THE EFFECT OF BMP4 AND MECHANICAL STIMULATION ON MUSCLE-DERIVED STEM CELLS: IMPLICATIONS FOR BONE AND ARTICULAR CARTILAGE REGENERATION

by

Karin A. Corsi

BS, McGill University, 2000

MS, University of Montreal, 2002

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This dissertation was presented

by

Karin A. Corsi

It was defended on

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and approved by

Kacey G. Marra, PhD, Assistant Professor, Departments of Surgery and Bioengineering

Partha Roy, PhD, Assistant Professor, Department of Bioengineering

Bruno Péault, PhD, Professor, Department of Pediatrics

Dissertation Director: Johnny Huard, PhD, Professor, Departments of Orthopaedic Surgery, Bioengineering and Molecular Genetics and Biochemistry

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The prevalence of bone and articular cartilage injuries is expected to increase with the aging population. As a possible therapeutic option, stem cell-based therapies are being investigated. It has previously been reported that muscle-derived stem cells (MDSCs) isolated from mouse skeletal muscle can undergo osteogenic and chondrogenic differentiation in vitro and in vivo when stimulated with bone morphogenetic protein 4 (BMP4). One goal of this project was to determine whether cell sex influences this differentiation potential. Using in vitro osteogenic assays, it was determined that male MDSCs (M-MDSCs) stimulated with BMP4 expressed osteogenic markers and displayed more mineralization than female MDSCs (F-MDSCs). In vivo, M-MDSCs expressing BMP4 and implanted into the hind limb of mice induced a more robust ectopic bone formation when compared to F-MDSCs. These results suggest that cell sex influences the osteogenic differentiation potential of MDSCs. In the second study, the signaling pathways involved during BMP4 stimulation were investigated to further characterize the osteogenic differentiation process. The phosphatidyl inositol 3-kinase and p38 MAPK pathways played a positive role in MDSC osteogenesis, while the extracellular signal-regulated kinase pathway was identified as a negative regulator of osteogenesis. These results suggest that the osteogenic differentiation of MDSCs could be manipulated by regulating these pathways. In the third study, the effect of BMP4 and transforming growth factor-β1 (TGF-β1) on the

chondrogenic differentiation of F- and M-MDSCs in vitro was investigated. All MDSCs tested underwent chondrogenic differentiation, with no significant sex-related differences observed. However, addition of TGF-β1 synergistically enhanced BMP4-induced chondrogenic differentiation. In the final study, the effect of mechanical stimulation on the proliferation and osteogenic differentiation of MDSCs was investigated by using both biaxial and uniaxial strain. Mechanical stimulation affected cell orientation, but did not significantly affect the proliferation or osteogenic differentiation of MDSCs. In conclusion, the BMP4-induced osteogenic and chondrogenic differentiation of MDSCs can be influenced by several factors including cell sex and growth factors and can be guided through the manipulation of cell signaling pathways. The results from this project support the continued investigation of MDSCs as a potential cell source for bone and articular cartilage tissue engineering.

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NOMENCLATURE

ALP, alkaline phosphatase

ASCs, adipose-derived stem cells

BMPs, bone morphogenetic proteins

BSA, bovine serum albumin

CM, chondrogenic medium

Col I, collagen type I

Col II, collagen type II

Col X, collagen type X

3D, three-dimensional

DMEM, Dulbecco's Modified Eagle's Medium

DMSO, dimethyl sulfoxide

ECM, extracellular matrix

ELISA, enzyme-linked immunosorbent assay

ERK, extracellular signal-regulated kinases

FBS, fetal bovine serum

FISH, fluorescence in situ hybridization

HS, horse serum

IGF-1, insulin-like growth factor-1

JNK, c-jun N-teminal kinases

MDCs, muscle-derived cells

MDSCs, muscle-derived stem cells

MSCs, mesenchymal stem cells

Ocn, osteocalcin

p38 MAPK, p38 mitogen-activated protein kinase

PBS, phosphate-buffered saline

PI3K, phosphatidyl inositol 3-kinase

PM, proliferation medium

qPCR, quantitative real-time PCR

RT, room temperature

Sca, stem cell antigen

TGF-β, ṭransforming growth factor-β

VEGF, vascular endothelial growth factor

PREFACE

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1.0 INTRODUCTION

In the United States, 1 in 7 Americans has a musculoskeletal impairment (1). Injuries to the bones and joints are a common occurrence and are particularly prevalent in trauma and sports injuries. Musculoskeletal conditions can also include disorders such as arthritis and osteoporosis, which mainly affect older patients. With an aging population and a prolonged life expectancy, an increase in damage to bone and articular cartilage is anticipated. Although the field of orthopaedics has developed significantly in the last century, with the emergence of new products and surgical techniques, certain injuries are particularly resistant to existing therapy and present a constant challenge.

Currently, we find ourselves in 'The Bone and Joint Decade', a worldwide effort to increase awareness among scientists, physicians and patients of the problems associated with the repair of bone and articular cartilage and to promote advancement in this field (2). One such advancement is the emergence of regenerative medicine in orthopaedic surgery. It focuses on therapies that will replace, repair or promote the regeneration of diseased or damaged tissue. This is achieved by investigating areas such as stem cell therapy, the implications of different growth factors to promote regeneration and the involvement of scaffolds. These different areas will be described in the following sections, with a particular emphasis on skeletal muscle-based regenerative medicine.

1.1 BASIC BIOLOGY OF BONE AND ARTICULAR CARTILAGE AND CURRENT THERAPIES AVAILABLE FOR THEIR REPAIR

To develop efficient therapies for bone and articular cartilage repair, a thorough understanding of the biological processes involved in musculoskeletal tissue formation and regeneration is necessary. The following sections will overview the biology of bone and articular cartilage, as well as the current treatment options available for their repair.

1.1.1 Bone biology and repair

Bone self-renews throughout life by achieving a balance between resorption and deposition. This equilibrium is maintained via the actions of two cell populations: the osteoclasts, which degrade the matrix, and the osteoblasts, which deposit new bone (3). In the case of minimal bone loss, osteoblasts will produce new bone if a matrix or framework is available to bridge the gap and facilitate mineral deposition. This matrix is usually provided by the initial blood clot and connective tissue. However, a large defect may lack a continuous connection between the ends of the fractured bones, thus preventing healing from taking place (4). In addition, poor vascularity to the site of injury due to the anatomy or to extensive periosteal stripping may also impair the healing process. The treatment of these relatively common nonunion fractures, which are often seen in patients with scaphoid fractures, high-energy injuries, and osteoporosis, presents a challenging problem in orthopaedics.

The gold standard for promoting bone formation is a bone autograft obtained from the pelvic region, but this procedure may lead to significant morbidity at the donor site

(5-8) and has led to the use of alternative bone graft substitutes, such as allografts, demineralized bone matrix, and calcium-based ceramic materials (9-11). Allografts and demineralized bone matrix have demonstrated successful applications, although their osteoinductive potential must be carefully monitored as it can be compromised during the processing techniques. Calcium-based ceramics are known as efficient bone fillers and also provide a promising scaffold into which osteoinductive substances can be incorporated. However, more effective treatment alternatives are constantly being sought.

1.1.2 Articular cartilage biology and repair

Articular cartilage is a highly organized structure. This specialized connective tissue is composed of chondrocytes, which develop from mesenchymal chondroprogenitor cells found in the bone marrow. Most cartilage exists as a transient intermediate structure: the chondrocytes proliferate, become hypertrophic, and are replaced by osteoblasts during the process of endochondral bone formation (12). However, articular chondrocytes present at the epiphysis of long bones do not go through the endochondral process but rather make up a permanent cartilage structure (13). The chondrocytes secrete and surround themselves with a highly organized avascular extracellular matrix (ECM) composed primarily of collagen type II (Col II) and hydrophilic proteoglycans, such as aggrecan. This structure has a high water content, which allows it to withstand large compressive forces and creates an almost frictionless surface for articulation. However, this dense ECM essentially immobilizes the chondrocytes, making them unable to migrate or participate in the healing process. The lack of vascularity in articular cartilage also impedes the migration of cells and renders the cartilage unlikely to repair itself.

The difficulties associated with articular cartilage repair are particularly evident in lesions that do not penetrate the underlying subchondral bone. To treat these injuries, clinicians employ the microfracturing technique, which involves drilling into the vascularized subchondral bone to permit a source of progenitor cells from the bone marrow to migrate and fill the defect (14-16). Although this process can mobilize mesenchymal stem cells (MSCs) to differentiate into cartilaginous repair tissue, it can also cause the formation of fibrocartilage. Fibrocartilage, unlike the original hyaline cartilage, is composed primarily of collagen type I (Col I), and will eventually degrade due to its poor biomechanical characteristics.

Transplantation-related treatments are also practiced by clinicians and include the osteochondral autograft and the autologous chondrocyte transplantation (16-19). Osteochondral autograft, or mosaicplasty, is a technique wherein osteochondral plugs are harvested from non–weight-bearing portions of the articular surface and press-fit into the defect. Autologous chondrocyte transplantation requires chondrocytes to be taken from a non-load-bearing area, expanded in culture, and then reimplanted either directly or within a scaffold under a periosteal flap from the patient. This requires two operations, resulting in twice the risk of intraoperative complications. Good to excellent clinical results have been reported, although some evidence of fibrocartilage has been observed 2 years post-operatively (20). Other limitations also exist with this seemingly successful therapy. The isolation of the chondrocytes by arthroscopy remains an invasive procedure and, because it is difficult to expand chondrocytes from older patients, this therapy is mainly available to younger individuals (21). The maintenance of the chondrocyte phenotype during the monolayer culture presents another problem. The chondrocytes adopt a fibroblastic

phenotype and the expression of Col II and aggrecan, both chondrocyte-specific genes, decreases with culturing while Col I expression increases (22). Also, the development of hypertrophic tissue often necessitates a second surgery to remove the excessive tissue (23). For patients who fail all reconstructive and grafting options, total joint replacements with artificial prostheses are possible alternatives.

Despite the numerous options available for the treatment of bone and articular cartilage injuries, there still remains progress to be made to treat injuries that exhibit delayed or impaired healing and that require replacement tissue that is indistinguishable from its native counterpart. For this reason, current efforts are directed toward the novel use of tissue engineering and gene therapy to promote bone and articular cartilage healing.

1.2 TISSUE ENGINEERING AND GENE THERAPY FOR BONE AND ARTICULAR CARTILAGE REPAIR

The first definition of tissue engineering was given by Langer and Vacanti who stated it to be "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" (24). Tissue engineering of bone and articular cartilage requires several essential elements. First, an efficient cell source may be needed to aid in the formation of the required tissue. Second, growth and differentiation factors can be administered to guide the cellular components and improve the regeneration process. Third, an appropriate scaffold may be needed to provide support and enable cell

attachment and deposition of the newly formed tissue. Lastly, proper mechanical stimulation may be required to promote the regeneration process.

1.2.1 Stem cells in orthopaedic tissue engineering: Identification of muscle-derived stem cells

Selection of the appropriate cell source for cell therapy depends largely on the application in which it will be used and the function it is expected to perform. One option is to select a specialized or tissue-specific progenitor cell that can replenish the cellular component that was damaged through injury. For example, chondrocytes would seem to be an ideal cell source for articular cartilage repair seeing as they are directly involved in articular cartilage formation. The transplantation of chondrocytes obtained from a non-load-bearing area into an injured area has been in clinical practice since 1987 (17, 19). Limitations associated with this approach were described in the previous section and have led to the establishment of alternative cell sources such as uncommitted cells that have the ability to differentiate towards various lineages.

Numerous progenitor cell sources have been discovered and are currently being investigated for orthopaedic applications. Human embryonic stem cells have been shown to differentiate towards bone and cartilage lineages (25-27). However, ethical concerns surrounding their use have increased the need to look at reservoirs of stem cells from postnatal tissue. Postnatal stem cells have been isolated from several tissues and display varying capacities to undergo differentiation (28). Among them, adult bone marrow-derived MSCs have received particular attention (29-32). MSCs are capable of differentiating in vitro and in vivo along multiple lineages that include bone; cartilage;

cardiac and skeletal muscle; and neural, tendon, adipose, and connective tissues. In addition to their multipotency, MSCs are relatively easy to isolate from the bone marrow, although the procedure is associated with some risk and morbidity. Stem cells harvested from adipose tissue are another interesting and readily available source. They have been shown capable to differentiate into adipocytes, osteoblasts, myoblasts, and chondrocytes (33-38). There is also recent evidence suggesting that these cells may also be able to undergo neuronal differentiation (37, 39-41). Other postnatal stem cells include but are not limited to cells isolated from the circulation or blood vessels (42, 43), from human placenta (44, 45), human umbilical cord (46-48), human amniotic fluid (49) and muscle (50-52). The availability of skeletal muscle and the relative ease of muscle cell isolation make this tissue an attractive source of cells for use in bone and articular cartilage tissue engineering applications.

Skeletal muscle tissue is composed primarily of myofibers, though it also contains other cells such as satellite cells, endothelial cells, fibroblasts, adipocytes, blood cells, and nerve. Using a modified preplate technique, various populations of mononucleated cells from digested skeletal muscle can be obtained on the basis of their differing abilities to adhere to collagen-coated flasks (51). In brief, skeletal muscle is isolated from mice, enzymatically digested and the cell suspension is plated onto a collagen-coated flask. Two hours after plating, the adherent cells are predominantly fibroblasts. The supernatant from this flask is transferred to a new collagen-coated flask and the procedure is repeated every 24 hours until preplate 6. A detailed procedure and schematic can be found in Appendix A. A subpopulation from preplate 6, termed ‘muscle-derived stem cells’ (MDSCs), has been shown to be positive for the stem cell markers CD34, Bcl-2, and stem

cell antigen (Sca)-1 (51, 53). MDSCs isolated from murine skeletal muscle demonstrate a capacity for long-term proliferation, self-renewal, and immune-privileged behavior (51, 54). Furthermore, they can undergo multilineage differentiation toward skeletal muscle, bone, cartilage and neural, endothelial, and hematopoietic tissues (51, 53-59). They are found in murine skeletal muscle at a ratio of 1 in 100,000 cells (51), a ratio similar to that of adult MSCs isolated from bone marrow aspirates (30). Although this suggests that a large muscle biopsy will be required to isolate sufficient cells for therapeutic applications, this limitation is overcome by the fact that MDSCs are easily expanded in vitro without loss of progenitor characteristics (60). The use of such early progenitor cells derived from skeletal muscle could improve the outcome of cell-mediated therapies focused on bone and articular cartilage repair.

1.2.2 Inductive factors for orthopaedic applications: Importance of Bone Morphogenetic Proteins

Another important component of tissue engineering is the use of growth factors, which are integral to the regeneration process due to the role they play in stimulating proliferation, migration, matrix synthesis, and cell differentiation. These activities could improve the treatment of orthopaedic diseases or injuries, but the applicability of inductive factors is limited by their unstable nature. Growth factors often need to be administered at a high dosage or via repeated injections because they have a relatively short half-life *in vivo*. Given these limitations, more viable alternatives are the administration of growth factors through gene therapy or through biomaterials that control their release. Of particular interest for osteogenesis and chondrogenesis are

factors belonging to the transforming growth factor-β (TGF-β) superfamily, such as bone morphogenetic proteins (BMPs).

The first indication of the existence of factors that can induce bone formation came in 1965 from the seminal work of Dr. Marshall Urist, who found osteoinductive factors in demineralized bone (61). These isolated factors are now known as BMPs and there are more than 20 that have been found (62). They are synthesized as precursor molecules, where the mature region is released from the propeptide region by cleavage. Many studies investigating the osteogenic and chondrogenic potential of stem cells have used BMP-2 or -4 (63-70). These two factors are highly homologous, differing solely in their amino terminal region. Interestingly, BMP4 is a regulatory molecule involved in mesoderm induction, skeletal formation, bone induction and fracture repair, which makes it an ideal candidate for bone and articular cartilage repair therapies. Larger proteins from this same family include BMP-5, -6, -7, and -8 (71). BMP3, which is also known as osteogenin and may be a negative modulator of bone formation, is less related to the other BMPs (72).

BMPs also play an essential role in the initialization of chondrocyte differentiation by mesenchymal cells, as evidenced by the absence of chondrogenesis in mice lacking the BMP receptor IB (73). BMP7 is known to be a potent stimulator in human articular chondrocytes for the synthesis of proteoglycans and Col II (74, 75). These findings reinforce the necessity of incorporating BMPs in the development of approaches to improve cartilage repair.

1.2.3 Current progress with MDSCs and BMPs for orthopaedic applications

The combination of stem cells such as MDSCs and growth factors such as BMPs has previously been studied, especially for their potential application in tissue engineering. It has been widely used in studies involving MDSCs. In fact, MDSCs genetically engineered to express BMP-2 or -4 have led to bone formation and healed critical-sized calvarial and long bone defects (53, 58, 59, 76, 77). In vivo tracking of the cells has demonstrated that greater than 95% of the implanted MDSCs expressing BMP2 were found in the newly formed bone (53). Some also appeared to colocalize with osteocalcin, suggesting that the cells had become osteogenic cells. These studies indicate that MDSCs genetically engineered to express BMP excel in 2 crucial activities for bone formation. First, they can function as osteoprogenitor cells. Second, they can act as gene delivery vehicles by secreting the growth factors necessary to recruit surrounding cells that can participate in new bone formation.

It is hypothesized that the recruitment of progenitor cells to further promote bone regeneration can also be achieved by increasing the blood supply to the site of injury. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor. Its synergistic effect on the promotion of bone formation by BMP-expressing MDSCs has been previously investigated (57, 77). In this study, MDSCs transduced to express BMP4 and VEGF were implanted into the muscle pockets or calvarial defects in mice. It was observed that BMP4 and VEGF work synergistically together to produce a greater amount of bone formation than observed after implantation of only BMP4-expressing MDSCs. Interestingly, the dosage of BMP4- to VEGF-expressing cells played a large role in the amount and quality of bone formation. A ratio of one-fifth VEGF to BMP4

resulted in complete bone union, whereas the presence of 5 times more VEGF than BMP4 led to only small patches of mineralized bone. A more recent study investigated the effect of BMP2 and VEGF on the ability of MDSCs to form bone in vivo (77). Similar results to the previous study with BMP4 and VEGF were obtained. Namely, VEGF in combination with BMP2 accelerated and enhanced the bone formation elicited by BMP2-expressing cells alone. Increased bone formation was also evident at specific ratios of VEGF- to BMP2-producing MDSCs. Five times more VEGF- than BMP2-expressing cells caused a decrease in bone formation. Interestingly, when comparing the 2 studies, VEGF had a greater effect on BMP4-induced bone formation than on BMP2-induced bone formation (57, 77).

While bone formation remains a very desirable outcome of regenerative medicine for orthopaedic surgery, regulating the bone that is formed and preventing its overgrowth is also essential. As well, another clinical problem faced by orthopaedic surgeons is heterotopic ossification of muscles, tendons, and ligaments (78). In both these cases, it will be necessary to develop methods to regulate or inhibit bone formation. Noggin, a BMP antagonist, will be a likely candidate to address both these issues. The ability of Noggin to inhibit heterotopic bone formation was evaluated by transducing MDSCs to express Noggin and co-implanting them with MDSCs transduced to secrete BMP4 into the hind limbs of mice (79). It was found that Noggin inhibited the amount of bone formation in a dose-dependent manner, with a greater ratio of Noggin to BMP4 leading to less bone. In an attempt to regulate bone formation, MDSCs expressing inducible BMP4 were co-implanted into critical-sized calvarial defects with MDSCs expressing Noggin (80). At specific dosages, this combinatorial therapy led not only to regulation of bone

formation but the bone that was formed in the BMP4 with Noggin group was histologically similar to normal bone. Both BMP4 and Noggin have been shown to be present at the fracture repair site during healing (81), suggesting that mimicking the physiological environment is an important part of developing new approaches to enhance and regulate tissue repair, regeneration, or replacement.

Cells isolated from skeletal muscle also have been tested for the repair of cartilage defects. Adachi *et al.* isolated muscle-derived cells (MDCs) and chondrocytes from New Zealand white rabbits and transduced the cells with a retrovirus encoding for the LacZ gene (82). The transduced cells were embedded in Col I gels, which were then grafted to the experimental full thickness osteochondral defects. Histological and histochemical evaluation showed that gels containing chondrocytes and those containing MDCs produced comparable outcomes. Both elicited better repair than the collagen gel alone. Furthermore, the repaired tissues in the MDC group were composed mainly of Col II, indicating that MDC-based *ex vivo* gene therapy can be used to deliver appropriate therapeutic genes to articular cartilage defects.

Genetic engineering of MDSCs with BMP4 has been used for articular cartilage repair (56). It was shown that MDSCs expressing BMP4 acquired a chondrocytic phenotype in vitro, in both monolayer and micromass pellet culture, whereas control MDSCs did not undergo chondrogenic differentiation. After implantation of BMP4-expressing MDSCs into full-thickness articular cartilage defects, donor cells could be seen within the defect and colocalized with Col II, a marker of chondrogenic differentiation. In addition, the repaired tissue in the defect was well integrated with the adjacent normal articular cartilage for up to 24 weeks. The group that received control

MDSCs did not display such a high degree of regeneration, suggesting that BMP4 is beneficial for cartilage repair. Thus, cell engineering of MDSCs to secrete BMP4 and other agents appears to be important for optimal regeneration of injured or diseased bone and articular cartilage tissue.

1.3 PROJECT OBJECTIVES

The overall goal of this project is to study the osteogenic and chondrogenic differentiation of female and male mouse MDSCs (F-MDSCs and M-MDSCs, respectively) under the influence of BMP4 and to further optimize their therapeutic potential by employing mechanical stimulation.

1.3.1 Objective #1: To evaluate the osteogenic potential of male and female mouse MDSCs when treated with BMP4, as well as the signaling pathways activated by BMP4.

Hypothesis: Both male and female populations of MDSCs will undergo osteogenic differentiation with BMP4 stimulation, but their osteogenic potential will be influenced by donor sex. As well, Smad-dependent and Smad-independent pathways will be involved in the BMP4-induced osteogenic differentiation of MDSCs.

Although the characterization of MDSCs indicates that they can participate in bone healing and that once genetically engineered, they are efficient in delivering osteogenic

factors such as BMPs to the sites of repair, many of these studies were performed with MDSCs isolated from female mice (53, 57-59, 76). A comparison of F- and M-MDSCs in terms of their osteogenic differentiation has never been reported. However, determining whether cell sex can influence the osteogenic differentiation of F- and M-MDSCs will be important in developing cellular therapies for bone healing. In the first part of this objective, we will treat F- and M-MDSCs with BMP4 and evaluate their osteogenic differentiation by observing the expression of osteogenic markers and their ability to undergo mineralization. Their ability to form bone in vivo will also be evaluated by implanting MDSCs transduced to express BMP4 into the intramuscular pocket of sex-matched and sex-mismatched normal mice.

Commitment to the osteogenic lineage by BMP4 treatment involves both Smad and non-Smad molecular signaling pathways, though the roles of specific pathways in MDSC differentiation remain unknown. The phosphatidyl inositol 3-kinase (PI3K) pathway, the p38 mitogen-activated protein kinase (p38 MAPK) pathway and the extracellular signal-regulated kinases (ERK) pathway, are 3 pathways previously implicated in osteogenesis (65, 83-88). Understanding the cell signaling pathways that are activated during BMP4 treatment and how they affect the osteogenic differentiation of MDSCs will allow for increased regulation of differentiation and enhanced control of cell growth in clinical applications. Hence, in the second part of this objective, MDSCs will be stimulated with BMP4 and the activation of the Smad, PI3K, ERK, and p38 MAPK pathways will be evaluated. As well, specific inhibitors to the different cell signaling pathways will be used to block the pathways and investigate the effect on

osteogenic gene and protein expression. This will help in defining the specific roles of the different pathways in the osteogenic differentiation of MDSCs.

1.3.2 Objective #2: To test the ability of F- and M-MDSCs to undergo chondrogenic differentiation when treated with BMP4 in the presence or absence of TGF-β1.

Hypothesis: The chondrogenic differentiation of MDSCs will display sex-related differences.

The chondrogenic differentiation of MDSCs in vitro has not been extensively studied, nor has the effect of cell sex been investigated. Therefore, in this objective F- and M-MDSCs will be cultured in chondroinductive medium and stimulated with BMP4 only or with BMP4 and TGF-ß1. To further investigate whether the addition of TGF-ß1 to BMP4 affects the rate of chondrogenic differentiation, MDSCs will be genetically engineered to express BMP4 and then cultured in chondroinductive conditions in the presence or absence of TGF-ß1. Chondrogenic differentiation will be assessed by Alcian Blue and Safranin O staining and by the expression of chondrogenic genes.

1.3.3 Objective #3: To investigate the effect of cyclic mechanical strain on the proliferation and osteogenic differentiation of MDSCs in vitro.

Hypothesis: Mechanical stimulation will promote the proliferation and osteogenic differentiation of MDSCs.

Mechanical stimulation has been shown to affect cellular proliferation in various cell types (89-97). In addition to proliferation and survival, mechanical stimulation can also affect cellular differentiation (92-94, 98-101). For example, the mechanical stimulation of human osteoblasts has led to a significant increase in their proliferation, but has decreased their expression of osteogenic markers (92). In another study, mechanical stimulation was able to maintain human embryonic stem cells in an undifferentiated state and did not affect their pluripotency (96). These studies suggest that mechanical strain may be used to enhance proliferation, while delaying the differentiation of the cells. However, other groups have reported a decrease in proliferation due to mechanical stimulation (89, 94). As well, in a study with human bone marrow stromal cells, uniaxial cyclic mechanical strain was shown to increase their expression of osteogenic markers when compared to unstretched controls (100). The variability seen in the results presented by different groups lie either with the cell used, the differentiation stage of the cell and more importantly with the mechanical strain regimen used. The effect of mechanical stimulation on MDSCs has never been investigated. In this objective, MDSCs will be subjected to either equibiaxial or uniaxial mechanical stimulation and the effect it has on their proliferation will be evaluated. In the second part of this objective, MDSCs will be treated with BMP4, stimulated with equibiaxial or uniaxial mechanical strain, and the osteogenic differentiation of MDSCs will be assessed.

2.0 THE OSTEOGENIC POTENTIAL OF POSTNATAL SKELETAL MDSCS IS INFLUENCED BY DONOR SEX

2.1 INTRODUCTION

MDSCs stimulated or genetically engineered to express BMP2 or BMP4 have undergone osteogenic differentiation in vitro, have led to ectopic bone formation in vivo in the muscle pocket, and healed bone defects of the skull and long bone (53, 57-59, 76, 77). Evaluating the differentiation potential of MDSCs and other stem/progenitor cells is necessary to define their clinical usefulness and to further increase our knowledge of stem cell biology. Yet, one important aspect that is poorly investigated is the effect of donor sex on the capacity of stem cells to differentiate into multiple lineages.

Sexual dimorphism has been studied in great detail in various mammalian systems. The greater bone size and bone mineral density in men than women is physically evident (102, 103). Prevalence of musculoskeletal problems is also influenced by sex, as osteoarthritis, osteoporosis, spinal disorders, and fractures all have a higher prevalence in females (104). At the cellular level, female and male chondrocytes have shown a differential response to 17ß-estradiol and testosterone (105, 106), and sex differences in response to progesterone have also been reported in cells derived from rat

lumbar vertebrae (107). At the mammalian gene expression level, hundreds to thousands of genes that differ between males and females have been identified (108, 109).

The lack of published reports on sex-related differences at the stem cell level are likely a result of many studies utilizing male donor cells to follow their fate in female hosts by fluorescence in situ hybridization (FISH)-analysis. Although therapies involving adult-derived stem cells will likely involve autologous transplantation, it will be beneficial to know whether a difference exists between the sexes. This difference may then be further investigated, and may provide a means to enhance the therapeutic potential of the stem cells.

Recently, it has been reported that MDSCs display sex-related differences in their ability to regenerate skeletal muscle (110). This study showed that F-MDSCs transplanted into dystrophic skeletal muscle led to a higher muscle regeneration and dystrophin delivery than M-MDSCs transplanted into skeletal muscle. Hence, the objective of this study was to determine whether the osteogenic differentiation of MDSCs is also subject to sexual dimorphism. To do so, F- and M-MDSCs were stimulated with BMP4 in vitro, and their osteogenic differentiation was assessed by expression of osteogenic proteins and genes as well as their ability to undergo calcification in a pellet culture assay. Also, single-cell derived colonies were obtained from both F- and M-MDSCs, stimulated with BMP4 and stained for alkaline phosphatase (ALP), an early osteogenic marker. This was done to determine whether more osteoprogenitor cells were present in the F- or M-MDSC populations tested. The F- and M-MDSCs were also transduced with a retrovirus to express BMP4 (MDSC-BMP4 cells), and used in the pellet culture and single-cell derived colony assays. Furthermore, F- and M-MDSC-

BMP4 cells were implanted in vivo to investigate whether sex-related differences existed in their potential to form ectopic bone. This study led to a series of interesting results regarding the effect of cell sex on a cell's osteogenic potential, and may have important implications in the development of cellular therapies for bone healing.

