Bone Morphogenetic Protein-2 (BMP2) upregulates osteoclast gene expression

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Dedication

This thesis is dedicated to my husband Karl, my parents, and my sisters for their love and support.

Abstract

Bone Morphogenetic Proteins (BMPs) induce bone formation by osteoblasts, but their direct role in bone resorption by osteoclasts remains to be characterized. Twisted Gastrulation (Twsg1) is a secreted BMP binding protein that inhibits BMPs from binding to their receptors. Mice lacking the Twsg1 gene (*Twsg1*$^{-/-}$) exhibit an osteopenic skeletal defect. Previous studies indicate that the osteopenic phenotype in *Twsg1*$^{-/-}$ mice is due to increased osteoclastogenesis and not due to reduced osteoblast function. This study hypothesizes that treatment of wild-type osteoclasts with BMP2 will increase osteoclast gene expression and that this gene expression will decrease with the addition of the known BMP inhibitor, Noggin. The results of this investigation show that the addition of BMP2 to RANKL upregulates Cathepsin K, Nfatc1, Acp5, DCSTAMP, and ATP6v6d02 gene expression levels. The addition of the more well understood BMP inhibitor, Noggin, downregulates these gene expression levels. These results indicate a possible direct mechanism of action for BMP2 on osteoclast activation.

Table of Contents

List of Tables

List of Figures

Introduction

The continual bone remodeling process is important for skeletal growth and bone maintenance throughout life. Bone remodeling involves two primary cell types. Osteoblasts build bone in areas where osteoclasts have previously resorbed bone. Several cytokines regulate bone remodeling, including bone morphogenetic proteins (BMP). BMPs are known to induce bone formation by osteoblasts, but their precise role in osteoclastic bone resorption remains to be identified. An understanding of how BMPs and their regulators influence the activity of bone cells is significant to discovering therapies for pathologic bone diseases. Twisted gastrulation (Twsg1), a 23.5 kilodalton secreted glycoprotein first identified in *Drosophila,* binds to and regulates BMP2 and BMP4. Twsg1 acts as an extracellular BMP antagonist by preventing BMPs from binding to their receptors, thus inhibiting BMP activity [1]. The Gopalakrishnan/Mansky/Petryk lab had previously shown that mice lacking the Twisted Gastrulation gene ($Twsg1^{-/-}$) show osteopenia, which is caused by enhanced osteoclastogenesis mediated by increased BMP signaling. However, controversy exists regarding whether BMPs can directly regulate osteoclast differentiation. The goal of my project was to determine the direct effect of BMP2 treatment on osteoclast gene expression and to determine if an increase in gene expression by BMP2 can be negatively regulated by treatment with Noggin, a known BMP inhibitor.

Review of the Literature

Bone

Bone remodeling is a dynamic process which requires constant turnover of cells to maintain skeletal homeostasis. Bone serves several important functions; it provides a site for muscle attachment, protects vital organs, maintains mineral balance by storing calcium and phosphate ions, and functions in hematopoiesis. Type I collagen makes up ninety percent of the organic component of bone, while the inorganic phase consists of hydroxyapatite crystals [2,3].

Bone Cells

Two predominant cell types are involved in bone remodeling. Osteoblasts, derived from mesenchymal stem cells, are responsible for bone formation. Osteoblasts are found on bone forming surfaces. They secrete bone matrix proteins and control osteoclast activity. Indirectly, osteoblasts maintain calcium balance and are responsible for osteoid mineralization. Bone is resorbed by osteoclasts, which are derived from the hematopoietic stem cell lineage. Osteoclasts fuse from single cells of the monocyte-macrophage lineage to become multinuclear osteoclasts containing four to twenty nuclei. Osteoclasts are found in contact with the bone surface in resorption sites [2]. Physiologic bone resorption involves the coupling of both osteoblasts and osteoclasts. Normally, bone resorption preceeds bone formation. When this highly regulated process becomes dysfunctional, the result is pathologic bone resorption in conditions such as

osteoporosis, rheumatoid arthritis, periodontal disease, and metastatic bone tumors [4].

Bone Morphogenetic Proteins (BMPs) and their Extracellular Inhibitors

BMPs, members of the transforming growth factor Beta (TGFβ) superfamily, are necessary for osteoblast differentiation, maintenance of mature osteoblasts, and endochondral ossification [5]. Of the fifteen BMPs identified in humans, BMP 2, 4, and 6 are secreted by osteoblasts and are autocrine regulators of bone formation. BMPs bind to serine/threonine kinase receptors on membranes of cells. By binding to receptors on osteoblasts, BMPs regulate Smad (signal-transducing molecules of the TGFβ superfamily) phosphorylation. Smad1, Smad5, and Smad8 are receptor-regulated Smads (R-Smads) that are activated by BMPs. Upon BMP binding, R-Smad1 and R-Smad5 are phosphorylated and bind to the only common mediator Smad, (C-Smad) Smad4. The R-Smad/C-Smad heterodimer re-locates to the nucleus where it induces transcription and gene expression (Figure 1) [6].

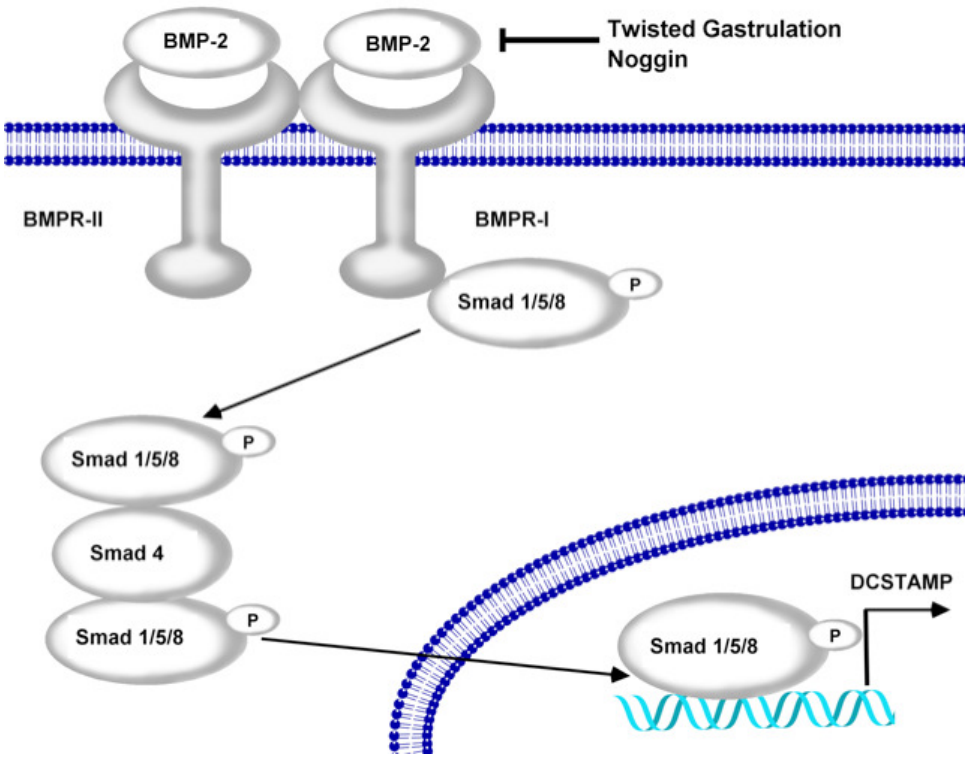

Figure 1: ***Diagramatic representation of BMP2 interaction with BMP2 receptors and Smads*****. BMP2 binds to BMP receptors I and II on cell surface membrane, which induces phosphorylation of Smad1/5/8, heterodimerization with Smad4, and translocation to the cell nucleus. Twisted Gastrulation and Noggin are extracellular proteins that inhibit BMPs from binding to their receptors.**

