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The comparative role of demineralized bone matrix placement on the periosteum versus in the muscle with and without bone morphogenetic protein 2

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Thesis

THE COMPARATIVE ROLE OF DEMINERALIZED BONE MATRIX
PLACEMENT ON THE PERIOSTEUM VERSUS IN THE MUSCLE WITH AND
WITHOUT BONE MORPHOGENETIC PROTEIN 2

by

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DEDICATION

I would like to dedicate this work to my parents who continuously support me through every endeavor. Without their help I would not be the person I am today.
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I would like to thank Dr. Louis C. Gerstenfeld and Dr. Beth Bragdon for their help throughout this project. They have taught me numerous laboratory skills that will guide me throughout my career. They have also served as a source of continuous guidance and advice.
THE COMPARATIVE ROLE OF DEMINERALIZED BONE MATRIX PLACEMENT ON THE PERIOSTEUM VERSUS IN THE MUSCLE WITH AND WITHOUT BONE MORPHOGENETIC PROTEIN 2

ALEXANDRA L. FEMIA

ABSTRACT

Demineralized bone matrix (DBM) is an allograft material used in orthopaedics that promotes endochondral bone formation. While the placement of DBM on either the periosteal surface of a bone or within a skeletal muscle promotes the recruitment of stem cells that can form skeletal tissues through the temporal progression of endochondral bone development, it remains unclear to what degree these processes are different between the two sites. In this study, we utilize a comparative in vivo model of endochondral ossification by implanting the DBM on the periosteum and in the muscle. Within the muscle we further compared the effects of DBM with and without Bone morphogenetic protein-2 (BMP-2), a primary morphogenetic factor involved in the differentiation of skeletal stem cells. The mice were harvested at various time points after DBM implantation in order to analyze the development of the bone. Analysis included X-ray imaging, microCT imaging, and mRNA expression. Plain x-ray and micro-CT imaging analysis showed mineralized bone formation in the implant on the periosteum and in the muscle with BMP-2, but no growth in the muscle when BMP-2 was not added to the DBM. The mechanisms for bone development were further analyzed by qRT-PCR to determine temporal patterns and levels of expression of various stem cell and differentiated skeletal cell associated genes. The stem cell gene expression varied
between implant placement locations suggesting different mechanisms for stem cell recruitment. Interesting, while DBM implants in the muscle without BMP did not induce mineralized tissue specific mRNA expression; specific stem cell and early skeletal cell lineage commitment genes were present. These results suggest that while DBM in muscle is capable of recruiting stem cells that higher BMP-2 levels are needed to promote the progression of cartilage to mineralized bone in muscle tissues.
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LIST OF ABBREVIATIONS

α-SMA.........................................................................................alpha smooth muscle actin
ACAN............................................................................................aggreecan
BMP..............................................................................................bone morphogenetic protein
BMP-2.........................................................................................bone morphogenetic protein 2
BMPRI......................................................................................bone morphogenetic protein receptor type 1
BMPRII.....................................................................................bone morphogenetic protein receptor type 2
BV ..............................................................................................bone volume
cDNA..........................................................................................complementary deoxyribonucleic acid
COL10A1......................................................................................collagen type X alpha 1
COL2A1......................................................................................collagen type II alpha 1
DBM............................................................................................demineralized bone matrix
DMP1.........................................................................................dentin matrix acid phosphoprotein 1
FGF..............................................................................................fibroblast growth factor
Hox..............................................................................................homeobox
ID1 ..............................................................................................DNA-binding protein inhibitor 1
LN2..............................................................................................liquid nitrogen
micro-CT......................................................................................micro-computed tomography
mRNA.............................................................................................messenger ribonucleic acid
MSTN............................................................................................myostatin
Nanog...........................................................................................nanog homeobox
NCAM1........................................................................neural cell adhesion molecule 1
OC.....................................................................................osteocalcin
Oct4....................................................................................octamer-binding transcription factor 4
PAX7....................................................................................paired box 7
PBS ...........................................................................phosphate buffered saline
PFA.............................................................................paraformaldehyde
PRX1...........................................................................paired related homeobox protein 1
qPCR........................................................................quantitative polymerase chain reaction
qRT-PCR........................................................................quantitative real-time polymerase chain reaction
RT....................................................................................real time
RUNX2...........................................................................runt-related transcription factor 2
Shh ...............................................................................sonic hedgehog
SOX2.............................................................................sex determining region Y-box 2
SOX9.............................................................................sex determining region Y-box 9
TGF-β............................................................................transforming growth factor β
VE-cadherin.....................................................................vascular endothelial cadherin
INTRODUCTION

Orthopaedics focuses on bone development and repair as an attempt to alleviate the high percentage of bone related injuries and ailments affecting U.S. citizens. In the United States, 6 million people break or fracture a bone each year (Physical Fields 1). In addition, 4.5 million women and 1 million men over the age of 50 suffer from osteoporosis (Amin 1). A better understanding of the processes underlying bone formation, remodeling, and repair is vital due to the large population that is affected by bone related conditions.

Bone development is a highly regulated process. Endochondral ossification, the method by which the appendicular skeleton is formed, utilizes a cartilage model prior to ossification. This development of cartilage and bone is facilitated through stem cells with skeletogenic potential and is orchestrated by a specific series of signaling processes that first recruit these stem cells and then lead them to proliferate and differentiate into their specific lineages (Provot et al. 658). While this overall process is well understood in embryonic development and primary bone formation, the mechanisms by which post-natal skeletogenic stem cells are recruited and their tissue origins are undefined.

Endochondral Ossification

The process of bone formation and growth can be carried out by one of two distinct processes: intramembranous ossification or endochondral ossification. Intramembranous ossification, primarily occurring in the skull and flat bones, occurs when mesenchymal cells directly differentiates into osteoblasts (Provot, et al., 658). The
remainder and majority of the skeleton develop via endochondral ossification. This process involves the formation of a cartilage template, which serves as a model onto which bone formation occurs with the cartilage intermediate phase being resorbed as osteoblast differentiation and bone formation progresses (Crombrugghe, et al. 721).

The development of the appendicular skeleton via endochondral ossification occurs in mesoderm cell layer-derived cells. This cell population appears early following the onset of the limb bud formation, and express sonic hedgehog gene (Shh), “which encodes a secreted protein involved in patterning and growth of the limb elements that is expressed in a localized region of the posterior limb mesenchyme” (Miclau, et al. 45). Shh is involved in the regulation of the expression of bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and with the induction of the Sox9 transcription factor which directs mesenchymal cells towards their eventual chondrogenic lineage (Miclau, et al. 45). In addition to Shh, Hox genes also affect the expression of BMPs and FGFs, ensuring that the correct location of the limb elements during limb bud formation, and accounting for some of the mesenchymal cell proliferation (Hollinger 3).

As the undifferentiated mesenchymal cells begin to condense, condensation initiates cell signaling that specifically mediates its activity through Sox9 (Miclau et al. 46). Sox9 is a member of the Sox family of transcription factors and is essential for the differentiation of chondrocytes from osteo/chondroprogenitor cells (Crombrugghe, et al. 721). It is expressed by proliferative chondrocytes, ensuring the expression of the cartilage matrix mRNAs, specifically type II collagen (Mackie et al. 55). Within the mesenchymal condensations besides Col2a1 the large proteoglycan aggrecan is also
normally expressed with both genes regulated by downstream enhancers of Sox9 (Crombrugghe et al. 723). Following condensation the expression of two other Sox transcription factors (Sox5 and Sox6) increases in order to facilitate the progression of chondrocyte differentiation and proliferation (Crombrugghe et al. 723). As chondrocyte proliferation progresses the terminal differentiation of this lineage is defined by cellular hypertrophy extracellular matrix mineralization and apoptosis with the ossification occuring in distinct zones of the developing long bone.

After the individual bones within limbs have been formed and at later stages of development, specific columnar arrangements of cells are retained farthest from the sites of midiphyseal regions where initial ossification occurred. These sites contain resting chondrocytes that give rise to the growth plates within the individual bones of the limb. During subsequent longitudinal growth chondrocytes move from the resting into a proliferative state, where they divide and grow in column shaped clusters. As they continue to develop and divide they enter a state of hypertrophic growth. During this phase the chondrocytes increase significantly in size and secrete and mineralize their extracellular matrix. The hypertrophic cells undergo apoptosis, leaving a scaffold of mineralized cartilaginous matrix. Blood vessels invade the region, bringing osteoprogenitor and hematopoietic cells to the site (Mackie et al. 55). The hematopoietic cells will become the site of the eventual bone marrow, while the osteoprogenitor cells differentiate into osteoblasts. The osteoblasts utilize the cartilage scaffold, secreting osteoid, forming bone onto of the original cartilage matrix (Kini and Nandeesh 36).
Stem Cell Expression

Stem cells have the ability to remain in an undifferentiated state while maintaining the capability to eventually differentiate. They are also defined by their capacity for self-renewal and to differentiate into one lineage (unipotent), multiple lineages (multipotent), or all three germ layers (pluripotent) (Punwar et al. 296). Mesenchymal stem cells have the ability to self-renew and differentiate into a variety of mesodermal tissues (Susanne et al. 4). In development, the earliest cells, totipotent cells, require specification to develop into over 200 different cell types. In a developing blastocyst, the embryonic stem cells affected are derived from the inner cell mass. In order to determine which lineage a cell will follow, transcription factors regulate gene expression in order to determine the cells fate. The exact process is still not completely understood (Boyer et al. 947).