2.2 METHODS

2.2.1 Isolation and culture of MDSCs

MDSCs were isolated from 3-week-old C57BL/10J mice using a modified preplate technique (51) A detailed description of this method can be found in Appendix A. A total of 6 F-MDSC populations and 6 M-MDSC populations were used in this study. The MDSCs were isolated from different litters and on different days. Animals were anatomically sexed at the time of isolation and the sex of the cells was later confirmed by FISH analysis. Cells were cultured on collagen coated flasks in proliferation medium (PM) containing phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 110 mg/L sodium pyruvate (Sigma-Aldrich), 584 mg/L L-Glutamine, 10% fetal bovine serum (FBS), 10% horse serum (HS), 1% penicillin/streptomycin (all from Invitrogen), and 0.5% chick embryo extract (Accurate Chemical Co.). Cells were trypsinized and replated at a density of 250 cells/cm^2 until ready to be assayed.

2.2.2 BMP4 stimulation of MDSCs

MDSCs were plated at a density of 1500 cells/cm^2 and were stimulated on the following day with PM supplemented with BMP4 (0-200 ng/ml). The MDSCs obtained fresh media changes every 48 hours. After 3 days of stimulation, the presence of ALP was evaluated by cytochemical staining (AP Kit 86-C, Sigma-Aldrich). To determine ALP enzymatic activity, cells were lysed with 0.1% Triton X in water at 2, 3, and 4 days following the initiation of BMP4 stimulation (50 ng/ml), and assayed using SIGMA FAST^{TM} p-nitrophenyl phosphate tablets (N-2770; Sigma-Aldrich). The relevant protocols recommended by the manufacturer were followed for both ALP assays. Protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce Chemical) with bovine serum albumin (BSA) as the standard. The enzyme activity is expressed as nanomoles of p-nitrophenyl phosphate per milligram of protein. Digital images of the ALP stain were taken with a Leica DMIRB microscope equipped with a Retiga 1300 digital camera (Q Imaging), using QCapture 2.68.2 software (Quantitative Imaging Corporation). Following this experiment, we selected 3 F- and 3 M-MDSC populations based on their levels of ALP activity at 2 days following the initiation of BMP4 stimulation. We chose a population from the lower, middle, and higher range of ALP activities within each sex, and used them for the remainder of the study.

2.2.3 Quantitative Real-Time PCR (qPCR) analysis of osteogenic markers

The F- and M-MDSCs (n=3/sex) were stimulated with BMP4 (50 ng/ml) for 12 hours before extraction of total RNA using the RNeasy kit (Qiagen). We performed qPCR

analysis with Taqman® One-step RT-PCR Master Mix (Applied Biosystems) as described previously (111). RNA samples (1 μL) were added to sequence-specific primers and Taqman® probes (200 nM per 10-μL reaction). All target genes were normalized to *18S* (primers from Applied Biosystems). The sequences of the target gene primers and probes can be found in Appendix B. All probes were labeled with FAM as the 5' reporter dye and TAMRA as the 3' quencher dye. The qPCR assays were carried out in triplicate on an ABI Prism 7900HT sequence detection system in the Genomics and Proteomics Core Laboratories of the University of Pittsburgh. Data was analyzed using SDS 2.1 Software from Applied Biosystems.

2.2.4 Retroviral transduction

A retroviral vector expressing human BMP4 and GFP (76) was used to transduce MDSCs overnight at a dilution of 1:1 with PM and in the presence of 8 μg/ml polybrene (MDSC-BMP4 cells). Cells were grown until the required number was reached for in vitro and in vivo experiments. A previously described BMP4 bioassay (112) and a human BMP4 enzyme-linked immunosorbent assay (ELISA) (R&D Systems) were used to determine the level of BMP4 being secreted by the transduced cells.

2.2.5 Cell proliferation

MDSC-BMP4 cells were plated in 96-well plates at a density of 1500 cells/cm^2 in PM with media changes every 2 days. After 4 days in culture, cellular proliferation was

evaluated using the CellTiter 96 AQ_{ueous} One Reagent (Promega) according to the manufacturer's instructions.

2.2.6 Osteogenic pellet culture

The F- and M-MDSCs were used in a pellet culture assay to test their ability to undergo osteogenic differentiation. Briefly, 2.5 x 10^5 cells were centrifuged at 500*g* for 5 minutes in 15 ml polypropylene conical tubes, then resuspended in 500 µl of osteogenic medium (phenol-red free DMEM supplemented with 110 mg/L sodium pyruvate, 584 mg/L L-glutamine, 10% FBS, 1% penicillin/streptomycin, 10^{-7} M dexamethasone, $5x10^{-5}$ M ascorbic-acid-2-phosphate and 10^{-2} M ß-glycerophosphate), supplemented with BMP4 (50 ng/ml), and centrifuged again. Medium was replaced every 3 days, and pellets were cultured for 14 days. MDSC-BMP4 cells were also made into pellets as described above, but they were cultured in osteogenic medium that was not supplemented with BMP4 for 7 and 14 days. At the designated endpoints, the pellets were fixed in formalin overnight, and paraffin embedded. To visualize mineralized matrix deposition and bone volume analysis, the paraffin embedded pellets were scanned using a µCT imaging system (vivaCT 40, Scanco Medical, Bassersdorf, Switzerland) with the following settings: 55 kVp of energy, 200 ms integration time, and an isotropic voxel size of 10.5 µm. Two-dimensional image slices were obtained and contour lines were drawn to define the volume of interest. An appropriate threshold was chosen for the bone voxels by visually matching thresholded areas to grayscale images. The threshold was kept constant throughout the analyses of each pellet. This led to a three-dimensional (3D) reconstruction of the bone within the pellets and provided quantitative data on bone

volume (mm^3) and density (mg HA/cm^3). The pellets were then sectioned to 5 μm sections and stained for von Kossa to detect mineralization. Images were captured with QCapture software using a Nikon Eclipse E800 microscope equipped with a Retiga EXi digital camera.

2.2.7 Single-cell sorting

The MDSCs and MDSC-BMP4 cells were cultured in PM, released from the collagen-coated flasks with 0.05% trypsin, and the cells were single-cell sorted into 96-well plates containing PM with a FACSAria cytometer (BD Biosciences Immunocytometry Systems). After 5 days in PM, and once colonies had reached 20-30 cells, medium was changed. The MDSCs received PM supplemented with BMP4 (50 ng/ml) and the MDSC-BMP4 cells received PM without the addition of any BMP4. After 3 days, all cells were fixed and stained for ALP. Colonies were scored as either "Low ALP+" if 50% or less of the cells making up the colony were ALP positive or "High ALP+" if 51% or more of the cells making up the colony were ALP positive.

2.2.8 In vivo bone formation

All animal experiments were conducted with the approval of the Animal Research and Care Committee of the Children's Hospital of Pittsburgh (Protocol 28-08). The F- and M-MDSC-BMP4 cells were trypsinized, counted, and a 20 μl suspension of 2.5 x 10^5 cells was seeded onto a 5 x 5-mm piece of sterile gelatin sponge (Gelfoam; Pharmacia & Upjohn). After the Gelfoam absorbed the cell suspension, PM was added to each well,

and the scaffolds were left in the incubator overnight. In our first experiment, 3 F-MDSC-BMP4 and 3 M-MDSC-BMP4 cells were implanted into the intramuscular pockets of sex-matched C57BL/10J mice. In our second experiment, the F- and M-MDSC-BMP4 populations that led to the greatest amount of bone formation in the previous experiment (F2-MDSC-BMP4 and M1-MDSC-BMP4, respectively) were subsequently implanted into sex-matched and sex-mismatched C57BL/10J mice. The mice received a MDSC-seeded scaffold into each hind limb, and the results from the 2 hind limbs were averaged for each mouse.

2.2.9 Radiographic analysis

Ectopic bone formation was monitored by X-ray examination of the mice on days 7, 14, and 21 (MX-20; Faxitron X-ray Corp). X-rays were scanned, and the bone area and density were calculated using Northern Eclipse imaging software (Empix Imaging).

2.2.10 Statistical analysis

All in vitro experiments were done in triplicate and repeated three times. Data are reported as mean ± SEM, and analyzed using either the Student *t*-test, One-way or Two-way ANOVA. Pairwise post-hoc analyses were performed using the Tukey test (SigmaStat, Jandel Corporation). *P* values of less than 0.05 were considered significant.

2.3 RESULTS

2.3.1 BMP4 stimulation of F- and M-MDSCs leads to the expression of osteogenic markers

Using a modified preplate technique (51), 6 male and 6 female populations of MDSCs were isolated from the skeletal muscle of 3-week-old C57BL/10J mice. To test the osteogenic potential of the cells, all 12 populations were stimulated with various concentrations of BMP4 (0-200 ng/ml) for 3 days, and then stained for the early osteogenic marker, ALP. We found that all 12 populations were able to respond to BMP4 stimulation, with the M-MDSCs displaying both a greater and a more intense staining for ALP than the F-MDSCs at all concentrations of BMP4 tested (Figure 2-1).

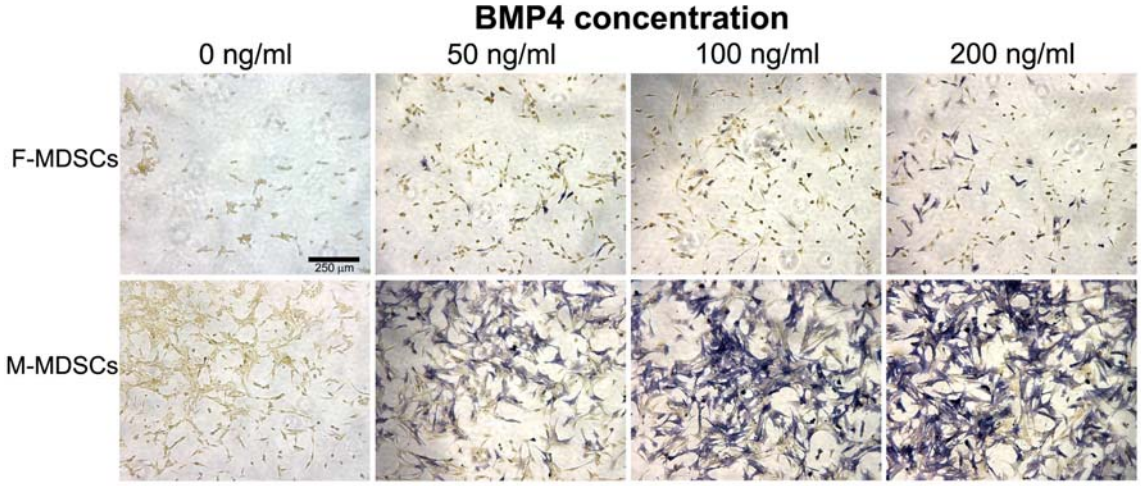

Figure 2-1. ALP staining of F- and M-MDSCs.
F- and M-MDSCs were stimulated with BMP4 (0-200 ng/ml) for 3 days and stained for ALP. Shown are representative images from 1 F-MDSC and 1 M-MDSC population. At all BMP4 concentrations tested, the M-MDSCs displayed a greater ALP staining than the F-MDSCs.

To account for any differences in the proliferation of F- and M-MDSCs, ALP activity was quantified and normalized to total protein content. In this study, cells were treated with a fixed amount of BMP4 (50 ng/mL), and ALP activity was quantified at 2, 3 and 4 days of stimulation. When all 12 populations were tested, we noted that M-MDSCs displayed a higher mean ALP activity than F-MDSCs at 2 days (Figure 2-2A) and 3 days (Figure 2-2B) after initiation of BMP4 stimulation. Though the M-MDSCs continued to have higher levels of ALP activity compared to the F-MDSCs at 4 days of BMP4 stimulation, the difference was no longer significant (Figure 2-2C). Also of note was our discovery that the M-MDSCs stimulated with BMP4 displayed a significant increase in their ALP activity when compared to their untreated control at all days tested, but this was only evident at 3 days in the F-MDSCs (Figure 2-2A-C, * $P < 0.05$)).

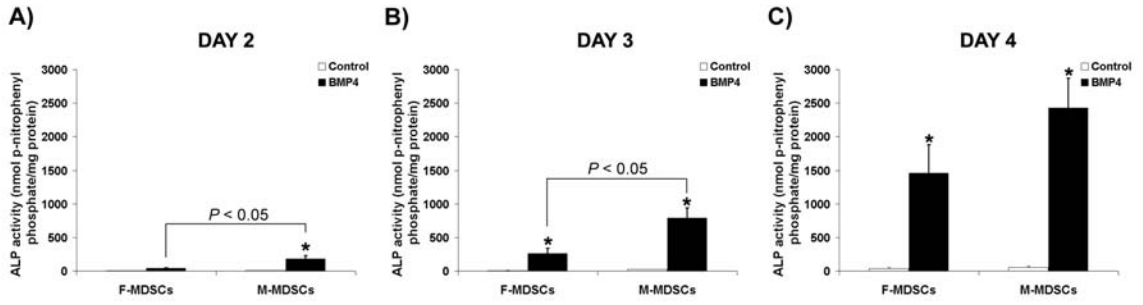

Figure 2-2. ALP activity of F- and M-MDSCs after 2, 3 and 4 days of stimulation with BMP4 (50 ng/ml).
(A) ALP activity of F- and M-MDSCs stimulated with BMP4 (50 ng/ml) for 2 days, (B) 3 days, and (C) 4 days. Bars represent the mean ± SEM; n=6 MDSC populations/sex. * indicates a significant difference from the untreated control of the same sex ($P < 0.05$). Statistical differences between F- and M- MDSCs are depicted on the graph ($P < 0.05$).

To determine whether BMP4 stimulation also upregulates *Alp* gene expression, qPCR was performed on 3 F- and 3 M-MDSC populations after 12 hours of BMP4 stimulation. In both sexes, *Alp* expression was significantly increased in comparison to the untreated control (Figure 2-3A, * $P < 0.05$), and, while there was no significant

difference between the results obtained from the F- and M-MDSCs post-BMP4 stimulation, there was a persistent trend toward higher *Alp* expression in the M-MDSCs compared to their female counterparts. Similar results were observed for *Runx2* gene expression, a transcription factor that has been shown to be essential for osteoblast differentiation (113, 114) (Figure 2-3B). Both F- and M-MDSCs exhibited an increase in *Runx2* gene expression over the untreated control (* $P < 0.05$).

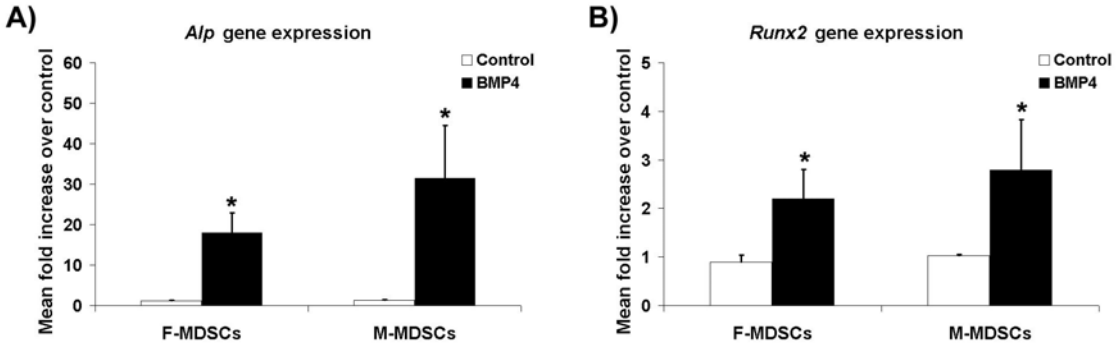

Figure 2-3. qPCR analysis of osteogenic genes in F- and M-MDSCs.
(A) *Alp* gene expression and **(B)** *Runx2* gene expression of F-MDSCs and M-MDSCs stimulated with BMP4 (50 ng/ml) for 12 hours is shown. Bars equal mean ± SEM; n=3 MDSC populations/sex.
* indicates a significant difference from the untreated control of the same sex ($P < 0.05$).

2.3.2 Osteogenesis in MDSC pellet cultures

The ability of MDSCs to form a mineralized matrix in vitro was assayed using a micromass culture system. After 14 days of culture, both F- and M-MDSCs demonstrated areas of mineralization within the pellets, although the extent of mineralization varied among the different cell populations. We determined that 2 out of the 3 F-MDSC populations and 2 out of the 3 M-MDSC populations tested had sparse areas of mineralization after 14 days of culture. The F-MDSC and M-MDSC populations with the greatest degree of mineralization (F3- and M3-MDSCs, respectively) are shown in Figure

2-4A and B. The 3D reconstructions demonstrate a larger bone volume in the M3-MDSC population than in the F3-MDSC population. In addition, quantification of bone volume (Figure 2-4C) and bone density (Figure 2-4D) for these two populations suggests that the M3-MDSCs underwent a greater degree of mineralization when compared to the F3-MDSCs. This was further confirmed upon staining sections throughout the pellets for von Kossa, an indicator of mineralized tissue (Figure 2-4E and F). The F3-MDSC population showed small areas of mineralization (Figure 2-4E), while the M3-MDSC population showed a larger and denser area of mineralization (Figure 2-4F).

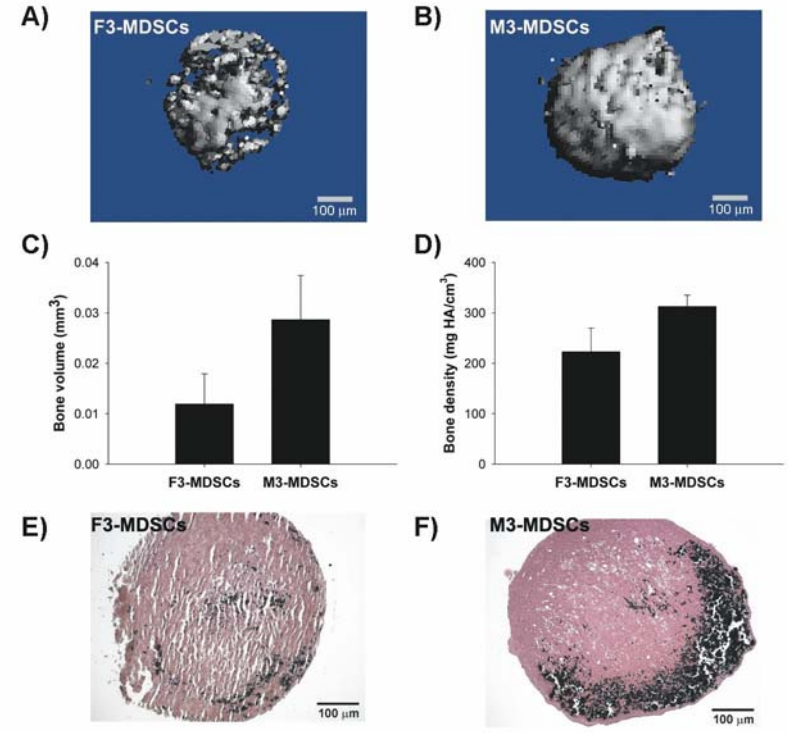

Figure 2-4. Osteogenic differentiation of MDSCs in pellet culture.
M-MDSCs formed more mineralized tissue than F-MDSCs when cultured as pellets in osteogenic medium supplemented with BMP4 for 14 days. Shown are µCT images of the (A) F-MDSC and (B) M-MDSC population displaying the greatest amount of mineralized tissue (F3-MDSCs and M3-MDSCs, respectively). (C) Bone volume and (D) bone density were greater in the M3-MDSCs than the F3-MDSCs. (E) von Kossa staining of F3-MDSC pellets and (F) M3-MDSC pellets showed a greater area of positive staining in the pellets made with M3-MDSCs.

Pellet culture assays with MDSCs transduced to express BMP4 (MDSC-BMP4 cells) also led to mineralized tissue in vitro. A detectable amount of calcification was observed in all populations tested, regardless of sex, and as early as 7 days after culture in osteogenic conditions. The F- and M-MDSC-BMP4 populations with the greatest degree of mineralization are shown in Figure 2-5A and B, respectively. A greater amount of bone was visible in the 3D reconstruction of the M-MDSC-BMP4 pellet (Figure 2-5B) than in the F-MDSC-BMP4 pellet (Figure 2-5A). The mean bone volume of all 3 MDSC-BMP4 populations tested at 7 and 14 days is shown in Figure 2-5C. Overall, a greater bone volume was seen in the M-MDSC-BMP4 cells than in the F-MDSC-BMP4 cells at both 7 and 14 days. Interestingly, this difference was less evident by 14 days, and may be explained by the fact that the F-MDSC-BMP4 cells underwent a mean fold increase of 4.74 ± 1.31 between 7 and 14 days, while the M-MDSC-BMP4 cells displayed a mean fold increase of 2.76 ± 0.78 in the same time frame. When comparing bone density between F- and M-MDSC-BMP4 cells, a higher bone density was seen in the M-MDSC-BMP4 cells than in the F-MDSC-BMP4 cells at both 7 and 14 days (Figure 2-5D). However, there was not a large change in bone density from 7 to 14 days in either the F- or M-MDSC-BMP4 cells (1.15 ± 0.06 and 1.33 ± 0.10, respectively). Histological analysis by von Kossa staining further confirmed the data obtained with the μCT. At 14 days, sections from the F- and M-MDSC-BMP4 pellets that showed the greatest area of von Kossa staining looked similar in terms of positive area, but the M-MDSC-BMP4 pellet displayed a more intense stain, suggesting a denser, more mineralized tissue (Figure 2-5E and F).

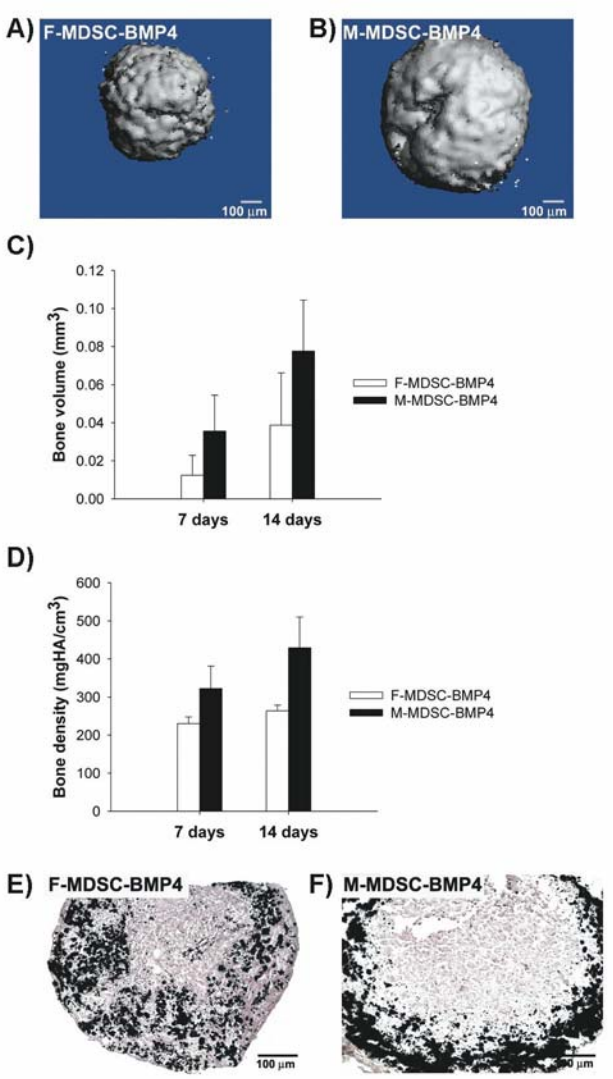

Figure 2-5. Osteogenic differentiation of MDSC-BMP4 cells in pellet culture.
M-MDSC-BMP4 cells formed more mineralized tissue than F-MDSC-BMP4 cells when cultured as pellets in osteogenic medium for 7 and 14 days. Shown are µCT images of the (A) F-MDSC-BMP4 cells and (B) M-MDSC-BMP4 cells displaying the greatest amount of mineralized tissue at 14 days. (C) Bone volume and (D) bone density were quantified at 7 and 14 days. Bars represent the mean ± SEM; n=3 MDSC-BMP4 populations/sex. (E) von Kossa staining of F-MDSC-BMP4 pellets and (F) M-MDSC-BMP4 pellets at 14 days showed a denser area of positive staining in the pellets made with M-MDSC-BMP4 cells.

2.3.3 M-MDSCs contain more BMP4 responsive cells than F-MDSCs

To determine whether the sex-related differences observed thus far are due to the presence of more osteoprogenitor cells within M-MDSC populations than F-MDSC populations, F- and M-MDSCs were single-cell sorted into 96-well plates, and colonies

were allowed to grow for 5 days in PM. The colonies that formed were tested for BMP4 responsiveness by stimulating them for 3 days with PM supplemented with BMP4 (50 ng/ml), and then staining them for ALP (Figure 2-6A). MDSC-BMP4 cells were also single-cell sorted and allowed to form colonies in PM for 8 days before being stained for ALP (Figure 2-6A).

The majority of the colonies formed in this assay were comprised of a mixture of ALP negative and positive cells (Figure 2-6B). To account for this, the colonies were either scored as "Low ALP+" (less than or equal to 50% of the cells making up the colony stained positive for ALP), or "High ALP+" (greater than 51% of the cells in the colony stained positive for ALP) (Figure 2-6B). Interestingly, a greater number of total colonies were obtained with the M-MDSC populations than with the F-MDSC populations (522 colonies versus 262 colonies, respectively). Within the F-MDSCs, there were significantly more Low ALP+ than High ALP+ colonies (Figure 2-6C, * $P < 0.05$), while M-MDSCs demonstrated an approximately equal percentage of Low ALP+ and High ALP+ colonies (Figure 2-6C). M-MDSCs also showed a trend toward a lower percentage of total colonies that were scored as Low ALP+ and a higher percentage of total colonies that were scored as High ALP+, when compared to F-MDSCs (Figure 2-6C).

Similar results to those previously presented with parental populations (Figure 2-6C) were obtained when the colonies were derived from MDSC-BMP4 cells (Figure 2-6D). As seen earlier, the colonies were comprised of a mixture of ALP positive and negative cells. F-MDSC-BMP4 cells had significantly more colonies that were scored as Low ALP+ than colonies that were scored as High ALP+ (Figure 2-6D, * $P < 0.05$). As

with the stimulated MDSCs, the M-MDSC-BMP4 cells had fewer colonies that were scored as Low ALP+ than their female counterparts, and more that were scored as High ALP+ (Figure 2-6D, # $P < 0.05$). A total of 256 colonies were obtained from F-MDSC-BMP4 cells, while 133 colonies were obtained from M-MDSC-BMP4 cells. This was due to one of the female populations (F2-MDSC-BMP4) having led to a total of 110 colonies.

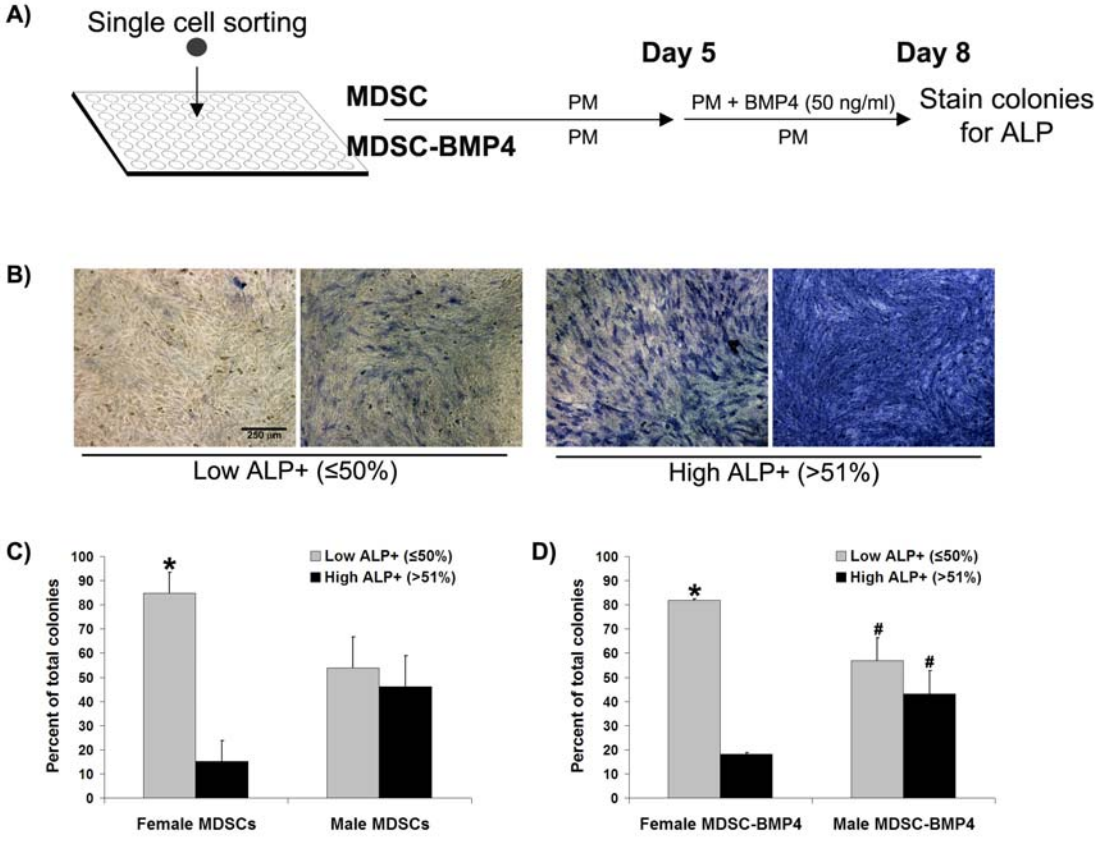

Figure 2-6. M-MDSCs contain more osteogenic cells than F-MDSCs.
(A) F- and M-MDSCs and F- and M-MDSC-BMP4 cells were single-cell sorted into 96 well plates, and, after 5 days in PM, were either stimulated with PM supplemented with BMP4 for 3 days (MDSCs), or given fresh PM (MDSC-BMP4). At Day 8, all colonies were stained for ALP. (B) Representative images of the different colonies obtained are shown. Colonies were scored as "Low ALP+" if they contained less than or equal to 50% ALP positive cells, or they were scored as "High ALP+" if they contained greater than 51% ALP+ cells. (C) The percentage of total colonies that were scored as either Low ALP+ or High ALP+ in the F- and M-MDSCs and (D) in the F-MDSC-BMP4 and M-MDSC-BMP4 are shown. Bars represent the mean ± SEM; n=3 MDSC populations/sex, where * indicates a significant difference between Low ALP+ and High ALP+ ($P < 0.05$), and where # indicates a significant difference between M- and F-MDSC-BMP4 within Low ALP+ or High ALP+ ($P < 0.05$).