Several proteins that inhibit BMPs from binding to their receptors have been identified, including Noggin, Chordin, and Twisted Gastrulation (Twsg1). Twsg1 is a secreted BMP-binding protein that specifically binds to BMP2 and BMP4 (Figure 1) [1]. Twsg1 has been shown to be an antagonist in BMP signaling in mammalian systems. Twsg1 acts as an antagonist by forming a ternary complex with Chordin and BMP, which prevents BMPs from binding to their receptors, thereby blocking BMP activity [7,15]

Bone Resorption

Several proteins are involved in an osteoclast cell's ability to resorb bone. Resorption occurs by a process of osteoclast attachment, polarization, formation of a sealing zone at the ruffled border, and ultimately resorption and apoptosis. The sealing zone is an actin ring that separates the acidic resorptive area from the extracellular space. The ruffled border is a "villous organelle" found in osteoclasts [8]. An acidic environment of pH 4.5, produced by hydrochloric acid secretion at the ruffled border, causes the hydoxyapatite in bone to be resorbed extracellularly [3,9].

Osteoclast Differentiation

Monocyte/macrophage precursors from the hematopoietic stem cell lineage differentiate into mature osteoclasts capable of resorbing bone. In order to differentiate, immature osteoclast precursors require RANK ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) to bind to receptor activator of nuclear factor (NF)-kB (RANK) and c-Fms receptors, respectively. RANK activation induces osteoclast-specific gene expression that supports the differentiation from immature bone marrow precursor cells to mononuclear TRAP+ preosteoclasts, which ultimately fuse together to form mature, multinuclear osteoclasts. [10,11]

Genes Involved in Osteoclast Differentiation and Fusion

A. Cathepsin K

Cathepsin K (Ctsk) is a proteinase that requires an acidic environment to degrade and remove Type I collagen. The type I collagen fragment (CTX-I) normally present during collagen breakdown is absent in Cathepsin K knockout osteoclast cells in culture. A defect in the Cathepsin K gene in humans, as well as a Cathepsin K knockout mouse, will cause a pycnodysostotic phenotype. Pycnodysostosis is characterized by lack of normal collagen breakdown and sclerotic bone that is prone to fracture [3].

B. Nuclear Factor of Activated T cells

Nuclear Factor of Activated T cells (NFAT) is a transcription factor, or master regulator of osteoclasts. NFAT is necessary for osteoclast differentiation and activation. RANK activation stimulates calcineurin to dephosphorylate and thus activate NFATc1 [4]. Genes such as cathepsin K, tartrate-resistant acid phosphatase (TRACP), calcitonin receptor (CTR), and osteoclast-associated receptor (OSCAR) are regulated by NFATc1. More recently, studies have shown that NFATc1 also upregulates dendritic cell-specific transmembrane protein (DC-STAMP) and the d2 isoform of vacuolar ATPase Vo domain (Atp6v0d2) [12]. NFATc1's importance in osteoclastogenesis is evident because osteoclasts fail to develop in in vitro studies involving NFATc1-/- stem cells, and because osteoclast precursors differentiate in the absence of RANKL at sites of ectopic NFATc1 expression [8].

C. Tartrate-resistant acid phosphatase

Tartrate-resistant acid phosphatase (TRACP) is expressed in a variety of tissues, but is found in highest levels in bone, liver, spleen, thymus and colon. The gene encoding the TRACP protein is known as Acp5. The overall bone phenotype of TRACP knockout mice illustrates an osteopetrotic one, with shorter and fatter limbs and axial skeletons and altered epiphyseal growth plates. Because TRACP is evident in many immune cells, the TRACP knockout mice also exhibit inefficient inflammatory and immune responses. TRACP is widely used as a marker enzyme of mature osteoclasts [13].

D. Dendritic cell-specific transmembrane protein and the d2 isoform of vacuolar ATPase Vo domain

Fusion of mononuclear osteoclast precursors into multinuclear osteoclasts is a crucial step in the differentiation process. RANKL binds to the RANK receptor on osteoclast precursor cells, causing fusion genes including the "d2 isoform of vacuolar ATPase Vo domain" (Atp6v0d2) and dendritic cell-specific transmembrane protein (DC-STAMP), to become activated through NFATc1. DCSTAMP is a protein comprised of seven transmembrane regions expressed on the surface of osteoclast cells [14,15,16]. Both DCSTAMP-/- and Atp6v0d2-/- mice exhibit an osteopetrotic phenotype due to the inability of osteoclasts to fuse [8]. Multinuclear osteoclasts are completely absent in DCSTAMP-/- mice [15,16]. In DCSTAMP-/- osteoclasts, resorption does occur, but it is less efficient, suggesting that unfused osteoclasts are not as active as multinuclear, fused

osteoclasts [9]. Similarly, an increase in the number of mononuclear TRAP+ osteoclasts is seen in Atp6v0d2-/- mice compared to wild-type mice. The reduction in bone resorption identified in Atp6v0d2-/- mice is partly due to the osteoclasts inability to fuse when the Atp6v0d2 gene is not present [17,5].

Receptor activator of nuclear factor (RANK) and Osteoprotegerin (OPG)

Osteoclast differentiation and activation indirectly relies on signaling by proteins produced by other cells. Both RANKL and OPG are proteins expressed by osteoblasts and stromal cells. Osteoclasts require RANKL and macrophage colony stimulating factor (M-CSF), secreted by osteoblasts and bone marrow stromal cells, to survive and mature. RANKL and OPG induce opposite effects on osteoclast activation and bone resorption.. RANKL is shown to induce differentiation of osteoclast precursors and to activate and maintain osteoclasts by binding to Receptor activator of nuclear factor (NF)-kB (RANK) on osteoclasts, thus initiating bone resorption. Both activation of RANK by RANKL and activation of c-Fms by M-CSF are required for this osteoclast activation. The RANKL protein expressed by osteoblasts binds to the RANK receptor on osteoclasts, thus causing osteoclast activation. OPG is a decoy receptor that binds to RANKL, inhibiting RANKL from binding to the RANK receptor on osteoclasts. Therefore, OPG inhibits osteoclastogenesis (Figure 2). RANKL, OPG, and RANK are tumor necrosis factor receptor-like (TNFR) proteins that are essential for the regulation of bone remodeling. The levels of RANKL and OPG proteins secreted by osteoblasts are dependent on systemic hormones and other

local factors [10,18]. Parathyroid hormone (PTH), 1,25 Dihydroxyvitamin D (1,25-OH)2D3, and calcitonin are systemic hormones shown to stimulate osteoclastic bone resorption at least partially by indirectly upregulating RANKL and downregulating OPG [18]. The ratio of RANKL and OPG expression by osteoblasts is involved in the positive and negative regulation of RANK, thus playing a crucial role in the dynamic process of bone remodeling [10,19].