Three central transcription factors identified in the differentiation of embryonic stem cells include Oct4, Sox2 and Nanog (Go et al. 1147). Oct4 is a member of the POU family of transcription factors, and recognizes the “consensus-binding motif ATGCAAT” in the target DNA strand (Wei et al. 441). It has been linked to the differentiation of embryonic stem cells in the inner cell mass to trophoectoderm (Boyer et al. 947). Sox2 is member of the HMG box family and binds specifically to the “consensus sequence (A/T)(A/T)CAA(A/T)G” (Go et al. 1147). The levels of both Oct4 and Sox2 are decisive for embryonic stem cell fate. Increase in expression of Oct4 leads to the differentiation of stem cells into endoderm and mesoderm (Wei et al. 442). Nanog has been linked to the development and differentiation of cells in the extra-embryonic
endoderm (Boyer et al. 947). In previous studies, Nanog has appeared to interact via BMP signaling pathways by interfering with BMP’s induction of mesenchymal stem cell differentiation (Bais et al. 13).

In the determination of osteoprogenitor and chondrogenetic stem cell lineages, three stem cells factors were identified and considered for the purpose of our study: Prx1, Pax7, and Gremlin. Prx1 is a member of the paired-homeobox gene family that is predominantly expressed in the mesoderm that specifies limb buds. It has an important role in the interaction that occurs between the perichondrium and chondrocytes, specifically by regulating their proliferation and differentiation within the bone by enhancing the DNA binding specificity (Berge et al. 3831). Pax7 is expressed by satellite cells, which are “quiescent mononucleated myogenic cell able to proliferate in response to injury and give rise to regenerated muscle” (Morgan et al. 1151). The process by which the satellite cells proliferate is highly regulated through growth and transcription factors. When muscle is induced to proliferate, Pax7 binds to distinct regions of DNA to activate genes required for cell proliferation. As such, it plays a crucial role for the process of myogenesis (Maltzahn et al. 16474). Pax7 is instrumental in bone repair, specifically in fracture healing. When a fracture occurs, the stem cells in the surrounding skeletal muscle are activated during the inflammatory response period. These satellite cells help to regulate the inflammatory process in order for bone regeneration to occur (Abou-Khalil et al. 2). Gremlin has recently been identified as an osteochondroreticular stem cell present in the bone marrow. These stem cells self-renew and differentiate into chondrocytes, osteoblasts, and reticular marrow stromal cells (Worthley et al. 269). It is
an antagonist for BMP-2, and is important for the development of bone (Worthley et al. 270). Identifying and understanding the earliest stem cell markers is important for understanding the origin of cells involved in osteogenesis and chondrogenesis.

The Role of Bone Morphogenetic Protein-2

BMPs are a subfamily of the Transforming Growth Factor-β (TGF-β) superfamily. They have been identified as important factors in the growth and differentiation of mesenchymal cells into chondrocytic and osteoblastic lineages. This process occurs through a series of signal transduction steps that influence and regulate gene expression (Moucha, et al. 175). BMPs are stored in the extracellular matrix of various bone tissue, osteoprogenitor cells, osteoblasts, chondrocytes and other bone related cells. During bone repair and remodeling the BMPs are released leading to the signal transduction and bone formation (Carreira et al. 338). The BMP focused on in this study is BMP-2 due to its role in skeletal repair and regeneration (Bragdon et al. 610).

BMP-2 signals through serine/threonine kinase receptors consisting of type I and type II receptors. The type I receptors include Alk3 and Alk6, and bind BMP ligands. The type II receptors have been identified as BMPR-II and type II/IIB activin receptors (Chen et al. 233). Once the ligand binds, type I receptors recruits the type II receptors which the type II receptors then phosphorylated the type I receptors initiating the BMP signaling cascade (Bragdon et al. 616). The type I receptor phosphorylates Smad1/5/8 proteins, which play a central role in the translocation of the BMP signal to the nucleus (Chen et al. 233). Once phosphorylated, Smad1 associates with related proteins (Smad 4) and
translocates into the nucleus where it regulates and affects gene transcription (Figure 1) (Chen et al. 234).

Because BMP-2 has such a powerful role in the formation of bone, there are a number of molecular mechanisms that regulate it. Two important BMP-2 antagonists include Gremlin and Noggin (Bragdon et al. 613). Gremlin, a member of the Dan family of BMP antagonists is able to bind and block BMP-2. Gremlin primarily functions by modulating early limb growth through inhibiting chondrogenesis and apoptosis (Merino, et al. 5519). Noggin binds directly to BMPs, creating a complex, which can no longer bind to the cell surface receptors, and stopping the signal from beginning. BMPs stimulate Noggin secretion in mesenchymal cells, but may act as a negative feedback control in cell differentiation (Zhu et al. 2). In addition to antagonists, there are a number of mechanisms to block the transduction. Overall, there are many mechanisms to regulate the expression of BMP-2. This regulation is important because of the degree of importance BMP-2 plays in bone formation and growth.
In orthopaedics, it is common to utilize bone grafts to supplement and enhance the healing process. There are a number of different graft options available including allografts, autografts, osteoinductive carriers and osteoinductive factors (Lee 218S). An osteoinductive graft has the ability to recruit stem cells and facilitates their differentiation while an osteoconductive graft has the ability to form a scaffold to which new bone can be formed (Lee 218S). An ideal bone graft has both osteoinductive and osteoconductive properties.

DBM is an “allograft bone void filler” and is processed directly from human tissue (DBM Demineralized Bone Matrix). It contains a number of important growth factors.
factors, proteins, and collagenous material. The high presence of type I collagen allows for DBM to act as a scaffold for the new bone formation, fulfilling its classification as an osteoconductive graft material (Pietrzak 346). Furthermore, through the process of demineralizing the bone, BMPs become unmasked and available for use. The BMPs have an osteoinductive affect, recruiting and affecting the differentiation of the mesenchymal cells (Pietrzak 346). Other factors present collagen IV, X, growth factors, calcium phosphate, and cellular debris (Gruskin 1066). DBM facilitates in the formation of new bone, but it does not precisely follow the same procedure as endochondral ossification. DBM induces mesenchymal stem cells to differentiate into chondrocytes, which form cartilage. The cartilage is then ossified following the resorption of cartilage, as opposed to occurring simultaneously (Lee 219S).

DBM is continuing to grow as an alternative to previously used grafting material (Gruskin 1074). While this trend is accompanied by positive results, there are still a number of questions regarding the underlying processes that occur in the development of DBM into ossified bone. Because DBM is a human derived tissue, it is therefore variable between the different donors based on a number of different factors including age, sex, processing, and sterilization (Holt 1124). While the composition is generally similar, containing BMPs, collagen type 1, and other growth factors, the concentrations vary considerable between donors (Holt 1124). When BMP was analyzed across ten samples, concentrations ranged from 22-110pg for BMP-2 and 44 to 125pg for BMP-7, illustrating the huge variability of DBM between samples (Bae et al. 1). The variability has profound effects on the potency and osteoinductive capabilities of the DBM when used clinically.
In order to test for the potency of DBM, it is typically implanted in the muscle of an animal model and ectopic bone formation is measured (Lee 218S). Because clinically DBM is used on the periosteum directly but its potency is measured in the muscle, questions persist as to if it has differing effects on skeletogenic cells at these different tissue sites. In addition, questions arise if there are in fact different populations of skeletogenic stem cells localized at these two different sites, if the quantities and temporal progression of bone formation will be different at the two tissue sites.

Clinically, there are instances where bone allograft material is needed to supplement natural healing and bone strength (Grafton DBM Clinical Overview 3). In these instances, demineralized bone matrix (DBM) can be utilized as an osteoinductive material to induce bone formation (DBX® Demineralized Bone Matrix 1). Although the processes by which DBM induces endochondral bone formation follows is similar to that of seen during development, the origins of the postnatal stem cell populations and exact processes of gene expression that contribute to these processes is far less understood.

In this study DBM placed directly on the periosteal femur surface versus in a muscle pouch was used to assess the magnitude of DBMs bone inductive properties at different tissue locations, the nature of the stem cells that are recruited at different tissue sites and the timing of specific genes expression indicative of endochondral bone developmental process. A second focus of this study was the role that bone morphogenetic protein 2 (BMP-2), plays in skeletogenic stem cell differentiation that is mediated by DBM. In this study we further compared DBM alone or combined with BMP-2 within the muscle pouch model. Using this model, we determined how BMP-2
augmented the osteoinductive processes within the muscle by comparing the effects of the two different treatments endochondral bone formation associated on gene expression in the muscle.
SPECIFIC AIMS

Aim 1. The objective of this study is to determine the effect of tissue site on DBM’s osteo-conductive and -inductive properties.

Aim 2. These studies further focused on the variable effects that BMP-2 addition had on the osteo-conductive and -inductive properties of DBM.

Methods: Variable effects of tissue site on DBM’s properties were assessed by DBM implantation on periosteum of the femur or within a muscle pouch in the quadriceps femoris muscle. In order to assess the effect of BMP-2 on DBM’s osteo-conductive and -inductive properties exogenous BMP-2 was added to DBM that were implanted in the muscle and compared to DBM without BMP-2 addition. The bone tissue formation was allowed to develop and was assessed from day 2-31 after surgery.

- Plain X-ray and quantitative microCT- analysis was carried out to analyze the spatial development of the implant relative to the femur. Bone volume data was then generated to compare the growth of mineralized tissue from implants placed in the muscle to those placed directly on the femur.

