SIGNIFICANCE OF BONE MORPHOGENETIC PROTEIN-2 EXPRESSION ON REGULATION OF OSTEOCLAST DIFFERENTIATION AND SPECIFIC GENE EXPRESSION

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF THE UNIVERSITY OF MINNESOTA

BY

DAVID PAULSON

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

KIM C. MANSKY, PhD

MAY 2019

ACKNOWLEDGMENTS

I would like to acknowledge Dr. Kim Mansky for supporting and guiding me through this research project. The amount of knowledge I gained on this topic from her mentorship was invaluable. Her willingness to help and teach made this project possible and successful.

I would like to thank Dr. Massimo Costalonga for his time dedicated to making sure my research progress was on track. He has improved my skills in both performing research and being able to critically evaluate the work and literature of others.

I would like to acknowledge Dr. Larry Wolff for agreeing to serve on the committee of my master's thesis. His kind heart and eagerness to help residents in any way he can are unmatched.

In addition, I would like to thank Dr. Jim Hinrichs for accepting me into the Advanced Education Program in Periodontology at the University of Minnesota.

DEDICATION

This master's thesis is dedicated to my family. To my wife Lindsay for her unwavering love, support, and patience with me over my eleven years of university education and training. Her willingness to let me pursue my goals and her commitment to our relationship has helped me achieve things I couldn't have done alone. To my parents, Steve and Julie, for their never-ending support and love. The opportunities and experiences they enabled me to attain have molded me into the person I am today, and I couldn't have accomplished nearly as much without them.

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BACKGROUND

Bone Biology

1. Composition

Bone comprises the largest proportion of the human body's connective tissue mass. It is considered a composite material as its extracellular matrix is made up of mineral, collagen, water, non-collagenous proteins, and lipids. Bone is unique in its physiology compared to other connective tissues in that its matrix is mineralized and is constantly turned over throughout the life of an individual. The mineral phase of bone is composed of nanocrystalline hydroxylapatite [$(CA_{10}(PO_4)_6(OH)_2$] with various substitutions of carbonate, magnesium, acid phosphate, and other trace elements which are dependent on the environmental and dietary factors of a particular individual. The functions of the mineral in bone are to strengthen the composite, provide mechanical resistance, and serve as a source of calcium, magnesium, and phosphate ions for skeletal homeostasis. (Rosen, 2013)

2. Osteoblasts/Osteocytes

There are three cell types present in bone tissue. One of these is the bone-forming osteoblast. Osteoblasts originate from mesenchymal stem cells which can also differentiate into muscle, chondrocytes, fat, ligament, and tendon. Bone morphogenetic proteins (BMPs) are thought to control the commitment of mesenchymal stem cells to the osteoblast lineage. (A. Bassi, 2011) Once differentiated, osteoblasts function to

mineralize the extracellular matrix. The process of mineralization involves osteoblasts secreting type I collagen and the enzyme alkaline phosphatase, and depositing of calcium and phosphate from the blood to bond with osteoid. (Karsenty & Wagner, 2002)

One fate of an osteoblast is to become engulfed in the bone mineral and become an osteocyte which is a terminally differentiated osteoblast. Osteocytes create a network amongst themselves by extending long processes to nearby osteocytes forming a canalicular network. The cell body remains in the lacuna, and the Haversian canals provide a vascular supply where nutrients and waste are exchanged. (Bellido, 2014)

3. Osteoclasts

Hematopoietic stem cells are able to differentiate into cells of two primary lineages, lymphoid and myeloid. Osteoclasts, the bone cells responsible for resorption, are derived from the myeloid lineage which includes granulocytes, monocytes, megakaryocytes, dendritic cells, erythrocytes, and platelets. These develop from progenitors which originate in the bone marrow. The commitment to a particular lineage is controlled by distinct transcription factors followed by differentiation in response to specific colony-stimulating factors. (Kawamoto, 2004) Osteoclasts, which are members of the monocyte-macrophage family, are multinucleated giant cells. (Suda et al., 1999) Bone marrow macrophages are the physiological osteoclast precursor. (Teitelbaum, 2000) In order for osteoclastogenesis to occur, two key cytokines are essential. One is receptor activator of nuclear factor-κB ligand (RANKL) and the other is macrophage-colony stimulating factor (M-CSF). M-CSF has been shown to promote proliferation and prevent apoptosis of osteoclast precursors. (Lee & States, 2006) RANKL stimulates osteoclast precursors to exit the cell cycle and terminally differentiate. (Ross, 2006)

Together, M-CSF and RANKL induce the expression of genes that characterize the osteoclast lineage, including but not limited to *c-Fos, Nfatc1, Dc-stamp, Acp5* and *Cathepsin K*. (Boyle, Simonet, & Lacey, 2003) The role of these genes in regulating osteoclast differentiation is discussed in the following sections.

3.1 Osteoclast Transcription Factors

c-Fos

One of the key regulators of osteoclast lineage determination and bone remodeling is the c-Fos oncoprotein. It is a component of the activator protein 1 (AP-1) transcription factor complex which is involved in regulating cell growth and proliferation, cellular differentiation, and apoptosis. c-Fos is active early in the osteoclast differentiation process. As evidence of their role in osteoclast differentiation, it was shown that hematopoietic cells lacking *c-Fos* are unable to differentiate into functional osteoclasts *in vivo*. Mice with a conditional knockout of *c-Fos* do not have differentiated, multinucleated osteoclasts and develop the bone disease osteopetrosis. (Grigoriadis et al., 1994)

Nfatc1

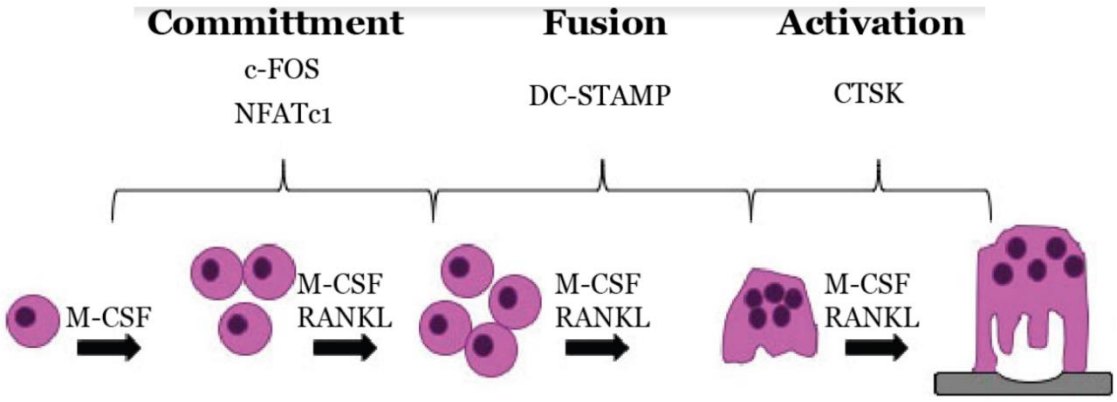
Nuclear factor of activated T-cells (NFATc1) is known as the master regulator of osteoclast differentiation. NFATc1 has been shown to regulate genes necessary for osteoclast differentiation and resorption. (Song et al., 2009) The presence of RANKL stimulation can induce the expression of NFATc1 the nuclear factor-kappa B (NF-κB) pathway. As further evidence to the key role of NFATc1 in osteoclast differentiation, it

was shown that embryonic stem cells deficient in NFATc1 fail to differentiate into osteoclasts in response to RANKL stimulation. Likewise, ectopic activation of NFATc1 in the absence of RANKL stimulation causes osteoclast precursors to undergo differentiation. Thus, NFATc1 acts as a master switch for regulation of osteoclast differentiation and functions downstream of RANKL in the differentiation process. (Takayanagi et al., 2002)

Dc-stamp

The fusion of multiple mononuclear osteoclasts into larger multinuclear cell is a key step in the differentiation and function of these cells. One of the primary genes involved in the fusion of osteoclasts is dendritic cell-seven transmembrane protein (DC-STAMP). This transmembrane protein is highly expressed in osteoclasts but not in macrophages, indicating its key role in the osteoclast lineage. DC-STAMP is expressed on the cell surface and is critical for cell-cell fusion. DC-STAMP is expressed midway through the differentiation process. (Xing, Xiu, & Boyce, 2012) Experiments have demonstrated that despite normal expression of osteoclast markers and cytoskeletal structure, the absence of DC-STAMP results in osteoclast cell fusion being completely halted. (Yagi et al., 2005)

Figure 1: Genes involved in osteoclast differentiation



3.2 Mechanism of Bone Resorption

As stated earlier under normal physiological conditions, osteoclasts are responsible for resorption of bone. Resorption begins by multinuclear osteoclasts tightly attaching to the bone surface by rearrangement of their cytoskeletal proteins to form a sealing zone. Within this sealing zone, the cytoplasmic membrane forms a ruffled border which increase the surface area available to contact the bone. Proteolytic enzymes and hydrogen and chloride ions are then secreted onto the bone surface. (Seeman, 2009) Hydrogen and chloride ions are responsible for dissolution of the mineralized hydroxyapatite matrix while the protease involved in collagen digestion is cathepsin K. The importance of cathepsin K was demonstrated by RANKL promoting its expression and by knockout mice for the gene displaying significant osteopetrosis. (Zaidi, Blair, Moonga, Abe, & Huang, 2003) The sealing zone allows for the degradation of the mineralized matrix while neighboring cells are simultaneously protected. (Boyce & Xing, 2008) During the process of bone resorption by the osteoclast, growth factors

embedded in the bone matrix are released, and these growth factors recruit osteoblasts to resorption sites as well as stimulate osteoblast activity which is vital to the process of homeostatic bone modeling and remodeling. (Charles & Aliprantis, 2014)

Acp5

Acp5 is the gene that encodes for tartrate-resistant acid phosphatase (TRAP). TRAP is an enzyme found in postmitotic osteoclast precursors and differentiated, multinucleated osteoclasts. The function of TRAP is to dephosphorylate bone matrix proteins like osteopontin and bone sialoprotein and allow osteoclast migration along the ruffled border. (Ek-Rylander, Flores, Wendel, Heinegard, & Andersson, 1994) TRAP activity is localized histochemically over these cells and is thus used as a marker of osteoclasts. (Minkin, 1982)