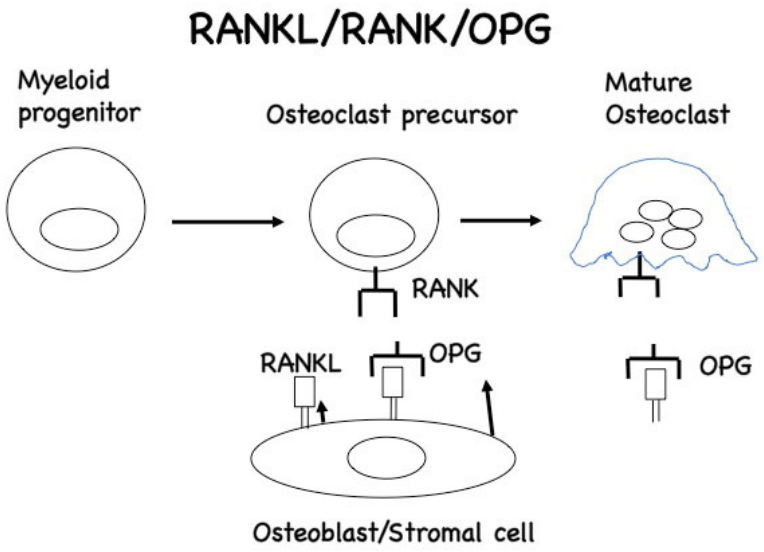

Figure 2: ***Interaction between RANKL/RANK/OPG*****. RANKL and OPG are expressed by osteoblasts and bone marrow stromal cells. RANKL binds to the RANK receptor on cells of the osteoclast lineage to induce differentiation and activation of osteoclasts. OPG is the decoy receptor that prevents RANKL from binding to its RANK receptor.**

Twisted Gastrulation Knockout Mice

To study its in vivo function in bone, *Twsg1* knockout mice have been generated. This research focuses on knockout mice from the 129Sv/Ev background, which are viable, fertile, and live a normal life span. *Twsg1*$^{-/-}$ mice are smaller in size compared to wild-type mice, have kinky tails, and exhibit

skeletal defects. The major skeletal defect in *Twsg1*$^{-/-}$ is osteopenia, or decreased bone. Femurs from *Twsg1*$^{-/-}$ mice contain significantly less cortical and trabecular bone compared to wild-type (WT), *Twsg1*$^{+/+}$ mice (Figure 3) [20]. Two possible mechanisms exist to cause altered bone metabolism; either osteoblast function decreases, or osteoclast function increases. Based on data from the Petryk/Gopalakrishnan/Mansky lab, the overall make-up and bone-forming ability of the osteoblast cells remains the same in both the *Twsg1*$^{-/-}$ and *Twsg1*$^{+/+}$ mice. Data from experiments involving tetracycline staining demonstrate that osteoblasts from both *Twsg1*$^{-/-}$ and *Twsg1*$^{+/+}$ mice form comparable amounts of bone. Because evidence supports that *Twsg1*$^{-/-}$ osteoblasts remain functional, the question of an altered osteoclast phenotype arises [20].

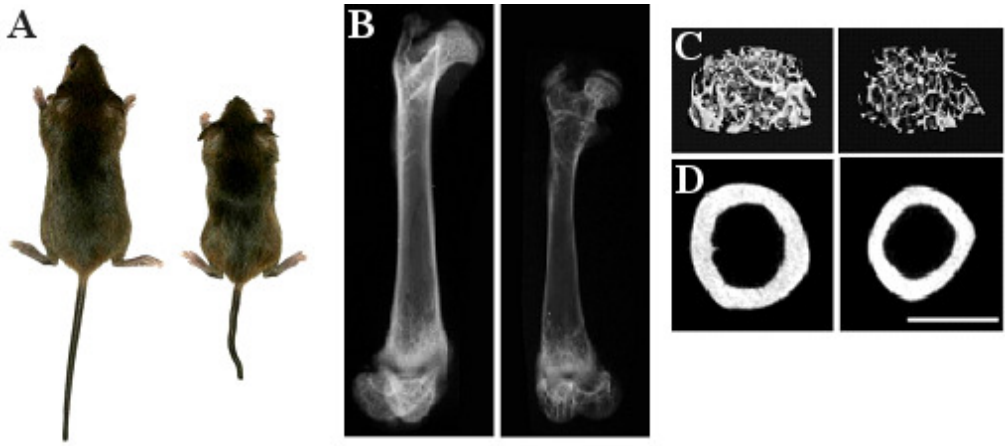

Figure 3: *Twsg1*$^{-/-}$ *mice are osteopenic*. Left side = WT mouse; Right side = *Twsg1*$^{-/-}$ mouse. A) *Twsg1*$^{-/-}$ mice are smaller, and have kinky tails. B) Radiographic representation of 1 month old WT and *Twsg1*$^{-/-}$ mouse femurs. C) CT comparing 3 month old WT and *Twsg1*$^{-/-}$ mouse trabecular and D) cortical bone.

Data from the Petryk/Gopalakrishnan/Mansky lab refutes that signaling by the RANKL/OPG mechanism is the cause for the increased osteoclastogenesis

observed in *Twsg1*$^{-/-}$ mice. More specifically, experiments evaluating RANKL and OPG show their similar mRNA expression levels between *Twsg1*$^{-/-}$ and WT mice [20]. Therefore, a direct mechanism of osteoclast activation and resorption to explain the increased osteoclastogenesis seen in *Twsg1*$^{-/-}$ mice remains a possibility.

Twsg1$^{-/-}$ mice have increased size and number of multinuclear, TRAP+ osteoclasts compared to osteoclasts from WT mice when cultured with M-CSF and RANKL. Enhanced fusion is suggested as a rationale for the increased number of nuclei per osteoclast in *Twsg1*$^{-/-}$ mice relative to WT mice. This data characterizes the unique, but overpowering role of osteoclasts in the *Twsg1*$^{-/-}$ mouse phenotype [20]. Published results from the Petryk/Gopalakrishnan/Mansky lab demonstrate that the osteopenic phenotype in *Twsg1*$^{-/-}$ mice is due to increased osteoclastogenesis and not due to impaired osteoblast function. They further showed that this enhanced osteoclastogenesis in *Twsg1*$^{-/-}$ mice is due to increased BMP signaling. They also showed that osteoclasts expressed BMP receptors and can be directly activated by BMPs. *Twsg1*$^{-/-}$ mice have increased size and number of osteoclasts relative to their wild-type counterparts. Multinuclear, tartrate-resistant acid phosphatase (TRAP) positive osteoclasts are more prevalent in the *Twsg1*$^{-/-}$ mice compared to the wild-type mice. Serum TRACP5b and carboxy-terminal collagen crosslinks (CTX) levels, representing increased number of osteoclasts and collagen breakdown respectively, are higher in *Twsg1*$^{-/-}$ mice relative to WT mice. Both NFATc1, Cathepsin K, and Acp5 genes are upregulated in *Twsg1*$^{-/-}$ osteoclasts compared to wild-type mice. These changes in

gene expression are further substantiated by the increased area of resorption pits on dentine discs identified in *Twsg1*$^{-/-}$ osteoclast cultures (Figure 4) [20].