- RNA was extracted from the implant, and qRT-PCR preformed. Analysis of genes specific to stem cell markers, osteogenesis, chondrogenesis, muscle development, angiogenesis, and BMP-2 signaling were compared.
• Statistical analysis was performed to determine significant difference in gene expressions between the DBM placed in the muscle and a previous analysis of the gene expression of DBM placed directly on the periosteum.

Overall goal of these studies: determine if the site of placement effected the overall quantity and/or temporal progression of bone formation, assess if the origin or nature of the stem cells that contribute to the development of bone were different based on tissue site of implantation, and determine the role that BMP-2 plays in stem cell recruitment and or differentiation.
METHODS

Animals & Surgery

All animal studies were approved by the Institutional Animal Care and Use Committee at Boston University. Mice were obtained from The Jackson Laboratory (Bar Harbor ME) and housed at the BU Animal facilities under standard conditions. All mice were male between 9-11 weeks and of the strain B6-129S7Rag1tmMom/J. Mice were either unilaterally or bilaterally implanted with 0.0500 ± 0.0003 µg of Grafton® DBM Putty which was inserted into the muscle surrounding the femur. Prior to insertion in the muscle, the putty was combined with 0.3µg of BMP-2. Preceding to, and following surgery mice were subcutaneously injected with a total of 0.1 mL of Buprenex® as pain medication and 0.01mL of Baytril® as antibiotics. Five mice were euthanized per time point at harvest days 2, 4, 8, 12, 16, 24, 31 post-surgery for RNA analysis. Mice harvested at days 8, 12, 24, and 31 received bilateral implants in order to perform MicroCT analysis as well.

Harvest and X-Ray Imaging

Mice were euthanized at a given time point by carbon dioxide inhalation followed by cervical dislocation. Immediately following death, mice were X-rayed using Faxitron MX-20 Specimen Radiography System at 30 kV for 40 seconds using Kodak BioMax XAR Scientific Film. The implant, surrounding muscle, and femur were collected separately for RNA extraction and gene analysis. The samples isolated for RNA analysis were stored at -80 °C. On harvest days 8, 16, 24, and 31 the left limb was recovered for
MicroCT imaging. The limb was placed in 4% PFA for one week for fixation, and stored in 1x PBS at 4°C.

**MicroCT Imaging**

MicroCT imaging and analysis was performed on five mice at post-operative day 8, 16, 24, and 31 using the SCANCO Medical µCT Scanner. The sample consisted of the limb with minimal muscle removed surrounding the implant and was scanned in a 20.5 mm conical tube. Scans were taken at 70kVp and 114µA with an integration time of 200ms. The imaging across time allowed for the temporal and spatial characterization of the DBM-induced mineralized tissue. Using SCANCO© Medical, the scans were manually contoured to include the implant in the muscle. The contour was analyzed using a total and bone volume script at a threshold of 240 based on a 45% evaluation of the cortical bone volume. The threshold was consistent with previous analysis and was set to detect the presence of mineralized bone.

**RNA Extractions**

RNA extraction was performed by tissue dissociation and chemical extraction. Samples were snap frozen in 0.75mL of QIAzol® Lysis Reagent using liquid nitrogen. A 5mm Qiagen® stainless steel bead was placed in each tube. Samples were lysed with the Qiagen® Tissue Lyser II using 2-minute intervals at 30Hz; snap freezing the sample again if the sample thawed before being completely lysed. Samples were transferred to a new 2mL tube with the addition 1mL of QIAzol® Lysis Reagent and samples incubated
on ice for 2 minutes. After 2 minutes, 200µL of Chloroform (Sigma-Aldrich®) was added and the samples vortexed. Again, samples were incubated on ice for 2 minutes followed by vortexing, and centrifuged for 15 minutes at 14000rpm and 4°C. The aqueous phase was transferred to a new 2mL tube, adding an equal volume of isopropanol (Sigma-Aldrich®). This new solution was then centrifuged for 30min at 4°C and 14000rpm.

The supernatant was removed and the pellet washed with 500µL of 70% ethanol (Sigma-Aldrich®), and centrifuged for 5 minutes at 4°C and 14000rpm. The supernatant was removed and the pellet was washed again. The ethanol was removed and the pellets were left to dry for 20-30 minutes. Once dry, pellets were re-suspended in 30-50 µL of RNase free water by slowly pipetting up and down. The extracted RNA was the stored at -80°C.

In order to ensure the quality and quantity of the extracted RNA, both spectroscopy and gel electrophoresis were used. For the spectroscopy, 1µL of RNA was dissolved in 99 µL of RNase free water. Using a Beckman Coulter™ DU®530 Life Science UV/Vis Spectrophometer, a 260nm/280nm ratio value in the range of 1.3-1.8 indicated an acceptable quality of RNA. The 260nm absorbance value was used to determine the concentration of RNA in the sample. For the gel, 1µL of RNA, 7µL of RNase free water, and 2µL of 6X Agarose Gel Loading Dye were mixed and loaded into 1% agarose gel. The gel was made with UltraPure™ agarose from Invitrogen and GelStar™ Nucleic Acid Gel Stain from Lonza Group to detect the presence of the nucleic
acid in the gel. The gel was run at 110V for 60-90 minutes. The presence of two bands under UV light indicates the RNA is intact and a relative idea of the concentration.

cDNA Production

RNAse free-water was added to 1µg of the extracted RNA in a 0.2mL PCR tube for a total volume of 10.4 µL. The following reagents were mixed in an eppendorf tube to create a mixture with a total volume of 19.6µL: 6.61µL of MgCl₂, 6.0µL of dNTP Mix, 3.0µL of 10X RT Buffer, 1.5µL of Random Hexamers, 0.6µL of RNAse Inhibitor, and 1.89µL of Taqman Reverse Transcriptase. All of the reagents were obtained from the TaqMan® Reverse Transcription Reagents kit from Applied Biosystems®. The master mix was then added to the 0.2mL PCR tube to bring the final volume to 30 µL. The samples were placed in the Eppendorf Mastercycler® Personal thermal cycler. The PCR cycle ran at the following settings: 25°C for 10 minutes, 37°C for 60 minutes, 95°C for 5 minutes, and finally, a 4°C hold. Following the PCR cycle, RNAse free water was used to make a 1:25 dilution of the cDNA, and the final samples were stored at -20°C.

qRT-PCR

For qRT-PCR, a 96-well qPCR plate was used, running doublets of each sample on a single plate. We combined 10 µL TaqMan® Universal PCR Master Mix from Applied Biosystems® and 1 µL TaqMan® Gene Expression Assays primer from Applied Biosystems® for a total of 11µL of per sample. The primers used in this study can be observed in Table 1. In addition to our DBM samples, non-operative femurs were used as
our controls. The 11µL of mix was combined with 9µL of cDNA to bring the total volume to 20µL per well. After the plate was filled, it was then centrifuged, covered with clear film and analyzed using ABI 7700 Sequence Detector® from Applied Biosystems®. The qRT-PCR reaction repeated the following cycle 40 times: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute. The threshold cycle (Ct) values were obtained giving a measure of the concentration of the target gene. The Ct value for the target gene was normalized by two different methods. The first method normalized the target gene Ct value to the 18s Ct value and then compared the fold expression to control femur. The second method normalized the target gene Ct value to 18s. In addition, the gene expression was determined for native muscle in order to visualize and quantify the fold increase from baseline.

Statistical Analysis

The statistical analysis of the data was accomplished using Microsoft® Excel® 2011 and JMP® 11.2.0. In order to calculate statistical significance, we used a multiple comparison t-test and Wilcoxon test with significance being p<0.05.
Table 1: qRT-PCR Primers. Primers used with their catalog numbers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Catalog Number</th>
</tr>
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<tr>
<td><strong>Normalization Primer</strong></td>
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</tr>
<tr>
<td>18s</td>
<td>Mm03928990_g1</td>
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<tr>
<td><strong>Stem Cell Primers</strong></td>
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</tr>
<tr>
<td>Sox2</td>
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</tr>
<tr>
<td>Prx1</td>
<td>Mm00440932_ml</td>
</tr>
<tr>
<td>Pax7</td>
<td>Mm01354484_m1</td>
</tr>
<tr>
<td>Nanog</td>
<td>Mm02384862_g1</td>
</tr>
<tr>
<td>Oct4 (Pou5fl)</td>
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</tr>
<tr>
<td>Gremlin</td>
<td>Mm00488615_s1</td>
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<td><strong>Cartilage Primers</strong></td>
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<td>Sox5</td>
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<tr>
<td>Col10a1</td>
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<td>Acan</td>
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<td>Col2a1</td>
<td>Mm00491889_m1</td>
</tr>
<tr>
<td><strong>BMP Associated Primers</strong></td>
<td></td>
</tr>
<tr>
<td>Noggin</td>
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</tr>
<tr>
<td>BMP2</td>
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<td>BMPR1b (Alk6)</td>
<td>Mm00432117_m1</td>
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<tr>
<td>BMPRII</td>
<td>Mm00432134_m1</td>
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<td><strong>Bone-Associated Primers</strong></td>
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<td>Osteocalcin (bglap)</td>
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</tr>
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<td>RUNX2</td>
<td>Mm00501578_m1</td>
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<tr>
<td>DMP1</td>
<td>Mm00803833_g1</td>
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<tr>
<td>Osterix (Sp7)</td>
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<td><strong>Muscle-Associated Primers</strong></td>
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<td>MSTN</td>
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</tr>
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<td>α-Sma (Acata2)</td>
<td>Mm00725412_s1</td>
</tr>
<tr>
<td><strong>Angiogenesis Primer</strong></td>
<td></td>
</tr>
<tr>
<td>VE-Cadherin (CDH5)</td>
<td>Mm03053719_s1</td>
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<td>SMA (Acta2)</td>
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RESULTS

X-ray Imaging

In a pilot study, DBM was combined with various concentrations of BMP-2 prior to being placed in the muscle. The concentration of BMP-2 was varied in order to determine the lowest concentration that induced bone formation. The concentrations tested were: 0.0µg, 0.1 µg, 0.3µg, 0.5µg, and 1.0µg. From the x-ray imaged (Figure 2), a concentration of 0.3µg was determined and used for the experiment.

![Figure 2: Effects of Varying Concentrations BMP-2 on DBM's Osteo -Conductive and -Inductive Effect.](image)

Bone. Growth in the Muscle with varying doses of BMP-2. The concentrations of BMP-2 are denoted in the figure. Arrow indicates mineralized tissue induced by the DBM and BMP-2.

Plain film X-rays were obtained at the time of harvest to visualize the mineralized tissue that formed due to the DBM implant. In Figure 3, the X-ray images of the DBM implant when placed on the periosteum is compared to the DBM placed within the muscle with and without BMP-2. The X-ray images showed mineralized tissue at the implant site.
when placed on the periosteum and in the muscle with BMP-2 as early as day 12 and continuing through day 31. The implant placed in the muscle without BMP-2 displayed no visible growth.
Figure 3: Effects of Tissue Placement and BMP-2 on DBM’s Osteoinductive and Osteoconductive Effect. Bone growth at each time point, DBM placed on the periosteum, in the muscle without BMP-2 and in the muscle with BMP-2. The yellow arrow indicates the presence of bone growth. The experimental group and time points are denoted in the figure.
**MicroCT40 Imaging and Analysis**

The total volume (TV) and the bone volume (BV) of each implant were quantified using the MicroCT40 and this data was compared to previous analysis of the DBM implant placed directly on the femur as well as in the muscle without BMP-2. When analyzing the samples, a threshold of 240 was determined in order to measure how the placement of the implant affected its mineralization. This threshold was determined by using 45% of the mean bone volume of the cortical bone. The analysis was performed at a lower threshold (35% of the mean bone volume) in order to determine if the inclusion of more material would lead to greater significance, but no difference was found between the two thresholds. As a result, a 240 threshold was used across all samples. For both previous experiments, MicroCT40 scans were taken and analyzed. The DBM implant on the periosteum induced detectable bone formation, but the implant in the muscle without BMP-2 displayed no detectable growth. The TV and BV comparison illustrates the comparison between the implant placed on the periosteum and in the muscle with BMP-2. The TV comparison, Figure 4, shows significantly increased total size of the implant in the muscle with BMP-2. This increased size remained consistent throughout days 16, 24, and 31. The BV comparison, Figure 3, shows increased mineralization of the DBM placed on the periosteum at days 12 through 31 compared to the DBM/BMP2 implant. Throughout the time points the DBM placed in the muscle with BMP-2 continues to increase in BV, but never remains significantly smaller. Using JMP software, an ANOVA was conducted to determine statistical significance of the DBM implant when placed on the periosteum versus in the muscle with BMP-2 through the time course.
The 3D renderings, Figure 5, of the femur and implant illustrated the spatial positioning of the implant relative to the femur, ensuring that no contact was made between the two. This is important to accurately determine the effect of the placement on the implant’s growth and development. The images allow for the TV and BV to be related to the implant’s size and visual density.

Figure 4: Effect of Tissue Placement and BMP-2 Addition on DBM’s Total and Bone Volumes. The top graph illustrates the comparative total volume between the three experimental groups. The bottom graph illustrates the comparative bone volume between three experimental groups. No visible growth is indicated with ND. The x-axis is the day post-harvest. The * indicates significance (p<0.05), error bars show SEM, n=5.
Figure 5: 3D Renderings of the Effect of Tissue Placement and BMP-2 Addition on DBM Growth. 3D renderings of the implant (orange) relative to the femur (gray) are displayed for days 16, 24, and 31 post-harvest. The line through the femur represents where the cut plane was taken. The smaller box is the cut plane (purple) looking through the femur.
q-RT-PCR Results

Although the DBM placed in the muscle with BMP-2 was able to induce the formation of bone shown by the BV, the development of this bone significantly lagged behind that of the DBM placed on the periosteal surface. In order to elucidate a possible mechanism between the implants and the relative gene expression for stem cell recruitment, endochondral ossification, and selected BMP signaling molecules were analyzed. Following analysis, the fold gene expression was illustrated in two ways: normalized to control femur and normalized to 18s. This method was applied in order to gain insight into the overall levels of gene expression relative to cell number in the volume of tissue that was analyzed. The expression of the genes in native muscle was also determined in order to compare relative increases in each experimental group. The two normalizations illustrated similar results in many cases, but there were some differences observed.

Stem Cell Recruitment

A number of early stem cell markers including: Sox2, Nanog, Oct4, Gremlin, Prx1, and Pax7 were analyzed in order to determine the earliest factors driving bone formation. In our analysis we not only looked at the DBM-induced expression in the muscle with BMP-2, but also compared it to previous studies where DBM was placed directly on the periosteum and in the muscle with no BMP-2.
Sox2, Nanog, and Oct4 showed very similar trends in the DBM implant placed on the periosteal surface. The DBM placed on the periosteum displayed increased expression at day 2 and day 4 followed by a decrease at days 8 through 31.

Sox2, when normalized to control femur, was significant at day 4 between the implant placed on the periosteum and the implant in the muscle both with and without BMP-2 (p=0.0304). When normalized to 18s, there was significance at day 2 between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0304).

Nanog, when normalized to control femur, displayed both a significant difference in expression between the implant placed on the periosteum versus the implant in the muscle with BMP-2 as well as the implant placed in the muscle with BMP-2 versus without BMP-2 at days 4, 12, 16, 14, 31 (p<0.002). At day 8 there was significant difference between all three experimental groups (p<0.001). When normalized to 18s, there were significant differences at days 8, 16, and 31 between the implant placed on the periosteum versus both the implants in the muscle with and without BMP-2 (p<0.003). In addition the peak at day 2 in the implant on the periosteum was significant from the implant placed in the muscle with BMP-2 (p=0.0304).

Oct4 expression showed similar trends of significance between the two normalizations. At days 4, 8, 12, 16, 24, and 31 there was significant differences in expression between the implant in the muscle with and without BMP-2 (p<0.03). When normalized to control femur there was also significant differences in expression at days 4, 8, 12, and 16 between the implant placed on the periosteum versus the implant placed in
the muscle without BMP-2 (p<0.03). When normalized to 18s, there was significant differences in expression at day 8 between the implant placed on the periosteum versus the implant placed in the muscle without BMP-2 (p=0.0058) and at day 31 between the implant placed on the periosteum versus the implant placed in the muscle BMP-2 (p=0.0367).

*Germlin*’s expression varied most dramatically between the two different methods used for the normalization of gene levels. When normalized to control femur, there is increased expression in the implant placed in the muscle without BMP-2 with a peak at day 8. Statistically, there is significant differences in expression at days 8, 12, 16, 24 between all three experimental groups (p<0.03) and at day 31 between the implant placed on the periosteum and both implants in the muscle (with and without BMP-2) with p=0.0200. When normalized to 18s, the implant in the muscle with BMP-2 displays increased fold expression throughout all time points. There are significant differences in expression at days 8 and 12 between all three experimental groups, and at days 12, 16, 24, and 31 between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p<0.02), as well as between the implant placed in the muscle with and without BMP-2 (p<0.03).

*Pax7* appears to have increased expression in the DBM implant on the periosteum compared to the implant placed in the muscle both with and without BMP-2. The gene expression in the implant placed on the periosteum is highest at days 2 and 4, with a small peak at day 24. When normalized to control femur, there is statistical significance at days 2, 4, 12, 16, 24, and 31 between the implant placed on the periosteum and the
implant placed in the muscle with BMP-2 (p<0.02). The DBM placed in the muscle without BMP-2 shows low expression throughout all time points, and when normalized to control femur displays statistical significance at days 4 through 31 (p<0.03). The DBM placed in the muscle with BMP-2 has highest expression at day 2 and day 8, with decreased expression at the remaining time points. When normalized to control femur this peak at day 8 is statistically significant from the implant placed in the muscle without BMP-2 (p=0.0122) as well as the implant placed on the periosteum. When Pax7 was normalized to 18s there were less points of significance between experimental groups, occurring at days 16, 24, and 31 between the implant places on the periosteum versus the implant placed in the muscle with BMP-2 (p<0.02) and between the implants placed in the muscle with and without BMP-2 at day 12 (p=0.0369).

Prx1 expression is within a close range of values between the three different placements. All three experiments peaks respectively at day 8 with a smaller peaks at days 16 and 31. These peaks are more visible when compared to control femur, but are still present with normalized to 18s. In both normalizations there is a significant difference in fold expression at days 2 and 4 between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.03) and at day 4 between the implants placed in the muscle with and without BMP-2 (p<0.03). There are additional points of significance when normalized to control femur specifically at days 8, 12, 16, and 31 between the implant places on the periosteum versus the implant placed in the muscle without BMP-2 (p<0.02) and at days 24 and 31 between the implants placed in the muscle with and without BMP-2 (p<0.03).
Chondrogenesis Gene Expression

Based on X-ray and MicroCT images, the expected time for chondrogenesis to occur was prior to day 12. The gene expression remained consistent with the imaging results, with peaks at day 8 indicating chondrogenesis occurring prior to the visualized mineralization. Various genes throughout the process of chondrogenesis were chosen for
analysis. The earliest marker, Sox9, displayed increased expression at day 8 in both normalizations. When normalized to control femur, at days 8, 12, 16 and 24 there was significance between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 (p<0.03) and between the implants placed in the muscle with and without BMP-2 (p<0.03). There was also significance at day 4 between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 (p=0.0304) and at day 31 between all three experimental groups (p=0.0122). When normalized to 18s, Sox9 only displayed significant differences in expression at day 4 (implant placed on the periosteum versus in the muscle with BMP-2 at p=0.0304), day 24 (all three experimental groups with p<0.03), and day 31 between the implant placed on the periosteum and both implants placed in the muscle with and without BMP-2.