2.3.4 Analysis of ectopic bone formation by F- and M-MDSC-BMP4 cells

To evaluate the osteogenic potential of MDSCs in vivo, 2.5 x 10^5 F- and M-MDSC-BMP4 cells were seeded onto a gelatin sponge, which was then implanted into the intramuscular pocket of sex-matched mice. No bone formation was evident after 7 days of implantation (data not shown). After 14 days, radiographs showed that the F-MDSC-BMP4 populations tested demonstrated more variability in the degree of bone formation than the M-MDSC-BMP4 populations tested (Figure 2-7A). This was also evident by the quantification of bone area (Figure 2-7B) and density (Figure 2-7C). While F2-MDSC-BMP4 cells led to a bone area which was significantly different than that seen with F3-MDSC-BMP4 cells, and a bone density which was significantly greater than the other two female populations tested, all M-MDSC-BMP4 populations led to bone that displayed a similar area and density (Figure 2-7B and C). Due to the variable results obtained with the female cells, the average bone area of the F-MDSC-BMP4 populations was similar to that of the M-MDSC-BMP4 populations (8.81 x 10^4 ± 2.34 x 10^4 pixels versus 9.52 x 10^4 ± 1.06 x 10^4 pixels, respectively). The overall bone density was greater in M-MDSC-BMP4 cells than F-MDSC-BMP4 cells (69.22 ± 1.72 versus 65.69 ± 6.56, respectively), but not statistically significant. Similar results were obtained at 21 days (data not shown).

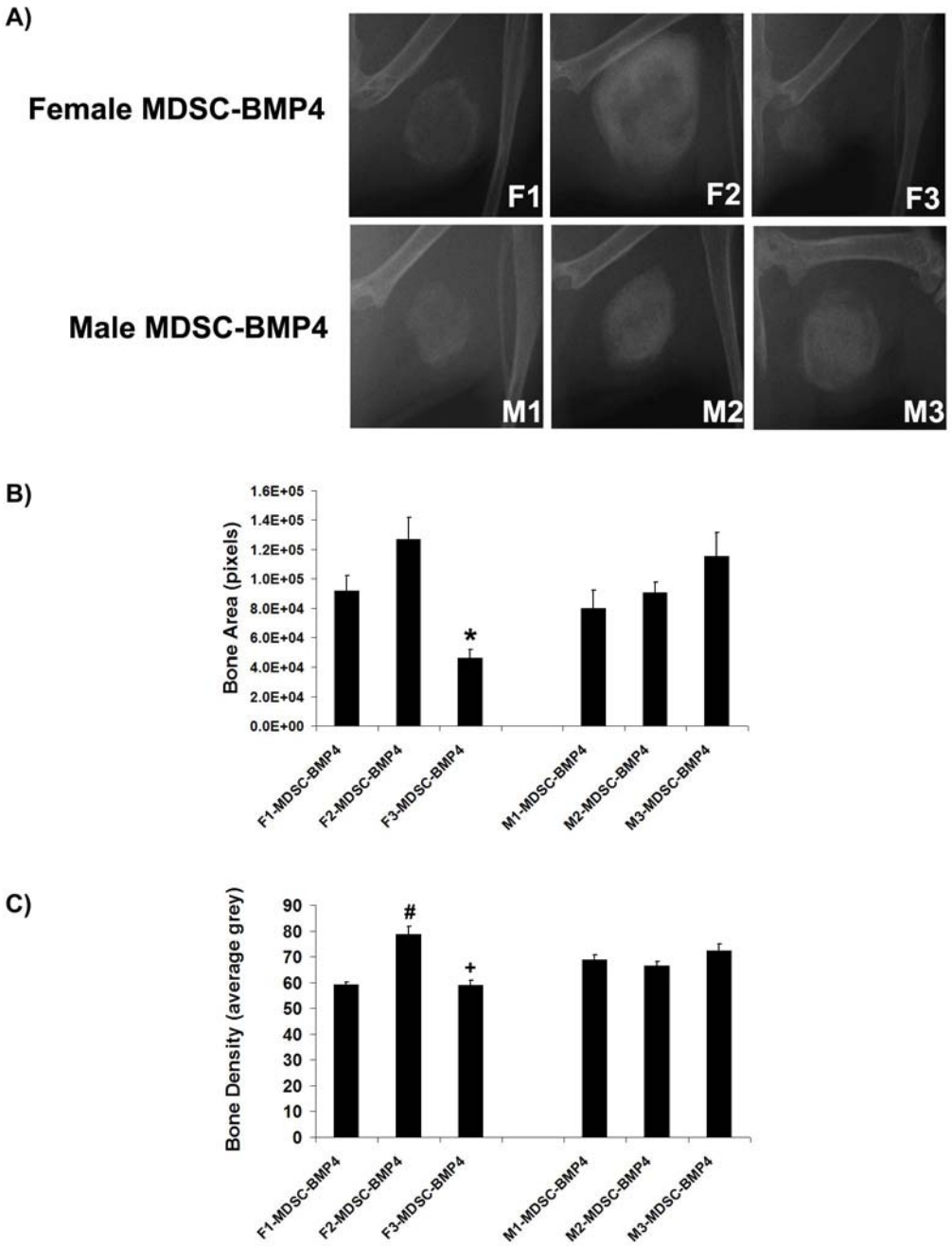

Figure 2-7. M-MDSC-BMP4 cells lead to a more consistent and denser ectopic bone formation than F-MDSC-BMP4 cells.

(A) Radiographs at 14 days post-implantation of 3 F- and 3 M-MDSC-BMP4 populations that were implanted into the intramuscular pocket of sex-matched mice. (B) The bone area for all six populations was quantified from the radiographs. Bars represent the mean ± SEM (n= 2-5/group), where * indicates a significant difference between F3-MDSC-BMP4 cells and F2-MDSC-BMP4 cells, as well as between F3-MDSC-BMP4 cells and M3-MDSC-BMP4 cells ($P < 0.05$). (C) The bone density of the F- and M-MDSC-BMP4 populations tested was also quantified from the radiographs. The density obtained with F2-MDSC-BMP4 cells was significantly greater than that obtained with F1-, F3-, M1- and M2-MDSC-BMP4 cells (# $P < 0.05$). As well, the bone density of F3-MDSC-BMP4 cells was significantly less than that of M3-MDSC-BMP4 cells (+ $P < 0.05$). n= 2-5/group.

Since MDSCs that are retrovirally transduced to express BMP4 and subsequently implanted into the muscle pocket of mice act both as a BMP4 delivery vehicle and as an osteoprogenitor cell, the number of cells at the implant site and the amount of BMP4 being secreted may affect the extent of bone formation seen in our study. To better understand the variability obtained in the in vivo study, we analyzed the proliferation rate and the amount of BMP4 being secreted by the MDSC-BMP4 populations in vitro. Cellular proliferation was evaluated with an MTS assay after 96 hours of culture in PM. M1-MDSC-BMP4 cells displayed a higher proliferation rate than all other MDSC-BMP4 cells tested (Figure 2-8A, * $P < 0.05$). In addition, F2-MDSC-BMP4 cells and M2-MDSC-BMP4 cells had a higher proliferation rate than F1-, F3-, and M3-MDSC-BMP4 cells (Figure 2-8A, # $P < 0.05$). It was also determined that the MDSC-BMP4 cells used in vivo secreted variable amounts of BMP4, with F1-MDSC-BMP4 cells secreting significantly more BMP4 than F3-, M1-, and M3-MDSC-BMP4 cells (Figure 2-8B, * $P < 0.05$).

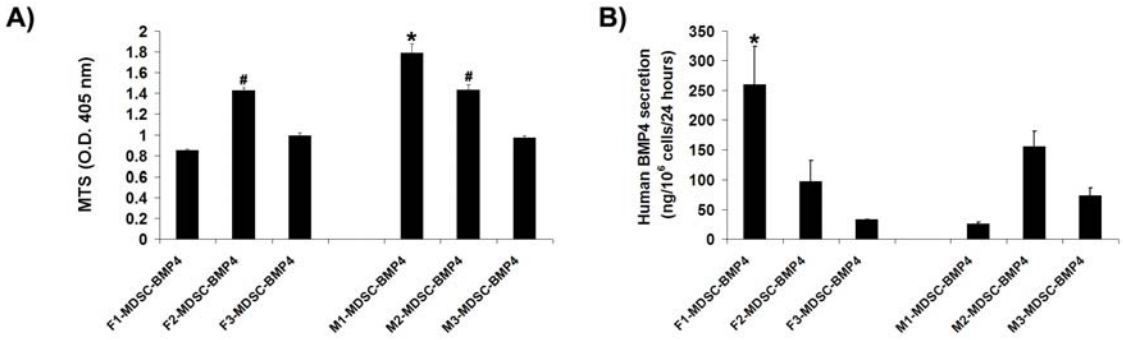

Figure 2-8. Proliferation and human BMP4 secretion of MDSC-BMP4 cells.
(A) The proliferation of the cells used in vivo was quantified in vitro using an MTS assay. Bars represent the mean ± SEM (n=3), * indicates a significant difference between M1-MDSC-BMP4 cells and all populations tested ($P < 0.05$) and # indicates that F2- and M2-MDSC-BMP4 cells were significantly greater than F1-, F3- and M3-MDSC-BMP4 cells ($P < 0.05$). (B) Human BMP4 secretion by MDSC-BMP4 cells was quantified by ELISA and normalized to ng of BMP4 per 10^6 cells per 24 hours. Bars represent the mean ± SEM; n=3. F1-MDSC-BMP4 cells secreted significantly more BMP4 than F3-, M1- and M3-MDSC-BMP4 cells (* $P < 0.05$).

2.3.5 The sex of the host affects ectopic bone formation by MDSC-BMP4 cells

To investigate whether the sex of the host also plays a role in vivo, we studied the influence of donor sex on bone formation by implanting a F- and a M-MDSC-BMP4 population (F2-MDSC-BMP4 and M1-MDSC-BMP4, respectively) into both female and male mice. This experiment resulted in our identification of the male mouse as being a better host for bone formation. The implantation of F-MDSC-BMP4 cells into a male host led to a mean fold increase in bone area of 1.86 ± 0.55 when compared to a female host (Figure 2-9A). A greater increase in bone area was also seen when M-MDSC-BMP4 cells were implanted into a male mouse rather than a female mouse (7.46 ± 1.96) (Figure 2-9A). The effect of host sex was not as pronounced for bone density, yet the male host led to a slightly higher bone density when compared to the female host, regardless of the sex of the cells that were implanted into the skeletal muscle pocket (Figure 2-9B).

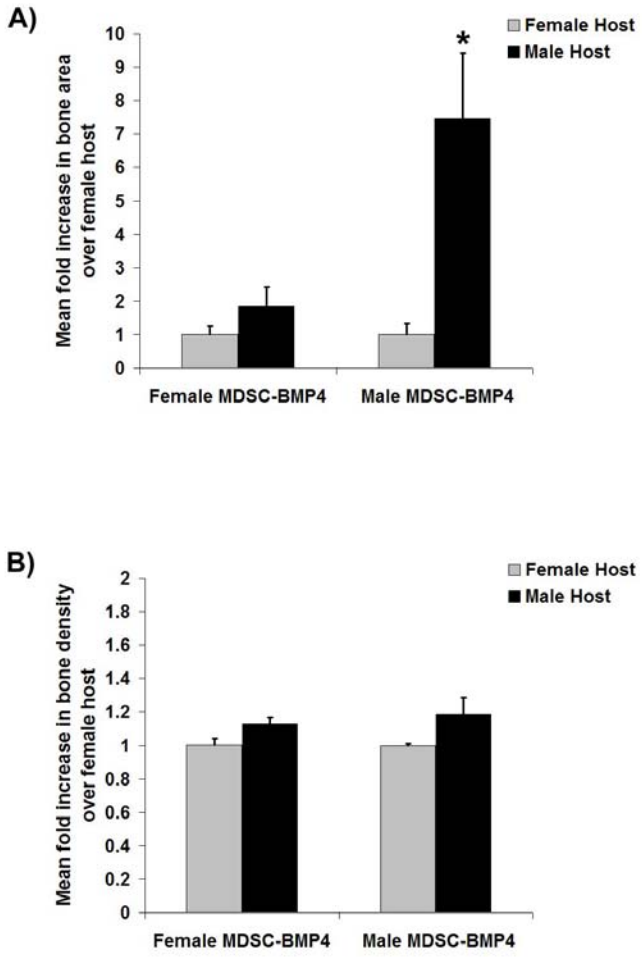

Figure 2-9. The male host promotes more ectopic bone formation than the female host, regardless of donor sex.

F- and M-MDSC-BMP4 cells were implanted into female and male hosts and radiographs were taken after 14 days. (A) Mean fold increase in bone area over the female host demonstrated that both F- and M-MDSC-BMP4 cells showed a larger bone area in the male host than the female host (* $P < 0.05$). (B) A mean fold increase in bone density over the female host was also observed with the use of a male host. n= 3/group.

2.4 DISCUSSION

The osteogenic differentiation of MDSCs in vitro, and their ability to form bone in vivo once retrovirally transduced to express BMP4 has previously been shown (53, 57-59). This previous work, however, was largely centered on populations isolated from female mice. Here, we have shown that both M- and F-MDSCs can undergo osteogenic differentiation, and demonstrated that the M-MDSC populations displayed a greater and more rapid osteogenic differentiation than their female counterparts.

ALP is an early osteogenic marker and is commonly used in osteogenic differentiation studies. By analyzing this marker at the gene expression and protein activity level, sex-related differences in MDSCs stimulated with BMP4 were identified. The initial effect of BMP4 on MDSCs led to interesting differences at 2-days post-stimulation. Not only did M-MDSCs have a greater ALP activity than F-MDSCs when stimulated with BMP4, but, unlike the F-MDSCs, M-MDSCs had ALP activity levels statistically greater than their untreated controls. This suggests that M-MDSCs may have a greater or more rapid response to BMP4 than F-MDSCs.

Another defining characteristic of osteogenesis is the ability of a cell to produce a calcified matrix. Standard assays utilize a monolayer culture and osteoinductive medium to induce bone nodule formation. The inability of MDSCs to remain attached to the culture dish after reaching confluence led us to try alternative methods to test calcification. By modifying a pellet culture method described by Muraglia and colleagues (115), we have shown for the first time that MDSCs can produce mineralized nodules in vitro, which suggests that these cells have the ability to undergo full osteogenic differentiation. The retroviral transduction of MDSCs to

express BMP4 further promoted their ability to produce mineralized matrix, as seen by calcified tissue that was detectable by μCT analysis after only 7 days of culture in osteogenic medium. Although some degree of variability was observed among the different populations tested within each sex, M-MDSCs displayed higher levels of calcification than F-MDSCs, further suggesting a sexual dimorphism in the osteogenic potential of MDSCs. Results from this assay also demonstrated that the M-MDSC-BMP4 cells responded to the osteogenic environment more rapidly than the F-MDSC-BMP4 cells. The M-MDSC-BMP4 cells underwent a greater degree of calcification in the first 7 days of culture and then continued to calcify at a slower rate, while the F-MDSC-BMP4 cells, which lagged behind in the first 7 days, increased their rate of mineralization from 7 to 14 days. These differences may be due to a greater number of osteoprogenitor cells present in M-MDSCs than F-MDSCs, which allows them to respond earlier to the osteogenic stimulus.

The heterogeneity within populations of stem and progenitor cells is also an important factor to consider when evaluating their osteogenic potential. Variations in the levels of ALP activity in human MSCs have been reported between donors (116, 117). Cellular heterogeneity is also found in stem and progenitor cells from skeletal muscle (118-120). In fact, some degree of variability among different MDSC populations of the same sex has recently been reported, although the overall results of this study indicated the existence of sex-related differences in the skeletal muscle regeneration of MDSCs (110). In the study presented in this chapter, we aimed to eliminate the heterogeneity of the population as a confounding factor by investigating clonal populations that were derived from a single MDSC. This allowed us to determine whether the differences observed in osteogenesis up to this point were the result of greater numbers of osteoprogenitor cells within the M-MDSCs than the F-MDSCs. Interestingly, many colonies

displayed a mixture of ALP positive and negative cells, a finding previously reported in fibroblastic colonies derived from a single precursor cell obtained from rabbit marrow (121). This is likely due to some cells within the colony having undergone osteogenic differentiation, while others were still proliferating. Since M-MDSCs had a greater percentage of their total colonies scored as "High ALP+" than F-MDSCs, this study confirmed earlier results seen with ALP activity, and further suggested that M-MDSCs contain more osteoprogenitors than their female counterparts.

The delivery of BMP through MDSC-based gene therapy has previously been shown to lead to bone formation (53, 57). We used this approach to determine whether sex-related differences were also present in the ability of MDSCs to form bone in vivo. While both F- and M-MDSC-BMP4 cells that we examined led to bone formation in vivo, a more constant bone area and density was seen in the M-MDSC-BMP4 cells than in their female counterpart. Unlike the in vitro studies presented thus far, the MDSC-BMP4 cells were put into an open environment, where the amount of bone being formed was due not only to the osteogenic potential of MDSCs, but was also the result of a balance between the amount of BMP4 being secreted, the proliferation and viability of MDSCs and their interaction with surrounding cells. Cells that proliferate to a higher degree may provide more BMP4 to their surroundings. This BMP4 will then recruit more cells to participate in bone formation. The in vitro proliferation of BMP4-transduced cells has been shown to be a key factor in their ability to lead to ectopic bone formation (122, 123). It was determined in this study, that the different MDSC-BMP4 populations examined differed in both their proliferative ability and their capacity to secrete BMP4 even though they were retrovirally transduced at the same time and in the same manner. However, some correlations between cellular proliferation and bone formation could be made.

The F2-MDSC-BMP4 cells led to a bone nodule that was larger in area and density than most of the other populations tested, especially the other F-MDSC-BMP4 populations. Interestingly, the F2-MDSC-BMP4 cells had the greatest number of total colonies in the single-cell derived colony assay, and they also demonstrated a higher rate of proliferation than the other F-MDSC-BMP4 cells tested in this study. Therefore, a greater proliferation rate in vitro may be indicative of greater bone formation in vivo.

To further examine the possibility that M-MDSCs contain more osteoprogenitor cells than F-MDSCs, we implanted the same BMP4 expressing MDSCs into the skeletal muscle of female and male mice. The greater bone area and density observed in the male host indicated that male skeletal muscle cells responded to BMP4 more readily than their female counterpart.

2.5 CONCLUSIONS

Overall, the results from the current study indicate that skeletal muscle from male mice contains more osteogenic cells than skeletal muscle from female mice. Hence, when isolating MDSCs from these skeletal muscles, more osteoprogenitor cells may be found within MDSCs obtained from male skeletal muscle than from female skeletal muscle, thus making more available for osteogenic differentiation (Figure 2-10). When MDSCs that have been genetically engineered to express BMP4 are implanted into female and male skeletal muscle, the area and density of the bone that is subsequently formed may be greater in the male host. This is likely due to the presence of a greater number of osteogenic cells in the male skeletal muscle that can subsequently respond to the BMP4 being secreted by the MDSCs. The finding that more osteoprogenitors or BMP4-responsive cells are found in M-MDSCs than F-MDSCs does not

imply that F-MDSCs are incapable of osteogenic differentiation. This was made evident by the ability of F-MDSCs to increase their ALP activity over time, and to undergo calcification in the osteogenic pellet studies.

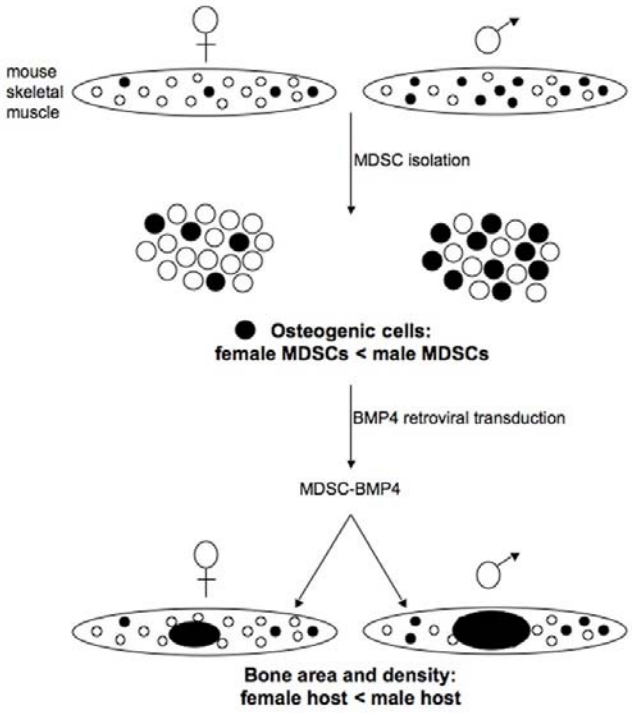

Figure 2-10. Schematic representation of the sex-related differences in MDSCs.
When MDSCs were isolated from female and male mouse skeletal muscle, it was found that M-MDSCs contained more osteogenic cells than F-MDSCs based on the presence of greater ALP, mineralization, and High ALP+ colonies found in M-MDSCs than F-MDSCs after BMP4 stimulation. Once retrovirally transduced to express BMP4 and implanted into the intramuscular pocket of female and male normal mice, a greater bone area and bone density was found in the male host than the female host, regardless of the sex of the implanted cell. This suggests that male skeletal muscle may contain more osteogenic cells than female skeletal muscle.

Sex-related differences have previously been reported in adipose-derived stem cells (ASCs), indicating that cells isolated from female mice display a greater adipogenic differentiation than those isolated from male mice (124). As well, they have recently been reported in MDSCs, with regards to their skeletal muscle regeneration efficiency (110). Here, we have demonstrated that sex-related differences could also be found in the osteogenic

differentiation of MDSCs, indicating that M-MDSCs display a greater osteogenic potential than F-MDSCs. Although the exact consequences of having a greater number of BMP responsive cells in M- than in F-MDSCs are currently unknown, it may be that cell-based therapies involving M-MDSCs will require less cells or less BMP4 than those based on F-MDSCs. We wish to pursue these studies in MDSCs isolated from human skeletal muscle from male and female sources, as well as from adolescent and adult donors. These future studies will extend our knowledge on the applicability of muscle stem cells for bone tissue engineering, and should encourage other investigators to study and report any sex-related differences, as they may clearly have significant clinical implications.

3.0 CHARACTERIZATION OF BMP4 SIGNALING IN MDSCS

3.1 INTRODUCTION

Stem cells play a key role in embryonic development, organogenesis and tissue regeneration in adults (125). To do so, they must maintain a balance between self-renewal and differentiation. Loss of this balance can lead to uncontrolled cellular proliferation or early differentiation, which can result in tumors, cancers and tissue defects (126). Due to their self-renewal potential and ability to differentiate towards various lineages, stem cells have become a key component of tissue engineering approaches. The differentiation of stem cells towards these different lineages is controlled by intrinsic signaling pathways, which are themselves regulated by extrinsic signals. Such extrinsic signals can be the binding of growth factors to cell surface receptors, which can lead to a cascade of intracellular events.

BMPs have been identified as biological factors involved in cell proliferation, differentiation and apoptosis. They play a key role in the osteogenic and chondrogenic differentiation of a variety of cells in vitro and are also known as inducers of bone and cartilage formation when implanted at ectopic sites. It is widely accepted that BMP signaling involves Smad proteins (127). However, there is increasing evidence that there are other signaling pathways that are activated by BMP stimulation. In either case, pathways activated by BMPs will regulate the expression of genes involved in osteogenic and chondrogenic differentiation, and

will allow the cell to which it first bound to adopt a more differentiated phenotype. Hence, understanding how signaling pathways regulate stem cells will be important in the development of stem cell-based therapeutic applications.

3.1.1 The Smad signaling pathway

BMPs exert their effect by binding to transmembrane serine/threonine kinase cell surface receptors, which lead to intracellular signaling and expression of target genes. There are 2 types of BMP receptors and they are known as Type I and Type II receptors (128). There are 3 types of Type I receptors, namely Alk2, Alk3 (Bmpr1a) and Alk6 (Bmpr1b), and only 1 Type II BMP receptor. Binding of BMP to a dimer containing a Type I receptor and a Type II receptor activates the main BMP signaling pathway, which utilizes helper molecules known as Smads. As can be seen in Figure 3-1, once BMP binds to the two types of transmembrane cell surface receptors, they come together, and the type II receptor phosphorylates the type I receptor, which in turn phosphorylates Smad1, 5 or 8 (R-Smad). Once phosphorylated, 2 R-Smads will form a complex with Smad4, which is found in the cytosol. Once this complex is formed, it can translocate to the nucleus where it will regulate gene expression (128-130). The Smad pathway also involves inhibitory elements, known as inhibitor Smads (I-Smads) (131-134). The I-Smads consist of Smad6 and Smad7, which are induced by members of the TGF-ß superfamily and they can block phosphorylation of R-Smads by competing with them for receptor interaction. This allows a negative feedback effect on the Smad signaling pathway. Although the Smad signaling pathway is known as the main BMP signaling pathway, other accessory pathways can also be involved in osteogenic differentiation, especially in the regulation of osteogenic gene expression.

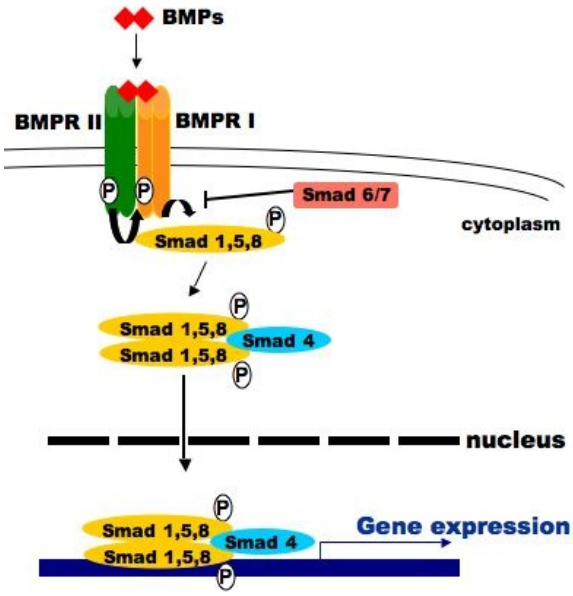

Figure 3-1. Schematic of Smad signaling.

3.1.2 Alternate pathways for BMP signaling

In addition to the Smad pathway, other pathways have been identified for BMP signaling. These include the PI3K and MAPK cascades (Figure 3-2) (65, 83-88).

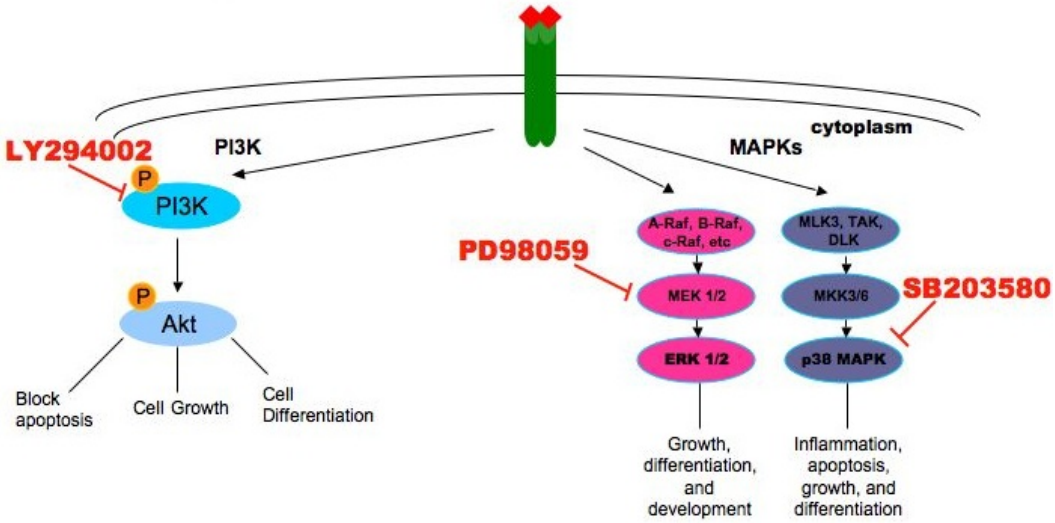

Figure 3-2. Schematic of non-Smad signaling pathways.
The PI3K/Akt, ERK ½, and p38 MAPK pathways are depicted in the schematic. Specific chemical inhibitors to these pathways are shown in red (LY294002, PD98059, and SB203580).