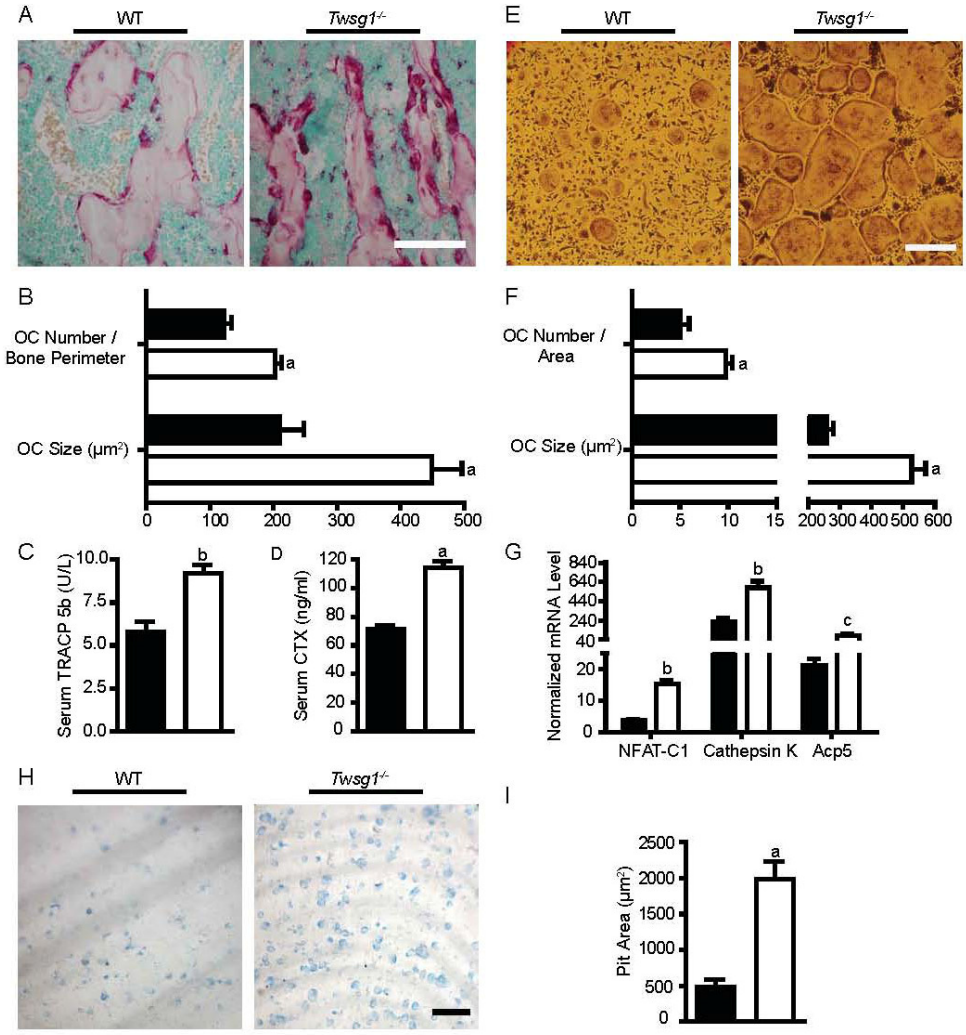

Figure 4: ***Increased osteoclastogenesis results in osteopenic phenotype of Twsg1*$^{-/-}$ *mice*****. Black bars represent WT; white bars represent *Twsg1*$^{-/-}$. A) TRAP-stained femur sections from WT and *Twsg1*$^{-/-}$ mice (Scale bar = 200 µm). B) Analysis of TRAP-stained femur sections from WT and *Twsg1*$^{-/-}$ mice. C) ELISA results measuring serum TRACP5b. D) ELISA results measuring serum CTX. E) TRAP stained osteoclasts after 7-days in culture differentiation of bone marrow cultures (Scale bar = 200µm). F) Analysis of TRAP-stained osteoclasts after 7 days in culture. G) NFATc1, Cathepsin K, and Acp5 (TRAP) gene expression levels from 3 day osteoclast cultures. H) Toluidine blue stained photomicrograph of resorption pits on dentine discs (Scale bar = 50µm). I) Quantification of resorption pit area of WT and *Twsg1*$^{-/-}$ osteoclasts.**

To determine the specific mechanism for increased osteoclastogenesis in *Twsg1*$^{-/-}$ mice, several experiments were performed. When *Twsg1*$^{-/-}$ osteoclasts were treated with increasing doses of the known BMP-2 and BMP-4 inhibitor Noggin, the osteoclasts became similar in number and size compared to WT mice. This result indicates that adding Noggin to osteoclast cultures from *Twsg1*$^{-/-}$ mice allows the osteoclast phenotype to be rescued. Noggin's only known function is as a BMP inhibitor, suggesting that BMP's are involved with the increased osteoclast function seen in *Twsg1*$^{-/-}$ mice [20].

To understand why lack of the Twsg1 protein alters osteoclasts, but seems not to affect osteoblasts, real time polymerase chain reaction (RT-PCR) was performed to determine expression of known BMP inhibitors Twsg1, Chordin, and Noggin. Twsg1 mRNA levels were significantly higher than Chordin or Noggin mRNA levels in osteoclasts, suggesting that loss of Twsg1 in osteoblasts could be compensated for by the presence of Noggin and Chordin. Further, WT osteoclasts were treated with BMP2 and sub-optimal RANKL concentrations, resulting in more numerous, and larger, multinuclear TRAP+ osteoclasts compared to RANKL treatment alone. No TRAP+ osteoclasts were seen with BMP2 treatment alone, but because the combination of BMP2 and RANKL together caused an increase in number and size of multinucleated TRAP+ osteoclasts, BMPs may enhance RANKL's affect on osteoclastogenesis (Figure 5). This finding demonstrates a direct effect of BMPs on osteoclast differentiation [20].

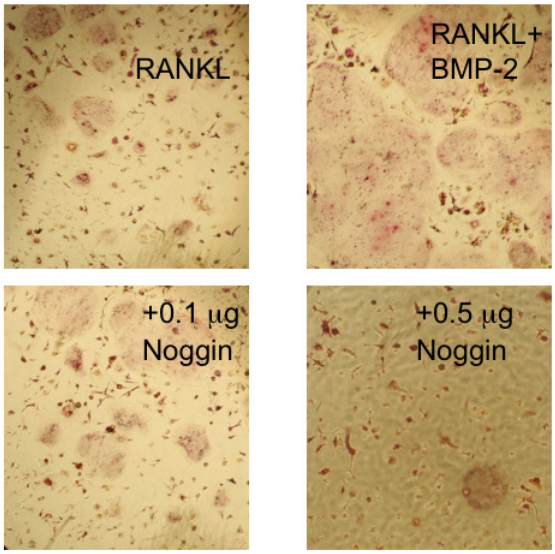

Figure 5: ***RANKL+ BMP2 increases size of osteoclasts while addition of Noggin rescues this phenotype*****. TRAP-stained WT osteoclasts treated with RANKL, RANKL + BMP2, +0.1µg Noggin, and +0.5µg Noggin from 7-day cultures.**

Bone morphogenetic proteins and Osteoclast Differentiation

It is widely known that BMPs directly regulate osteoblasts, but current research on whether BMPs directly regulate osteoclasts remains controversial. One review cites many separate studies supporting BMP's role in formation and activation of osteoclasts, while conflicting evidence from other studies does not support this data. Kaneko et al revealed a direct mechanism by which BMP2 and BMP4 stimulated osteoclasts by using in vitro osteoclast cells cultured from long bones of rabbits. They found that BMPs caused resorption pits without exogenous RANKL, identified BMP receptors IA and II on the mature osteoclast cell membranes, and found upregulated osteoclast gene expression (21, 22). Osteoclasts derived from mouse bone marrow were shown by Itoh et al to express BMP receptor IA mRNA. These authors also found that RANKL and M-CSF1 treated osteoclasts exhibited increased differentiation when BMP2 was added,

leading to their conclusion that BMPs have a direct role on osteoclastogenesis (23, 22). Results by Itoh et al support findings from the Mansky/Gopalakrishnan lab. Conversely, an in vitro study conducted by Kanatani et al revealed that BMP2 could only stimulate osteoclast cells in the presence of stromal cells, suggesting that BMP indirectly induces osteoclastogenesis via the pathway in which stromal cells produce RANKL [24, 22]. Similarly, Abe et al demonstrated an indirect effect of BMPs on osteoclastogenesis via the stromal cell and osteoblast-mediated RANKL signaling pathway (25, 22).