Ctsk

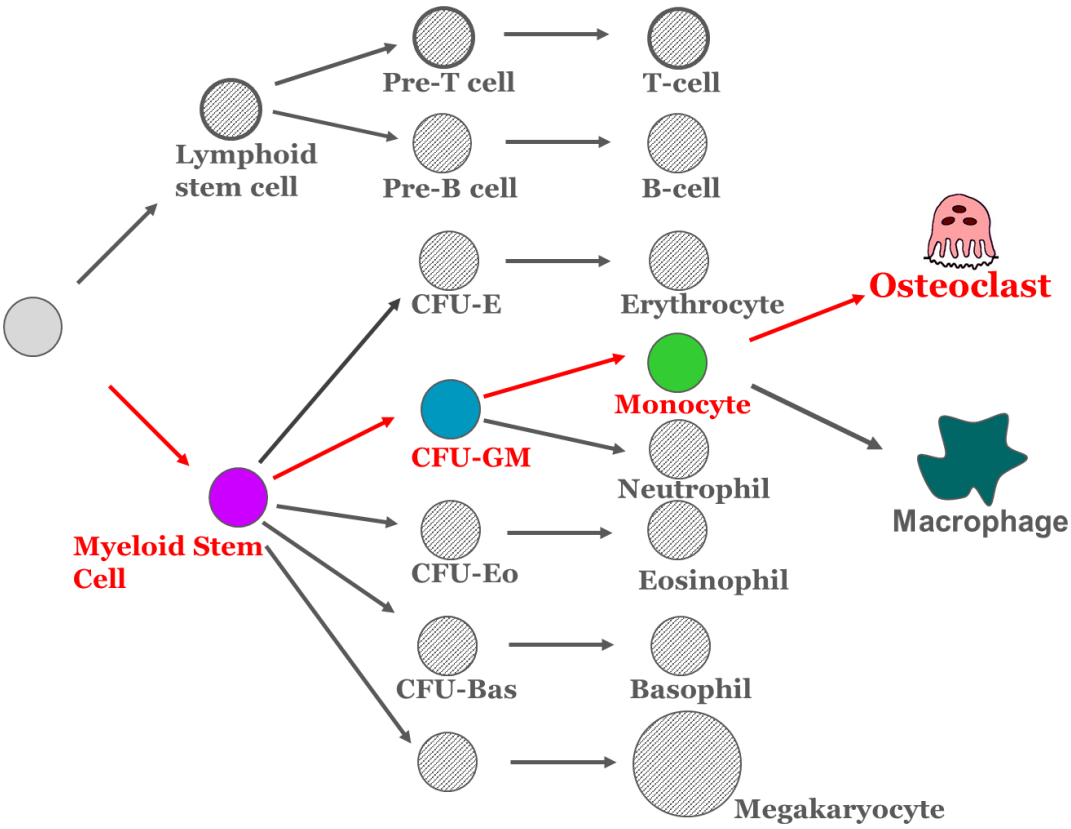
Cathepsin K (CTSK) is a protease enzyme which plays a major role in osteoclast-directed bone resorption. It is secreted by osteoclasts into the sealed osteoclast-bone cell interface and degrades type I collagen and other matrix proteins. (Drake, Clarke, Oursler, & Khosla, 2017) This protein is expressed later in the differentiation process. A mouse expressing a global knockout of CTSK leads to decreased bone resorption which results in osteopetrosis. (Lotinun et al., 2013)

3.3 Homeostasis

Bone homeostasis relies on the proper balance of osteoblast and osteoclast function. It is osteoblasts and/or osteocytes that produce the cytokines M-CSF and RANKL to promote osteoclast differentiation and maintain this balance. (Xiong et al., 2011) Bone

remodeling occurs over a period of several weeks in a temporary anatomical structure known as a basic multicellular unit. Within this structure a unique microenvironment is generated which facilitates coupling of osteoclast resorption and osteoblast formation. This functions to ensure there is minimal net change in bone volume during the remodeling process. (Raggatt & Partridge, 2010) Bone remodeling can be broken down into four distinct phases. The first phase is the activation phase during which detection of an initiation signal takes place. This signal can come in several forms including direct mechanical strain on the bone or hormone action on bone cells in response to systemic changes. Next is the resorption phase in which osteoclasts initiate resorption of the organic and mineral components of the bone. This phase lasts two to four weeks. Upon reaching a certain size of resorption area, the resorption process is terminated by apoptosis of the osteoclasts. The reversal phase prepares the resorbed surface for new matrix deposition by recruiting macrophage-like cells to smooth the surface. Finally, the formation phase takes place when osteoblasts lay down new bone. Throughout this phase some osteoblasts will become engulfed in the bone and differentiate into osteocytes and remain in the lacunae. The formation phase can last four to six months until osteoblasts undergo apoptosis. (Wittkowske, Reilly, Lacroix, & Perrault, 2016)

Figure 2: Myeloid cell lineage



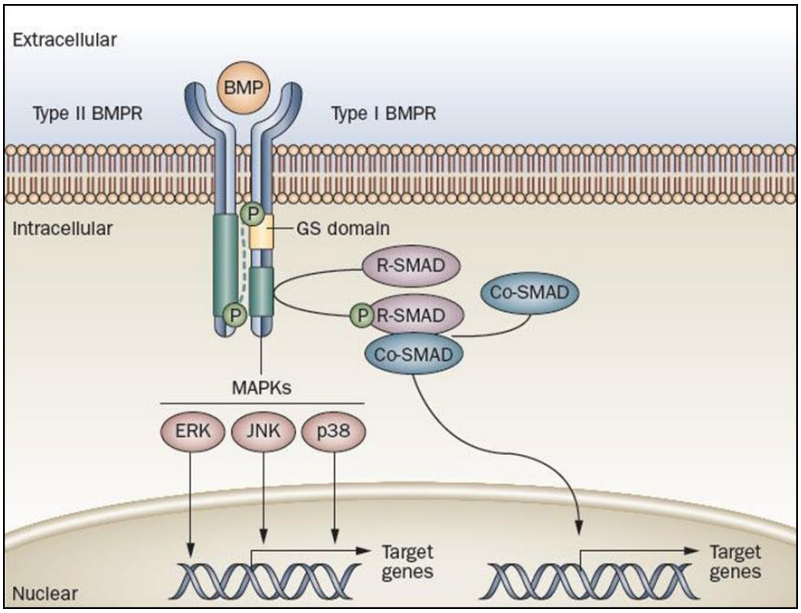
3.4 Bone Morphogenetic Proteins / Signaling

Bone morphogenetic proteins (BMPs) are multifunctional cytokines belonging to the transforming growth factor-beta (TGF-β) superfamily. They are responsible for stimulating angiogenesis and migration, proliferation, and differentiation of mesenchymal stem cells into cartilage and bone forming cells. BMPs are known to operate through distinct mechanisms and signaling pathways. The canonical signaling cascade for BMPs

begins when a signal is transmitted across the plasma membrane by the formation of heteromeric complexes of specific type I and type II serine/threonine kinase receptors. Following the activation of specific type II receptors, the type I receptor is phosphorylated which initiates intracellular signaling. Consequently, specific Smad proteins, R-Smads, are phosphorylated and these activated R-Smads form a complex with co-Smad, Smad4. Once formed, this complex will translocate into the nucleus to direct transcription of the DNA into corresponding RNA. This results in targeted gene expression. (G. Chen, Deng, & Li, 2012)

The non-canonical pathway is a second mechanism for BMP ligands to activate BMP signaling. It occurs when BMP receptors are not dimerized but binding of BMP ligand to BMPR-1A recruits BMPR-2 into a complex. This signaling complex has been shown to initiate non-SMAD pathways or non-canonical signaling pathways such as mitogen activating protein kinases or MAPKs.

Figure 3: BMP signaling



BMP Receptors

Osteoclasts express several BMP receptors that are responsible for detecting and activating BMP signals resulting in various effects on osteoclastogenesis. BMP receptor type 1A (BMPR-1A) is activated in the late stages of osteoclast differentiation and is involved in regulation of osteoclast and osteoblast coupling. Conditional deletion of this gene in osteoclasts results in increased markers of bone resorption while the conditional deletion of this gene in osteoblasts results in increased bone formation. (Okamoto et al., 2011) In addition, it was shown that osteoclasts derived from mice having a conditional deletion of BMPR-1A in their myeloid cells had a decreased ability to form multinuclear TRAP positive cells. (A. Li et al., 2017) These findings demonstrate the biphasic effect of BMPs on osteoclast differentiation.

Another important receptor in BMP signaling is BMP receptor type 2 (BMPR-2). Deletion of this gene in myeloid lineage cells including osteoclasts results in mice that are osteopetrotic with an increase in both bone volume and trabeculae due to a decrease in osteoclast differentiation and activity. While these mice had changes in the noncanonical (MAPK)l pathway, none were observed in the canonical (SMAD) pathway. This suggests that these pathways are utilized at different stages of osteoclast differentiation. (Broege et al., 2013)

3.5 BMPs and Osteoclasts

It has been demonstrated that osteoclasts express BMP-2 in both early lineage cells (bone marrow macrophages) and mature osteoclasts. (Itoh et al., 2001) In the early lineage cells, BMP-2 can either increase or decrease cell proliferation depending on the concentration present. However, this stimulation by BMP-2 does not alter lineage commitment. It is believed that this variable response to BMP-2 stimulation may be a technique of controlling maintenance or cell expansion of early lineage cells. (Bhatia et al., 1999)

A series of published studies from the Mansky/Gopalakrishnan lab at the University of Minnesota have led to discoveries which serve as the basis of the research presented in this thesis. Since BMPs exert their physiological activities through transmembrane receptors, their signaling is subject to regulation at both the intracellular and extracellular levels. (Gazzerro & Canalis, 2006) Twisted gastrulation (TWSG1) is an example of an extracellular proteins involved in regulating BMP signaling. Extracellular modulation of BMP signaling by TWSG1 has been shown to occur because TWSG1 physically interacts with cell surface receptors used by BMPs. This direct antagonism limits the accessibility of BMPs to their receptors. In addition, TWSG1 is one of the major modulators of BMP signaling in the extracellular space. The binding of this secreted protein to BMPs is required for its inhibitory effects on osteoclasts. (Huntley et al., 2015) Mice deficient for TWSG1 developed osteopenia which was a result of marked enhancement of

osteoclastogenesis. This phenomenon was due to increased cell fusion, differentiation, and function of osteoclasts. The osteoclasts in the TWSG1 knockout mice were significantly larger in size than those in wild-type controls. Furthermore, the enhanced osteoclastogenesis in the TWSG1 knockout mice was reversed with exposure to Noggin, a BMP antagonist. The authors were also able to increase RANKL-mediated osteoclastogenesis by treating wild-type osteoclasts with rhBMP-2. These data suggested that the enhanced osteoclastogenesis is a result of the increased BMP signaling. (Rodriguez et al., 2009)

In another study, BMP-2 was found to enhance differentiation of multinucleated osteoclasts when added to cultures with suboptimal levels of RANKL. This enhancement is not a result of changes in the proliferation or survival of the cells but is accredited to an increase in expression of genes involved in osteoclast differentiation and fusion. When osteoclast cultures are treated with BMP-2, expression of RANKL was not significantly altered indicating the enhancement of osteoclastogenesis is not mediated through increased RANKL expression. In the absence of RANKL, the addition of BMP-2 did not induce osteoclast formation, confirming that BMP signaling is not adequate to induce osteoclast differentiation. Osteoclasts express BMP-2 receptors and the differentiation of osteoclasts is promoted by an autocrine signaling mechanism. (Jensen et al., 2010)

The next publication aimed to overexpress TWSG1 in osteoclasts in order to inhibit osteoclast activity. Overexpression of this gene, which is an inhibitor of BMP-2 signaling, led to a decreased size and number of TRAP-positive osteoclasts, expression of osteoclast genes, and resorption ability. The authors also demonstrated that by adding exogenous BMP-2 to the osteoclasts overexpressing TWSG1, the size of the osteoclasts

was able to be rescued. They were also able to restore the size of enhanced osteoclasts found in TWSG1 deficient mice by infecting them with TWSG1 via an adenovirus. Furthermore, the rescue of these osteoclasts was reversed by addition of exogenous BMP-2. These experiments confirmed the inhibitory role of TWSG1 in osteoclast differentiation by disrupting BMP signaling. (Pham et al., 2010)

The final study from the lab investigated the role of BMP signaling in osteoclastogenesis by eliminating BMP receptor 2 using a conditional knockout. As stated above in the BMP receptor section, a significant decrease in both size and number of multinucleated osteoclasts was found in the BMP receptor 2 knockout mice compared to the wild type controls. Bone marrow macrophages, a precursor cell of osteoclasts, are severely inhibited in their ability to differentiate into mature multinucleated osteoclasts even in the present of M-CSF and RANKL when their BMP receptor 2 is knocked out. The skeletal phenotype of these mice was also altered to have increased bone mass due to the reduced bone resorption. (Broege et al., 2013)

Indirect Effect of BMPs on Osteoclasts

The above research has shown that BMPs can directly regulate osteoclast differentiation. However, earlier studies demonstrated that BMPs regulate osteoclast differentiation indirectly through actions on chondrocytes, osteoblasts, and osteocytes. Starting in 1995, one group demonstrated that cultures of rat bone marrow cells treated with osteogenic protein-1 (OP-1) enhanced the ability of vitamin D_3 to induce formation of TRAP positive osteoclasts *in vitro.* (Hentunen et al., 1995) Both murine and chicken chrondrocytes have been shown to express RANKL RNA and protein which was significantly enhanced by BMP2/SMAD1 activation. This RANKL

protein expression from chrondrocytes may act to regulate osteoclast differentiation at growth plates in order to remove calcified matrix through BMP induction. (Usui et al., 2008) Another study demonstrated that BMP-2 would stimulate mature osteoclasts in the presence but not absence of stromal cells, suggesting that BMP-2 stimulates bone resorption by osteoclasts indirectly through stromal cells. (Kanatani et al., 1995) Further evidence of the involvement of stromal cells was shown by studies that demonstrate BMP-2 stimulating bone resorption by osteoclasts through the modulation of RANKL RNA expression. (Itoh et al., 2001) Other groups have demonstrated that BMP2 can modulate the expression of osteoprotegerin (OPG) by osteoblasts and thereby regulate osteoclast differentiation. (Kamiya et al., 2016) Inflammatory conditions may also be affected by BMP-2. When BMP-2 and IL-1□ are present in combination, the expression of RANKL RNA by osteoblasts is upregulated. This indirectly enhances osteoclast differentiation and may enhance bone resorption during these inflammatory states. (Koide et al., 1999)

Analysis in a mouse model with deletion of *Bmpr1a* in osteoblasts demonstrates an increase in bone mass due to disruption in the ratio of RANKL to OPG resulting in a decrease in osteoclast differentiation and activity. (Shi, Zhang, Louie, Mishina, & Sun, 2016) Until more recently the critical role of osteocytes in regulating skeletal development were not well understood. In a mouse model using *Dmp1-Cre* to disrupt *Bmpr1a* in osteocytes, the authors determined that similar to the mice null for *Bmpr1a* in osteoblasts, the expression of OPG bone RNA was increased and RANKL bone RNA was decreased leading to a sharp reduction in osteoclast differentiation and

activity. (Kamiya et al., 2008) All this data collectively suggests that BMPs can act on other cells of the skeleton and indirectly regulate osteoclast differentiation and activity.

3.5 Development of Recombinant BMPs

The first coding sequences for bone morphogenetic protein family members were cloned and expressed in the late 1980s. The identification and isolation of BMPs in bone matrix was difficult to obtain due to the proteins being tightly bound to components of the extracellular matrix. Recombinant technologies allowed BMPs to be created for therapeutic use. (Rao, Ugale, & Warad, 2013) Molecular cloning of their cDNA and their expression in Chinese hamster ovary cells allowed the recombinant proteins to be obtained in large quantities for preclinical and clinical evaluation and therapeutic use. (Carreira et al., 2014) While at least twenty types of BMPs have been identified in humans, only BMP-2 and BMP-7 have been approved by the FDA for therapeutic use in medicine. BMP-7 is typically used in cases of non-union fractures and may be combined with autologous bone grafting or used alone. (Gautschi, Frey, & Zellweger, 2007) For surgeons who perform bone grafting procedures, these proteins offer a commercially available osteoinductive autograft replacement. The appeal is to avoid having to harvest autogenous bone from a second surgical site and the associated complications which made the identification and development of rhBMP-2 a significant advancement in the field of dentoalveolar bone grafting.

Periodontology

Epidemiology

Some of the most prevalent conditions of the oral cavity are plaque-induced periodontal diseases. Gingivitis, the more mild and reversible form of periodontal disease, affects 50-90% of adults in the United States. (Y. Li et al., 2010) Periodontitis, the more destructive and irreversible form of periodontal disease, has been estimated to affect 47% of the adult population in the United States. (Eke, Dye, Wei, Thornton-Evans, & Genco, 2012) The vast prevalence and widespread health and economic burden that this disease process poses makes the research and advancement of knowledge in the field valuable to millions of individuals.

Etiology

Periodontitis is a chronic inflammatory disease which results from the interaction between periodontal pathogens and the susceptible host's immune response. The presence of certain microbes and their by-products elicit an inflammatory response in the surrounding periodontal tissues. (Offenbacher, 1996) While protective in nature, this host response can eventually result in local tissue destruction if either hypo- or hyper-responsiveness of the system occurs. The primary determinants of the individual host response are both genetic and environmental. (Preshaw, Seymour, & Heasman, 2004) Ultimately, periodontitis leads to destruction of alveolar bone, periodontal ligament and connective tissue, cementum, and gingiva.

Periodontal Tissue Destruction by the Host Immune Response

The host immune response and its interaction with the periodontal bacteria present results in various cytokines and chemokines being released which play a role in osteoclastogenesis. Rather than a select few microorganisms being thought of as the main pathogens and initiators of periodontal disease, a newer model of pathogenesis has been developed. According to this model termed polymicrobial synergy and dysbiosis, periodontitis is initiated by a synergistic and dysbiotic microbial community. Microbes present in the subgingival environment of the periodontitis lesion have various gene combinations and virulence factors that enable them to disrupt or evade immune surveillance by the human host by fulfilling distinct roles. The result of this immune subversion is a dysbiotic microbial community which disrupts the homeostasis of the periodontal tissue microenvironment through various virulence factors including co-adhesion, production of toxic proteolytic enzymes, and proinflammatory ligands. (Hajishengallis & Lamont, 2012) Together, the dysbiotic microflora are able to sustain a proinflammatory state which elicits the host response, eventually resulting in tissue destruction if left unresolved. The breakdown of the tissue creates products that serve the nutritional needs of the community and allow further growth of the virulent organisms. (Gaffen & Hajishengallis, 2008)

The direct modulators of osteoclast in periodontitis are RANKL, tumor necrosis factor alpha (TNF-α), and interleukin 1 (IL-1). In addition, bacteria involved in the pathogenesis of the disease may produce various forms of LPS and TLR activating ligands that can directly stimulate osteoclastogenesis. (Novack & Mbalaviele, 2016) Toll-like receptors (TLRs) are transmembrane glycoproteins that function to recognize

microbes and play a role in signaling the activation of osteoclast differentiation. (Jimenez-Dalmaroni, Gerswhin, & Adamopoulos, 2016) TNF-α and IL-1 are pro-inflammatory cytokines which participate in the immune response by recruitment and activation of adaptive immune cells. (Ebersole & Cappelli, 2000) RANKL, which is necessary for the complete differentiation of osteoclast precursor cells, is expressed primarily by activated B and T cells in the periodontal lesion. The increased source of RANKL plays a primary role in the enhancement of the bone resorption process in periodontal disease. (B. Chen et al., 2014)

Applications of Bone Morphogenetic Proteins in Periodontology

Clinical Applications

There is currently only one Food and Drug Administration (FDA) approved product for the use in oral and maxillofacial surgery and dental regeneration which contains bone morphogenetic proteins. INFUSE® Bone Graft is an alternative to autogenous bone grafting and consists of recombinant human Bone Morphogenetic Protein-2 (rhBMP-2) placed on a resorbable collagen sponge. It is indicated for use in sinus augmentation and localized alveolar ridge augmentations for defects associated with extraction sockets. Clinical research in oral and maxillofacial uses of INFUSE® have demonstrated that at a concentration of 1.5mg/cc, significant bone formation is induced which is biologically similar to native bone and is suitable for dental implant placement. Osseointegration of these dental implants occurs and functional loading of them with a prosthesis is

successful. (McKay, Peckham, & Badura, 2007) In sinus augmentation, rhBMP-2 was found to increase the overall mean vertical height of the available bone by 8.51mm without any serious immunologic or adverse effects. (Boyne et al., 1997) Alveolar ridge preservation after tooth extraction and localized alveolar ridge augmentation have also been demonstrated to be safe and feasible treatments with the use of rhBMP-2. (Howell et al., 1997)

Mechanism of Action

The mechanism of action of rhBMP-2 is through osteoinduction. It stimulates the recruitment and differentiation of bone-forming cells which induce new bone formation or aid in the healing of existing bone. Once rhBMP-2 is implanted, the migration of mesenchymal stem cells to the site of implantation occurs. The rhBMP-2 and the absorbable collagen sponge provide an environment where the stem cells are able to multiply. (Wilke, Traub, Kienapfel, & Griss, 2001) They then differentiate into osteoblasts due to the rhBMP-2 binding to specific receptors on the stem cell surface. Once differentiated, osteoblasts are able to produce new mineralized tissue which replaces the absorbable collagen sponge. (Puleo, 1997) Concurrently, angiogenesis is taking place. In response to the local environmental and mechanical forces, the bone is continually remodeled and results in normal trabecular bone. (Schmitt, Hwang, Winn, & Hollinger, 1999)

Complications and Adverse Events

While rhBMP-2 does show promise as an alternative to autogenous bone grafting, it is not without a potential for adverse side effects and events. Clinical trials demonstrated that the most frequent adverse events associated with INFUSE® are mouth pain (85.0%), oral edema (67.5%), facial edema (67.5%), and oral erythema (47.5%). The significant amount of edema present with the use of this product is likely due to the recruitment of fluid and cells into the area being treated. The immune response of the patients in these clinical trials were assessed for the presence of antibodies using an enzyme-linked immunosorbent assay (ELISA) before and after the use of rhBMP-2. Of the 184 patients tested, 4 of them (2.2%) had a positive antibody response to rhBMP-2. It is theoretically possible that the antibodies made in response to rhBMP-2 could neutralize endogenous BMP-2, but this was not seen in any of the patients. (Medtronic, 2012)

RATIONALE AND SIGNIFICANCE

At the center of the alveolar bone destruction present in inflammatory, plaque-induced periodontitis is enhanced osteoclastogenesis and the subsequent pathologic resorption of bone by these osteoclasts. Advancing the knowledge in the fields of bone biology and periodontology are paramount to developing therapies that can help prevent and/or reverse periodontal tissue destruction. Multiple publications from the Mansky/Gopalakrishnan lab have established BMPs as important *in vivo* regulators of osteoclast formation and demonstrated that aberrant activation of BMP signaling in osteoclasts promotes bone resorption in mice. (Broege et al., 2013; Jensen et al., 2010; Pham et al., 2010; Rodriguez et al., 2009) This disproportionate bone loss is similar to the pathological bone loss of human diseases such as osteoporosis, rheumatoid arthritis, osteolytic cancers, and periodontitis. These disorders which cause osteoclast-mediated bone destruction create a serious burden on both patient morbidity and the economic costs associated with treating them. There is a great need for new treatments aimed at osteoclasts. BMPs have been suggested as potential therapeutic targets with the prediction that inhibiting their signaling will impair osteoclast formation and reduce bone loss. Bone formation and bone resorption must be precisely balanced in order to sustain normal skeletal homeostasis. BMPs are important positive regulators of bone formation. The effectiveness of BMPs in promoting bone formation has been documented. However, what is still poorly understood is the mechanisms by which BMPs regulate bone resorption through the direct regulation of osteoclast differentiation and activity. The significance of this current study will be to add to the existing knowledge of BMP regulation and function which may eventually help

refine current therapies and contribute to improved preventive, therapeutic, and bone regenerative strategies for diseases associated with increased bone loss.

MATERIALS AND METHODS

Breeding of *Bmp2$^{fl/fl}$;LysM-Cre*

Bmp2$^{fl/fl}$ mice were obtained from Dr. Stephen Harris, University of Texas-San Antonio (Ma & Martin, 2005). These mice were crossed with B6.129-*Lyzstm1(cre)Ifo/J* mice (*LysM-Cre)* which expresses CRE recombinase in cells of the myeloid lineage (Jackson Labs (Clausen, Burkhardt, Reith, Renkawitz, & Forster, 1999). The use and care of these mice was reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee, IACUC protocol number 1806-36053A. Mice were euthanized by asphyxiation with CO_2.

Primary Osteoclasts Cell Culture

Primary bone marrow macrophages were harvested from the femurs and tibiae of wild-type or *Bmp2$^{fl/fl}$;LysM-Cre* littermates and adherent tissue was removed. Primary bone marrow macrophages from the mice were then isolated from the femora and tibiae. The ends of the femora and tibiae were cut and the bone marrow was flushed out from the inner compartments. Red blood cells were lysed from the flushed marrow using red cell blood lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, ph 7.4). The resulting cells were then plated and cultured overnight in 10 cm tissue culture dishes (TPP, MidSci) in osteoclast media (phenol red-free alpha-MEM (Gibco) with 5% heat inactivated fetal bone serum (Hyclone), 25 units/mL penicillin/streptomycin (Invitrogen),

400 mM L-Glutamine (Invitrogen), and supplemented with 1% CMG 14-12 (culture supernatant containing M-CSF). Cell populations that were non-adherent, including osteoclast precursor cells, were then removed and replated in 12-well cell culturing plates (TPP, MidSci) at a concentration of 2 x 10^5 cells/well in osteoclast media supplemented with 1% CMG culture supernatant containing M-CSF. Subsequently, every two days, cells were refed with 1% CMG plus 10 ng/mL of RANKL (R&D Systems) to initiate osteoclastogenesis.

Selecting for Osteoclasts

This process selects for osteoclast precursors because T-cells and B-cells do not recognized M-CSF. Stromal cells adhere to the plates while the osteoclasts/macrophages are floating. TRAP staining then further distinguishes osteoclasts because they are the only TRAP-positive cell present. Sorting is not necessary because the knockout is conditional only in myeloid lineage cells and is not a global knockout. This is consistent with protocols used throughout the literature.

Tartrate Resistant Acid Phosphatase (TRAP) Staining

After culturing the primary osteoclasts as described above, the cells were fixed with 4% paraformaldehyde for 20 minutes. Primary osteoclasts expressing TRAP were stained using the Naphthol AS-MX phosphate and Fast Violet LB salt protocol (BD Biosciences Technical Bulletin #445). The composition of this stain included tartrate 5 mg, Naphthol AS-MX phosphate, 0.5 mL M, M-Dimethyl formamide, 50 mL acetic acid buffer (1 mL

acetic acid, 6.8 g sodium acetate trihydrate, 11.5 g sodium tartrate in 1 L water) and 25 mg Fast Violet LB salt. Cells that were stained were imaged and photographed using bright field light microscopy at 4x magnification. The pictures were analyzed using NIH ImageJ to measure the size in area and number of the TRAP positive osteoclasts. Mean cell count and mean average size in micrometers were calculated from the three images taken per sample.

RNA Isolation and Real-Time PCR

RNA was harvested from primary osteoclasts plated in duplicate using TRIZOL Reagent (Ambion, Life Technologies) and quantified using UV spectroscopy. cDNA was then prepared from 1 ug of purified RNA using iScript cDNA Synthesis Kit (Bio-Rad) as per manufacturer's protocol. Quantitative real-time PCR was performed in duplicate using CFX Connect Real-Time PCR system (Bio-Rad). Each reaction contained 10 μl iTaq Universal Sybr Green Supermix, 8.8 μl DEPC water, 500 nM forward and reverse primers, and 1 μl cDNA for a total of 20 μl per reaction. The PCR conditions were as follows: 95°C for 3 minute, and 40 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, followed by melting curve analysis (95°C for 5 seconds, 65°C for 5 seconds, and then 65°C to 95°C with 0.5°C increase for 5 seconds). *C-fos* (Forward) 5'-CCA AGC GGA GAC AGA TCA ACT T (Reverse) 5'-TCC AGT TTT TCC TTC TCT TTC AGC AGA; *Nfatc1* (Forward) 5' -TCA TCC TGT CCA ACA CCAAA; (Reverse) 5' -TCA CCC TGG TGT TCT TCC TC; *Cathepsin K* (Forward) 5'-AGG GAA GCA AGC ACT GGA TA; (Reverse) 5'-GCT GGC TGG AAT CAC ATC TT; *Dc-stamp* (Forward) 5'-GGG

CAC CAG TAT TTT CCT GA; (Reverse) 5' -TGG CAG GAT CCA GTA AAA GG. Experimental genes were normalized to *Hprt* (Forward) 5'-GAG GAG TCC TGT TGA TGT TGC CAG and (Reverse) 5'-GGC TGG CCT ATA GGC TCA TAG TGC. All measurements were performed in duplicate and analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Each experiment for cell count and size was run in triplicate and performed three times. Each experiment for gene expression was run in duplicate and performed three times. All results are expressed as mean ± standard deviation. Student's unpaired t-test were used to compare data using GraphPad Prism version 8. A p-value of $p < 0.05$ indicates significance. Descriptive statistics were used to present the results of the sample's characteristics including mean, standard deviation, range, and difference of means for cell count, average cell size, and relative expression of genes.

Hypothesis testing to determine the probability that a given hypothesis is true using statistics consists of four steps. The first is formulating a null and alternative hypothesis. The null hypothesis is that there is no difference between the two groups, or that the observations seen are a result of pure chance. The alternative hypothesis is that the observations seen show a real effect. The second step is identifying a test statistic that can be used to assess the truth of the null hypothesis. The test statistic used in the research being presented is the t-value. The t-value measures the size of the difference relative to the variation in the sample data. More specifically, a 2-sample t-test is being used to find evidence of a significant difference between the population means of the

control and test groups. The third step is computing a p-value. The p-value is the probability that a test statistic at least as significant as the one observed would be obtained assuming the null hypothesis is true. The final step is to compare the p-value to an acceptable significance value (known as the alpha value or α). If $p \leq \alpha$, then the observed effect is considered statistically significant, the null hypothesis is taken as false, and the alternative hypothesis is taken as valid. A large p-value provides more evidence toward the null hypothesis and a smaller p-value provides stronger evidence against the null hypothesis.

Two types of errors can occur when performing hypothesis testing. The first is the type I error which is the incorrect rejection of the null hypothesis. When this error is made, the statistics reveal a significant difference when there actually is not a significant difference. This is also known as a false positive. The other error that can be made is the type II error which is the incorrect retention of a null hypothesis that is false. When this error is made, the statistics reveal no significant difference when there actually is a significant difference. This is also known as a false negative.

The statistical power of any test of statistical significance is the probability that it will reject a false null hypothesis. In other words, power is the likelihood that a test will detect an effect where there is an effect to be detected. When the statistical power is high, the probability of making a type II error goes down. In this research, a sample size of 9 and 18 in two groups will have 80% power to detect an effect size of 1.19 (ratio of mean difference and standard deviation).

RESULTS

Osteoclasts null for BMP2 expression are smaller than wildtype osteoclasts

As presented in the introduction, multiple researchers have demonstrated that BMP signaling enhances RANKL stimulated osteoclast differentiation and that osteoclasts express BMPs. (Itoh et al., 2001) Research from the Mansky lab had demonstrated that osteoclasts express Bmp2 mRNA throughout differentiation. (Jensen et al., 2010) However, there has been no *in vivo* research to date demonstrating the significance of BMP2 expression by osteoclasts on the skeleton. In an attempt to address this research question, bone marrow macrophages were cultured in the presence of M-CSF and RANKL to generate osteoclast cultures. Osteoclasts were fixed and stained for TRAP. Using the computer program NIH Image J, osteoclast size and number were quantitated. Before analysis of size and number of TRAP stained cells, parallel cultures of osteoclasts were analyzed by RT-qPCR to verify loss of *Bmp2* expression (data not shown). TRAP positive multinucleated osteoclasts were measured on day two, three, and four after RANKL stimulation. Both the total number of cells present and the average size of the cells were evaluated. The data shown are presented in the following graphical representations and summary tables.

Figure 4: TRAP stained images from days 2, 3, and 4

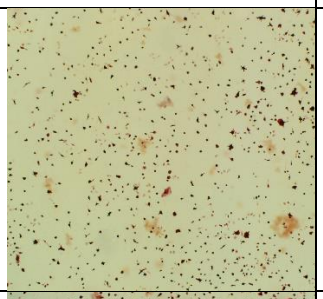
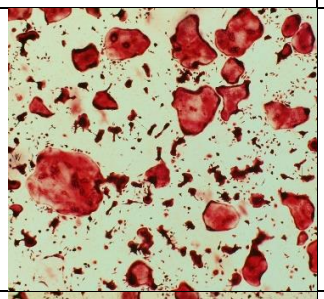
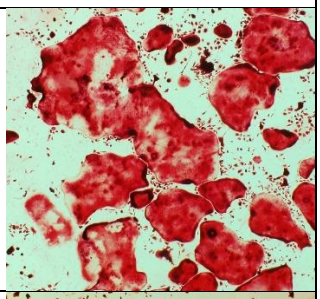
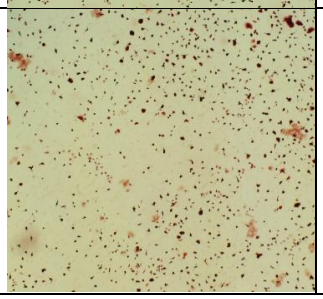
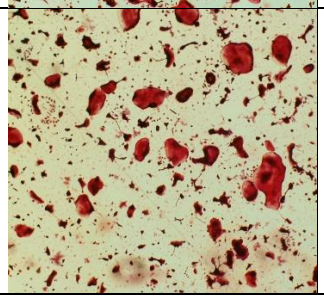
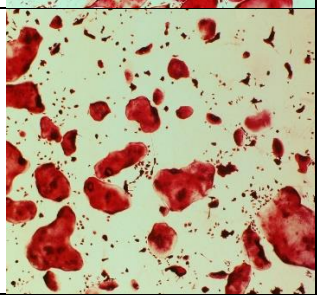
TRAP Stained Cells	Day 2	Day 3	Day 4
Wild-type			
Bmp2$^{fl/fl}$;LysM-Cre			

Figure 5: Bone marrow macrophages (BMMs) were flushed from WT or BMP2cKO mice. BMMs were stimulated with M-CSF and RANKL for indicated days. TRAP stained images were quantified for number of TRAP positive cells at 2 days, 3 days or 4 days. Samples were compared using an unpaired, two-tailed student's t-test.

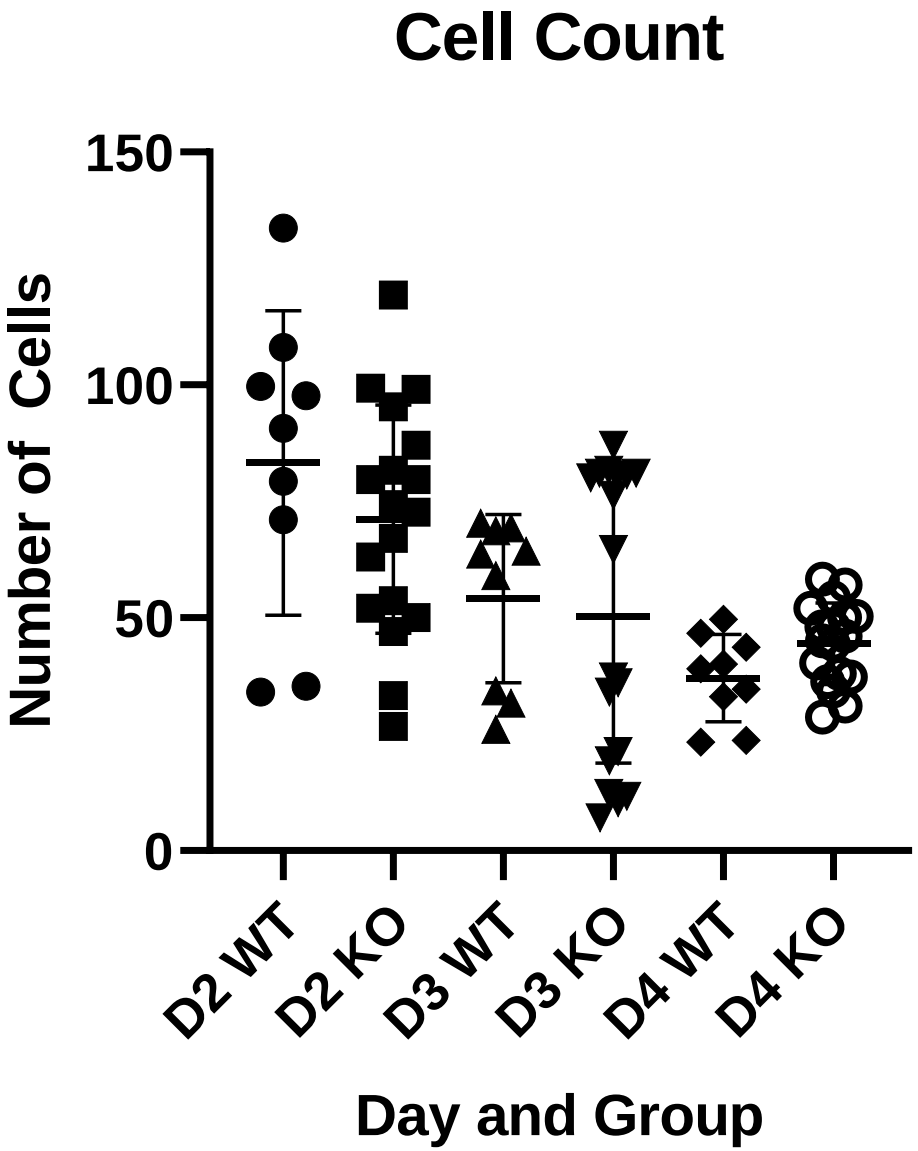


Table 1: Cell counts

Cell Count	n	Mean	SD	Range	Difference of Means	p-value
Day 2 WT	9	83.26	32.74	34.00 – 133.70	-12.11	0.2892
Day 2 KO	18	71.15	24.48	26.67 – 119.30		
Day 3 WT	9	54.15	18.07	26.00 – 70. 33	-4.09	0.1176
Day 3 KO	18	50.13	31.31	7.00 – 87.00		
Day 4 WT	9	37.07	9.34	23.33 – 49.67	7.33	0.0557
Day 4 KO	18	44.41	8.76	28.67 – 58.33		

The results of the cell counts from both the wild type and Bmp2 null osteoclasts for days 2, 3, and 4 are presented in Table 1. The sample size for all the wild type groups was 9 and the sample size for all of the Bmp2 null groups was 18. In both groups, the mean number of cells progressively decreased from day 2 through day 4. While there were no statistically significant differences ($p < 0.05$) between the two groups at any of the three timepoints, there was a trend at day 4 of the wild type cell count being less than the knockout group which approached statistical significance ($p = 0.0557$).

Figure 6: Bone marrow macrophages (BMMs) were flushed from WT or BMP2cKO mice. BMMs were stimulated with M-CSF and RANKL for indicated days. TRAP stained images were quantified for size of TRAP positive cells at 2 days, 3 days or 4 days. Samples were compared using an unpaired, two-tailed student's t-test.

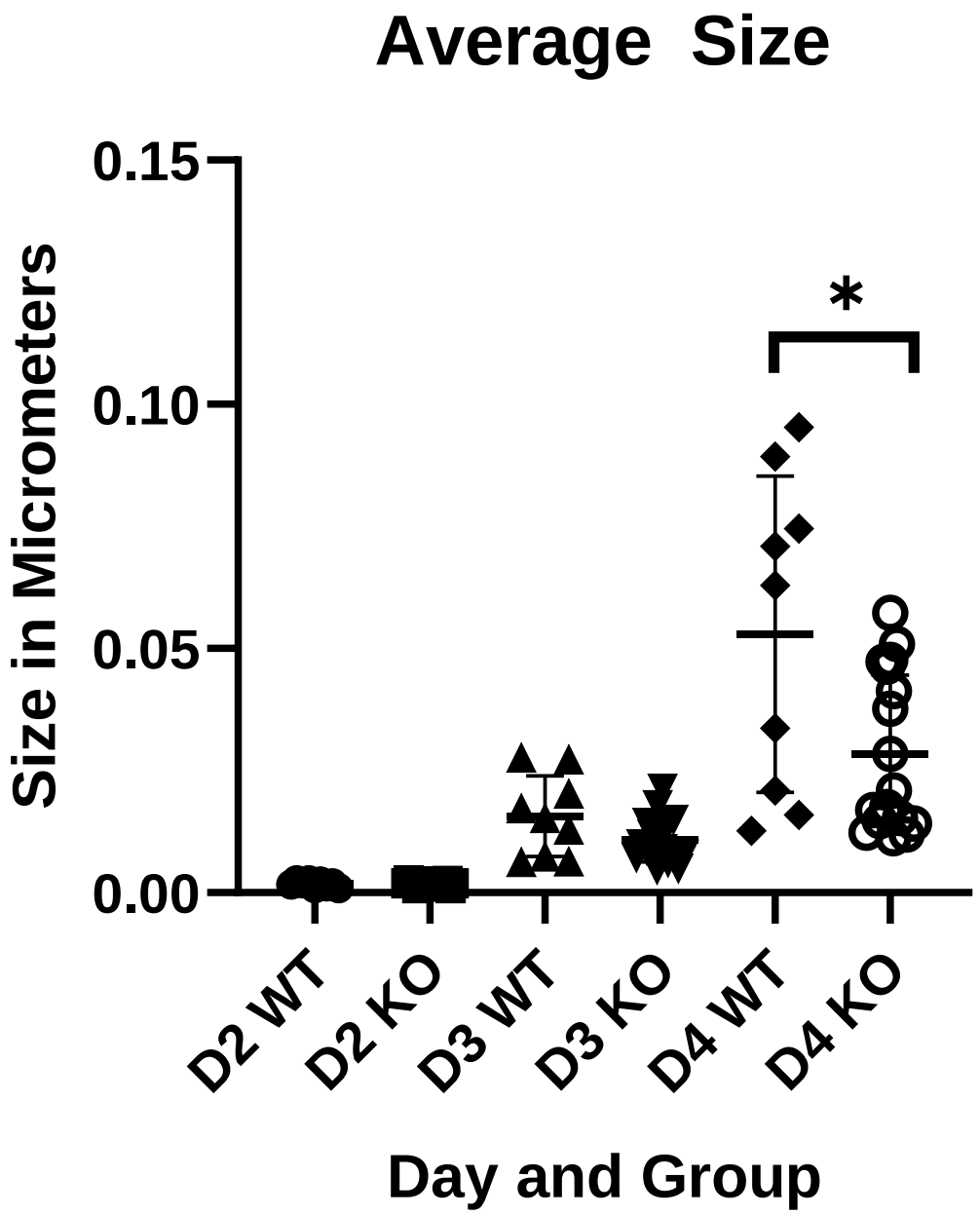


Table 2: Average size

Average Size (micrometers)	n	Mean	SD	Range	Difference of Means	p-value
Day 2 WT	9	0.0018	0.0006	0.0001 – 0.0026	2.278e-005 ± 0.0002144	0.9163
Day 2 KO	18	0.0019	0.0004	0.0001 – 0.0025		
Day 3 WT	9	0.0157	0.0083	0.0063 – 0.0277	-0.004828 ± 0.002457	0.0606
Day 3 KO	18	0.0109	0.0046	0.0047 – 0.0213		
Day 4 WT	9	0.0529	0.0324	0.0127 – 0.0953	-0.02466 ± 0.009263	0.0134*
Day 4 KO	18	0.0283	0.0162	0.0111 – 0.0573		

The results of the cell size from both the wild type and *Bmp2* null groups for days 2, 3, and 4 are displayed in Table 2. As stated for Table 1 and Figure 5, the sample size for all the wild type groups was 9 and the sample size for all of the knockout groups was 18. In both groups, the mean cell size progressively increased from day 2 through day 4. There were no statistically significant differences ($p < 0.05$) between the groups at day 2 or day 3; however, at day 3 the difference in cell size approached statistical significance ($p=0.0606$) with the wild type cells being larger than the knockout cells. At day 4, the wild type cells had a statistically significant larger mean cell size when compared to the *Bmp2* null osteoclasts ($p=0.0134$).

Bmp2 null osteoclasts have no changes in gene expression

To begin to understand mechanistically how loss of Bmp2 expression leads to a decrease in osteoclast size, real time quantitative polymerase chain reaction (RT-qPCR) was used to detect and quantify RNA transcripts of four specific genes. As presented in the introduction, the expression of the examined genes are important for osteoclast formation and function.

Figure 7: qRT-PCR comparing expression of *c-Fos* from WT and BMP2cKO mice after 2, 3 or 4 days of RANKL treatment. (Data shown are the mean ± SD of three independent experiments in which gene expression was measured from three wells of each genotype, with each PCR reaction performed in duplicate. Expression of each gene is graphed relative to *Hprt*.

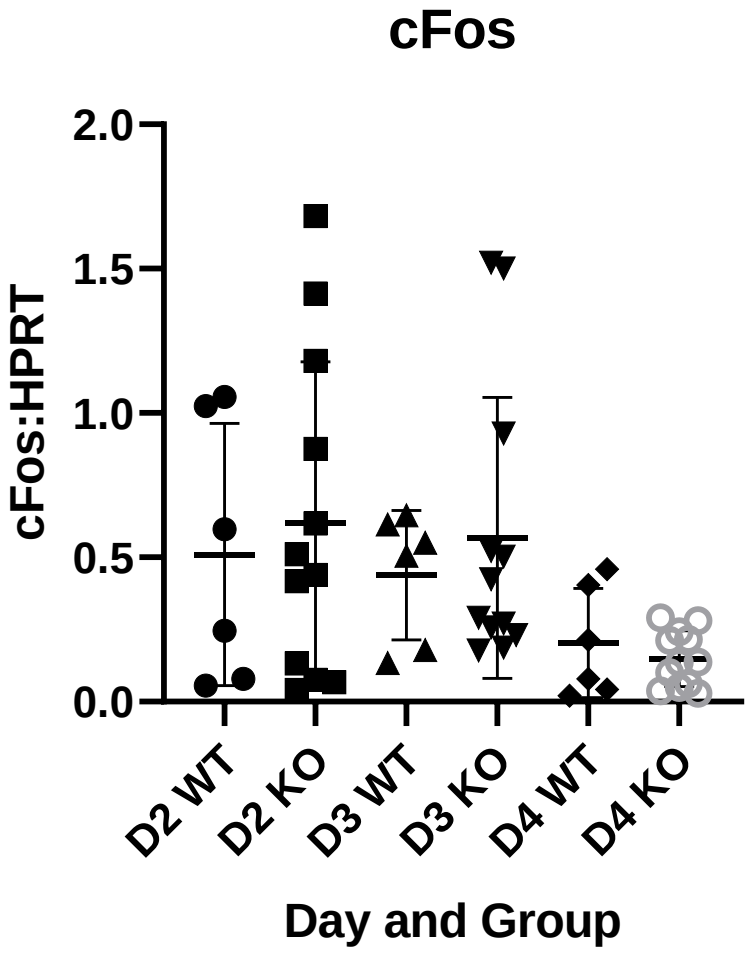


Table 3: *c-Fos* data

Relative Expression of cFos:HPRT	**n**	**Mean**	**SD**	**Range**	**Difference of Means**	**p-value**
Day 2 WT	6	0.5097	0.4540	0.0559 – 1.055	0.1117 ± 0.2630	0.6766
Day 2 KO	12	0.6214	0.5557	0.0409 – 1.105		
Day 3 WT	6	0.4380	0.2242	0.1335 – 0.6462	0.1296 ± 0.2114	0.5484
Day 3 KO	12	0.5676	0.4870	0.1768 – 1.5210		
Day 4 WT	6	0.2036	0.1896	0.0216 – 0.4585	-0.05409 ± 0.06634	0.4269
Day 4 KO	12	0.1495	0.1495	0.0285 – 0.2912		

The results of the gene expression of *c-Fos* measured from RT-qPCR from both the wild type and knockout groups for days 2, 3, and 4 are displayed in Table 3. The mean is reported as the relative expression of the target gene, *c-Fos*, to the housekeeping gene, *Hprt* (cFos:HPRT). The sample size for all the wild type groups was 6 and the sample size for all of the *Bmp2* null groups was 12. In both groups, the relative expression of *c-Fos:Hprt* decreased from day 2 through day 4. There were no statistically significant differences ($p < 0.05$) between the groups at any of the timepoints measured.

Figure 8: qRT-PCR comparing expression of *Ctsk* from WT and BMP2cKO mice after 2, 3 or 4 days of RANKL treatment. (Data shown are the mean ± SD of three independent experiments in which gene expression was measured from three wells of each genotype, with each PCR reaction performed in duplicate. Expression of each gene is graphed relative to *Hprt*.

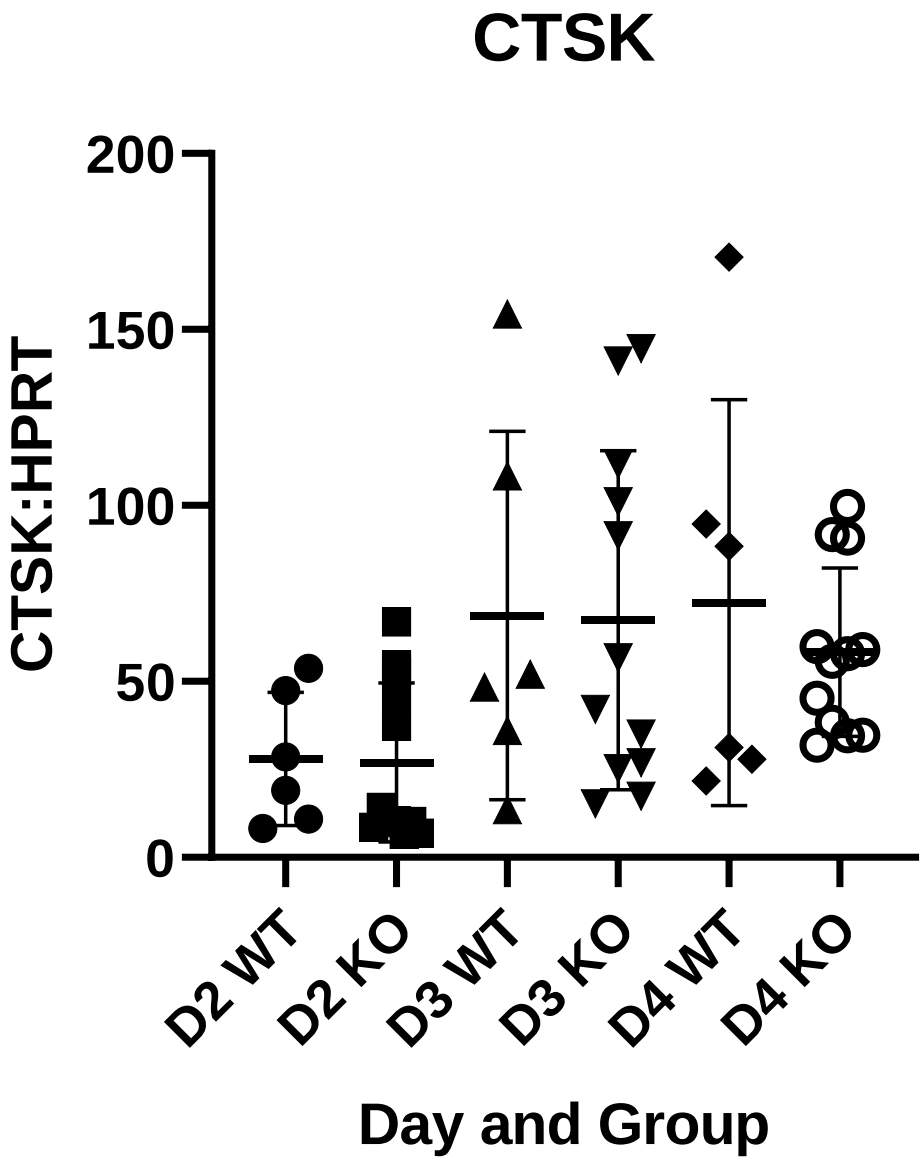


Table 4: *Ctsk* data

Relative Expression of CTSK:HPRT	**n**	**Mean**	**SD**	**Range**	**Difference of Means**	**p-value**
Day 2 WT	6	27.99	18.95	8.282 – 53.73	-1.015 ± 10.78	0.9261
Day 2 KO	12	26.98	22.64	6.635 – 66.95		
Day 3 WT	6	68.81	52.36	13.50 – 154.3	-1.410 ± 24.74	0.9553
Day 3 KO	12	67.40	48.13	15.30 – 144.5		
Day 4 WT	6	72.41	57.71	21.71 – 170.7	-14.09 ± 18.93	0.4675
Day 4 KO	12	58.32	23.90	31.89 – 99.73		

The results of the gene expression of Ctsk measured from RT-qPCR from both the wild type and *Bmp2* null groups for days 2, 3, and 4 are displayed in Table 4. The mean is reported as the relative expression of the target gene, *Ctsk*, to the housekeeping gene, *Hprt* (*Ctsk:Hprt*). The sample size for all the wild type groups was 6 and the sample size for all of the knockout groups was 12. In both groups, the relative expression of *Ctsk:Hprt* increased significantly after day 2. There were no statistically significant differences ($p < 0.05$) between the groups at any of the timepoints measured.

Figure 9: qRT-PCR comparing expression of *Dc-stamp* from WT and BMP2cKO mice after 2, 3 or 4 days of RANKL treatment. (Data shown are the mean ± SD of three independent experiments in which gene expression was measured from three wells of each genotype, with each PCR reaction performed in duplicate. Expression of each gene is graphed relative to *Hprt*.

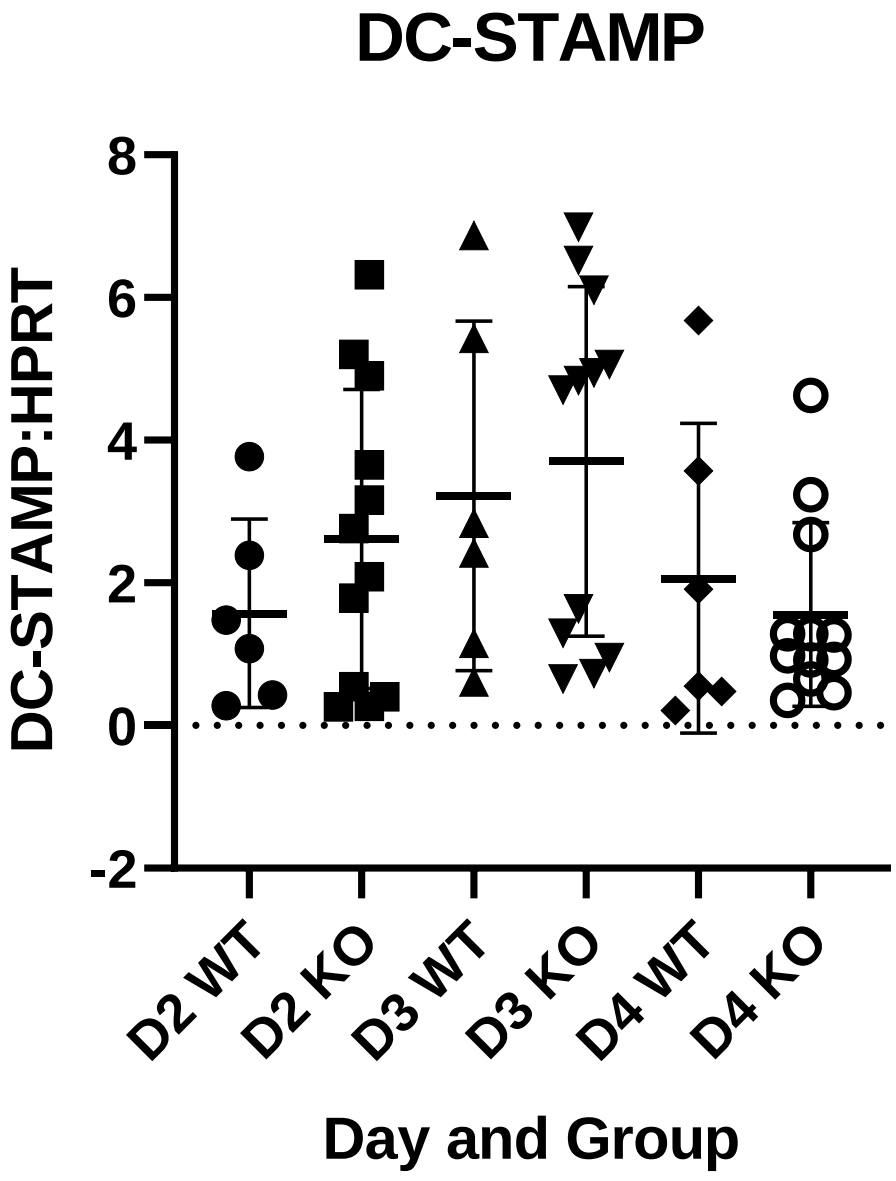


Table 5: *Dc-stamp* data

Relative Expression of DC-STAMP:HPRT	**n**	**Mean**	**SD**	**Range**	**Difference of Means**	**p-value**
Day 2 WT	6	1.572	1.320	0.2832 – 3.771	1.042 ± 0.9433	0.2855
Day 2 KO	12	2.614	2.094	0.2658 – 6.320		
Day 3 WT	6	3.220	2.451	0.6134 – 6.869	0.4833 ± 1.225	0.6983
Day 3 KO	12	3.703	2.449	0.6507 – 6.989		
Day 4 WT	6	2.066	2.171	0.2095 – 5.676	-0.5088 ± 0.8090	0.5383
Day 4 KO	12	1.557	1.290	0.3548 – 4.627		

The results of the gene expression of *Dc-stamp* measured from RT-qPCR from both the wild type and knockout groups for days 2, 3, and 4 are displayed in Table 5. The mean is reported as the relative expression of the target gene, *Dc-stamp*, to the housekeeping gene, *Hprt* (*Dc-stamp:Hprt*). The sample size for all the wild type groups was 6 and the sample size for all the *Bmp2* null groups was 12. In both groups, the relative expression of DC-STAMP:HPRT was the highest on day 3. There were no statistically significant differences ($p < 0.05$) between the groups at any of the timepoints measured.

Dc-stamp was about twice as high on day 3 as on days 2 or 4. The gene *Ctsk*, responsible for producing protease enzymes involved in resorption, is activated later in differentiation and evidence of this was seen as the levels of expression were two to three times higher on days 3 and 4 than on day 2.

Figure 10: qRT-PCR comparing expression of *Nfatc1* from WT and BMP2cKO mice after 2, 3 or 4 days of RANKL treatment. (Data shown are the mean ± SD of three independent experiments in which gene expression was measured from three wells of each genotype, with each PCR reaction performed in duplicate. Expression of each gene is graphed relative to *Hprt*.

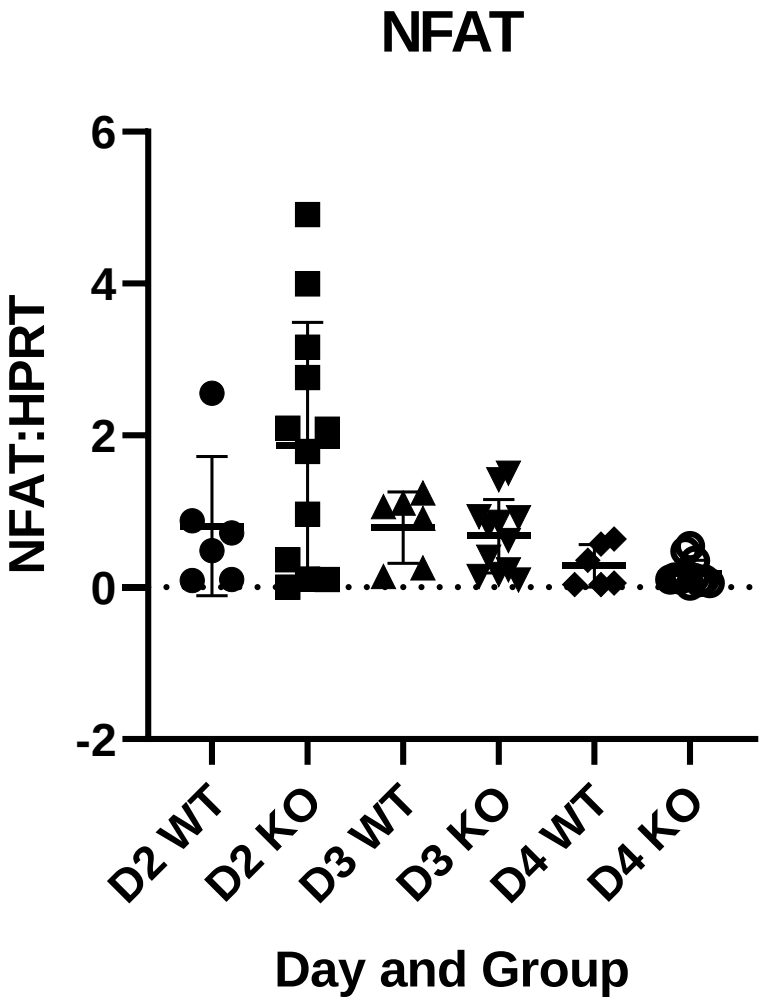


Table 6: *Nfatc1* data

Relative Expression of NFAT:HPRT	**n**	**Mean**	**SD**	**Range**	**Difference of Means**	**p-value**
Day 2 WT	6	0.8046	0.9164	0.08717 – 2.558	1.058 ± 0.7216	0.1621
Day 2 KO	12	1.862	1.627	0.0002934 – 4.908		
Day 3 WT	6	0.7877	0.4698	0.1431 – 1.244	-0.1141 ± 0.2394	0.6400
Day 3 KO	12	0.6736	0.4828	0.09706 – 1.500		
Day 4 WT	6	0.2819	0.2783	0.03349 – 0.6351	-0.09735 ± 0.1058	0.3711
Day 4 KO	12	0.1846	0.1729	0.02547 – 0.5434		

The results of the gene expression of *Nfatc1* measured from RT-qPCR from both the wild type and *Bmp2* null groups for days 2, 3, and 4 are displayed in Table 6. The mean is reported as the relative expression of the target gene, *Nfatc1*, to the housekeeping gene, *Hprt* (*Nfatc1*:*Hprt*). The sample size for all the wild type groups was 6 and the sample size for all of the *Bmp2* null groups was 12. In both groups, the relative expression of *Nfatc1*:*Hprt* decreased from day 2 through day 4. There were no statistically significant differences ($p < 0.05$) between the groups at any of the timepoints measured.

DISCUSSION

In the present investigation, the first outcome evaluated was the number of cells present at each day during the differentiation process. Determining whether the number of cells present affects cell fusion and development of multinucleated osteoclasts was the objective of obtaining this information. While there were no significant differences between the *Bmp2* knockout and wild-type groups at any of the three timepoints, both groups followed a similar trend of decreasing number of cells over time. This can be explained by the normal process of osteoclast differentiation. Upon stimulation of monocytes with RANKL, the monocytes begin to differentiate and fuse forming giant multinucleated osteoclast cells. This process requires four to five days to accomplish and then the osteoclasts can be observed in culture for only two to four days before they die by apoptosis. (Akchurin et al., 2008) Day 2 is right before cell fusion and mean cell count of the wild-type group was 83.26 and the knockout group was 71.15. This difference was not statistically significant. ($p=0.2892$). It is not expected to see a difference in osteoclast size at this timepoint because none of the cells have undergone fusion and thus are all still mononuclear monocytes. At day 3 as the individual cells begin to fuse and start forming multinucleated cells, the mean cell count of the wild-type group dropped to 54.15 and the knockout group dropped to 50.13. This difference was also not statistically significant ($p=0.7260$). The decrease in number of cells in both groups is expected because the mononuclear cells are fusing to become multinuclear. At day 4 when the osteoclasts are fully differentiated, the mean cell count dropped to 37.07 and 44.41 for the wild-type and knockout groups, respectively. The further decrease in number of cells in both groups from day 3 is also expected because the cells are

continuing to fuse and becoming larger. While this difference was not statistically significant, it did approach the α level ($p<0.05$) of significance with a p-value of 0.0557. This difference correlates well with the average cell size difference found between the groups at day 4. The wild-type cells are significantly larger than the knockout cells, so there are fewer of them present in any one microscope image taken.

The second outcome evaluated was the average size of the osteoclasts at each time period measured in area (mm^2). Determining how the presence of BMP-2 affected the fusion and size of differentiated multinucleated osteoclasts was the objective of obtaining this information. At day 2, there were no statistically significant differences between the average size of the wild-type ($0.0018mm^2$) and knockout ($0.0019mm^2$) groups with a p-value of p=0.9163. It is expected that the cell size is unchanged at day 2 because no cell fusion has occurred yet, and all of the cells are still mononuclear. At day 3, the cell-cell fusion process has begun *in vitro* and multinucleated cells begin to form. The mean size of the wild-type cells was $0.0157mm^2$ and of the knockout cells was $0.0109mm^2$. The difference of these values approached statistical significance with a p-value of p=0.0606. The microscopic images taken and displayed in the findings of this paper show evidence of the increased size of the wild-type cells compared to the knockout cells. At day 4, most of the osteoclasts are fully differentiated and fusion has occurred. There was a statistically significant difference between the mean size of the wild-type cells ($0.00529\mu m^2$) and the knockout cells ($0.0283\mu m^2$) with a p-value of p=0.0134. Images of the cells under brightfield microscopy at 4x magnification reveals cells expressing BMP-2 are greatly larger in size.

The significant difference in osteoclast size is a noteworthy finding because it confirms data generated demonstrating that recombinant BMP2 added to osteoclast cultures enhances the size of the osteoclasts. A related study found an increased number of nuclei per cell in murine cells that were deficient for TWSG1, which is a BMP antagonist. They attributed the larger phenotype of the multinucleated osteoclasts to excessive fusion of mononuclear progenitors. Also in line with the results of the present study, they found little effect on overall number of TRAP-positive cells. (Jensen et al., 2010) Future studies may be interested in investigating how the number of nuclei in osteoclasts at different points of differentiation is affected by the knockout of the BMP-2 gene. A staining technique that could be used for this purpose is 4′,6-diamidino-2-phenylindole (DAPI). DAPI is a fluorescent stain that can visualize nuclear DNA in both living and fixed cells. (Tarnowski, Spinale, & Nicholson, 1991)

When looking at gene expression of the four genes investigated between the test and control groups at each day, no statistically significant differences were found. However, predictable observations were made in regard to the relative expression of each gene at different days. The trends were similar for both the wild-type and knockout groups. The expression of *c-Fos* was two to four times greater on days 2 and 3 than on day 4. This comes as no surprise since *c-Fos* is a transcription factor important to committing cells to the osteoclast lineage and is active early on in the differentiation process. *Nfatc1* which is also found early in the differentiation process was found to be expressed at levels of three to ten times higher on day 2 or 3 than on day 4. The gene responsible for fusion of osteoclasts into larger multinuclear cells is *Dc-stamp* and is most highly expressed midway through differentiation. This was confirmed by our experiments in which the

relative expression of *Dc-stamp* as about twice as high on day 3 as on days 2 or 4. The gene *Ctsk*, responsible for producing protease enzymes involved in resorption, is activated later in differentiation and evidence of this was seen as the levels of expression were two to three times higher on days 3 and 4 than on day 2.

There could be several reasons for no significant differences being found between the test and control groups for the relative expression of the four selected genes. The sample size is relatively small at only 6 and 12 samples per control and test groups, respectively. Increasing the sample size may allow a difference to be detected if it in fact exists. Another observation is that the standard deviations are very high in respect to the mean values. In most cases, the standard deviation was at least half of the mean value and in some cases the standard deviation even exceeded the mean.

These four genes were selected to be investigated because they are all known to be involved in osteoclast differentiation. The effect of the presence or absence of BMP-2 expression on these genes was unknown and is why they were selected for investigation. From the current data, it is not possible to state that there is a difference in relative gene expression for mice null of BMP-2. Therefore, the null hypothesis is accepted as true.

There are numerous other genes involved in this process and it is possible that investigating other genes might provide more insight into how BMP-2 affects osteoclast differentiation. In future studies, a wider array of genes could be included to determine which are being affected. One method in which this could be accomplished is RNA sequencing (RNA-Seq). This technique covers a wide range of transcript abundance and can identify mRNA transcripts at a single nucleotide resolution. Advantages of RNA-Seq include being able to look at changes in gene expression over time and the

differences in gene expression in different groups. (Pimentel, Bray, Puente, Melsted, & Pachter, 2016) Another technique that could be considered is a DNA microarray. This technique is employed to measure the expression levels of a large number of genes simultaneously. DNA microarrays are also versatile in the fact that they can detect DNA or RNA (in the form of cDNA after reverse transcription). This laboratory method has been used successfully in other studies to profile the gene expression of osteoclast differentiation already. (Rho et al., 2002)

In the present study, the most significant finding was the difference in the average cell size between the control and knockout groups. From this finding, it is evident that osteoclast fusion is one of the processes most greatly affected by the absence of BMP-2. Future investigations may focus more closely on other genes that are known to be involved in the fusion of osteoclasts during differentiation. Several candidates to explore further are triggering receptor expressed on myeloid cells 2 (TREM2), ATPase H+ transporting v0 subunit d2 (ATP6v0d2), cellular communication network factor 2 (CCN2), CD9 molecule (CD9), and macrophage fusion receptor (MFR). TREM2 induces fusion of pre-osteoclasts into multinucleated cells and in its absence osteoclast development and fusion are impaired. (Helming et al., 2008) ATP6v0d2 is a component of the ATPase pump and is required for osteoclast fusion under basal conditions. Without this gene, it has been shown that the fusion of pre-osteoclasts to mature osteoclasts is inhibited. In addition, bone formation is significantly increased in mice that have this gene knocked out because it is found only in osteoclasts and not in osteoblasts. (Xing et al., 2012) CCN2 is a connective tissue growth factor that promotes endochondral ossification. Studies have demonstrated RANKL-induced

osteoclastogenesis is impaired in cells void of this gene and that they can be rescued by the addition of recombinant CCN2. The combination of recombinant CCN2 and RANKL greatly enhances TRAP-positive multinucleated osteoclast cell formation. (Nishida, Emura, Kubota, Lyons, & Takigawa, 2011) CD9 is a protein of the tetraspanin superfamily. It is implicated in a variety of cell processes which includes fusion. Researchers demonstrated that blockage of CD9 by neutralizing antibodies reduces osteoclast formation while its over-expression promotes cell fusion. (Ishii et al., 2006) MFR was the first molecule identified to be critical for macrophage fusion. Its interaction with CD47, an integrin-associated protein that binds to the receptor of MFR, is involved in cell-cell recognition at the time before cell-cell fusion. Blockage of this pathway results in reduced formation of TRAP-positive multinucleated osteoclasts in cultures of murine bone marrow cells. (Lundberg et al., 2007)

The maintenance of the skeletal system and the balance between bone formation and resorption is a key biological process carried out by all vertebrates. Regulation of these processes are influenced by certain autocrine and paracrine factors like BMPs. It is recognized that BMPs enhance bone formation, but their interactions on osteoclasts and bone resorption is not clearly understood. The results of this study contribute to the growing body of evidence that BMP signaling has an influence on the differentiation and biologic activity of osteoclasts. BMP-2 has already been shown to cause both a dose- and time-dependent increase in bone resorption by osteoclasts. It also elevates the mRNA expression of carbonic anhydrase II which is a key enzyme for degrading inorganic bone matrices. (Kaneko et al., 2000) Specific members of the BMP family, one of which is BMP-2, can directly act on osteoclast precursor cells stimulating them to form TRAP-

positive multinucleated cells. (Kanatani et al., 1995) More evidence of the stimulatory effects of BMP on osteoclast formation and function was revealed when researchers found that BMP-2 may increase bone resorption rather than bone formation if added to an inflammatory environment. They showed that the combination of BMP-2 with IL-1a caused an increase in the formation of cyclooxygenase-2 and RANKL mRNA in osteoblasts. The enhancement of these factors in osteoblasts was seen phenotypically as an increase in the differentiation of osteoclasts. (Koide et al., 1999)

Going forward, this research may be used to better understand the effect BMP-2 has on the relationship between bone resorption and bone formation in both osteoclasts and osteoblasts. The data presented here demonstrates that osteoclast fusion and differentiation is affected by the absence of BMP-2. Future research may be aimed at determining if addition of exogenous BMP-2 is able to rescue the phenotype of these osteoclasts. In addition, it is unclear from the current research and evidence if the altered phenotype of the osteoclasts observed in this study affect their ability to resorb bone.

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