The Effect of BMP 4, 6, and 7 on Osteoclast Differentiation and Resorption

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I would like to thank Dr. Kim Mansky for her constant support, encouragement, expertise, and willingness to let me work in her laboratory. I would also like to thank Dr. Raj Gopalakrishnan and Dr. Brent Larson for their advice and time in the completion of this project and the members of the Mansky/Gopalakrishnan laboratory for their helpful guidance.

Dedication

This thesis is dedicated to my family. Specifically to my amazing parents who showed me what it means to work hard and follow my dreams. A special thank you to my husband for his support and unconditional love.

Abstract

Previous research by the Mansky lab has demonstrated that BMP2 enhances RANKL-mediated osteoclast differentiation and activity. To investigate the effects of other BMPs on osteoclast differentiation, I treated bone marrow macrophages with RANKL and increasing concentrations of BMP4, 6 or 7 during osteoclast differentiation. Osteoclasts were TRAP stained and measured and counted using NIH Image J. My results demonstrated that BMP4 and BMP6 increased osteoclast size but not osteoclast number; however, BMP7 increased osteoclast number but not size. Secondly I treated mature multinuclear osteoclasts with increasing concentrations of BMP 4, 6 and 7 to determine the effect of BMP on osteoclast activity. An increase in osteoclast activity was only measured with the addition of BMP7 to mature osteoclasts. My experiments demonstrate that similar to BMP2, BMP4, 6 and 7 enhance RANKL mediated osteoclast differentiation; however, the mechanism by which BMPs enhance differentiation still needs to be explored.

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Introduction

Bone is a vital component of one's structural support and muscle attachment. As one of the few organs that continue to regenerate into adulthood, bone resorption and formation in normal circumstances occurs in cycles to maintain homeostasis (1). Maintenance of this homeostasis is vital for bone health and stability. When this balance is disturbed, whether due to trauma, autoimmune diseases, neoplasms, hormonal changes, or numerous other factors, it can result in a destructive manifestation that negatively impacts quality of life (2). This homeostasis occurs when the cells involved in building and breaking down bone complement each other and allow for bone remodeling. Bone remodeling is a lifelong process necessary to repair bone damage that involves bone cells, as well as proteins such as bone morphogenetic proteins (BMPs), and signaling pathways to achieve a balanced activity between bone resorption and apposition in order to maintain proper bone mass (3).

Review of Literature

Osteoblasts

Osteoblasts are cells that originate from the mesenchymal lineage and are responsible for bone formation. When groups of these cells come together, they form an osteon and are then able to synthesize dense, crosslinked collagen and other specialized proteins (osteocalcin and osteopontin) that ultimately form bone. The coupling of bone resorption with bone formation in skeletal remodeling is primarily determined and regulated by osteoclast and osteoblast intracellular communication (4). The main purpose of the remodeling process is to resorb

away old bone by osteoclasts and subsequently lay down new bone by osteoblasts (4).

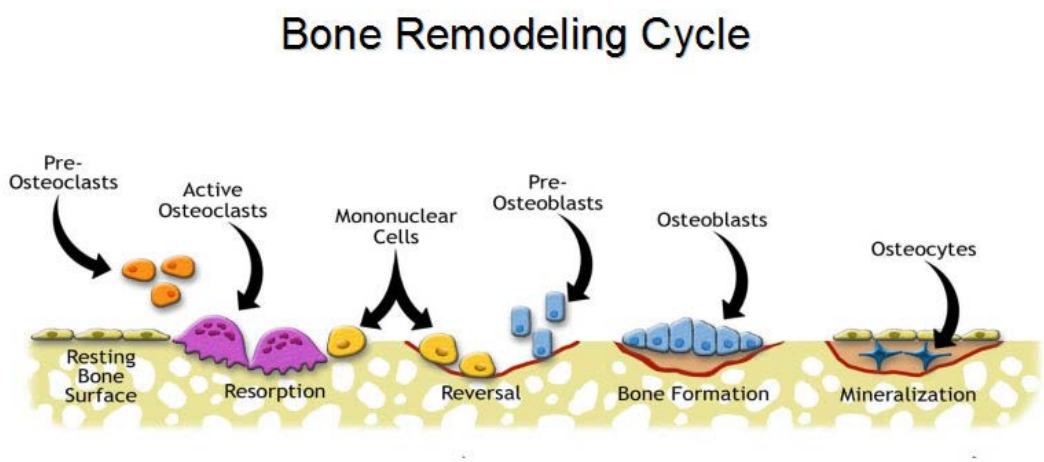


Figure 1. Bone remodeling cycle (5).

Bone formation and resorption is a continuous, life long process with 5-25% of bone surface undergoing bone remodeling at any given time. The four major stages are: activation, resorption, reversal, and formation (4). The remodeling cycle is initiated when quiescent bone surface covered with bone lining cells are activated and recruit osteoclast precursor cells (4). Once these cells fuse to the bone surface, they form into mature osteoclasts and begin resorbing bone. Resorption occurs by osteoclasts creating an acidic microenvironment and with the help of specific enzymes, degrading the organic bone matrix. During the reversal phase, preosteoblasts enter the newly resorbed cites and take residence in the resorption pits (4). Once there, these cells mature into osteoblasts and begin the bone formation process. During this process, some osteoblasts may become trapped in the mineralized matrix and become osteocytes (4). Osteocytes are the most common type of bone cell and responsible for

molecular synthesis and modification as well as signal transmission to maintain bone function.

Osteoblasts are single nucleated cells of mesenchymal origin but work in groups of cells when activated to produce bone. They produce multiple specialized proteins including osteocalcin and osteopontin (1), which comprise the organic matrix of bone. Their main function is to fabricate and secrete an un-mineralized protein matrix called osteoid (1). Once embedded in bone, they are called osteocytes. Osteoblasts and osteocytes communicate and regulate osteoclast differentiation (6). Such mediators include RANKL (Receptor activator of nuclear factor kappa-B ligand) and OPG (osteoprotegerin, also known as osteoclastogenesis inhibitory factor, (6).

Osteoclastogenesis

Osteoclasts are multinucleated cells that originate from hematopoietic stem cells (HSCs), which through a series of steps, commit to the monocyte/macrophage lineage and eventually mature to multinuclear osteoclasts (2). Histologically, the cytoplasm of osteoclasts is characterized by a foamy appearance due to a high concentration of vacuoles and vesicles containing acid phosphatase filled lysosomes. As mononuclear precursor cells, they begin to differentiate and fuse with other precursor cells to become multi-nucleated cells (7). Osteoclasts are responsible for bone resorption by degrading extracellular matrix and acidification of their environment (4). The survival and activation of mature osteoclasts is dependent upon numerous hormones and cytokines. One important cytokine RANKL as discussed above has shown to increase the

osteoclast life cycle and permit cell function (2). As a membrane-residing protein on osteoblasts and their precursors, RANKL activates RANK on osteoclast precursors stimulating osteoclast differentiation and activity through numerous pathways (8). M-CSF another essential factor for osteoclast differentiation is produced by stromal/osteoblasts cells and is required for osteoclast development and survival (9). Together, RANKL and M-CSF are required for proper osteoclast differentiation (10).

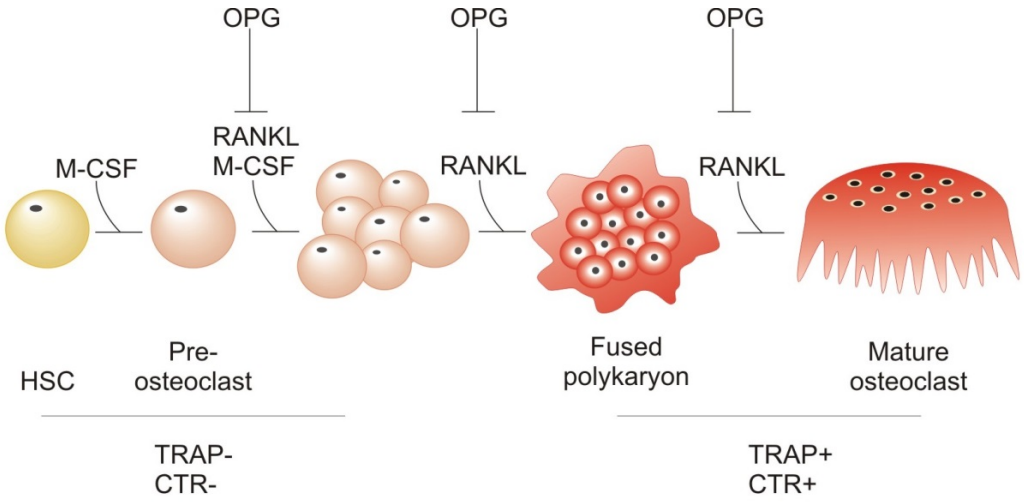


Figure 2. Differentiation of HSCs (haematopoietic stem cells) through osteoclast lineage via preosteoclasts, which fuse to form multinucleated cells, before maturing into osteoclasts. This process requires both M-CSF (macrophage-colony stimulating factor) and RANKL (receptor activator nuclear factor kappa B ligand). OPG (osteoprogerin) is required for inhibition. Expression of TRAP (tartrate resistant acid phosphate) and CT (calcitonin) receptors are induced with RANKL stimulation.

RANKL regulatory pathway

As a member of the TNF (tumor necrosis factor) superfamily, RANKL has traditionally thought to be expressed primarily by bone marrow stromal cells and osteoblasts to stimulate osteoclast differentiation upon binding to the RANK receptor on osteoclast precursor cells (8). Yet, recent research has shown the

important role osteocytes play in the production and secretion of RANKL (11, 12) Both in vivo and in vitro, the loss of RANKL results in the absence of osteoclastogenesis and an osteopetrotic phenotype (11, 12). Based on mouse models, osteoblasts are hypothesized to regulate osteoclast differentiation in developing animals while osteocytes regulate osteoclast differentiation in mature animals (11). A decoy receptor that sequestrates RANKL from binding RANK when activation is undesired is osteoprotegrin (OPG) (13). If OPG is overly expressed, this too can lead to RANKL inactivation and lack of osteoclastogenesis (13). Systemic hormones such as parathyroid hormone, vitamin D3, calcitonin, as well as other local autocrine and paracrine factors (TNF-α, IL-1, etc.) can regulate osteoclastogenesis (14).

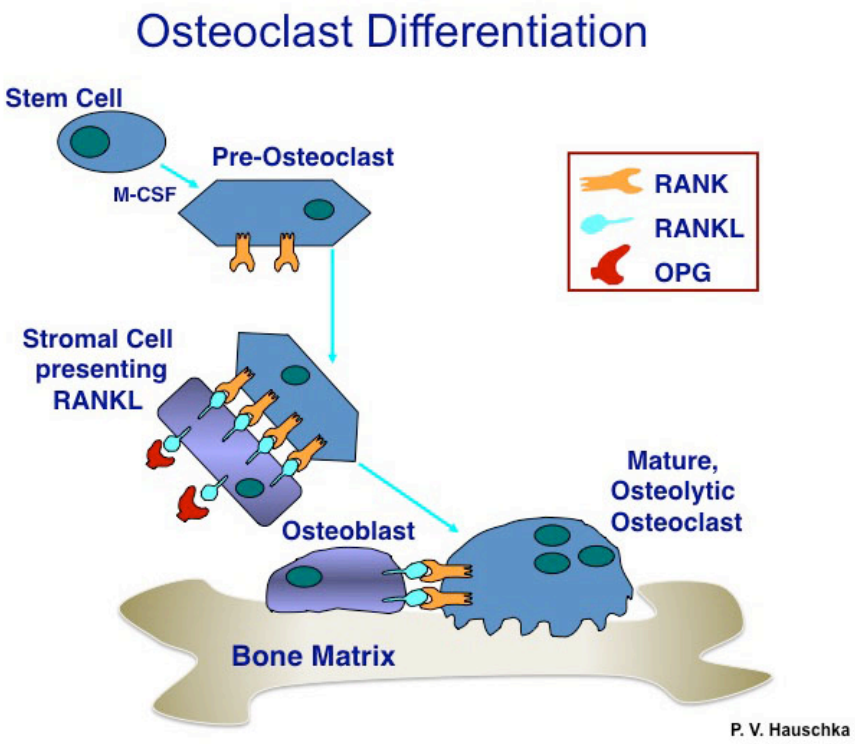


Figure 3. RANKL is expressed on the surface of osteoblast and stromal cells that release OPG into the extracellular matrix. RANKL binds RANK on the surface of osteoclast precursors and stimulates osteoclast differentiation and activation once cell to cell contact is achieved. OPG acts as a decoy receptor to prevent binding of RANKL to RANK.

Osteoclast Role in Dentistry and Orthodontics

The role of osteoclasts is paramount in the field of dentistry. Although the remodeling process of bone is constant and ongoing in the maxilla, mandible, and alveolar processes, there are several instances where osteoclast function is required in order to achieve a desired outcome set by the practitioner and/or maintain homeostasis (15).

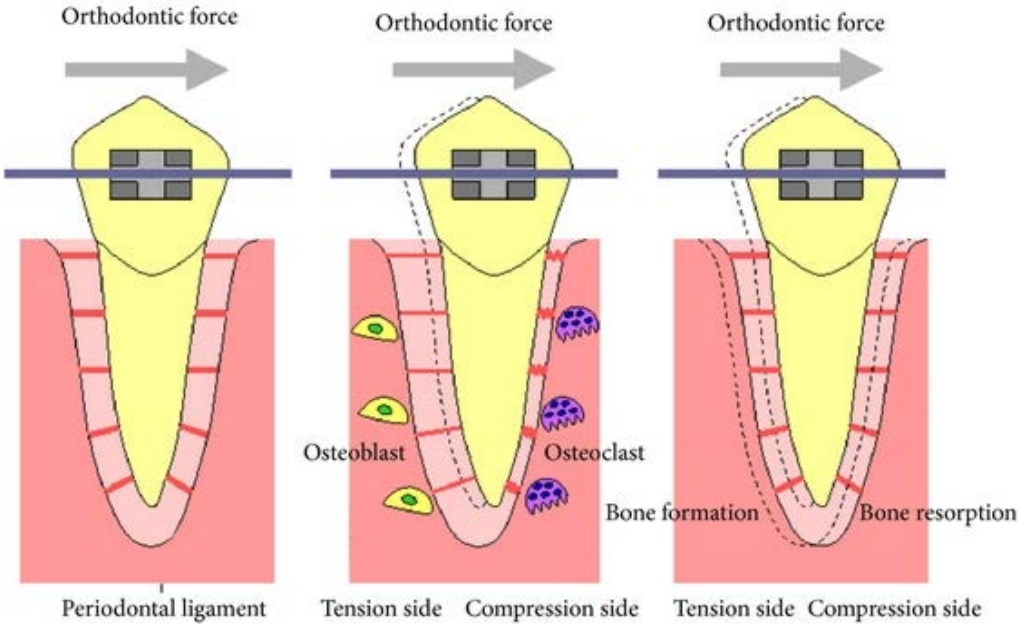


Figure 4. Orthodontic Force resulting in bone remodeling (16).

Such circumstances included the extraction of teeth, whether to discard an unrestorable tooth, or create space for comprehensive orthodontic treatment where the patient presents with severe crowding. In such cases, the tooth socket (bone) is exposed to the oral cavity and only separated by a thin biofilm. Over time, this portion of exposed bone houses resorption lacuna where osteoclasts separate the dead bone from the underlying healthy bone (M). This process allows for the removal of necrotic bone and initiation of new bone mineralization in the socket site. Osteoclasts also play a key role in orthodontic tooth movement.

Bone Morphogenetic Proteins (BMPs):

BMPs are members of the TGF-β superfamily of polypeptides and regulate cell differentiation, proliferation, and apoptosis at the cellular level in osteoblasts, osteoclasts, and chondrocytes in both embryonic and postnatal stages (3). They

were first discovered forty years ago by Urist as an osteoinductive component of demineralized bone matrix (17). The BMP family also plays a role in organogenesis of the heart, gut, lung, teeth, kidney, and skin as well as cartilage and bone (18). Within the TGF-β superfamily of growth factors, BMPs are the largest group and distinguished from other members in the group by containing two extra conserved cysteine amino acids as opposed to the standard seven that are folded into a cysteine knot (18). BMPs' general role in the pathophysiology of fracture healing and skeletal development has been broadly explored (19). BMPs critical presence in osteoclast and osteoblast development implies they also play a key role in bone remodeling (3, 19).

BMP regulation and signaling pathway:

There are several antagonists (both intracellular and extracellular) that regulate BMP activity. Intracellular antagonists that regulate BMP signaling include Smad6, Smad7, Smad8d, Smurf1, and Smurf2 (18) while extracellular antagonists include noggin, chordin, twisted gastrulation, gremlin, and follistatin (18). There are multiple ligands and receptors involved in the BMP signaling pathway. It is important to note that BMPs can behave in both a paracrine and autocrine model (18); however, the mechanism by which they regulate osteoclast differentiation and activity is unknown. The signaling initiation begins when BMPs bind to two surface receptors - Type 1R/BMPRII and Type IR/ALK2, 3, 6. These receptors activate the SMAD or canonical pathway or MAPKs (mitogen-activated protein kinase) or noncanonical signaling transduction pathways (3, 20).

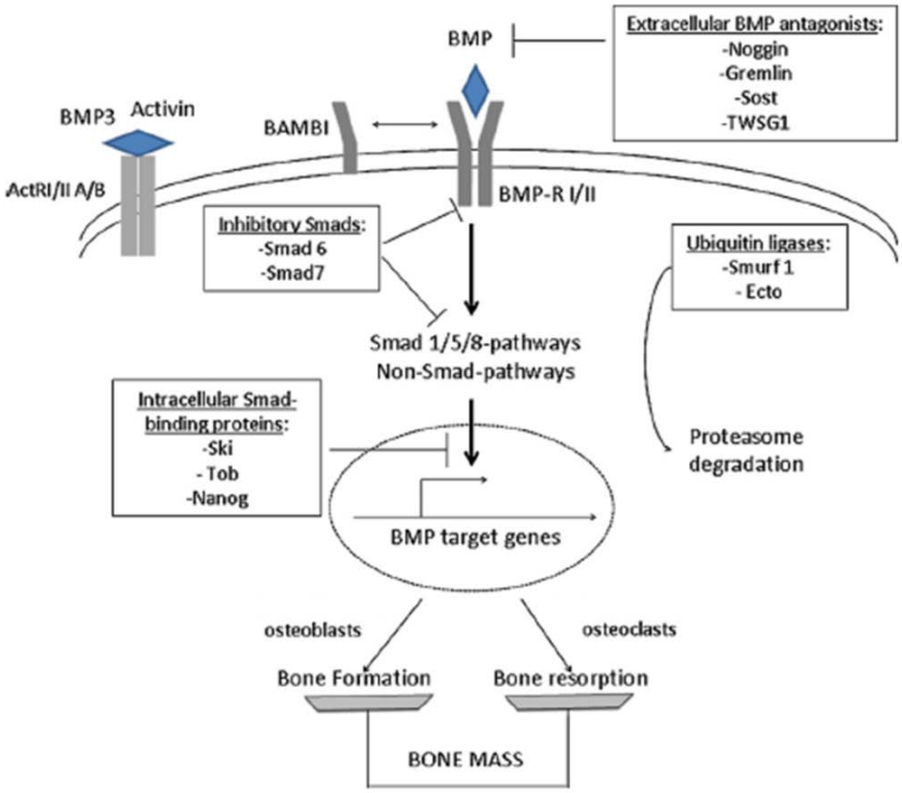


Figure 5. Extracellular and intracellular inhibitors modulate BMP's signaling pathway once BMP binds to a surface BMP receptor I and II. This downstream signaling pathway includes the canonical Smad and non-canonical Smad independent pathways and eventually results in regulating osteoblast and osteoclast differentiation and activation genes.

BMP and Osteoclast differentiation:

BMP2 does not only affect osteoblast differentiation and activity, but as the Mansky/Gopalakrishan lab and others have shown that BMP2 increases osteoclast differentiation and function (21-25). Both early lineage cells (bone marrow macrophages) and mature osteoclasts express BMP2 (25, 26). In early hematopoetic stem cells, BMP2 can increase or decrease cell proliferation, depending on concentration, but BMP stimulation does not affect lineage commitment (27). In lineage committed OCLs, BMP2 acts to enhance survival

and proliferation, and its phenotypic effect can be potentiated by IL-1A, prostaglandin, vitamin D3, and PTH (26, 28, 29). Both BMP2 and 4 are highly expressed by osteoclasts at fracture sites (30). Overexpression of BMP4 in bone leads to osteopenia due to increased osteoclast number (31). Due to its ability to induce bone formation, although less potently than BMP 2/6/9, BMP7 has been approved by the FDA for use in lumbar fusions. BMP7 like BMP2 and BMP4 results in enhanced resorption at fracture site (32).

BMPs and their clinical use

BMPs have also shown to increase the rate of healing and reduced secondary intervention in anterior lumbar interbody spine fusions, open tibia fractures, reoperation of failed posterolateral spine fusions, and recalcitrant nonunions in preclinical studies (33). In 2004, the FDA approved BMP-2, marketed as Infuse (Medtronic Sofamor Danek) for treating open tibial shaft fractures. Additionally, BMP-2, when delivered as an absorbable collagen sponge, can treat metaphyseal core defects resulting in bone resorption followed by bone formation in nonhuman primates (33). In one clinical study using rh-BMP2 in transforaminal lumbar interbody fusion almost all subjects contained active resorption (92%), and 69% of patients showed lumbar level defects (34). As related to the dental profession, BMP-7, when placed directly in a Class III periodontal defect furcation in dogs, showed marked stimulation of regenerative cementum, osteogenesis and increased attachment formation (35). Research conducted with isolated mice molars pretreated with BMP-2 and BMP-4 notably displayed

accelerated alveolar bone development (36). Although the existing published research regarding BMP's has the potential to have significant clinical implications, there is still a necessity to learn more about these potent proteins.

Hypotheses*:*

Because there is much unknown about how specific BMPs directly regulate osteoclast differentiation, this research will address the following hypothesis:

1. Treating osteoclasts with RANKL+ increasing concentrations of BMP 4, 6, or 7 during osteoclast differentiation will enhance differentiation compared to RANKL only treated osteoclasts.

2. Treating mature multinuclear osteoclasts with RANKL+ increasing concentrations of BMP 4, 6 or 7 will result in increased osteoclast activity (resorption) compared to RANKL only treated osteoclasts.

If my hypotheses are correct, I expect to see an increase in the size of the osteoclasts treated with BMP4, 6 and/or 7 compared to RANKL only treated osteoclasts. We may not see an increase in osteoclast number since in the Mansky's lab experience as the size of the osteoclasts increase due to increase fusion then the number of osteoclasts actually decreases. Lastly we expect that mature (i.e. multinuclear) osteoclasts when treated with either BMP 4, 6 and/or 7 will have more activity than osteoclasts treated with RANKL only. If confirmed, implications to this outcome would be important for understanding the mechanisms by which different BMPs regulate osteoclast differentiation and for the clinical applications associated with the use of BMPs.

Materials and Methods*:*

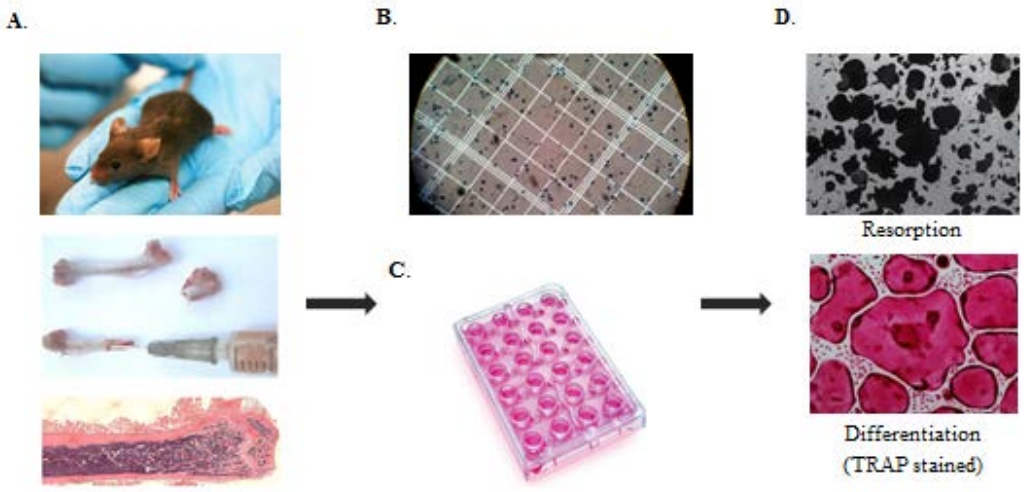


Figure 6. Methods A. Population being studied wild type mice, mice femur dissected, bone marrow cells harvested. B. Cells counted using counting chambers. C. Cells plated in each well for either differentiation (coated plastic) or resorption (calcium/phosphate coated). D. Resorption and differentiation wells fed with RANKL and MCSF along with varying degrees of BMP concentration.

Harvesting of bone marrow/Primary OCLs

Primary bone marrow macrophages were harvested from the femurs and tibiae of 4-week-old C57Bl6 mice. The femurs and tibiae were dissected and adherent tissue was removed. The ends of the bones were cut and the marrow was flushed from the inner compartments. Red blood cells (RBC) were lysed from the flushed bone marrow tissue with RBC lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH7.4) and the remaining cells were plated on 100 mm plates and cultured overnight in osteoclast medium (phenol red-free Alpha-MEM (Gibco) with 5% heat-inactivated fetal bovine 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). The

non-adherent cell population, including osteoclast precursor cells, was then carefully separated and re-plated at approximately 200,000 cells/well in a 12 well plate with osteoclast medium supplemented with 1% CMG 14-12 culture supernatant. Two days later, this medium was replaced with medium containing 1% CMG 14-12 culture supernatant and 20 ng/mL RANKL (R&D Systems) to stimulate osteoclast differentiation. For osteoclast resorption assays, experiments were performed and quantitated using calcium phosphate plates (Corning).

TRAP Stain

Primary osteoclasts were fixed with 4% paraformaldehyde (PFA) and washed with PBS. The cells were then stained for tartrate resistant acid phosphatase (TRAP) expression with 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 were then observed and captured with light microscopy and the measurements were analyzed using NIH Image J.

Resorption Assays

Primary osteoclasts were plated on Corning Osteo Assay Surface plates at a density of 100,000 cells per well. Cells were allowed to fully differentiate with RANKL until multinuclear cells appeared. Differentiation medium was supplemented with 200 ng/ml BMP2 or indicated doses of BMP 4, 6 or 7 (50-200 ng/ml, R&D Systems) for 24 hours. For all the resorption assays, the media was completely removed 24 hours after the addition of BMPs and 100µL/ well of 10% bleach was added and allowed to incubate at room temperature for 5 minutes.

The bleach solution was then aspirated and the wells were washed twice with 150μL of dH_2O. The plate was then allowed to air dry completely at room temperature for 3-5 hours. The wells were observed at 4x magnification for the formation of resorption pits and images were captured with light microscopy. Images were measured and analyzed using NIH Image J. For Image J analysis, images were converted to binary (black and white) and parameters in Image J were set so that only osteoclasts that were multinuclear (greater than 3 nuclei) were counted and measured.

Statistics

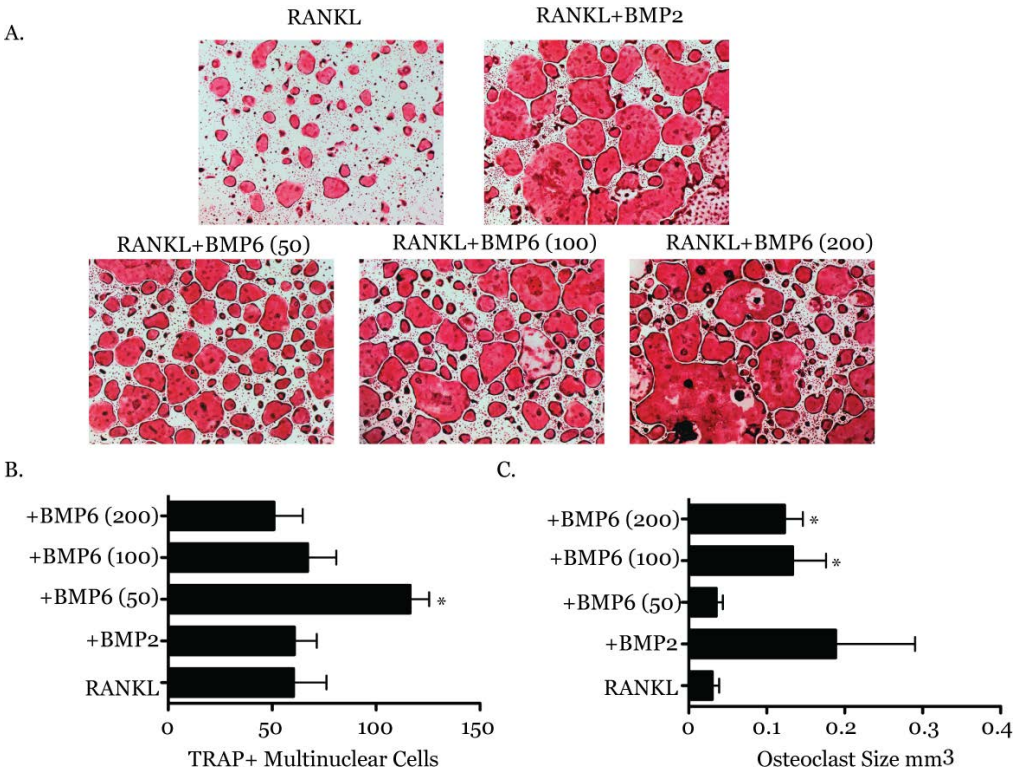
All experiments were completed in triplicate and performed at least three times. The data shown are representative of the mean $\pm$ SD of all experiments. One way ANOVA analysis followed by a Tukey's multiple comparison test were used to compare data; $p \leq 0.05$ indicates significance. Statistical analysis was performed using Prism 5 software for Mac OSX.

Results

Previous results from the Mansky/Gopalakrishnan lab have demonstrated that BMP2 enhances osteoclast differentiation through enhancement of osteoclast fusion (21-23, 25, 37). A limitation to the Mansky/Gopalakrishnan's previous studies is that they only tested BMP2's effect on osteoclast differentiation and no other BMP ligands. Preliminary studies from the Mansky/Gopalakrishan lab have shown that osteoclasts express RNA for BMP ligands 4 and 6 as well as BMP2 (Mansky, personal communication). In order to determine how other BMP ligands affect osteoclasts, bone marrow cells were isolated from wild-type mice

and osteoclasts were cultured. To measure the effect of BMP 4, 6 or 7 on osteoclast differentiation osteoclasts were treated with three different concentrations of each BMP ligand (50, 100, 200 ng/mL) and compared to RANK-L alone. BMP2 + RANK-L was used as a positive control. The cells were then photographed and NIH Image J was used to determine the size or number of cells. Each BMP ligand was compared to the control and at each different concentration level.

Figure 7. BMP6 enhances osteoclast differentiation. BMMs were differentiated in the presence of M-CSF and RANKL, BMP2 (200 ng/mL) or various concentrations of BMP6. Osteoclasts were TRAP stained, imaged and quantified. Only cells with 3 or more nuclei were quantified. (A) Representative image of TRAP stained cells (B) osteoclast number and (C) osteoclast size.



To analyze osteoclast number and size, each well was photographed using a microscope equipped with a digital camera. The captured image was analyzed by the NIH Image J software. Triplicate photographs were taken from each well at

4x magnification. Area measures were completed for both control and cells treated with the varying BMP ligands. As shown in Figure 7-9 and as previously reported, osteoclasts treated with BMP2 compared to RANKL only treated cells showed an increase in osteoclast size (Figure 7-9C, $p<0.05$) but no significant change in osteoclast number (Figure 7-9B).

To examine the effect of BMP6 on osteoclast differentiation, osteoclast precursors were platted in the presence of RANKL and varying (increasing) doses of the BMP6. Osteoclasts were TRAP stained, imaged, and analyzed using NIH Image J software to assess osteoclast size and number. As you can see in Figure 7B, BMP6 at the lower dose of 50 ng/mL increased the number of TRAP+ multinuclear cells, while at higher doses (100 and 200 ng/mL), BMP6 increased the size of the osteoclasts similar to that measured with BMP2 (Figure 7C).

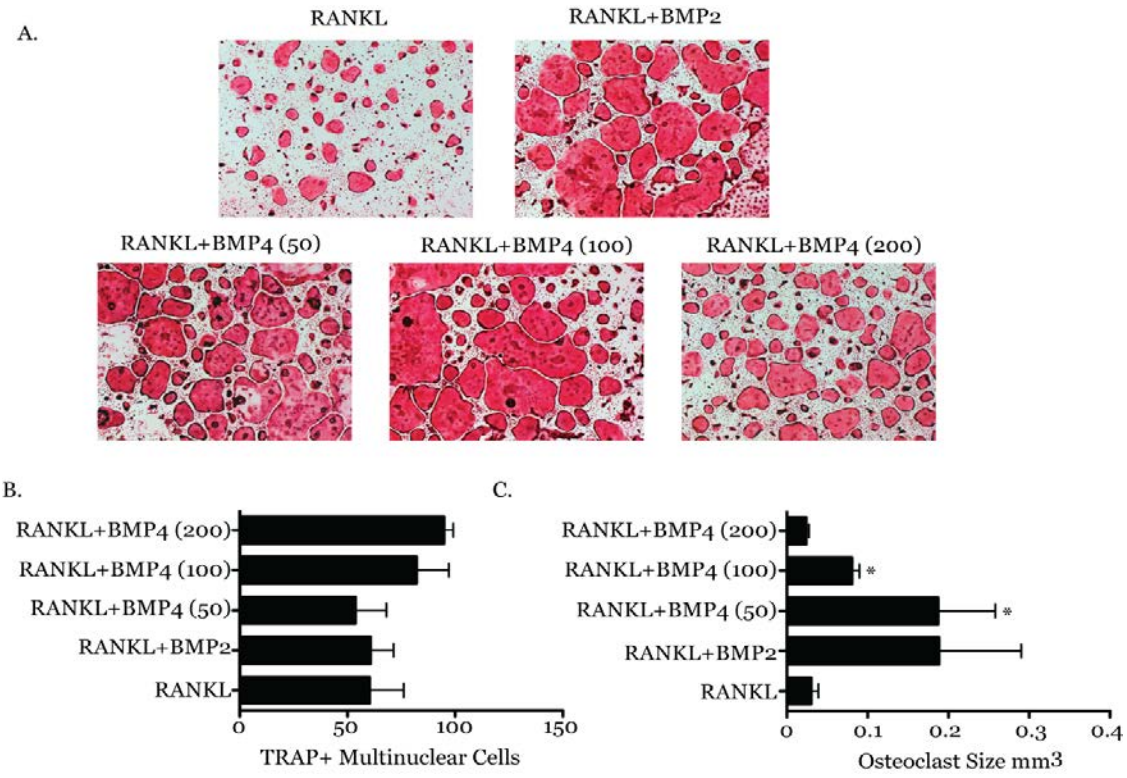


Figure 8. BMP4 Enhances Osteoclast Differentiation. BMMs were differentiated in the presence of M-CSF and RANKL, BMP2 (200 ng/mL) or various concentrations of BMP4. Osteoclasts were TRAP stained, imaged and quantified. Only cells with 3 or more nuclei were quantified. (A) Representative image of TRAP stained cells (B) osteoclast number and (C) osteoclast size.

To examine the effect of BMP4 on osteoclast differentiation, osteoclast precursors were plated in the presence of RANKL and varying (50, 100 or 200 ng/mL) concentrations of the BMP4. Osteoclasts were TRAP stained, imaged, and analyzed using NIH Image J software to measure osteoclast size and number. As you can see in Figure 8, BMP4 at the lower dose of 50 and 100 ng/mL increased the size of TRAP+ multinuclear cells compared to RANKL only treated cells (Figure 8C), while there was no statistically difference in osteoclast number when comparing osteoclasts treated with RANKL alone and osteoclasts treated with RANKL and BMP4.

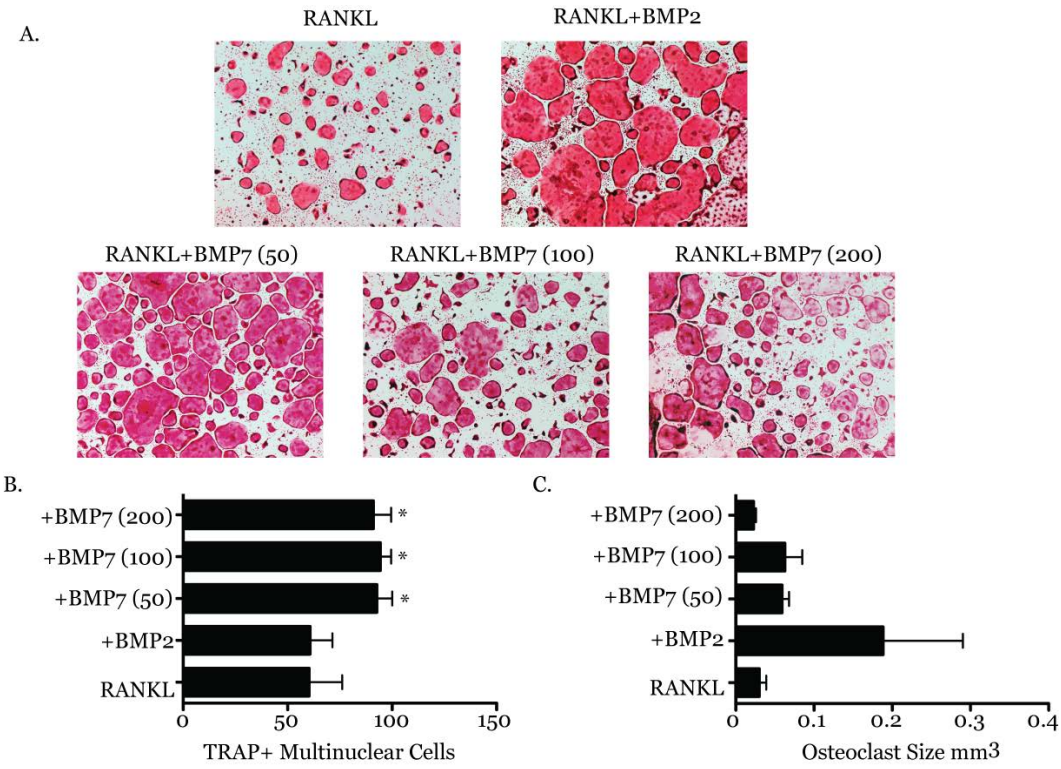


Figure 9. BMP7 Enhances Osteoclast Number. BMMs were differentiated in the presence of M-CSF and RANKL, BMP2 (200 ng/mL) or various concentrations of BMP4. Osteoclasts were TRAP stained, imaged and quantified. Only cells with 3 or more nuclei were quantified. (A) Representative image of TRAP stained cells (B) osteoclast number and (C) osteoclast size.

To examine the effect of BMP7 on osteoclast differentiation, osteoclast precursors were platted in the presence of RANKL and varying (50, 100 and 200 ng/mL) concentrations of the BMP7. Osteoclasts were TRAP stained, imaged, and analyzed using NIH Image J software to assess osteoclast size and number. As you can see in Figure 9, there is an increase in the number of osteoclasts in the presence of BMP7 and RANKL (Figure 9B) when compared with RANKL alone but no significant effect on osteoclast size in the presence of BMP7 compared to RANKL alone (Figure 9C).

In the second part of my study, I wanted to separate the effects of BMPs on osteoclast differentiation and activity by determining if BMP 4, 6 and 7 enhanced osteoclast activity on mature osteoclasts. For this part of the study, BMMs (bone marrow macrophages) were differentiated on calcium/phosphate coated plates into osteoclasts in the presence of M-CSF and RANKL until multinuclear cells appeared. Once multinuclear or mature cells appeared, BMP 4, 6 or 7 were added at various concentrations for 24 hours. Plates were treated to remove cells, imaged and quantified using NIH Image J.

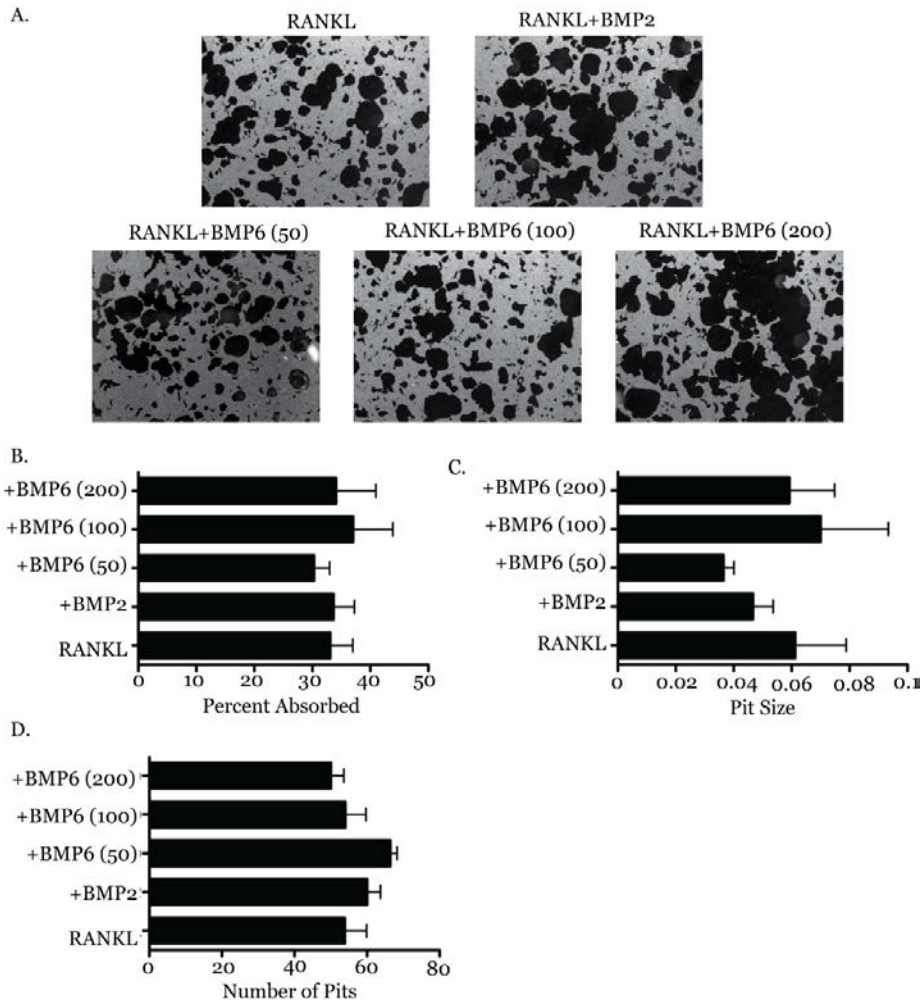


Figure 10. BMP 6 Effects on Osteoclast Resorption. BMMs were flushed from mice and plated on calcium phosphate coated plates in the presence of M-CSF and RANKL. (A-D) Various concentrations of BMP6 were added to multinuclear osteoclasts for 24 hours. (A) Representative images of calcium phosphate coated wells. Quantification of (B) percent area resorbed, (C) average size of pits, (D) number of pits.

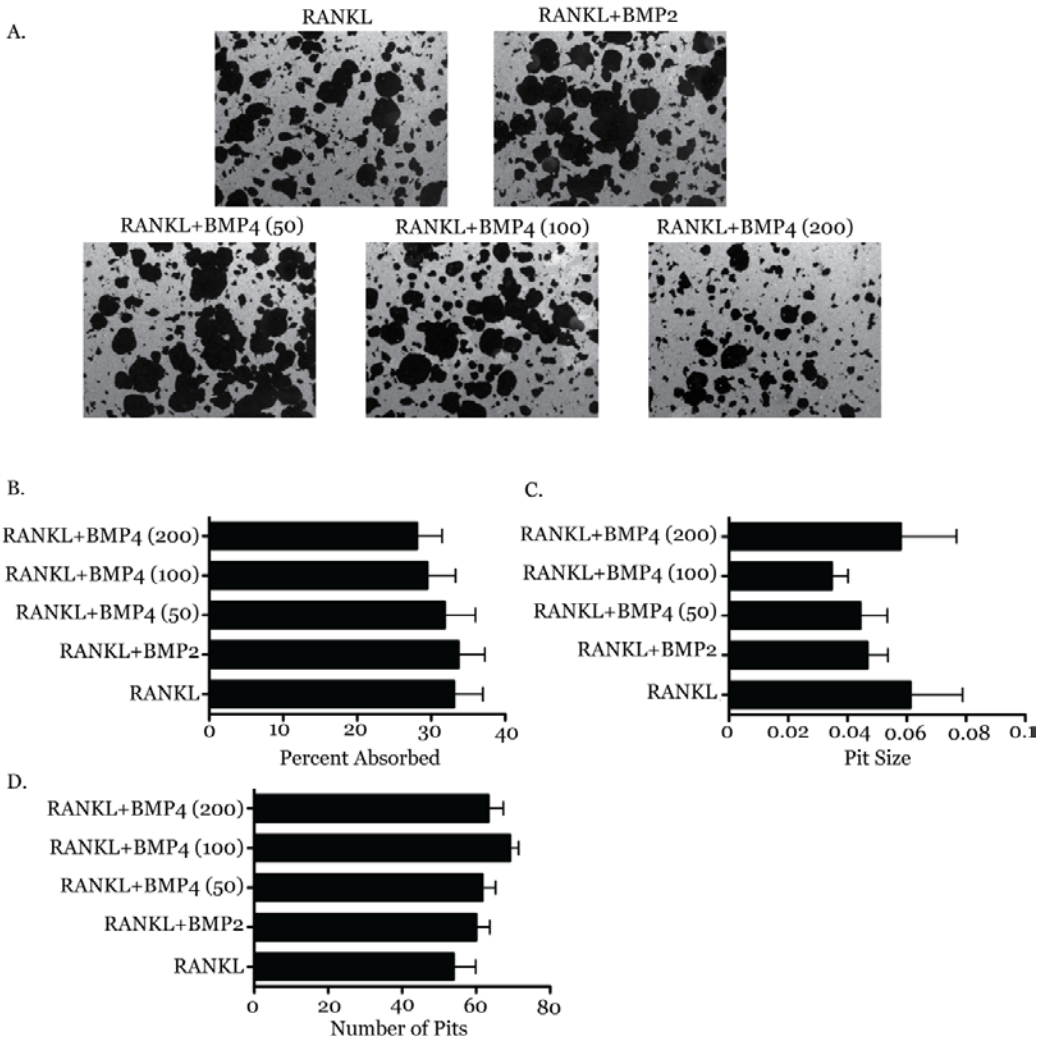


Figure 11. BMP4 Effects on Osteoclast Resorption. BMMs were flushed from mice and plated on calcium phosphate coated plates in the presence of M-CSF and RANKL. (A-D) Various concentrations of BMP4 were added to multinuclear osteoclasts for 24 hours. (A) Representative images of calcium phosphate coated wells. Quantification of (B) percent area resorbed, (C) average size of pits, (D) number of pits.

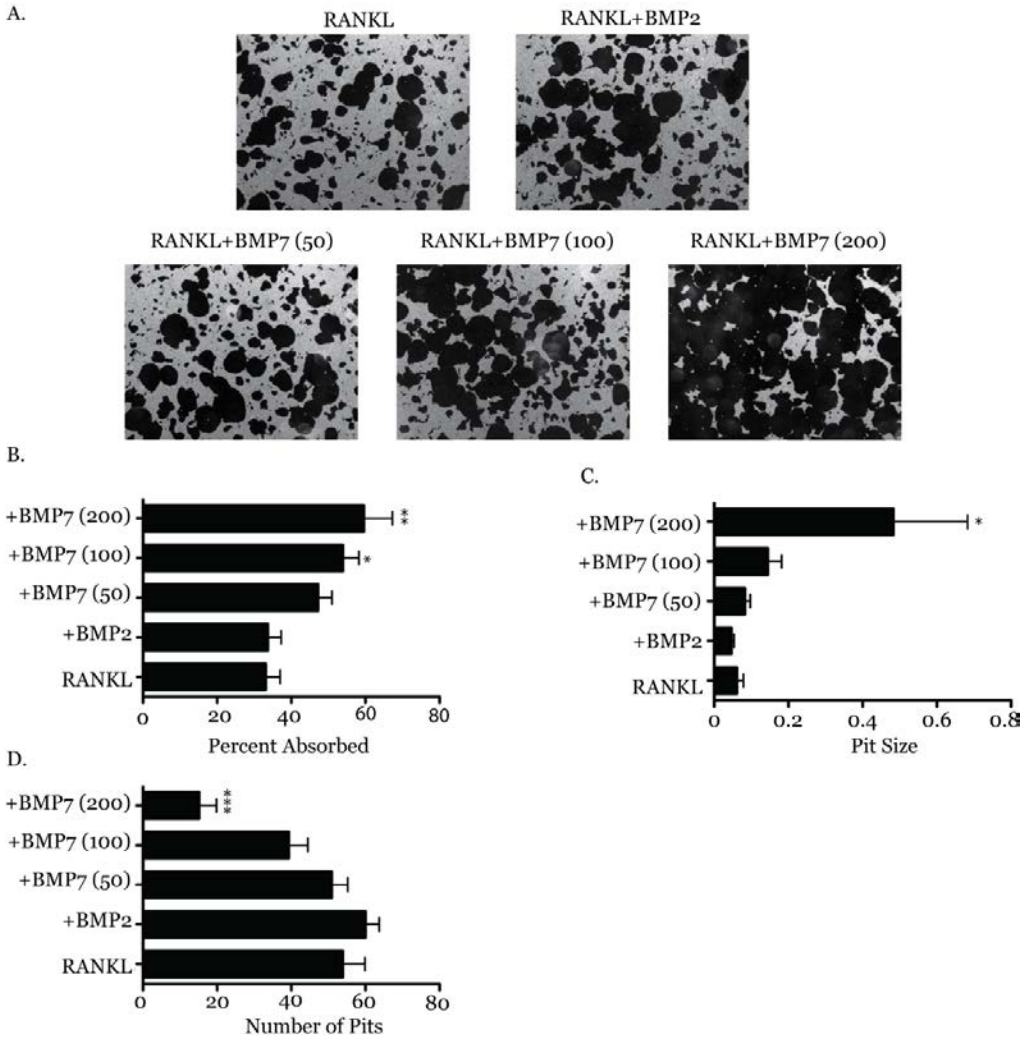


Figure 12. BMP7 Effects on Osteoclast Resorption. BMMs were flushed from mice and plated on calcium phosphate coated plates in the presence of M-CSF and RANKL. (A-D) Various concentrations of BMP7 were added to multinuclear osteoclasts for 24 hours. (A) Representative images of calcium phosphate coated wells. Quantification of (B) percent area resorbed, (C) average size of pits, (D) number of pits.

As is shown in Figures 10 and 11, there is no statistically significant difference between BMP6 (Figure 10B-C) or BMP4 (Figure 11B-C) treated osteoclasts compared to RANKL only treated when measuring osteoclast activity. However in Figure 12 treatment of mature osteoclasts with BMP7 significantly enhances osteoclast activity compared to RANKL treated osteoclasts. There is an

approximate two-fold increase in percent resorbed as the BMP7 concentration level increases as well as an increase at 200 ng/mL in pit size. It appears as the areas of resorption increased in size, the pit number decreased which could be due to the coalescing of the pits as seen in Figure 12A RANKL + BMP7 (200).

Discussion:

Previously the Mansky/Gopalakrishnan lab had published that addition of exogenously added BMP2 to osteoclast cultures enhanced RANKL activation of osteoclast differentiation (25). To further support the direct role of BMPs to regulate osteoclasts, the Mansky/Gopalakrishnan went on to further demonstrate that loss of BMP receptor type II in osteoclasts leads to a decrease in osteoclast differentiation and an osteopetrotic phenotype in null mice (22). Previous research from other labs had shown the role of BMPs in regulating osteoclasts through regulation of osteoclast precursors (M-CSF), differentiation factors (RANK-L), and inhibitory factors (OPG). Up to this point the studies from the Mansky/Gopalakrishnan lab have focused on the effect of BMP2 on osteoclast differentiation and activity. My research project focused on the effect of BMP 4, 6 and 7 on osteoclast differentiation and activity. I chose to experimentally test BMP 4, 6 and 7 because besides BMP 2, BMP 4, 6 and 7 are the most highly expressed BMPs in the bone. I experimentally tested whether addition of BMP 4, 6 or 7 to RANKL treated osteoclasts would enhance osteoclast differentiation. Based on my results, I determined that BMP 4 and BMP 6 enhanced the size of TRAP positive osteoclasts (Figure 7-8) but not osteoclast number. However, when I tested the addition of BMP7 to osteoclast cultures, I found that BMP7

increased number of TRAP positive osteoclasts but not osteoclast size (Figure 9). This data suggests that BMP7 may act to enhance osteoclast differentiation through a different mechanism compared to BMPs 2, 4 and 6.

I also determined whether there is an increase in osteoclast activity when BMP 4, 6 or 7 where added to mature osteoclasts. As shown in figures 10-12, I only measured an increase in osteoclast activity compared to RANKL treated osteoclasts with the addition of BMP 7 (see Figure 12). One caveat to the experiments looking at the effects of BMPs on osteoclast activity is that I did not measure an increase in activity of mature osteoclasts with the addition of BMP2 as previously published by the Mansky/Gopalakrishnan lab (23). It is not clear at this time why I was not able to measure an increase in activity with BMP2 but the Mansky lab is in the process of repeating those experiments under different conditions.

The goal of these experiments was to gain a better understanding of BMP's and their influence on osteoclasts. BMP's have been shown to be effective in treating healing fractures, bone defects in long bones and the cranium and even alveolar bone regeneration in animal studies (20). The FDA has only approved limited use of recombinant BMPs clinically since the full scope of BMP's effect on other cells is not fully understood. Long bone nonunion fractures, lumbar spinal fusion, and alveolar ridge/sinus augmentation are the few applications rhBMP are currently approved for in the clinic. It is important to note that in the case of lumbar spinal fusion application, conflicting reports have been published where the outcome was undesirable such as ectopic bone formation (32). Besides

bone metabolic disorders, gained knowledge about BMPs can influence the way we practice orthodontics. Influencing bone resorption and deposition may be used in various ways such as increasing tooth movement rates and increase bone healing/integration rates after bone grafting (especially for our cleft lip/palate patients). Ultimately, the use of BMPs has the potential to reduce treatment time for our orthodontic patients and increase the success rates of certain procedures that involve bone remodeling and regeneration.

Conclusion

There is lack of understanding about the pathway from osteoclast precursor cells committing to the lineage and fusion between differentiated osteoclasts. BMP6 and BMP4 were able to enhance osteoclast size during differentiation with BMP4 more active at lower doses when compared to BMP6. BMP7 appears to enhance osteoclast in number rather than size and this may suggest a different mechanism of action when compared to BMP4 and BMP6. BMP 4 and 6 do not appear to affect osteoclast resorption. BMP7 appears to increase resorption at higher doses when compared to RANKL. Experiments exploring mechanisms to understand how BMPs increase osteoclast size, number and activity are currently ongoing in the Mansky/Gopalakrishnan lab.

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