Hypothesis

The hypothesis of my project is that treatment of wild-type osteoclasts with BMP2 will increase osteoclast gene expression and that this gene expression will decrease with treatment of the known BMP inhibitor, Noggin.

Specific Aims

1. To determine the direct effect of BMP2 treatment on osteoclast gene expression.
2. To determine the effect of a key BMP inhibitor, Noggin, on the direct effect of BMP2 on osteoclast gene expression.

Materials and Methods

Primary Osteoclast Cell Cultures

Bone marrow was flushed from femurs of WT mice and cultured in alpha-MEM media for three days in the presence of 50ng/ml M-CSF (macrophage-colony stimulating factor) on non-tissue culture dishes. M-CSF selects for the osteoclast population because it promotes survival and proliferation of osteoclasts, but not other bone marrow cells. The adherent cell population containing the osteoclasts was then cultured in dishes with various combinations of M-CSF (30ng/ml), RANKL (30ng/ml), BMP2 (30ng/ml), and Noggin (1ug/ml), all from R&D Systems, for 3 days. The amounts of M-CSF, RANKL, BMP2 and Noggin that produced ideal osteoclast differentiation were previously identified by doing a dose-response curve.

Quantification of gene expression

RNA from the cultured cells was harvested with TriZol Reagent (Invitrogen) and quantified by UV spectroscopy. Reverse Transcriptase polymerase chain reaction (RT-PCR) using the iScript cDNA synthesis kit (Bio-Rad) was used to make cDNA. This cDNA was then used to measure gene expression by real-time PCR (RT-PCR) using the iCycler thermal cycler machine. The thermal cycler detects SYBR green (Bio-Rad IQ SYBRGreen Supermix), a dye that fluoresces only when bound to double stranded DNA (Figure 6).

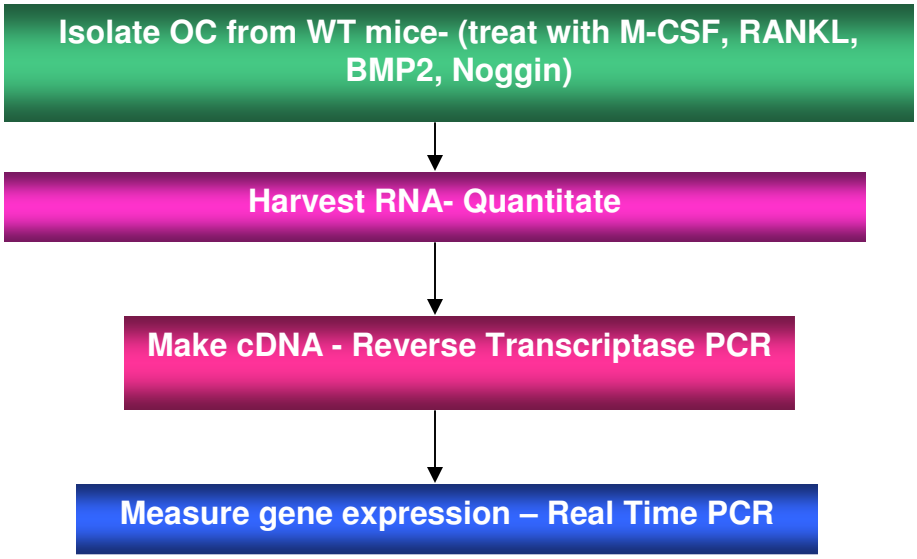

Figure 6: ***Flowchart representing sequence of experiments*****. Osteoclasts are isolated from WT mice and treated with various combinations of M-CSF, RANKL, BMP2, and Noggin. RNA is harvested from the osteoclasts and quantified by UV spectroscopy. This RNA is used to make cDNA by Reverse Transcriptase PCR. Finally, gene expression levels are measured with Real Time PCR.**

RT-PCR is a tool that allows relatively quick and precise quantification of mRNA levels where genes of interest are amplified and measured in real time. The housekeeping gene, L4, acts as an internal standard that all other genes are compared against. L4, a ribosomal gene, remains the same in every cell and thus operates as a control to account for small pipetting errors, and altered efficiency of cDNA preparation and purification between samples.

Nucleotides, forward and reverse primers specific for the target gene, cDNA, and the SYBRGreen Supermix make up the reaction mixture for each sample. Osteoclast treatment groups were cultured in triplicate. Subsequently, RT-PCR using cDNA from each osteoclast treatment group was run in triplicate

for each of the five genes of interest and the internal standard, the L4 gene (Figure 7).

Real-Time PCR Primer Sequences

Gene	Forward	Reverse
NFATc1	TCATCCTGTCCAACACCAAA	TCACCCTGGTGTTCTTCCTC
Cathepsin K	AGGGAAGCAAGCACTGGATA	GCTGGCTGGAATCACATCTT
TRAP	CGTCTCTGCACAGATTGCA	GAGTTGCCACACAGCATCAC
DCSTAMP	GGGCACCAGTATTTTCCTGA	TGGCAGGATCCAGTAAAAGG
ATP6v0d2f	TCAGATCTCTTCAAGGCTGTGCTG	GTGCCAAATGAGTTCAGAGTGATG
L4	CCTTCTCTGGAACAACCTTCTCG	AAGATGATGAACACCGACCTTAGC

All Sequences are 5′-3′.

Figure 7: *Real-Time Primer Sequences*. Real-Time Primer Sequences for the housekeeping gene and each of the five osteoclast genes of interest.

Parameters used for all RT-PCR reactions consisted of the following cycles: 95 degrees for 3 minutes, 94 degrees for 15 seconds, 60 degrees for 30 seconds, 72 degrees for 30 seconds, Repeat 2 through 4 for 45 times, 12 degrees for infinity.

Threshold cycle (Ct) values are gathered after each RT-PCR run from the MyIQ software and a series of calculations comparing each target gene to the reference gene L4 are computed using Microsoft Excel. These calculations ultimately lead to the Average Expression and Fold Change values that are entered into GraphPad for statistical analysis.

Statistical Analysis

Statistical analysis included Student's t-tests to measure significance between RANKL and RANKL+BMP2 treatment groups and between RANKL+BMP2 and RANKL+BMP2+Noggin treatment groups. One-way analysis of variance (ANOVA) measured significance between each of the treatment groups in both the experiments with either four or five treatment groups. Further statistical analysis using Tukey's Multiple Comparison Test identified which of the treatment group comparisons from the ANOVA were statistically significant.

Results

Specific Aim 1: To determine the direct effect of BMP2 treatment on osteoclast gene expression.

The first aim was to determine the effect of BMP2 treatment on osteoclast gene expression. WT osteoclasts were treated with CSF-1, RANKL, BMP2, and RANKL+BMP2 and changes in gene expression levels were evaluated by RT-PCR. The osteoclast target genes measured by RT-PCR included Cathepsin K, NFATc1, Acp5, DCSTAMP, and Atp6vOd2 compared to L4, the internal housekeeping gene. RANKL treatment induced an upregulation of all of the five genes of interest, whereas BMP2 alone did not increase gene expression for any of the five genes. The RANKL+BMP2 treatment group did produce enhanced expression of all five genes of interest beyond that of RANKL alone. Numerical

values representing graphed fold activation values are listed in Table 1. Average fold increases in gene expression levels comparing RANKL+BMP2 treated osteoclasts to RANKL treated osteoclasts were as follows: Ctsk (1.74 fold), Nfatc1 (3.54 fold), Acp5 (1.95 fold), DCSTAMP (2.13 fold), and Atp6vOd2 (1.58 fold) (Table 2).

Real-time PCR results showing average fold change in activation of the five genes is graphed with error bars that correspond to the mean triplicate results for each of the osteoclast treatment groups (Figure 8a-e).

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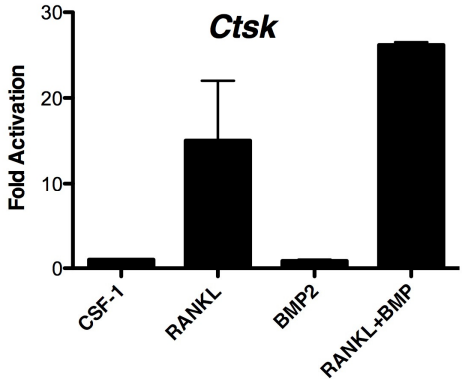

Figure 8a: ***Average fold activation of Cathepsin K gene*****. Changes in gene expression levels for wild-type osteoclasts treated with CSF-1, RANKL, BMP2, and RANKL + BMP2.**

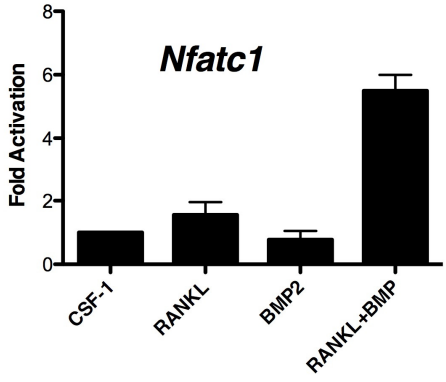

Figure 8b: ***Average fold activation of Nfatc1 gene*****. Changes in gene expression levels for wild-type osteoclasts treated with CSF-1, RANKL, BMP2, and RANKL + BMP2.**

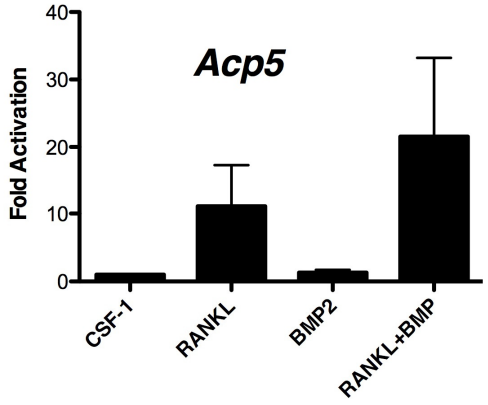

Figure 8c: ***Average fold activation of Acp5 gene encoding TRAP protein*****. Changes in gene expression levels for wild-type osteoclasts treated with CSF-1, RANKL, BMP2, and RANKL + BMP2.**

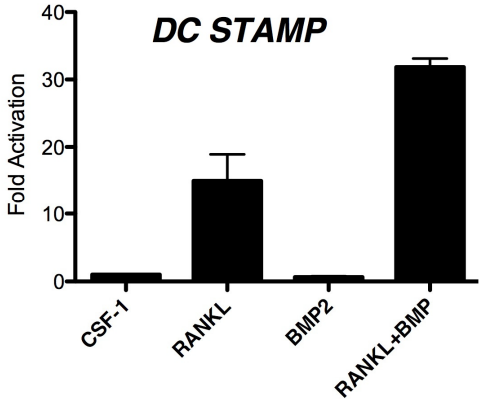

Figure 8d: ***Average fold activation of DCSTAMP gene*****. Changes in gene expression levels for wild-type osteoclasts treated with CSF-1, RANKL, BMP2, and RANKL + BMP2.**

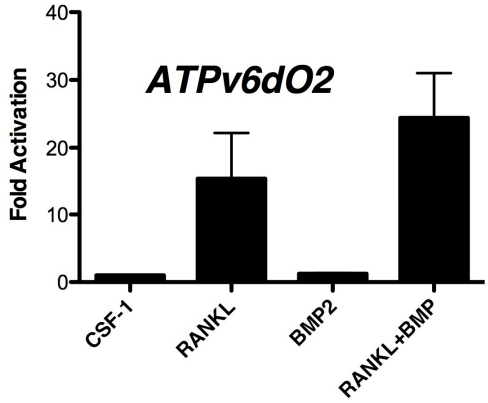

Figure 8e: ***Average fold activation of ATPv6d02 gene*****. Changes in gene expression levels for wild-type osteoclasts treated with CSF-1, RANKL, BMP2, and RANKL + BMP2.**

	CSF-1	RANKL	BMP2	RANKL + BMP2
CathepsinK	1.0	15.04	0.86	26.18
Nfatc1	1.0	1.58	0.599	4.986
Acp5	1.0	11.07	1.29	21.56
DCSTAMP	1.0	14.97	0.64	31.84
Atp6	1.0	15.45	1.25	24.41

Table 1: ***Average fold activation in gene expression.*** **Average fold activation in gene expression of the treatment groups compared to CSF-1.**

Unpaired t-tests were calculated to evaluate the difference in gene expression levels between RANKL and RANKL + BMP2 treated osteoclasts for each of the five genes of interest. P-values of less than 0.05 are considered statistically significant. Two of the five genes exhibited a t-test of statistical significance under this analysis. Nfatc1 and DCSTAMP had statistically significant p-values of 0.0036 and 0.0152, respectively. The p-values comparing RANKL versus RANKL + BMP2 were not statistically significant for Ctsk (p=0.1855), Acp5 (p=0.4720), and Atp6v0d2 (p=0.3963) (Table 2).

	Average Fold Increase	t-test p-value	Significance
Cathepsin K	**1.74**	**0.1855**	**No**
Nfatc1	**3.54**	**0.0036**	**Yes ****
Acp5	**1.95**	**0.4720**	**No**
DCSTAMP	**2.13**	**0.0152**	**Yes ***
Atp6vOd2	**1.58**	**0.3963**	**No**

Table 2: ***Analysis of changes in gene expression levels*****. Average fold increase, p-values, and significance comparing RANKL treated vs RANKL+BMP2 treated osteoclasts. *indicates significance where 0.01<p<0.05, **indicates high significance where 0.001<p<0.01, *** indicates extreme significance where p<0.001.**

Analysis of Variance with Tukey's Multiple Comparison Test was also calculated to analyze differences in gene expression between all four of the following treatment groups: CSF1, RANKL, BMP2, and RANKL+BMP2. Overall changes in average Cathepsin K, NFATc1, DCSTAMP, and ATP6vOd2 expression calculated using ANOVA were significant between the four treatment groups, with p-values of 0.0023, 0.0001, 0.0001, and 0.0201, respectively. The p-value for Acp5 is 0.1690, and is not statistically significant (Table 3).

Gene	ANOVA p-value	Significance
Cathepsin K	0.0023	Yes **
Nfatc1	0.0001	Yes ***
Acp5	0.1690	No
DCSTAMP	0.0001	Yes ***
ATP6vOd2	0.0201	Yes *

Table 3: *Summary ANOVA results*. Overall ANOVA results indicate p-value and significance for comparing each of the five genes of interest among the four treatment groups (CSF-1, RANKL, BMP2, and RANKL+BMP2). *indicates significance where 0.01<p<0.05, **indicates high significance where 0.001<p<0.01, * indicates extreme significance where p<0.001.**

Further analysis using Tukey's Multiple Comparison Test showed significance between several of the treatment groups for each gene. Most notable is the comparison between RANKL and RANKL+BMP2 treatment groups. Similar to the student's t-test results, both Nfatc1 and DCSTAMP genes were significantly upregulated when treated with RANKL+BMP2 compared to RANKL alone. Several other comparisons were made between these two treatment groups, all of which are summarized (Table 4).

	Cathepsin K	Nfatc1	Acp5	DCSTAMP	Atp6vOd2
CSF-1 vs RANKL	No	No	No	Yes **	No
CSF-1 vs BMP2	No	No	No	No	No
CSF-1 vs RANKL+BMP2	Yes **	Yes ***	No	Yes ***	Yes *
RANKL vs BMP2	No	No	No	Yes **	No
RANKL vs RANKL+BMP2	No	Yes ***	No	Yes **	No
BMP2 vs RANKL+BMP2	Yes **	Yes ***	No	Yes ***	Yes *

Table 4: *Summary of Tukey's Multiple Comparison Tests.* Significance of comparisons between treatment groups labeled in left-hand column for the five genes labeled in each of the rows. Blue represents notable significance between RANKL vs RANKL+BMP2 treatment groups. *indicates significance where 0.01<p<0.05, **indicates high significance where 0.001<p<0.01, * indicates extreme significance where p<0.001.**

Specific Aim 2: To determine the effect of a key BMP inhibitor, Noggin, on the direct effect of BMP2 on osteoclast gene expression.

The second aim was to determine if increase in gene expression by BMP2 was negatively regulated by treatment with Noggin, a known BMP inhibitor. Real-time PCR results showing average fold change in activation of the five genes are graphed with error bars that correspond to the mean triplicate results for each of the five osteoclast treatment groups. Treatment groups include the previous four treatment groups in addition to the RANKL+BMP2+Noggin treatment group. Similar to the results above, RANKL causes an increase in fold activation of all five genes, and BMP2 treatment alone does not induce upregulation of any of the five genes. Again, treatment of osteoclasts with RANKL+BMP2 causes a considerable increase in average fold gene activation compared to RANKL treatment alone. The increase in gene expression by RANKL+BMP2 was negatively regulated by treatment with Noggin for each of the five target osteoclast genes. Real-time PCR results showing average fold change in activation of the five genes is graphed with error bars that correspond to the mean triplicate results for each of the osteoclast treatment groups (Figure 9a-e). Figure 9a-e graphically represents the numerical fold activation values in Table 5. Average fold increases in gene expression levels comparing RANKL+BMP2 treated osteoclasts to RANKL treated osteoclasts were as follows: Ctsk (1.60 fold), Nfatc1 (3.04 fold), Acp5 (1.95 fold), DCSTAMP (1.99 fold), and Atp6vOd2 (1.58 fold).

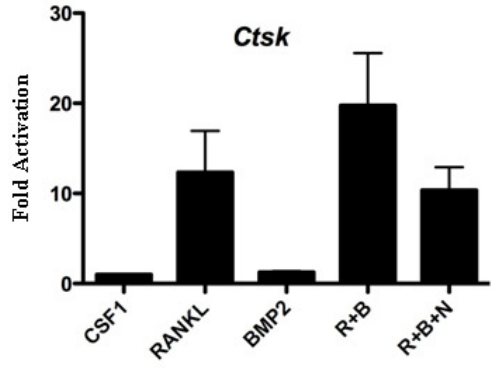

Figure 9a: ***Average fold activation of Cathepsin K gene*****. Changes in gene expression levels for wild-type osteoclasts treated with CSF-1, RANKL, BMP2, RANKL+BMP2, and RANKL+BMP2+Noggin**

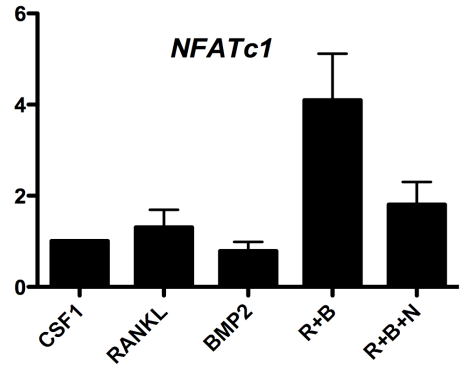

Figure 9b: ***Average fold activation of NFATc1 gene*****. Changes in gene expression levels for wild-type osteoclasts treated with CSF-1, RANKL, BMP2, RANKL+BMP2, and RANKL+BMP2+Noggin**

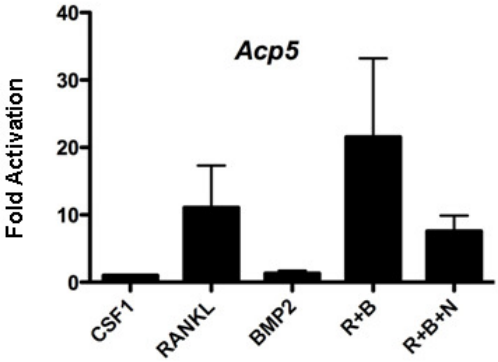

Figure 9c: ***Average fold activation of ATPv6d02 gene*****. Changes in gene expression levels for wild-type osteoclasts treated with CSF-1, RANKL, BMP2, RANKL+BMP2, and RANKL+BMP2+Noggin**

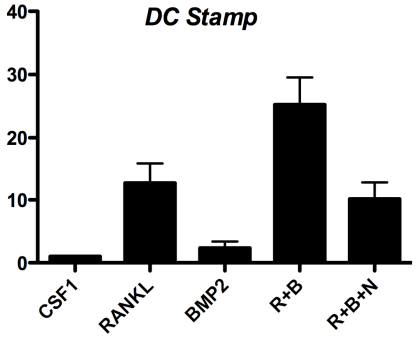

Figure 9d: ***Average fold activation of ATPv6d02 gene*****. Changes in gene expression levels for wild-type osteoclasts treated with CSF-1, RANKL, BMP2, RANKL+BMP2, and RANKL+BMP2+Noggin.**

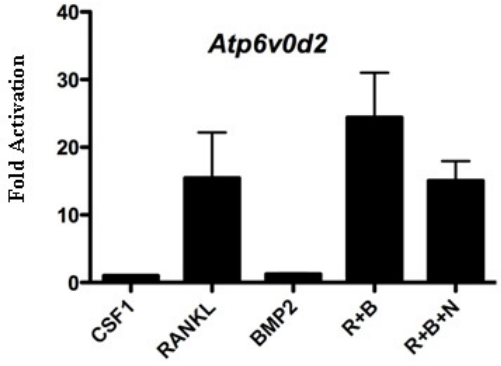

Figure 9e: ***Average fold activation of ATPv6d02 gene*****. Changes in gene expression levels for wild-type osteoclasts treated with CSF-1, RANKL, BMP2, and RANKL+BMP2, and RANKL+BMP2+Noggin.**

	CSF-1	RANKL	BMP2	R+B	R+B+N
CathepsinK	1.0	12.34	1.25	19.76	10.36
Nfatc1	1.0	1.35	0.78	4.10	1.81
Acp5	1.0	11.07	1.29	21.56	7.59
DCSTAMP	1.0	12.68	2.30	25.25	10.16
Atp6	1.0	15.45	1.25	24.40	15.05

Table 5: ***Average fold activation in gene expression.*** **Average fold activation in the expression of the treatment groups compared to CSF-1.**

Similar to the first group, RANKL versus RANKL+BMP2 showed statistically significant upregulation in gene expression for Nfatc1 and DCStamp, with unpaired t-test p-values of 0.0214 and 0.0294, respectively. Cathepsin K (p=0.1862), TRAP (p=0.2360), and ATP6 (p=0.1981) were not statistically significant for the RANKL versus RANKL+BMP2 treatment groups.

Unpaired t-tests were also calculated to determine the change in gene expression levels between RANKL+BMP2 versus RANKL+BMP2+Noggin treatment groups. These comparisons had the following p-values, Nfatc1

(p=0.0448)), DCSTAMP (p=0.0121), Ctsk (p=0.1063), Acp5 (p=0.1525), and Atp6v0d3 (p=0.1324), of which Nfatc1 and DCStamp are statistically significant. Average fold decreases in gene expression comparing RANKL + BMP2 versus RANKL + BMP2 + Noggin treatment groups are as follows: Ctsk (1.91 fold), Nfatc1 (2.27 fold), Acp5 (2.84 fold), DCSTAMP (2.49 fold), and Atp6v0d2 (1.62 fold). All p-values and fold changes are summarized in Table 6.

	Average Fold Decrease	t-test p-value	Significance
Cathepsin K	**1.91 fold**	**0.1063**	**No**
Nfatc1	**2.27 fold**	**0.0448**	**Yes ***
Acp5	**2.84 fold**	**0.1525**	**No**
DCSTAMP	**2.49 fold**	**0.0121**	**Yes ***
Atp6vOd2	**1.62 fold**	**0.1324**	**No**

Table 6: ***Analysis of changes in gene expression levels*****. Average fold decrease, p-values, and significance comparing RANKL+BMP2 vs RANKL+BMP2+Noggin treated osteoclasts. *indicates significance where 0.01<p<0.05, **indicates high significance where 0.001<p<0.01, *** indicates extreme significance where p<0.001.**

Analysis of Variance with a Tukey's Multiple Comparison Test was calculated to evaluate differences in gene expression between all five of the following treatment groups: CSF1, RANKL, BMP2, RANKL+BMP2, and RANKL+BMP2+Noggin. Overall changes in average Cathepsin K, Nfatc1, DCSTAMP, and ATP6vOd2 expression calculated using ANOVA were significant between the five treatment groups, with p-values of, 0.0162, 0.0040, 0.0001 and 0.0153, respectively. Acp5 was not statistically significant, with a p-value of 0.1727 (Table 7).

Gene	ANOVA p-value	Significance
Cathepsin K	0.0162	Yes *
Nfatc1	0.0040	Yes **
Acp5	0.1727	No
DCSTAMP	0.0001	Yes ***
ATP6vOd2	0.0153	Yes *

Table 7: ***Summary ANOVA results*****. Overall ANOVA results indicate p-value and significance for comparing each of the five genes of interest among the five treatment groups (CSF-1, RANKL, BMP2, RANKL+BMP2, and RANKL+BMP2+Noggin). *indicates significance where 0.01<p<0.05, **indicates high significance where 0.001<p<0.01, *** indicates extreme significance where p<0.001.**

Further analysis using Tukey's Multiple Comparison Test showed significance between some of the treatment groups for each gene. Several other comparisons were made between two treatment groups, all of which are summarized (Table 8).

	Cathepsin K	Nfatc1	Acp5	DCSTAMP	Atp6vOd2
CSF-1 vs RANKL	No	No	No	No	No
CSF-1 vs BMP2	No	No	No	No	No
CSF-1 vs RANKL+BMP2	Yes *	Yes **	No	Yes ***	Yes *
CSF-1 vs R+B+N	No	No	No	No	No
RANKL vs BMP2	No	No	No	No	No
RANKL vs RANKL+BMP2	No	Yes *	No	Yes *	No
RANKL vs R+B+N	No	No	No	No	No
BMP2 vs RANKL+BMP2	Yes *	Yes **	No	Yes ***	Yes *
BMP2 vs R+B+N	No	No	No	No	No
R+B vs R+B+N	No	No	No	Yes *	No

Table 8: ***Summary of Tukey's Multiple Comparison Tests*****. Significance of comparisons between treatment groups labeled in left-hand column for the five genes labeled across each of the rows. . *indicates significance where 0.01<p<0.05, **indicates high significance where 0.001<p<0.01, *** indicates extreme significance where p<0.001.**

Discussion

A general trend is apparent when comparing changes in gene expression levels among the WT osteoclast treatment groups. While M-CSF allows survival and proliferation of osteoclasts, these results show an appreciable upregulation of all five target genes as osteoclasts begin to differentiate when RANKL is added to the culture media. BMP2 treatment alone does not produce an increase in osteoclast gene expression levels. On the other hand, the treatment of osteoclasts with both RANKL and BMP2 consistently results in a superior upregulation of each of the five osteoclast genes, indicating a possible synergistic effect of RANKL and BMP2 on osteoclast activation compared to RANKL alone. The addition of the known BMP inhibitor Noggin reliably initiates the downregulation of osteoclast gene expression levels in the RANKL+BMP2+Noggin treated osteoclasts because Noggin inhibits BMP2 from binding to its receptor.

These results are consistent with some of the data previously collected in the Petryk/Gopalakrishnan/Mansky lab in which osteoclasts are larger and contain more nuclei in *Twsg1*$^{-/-}$ mice compared to WT mice. Perhaps this phenotype of more nuclei per osteoclast results from enhanced fusion in the process of maturing from a mononuclear osteoclast precursor into a multinuclear cell. DCSTAMP is a gene that regulates fusion. It is possible that the augmentation of RANKL by BMP2 increases gene expression involved in fusion as seen in the results of this study. Enhanced fusion by upregulation of DCSTAMP may provide a possible explanation for the increased number of nuclei per cell seen in *Twsg1*$^{-/-}$ osteoclasts. NFatc1 was also found to cause a statistically significant increase in

BMP2's ability to enhance RANKL induced gene expression. Nfatc1 is a master regulator for osteoclast gene expression and is known to activate other key genes involved in osteoclast differentiation. Nfatc1 is regulated on many levels; this may explain the lower fold activation changes seen in Nfatc1 compared to the downstream genes that it regulates.

Of particular interest is the mechanism by which BMP2 enhances osteoclast gene expression levels which subsequently induce osteoclast activation and bone resorption. As discussed previously, reports differ in terms of the data presented to confirm or refute a direct or indirect role for BMPs on osteoclast activation. Results of this study coincide with previous research depicting a direct action of BMPs on osteoclast cells, exclusive of the osteoblast-mediated RANKL/OPG pathway. Some reports provoke another question related to the circumstances under which BMPs may directly induce osteoclast activation. Pathologic bone resorption seen in conditions such as periodontal disease or rheumatoid arthritis may lead to an increase in tumor necrosis factor alpha (TNF-α), which may act with RANKL to increase osteoclast activation. Thus, the upregulation of TNF-α and RANKL during inflammatory situations may disrupt the normal coupling between osteoblasts and osteoclasts [26].

Future experiments should include the quantification of gene expression levels when Twsg1 is added in place of Noggin to the osteoclast cultures. In this study we used Noggin because we understand its role as an extracellular BMP inhibitor more completely than Twsg1. Preliminary data suggest that Twsg1 is secreted by osteoblasts, but that it is not expressed into the extracellular space and

is present localized to the membrane in osteoclast cells. Therefore, one experiment would be to infect osteoclasts with an adenovirus containing Twsg1 instead of treating osteoclasts extracellularly with the protein.

Knowledge of how local signaling molecules and their regulators interact in bone is pertinent to our understanding of skeletal biology. The discovery of mechanisms by which osteoblasts and osteoclasts function may lead to therapeutic applications that relieve pathologic bone diseases. Osteoporosis, malignancies, periodontal disease, arthrititis, and fractures are some of the major osteopenic conditions affecting bone, of which osteoporosis alone affects twenty-eight million adults in the United States [11]. While it is certain that BMPs directly induce osteoblast differentiation and function, identifying either a direct or indirect method of osteoclast activation by BMP2 is relevant to understanding how BMP therapies may be used in the future. BMPs have been shown to be effective in treating long and craniofacial bone defects, healing fractures, and regenerating alveolar bone in animal studies. