentioned, MSCs harvested from bone are a mixture of progenitors with the ability to develop into osteoblasts, chondrocytes or adipocytes. Thus, it makes sense that it would be inefficient to implant a mixture of cells into a bone defect site and hope for the best. The differentiation of these cells in vitro, with the idea of implanting these committed

osteoprogenitors back into the patient, makes this study novel. To our knowledge, no other study has stimulated isolated osteoprogenitors to ensure osteoblastic lineage commitment. Therefore, the future aim of this study is to provide maximal bone filling defects by ensuring a maximal addition of preosteoblastic cells to the defect site via differentiation in vitro.

4.9 Summary and concluding remarks

The study presented herein represents the first attempt in stimulating human bone derived osteoprogenitors with doxycycline in vitro to determine the extent of differentiation towards the osteoblast phenotype.

In comparison with the more commonly studied and well characterized BMP-2, we found that doxycycline had similar stimulatory osteoinductive effects. Doxycycline at 10 μM and 50 μM was as efficient as BMP-2 in producing similar results in regards to nodule formation, increased ALP activity and increased OCN gene expression. The only difference was the toxicity associated with high levels of doxycycline (100 μM). This occurrence however was not problematic in that the desired effects were achieved with low dosage doxycycline. Therefore, even though BMP-2 is a successful stimulatory factor for osteoblast differentiation, doxycycline is equally as successful and should not be overlooked as an alternative.

Furthermore, two isolation techniques for harvesting osteoprogenitors from human cancellous bone were compared. It was found that enzyme digestion yielded a

maximum number of cells in a shorter time period, making it a superior method when compared to explant culturing methods.

In conclusion, when a viable source of osteoprogenitors is required, enzyme digestion of cancellous bone should be considered. Moreover, for the stimulation of these cells into mature osteoblasts with the ability to form bone, doxycycline should be considered as it has osteoinductive potential comparable to the commonly known BMP-2.

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