NCam1 displayed a peak at day 8 for all three experimental groups as well as a subsequent increase in expression at day 16. The implant placed in the muscle with BMP-2 continued to increase in expression in days 24 through 31 with the other groups decreasing. When normalized to control femur, there were significance between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 at day 12 (p=0.0216) and day 24 (p=0.0122) and between all three experimental groups at day 31 (p<0.03). When normalized to 18s, there was significance between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 and between the implants placed in the muscle with and without BMP-2 a day 8 (p<0.03). At day 12 there was significance between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0216). At day 16, there was significance
between the implant placed on the periosteum and both implants placed in the muscle with and without BMP-2 (p=0.012). At days 24 and 31 there was significance between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p<0.02) and between the implants placed in the muscle with and without BMP-2 (p=0.0122).

*Col2a1* is important for the clustering of chondrocytes during chondrogenesis and displays the same day 8 peaks, with the largest being in the implant in the muscle with BMP-2. When normalized to control femur, there is significance between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 at days 8, 16, 24, and 31 (p=0.0122) and significance between the implants placed in the muscle with and without BMP-2 at days 8, 24, and 31 (p<0.02). When normalized to 18s, the same groups remain significant at days 24 and 31. There is also significance between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 at days 12 and 16 (p=0.0216 and p=0.0122), between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 at day 8 (p=0.0348), and between the implants placed in the muscle with and without BMP-2 also at day 8 (p=0.0216).

Aggrecan (*Acan*) follows as the next marker for the progression of chondrogenesis, again with a peak at day 8 throughout all three experimental groups. When normalized to control femur, there was significance between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 at day 8 (p=0.0367), between all three experimental groups at day 16 (p=0.0122) and between the implant placed on the periosteum and the implant placed in the muscle without BMP-2.
(p=0.0122) and between the implants placed in the muscle with and without BMP-2
(p=0.0122) at days 24 and 31. When normalized to 18s, there were similar trends with
significant differences occurring between the implant placed on the periosteum and the
implant placed in the muscle without BMP-2 and between the implants placed in the
muscle with and without BMP-2 at days 8, 12, and 24 (p<0.01). There was also
significance between all three experimental groups at day 16 (p=0.0122) and between the
implant placed on the periosteum and the implant placed in the muscle without BMP-2 at
day 31 (p=0.0122).

The final chondrogenetic gene analyzed was Col10a. This was the latest marker
of chondrogenesis analyzed, and displayed a peak at day 8 for the implant placed on the
periosteum and the implant placed in the muscle with BMP-2, and a peak at day 12 for
the implant placed in the muscle without BMP-2. For both normalizations there was
significance between the implant placed on the periosteum and the implant placed in the
muscle with BMP-2 at days 8 and 16 (p<0.03) and between the implant placed on the
periosteum and the implant placed in the muscle without BMP-2 (p<0.03) and between
the implants placed in the muscle with and without BMP-2 at day 24 (p<0.02). The two
normalizations differed at day 8. When normalized to control femur, there was
significance between all three experimental groups (p<0.03) compared to the 18s
normalization where there was significance between the implant placed on the
periosteum and the implant placed in the muscle without BMP-2 (p=0.0216) and between
the implants placed in the muscle with and without BMP-2 (p=0.0122).
Figure 7: qRT-PCR Analysis of Chondrogenesis Genes when DBM Tissue Placement is Varied and BMP-2 Added. The graphs display two different normalizations comparing the fold expression between the three experimental groups. The graphs on the left display the fold expression when normalized to control femur. The graphs on the right display the fold expression when normalized to 18s, also note the logarithmic scale on the y-axis. An ANOVA and Wilcoxon test were performed between the three experimental groups at each time point post-harvest. Significance was determined if p<0.05. “A” signifies significance between the implant placed on the periosteum versus muscle with BMP-2, “B” signifies significance between the implant placed on the periosteum versus muscle without BMP-2, “C” signifies significance between the implants placed in the muscle with and without BMP-2. “D” signifies significance between all three experimental groups.
Angiogenesis Gene Markers

Bone is a constantly remodeling organ and is therefore very dependent of having a constant source of nutrients. As such, angiogenesis is a necessary component to the formation and remodeling of bone. Two markers of angiogenesis, SMA and VE-Cadherin were analyzed to determine the fold expression in the three different implants.

SMA expression differed between the two normalizations. When normalized to control femur, there was a large peak at day 4 in both the implant placed on the periosteum and the implant in the muscle with BMP-2. The implant in the muscle without BMP-2 has a slight increase at day 8, and all three groups decreased in expression in days 12 through 31. There was significance between the implants placed in the muscle with and without BMP-2 at day 8 (p=0.0216) and days 16 and 31 between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 (p=0.0216 and p=0.0122) and between the implants placed in the muscle with and without BMP-2 (p=0.0200 and p=0.0200). When normalized to 18s, there were more instances of significance between experimental groups across all time points. Specifically, at days 4 and 24 between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 (p=0.0304 and p=0.0122) and between the implants placed in the muscle with and without BMP-2 (p=0.0304 and p=0.0122), days 12 and 16 between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0216 and p=0.0367) and the implant placed on the periosteum and the implant placed in the muscle without BMP-2 (p=0.0216 and p=0.0367). Furthermore, at day 8 there was significance between all three experimental groups (p<0.02) and at day 31.
between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0216) and between the implants placed in the muscle with and without BMP-2 (p=0.0200).

*VE-Cadherin* showed similar trends in both normalizations between the implant placed on the periosteum and the implant placed in the muscle without BMP-2, with high fold expression at days 2 and 8, and a largest decrease at day 4. The implant placed in the muscle with BMP-2 displays a different pattern peaking at day 8 and 12, with decreased expression at days 2 and 8. When normalized to control femur, there was significance at day 8 between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0230) and at day 31 between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0122) and between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 (p=0.0122).

When normalized to 18s, at day 8 there was significance between all three experimental groups with p<0.03. At days 12 and 16 there was significance between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 and significance between the implants placed in the muscle with and without BMP-2 (p=0.0122). At day 24 there was only significance between the implants placed in the muscle with and without BMP-2 (p=0.0216). At day 31, there was significance between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0122) and between the implants placed in the muscle with and without BMP-2 (p=0.0122).
Osteogenic Gene Markers

From the X-ray and microCT40 analysis, bone formation and mineralization became apparent at day 12. To analyze the mineralization further, various Osteogenic markers were analyzed.

The expression of Runx2 in the implant placed on the periosteum is relatively low compared to that of the implant placed in the muscle both with and without BMP-2. With BMP-2, the implant placed in the muscle shows increased expression in days 2 through 8,
maintaining that level through day 31. With no BMP-2, the implant placed in the muscle begins with a higher level of expression at day 4 and gradually decreases. For the normalization to control femur, there was significance between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 at days 8, 12, 16, and 24 (p<0.01), between the implants placed in the muscle with and without BMP-2 at days 4, 8, 16, and 24 (p<0.03), and between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 at day 4 (p=0.0304). In addition, there was significance between all three experimental groups at day 31 (p<0.02). When normalized to 18s, there is significance between all three experimental groups at days 8 and 24 (p=0.0304 and p=0.0367), between the implants placed in the muscle with and without BMP-2 at day 8 (p=0.0122), between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 at day 16 (p=0.0367), and between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 at day 31 (p=0.0367).

*Osterix* appears to have increased expression in the implant placed in the muscle with BMP-2 in both normalizations. When normalized to control femur, there was significance between the implants placed in the muscle with and without BMP-2 at days 4 through 31 (p=<0.03), between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 at days 4, 16, 24, and 31 (p<0.03), and between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 at days 8 and 12 (p=0.0122). When normalized to 18s, there was significance between the implants placed in the muscle with and without BMP-2 at days 8, 12, 24, and 31.
(p<0.0122), between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 at days 12, 24, and 31 (p=0.0122), and between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 at day 8 (p=0.0058).

*Osteocalcin* continues to follow the trend of *osterix*, with increased expression in the implant placed in the muscle with BMP-2. In all three experimental groups, expression does not increase until day 12, which is when mineralization was observed in the X-ray and microCT images. The fold expression was very similar between the two normalizations, with significance occurring between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 and between the implants placed in the muscle with and without BMP-2 at days 12, 24, and 31 (p<0.01), and at day 8 between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 (p=0.0058). When normalized to control femur, there is also significant difference at day 16 between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0122).

The final Osteogenic gene, *DMP-1* (DMP), is the latest of the markers analyzed. It continues to follow the trend of increased expression in the implant placed in the muscle with BMP-2. When normalized to control femur, there was significance between all three experimental groups at days 12, 16, and 31 (p<0.02), between the implants placed in the muscle with and without BMP-2 at days 8 and 24 (p=0.0122 and p=0.0122), between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 at day 8 (p=0.0149), and at day 24 between the implant placed on the
periosteum and the implant placed in the muscle with BMP-2 (p=0.0122). In the
normalization, there were again significant differences at the later time points.
Specifically, at days 12, 16, 24, and 31 there was significance between the implant placed
on the periosteum and the implant placed in the muscle with BMP-2 (p<0.03), at days 12
and 31 between the implants placed in the muscle with and without BMP-2 (p=0.0122
and p=0.0122), and at day 24 between the implant placed on the periosteum and the
implant placed in the muscle without BMP-2 (p=0.012).
Figure 9: qRT-PCR Analysis of Osteogenic Genes when DBM Tissue Placement is Varied and BMP-2 Added. The graphs display two different normalizations comparing the fold expression between the three experimental groups. The graphs on the left display the fold expression when normalized to control femur. The graphs of the right display the fold expression when normalized to 18s, also note the logarithmic scale on the y-axis. An ANOVA and Wilcoxon test were preformed between the three experimental groups at each time point post-harvest. Significance was determined if $p<0.05$. “A” signifies significance between the implant placed on the periosteum versus muscle with BMP-2, “B” signifies significance between the implant placed on the periosteum versus muscle without BMP-2. “C” signifies significance between the implants placed in the muscle with and without BMP-2. “D” signifies significance between all three experiential groups.
**BMP-2 Signaling Expression**

BMP signaling plays an important role in the formation of bone. From our X-ray and microCT images, there was no bone formation in the DBM placed within the muscle without exogenous BMP-2. BMP-2, its receptors, and a number of downstream signaling molecules were analyzed to see the expression in all three experimental groups in order to determine what role BMP-2 plays in ectopic bone formation.

The first BMP gene analyzed was *BMP-2*. BMP-2 was directly mixed with one of the implants placed in the muscle and was therefore very important to analyze its expression in all three groups. When normalized to control femur, the implant placed on the periosteum has the highest fold expression. There was significant difference in fold expression between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 at days 2, 4, 8, 16, 24, and 31 (p<0.03). There was also significance between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 at days 8, 12, 16, 24, and 31 (p<0.03). When normalized to 18s, the difference remained between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 at days 8, 12, 16, and 31 (p<0.01), but there was also significance between the implants placed in the muscle with and without BMP-2 at days 8, 12, 16, and 31 (p<0.01). At day 8, there was significance between all three experimental groups (p<0.03).

Following the signaling pathways of BMP-2, the receptors BMPR-1a (Alk3) and BMPR-1b (Alk6) were analyzed. BMPR-1a, when normalized to control femur, displayed significant difference in expression between the implant placed on the
periosteum and the implant placed in the muscle with BMP-2 and between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 at days 4, 8, 12, 16, and 24 (p<0.03). At days 2 and 31, there was also significance between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0304 and p=0.0367). When normalized to 18s there were similar trends of significance occurring specifically between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 at days 2, 4, 24, and 31 (p<0.03). The implant placed on the periosteum and the implant placed in the muscle without BMP-2 displayed significance at days 4 and 24 (p=0.0304 and p=0.0122) and all three experimental groups at day 16 (p<0.03).

For BMPR-1b, there was a much higher fold expression in the implant placed on the periosteum for all times points in both normalizations. When normalized to control femur there was significance between all three experimental groups at days 4 through 31 (p<0.03) and between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 at day 2 (p=0.0304). When normalized to 18s, there was significance between the implant placed on the periosteum and the implants placed in the muscle both with and without BMP-2 at days 4 through 31 (p<0.03) and at day 2 between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0304). The data between the two normalizations suggests that the receptors are more active in the implant when placed on the periosteum due to the higher fold expression.

BMPR-2 displays the same trends as displayed by receptors 1a and 1b, with increased expression in the implant when placed on the periosteum. When normalized to
control femur, this is evident due to the significant difference in fold expression between the implant placed on the periosteum and both implants in the muscle with and without BMP-2 at all time points (p<0.03). The expression differs when normalized to 18s, with significance occurring only at day 16 between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 (p=0.0122) and between the implants placed in the muscle with and without BMP-2 (p=0.0122) and at day 24 between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 (p=0.0122).

Downstream in the BMP-2 signaling pathway, \( ID1 \), expression was analyzed to further attempt to understand the comparative role BMP-2 plays in the growth of DBM in the different experimental groups. In both normalizations, \( ID1 \) expression is increased in the implant placed in the muscle with BMP-2. When normalized to control femur, there was significance between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 and between the implants placed in the muscle with and without BMP-2 at days 4, 24, and 31 (p<0.03). At days 8 and 12 there was significance between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 and between the implants placed in the muscle with and without BMP-2 (p<0.01). At day 16 there was significance between all three experimental groups (p=0.0122). When normalized to 18s there was only significant differences at day 24 between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0122) and at day 31 between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0122) and between the implant placed on the periosteum and
the implant placed in the muscle without BMP-2 (p=0.0367).

*Noggin*, a BMP-2 antagonist, was analyzed and displayed very similar results to that of the BMP-2 receptors, with an increase in expression in the implant place on the periosteum at all time points. When normalized to control femur, there was significance between all three experimental groups at all time points (p<0.03). When normalized to 18s, there was significance between the implant placed on the periosteum and the implant placed in the muscle both with and without BMP-2 at days 4, 8, 16, 24, and 31 (p<0.03) with significance between all three experimental groups at day 12 (p=0.0122).

The final gene analyzed with the BMP-2 signaling genes was *MSTN*. Although it is primarily a marker in skeletal muscle, it is a member of the TGF-β super family along with BMPs, and was therefore analyzed within this category. When normalized to control femur, *MSTN* displays a large peak at day 24 in the implant placed on the periosteum, with overall increased expression throughout all time points. At days 8, 16, 24, and 31 there was significance between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p<0.01). At days 12 and 24 there was significance between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 (p=0.0122). At days 8 and 24 there was significance between the implants placed in the muscle with and without BMP-2 (p=0.0122). When normalized to 18s, the fold expression was much closer between experimental groups with significance only occurring at two time points. At day 12, there was significance between the implant placed on the periosteum and the implant placed in the muscle without BMP-2 (p=0.0367) and between the implants placed in the muscle with and without BMP-2
(p=0.0216). At day 24, there was significance between the implant placed on the periosteum and the implant placed in the muscle with BMP-2 (p=0.0200) and between the implants placed in the muscle with and without BMP-2 (p=0.0200).
Figure 10: qRT-PCR Analysis of BMP-2 Signaling Genes when DBM Tissue Placement is Varied and BMP-2 Added. The graphs display two different normalizations comparing the fold expression between the three experimental groups. The graphs on the left display the fold expression when normalized to control femur. The graphs of the right display the fold expression when normalized to 18s, also note the logarithmic scale on the y-axis. An ANOVA and Wilcox test were performed between the three experimental groups at each time point post-harvest. Significance was determined if p<0.05. “A” signifies significance between the implant placed on the periosteum versus muscle with BMP-2, “B” signifies significance between the implant placed on the periosteum versus muscle without BMP-2. “C” signifies significance between the implants placed in the muscle with and without BMP-2. “D” signifies significance between all three experiential groups.
DISCUSSION

Effect of Tissue Site of DBM Implantation on Extent of Bone Formation

The DBM placed on the periosteal surface of the femur and the DBM implanted in the muscle with BMP-2 both induced ectopic bone formation while the DBM in the muscle with no additional BMP-2 did not. While throughout the time course, the BV of the implant in the muscle with BMP-2 does increase, most noticeably between days 24 and 31, the BV of the implant placed on the periosteum was consistently and significantly greater than that of the implant placed in the muscle with BMP-2 at earlier times. The TV of the implant in the muscle with BMP-2 was consistently and significantly greater than that of the implant on the periosteum. This BV/TV comparison suggests that while the implant in the muscle with BMP-2 is larger in size, it does not mineralize to the extent that the implant on the periosteum does. Figure 4 supports this with a visualization of both the size and relative densities of the implants. It appears that the implant in the muscle with BMP-2 increases in size, but forms a shell with the most mineralization occurring in the outer portion of the implant. Thus the implant on the periosteum while having an overall smaller TV is smaller is more compact and forms a more uniform volume of mineralized tissue in the new bone that is formed.

There was no detectable mineralization in the implant in the muscle without BMP-2. This suggests that BMP-2 is required for the induction of bone formation. This conclusion remains consistent with other studies, such as Jang et al., where it was concluded that activating DBM with recombinant BMP-2 enhanced the osteogenesis.
Further analysis using qRT-PCR was required in order to better determine the mechanisms leading to the recruitment, development, and growth of the implant in the varying experimental groups.

Comparison of Various Means of Evaluating Gene Expression Levels.

When examining the mRNA expression levels multiple approaches were taken to normalize the qRT-PCR results. Normalization was either as a fold to the base line level in bone or simply to the genes expression level per ratio to 18S rRNA. A third assessment of the baseline levels of each gene in muscle was also made. By normalizing to 18s, the gene expression is being normalized to the number of cells in the specific sample. This is based on the assumption that rRNA levels are constant per cell. This normalization is valuable in comparing directly between the experimental groups to determine where expression was the highest and at what time point and providing a direct comparisons of the genes expression in the different tissues. Conversely when assessed as a fold of the bone this provides a means of showing the levels of induction of the individual genes over that seen in cells in quiescent bone. Finally by accessing the baseline in muscle we can determine if the observed genes appeared to be expressed de novo in this tissue. Such comparisons are very useful in determining differences in the stem cell associated markers and possibly populations of stem cells recruited to the different sites. These assessments are also useful in assessing the overall quantities of new skeletal cell induction and times of these cells differentiation.
**Implant Placement Alters Stem Cell Gene Expression**

*Sox2, Oct4, and Nanog* are three of the earliest markers and are associated with embryonic stem cells (Go *et al.* 1147). The qRT-PCR results, for both normalizations, display a distinct pattern of expression of these genes between the DBM placed on the periosteum versus the DBM placed in the muscle with and without BMP-2. The day 2 increase in the implant on the periosteum is sharply contrasted to the implants in the muscle. This would suggest that there may be an extremely early population of stem cells in the periosteum that is recruited that contributes to bone development in response to the DBM placement on the periosteum. The implants in the muscle with and without BMP-2 showed no early induction with their levels largely remaining the same across the whole time course.

The osteochondrogenetic marker, *Gremlin*, displays two different patterns of expression when normalized to control femur versus normalized to 18s. When normalized to control femur, there is a major peak at day 8 in the implant in the muscle without BMP-2. Since *Gremlin* defines a population of stem cells that generate osteoblasts and chondroblasts, it could be inferred that this peak is related to an increase chondrocyte activity (Worthley 269). The DBM in the muscle with no BMP-2 does not induce mineralized bone, therefore the peak could be an indication that the implant is undergoing chondrogenesis and recruiting cells to proceed with osteogenesis, but it unable to continue with the process. When normalized to 18s, expression is increase in both implants in the muscle relative to the implant on the periosteum. The largest expression is seen in the implant in the muscle with BMP-2, specifically in days 12
through 31. During this time, increased mineralization of the implant in the muscle with BMP-2 is occurring, suggesting that there would be an increase in Osteogenic cell activity supported by the rise in Gremlin signaling.

The normalizations for Prx1 varied for the implant in the muscle with BMP-2. When normalized to control femur, all three experimental groups follow similar patterns and levels of expression peaking at day 8 and 16. These peaks correlate with the time in which chondrogenesis appears to be occurring in the implants. Since Prx1 is involved with the proliferation and differentiation of chondrocytes, this peak further supports chondrogenesis is occurring at day 8. (Berge et al. 3831). This also suggests that the process of chondrocyte signaling and recruitment is consistent between experimental groups. The groups vary between days 24 and 31 when there is a large increase in expression in the implant in the muscle with BMP-2. Further analysis of the histology could indicate if there is a reservoir of Prx1 positive cells in the implant at these later time points. When normalized to 18s, the expression changes drastically for the implant in the muscle with BMP-2, specifically in relation to the other experimental groups and at later time points. Labeling and comparing Prx1 positive cells between all three experimental groups, specifically at day 31, could help to better determine which normalization is a better representation of the population of cells present.

Pax7, identified as a myogenic stem cell, interacts with satellite cells in the muscle to cause proliferation (Morgan et al. 1151). Contrary to our initial expectations, Pax7 expression is higher in the DBM on the periosteum versus in the muscle (both with and without BMP-2) in both normalizations. This could be a result of two different
mechanisms working depending on the location of the implant. Furthermore, the implant on the periosteum could have increased expression because it is recruiting cells from the muscle. The implants in the muscle could require less of a signal due to the closer proximity to *Pax7* responsive cells.

*Cartilage Gene Expression and Endochondrial Ossification*

The model of endochondral ossification, with chondrogenesis occurring prior to ossification appears to be present in all three of the experimental groups. *Sox9*, the gene responsible for chondrocyte lineage commitment from mesenchymal cells (Provot et al. 659) shows significant increase in all three experimental groups at day 8. *Col2a1*, *Acan*, and *Col10a* are specific late cartilage markers, which are used to assess the progression of chondrogenesis, specifically in endochondral ossification (Mwale et al. 1791). The three markers, again, increase at day 8 in all three experimental groups. The peak indicates chondrocyte commitment and differentiation, is at day 8. This is consistent with our X-ray and MicroCT data where mineralized tissue first appears at day 12, indicating cartilage would be developing in the previous time points. The qRT-PCR graphs all decrease steadily from days 8 to day 31, which is the time when osteogenesis would be occurring.

Of the chondrogenic markers, *NCam1* is the only gene, which slightly differentiates from the pattern of peak expression at day 8. The expression is relatively high at both day 4 and day 8 followed by slight decreases though day 31. *NCam1*, a member of the immunoglobulin superfamily, is involved in cell-to-cell interaction as well
as matrix interactions during development (Wu et al. 577). The declining trend remains consistent with the results of Wu et al., observing a decline in NCam1 expression as condensed pre-chondrocytes differentiates into chondrocytes (Wu et al., 577). The gene expression remains consistent with expectation based on the microCT images and the process of endochondral ossification. While the level of expression is higher in the muscle, both placements follow similar trends.

Angiogenesis Expression

α-Sma expression, when normalized to control femur, is increased at days 2 and 4 in the implant placed on the periosteum and the implant placed in the muscle with BMP-2 compared to the implant placed within the muscle without BMP-2. By day 8, the expression decreases and proceeds to follow the same pattern and level of expression as that of the muscle implant both with and without BMP-2. α-Sma is a marker of myofibroblasts. The differentiation of myofibroblasts leads to the synthesis of smooth muscle cells, which make up blood vessels (Abdalla et al.). The rise of SMA in the implant placed on the periosteum and the implant placed in the muscle with BMP-2 could relate to their abilities to mineralize at later time points. Increased α-Sma suggests the development of vessels that would be vital for the development of bone. The implant in the muscle with BMP-2 has lower fold expression, and does not develop and mineralize into bone. The normalization to 18s differs in that the expression in the muscle without BMP-2 is increased. The expression could be increased as an attempt for the implant to recruit smooth muscle cells to develop in order to form a blood source for implant. When
it is unable to do so, the expression decreases as seen in days 12 through 16, and the implant is unable to mineralize.

VE-Cadherin expression, when normalized to control femur, is very similar in the implant on the periosteum and in the muscle with no BMP-2. The implant in the muscle with BMP-2 has the same relative levels of expression, but follows a different pattern throughout the initial time points. It remains consistently lower than both other experimental groups until day 31 where it increases. VE-cadherin’s primary role is in the adhesion of endothelial cells. For vascularization, they play a crucial role in embryonic angiogenesis (Vestweber 223). As such, the increase at days 2 and 8 could correlate with increasing vascularization to assist in the chondrogenesis that is also occurring at day 8. The normalization to 18s displays very comparable levels between the three experimental groups with the lowest expression being in the muscle with no BMP-2. Again this suggests that the implant in the muscle with no BMP-2 was not able to induce the differentiation of myofibroblasts, and therefore the signaling for adhesion would be decreased.

**Bone Gene Expression**

*Runx2* is an osteoblastic transcription factor that stimulates the differentiation of skeletal myocytes into osteoblasts (Gersbach 874). It has also been demonstrated that *Runx2* has a significant role during chondrocyte hypertrophy in endochondral ossification (Kim *et al.* 2). The gene expression of *Runx2* suggests that the implant in the muscle without BMP-2 attempts to induce chondrocyte hypertrophy and differentiation of
myocytes through the high expression at day 4. The expression decreases through day 8, as the implant is unable to induce the differentiation to form cartilage. The implant in the muscle with BMP-2 shows a similar pattern to that of the periosteal implant, but with higher fold expression. This higher expression, especially at the later time points could account for the muscle implant with BMP-2’s increase in bone volume in days 24 through 31.

*Osterix* expression remains very consistent between the two normalizations with the highest expression being seen in the implant in the muscle with BMP-2 and lower expression in the muscle without BMP-2. The increase in the implant in the muscle with BMP-2 as compared to the implant on the periosteum correlates with the slower mineralization of the implant in the muscle versus periosteum. At day 16, the implant on the periosteum has already reached near its maximum bone volume while the implant in the muscle with BMP-2 is continuing to mineralize at a slower rate. As a result, the expression remains higher in the muscle with BMP-2 while the implant on the periosteum decreases at day 16.

*Osteocalcin*, a small protein secreted by osteoblasts, is a marker for the mineralization of bone (Caetano-Lopes *et al.* 107). Osteocalcin activity is increased in the implant in the muscle with BMP-2 specifically in days 16 through 31. The bone volume measurements follow this trend as the implant in the muscle with BMP-2 increases in volume in the later time points. This expression differs from both the DBM on the periosteum as well as the implant in the muscle with no BMP-2, which show minimal expression.
**DMP1**, dentin matrix protein 1, is associated with the later stages of skeletogenesis, specifically the mineralization of bone (Sun et al. 199). In the normalization to control femur, the increased expression of DMP1 in both the DMB implant in the muscle with BMP-2 and the implant on the periosteum correlate to bone development in both cases. The expression is higher in the implant in the muscle with BMP-2, which could a result of the slower bone formation. Expression is increased in days 16 through 31 in an attempt to catch up to the developing periosteal implant. The lack of expression in the implant in the muscle with no BMP-2 is supported by the lack of bone formation seen in the MicroCT and X-ray analyses. In the normalization to 18s, the same trends remain, except at day 24 when the implant in the muscle without BMP-2 increases above that of the implant in the periosteum. This increase is different than what we expected, as there was no mineralized bone detected in the implant in the muscle without BMP-2.

**BMP-2 Signaling Expression Limited to the Periosteum**

BMP-2 signaling follows very comparable expression throughout the signaling pathway. In general, for BMP-2 in the periosteum, there is an increased level of expression through the entire time course as compared to in the muscle where there is essentially no expression. This pattern continues through the BMPR1a, BMPR1b and BMPR2, but there is a much larger fold change in BMPR1b. The BMP-2 signaling in the implant on the periosteum is consistent with the development and mineralization of the implant. It is interesting that the implant in the muscle has low fold expression even when
BMP-2 is added. The implant in the muscle with BMP-2 may have a reduced need to signal endogenous BMP-2 resulting in the decreased expression.

Noggin increases in the periosteum at day 24 when BMP-2 shows declining expression. This differs from Noggin’s expression in the muscle with BMP-2 where there is comparable expression throughout the time course. As a result, Noggin’s role as a BMP-2 antagonist appears to be important in the periosteal implant, having less of an affect in the muscle. Expression of ID1 is increased in days 2 through 4, steadily decreasing and remaining constant in days 12 through 31 in the implant in the muscle with BMP-2. This differs from the implant on the periosteum and the implant in the muscle with no BMP-2 where there is very low expression. MSTN, a member of the TGF-β superfamily that has an important role in negatively regulating muscle growth, peaks in all three groups (Rios et al. 993). In the implant on the periosteum and implant in the muscle without BMP-2, there is a peak at day 24 with a higher expression in the implant on the periosteum. This peak correlates with noggin, which also peaks at day 24 in the implant on the periosteum. The implant in the muscle with BMP-2 does not reach the same fold expression and appears to have relatively low expression. This correlates with Lui et al. who reported that muscle genes are down regulated in response to BMPs (Lui et al. 73).

Conclusion & Future Work

The X-ray and MicroCT images provide support that DBM is a viable option for bone formation in vivo. Based on the gene expression, a model of endochondral
ossification is accepted in the formation of ectopic bone, with a peak in chondrogenetic expression prior to osteogenesis.

The gene expression showed a large degree of variation between the different placements of DBM in the muscle and on the periosteum. Based on microCT analysis, DBM in the muscle required BMP-2 in order to induce bone formation. The final volume and distribution of the bone appears to differ between the two placements. When placed on the periosteum, the total volume is much smaller, but the overall bone volume is increased. This suggests a smaller, more solidified bone formation. The implant in the muscle with BMP-2 has a decreased bone volume, but a significantly larger total volume. In future analysis, the bone volume could be determined for the core of the implant versus the exterior in order to determine the density and distribution of the bone formation in the muscle with BMP-2.

The gene expression differed between the implant on the periosteum and the implant in the muscle with and without BMP-2 added. The implant on the periosteum and the implant in the muscle with BMP-2 were able to induce bone formation while the implant in the muscle without BMP-2 was not. This proved interesting, as all three groups had some degree of expression of the early stem cell markers. The patterns of expression differed between the implant on the periosteum and the implants in the muscle suggesting the preliminary mechanisms leading to the recruitment of stems cells and differ between the two tissue locations. It appears that when placed on the periosteum, the implant is able to induce early stem cell markers, whereas the implant in the muscle with BMP-2 is developing through an alternative mechanism. All three experimental groups
were able to induce the formation of cartilage as seen in the cartilage gene markers. When the cartilage was progressing to bone, the DBM in the muscle without BMP-2 was able to signal, but the cells were unable to progress. The DBM in the muscle with BMP-2 and the implant on the periosteum continued to ossify and mineralize to detectable volumes of bone.

Overall, this study provided further information into the effect of tissue placement on the quality of progression of bone formation. The most optimal implant growth occurred when placed directly on the periosteum, but growth was inducible in the muscle when BMP-2 is added. The placement of the implant in different tissues not only affected the size and volume of the implant, but also the gene expression. For stem cell recruitment, it is evident that different mechanisms are occurring to progress bone formation in the different tissue locations. Due to the low expression from the stem cell genes analyzed, future studies are needed to determine what mechanism is occurring when the implant is placed in the muscle. Specific indicator mice can be used to identify cells where stem cell makers are expressed, for example Prx1, Pax7, and Sox2 and follow their temporal and spatial movement. For the analysis of the effect of BMP-2, it is concluded that the addition of BMP-2 is required for the formation of bone. When placed on the periosteum, endogenous BMP-2 is present leading to the development and progression of the implant to form bone. When placed in the muscle, the implant progressed to form bone only with the addition of BMP-2. The gene expression supported this with increased expression in the implant placed on the periosteum and in the muscle with BMP-2. To further analyze the role BMP-2 plays in DBM’s ability to recruit stem
cells versus promote differentiation in different tissue locations, noggin, a BMP-2 antagonist, can be added to the DBM and placed on the periosteum and in the muscle. This would further illustrate whether DBM when placed on the periosteum retains its ability to induce various stem cell associated genes or whether this effect is dependent on BMP-2. The question of retention of BMP2 in DBM and its spatial presentation at implant sites will further be analyzed by quantum dots used tag BMP-2 within the various DBM implant sites.
REFERENCES


CURRICULUM VITAE

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EDUCATION

Boston University School of Medicine | Boston, MA | September 2013 – May 2015
Master of Science in Medical Sciences
Relevant Coursework: Human Physiology, Biochemistry, Histology, Pathology, Biomedical Informatics, Biostatistics

College of the Holy Cross | Worcester, MA | August 2009 – May 2013
Bachelor of Arts in Mathematics
Relevant Coursework: Introduction to Biology, Organic Chemistry, General Chemistry, Physics, Calculus, Multivariable Calculus, Medical Ethics
Directed Project: Modeling Negative Curvature of Beta Sheet Proteins
Honors: Dean’s List in 2012 and 2013

RESEARCH & WORK EXPERIENCE

Boston University School of Medicine: Department of Orthopaedic Surgery
Boston, MA | July 2014 – Current | Graduate Researcher
• Completed a master’s thesis pertaining to the role of bone morphogenetic protein 2 (BMP-2) in ectopic bone growth in muscle

University of Buffalo: Orthopaedics and Sports Medicine
Buffalo, NY | June 2013 – August 2013 | Undergraduate Literature Reviewer
• Performed a literature review on allografts versus autographs in elbow instability
• Familiarized myself with research techniques, various databases, and relevant literature

Massachusetts General Hospital: Department of Clinical Cardiology
Boston, MA | May 2012 – August 2012 | Research Assistant
• Prepared, aliquoted, and handled blood samples for a multisite study
• Enrolled patients, collected specimens, and performed data entry for BIONICS-Heart Failure study
• Created a database for the North South East West Study
• Attended a certification course in phlebotomy
• Observed patients the Cardiac Intensive Care Unit
Excelsior Orthopaedics
Buffalo, NY | May 2011 – August 2011 | Student Shadow
• Shadowed doctors, physicians assistants, and physical therapists
• Observed several surgical procedures

RESEARCH SKILLS
• Laboratory mouse handling including intraperitoneal and subcutaneous injections
• Surgical techniques including anesthesia and suturing
• X-Ray imaging and development
• Scanning using a MicroCt40 and image analysis
• Extensive experience with RNA extraction, cDNA production, and qRT-PCR
• Knowledge of agarose Gel Electrophoresis running and analysis
• Experience with Microsoft PowerPoint and Excel, as well as the presentation of scientific findings
• Proficient in MATLAB

LEADERSHIP & VOLUNTEER ACTIVITIES

Massachusetts General Hospital: Patient Escort Services
Boston, MA | March 2014 – Current | Volunteer
• Assist patients entering and being discharged from the hospital
• Work on a team with peers to coordinate the successful transport of patients

American Mathematical Society: Spring Eastern Sectional Meeting
Chestnut Hill, MA | April 2013 | Student Organizer and Presenter
• Presented on Modeling Negative Curvature of Beta-Sheet Proteins
• Reviewed undergraduate submissions for acceptance to the conference
• Chaired and ran presentations throughout the session

College of the Holy Cross: Student Programs for Urban Development (SPUD)
Worcester, MA | May 2012 – May 2013 | Educational Opportunities Intern
• Accountable for seven volunteer sites, seven program directors, and over a hundred volunteers
• Worked with campus groups and program directors to promote social justice and action within the Holy Cross community
• Developed teamwork and organizational skills working intimately with four fellow interns to coordinate the largest student organization on campus

City View Afterschool Program
Worcester, MA | September 2009 – May 2013 | Program Director and Volunteer
• Volunteered weekly with elementary and middle school children
• Provided students with one-on-one attention for both tutoring and games
• Transported thirty volunteers to and from the site

**Students Helping Children Across Borders, Inc.: Working for Worcester**
Worcester, MA | September 2012 – May 2013 | *Site Coordinator*

• Developed and implemented a city-wide project to build community infrastructure for Worcester’s families and children
• Partnered with local business and colleges to execute a city wide effort
• Coordinated and planned a cafeteria remodeling and grounds clean up of Elm Park Elementary School

**College of the Holy Cross: Arrupe Immersion Program**
Tanzania | January 2012

• Visited Project Partners of International Partners in Mission
• Socialized with orphans of HIV/AIDS and Malaria victims at the Mgolole children’s orphanage
• Learned the history and culture of Tanzania at the University of Dar es Salaam
• Developed a deeper understanding for the important of cross cultural exchange and solidarity through building relationships with members of the community

**College of the Holy Cross: Department of Mathematics**
Worcester, MA | September 2011- May 2013 | *Student Advisory Committee*

• Distributed and reviewed student course evaluations
• Involved in the hiring and promotion of new and existing professors

**College of the Holy Cross: Spring Break Immersion**
Lynchburgh, VA | March 2008 | *L’Arche USA Volunteer*

• Spent a week living and working with L’Arche community members
• Formed relationships with adults living with disabilities

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**PUBLICATIONS**


Berardinis, B. D., Magrini, L., Gaggin, H. K., Belcker, A., Zancla, B., Femia, A., ...
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