3.1.2.1 PI3K/Akt pathway

Akt is a serine/threonine kinase, which is activated by various extracellular stimuli including insulin and insulin-like growth factor through the PI3K pathway (135). The PI3K-Akt pathway has been implicated in the differentiation of myoblasts, chondrocytes, osteoblasts and adipocytes (84, 136-140). In fact, *Runx2*, an important transcription factor for osteoblast and chondrocyte differentiation, couples with Akt signaling to regulate osteogenic and chondrogenic differentiation and migration (140). It has also been shown that BMP2 can stimulate PI3K activity in osteogenic cells and its inhibition with the specific inhibitor Ly294002 prevented BMP2-induced ALP activity (84). As well, lack of both *Akt1* and *Akt2* genes results in a severely delayed bone development (141).

In addition to its role in differentiation, Akt is also known for its role in cell survival (142). Hence, Akt is necessary for cell survival and differentiation and is a pathway that should be analyzed in better understanding how BMP4 signals and affects the differentiation of stem cells.

3.1.2.2 MAPK cascades

The MAPK family responds to extracellular stimuli through a group of serine/threonine kinases. The family includes the extracellular signal-regulated kinases (ERKs) and two stress-activated protein kinases, known as the c-jun N-terminal kinases (JNKs) and p38 MAPK. These serine/threonine kinases are regulated by two additional protein kinases, which are activated in series. A MAPK Kinase Kinase (MAPKKK) phosphorylates a MAPK Kinase (MAPKK), which in turn phosphorylates a MAPK, such as ERK, JNK and p38 MAPK.

The p38 MAPK pathway has been linked to osteogenesis by various groups studying human osteoblastic cells (143, 144), mouse MC3T3-E1 preosteoblastic cells (145), and mouse

C2C12 cells (65, 146). Gallea *et al*. showed that both the p38 MAPK and ERK cascades are activated by stimulation of C2C12 cells with BMP2, but not the JNK cascade (65). Blocking the p38 MAPK pathway with different doses of a p38-specific inhibitor known as SB203580 led to a dose dependent decrease in ALP activity and *osteocalcin* (*Ocn*) mRNA expression, both indicators of osteogenic differentiation. Instead, inhibition of the ERK cascade by its selective inhibitor PD98059 led to a slight increase in ALP activity, but a decrease in *Ocn* gene expression. Although not activated in C2C12 cells, JNK has been linked to BMP2 signaling in mouse MC3T3-E1 and primary cultured calvaria-derived osteoblastic cells, where it was found to be a positive regulator of Ocn synthesis (145). These results suggest that MAPKs play crucial roles in controlling the BMP2-induced osteoblastic differentiation of a variety of cells.

BMP4 signaling through the p38 MAPK and ERK cascades has also been reported in MC3T3-E1 cells (147). The p38 MAPK pathway was found to be a positive regulator of BMP4 stimulated Ocn synthesis, while the ERK pathway was found to be a negative regulator of BMP4-induced Ocn synthesis.

It has also been shown that non-Smad pathways may interact with the Smad pathway. In fact, both the Smad and the p38 MAPK pathway can converge on the *Runx2* gene (148). As well, ERK can phosphorylate serine residues in the linker region of Smad1 and this blocks its translocation to the nucleus and transcriptional activity, indicating a negative regulation of the Smad pathway by ERK (149). Akt, which is part of the PI3K pathway, has also been shown to regulate Smad translocation to the nucleus (84). From these studies, it is clear that different cell types may have different pathways that are activated by BMP stimulation, and the role that these pathways play in determining the cells' fate may also vary by cell type. Overall, the role that

these non-Smad pathways play in osteogenic differentiation and their cooperation, if any, with the Smad pathway remain to be clearly identified.

MDSCs have been shown capable of osteogenic differentiation in vitro, have been used for bone formation in vivo, and show promise in the developing field of tissue engineering (150). As was demonstrated in the previous chapter, BMP4 can induce the osteogenic differentiation of both F- and M-MDSCs. To date, signaling pathways involved in the BMP4-induced osteogenesis of MDSCs have never been investigated. Elucidating the role of specific signaling pathways in the BMP4-induced osteogenic differentiation of MDSCs may allow for increased regulation of differentiation, which may in turn lead to novel approaches for bone tissue engineering. Therefore, the objective of this study was to characterize the BMP4 signaling pathways involved in the osteogenic differentiation of MDSCs. To do so, a female MDSC population (F1-MDSC) that did not readily respond to BMP4 in terms of upregulation of ALP activity was compared to a male MDSC population (M1-MDSC) that quickly responded to BMP4 by increasing its ALP activity. Both MDSCs were stimulated with BMP4 to determine its ability to activate the Smad, PI3K, ERK and p38 MAPK cascades. These signaling pathways were chosen based on their involvement in the osteogenic differentiation of other cell types. As well, by blocking the non-Smad pathways with their specific inhibitors during BMP4 treatment, and evaluating the expression of osteoblast-related genes by qPCR and measuring ALP activity, the contribution of each pathway to the osteogenic differentiation of MDSCs was determined.

3.2 MATERIALS AND METHODS

3.2.1 Isolation and culture of MDSCs

MDSCs were isolated from 3-week-old C57BL/10J mice using a modified preplate technique (51). Animals were anatomically sexed at the time of isolation and the sex of the cells was later confirmed by FISH analysis. Cells were cultured in phenol red-free PM consisting of DMEM (Invitrogen) supplemented with 110 mg/L sodium pyruvate (Sigma-Aldrich), 584 mg/L L-Glutamine, 10% FBS, 10% HS, 1% penicillin/streptomycin (all from Invitrogen), and 0.5% chick embryo extract (Accurate Chemical Co.) at 37°C in a humidified atmosphere of 5% CO_2 in air. For all assays, cells were plated at a density of 1500 cells/cm^2 and on the following day were treated with or without BMP4 (50 ng/ml) or the inhibitors PD98059 (Biomol International), SB203580 (Biomol International) or Ly294002 (Cell Signaling), which are specific inhibitors for the ERK, p38 MAPK and PI3K pathways, respectively. Inhibitors were dissolved in dimethyl sulfoxide (DMSO) before use, and the control cultures received a concentration of 25 μm of DMSO, which is equivalent to the highest concentration found in the treated cultures. In all assays, cells were incubated with the inhibitors for 1 hour before addition of BMP4.

3.2.2 Protein isolation and Western blot analysis

Cell cultures were stimulated with BMP4 for the indicated time periods, were washed twice with ice-cold phosphate-buffered saline (PBS), and lysed using cell lysis buffer (Cell Signaling) supplemented with protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Sigma-Aldrich). Cells were incubated with the lysis buffer on ice for 5 minutes, scrapped off the

plate, centrifuged at 2500 rpm for 5 minutes and the supernatant collected. Protein concentrations were determined using the Micro BCA™ protein assay (Pierce). Equal amounts of protein extracts were fractioned on a 10% sodium dodecyl sulfate-polyacrylamide gel and electroblotted onto Trans-Blot® Transfer Medium (BioRad). The membranes were incubated in blocking buffer consisting of 5% milk powder in PBS/Tween (0.5% Tween-20) for 1 hour at room temperature (RT). Primary antibody was diluted 1:1000 in 5% BSA (Sigma-Aldrich) in PBS/Tween and incubated with the membrane overnight at 4°C. Primary antibodies to Phospho-Smad1 (Ser463/465)/Smad 5 (Ser463/465)/Smad 8 (Ser426/465), Phosho-p38 MAPK (Thr180/Tyr182), Phospho-p44/42 MAPK (ERK1 and ERK2) (Thr202/Tyr204) and Phospho-Akt (Ser473) were obtained from Cell Signaling. The membrane was then incubated for 1 hour at RT with an anti-rabbit IgG horse-radish-peroxidase-linked antibody (1:2000). Signals on the blot were detected by SuperSignal® West Pico Chemiluminescent Substrate (Pierce). Blots were stripped in Restore™ Western Blot Stripping Buffer (Pierce) and probed for mouse anti-β-actin (1:8000, Sigma-Aldrich) followed with a goat anti-mouse horse-radish-peroxidase-conjugated antibody (1:10 000, Sigma-Aldrich).

3.2.3 Cell proliferation

Cell proliferation was measured in 96-well microtiter flat-bottomed plates. Inhibitors were tested at concentrations of 25 and 50 μM (PD98059 and SB203580) or 10 and 25 μM (Ly294002) and medium was refreshed every 48 hours. Cell viability was assayed with the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) on Days 0, 2 and 4 in accordance with the protocol recommended by the manufacturer.

3.2.4 qPCR analysis of osteogenic genes

MDSCs were stimulated as described above for 24 hours, after which total cellular RNA was collected using the RNAeasy kit (Qiagen). Real-time qPCR analysis was performed with Taqman® One-step RT-PCR Master Mix (Applied Biosystems) as described previously (111). RNA samples (1 μL) were added to sequence-specific primers and Taqman® probes (200 nM per 10-μL reaction). All target genes were normalized to *18S* (primers from Applied Biosystems). The sequences of the target gene primers and probes can be found in Appendix B. All probes were labeled with FAM as the 5' reporter dye and TAMRA as the 3' quencher dye. qPCR assays were carried out in triplicate on an ABI Prism 7900HT sequence detection system in the core facility of the Genomics and Proteomics Core Laboratories of the University of Pittsburgh. Data was analyzed using SDS 2.1 Software from Applied Biosystems.

3.2.5 ALP activity

The presence of ALP was evaluated by cytochemical staining (AP Kit 86-C, Sigma Diagnostics) and by analysis of ALP enzymatic activity. For ALP enzymatic activity, cells were lysed in 0.1% Triton-X in water and assayed using SIGMA *FAST*™ p-Nitrophenyl phosphate tablets (N-2770; Sigma-Aldrich). ALP activity was normalized per mg protein using the Micro BCA™ protein assay (Pierce) and expressed as nanomoles of p-nitrophenyl liberated per microgram of total cellular protein.

3.2.6 Statistical analysis

All in vitro experiments were done in triplicate and repeated three times. Data are reported as mean ± SEM, and analyzed using ANOVA. Pairwise post-hoc analyses were performed using the Tukey test (SPSS statistical software). *P* values of less than 0.05 were considered significant.

3.3 RESULTS

3.3.1 Characterization of F1-MDSCs and M1-MDSCs

To determine the effect of BMP4 on the osteogenic differentiation of F1- and M1-MDSCs, the cells were plated at a density of 1500 cells/cm^2 and stimulated on the following day with BMP4 at a concentration of 50 ng/ml, which was the same concentration used in the studies presented in Chapter 2. *Alp* gene expression was analyzed 24 hours after the initiation of BMP4 stimulation and ALP activity was quantified 3 days after the initiation of BMP4 stimulation. It was determined that *Alp* gene expression was significantly different from untreated control in both MDSCs (Figure 3-3A, $*P < 0.05$). As well, *Alp* gene expression was greater in the M1-MDSC population than the F1-MDSC population in both the control and BMP4 stimulated cultures (Figure 3-3A, $P < 0.05$). The ALP activity of the two MDSCs tested also demonstrated the same trend as the gene expression analysis (Figure 3-3B). Both F1-MDSCs and M1-MDSCs were significantly greater than their untreated control (Figure 3-3B, $*P < 0.05$). As well, 3 days after the initiation of BMP4 stimulation, M1-MDSCs displayed a higher ALP activity than F1-MDSCs (Figure 3-3B, $P < 0.05$).

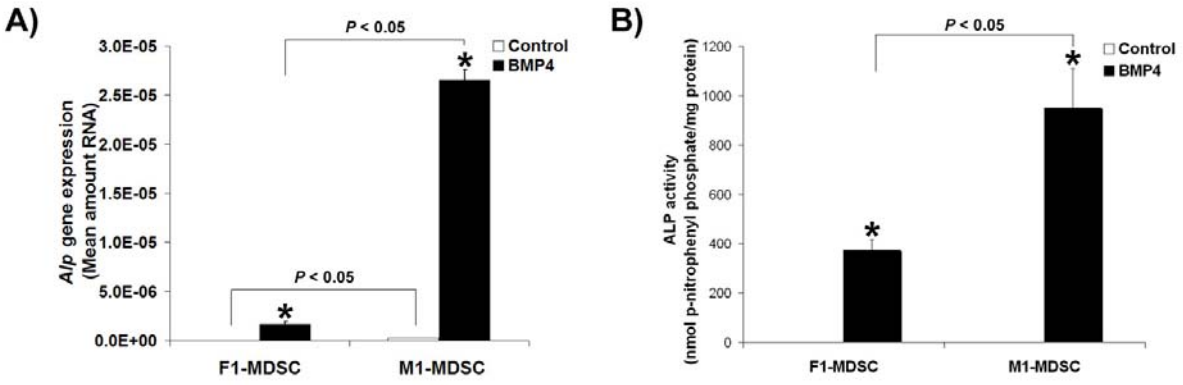

Figure 3-3. (A) *Alp* gene expression and (B) ALP activity of F1- and M1-MDSCs.
Bars represent the mean ± SEM, n=3. * indicates a significant difference from the untreated control ($P < 0.05$). Statistical differences between F1- and M1-MDSCs are depicted on the graph ($P < 0.05$).

In addition to investigating the effect of BMP4 on the early osteogenic differentiation of the two MDSC populations tested in this study, its effect on proliferation was also determined. As shown in Figure 3-4, without BMP4 stimulation (solid lines), M1-MDSCs had a higher proliferation rate than F1-MDSCs at Day 2 and Day 4 ($P < 0.05$). As well, M1-MDSCs stimulated with BMP4 (blue dashed line) displayed a greater proliferation rate than F1-MDSCs stimulated with BMP4 (red dashed line) at Day 2 and Day 4 ($P < 0.05$). Interestingly, the proliferation of M1-MDSCs was not affected by BMP4 stimulation, while F1-MDSCs significantly increased their proliferation at 2 days and 4 days after initiation of BMP4 stimulation ($P < 0.05$).

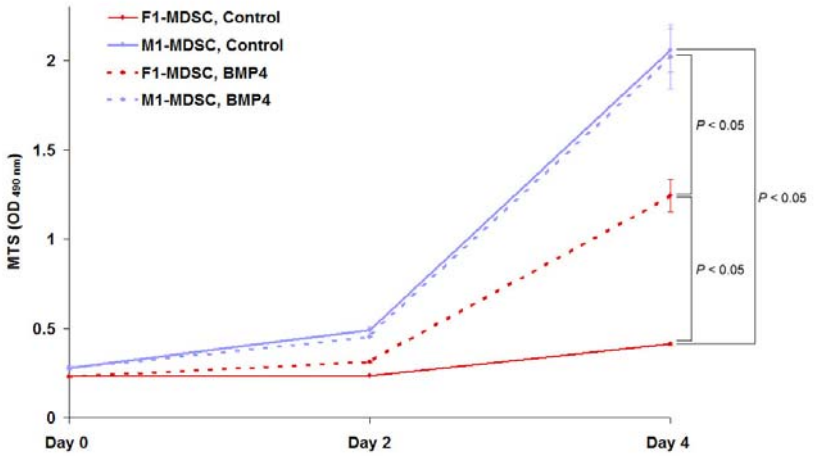

Figure 3-4. Effect of BMP4 on the proliferation of F1- and M1-MDSCs.
Significant differences between groups are depicted on the graph ($P < 0.05$) and represent significant differences at Day 2 and Day 4.

3.3.2 Activation of the Smad pathway

To determine whether the Smad signaling pathway is activated in MDSCs following treatment with BMP4, cell lysates from F1- and M1-MDSCs were obtained after 0, 30 minutes, 1 hour, 2 hours, 3 hours and 6 hours of BMP4 stimulation and analyzed by western blot (Figure 3-5). In both cell types there was a band evident at all time points, including the unstimulated control. The intensity of phospho-Smad 1/5/8 seems to peak at 1 hour of BMP4 stimulation followed by a decrease in intensity at longer time points.

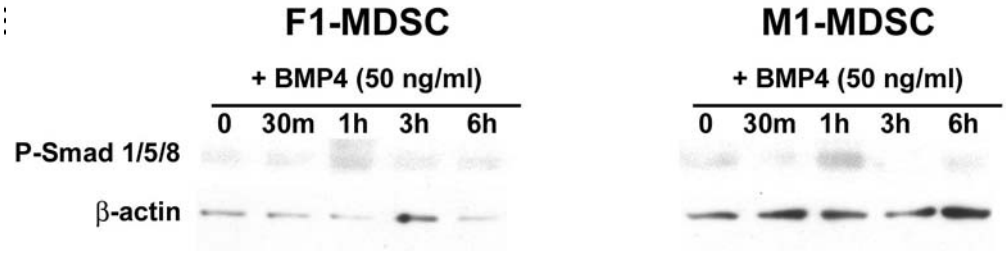

Figure 3-5. Western blot for phosphorylated Smad 1/5/8.
F1- and M1-MDSCs were stimulated with BMP4 (50 ng/ml) for 0, 30 minutes, 1 hour, 3 hours, and 6 hours and analyzed by western blot analysis.

3.3.3 Activation of non-Smad pathways

To determine if BMP4 activates pathways other than the Smad pathway, the lysates used for the phospho-Smad western were also used to detect the phosphorylation of Akt, ERK and p38 MAPK. As can be seen in Figure 3-6, all 3 pathways were activated. Akt was phosphorylated at all time points, ERK 1/2 was phosphorylated after 30 minutes of BMP4 stimulation and phosphorylation of p38 MAPK was evident at all time points in F1-MDSCs but a visible band was only seen at 6 hours of BMP4 stimulation in M1-MDSCs. The blots were then stripped and probed for β-actin.

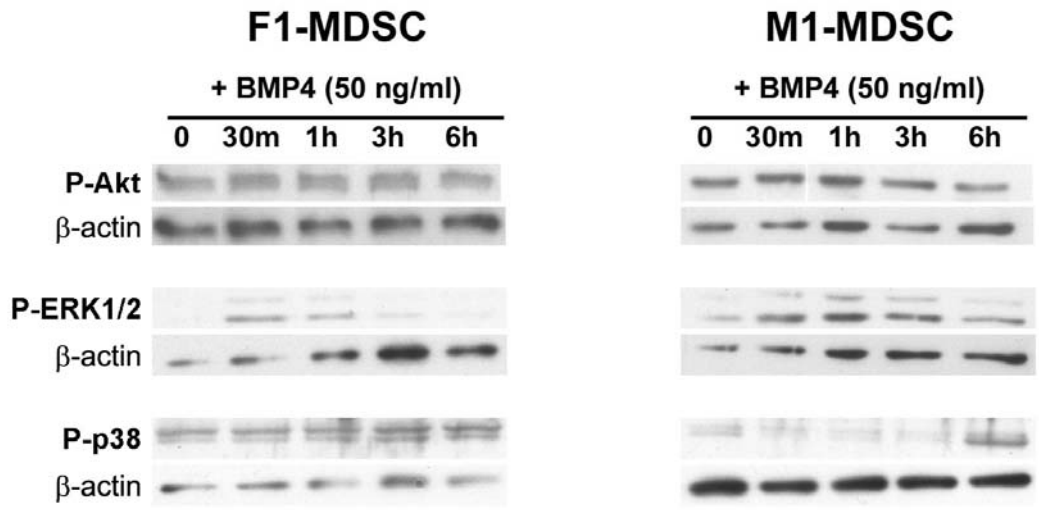

Figure 3-6. Western blot for phosphorylated Akt, ERK1/2, and p38.
F1- and M1-MDSCs were stimulated with BMP4 (50 ng/ml) for 0, 30 minutes, 1 hour, 3 hours, and 6 hours and analyzed by western blot analysis.

3.3.4 Effect of pathway inhibitors on proliferation

Specific inhibitors to the PI3K, the ERK and the p38 MAPK pathways (Ly294002, PD98059 and SB203580, respectively) were added to untreated or BMP4 stimulated cells and the cell viability was measured after 4 days (Figure 3-7A-F). As was seen earlier in Figure 3-4, BMP4 stimulated

cells were significantly different from untreated control cells in F1-MDSCs but not in M1-MDSCs (*$P < 0.05$).

Blocking the PI3K pathway with Ly294002 in F1-MDSCs did not decrease cell viability compared to untreated control (Figure 3-7A). However, adding the inhibitor to the BMP4 treated cells decreased their viability when compared to cells that only received BMP4 (Figure 3-7A, #$P < 0.05$ vs. BMP4). This decrease was even more pronounced when the concentration of Ly294002 was increased from 10 μm to 25 μm (Figure 3-7A, +$P < 0.05$, BMP4 + Ly 10 vs. BMP4 + Ly 25), suggesting that this concentration may be highly toxic to the cells. Inhibition of the PI3K pathway in M1-MDSCs also displayed a decrease in cell viability and this was evident when control cells were incubated with Ly294002 (Figure 3-7B, *$P < 0.05$ vs. Control) and when BMP4 stimulated cells were incubated with the same inhibitor (Figure 3-7B, #$P < 0.05$ vs. BMP4).

Inhibition of the ERK pathway had no effect on the proliferation of untreated F1-MDSCs (Figure 3-7C). However, the increase in proliferation seen with the addition of BMP4 to the medium was significantly decreased when PD98059 was added at a concentration of 50 μm (Figure 3-7C, #$P < 0.05$ vs. BMP4). This concentration also led to a significant decrease in cell viability when compared to PD98059 at a dose of 25μM (Figure 3-7C, +$P < 0.05$, BMP4 + PD 25 vs. BMP4 + PD 50), possibly due to the toxicity of the inhibitor at a concentration of 50 μm. Interestingly, inhibiting the ERK pathway had no effect on the viability of M1-MDSCs (Figure 3-7D).

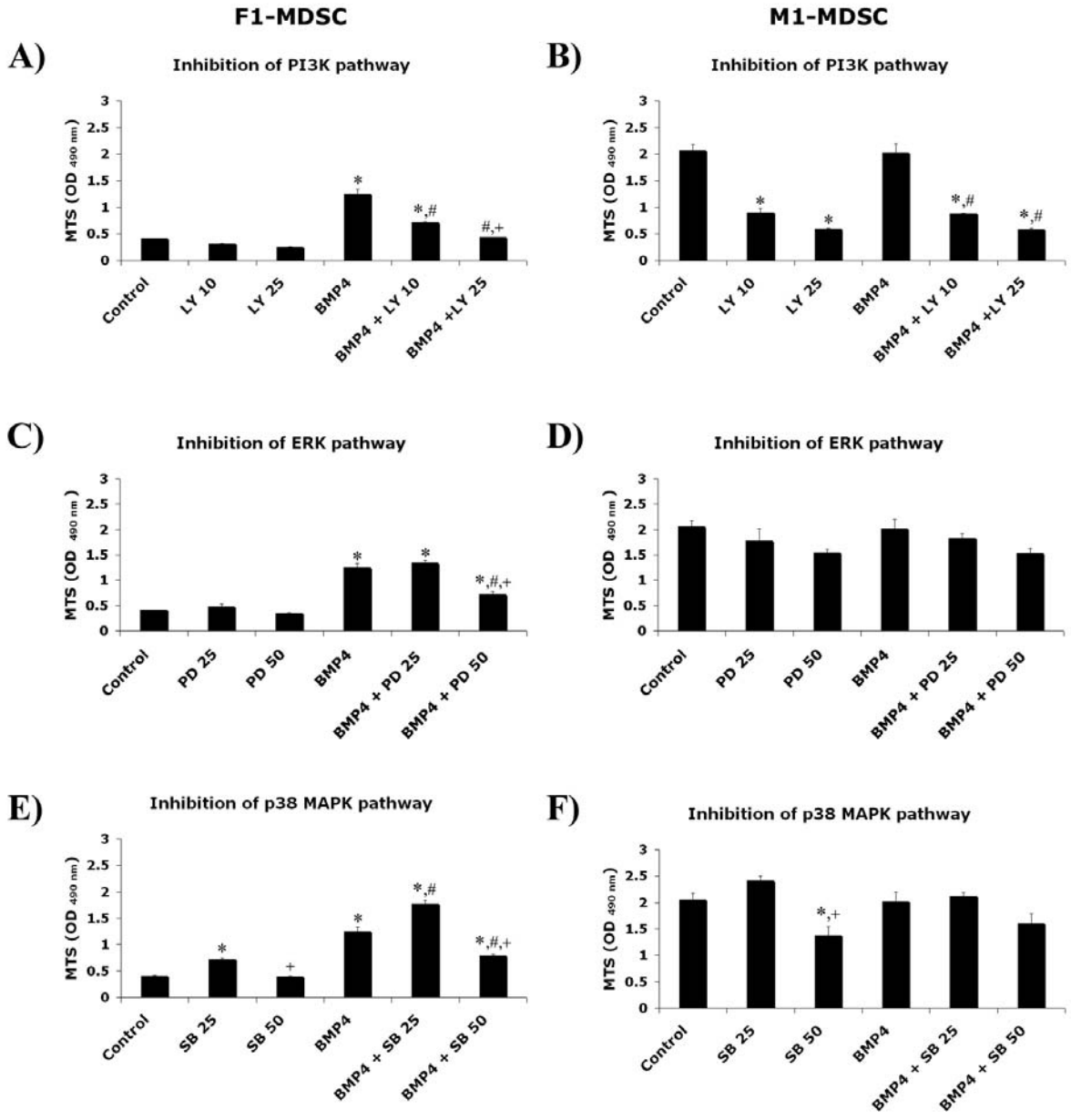

Figure 3-7. Viability of F1- and M1-MDSCs after inhibition of the PI3K, ERK or p38 MAPK pathways. Viability was assessed with an MTS assay after 4 days of incubation in PM with or without BMP4 and supplemented with (A,B) Ly294002 (10 μm, 25 μm), (C,D) PD98059 (25 μm, 50 μm) or (E,F) SB203580 (25 μm, 50 μm). * indicates a significant difference compared to Control ($P < 0.05$). # indicates a significant difference compared to BMP4 ($P < 0.05$). + indicates a significant difference compared to the lower concentration of inhibitor ($P < 0.05$).

The other pathway that was studied for its role in proliferation was the p38 MAPK pathway. It was found that p38 MAPK does play a role in proliferation, as its inhibition by SB203580 at a concentration of 25 μm increased cell proliferation in F1-MDSCs compared to control (Figure 3-7E, $*P < 0.05$ vs. Control). A significant increase in proliferation was also observed when the same concentration of inhibitor was added to BMP4 stimulated F1-MDSCs (Figure 3-7E, $\#P < 0.05$ vs. BMP4). At a concentration of 50 μm, SB203580 decreased the viability of both untreated and BMP4 treated F1-MDSCs ($+P < 0.05$, SB 25 vs. SB 50 and BMP4 + SB 25 vs. BMP4 + SB 50). In the M1-MDSC population, inhibition of the p38 MAPK pathway had no effect on proliferation (Figure 3-7F). The only difference observed was the decrease in viability with a SB203580 dose of 50 μm when compared to Control or SB 25 ($*P < 0.05$ vs. Control, and $+P < 0.05$, SB 25 vs. SB 50). This may likely be a result of the toxicity associated with this higher concentration of inhibitor.

Since PD98059 and SB203580 at a concentration of 50 μm were considered toxic to the cells, the rest of the studies presented in this chapter used a dose of 10 μm for Ly294002, 25 μm for PD98059 and 25 μm for SB203580.

3.3.5 Effect of pathway inhibitors on osteogenic differentiation

To determine the involvement of the PI3K, ERK and p38 MAPK pathways on the osteogenic differentiation of MDSCs, cells were incubated with or without the pathway inhibitors and BMP4 for various time points. qPCR was used to determine any changes in the expression of osteogenic genes such as *Runx2*, *Osterix* (*Osx*), *Alp* and *Smad 6*. *Runx2* and *Osx* are transcription factors implicated in osteogenic differentiation (151, 152). *Alp* is an early marker of osteogenic

differentiation and *Smad6* is the inhibitory molecule for Smad signaling. As well, the ALP activity of F1- and M1-MDSCs was also tested.

3.3.5.1 Osteogenic gene expression analysis

MDSCs were treated with or without BMP4 and inhibitors for 24 hours before analysis of gene expression by qPCR. Addition of BMP4 to both MDSC populations led to an increase in *Runx2*, *Osx*, *Alp* and *Smad6* gene expression when compared to the untreated control (Figure 3-8A, B, C, and D, respectively, * $P < 0.05$ vs. Control). The only increase that was not statistically significant was the increase in *Runx2* gene expression in M1-MDSCs (Figure 3-8A).

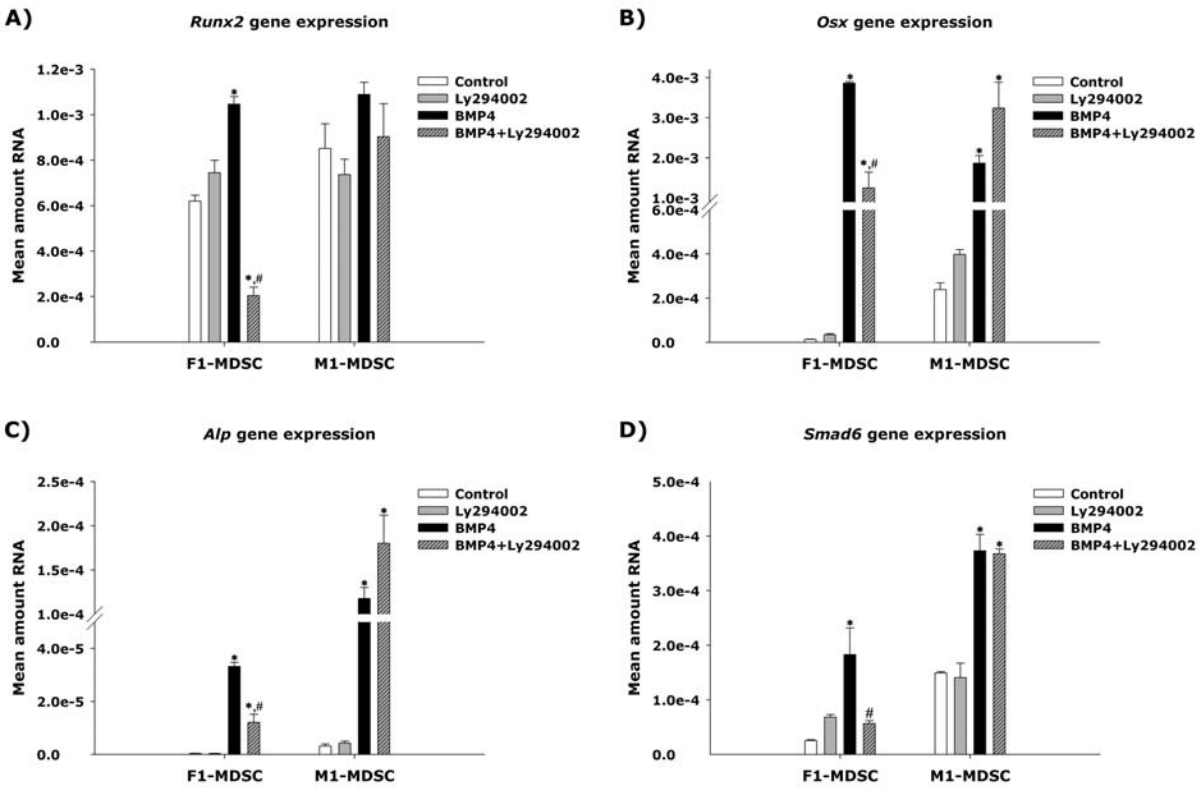

Figure 3-8. Effect of PI3K pathway inhibition with Ly294002 (10 μm) on the gene expression of *Runx2, Osx, Alp* and *Smad6*.
* indicates a significant difference from Control ($P < 0.05$). # indicates a significant difference from BMP4 ($P < 0.05$).

To determine the role of the PI3K pathway in the osteogenic differentiation of MDSCs, the pathway was inhibited with Ly294002 (Figure 3-8A-D). This led to different responses in F1- and M1-MDSCs. While the addition of Ly294002 to BMP4 in M1-MDSCs did not affect gene expression compared to BMP4 alone, it led to a decrease in the expression of all genes tested in F1-MDSCs (Figure 3-8A-D, #$P < 0.05$ vs. BMP4). This decrease was especially evident in *Runx2* gene expression, where addition of Ly294002 to BMP4 resulted in a mean amount of RNA significantly lower than the untreated control (Figure 3-8A, *$P < 0.05$ vs. Control).

The analysis of osteogenic gene expression in F1- and M1-MDSCs also revealed a role for the ERK pathway in their response to BMP4. The increase in *Runx2* gene expression seen when F1-MDSCs were stimulated with BMP4 was significantly decreased when they were stimulated with BMP4 and the specific inhibitor to the ERK pathway, PD98059 (Figure 3-9A, #$P < 0.05$ vs. BMP4). Inhibition of the ERK pathway in M1-MDSCs had no effect on *Runx2* gene expression (Figure 3-9A). In the case of *Osx* gene expression, inhibition of the ERK pathway led to a decreased *Osx* gene expression in F1-MDSCs, but an increased *Osx* gene expression in M1-MDSCs when compared to cells receiving BMP4 only (Figure 3-9B, #$P < 0.05$ vs. BMP4). Differences between F1- and M1-MDSCs were also observed in *Alp* gene expression. Firstly, addition of PD98059 to F1-MDSCs significantly increased their expression of *Alp* compared to control cells kept in PM (Figure 3-9C, *$P < 0.05$ vs. Control). In fact, the expression of *Alp* was at a level similar to that of BMP4 treated F1-MDSCs. This increase in *Alp* gene expression due to inhibition of the ERK pathway, but without BMP4 stimulation was not seen in M1-MDSCs (Figure 3-9C). When the ERK pathway inhibitor was added to BMP4 treated cells, no change in *Alp* expression was seen in F1-MDSCs, but a significant increase was

seen in M1-MDSCs (Figure 3-9C, #$P < 0.05$). Lastly, inhibition of the ERK pathway did not effect *Smad6* gene expression in both F1- and M1-MDSCs (Figure 3-9D).

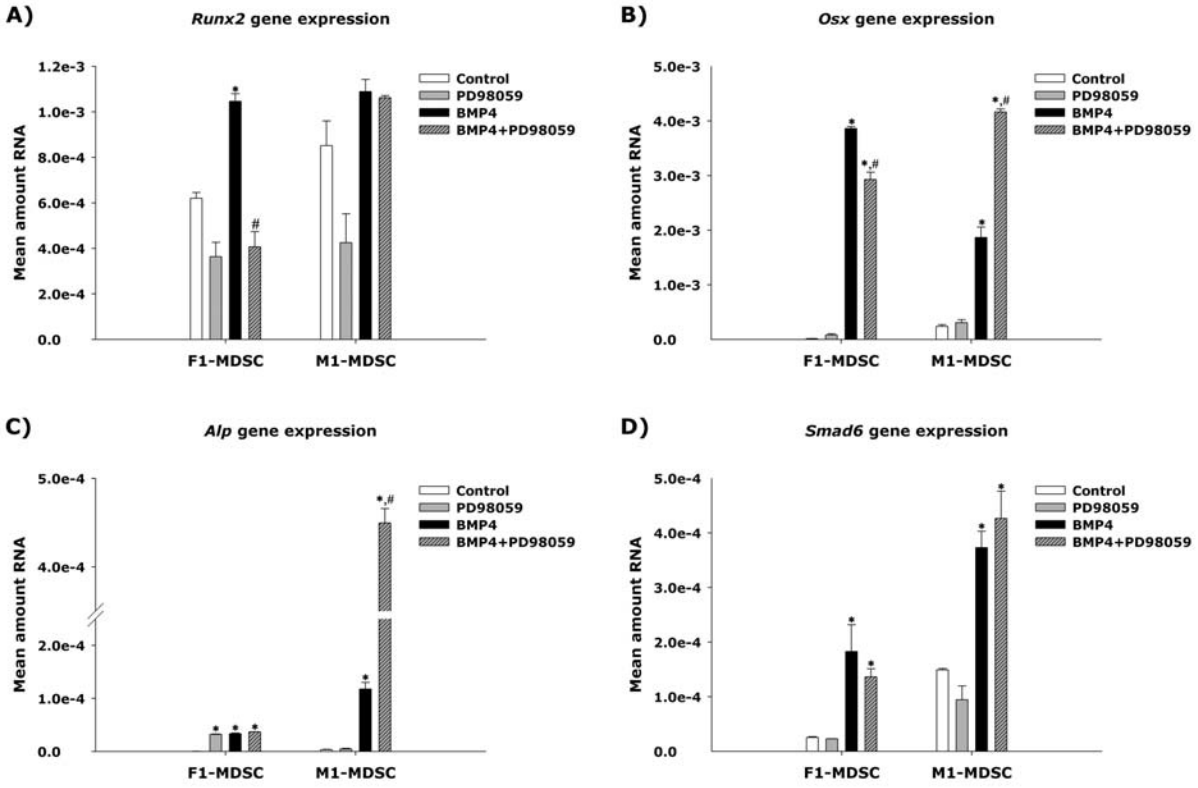

Figure 3-9. Effect of ERK pathway inhibition with PD98059 (25 μm) on the gene expression of *Runx2, Osx, Alp* and *Smad6*.
* indicates a significant difference from Control ($P < 0.05$). # indicates a significant difference from BMP4 ($P < 0.05$).

The p38 MAPK pathway was also investigated for its role in the osteogenic differentiation of F1- and M1-MDSCs. The inhibition of this pathway in control cells had no effect on *Runx2*, *Osx*, *Alp* and *Smad6* gene expression in F1-MDSCs (Figure 3-10A-D). However, addition of the p38 MAPK specific inhibitor SB203580 to M1-MDSCs cultured in PM led to a decrease in *Runx2* gene expression (Figure 3-10A, *$P < 0.05$ vs. Control). When the inhibitor was added to cells treated with BMP4, a decrease in *Runx2*, *Osx*, *Alp*, and *Smad6* gene expression was observed in both F1- and M1-MDSCs (Figure 3-10A-C, #$P < 0.05$ vs. BMP4).

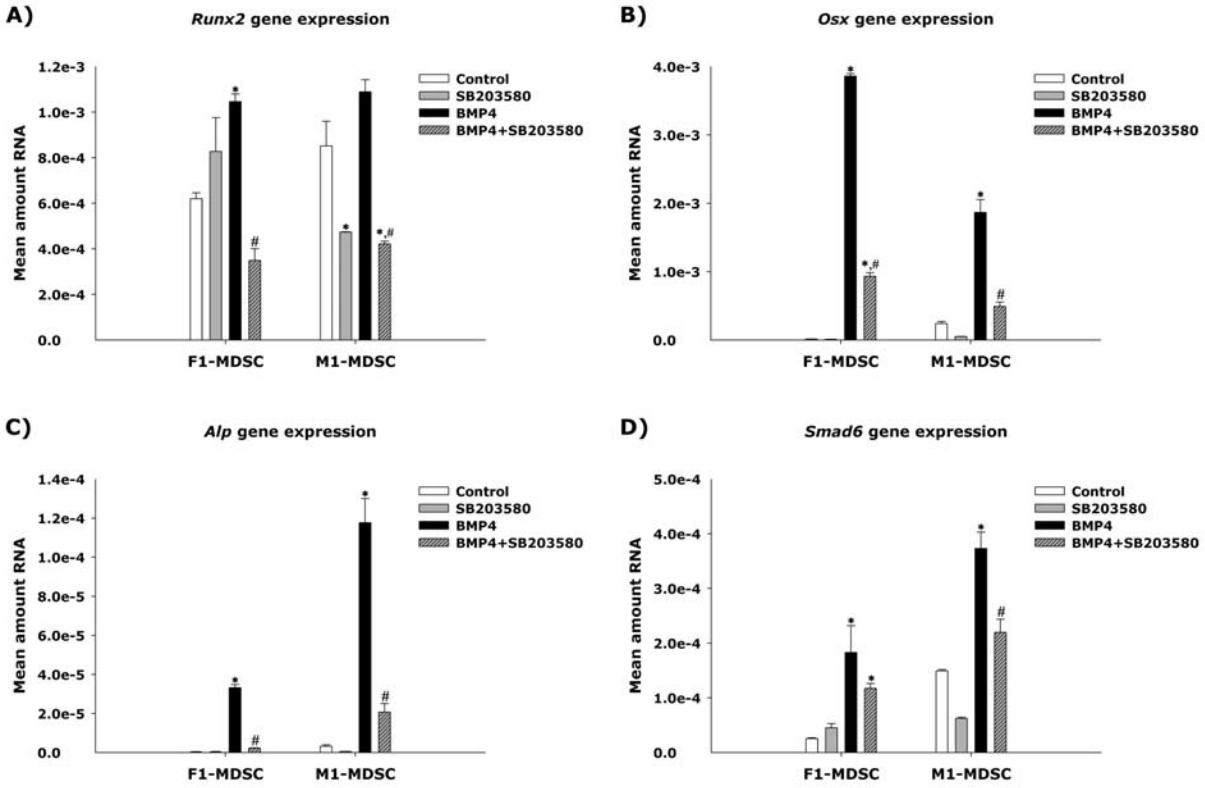

Figure 3-10. Effect of p38 MAPK pathway inhibition with SB203580 (25 μm) on the gene expression of *Runx2, Osx, Alp* and *Smad6*.
* indicates a significant difference from Control ($P < 0.05$). # indicates a significant difference from BMP4 ($P < 0.05$).

3.3.5.2 ALP activity

The role of the different pathways in the induction of ALP activity was also investigated by using specific inhibitors to the PI3K, ERK, and p38 MAPK pathways. F1-MDSCs had non-detectable levels of ALP activity when no BMP4 was added to the PM (Figure 3-11A). This remained true even when the PM was supplemented with the different inhibitors. Inhibition of the PI3K, ERK, and p38 MAPK cascades in M1-MDSCs that were not treated with BMP4 also had no effect on their ALP activity (Figure 3-11B). As expected, when BMP4 was added to the culture medium, an increase in ALP activity was observed in both F1- and M1-MDSCs compared to their untreated control (Figure 3-11A and B, *$P < 0.05$ vs. No BMP4 Control). This

increase in ALP activity when compared to untreated control cells was also evident when the inhibitors Ly294002, PD98059, and SB203580 were added to BMP4 treated MDSCs, except for the addition of SB203580 to F1-MDSCs (Figure 3-11A and B, *$P < 0.05$ vs. No BMP4 Control).

Inhibition of the PI3K pathway with Ly294002 in BMP4 treated cells did not affect the ALP activity of F1-MDSCs, but decreased the ALP activity of M1-MDSCs (Figure 3-11A and B, #$P < 0.05$ vs. BMP4 Control). Inhibition of the ERK pathway with PD98059 led to an increase in ALP activity in both F1- and M1-MDSCs treated with BMP4 (Figure 3-11A and B, #$P < 0.05$ vs. BMP4 Control). Lastly, inhibition of the p38 MAPK pathway with SB203580 in F1- and M1-MDSCs treated with BMP4 led to a decrease in ALP activity (Figure 3-11A and B, #$P < 0.05$ vs. BMP4 Control).

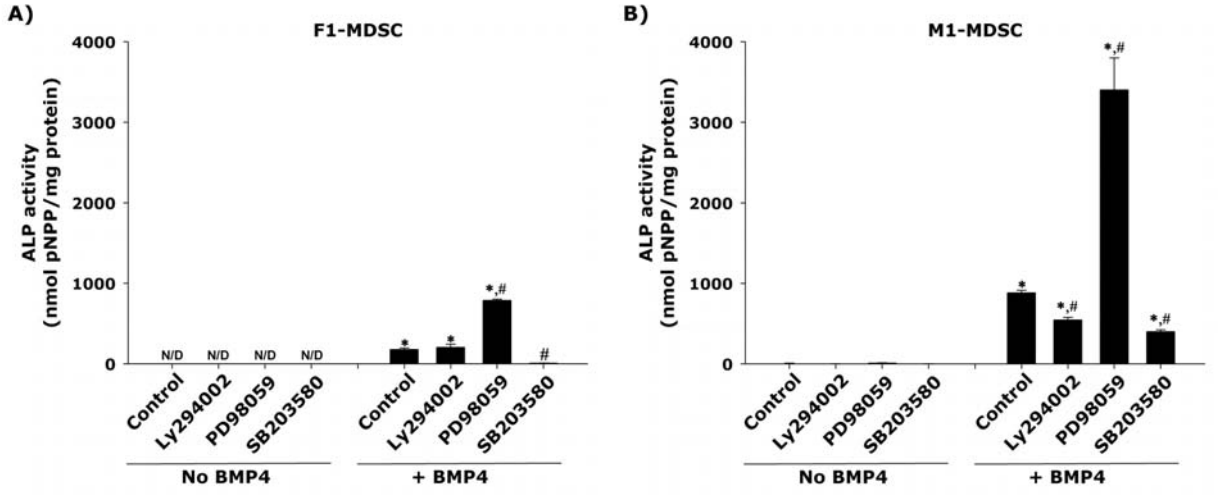

Figure 3-11. (A) ALP activity of F1-MDSCs and (B) M1-MDSCs in the presence or absence of BMP4 and specific inhibitors Ly294002, PD98059, and SB203580 to the PI3K, ERK and p38 MAPK pathways, respectively.
* indicates a significant difference from No BMP4 Control ($P < 0.05$). # indicates a significant difference from +BMP4 Control ($P < 0.05$).

The ALP activity results presented in Figure 3-11 were further confirmed by ALP staining of F1- and M1-MDSCs treated with Ly294002, PD98059 and SB203580 and then stimulated with BMP4 (Figure 3-12). Inhibition of PI3K with Ly294002 and p38 MAPK with

SB203580 led to a decrease in ALP staining when compared to cells having received BMP4 only, as was previously seen with the ALP activity. As well, an increase in ALP staining was seen when the ERK pathway was inhibited with PD98059. As previously shown with the quantification of ALP activity, this was more pronounced in M1-MDSCs than in F1-MDSCs.

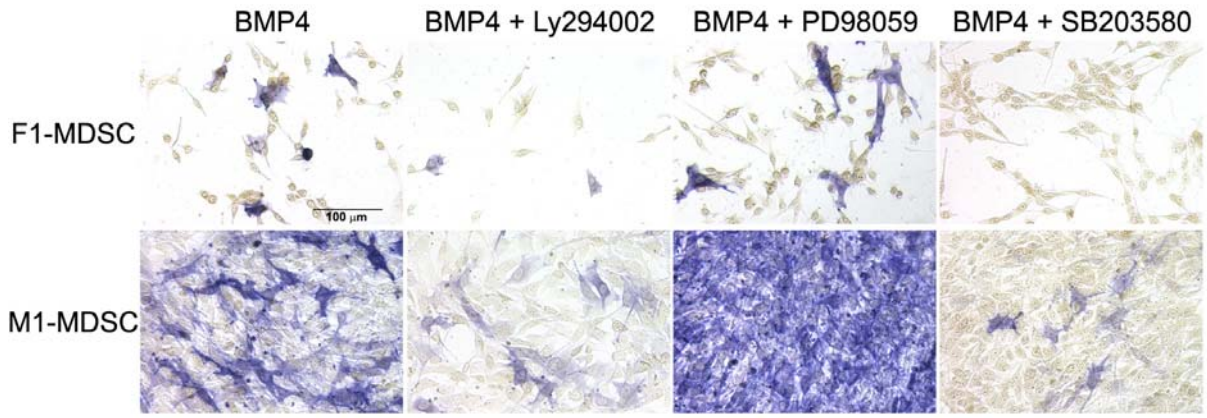

Figure 3-12. ALP stain of F1- and M1-MDSCs stimulated with BMP4 and inhibitors to the PI3K, ERK and p38 MAPK pathways.

3.4 DISCUSSION

In this study, the BMP4 signaling of MDSCs was characterized by studying a MDSC population that does not readily respond to BMP4 by increasing its *Alp* gene expression or ALP activity (F1-MDSC) versus that of a MDSC population that quickly undergoes osteogenic differentiation when stimulated with BMP4 (M1-MDSC). It was first determined that BMP4 signals through the Smad pathway in both MDSCs tested. As well, it can activate other accessory pathways such as the PI3K, ERK and p38 MAPK pathways. By taking advantage of chemical inhibitors to PI3K, ERK and p38 MAPK, such as Ly294002, PD98059, and SB203580, respectively, it was possible to elucidate the involvement of each pathway in the BMP4-induced osteogenesis of MDSCs.

In studying the osteogenic differentiation of MDSCs induced by BMP4, it was also important to determine how this growth factor affected cell proliferation, and the involvement of the different pathways analyzed in this study. It was determined that M1-MDSCs have a greater proliferation rate than F1-MDSCs when in PM. However, when BMP4 was added to the PM, F1-MDSCs had a significant increase in proliferation, while M1-MDSCs were unaffected. This may be explained by the existence of an inverse relationship between proliferation and differentiation, where molecules that promote differentiation, may also prevent cell cycle re-entry. While M1-MDSCs quickly respond to BMP4 by undergoing osteogenic differentiation rather than increasing their proliferation, F1-MDSCs do not readily undergo differentiation but increase their proliferation. By using chemical inhibitors, it was also determined that the proliferation of MDSCs was affected by inhibition of the PI3K and p38 MAPK pathway but not by inhibition of the ERK pathway. Both F1- and M1-MDSCs had a decreased proliferation when the PI3K pathway was blocked and F1-MDSCs displayed an increased proliferation when the p38 MAPK pathway was blocked. Reduced proliferation after blockade of PI3K with Ly294002 has

previously been reported and is anticipated since the PI3K/Akt pathway is a key regulator of cell survival (142). As well, the p38 MAPK pathway is known to induce exit from the cell cycle and to lead to the differentiation of various cell types (153, 154). Its inhibition by the specific inhibitor SB203580, which was used in this study, has increased the proliferation of mammalian cardiomyocytes, which are considered to be terminally differentiated and incapable of proliferation (155). In the same study, inhibition of PI3K blocked cardiomyocyte proliferation. Hence, in MDSCs, PI3K is crucial for cell survival and p38 MAPK may function as a negative regulator of proliferation.

The role of the PI3K, ERK and p38 MAPK pathways was also investigated in the osteogenic differentiation of MDSCs. Genes such as *Runx2*, *Osx*, *Alp* and *Smad6* were investigated, as they are key indicators of osteogenic differentiation. Inhibition of the PI3K pathway by its specific inhibitor Ly294002 led to interesting differences in both gene expression and ALP activity. At the gene expression level, the PI3K pathway was important for the expression of osteogenic genes in F1-MDSCs, but not in M1-MDSCs. However, at the protein level, ALP activity was significantly decreased in M1-MDSCs when PI3K was inhibited in BMP4 stimulated cells, but not in F1-MDSCs. The difference observed may have to do with the time point chosen for analysis after BMP4 stimulation. Gene expression levels were investigated 24 hours after initiation of BMP4 stimulation, while ALP protein activity was measured after 3 days of BMP4 stimulation. Perhaps the F1- and M1-MDSCs do not use the PI3K pathway with the same timing. However, since the inhibition of the PI3K pathway affected both F1-MDSCs and M1-MDSCs, it is reasonable to assume that this pathway plays a role in the osteogenic differentiation of MDSCs. Ghosh-Choudhury *et al*. were the first to report that activation of the BMP receptor by BMP2 led to the activation of the PI3K/Akt pathway and that it did in fact play

a role in osteoblast differentiation (84). The PI3K was also shown to be important for the ALP activity of Runx2 stable transfectants of MC3T3-E1 cells (136). In human MSCs, the PI3K pathway was required for the BMP-induced expression of early osteogenic genes such as *Alp* (86). PI3K activity was also shown to be important for the osteoblastic differentiation induced by BMP7 (87). Taken together, the findings from this study suggest that PI3K plays an important role in BMP4-induced signaling of MDSCs, and that further studies must be undertaken to fully elucidate its contribution to osteogenesis.

The ERK pathway was also shown to be involved in the osteogenic differentiation of MDSCs. Its inhibition decreased the gene expression of *Runx2* and *Osx* in F1-MDSCs, but increased the *Osx* gene expression in M1-MDSCs. Although the difference is significant when comparing mean amount of RNA levels, the actual fold change in *Osx* gene expression between BMP4 treated cells and BMP4 + PD98059 treated cells was 0.78 ± 0.07 in F1-MDSCs and 2.0 ± 0.03 in M1-MDSCs. This does not suggest a large difference between F1- and M1-MDSCs. Interestingly, inhibition of the ERK pathway increased the *Alp* gene expression of F1-MDSCs which were not stimulated with BMP4, and this to levels similar to that seen when the same cells were stimulated with BMP4. However, it did not increase *Alp* gene expression in BMP4-stimulated F1-MDSCs. These results may relate to the inhibitory effect ERK has on Smad signaling (149). Addition of the inhibitor to F1-MDSCs in PM may have removed the ERK inhibition on Smad signaling and increased the expression of *Alp*. Addition of BMP4 to the same cells directly activated the Smad pathway and also led to an increase in *Alp* gene expression. However, this increase may have been the maximum response these cells could reach and addition of the ERK inhibitor to BMP4 could not further increase their *Alp* gene expression. The M1-MDSCs, which were chosen in this study for their ability to readily respond to BMP4,

displayed an increased *Alp* gene expression as a result of BMP4 stimulation, and an even further increase when the ERK inhibitor was added. In this case, they may not have reached their maximum expression level with BMP4 stimulation alone. The differences seen between F1- and M1-MDSCs at the gene expression level again suggest that at the 24-hour time point, F1- and M1-MDSCs may not be responding to the BMP4 and ERK inhibitor in the same manner. However, after 3 days of stimulation with BMP4 in the absence or presence of PD98059, similar results were obtained in both MDSC populations tested. Inhibition of the ERK pathway led to an increase in the ALP activity of BMP4 stimulated MDSCs. The inhibition of the ERK pathway has also led to an increase in ALP activity in C2C12 cells and human MSCs, suggesting that high ERK activity negatively regulates BMP2-stimulation of ALP (65, 86). It has also been shown to be a negative regulator of Ocn synthesis in MC3T3-E1 cells (147). Hence, from the results presented in this study, the ERK pathway is a negative regulator of the BMP4-induced osteogenesis of MDSCS.

Studies investigating the role of the p38 MAPK pathway in osteogenic differentiation are at times disputable. While one study looking at the inhibition of p38 MAPK activity in C2C12 cells treated with BMP2 reported an increased osteogenic effect, suggesting an inhibitory effect of p38 MAPK on osteogenesis (146), another study concluded that p38 MAPK activation was necessary but not sufficient to stimulate the expression of Ocn and ALP in BMP2 stimulated cells (65). On the other hand, studies employing primary calvarial osteoblasts, primary bone marrow osteoprogenitor cells, normal human osteoblasts, and the murine osteoblast line MC3T3-E1 found that p38 MAPK is necessary for BMP-induced osteogenic differentiation (143-145, 147, 156). Recently, it has also been found that the p38 MAPK pathway plays a positive role in BMP-induced *Osx* expression (157). In this study, all osteogenic genes, which were upregulated

when F1- and M1-MDSCs were stimulated with BMP4, saw their expression decrease when p38 MAPK was inhibited with SB203580. This decrease was also observed in *Smad6* gene expression, an inhibitory molecule of Smad signaling, suggesting that the p38 MAPK pathway may play a role in the expression of this gene. Since few studies have investigated the effect of p38 MAPK on *Smad6* gene expression, it may be interesting to further investigate this relationship in MDSCs. Overall, these results suggest that the p38 MAPK pathway is necessary for the osteogenic differentiation of MDSCs.

This study was the first step towards understanding how BMP4 signals in MDSCs. It demonstrated that the PI3K pathway and more specifically the p38 MAPK pathway play an important role in the osteogenic differentiation of MDSCs (Figure 3-13). On the other hand, the ERK pathway was shown to have an inhibitory role (Figure 3-13). The osteogenic differentiation of MDSCs may involve the integration of multiple signaling pathways and understanding how these pathways affect differentiation may have important implications in the development of stem cell-based therapies for bone tissue engineering. For example, the inhibition of the ERK pathway has been used to increase the osteogenic differentiation of poorly responsive human MSCs (86). This may be an interesting approach for low osteogenic cells such as F1-MDSCs. In fact, by blocking their ERK activity, these cells had a significant increase in ALP activity. Future studies may involve preconditioning MDSCs with the ERK pathway inhibitor to increase their osteogenic differentiation in vitro and possibly enhancing their ability to form bone in vivo. Interestingly, the ERK inhibitor PD98059 has been used in vivo. The inhibitor was administered 30 minutes before brain trauma and led to a reduced cortical lesion 7 days after the initial brain trauma (158). Hence, it is possible to further pursue these studies *in vivo.*

As well, to better understand the differences between F1- and M1-MDSCs in terms of their response to BMP4 stimulation, future work should also investigate the BMP receptors on the surface of these cells. It will be interesting to determine whether M1-MDSCs have more BMP receptors than F1-MDSCs.

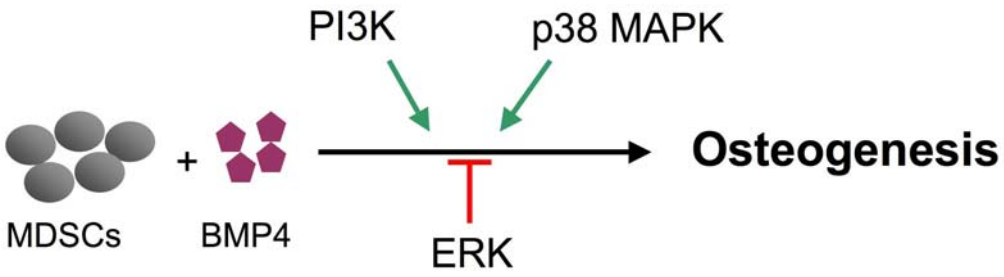

Figure 3-13. Overview of the pathways involved in the BMP4-induced osteogenesis of MDSCs.
The PI3K and p38 MAPK pathways play a positive role in the BMP4-induced osteogenesis of MDSCs, while the ERK pathway is a negative regulator of osteogenesis.

3.5 CONCLUSION

In summary, BMP4 stimulation of MDSCs leads to the activation of the Smad pathway as well as the PI3K, ERK and p38 MAPK pathways. The PI3K and p38 MAPK pathways play a positive role in osteogenesis, while the ERK pathway is a negative regulator of osteogenic differentiation in MDSCs (Figure 3-13). A further understanding of the molecular interaction between the Smad and non-Smad pathways will clarify BMP4-induced osteogenic differentiation in MDSCs and may lead to the identification of therapeutic targets for bone tissue engineering.

4.0 EFFECT OF BMP4 AND TGF-β1 ON THE CHONDROGENESIS OF FEMALE AND MALE MDSCS

Articular cartilage has the important role of providing an almost frictionless surface for joint movement. It can be affected by traumatic injuries and age-related diseases, and due to its limited ability for self-repair after trauma; its treatment remains a current challenge for orthopaedic surgeons. This is especially true of partial thickness defects that do not penetrate the subchondral bone, thus preventing cells from coming into the defect and helping the repair process (159). Numerous preclinical and clinical studies have attempted to promote cartilage repair with procedures such as autologous chondrocyte transplantation (17, 18), microfracture chondroplasty (15, 160), abrasion arthroplasty (161), and osteochondral grafts (162, 163). Although successful, some treatments are plagued with fibrous tissue formation, while others require removing articular cartilage from non load-bearing areas, thus limiting the amount that can be obtained. Experimental approaches have increasingly focused on regenerative medicine techniques, with a particular emphasis on stem cell-based therapies.

Numerous cell sources have been studied for their ability to undergo chondrogenic differentiation in vitro. Among the most popular are MSCs (30, 164-167), although other sources have included stem cells harvested from adipose tissue (34, 36-38, 168-170), human placenta (45), umbilical cord blood (171-173) and skeletal muscle (56, 82, 174).

To develop efficient cell-based therapies, it will be imperative to understand the various signaling factors that induce chondrogenesis and that will maintain the chondrogenic phenotype. Of particular interest are growth factors from the TGF-β family, as they have been implicated in articular cartilage development. TGF-β1 has promoted the chondrogenic differentiation of limb embryonic mesenchymal cells (175). As well, MSCs have been induced towards chondrogenic differentiation in vitro with cultures containing TGF-β1, -β2 or -β3 (165-167, 176). As well, the combination of TGF-β3 with BMP-2, -4 or -6 has also enhanced the chondrogenic differentiation of MSCs, suggesting that BMPs may also be important for articular cartilage tissue engineering (177-179). Although current cell-based approaches rely on growth factor stimulation to undergo chondrogenesis, when implanted into articular cartilage defects, the cells may not be exposed to sufficiently high concentrations of growth factor to lead to optimal articular cartilage repair (180). In this case, gene therapy may be a viable alternative that provides a sustained release of the growth factors needed to induce chondrogenesis, and lead to a better repair of articular cartilage (181).

Recently, genetic engineering of MDSCs with BMP4 has been used for articular cartilage repair (56). After implantation of BMP4-expressing MDSCs into full-thickness articular cartilage defects made in the rat knee, donor cells could be seen within the defect and colocalized with Col II, a marker of chondrogenic differentiation. In addition, the repaired tissue in the defect was well integrated with the adjacent normal articular cartilage for up to 24 weeks. The group that received control MDSCs did not display such a high degree of regeneration, suggesting that MDSCs and BMP4 are beneficial for cartilage repair. Also, this study was performed with two populations of F-MDSCs, and the ability of MDSCs to undergo chondrogenic differentiation in vitro when stimulated with BMP4 and/or TGF-β1 was not thoroughly investigated. Hence, M-

MDSCs have thus far not been tested for their ability to differentiate towards the chondrogenic lineage. As well, it has recently been reported that F- and M-MDSCs display different capacities for skeletal muscle regeneration, suggesting that sex-related differences may exist in the dfferentiation potential of MDSCs (110).

The aim of this study was to evaluate the effect of BMP4 and TGF-β1 on the chondrogenic differentiation of both F- and M-MDSCs in vitro. Three F-MDSC and 3 M-MDSC populations were cultured as pellets in chondroinductive medium supplemented with BMP4, TGF-β1, or both. In order to also test the feasibility of using gene therapy to induce chondrogenic differentiation, the MDSCs were transduced with a BMP4 retrovirus and cultured in chondroinductive medium in the presence or absence of TGF-β1. Chondrogenic differentiation was assessed by histology and gene expression. Results from this study may provide valuable information on the growth factors necessary for the chondrogenic differentiation of MDSCs, a potential cell source for articular cartilage tissue engineering.

4.1 MATERIALS AND METHODS

4.1.1 Isolation and culture of MDSCs

MDSCs were isolated from 3-week-old C57BL/10J normal mice using a modified preplate technique (51). Animals were anatomically sexed at the time of isolation and the sex of the cells was later confirmed by FISH analysis. Three F-MDSC populations (F1-, F2- and F3-MDSC) and 3 M-MDSC populations (M1-, M2-, M3-MDSC) were used in this study. Cells were cultured on collagen coated flasks in phenol red-free PM consisting of DMEM (Invitrogen) supplemented

with 110 mg/L sodium pyruvate (Sigma-Aldrich), 584 mg/L L-Glutamine, 10% FBS, 10% HS, 1% penicillin/streptomycin (all from Invitrogen), and 0.5% chick embryo extract (Accurate Chemical Co.) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were trypsinized and replated at 225 cells/cm^2 until a sufficient number of cells was available for the chondrogenic assays.

4.1.2 Retroviral transduction of MDSCs to express BMP4

All 6 MDSC populations were retrovirally transduced with a vector expressing human BMP4 and GFP (76). MDSCs were incubated with the retrovirus overnight at a dilution of 1:1 with PM in the presence of 8 μg/ml polybrene. The 3 female populations will be referred to as F1-, F2- and F3-MDSC-BMP4, while the 3 male populations will be referred to as M1-, M2- and M3-MDSC-BMP4.

4.1.3 Pellet culture

Both untransduced and transduced MDSCs were used in a pellet culture assay, which is a standard assay to study chondrogenic differentiation in vitro (165). A total of 2.5 x 10^5 cells were placed in a 15-ml conical polypropylene tube, centrifuged at 500*g* for 5 minutes and resuspended in 500 μl of chondrogenic medium (CM) that contained phenol-red free DMEM supplemented with 110 mg/L sodium pyruvate, 584 mg/L L-Glutamine, 1% penicillin/streptomycin, 10^{-7} M dexamethasone, 50 μg/ml ascorbate-2-phosphate, 40 μg/ml proline, and 1% BD™ ITS+Premix (Becton-Dickinson; consisting of 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml BSA, and 5.35 μg/ml linoleic acid). Cells then underwent another

round of centrifugation. The pellets made with MDSCs were cultured in either CM supplemented with BMP4 (50 ng/ml, R&D Systems) or CM supplemented with both BMP4 (50 ng/ml) and TGF-β1 (10 ng/ml, R&D Systems). The pellets made with MDSC-BMP4 cells were cultured in either CM only or CM supplemented with TGF-β1 (10 ng/ml). All pellets were cultured for 14 or 21 days and the medium was changed every 2–3 days.

4.1.4 Histology

After 14 and 21 days of culture, pellets were fixed in 10% neutral-buffered formalin for a minimum of 24 hours at RT. After dehydration in graded alcohols, the pellets were paraffin embedded and cut at a thickness of 5 μm using a Reichert-Jung Biocut 2030 (Leica Instruments, UK). Sections were then stained with Alcian Blue to detect the presence of sulfated polysaccharides in the ECM or Safranin O to detect the presence of proteoglycans. Standard protocols for each of these stains were followed. Images were captured with QCapture software using a Nikon Eclipse E800 microscope equipped with a Retiga EXi digital camera.

4.1.5 Quantitative real-time PCR

qPCR was used to evaluate the expression of genes involved in chondrogenesis. RNA was isolated from MDSC-BMP4 pellets cultured for 21 days in CM supplemented with TGF-β1 (10 ng/ml) and from M1-MDSC-BMP4 pellets cultured for 21 days in CM only. To isolate total RNA, 4 pellets from each group were pooled together, rinsed in PBS and incubated in 500 μl of RNA later (Qiagen) for 1 hour at room temperature before homogenization with a Turrax T8 homogenizer. The suspension was then passed through a QIAshredder spin column (Qiagen)

followed by RNA isolation with the RNeasy kit (Qiagen). qPCR analysis was performed with Taqman® One-step RT-PCR Master Mix (Applied Biosystems) as described previously (111). RNA samples (1 μl) were added to sequence-specific primers and Taqman® probes (200 nM per 10-μl reaction). All target genes were normalized to *18S* (primers from Applied Biosystems). The sequences of the target gene primers (*Sox9*, *Col II* and *Collagen Type X* (*Col X*)) can be found in Appendix B. All probes were labeled with FAM as the 5' reporter dye and TAMRA as the 3' quencher dye. Three samples from each group were isolated (each containing 4 pellets pooled together) and qPCR assays on these samples were carried out in triplicate on an ABI Prism 7900HT sequence detection system in the core facility of the Genomics and Proteomics Core Laboratories of the University of Pittsburgh. Data was analyzed using SDS 2.1 Software from Applied Biosystems.

4.1.6 Statistical analysis

Data are reported as mean ± SEM and analyzed using a one-way ANOVA (SigmaStat, Jandel Corporation). Pairwise post-hoc analysis was performed using the Dunn's test. *P* values of less than 0.05 were considered significant.

4.2 RESULTS

4.2.1 Chondrogenesis in MDSC pellet cultures

To determine whether BMP4 or TGF-β1 alone were sufficient for chondrogenic differentiation, MDSCs were cultured as pellets in CM only, CM supplemented with BMP4 at a dose of 50 ng/ml or CM supplemented with TGF-β1 at a dose of 10 ng/ml. The pellets were also cultured in CM supplemented with both BMP4 (50 ng/ml) and TGF-β1 (10 ng/ml) to establish whether combining the two growth factors had a greater effect on chondrogenesis than administration of each factor separately. Cell pellets were obtained by centrifugation and first appeared as a flattened sheet of cells at the bottom of the tube. By the following day, the edges of the flattened sheet began to fold up and towards the center of the tube. In general, 3 days after centrifugation, the pellet was a spherical mass, and remained this way for the rest of the culture period. Histological evaluation of chondrogenic differentiation was based on staining for Alcian Blue. Representative images of the 4 groups are shown in Figure 4-1. MDSC pellets cultured in CM only did not display matrix formation, nor did they stain highly for Alcian Blue. Addition of BMP4 to the CM led to lightly positive Alcian Blue staining, with some cells within lacunae, a morphology associated with chondrocytes. A similar staining pattern was seen when the CM was supplemented with TGF-β1. However, addition of both BMP4 and TGF-β1 to the CM produced high levels of Alcian Blue positive matrix. The Alcian Blue positive area in this group represents the typical appearance of chondrocytes, where cells can be found in lacunae surrounded by matrix. Interestingly, this highly chondrogenic area was not seen throughout the pellet, but rather as a condensation that was forming on one side of the pellet. This initial study prompted the

investigation of the effect of TGF-β1 on the BMP4-induced chondrogenic differentiation of MDSCs.

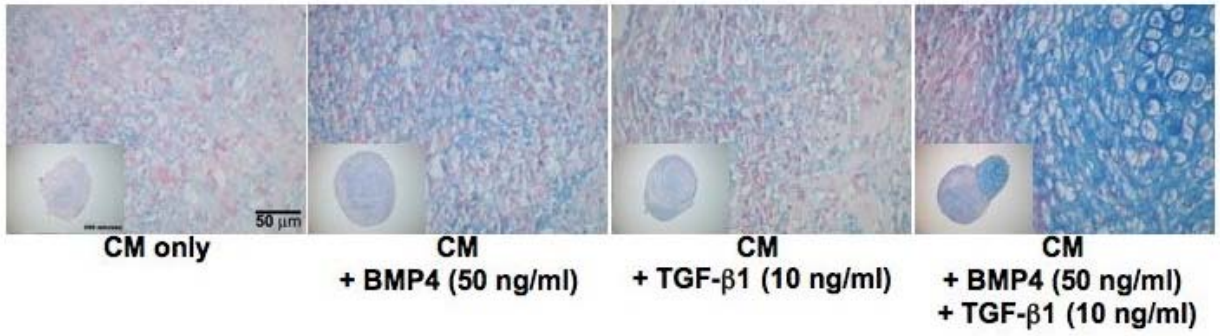

Figure 4-1. Effect of growth factors on the chondrogenesis of MDSC pellets.
MDSCs were cultured as pellets for 14 days in CM only, CM + BMP4 (50 ng/ml), CM + TGF-β1 (10 ng/ml), or CM + BMP4 (50 ng/ml) + TGF-β1 (10 ng/ml). Shown are representative images of sections that were stained with Alcian Blue. Scale bar on inset image = 250 μm.

4.2.2 Effect of TGF-β1 on the chondrogenesis of F- and M-MDSC pellets stimulated with BMP4

F- and M-MDSCs were cultured as pellets for 14 and 21 days before being processed for histology. One group received CM supplemented with BMP4 (Figure 4-2), while the other received CM supplemented with both BMP4 and TGF-β1 (Figure 4-3). The majority of the cells, except for F1-MDSCs, generated a solid 3-dimensional tissue construct that could easily be processed for histology. The pellets made with F1-MDSCs did not generate a solid pellet, and easily fell apart during medium changes and histological processing. F-MDSCs stimulated with BMP4 displayed small areas of Alcian Blue positive staining at 14 and 21 days of culture (Figure 4-2, left panel). M-MDSCs cultured in the same conditions displayed more Alcian Blue staining,

especially at 21 days of culture (Figure 4-2). The population with the greatest degree of Alcian Blue staining was the M1-MDSC population at 21 days of culture.

In general, pellets treated with CM supplemented with both BMP4 and TGF-β1 (Figure 4-3) displayed a more pronounced chondrogenic differentiation than those stimulated with BMP4 only (Figure 4-2). Although F1-MDSCs did not show any positive Alcian Blue staining at 14 and 21 days (Figure 4-3, left panel), F2- and F3-MDSC pellets displayed numerous cells within lacunae and some areas of positive Alcian Blue matrix at these same time points (Figure 4-3, left panel). However, these chondrogenic areas were not present throughout the whole section of the pellet. On the other hand, M1-MDSCs produced a highly dense culture, with Alcian Blue positive areas throughout the pellet, and this was evident at both 14 and 21 days of culture when TGF-β1 was added to BMP4 (Figure 4-3, right panel). The areas of large matrix deposition and hypertrophic chondrocytic cells were concentrated all along the peripheral zone of the M1-MDSC pellets. M2- and M3-MDSC pellets also contained areas of Alcian Blue positive staining. There was enhanced matrix deposition and lacunae containing cells at 21 days of culture when compared to 14 days. These results suggest that BMP4 and TGF-β1 have a synergistic effect on the chondrogenic differentiation of F- and M-MDSCs. Although there was not a clear difference between F- and M-MDSCs in terms of their chondrogenic differentiation, it was determined that M1-MDSCs have a high chondrogenic capacity when compared to all MDSC populations tested.

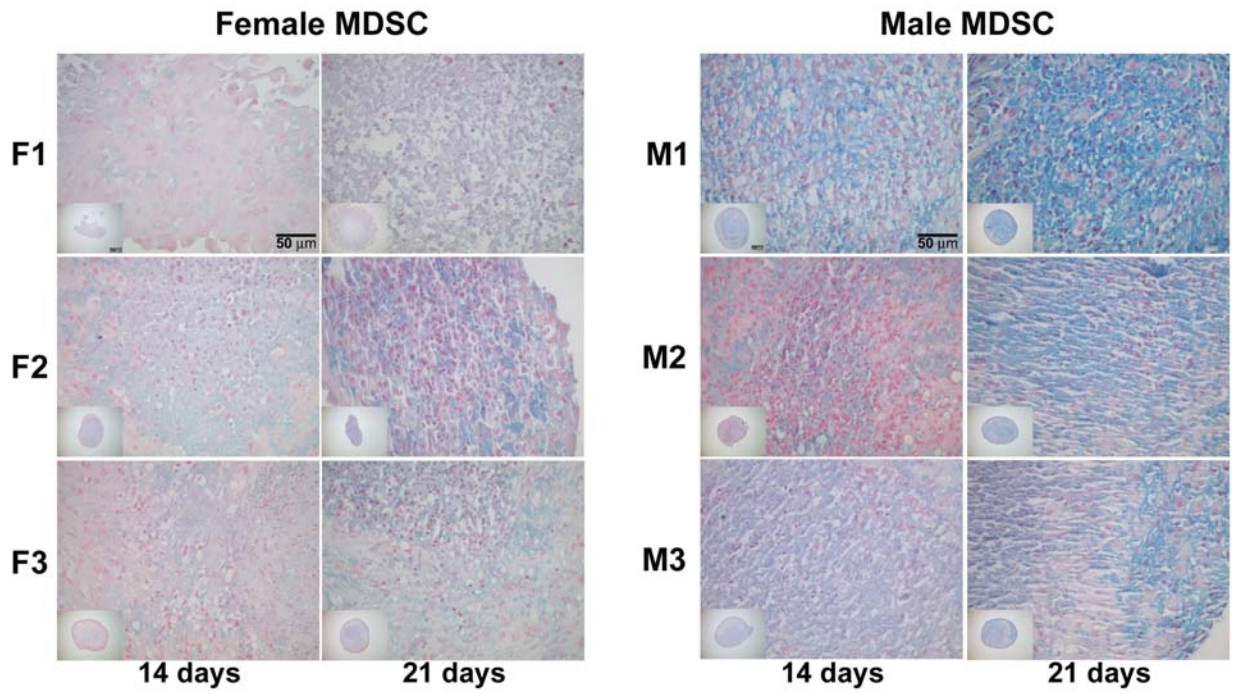

Figure 4-2. Alcian Blue staining of MDSC pellets cultured in CM supplemented with BMP4 (50 ng/ml) for 14 and 21 days. Scale bar on inset image = 250 µm.

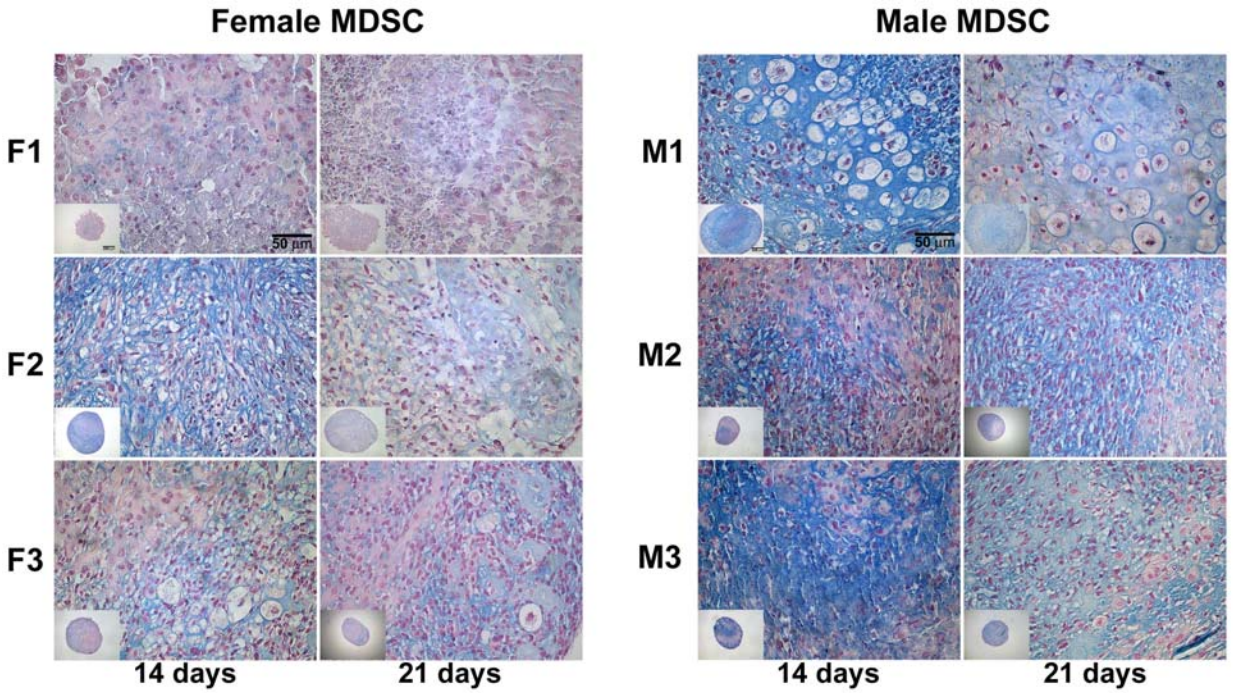

Figure 4-3. Alcian Blue staining of MDSC pellets cultured in CM supplemented with BMP4 (50 ng/ml) and TGF-β1 (10 ng/ml) for 14 and 21 days. Scale bar on inset image = 250 µm.

4.2.3 Effect of TGF-β1 on the chondrogenesis of MDSC-BMP4 pellets

To determine whether BMP4 could be provided through genetic engineering of MDSCs and whether TGF-β1 also enhances their chondrogenesis, all 6 MDSC populations were retrovirally transduced with a BMP4 retrovirus in parallel and cultured as pellets in CM only (Figure 4-4) or CM supplemented with TGF-β1 (Figure 4-5). It was determined that BMP4 transduction enhanced the chondrogenesis of all MDSC populations tested, when compared to the previous experiment with BMP4 stimulation (Figures 4-2 and 4-3). The F1-MDSC population, which did not display any Alcian Blue positive staining when stimulated with BMP4 (Figure 4-2) or BMP4 and TGF-β1 (Figure 4-3), was found to have Alcian Blue positive areas as early as 14 days when genetically engineered to express BMP4 and cultured in CM only (Figure 4-4, left panel) or CM supplemented with TGF-β1 (Figure 4-5, left panel). The MDSC-BMP4 cells displaying the greatest degree of chondrogenesis when cultured in CM only, as indicated by positive Alcian Blue staining, were F2-, M1- and M3-MDSC-BMP4 cells. As before, only pellets made with M1-MDSC-BMP4 cells displayed cells within lacunae throughout the whole area of the pellet.

Addition of TGF-β1 to the CM also led to chondrogenic differentiation of all populations tested. It substantially enhanced the chondrogenesis of M1-MDSC-BMP4 pellets (Figure 4-5, right panel), which displayed extensive matrix deposition that stained highly for Alcian Blue and contained numerous cells within lacunae. In fact, the pellets made with M1-MDSC-BMP4 cells and cultured in CM with TGF-β1 are the only pellets that stained positive for Safranin O, which detects the presence of proteoglycans. This was evident at both 14 and 21 days (Figure 4-6A and B, respectively). These results suggest that gene therapy may be useful for articular cartilage tissue engineering and TGF-β1 may promote BMP4-induced chondrogenesis.

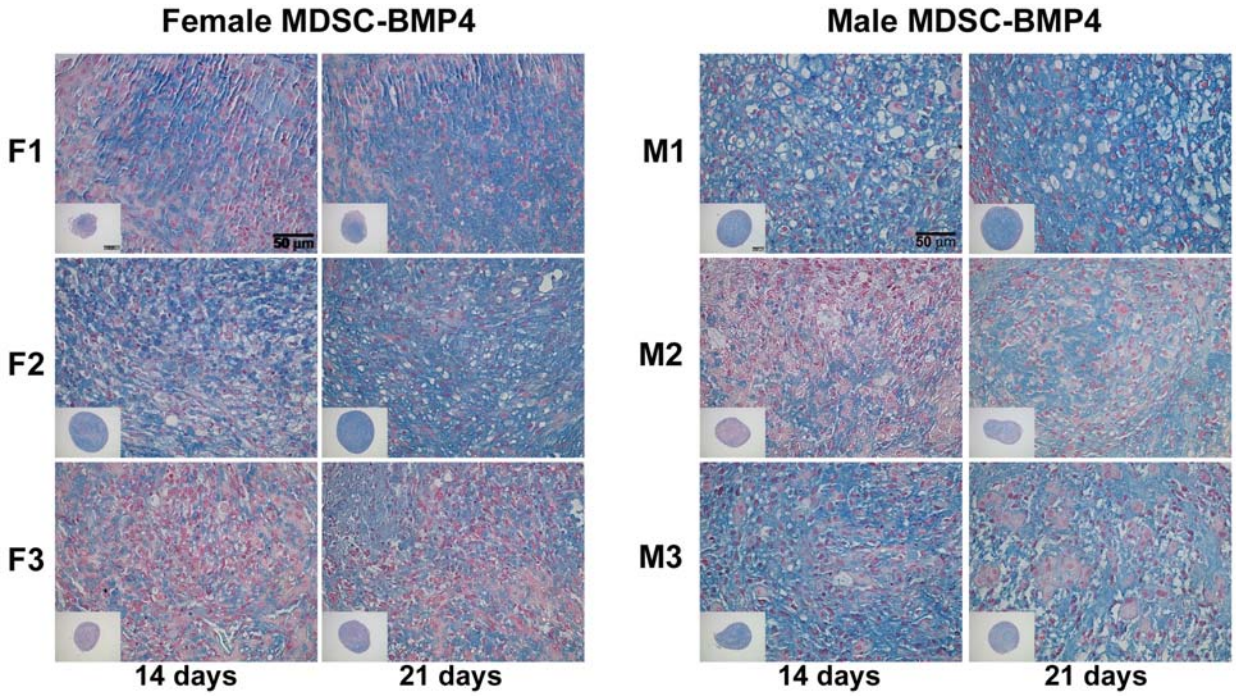

Figure 4-4. Alcian Blue staining of MDSC-BMP4 pellets cultured in CM for 14 and 21 days. Scale bar on inset image = 250 µm.

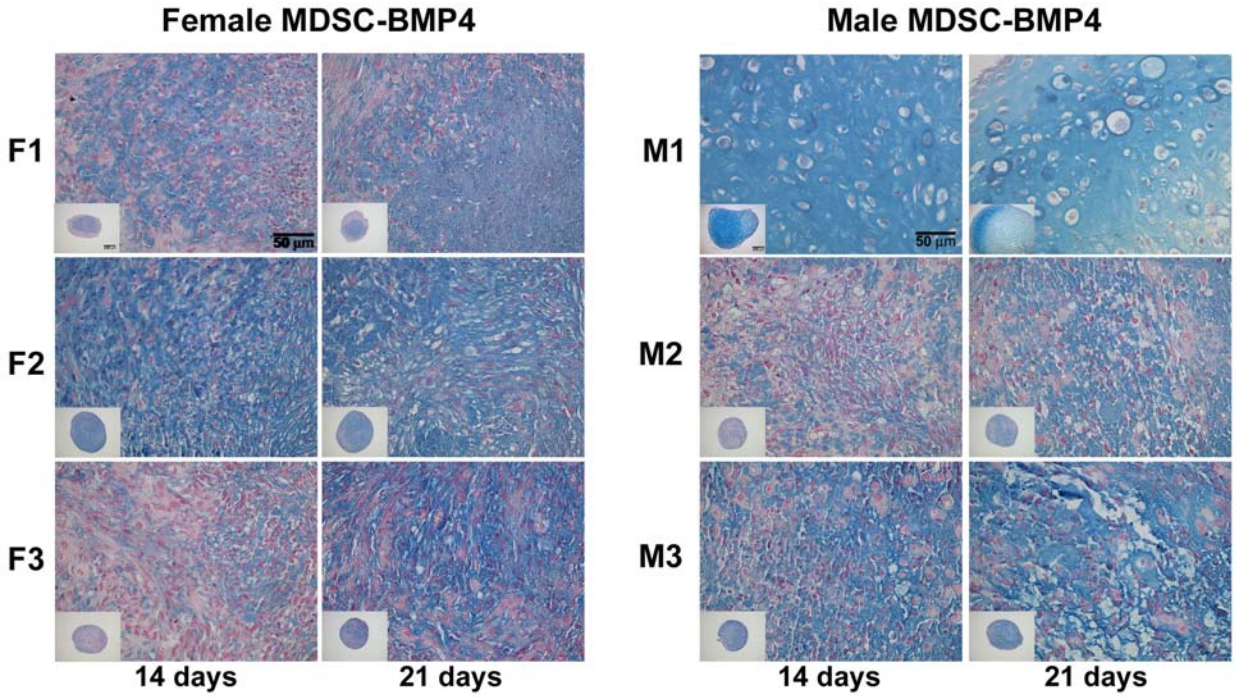

Figure 4-5. Alcian Blue staining of MDSC-BMP4 pellets cultured in CM supplemented with TGF-β1 (10 ng/ml) for 14 and 21 days. Scale bar on inset image = 250 µm.

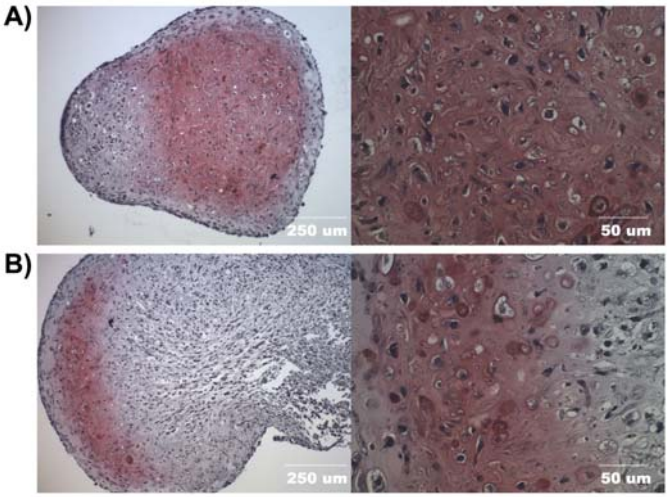

Figure 4-6. (A) Safranin O staining of M1-MDSC-BMP4 pellets cultured in CM supplemented with TGF-β1 for 14 days and (B) 21 days. The right panel is a higher magnification of the left panel.

4.2.4 Effect of TGF-β1 on gene expression during in vitro chondrogenesis of M1-MDSC-BMP4 cells

Due to their superior ability for chondrogenesis in pellet culture, M1-MDSC-BMP4 cells were chosen to further study the effect of TGF-β1 on the chondrogenesis of MDSCs. To do so, M1-MDSC-BMP4 pellets were cultured in CM only or CM supplemented with TGF-β1 for 21 days and RNA was isolated for qPCR analysis. The expression of *Sox9*, *Col II* and *Col X* were determined as they are implicated in chondrogenesis. Culturing the pellets in the presence of TGF-β1 led to a 3.86 ± 1.84 fold increase in the expression of *Sox9*, a key transcription factor in chondrogenesis, when compared to the same pellets cultured in CM only (Figure 4-7). The expression of *Col II* and *Col X* was also increased when TGF-β1 was added to the CM (91.08 ± 64.63 and 6.56 ± 2.41, respectively) (Figure 4-7). The variability seen in the *Col II* expression was due to one sample displaying a mean fold increase of 155.06 over CM only. Although some

variability was present between samples, these results suggest that TGF-β1 promoted the expression of chondrogenic genes in M1-MDSC-BMP4 pellets when compared to CM only.

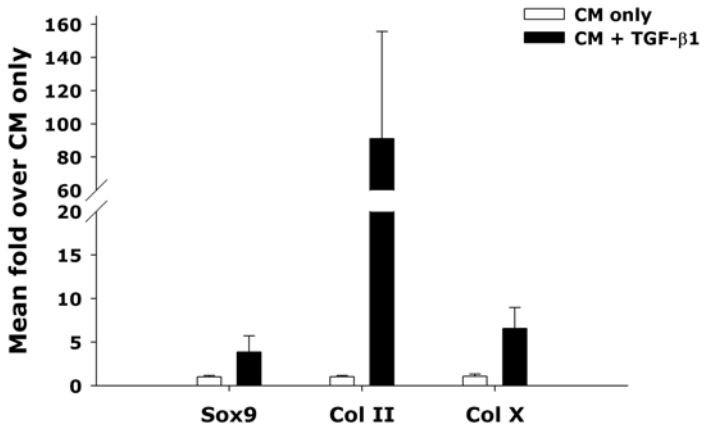

Figure 4-7. Expression of *Sox9, Col II,* and *Col X* in M1-MDSC-BMP4 pellets cultured in CM only or CM supplemented with TGF-β1 (CM + TGF-β1) for 21 days.

4.2.5 Gene expression during in vitro chondrogenesis of MDSC-BMP4 cells stimulated with TGF-β1

Since addition of TGF-β1 to the CM was shown to be the best chondroinductive condition for M1-MDSC-BMP4 cells (Figure 4-7), all 6 populations of MDSC-BMP4 cells were cultured as pellets in CM supplemented with TGF-β1 for 21 days and chondrogenic gene expression was analyzed with qPCR. All populations expressed some level of *Sox9*, with F3- and M1-MDSCs having higher levels than the other MDSC populations (Figure 4-8A). As expected from the histology results presented earlier, the expression of *Col II* was highest in M1-MDSC-BMP4 pellets (Figure 4-8B). F2- and M2-MDSC-BMP4 pellets expressed similar levels of *Col II*, while F1-, F3-, and, M3-MDSC-BMP4 pellets expressed low levels (Figure 4-8B). The expression of *Col X*, which is often associated with hypertrophic chondrocytes, was highly expressed in M1-

MDSC-BMP4 pellets (Figure 4-8C). *Col X* expression was also observed in F1-, M2- and M3-MDSC-BMP4 pellets, but was not detectable in F2- and F3-MDSC-BMP4 pellets (Figure 4-8C). From these results, it is evident that MDSC-BMP4 cells do not undergo chondrogenic differentiation at the same rate, or to the same extent. Although there is a degree of variability among the different populations tested, the M1-MDSC-BMP4 cells were again found to display the highest chondrogenic capacity, based on their high expression of *Col II*.

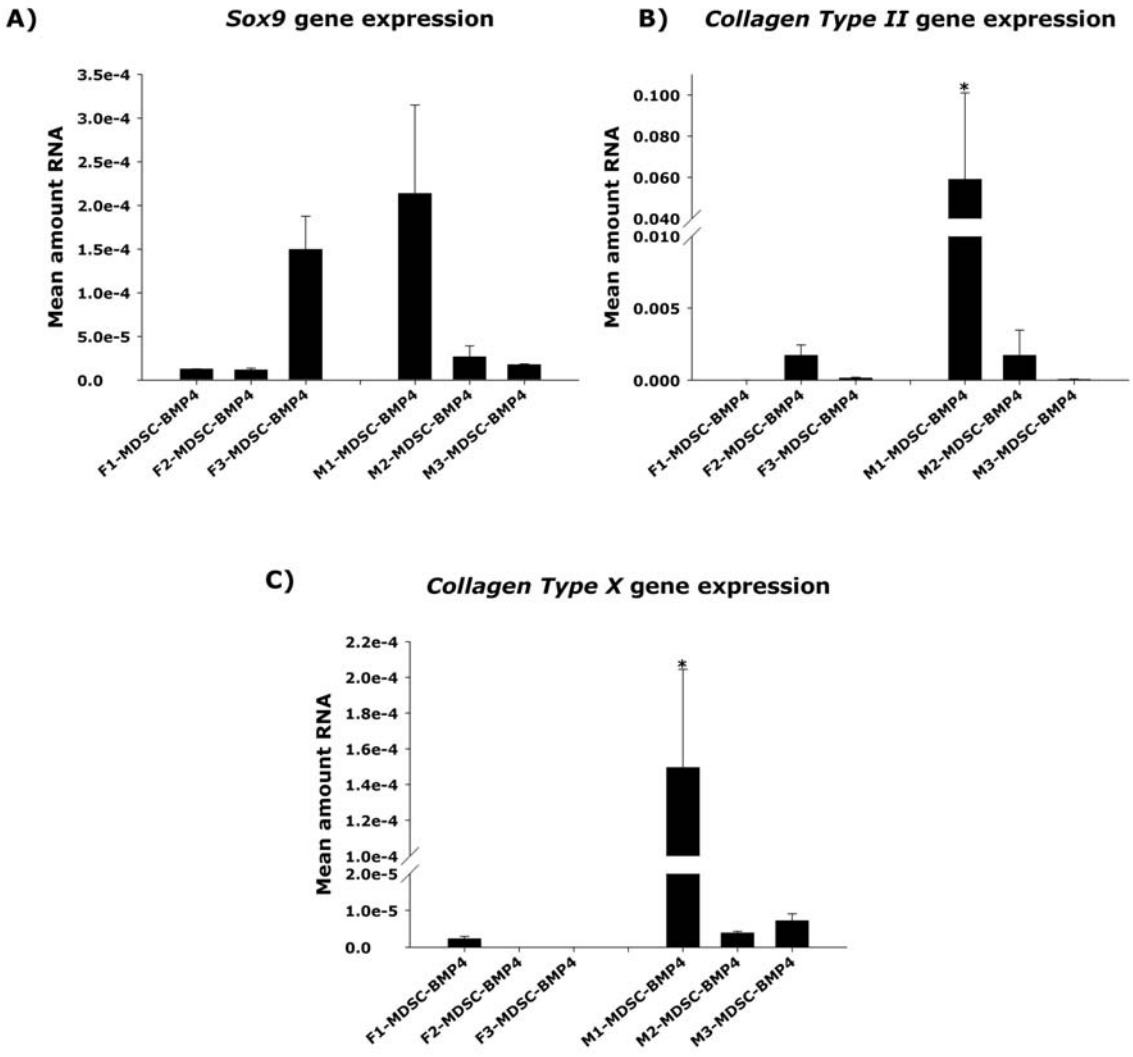

Figure 4-8. Gene expression in MDSC-BMP4 pellets cultured in CM supplemented with TGF-β1 for 21 days.
* indicates a significant difference between M1-MDSC-BMP4 and F1-MDSC-BMP4 ($P < 0.05$).

4.3 DISCUSSION

This study investigated the effects of BMP4, TGF-β1 or a combination of both on the chondrogenic differentiation of MDSCs in a pellet culture system in vitro. It was shown that MDSCs isolated from both female and male mice undergo chondrogenic differentiation when cultured as a pellet in chondroinductive conditions containing BMP4. This chondrogenesis was further enhanced when TGF-β1 was added to BMP4. As well, it was shown that genetically engineering MDSCs to express BMP4 had a greater chondrogenic effect than BMP4 stimulation alone. Lastly, unlike the results presented in Chapter 2 with regards to the osteogenic differentiation of MDSCs, or those recently published about the skeletal muscle regeneration capacity of MDSCs (110), cell sex did not seem to influence the ability of MDSCs to undergo chondrogenic differentiation.

BMP4 has been implicated in chondrogenesis in vitro and in vivo (68, 182-185). Previous reports have also suggested that TGF-β1 can stimulate mesenchymal cell repair of full-thickness articular cartilage defects (186). The enhanced chondrogenesis seen when BMPs and TGF-βs are combined has also been reported in studies using rabbit and human MSCs (177-179, 187, 188). As well, TGF-β1 combined with BMP2 improved the in vitro chondrogenesis of rat periosteal cells (189). It has also been reported that the combined effect of TGF-β3 and BMP2 was greater than each growth factor administered separately (190). Kuroda *et al*. have previously reported that the addition of TGF-β1 to the CM in pellet cultures of F-MDSCs led to a greater Alcian Blue staining than pellets cultured in CM only (56). It was also shown that genetically

engineering MDSCs to express BMP4 increased chondrogenesis in vitro compared to untransduced cells in the same culture conditions. Hence, the observed chondrogenesis of MDSCs in this study is in accordance with the chondrogenesis previously observed in vitro with F-MDSCs and it further confirms a synergistic effect of TGF-β1 and BMP4 on chondrogenic differentiation. Cell-based therapies for articular cartilage repair involving MDSCs may need to incorporate both these growth factors.

Methods to deliver these growth factors to articular cartilage defects may include direct administration of the recombinant protein, having it slowly released by a scaffold, or employing gene therapy. The usefulness of gene therapy for articular cartilage repair has previously been shown with MDSCs (56), as well as with MSCs (191, 192) and fibroblasts (193, 194). Gene therapy in vitro has also been reported (195, 196). In fact, adenoviral-mediated delivery of TGF-β1, BMP2 and insulin-like growth factor-1 (IGF-1) has led to the chondrogenesis of rabbit MSCs in pellet culture, yet the level of transgene expression, its duration, and the viral load influenced the outcome (196). Interestingly, this study also reported that the delivery of BMP2 and TGF-β1 to the culture medium by adenoviral-mediated expression was superior to the delivery of the recombinant forms of these 2 proteins for chondrogenesis. This is in accordance with the current study, where MDSCs retrovirally transduced to express BMP4 displayed a greater chondrogenesis than MDSCs stimulated with the BMP4 protein. These results suggest that a constant presence of the growth factors, at the correct concentrations, provides an optimal environment for the cells to undergo chondrogenesis. Although gene therapy may provide such an environment, it should also be noted that drug-releasing biomaterials are also being extensively studied for this purpose. The release of TGF-β1 has been demonstrated with various polymeric systems, and some groups have shown improved articular cartilage repair in vivo

(197-200). Hence, future studies with MDSCs may benefit from using gene therapy for the delivery of growth factors and also looking into combining MDSCs with polymers that release BMP4 and TGF-β1.

Analyzing the expression of chondrogenic genes provides a detailed account of the state of chondrogenic differentiation. In this study, the expression of the genes *Sox9*, *Col II*, and *Col X* was measured by qPCR. The transcription factor SOX9 has been reported to play a key role in chondrogenesis (12), as it drives the expression of *Col II*, *Collagen Type 9*, *Collagen Type 11*, and *aggrecan* genes (201-203). All MDSCs tested expressed some level of *Sox9*, suggesting that they all have the potential to undergo chondrogenesis, and this was seen by histological evaluation of the pellets with Alcian Blue. The *Col II* gene, another key marker of chondrogenesis, was also expressed by all MDSCs, although to varying degrees. Interestingly, the cells expressing the highest level of *Col II* were also the ones showing the greatest chondrogenic differentiation in the histological analysis. The correlation between histology and gene expression was not as evident for the other cell populations, but seeing as both these assays provide valuable information on chondrogenesis, it is recommended that both be used in future chondrogenic differentiation assays.

The present study investigated the chondrogenesis of both F- and M-MDSCs. Based on the 6 MDSC populations tested; cell sex did not appear to have a significant effect on the in vitro chondrogenic differentiation of MDSCs. In fact, a certain degree of variability among the different populations tested was evident in this study. This variability in different populations of F- and M-MDSCs has been reported previously (110). Further investigation is needed to understand the variability observed in the current study, but once differences are understood,

they may facilitate the identification of markers for the rapid isolation of optimal chondroprogenitor cells.

The pellet culture system used in this study is the standard assay for chondrogenesis in vitro, however there is not a clear correlation between in vitro and in vivo chondrogenesis. It was previously shown that F-MDSCs transduced to express BMP4 displayed chondrogenic differentiation in vitro, in a pellet culture, and enhanced articular cartilage repair in vivo when implanted into the defect with fibrin glue (56). In the current study, it may be suggested that M1-MDSCs will lead to optimal healing of an articular cartilage defect in vivo when compared to cells such as F1-MDSCs, which did not readily undergo chondrogenic differentiation. However, M1-MDSC-BMP4 cells displayed extensive hypertrophy. This was evident by the large lacunae within the matrix and the high expression of *Col X* after 21 days of culture. High expression of *Col X* has also been reported in long-term pellet cultures of MSCs in CM containing TGF-β (165, 167, 176, 204). Seeing as hypertrophic chondrocytes indicate the initiation of calcification, these results may be concerning when thinking of using the cells for implantation in vivo. However, the differentiation will be highly dependent on the microenvironment in which they find themselves, and implanting such cells into an articular cartilage defect, devoid of vascularity may further promote articular cartilage formation. Hence, future studies that compare F1- and M1-MDSCs in an articular cartilage defect model are needed to further determine whether the in vitro pellet culture system is a good screening tool to identify cells that will lead to high tissue regeneration in vivo.

4.4 CONCLUSION

In conclusion, the findings presented in this chapter indicate that F- and M-MDSCs have the ability to undergo chondrogenic differentiation in vitro. This process does not appear to be influenced by cell sex, although a greater number of populations may need to be tested in vitro an in vivo to confirm this. As well, BMP4 and TGF-β1 were found to be key regulators of the chondrogenic differentiation of MDSCs. Hence, MDSCs and the combined use of BMP4 and TGF-β1 should be further pursued as a potential therapeutic option for articular cartilage tissue engineering.

5.0 THE EFFECT OF MECHANICAL STIMULATION ON THE PROLIFERATION AND OSTEOGENIC DIFFERENTIATION OF MDSCS

5.1 INTRODUCTION

Stem cells have emerged as a key component of regenerative medicine, where they can be used for transplantation to ameliorate diseased or damaged tissue. Among these, skeletal MDSCs have been shown to be a potential cell source for orthopaedic tissue engineering (150). As seen in previous chapters, MDSCs under the influence of BMP4 can undergo osteogenic and chondrogenic differentiation (Chapters 2-4). These cells are also very promising since skeletal muscle is an easily accessible source of tissue from which to obtain a biopsy and isolate a large number of cells. To increase the therapeutic potential of stem cells, two aspects may need to be further investigated. First, for stem cells to be used in transplantation studies, they may need to be expanded to obtain clinically relevant numbers. As well, it may be valuable to develop methods that could optimize the differentiation potential of stem cells.

MDSCs isolated from mouse skeletal muscle have been expanded to over 200 population doublings with no loss of progenitor characteristics (60). As well, MDSCs have been successfully expanded with the use of epidermal growth factor, basic fibroblast growth factor, IGF-1, and stem cell factor (205). Although successful, this may not be a cost-efficient method of expansion. An alternative expansion technique for stem cells may be the use of mechanical

stimulation (91). In fact, human pulmonary epithelial cells, which received an equibiaxial strain at an elongation of 20% and a frequency of 1 Hz, saw an increase in their cellular proliferation during strain when compared to unstrained cells (206). As well, an equibiaxial strain of 10, and 15% elongation at a frequency of 1 Hz also increased the proliferation of bone marrow stromal cells (93). Many studies performed on osteoblasts also suggest that their response to mechanical strain typically involves an increased rate of proliferation (92, 207-211). However, other studies have reported the opposite effect of mechanical stimulation on proliferation. Human osteoblastic periodontal ligament cells displayed a decreased cellular proliferation in response to mechanical strain (212). Human ASCs cultured for 7 days with a 10% uniaxial cyclic strain at 1 Hz also displayed a decrease in cell proliferation during the straining regimen (94). Application of a 3% equibiaxial cyclic strain at 0.25 Hz also inhibited proliferation of human MSCs (88). Interestingly, it has also been reported that mechanical stimulation had no effect on cellular proliferation of human MSCs (213). These studies suggest that mechanical stimulation can affect cellular proliferation, although it may depend on the cell type or straining regimen used.

The effect of mechanical stimulation on the osteogenic differentiation of cells has also been shown to either promote or inhibit the differentiation process. An increase in osteogenic markers as a result of mechanical strain has been reported in hMSCs, rat calvarial osteoblasts, and calcifying vascular cells (88, 99, 100, 213). However, Kaspar *et al*. have also reported the opposite when studying the effect of mechanical stimulation on human osteoblasts (92). They observed a significant increase in the proliferation of human osteoblasts, yet a decrease in their ALP activity and Ocn synthesis following mechanical strain. A significant decrease in the ALP activity of rat MSCs due to mechanical strain has also been reported (214). Interestingly, when culturing human embryonic stem cells with mechanical strain, it was found that the mechanical

stimulus could maintain the cells in an undifferentiated state and it did not affect their pluripotency (96).

Satellite cells, a myogenic cell population distinct from MDSCs, have been subjected to mechanical stimulation in vitro (215). Although they did not display an increase in proliferation once subjected to strain, it was observed that a greater percentage of the cells in the strained group had entered the cell cycle earlier than the unstrained group. To date, it has not been determined whether MDSCs can also be activated by mechanical stimulation. Consequently, the objective of this study was to investigate the effect of mechanical stimulation on the proliferation and osteogenic differentiation of MDSCs. To do so, MDSCs were subjected to either equibiaxial or uniaxial strain. Their response to strain was analyzed by observing cell orientation, proliferation, marker profile, secretion of growth factors and their ability to respond to BMP4.

5.2 MATERIALS AND METHODS

5.2.1 Isolation and culture of MDSCs

MDSCs were isolated from a 3-week-old C57BL/10J male mouse using a modified preplate technique (51). The mouse was anatomically sexed at the time of isolation and the sex of the cells was later confirmed by FISH analysis. Cells were cultured in phenol red-free PM consisting of DMEM (Invitrogen) supplemented with 110 mg/L sodium pyruvate (Sigma-Aldrich), 584 mg/L L-Glutamine, 10% FBS, 10% HS, 1% penicillin/streptomycin (all from Invitrogen), and 0.5% chick embryo extract (Accurate Chemical Co.) at 37°C in a humidified atmosphere of 5% CO_2 in air.

5.2.2 Mechanical stimulation

Cells were plated at a density of 50 000 cells/well on either BioFlex™ or UniFlex™ six-well culture dishes (Flexcell® International Corp., Hillsborough, NC). The plates have flexible silicone rubber bottoms that are coated with Col 1. Both the BioFlex™ (equibiaxial strain) and the UniFlex™ (uniaxial strain) plates have a well area of 9.63 cm^2. However, the wells in the UniFlex™ plate have a rectangular strip across the middle of the well, where the cells attach and experience the uniaxial strain. MDSCs were allowed to attach overnight and the following day; each well received 2 ml of fresh PM. A Flexercell® Tension Plus™ System (FX-4000T, Flexcell® International Corp.) was used to deliver either a 10% equibiaxial strain or a 10% uniaxial strain, both at a frequency of 0.25 Hz (2 seconds on, 2 seconds off) for 24 hours. The culture dishes were put on top of either 25-mm circular loading posts (equibiaxial strain) or Arctangle™ loading posts (uniaxial strain). This allowed the flexible membranes to distend when air vacuum suction was applied to the bottom of the plates. Unstrained (control) plates were cultured in the same manner but did not receive mechanical stimulation.

5.2.3 Cell orientation

At the end of the straining regimen, unstrained and strained cells were fixed with 10% formalin for 5 minutes and washed with PBS. The flexible membranes were cut out from the well and placed on a slide in the same orientation they were in the well. Images of areas on the center of the loading post and on the edge of the loading post were captured with QCapture software using a Nikon Eclipse E800 microscope equipped with a Retiga EXi digital camera.

5.2.4 Cell proliferation

To assess the effect of mechanical strain on cell proliferation, unstrained and strained cells were harvested at the end of the experiment with 0.05% trypsin (Gibco) and counted with a hemocytometer. Replicate wells were left untouched, and remained in the incubator at 37°C in a humidified atmosphere of 5% CO_2 in air for an additional 24 hours after the end of the straining regimen. They were then harvested with 0.05% trypsin and counted with a hemocytometer. This provided data regarding the proliferation of MDSCs during the straining regimen and the percent change in cell number during the 24 hours following mechanical stimulation.

5.2.5 Marker profile analysis

MDSCs harvested from unstrained and strained wells at the end of the mechanical stimulation regimen were labeled to determine the expression of the cell surface proteins CD34 and Sca-1, which are stem cell markers expressed by MDSCs. MDSCs were labeled with rat anti-mouse Sca-1 (phycoerythrin) and CD34 (biotin) monoclonal antibodies. A separate fraction of the cells was treated with isotype control antibodies. Both fractions were then labeled with streptavidin-allophycocyanin and 7-amino-actinomycin D was added to exclude nonviable cells from the analysis. All reagents were obtained from BD Biosciences PharMingen. The percentage of Sca-1 and CD34 cells was determined by flow cytometry with a FACSAria cytometer (BD Biosciences Immunocytometry Systems).

5.2.6 Secretion of growth factors

At the end of the straining regimen, the medium from each well was collected, centrifuged at 2500 rpm for 5 minutes to remove any cellular debris, and the supernatant was transferred to a new tube that was then frozen at -80°C until analysis. Supernatants were assayed for mouse VEGF and mouse BMP2 with commercially available ELISA kits (R&D Systems). The amount of growth factor present in the culture medium was normalized to the number of cells that were collected from the same well.

5.2.7 Osteogenic differentiation after mechanical stimulation

To determine whether mechanical stimulation had an effect on the ability of MDSCs to undergo osteogenic differentiation, unstrained and strained cells were replated at a density of 1500 cells/cm^2 and stimulated with BMP4 (50 ng/ml) for 3 days. The presence of ALP was evaluated by cytochemical staining (AP Kit 86-C, Sigma Diagnostics) and by analysis of ALP enzymatic activity. For ALP enzymatic activity, cells were lysed in 0.1% Triton-X in water and assayed using SIGMA *FAST*™ p-Nitrophenyl phosphate tablets (N-2770; Sigma-Aldrich). ALP activity was normalized per mg protein using the Micro BCA™ protein assay (Pierce) and expressed as nanomoles of p-nitrophenyl liberated per microgram of total cellular protein.

5.2.8 Effect of mechanical stimulation on osteogenesis

In a separate experiment, MDSCs were plated on either BioFlex™ or UniFlex™ six-well culture dishes as described above and allowed to adhere overnight. The following day, MDSCs received

fresh PM supplemented with BMP4 (50 ng/ml) and were subjected to either a 10% equibiaxial strain or a 10% uniaxial strain, both at a frequency of 0.25 Hz for 48 hours. Unstrained (control) plates were cultured in the same manner but did not receive mechanical stimulation. At the end of the straining regimen, cells were lysed in 1 ml of 0.1% Triton-X in water and stored at -20°C until assayed for ALP activity as described above.

5.2.9 Statistical analysis

All experiments were performed three times in triplicate. Data are reported as mean ± standard error of mean. Comparisons between unstrained and strained cells were analyzed with a Student's *t*-test. *P* values of less than 0.05 were considered significant.

5.3 RESULTS

5.3.1 Effect of mechanical stimulation on the cellular orientation of MDSCs

MDSCs subjected to equibiaxial strain and located at the center of the well did not display a different orientation than the unstrained MDSCs (Figure 5-1A). When uniaxial strain was applied, images taken at the center of the well displayed a greater number of MDSCs oriented perpendicular to the direction of strain (Figure 5-1B). However, this was not the case with all the MDSCs. Interestingly, images taken of MDSCs located right off the edge of the loading post displayed the majority of the cells in that area had an orientation that was perpendicular to the direction of strain, and this was evident for both types of strains used in this study (Figure 5-1C).

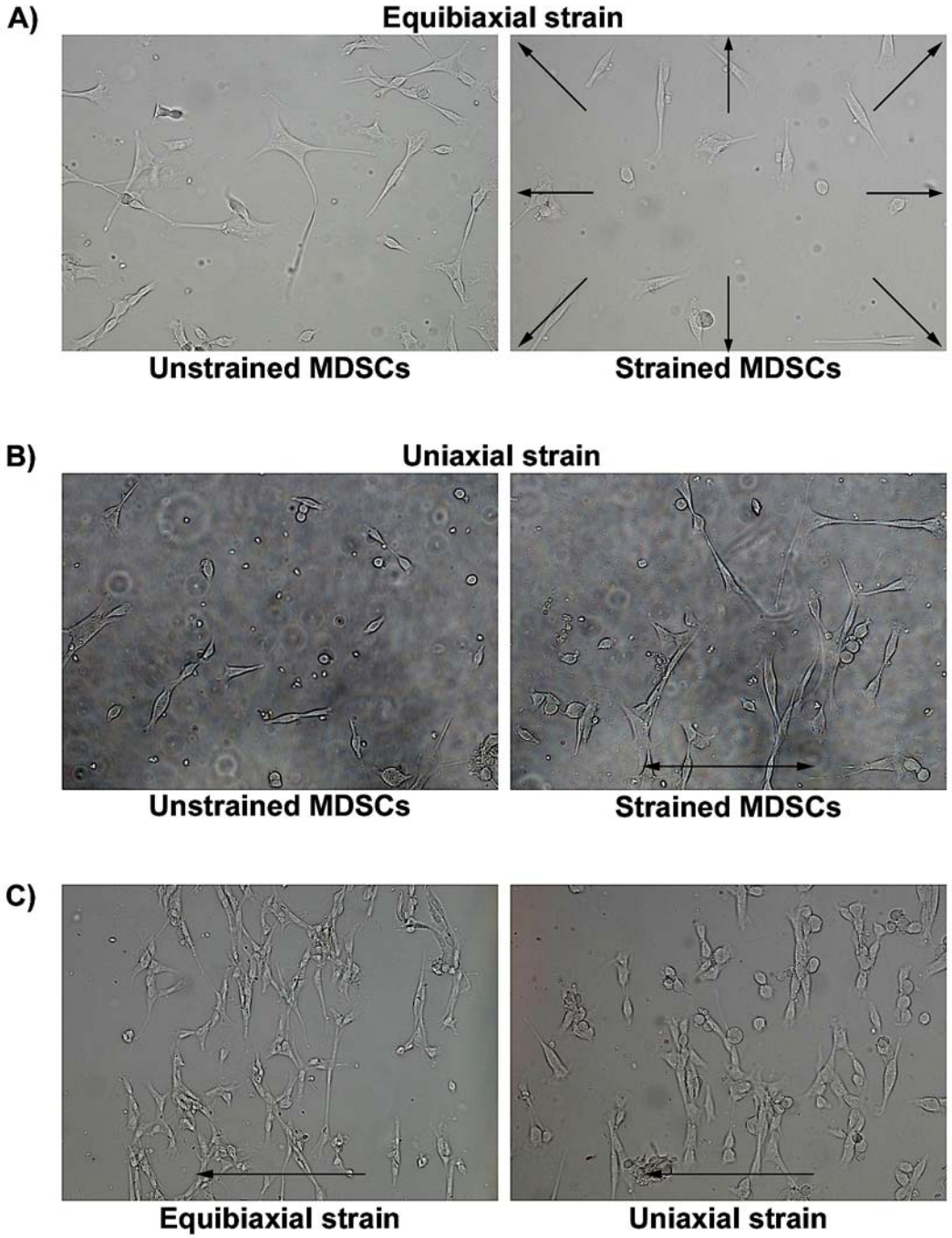

Figure 5-1. Cell orientation following mechanical stimulation.
(A) Unstrained MDSCs and strained MDSCs subjected to equibiaxial strain and located at the center of the well displayed a random orientation. (B) Unstrained MDSCs in the uniaxial strain group also displayed a random orientation, while MDSCs subjected to uniaxial strain and located at the center of the well had some cells that oriented perpendicular to the direction of strain. (C) Images of MDSCs located on the area adjacent to the edge of the loading post and subjected to either equibiaxial or uniaxial strain displayed an orientation perpendicular to the direction of strain. Arrows represent the direction of strain. Images were all taken at an original magnification of 20x.

5.3.2 Effect of mechanical stimulation on the proliferation of MDSCs

At the end of mechanical stimulation, it was found that equibiaxial strain led to a small decrease in the number of cells collected from the strained wells when compared to the unstrained wells (Figure 5-2). The same decrease in cell number was seen after uniaxial stimulation (Figure 5-2). However, these differences were not statistically significant.

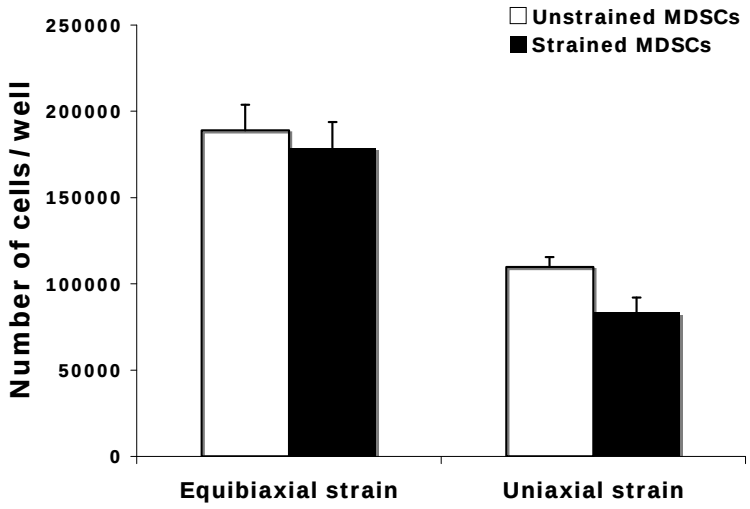

Figure 5-2. Effect of equibiaxial and uniaxial strain for 24 hours on the proliferation of MDSCs.

Since mechanical stimulation did not significantly effect proliferation during the straining regimen, but has previously been shown to activate other muscle cells (215), the effect on proliferation after the end of mechanical stimulation was also investigated. Hence, at the end of the straining regimen, unstrained and strained wells were cultured for an additional 24 hours before being harvested and counted. The number of cells obtained after 24 hours was compared to the number of cells at the end of the straining regimen and reported as the percent change in cell number during the 24 hours following mechanical stimulation (Figure 5-3). This provided information on the ability of the cells to proliferate once mechanical stimulation was removed.

As seen in Figure 5-3, MDSCs stimulated for 24 hours with an equibiaxial strain displayed a slightly higher percent change in cell number than their unstrained control. However, MDSCs subjected to uniaxial strain had a lower percent change in cell number during the 24 hours following the end of the straining regimen than the unstrained MDSCs. Although these results were not statistically significant, they suggest that MDSCs may respond better to an equibiaxial strain than a uniaxial strain.

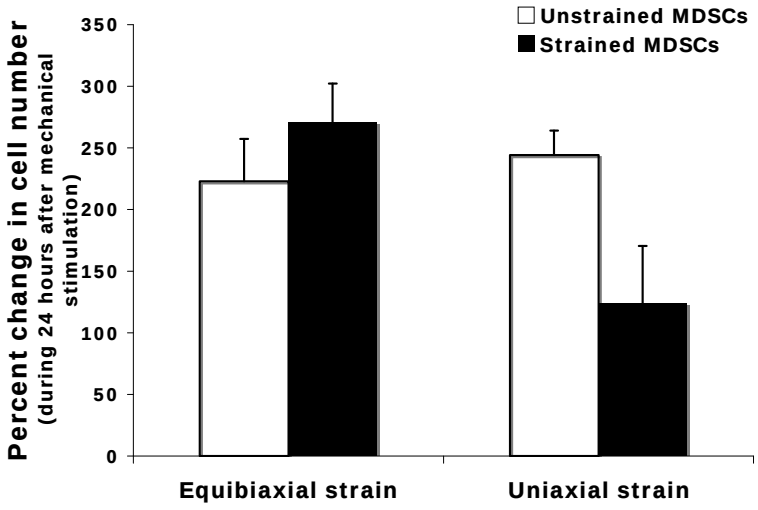

Figure 5-3. Effect of mechanical stimulation on the proliferation of MDSCs 24 hours following the end of the straining regimen.

5.3.3 Effect of mechanical stimulation on the marker profile of MDSCs

Previous observations have suggested that the stem cell characteristics of MDSCs are related to the expression of the cell surface markers CD34 and Sca-1 (51, 54). Hence, the effect of mechanical stimulation on the marker profile of MDSCs was investigated by flow cytometry at the end of the mechanical straining regimen. Interestingly, while CD34 expression was not affected by mechanical stimulation, Sca-1 expression was affected by both equibiaxial and

uniaxial strain (Figure 5-4A and B). MDSCs subjected to equibiaxial strain had a higher percentage of cells that were positive for Sca-1 than their unstrained control (Figure 5-4A). The opposite was seen with uniaxial strain, which led to a decrease in the percentage of Sca-1 positive cells in the strained MDSCs compared to the unstrained MDSCs (Figure 5-4B). There was no change in the percentage of cells positive for both CD34 and Sca-1 following mechanical stimulation (Figure 5-4A and B).

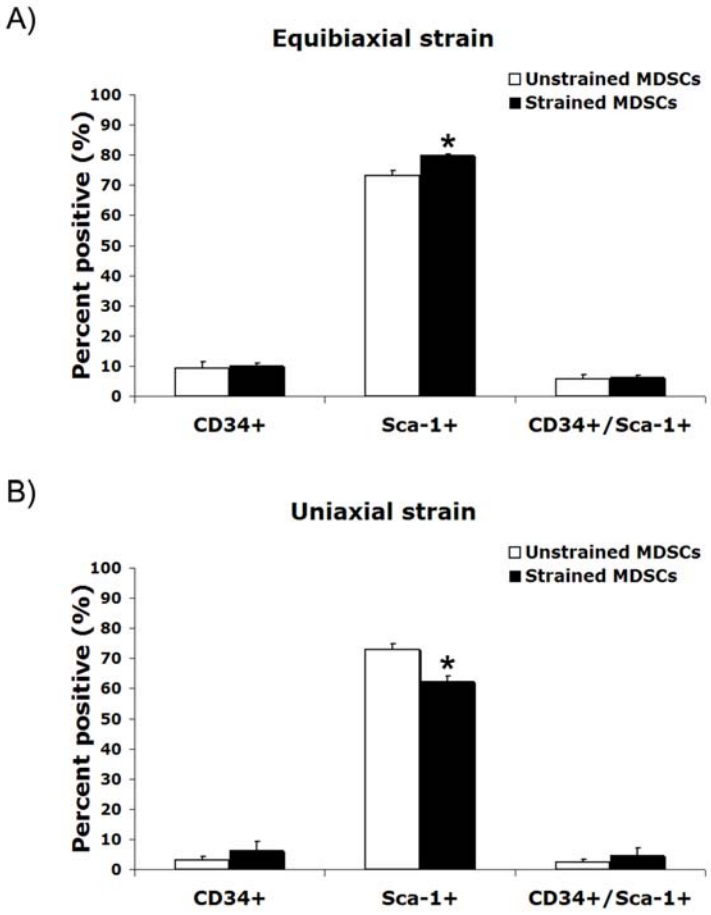

Figure 5-4. CD34 and Sca-1 expression of unstrained and strained MDSCs.
* indicates a significant difference from unstrained MDSCs ($P < 0.05$).

5.3.4 Effect of mechanical stimulation on BMP2 and VEGF expression

To determine if mechanical stimulation affected the release of growth factors by MDSCs, the culture medium was collected at the end of the straining regimen and an ELISA was used to quantify growth factors such as BMP2 and VEGF. It was determined that the samples collected had non-detectable levels of BMP2. However, a trend of higher levels of VEGF in the culture medium of strained MDSCs was observed with both equibiaxial and uniaxial strain (Figure 5-5).

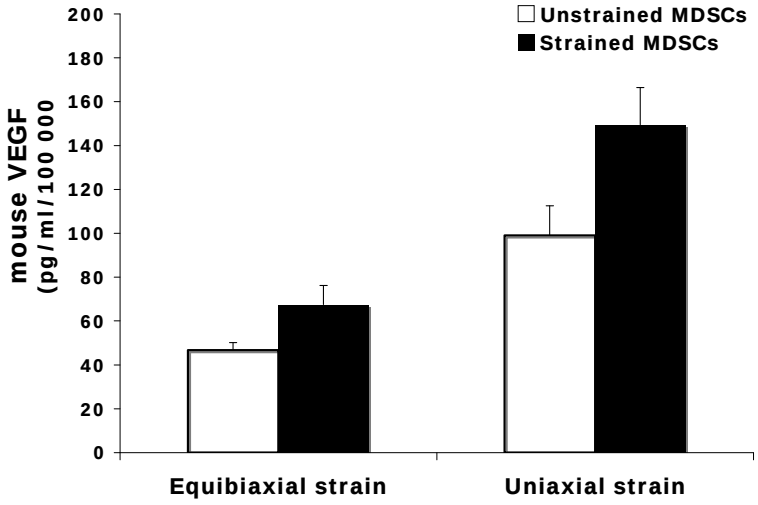

Figure 5-5. VEGF expression in the culture medium of unstrained and strained MDSCs.

5.3.5 Effect of mechanical stimulation on the osteogenesis of MDSCs after strain

At the end of the straining regimen, MDSCs were replated, stimulated with BMP4 for 3 days and the ALP activity was analyzed to determine whether mechanical stimulation had affected the ability of MDSCs to undergo osteogenic differentiation. Both unstrained and strained MDSCs responded to BMP4 and led to a positive staining for ALP (Figure 5-6A). As well, no apparent difference was observed between the unstrained and strained groups or between equibiaxial and

uniaxial strain. Quantification of ALP enzymatic activity also did not show any statistically significant differences between unstrained and strained cells (Figure 5-6B).

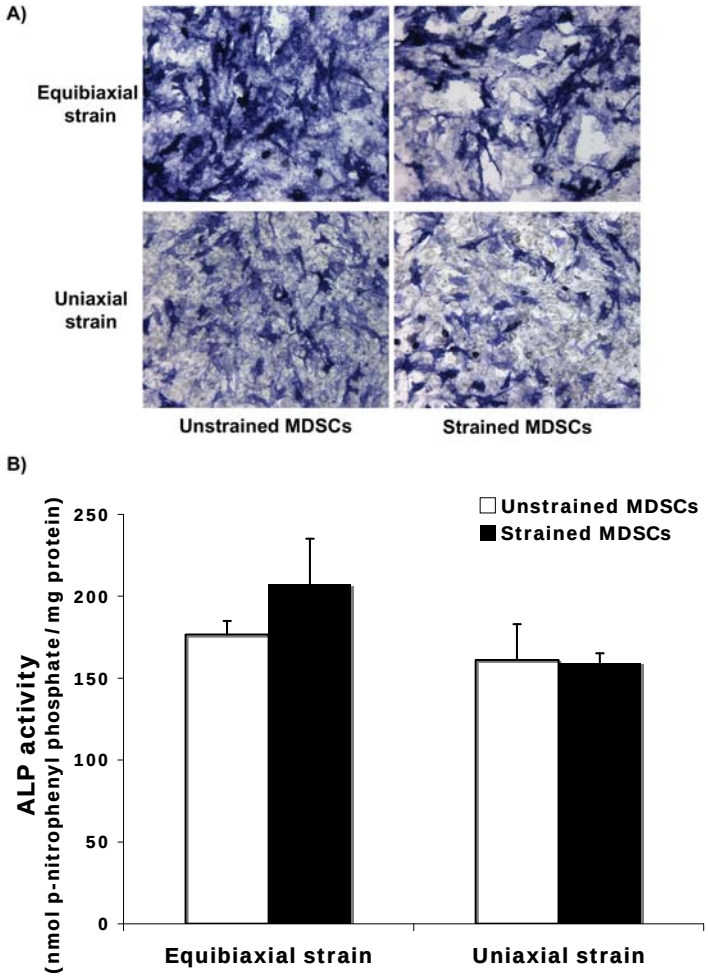

Figure 5-6. (A) ALP staining and (B) ALP activity of unstrained and strained MDSCs that were replated after strain and stimulated with BMP4 (50 ng/ml) for 3 days.
Images were taken at an original magnification of 20X.

5.3.6 Effect of mechanical stimulation on the osteogenic differentiation of MDSCs

Lastly, the capacity of MDSCs to undergo osteogenic differentiation during strain and how it is affected by mechanical stimulation was also studied. In this case, MDSCs were stimulated with BMP4 for 48 hours in the presence or absence of mechanical stimulation. At the end of the

straining regimen, the cell lysates were tested for ALP activity. The results indicated that both equibiaxial and uniaxial strain led to a decreased ALP activity compared to the unstrained MDSCs, although not statistically significant (Figure 5-7).

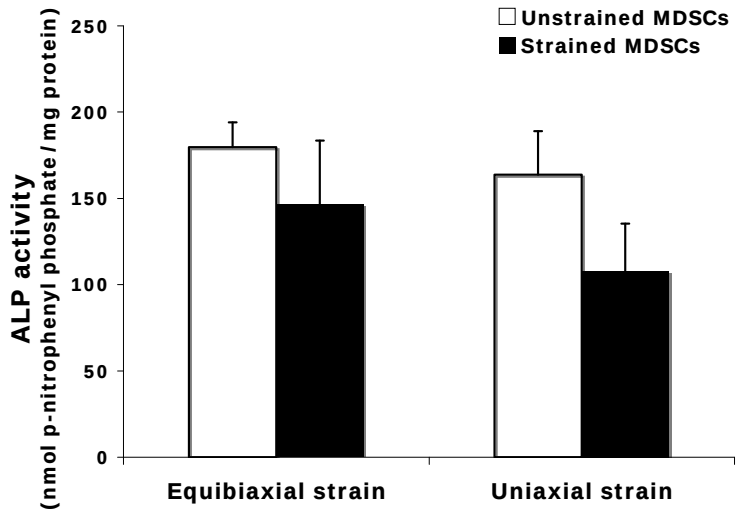

Figure 5-7. ALP activity of MDSCs stimulated with BMP4 (50 ng/ml) and subjected to either equibiaxial or uniaxial strain for a period of 48 hours.

5.4 DISCUSSION

Mechanical stimulation has increased the proliferation and osteogenic differentiation of numerous cell types (93, 99, 206, 213), suggesting that it could be a novel tool to promote the expansion of stem cells for clinical applications and to promote their osteogenic differentiation. This is the first study that investigated the effect of mechanical stimulation on the proliferation and osteogenic differentiation of MDSCs. Two types of strains were applied in this study, an equibiaxial strain and a uniaxial strain. Both straining regimens consisted of a 10% elongation at a frequency of 0.25 Hz for 24 hours to test cellular reorientation, proliferation, marker profile,

VEGF secretion and ability to undergo osteogenesis. In a second study, the same straining regimen was applied for 48 hours in the presence of BMP4 to determine the effect of mechanical stimulation on osteogenesis.

Cyclic strain of various magnitudes has previously been shown to cause cells to reorient in a direction that is perpendicular to the direction of applied strain (93, 94, 216). This change in orientation was also observed in the present study, although it was most evident on the off-loading post region. These results indicated that MDSCs did sense the straining regimens used in this study.

Although many studies have reported an increase in proliferation as a result of mechanical stimulation, the results presented in the current study indicated that mechanical stimulation did not significantly affect the proliferation of MDSCs. However, there was a trend that suggested a slight reduction in the number of cells obtained after mechanical stimulation, when compared to the unstrained control cells, and this was particularly evident when a uniaxial strain was applied. A decrease in cellular proliferation as a result of a 10% uniaxial strain has recently been reported in human ASCs, although the frequency used was not the same as the current study (94). Interestingly, the lack of increased proliferation during uniaxial mechanical stimulation has also previously been reported with satellite cells, another muscle cell type (215). In another study, Koike *et al.* subjected bone marrow stromal cells to an equibiaxial strain ranging from 0.80% to 15% for 24 or 48 hours and did not see a change in cell numbers at elongations of less than 10% (93). However, unlike the MDSCs, the bone marrow stromal cells had a significant increase in proliferation when the equibiaxial strain reached 10% and 15% elongation. These studies suggest that the magnitude and frequency of the strain used may have a

determining role on cellular proliferation. It may be possible that the straining regimen used in the current study was not optimal to promote the proliferation of MDSCs.

The regenerative potential of MDSCs has often been associated with their marker profile (51, 54). For this reason, the effect of mechanical stimulation on the expression of CD34 and Sca-1 was investigated in this study. Interestingly, an increase in Sca-1 expression was observed after equibiaxial strain and a decrease was observed after uniaxial strain. This suggests that MDSCs may have a better response to equibiaxial strain than uniaxial strain.

VEGF is a potent angiogenic factor that is expressed by osteoblasts during normal bone development. It has previously been shown that MDSCs transduced to express VEGF can enhance the bone formation of MDSCs expressing either BMP2 or BMP4 (57, 77). Also, mechanical stimuli have led to an increase in VEGF expression in rat calvarial osteoblasts, and human gingival and periodontal ligament fibroblasts (99, 217). As such, it was hypothesized in this study that mechanical stimulation would increase the VEGF expression of MDSCs. Although a trend of higher levels of VEGF was observed in the medium collected from strained MDSCs than unstrained MDSCs, the difference was not statistically significant. Hence, the mechanical stimulation applied to MDSCs in this study was not optimal for increasing VEGF secretion by these cells.

The use of a technique such as mechanical stimulation to expand stem cells may require cells to maintain their potential for differentiation. In order to test whether mechanical stimulation affected the ability of MDSCs to respond to BMP4, unstrained and strained cells were replated and stimulated with BMP4 for 3 days. The results indicated that strain did not have a negative effect on the ability of MDSCs to undergo osteogenic differentiation. Also,

mechanical strain during BMP4 stimulation of MDSCs did not show a significant change in their ALP activity.

5.5 CONCLUSION

This study investigated the effect of mechanical stimulation on MDSCs. Although the results obtained did not correspond to the hypothesis that mechanical stimulation could be used as a tool to expand MDSCs and promote their osteogenic differentiation, it should be noted that the straining regimen used consisted of only one magnitude and frequency. It is possible that the current straining regimen may not be optimal for MDSCs. Future studies should concentrate on applying a range of magnitudes and frequencies of strain to MDSCs. As well, other variables such as the duration of the experiment and an intermittent versus continuous strain should also be investigated.

6.0 OVERALL CONCLUSIONS

In previously published studies, the characterization of MDSCs indicated that they could undergo osteogenic differentiation in vitro. In addition, when MDSCs were retrovirally transduced to express BMP4 and then implanted into bone and articular cartilage defects, the cells could readily participate in the healing and repair of the defects. These studies were, in retrospect, mainly performed with MDSCs isolated from female mice. Here, we investigated whether F- and M-MDSCs could have a similar capacity for osteogenic and chondrogenic differentiation. Furthermore, we explored the signaling pathways activated by BMP4 in MDSCs that regulated osteogenic differentiation of the cells. In additional studies, we investigated the effect of mechanical stimulation on MDSC proliferation and differentiation. Taken together, the overall goal of this project was to better characterize MDSCs by investigating the influence of sex, cell signaling pathways, and mechanical stimulation on their stem cell behavior in relation to orthopaedic applications.

The study presented in Chapter 2 directly compared F- and M-MDSCs for their osteogenic differentiation. It was shown that the osteogenic potential of MDSCs is influenced by donor sex when stimulated with BMP4. M-MDSCs expressed osteogenic markers and underwent mineralization more readily than F-MDSCs. Based on these findings, we hypothesize that M-MDSCs may contain a greater number of osteoprogenitor cells than F-MDSCs. These findings are interesting with regards to the biology of stem cells, since few studies that investigate the

differentiation potential of stem cells report the sex of the cells used. We hope that this study will encourage other investigators to test numerous cell populations and from both sexes when undertaking differentiation studies with stem cells. The reproducibility of the isolation technique and sex-related differences are both important factors that should be taken into account when researching cell-based therapies. Understanding the mechanism behind these differences may then allow the development of more efficient therapies.

The experiments presented in Chapter 2 that focused on the osteogenesis of MDSCs in vitro also introduced a novel assay to test mineralization. The standard assay is to grow the cells in a monolayer culture and evaluate bone nodule formation. The MDSCs used in this study consistently detached from the plate when the culture reached complete confluence, causing this method to be inefficient. In our technique, MDSCs were cultured as pellets in osteogenic medium to induce mineralization and this was quantified by μCT. Few studies have used the pellet culture for osteogenesis (115, 218); however, to the best of our knowledge, we are the first to have analyzed the osteogenic pellet culture by μCT. Such a novel approach will likely become a standard assay in our laboratory.

The second study in this project, presented in Chapter 3, further characterized the differentiation process by investigating the signaling pathways involved in the regulation of BMP4-induced osteogenic differentiation. It was determined that the PI3K and p38 MAPK pathways act as positive regulators of osteogenic differentiation, while the ERK pathway is a negative regulator of osteogenic differentiation. This was the first study that looked at cell signaling pathways involved in the BMP4-induced osteogenesis of MDSCs. It provided further characterization of MDSCs, but more importantly, this study provided avenues to better regulate their osteogenic differentiation. The identification of the ERK pathway as a negative regulator of

osteogenesis may be an advantageous target in the development of cellular therapies for orthopaedic applications. Stimulating MDSCs ex vivo with the ERK inhibitor, PD98059, and then implanting them in vivo and evaluating their ability to form bone when compared to an unstimulated control is an interesting future direction to these cell signaling experiments.

The third study investigated the effect of BMP4 and TGF-β1 on the chondrogenic differentiation of F- and M-MDSCs. It was found that both F- and M-MDSCs undergo chondrogenic differentiation when stimulated or genetically engineered to express BMP4. Also, TGF-β1 synergistically enhanced the BMP4-induced chondrogenic differentiation of MDSCs. Although cell sex was not found to influence the chondrogenic differentiation of MDSCs, the importance of testing MDSCs from different isolations was again brought forth. The chondrogenic differentiation seen in the different populations was variable; however it permitted the identification of populations that were poor chondroprogenitor cells and populations that were highly chondrogenic. Future studies that investigate the differences that exist between these populations may lead to the identification of markers that could help identify highly chondrogenic cells from stem cell isolations.

In the chondrogenic differentiation studies, the pellet culture was used since it is the standard assay to test chondrogenesis in vitro. Few studies correlate the results obtained in vitro with in vivo performance. A future study comparing a low and high chondroprogenitor, as determined in vitro, in an articular cartilage defect made in nude rats should validate the results obtained with the in vitro pellet culture technique.

The potential use of mechanical stimulation to promote cellular proliferation and osteogenic differentiation was also investigated in this project. Although the straining regimen used in this study did not promote the proliferation or osteogenic differentiation of MDSCs, this

potential expansion method should be further investigated with regimens of different strain magnitudes and frequencies.

In conclusion, this project provided valuable information on the basic biology and therapeutic potential of murine MDSCs. As we continue to move forward in 'The Bone and Joint Decade', the identification and isolation of an 'MDSC-like' population in human skeletal muscle will be important to the clinical relevance of these findings. Researchers in Dr. Huard's laboratory are currently investigating the existence of these cells within human skeletal muscle. Due to the results presented in this project, it is suggested that once the equivalent to mouse MDSCs can be obtained from human skeletal muscle, the osteogenic and chondrogenic potential should be compared in cells obtained from female and male donors. In addition, it will be interesting to compare this in various age groups. The study of cell signaling pathways and the use of mechanical stimulation for cell expansion are all avenues that should be further investigated with human MDSCs. Such work will have a great impact in the development of tissue engineering therapies for bone and articular cartilage repair.

APPENDIX A

ISOLATION OF MDSCS USING THE PREPLATE TECHNIQUE

All the cells used throughout this study were MDSCs isolated from mouse skeletal muscle using a modified preplate technique developed in the laboratory of Dr. Johnny Huard and reported in Qu-Peterson *et al.* (51). Three-week old C57BL/10J mice were anatomically sexed at the time of isolation and a biopsy from their hindlimb skeletal muscle was obtained. The muscle was then minced and enzymatically digested in 0.2% collagenase-type XI for 1 hour. After centrifugation, the cells were incubated in dispase (2.4 units/ml of HBSS) for 45 minutes. They were then transferred to 0.1% trypsin-EDTA for 30 minutes, centrifuged, resuspended in PM and put into a collagen-coated T25 flask. As seen in Figure A-1, some cells attach to this first flask and those that do not are transferred to a new flask 2 hours later. The cells found in the first flask are known as preplate 1 or PP1 and contain mostly fibroblastic cells. PP2 to PP6 are obtained by transferring the supernatant to a new flask every 24 hours. PP2 to PP5 are known as early preplate cells or EP cells. MDSCs were then isolated from preplate 6 by taking the cells and replating them in a collagen-coated 12-well culture dish. After 2 weeks in culture, colonies began to form and were transferred to flasks for expansion.

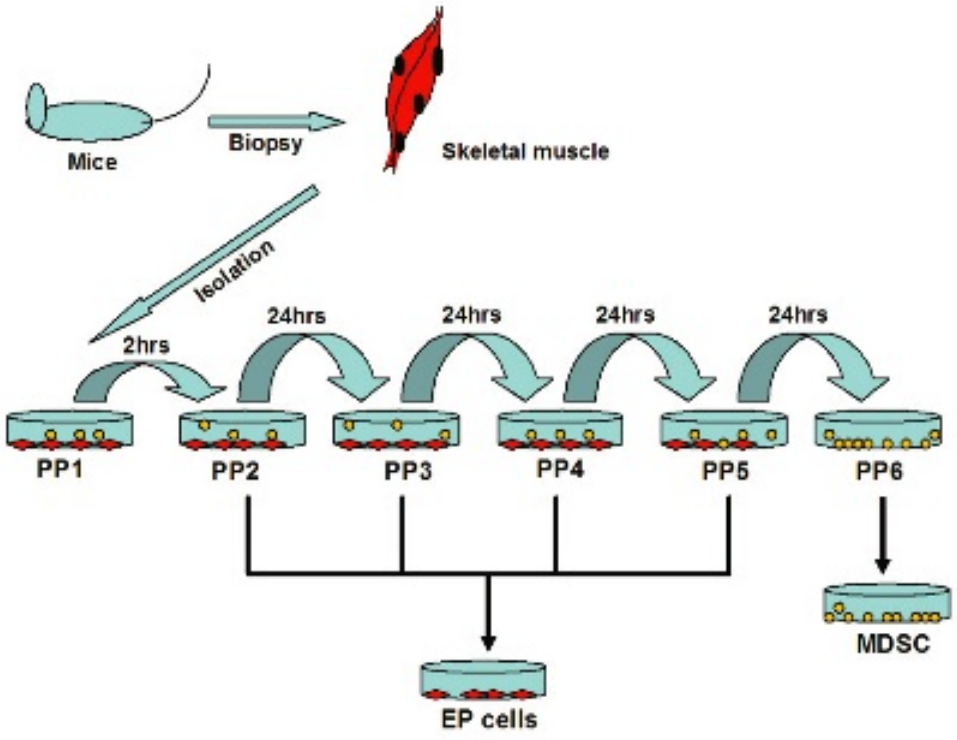

Figure A-6-1. Illustration of the preplate technique used to isolate MDSCs from the skeletal muscle of mice.

APPENDIX B

INFORMATION FOR QUANTITATIVE REAL-TIME PCR ANALYSIS

All target gene probes used in this project have FAM as the 5' reporter dye and TAMRA as the 3' quencher dye. *18S* has JOE/TAMRA. The sequences for both the primers and probes can be found in Table A-1 below. Of note, the primers for the *Smad6* gene were originally designed against the human gene. However, that section of the gene is homologous in mouse. When the primers were used in the studies presented in Chapter 3, *Smad6* gene expression was upregulated in MDSCs stimulated with BMP4, confirming that they could also detect mouse *Smad6*.

Table B.1. Sequences for primers and probes used in qPCR

Gene	Genbank #	Forward primer	Reverse primer	Taqman® probe
Mouse *Alp*	XM 124424	CCGATGGCACACCTGCTT	GAGGCATACGCCATCACATG	CGGCGTCCATGAGCAGAACTACATTCC
Mouse *Runx2*	NM 009820	AAATGCCTCCGCTGTTATGAA	GCTCCGGCCCACAAATCT	AACCAAGTAGCCAGGTTCAACGATCT
Mouse *Osx*	NM 130458	CCCTTCTCAAGCACCAATGG	AGGGTGGGTAGTCATTTGCATAG	CAGGCAGTCCTCCGGCCCC
Human *Smad6*	NM 005585	GCCACTGGATCTGTCCGATT	CACCCGGAGCAGTGATGAG	CACATTGTCTTACACTGAAACGGAGGCTACCA
Mouse *Sox9*	NM 011448	CGGCTCCAGCAAGAACAAG	TGCGCCCACACCATGA	ACGTCAAGCGACCCATGAACGC
Mouse *Col II*	NM 031163	AAGTCACTGAACAACCAGATTGAGA	AAGTGCGAGCAGGGTTCTTG	ATCCGCAGCCCCGACGGCT
Mouse *Col X*	X67348	TACTTACACGGATGGAGACCATGTT	ATCCAGTTGACTACTGGTGCAATTT	AACCCTCTTTTCGGATTAACCCTGCGA
18S	?	Proprietary of ABI	Proprietary of ABI	Proprietary of ABI

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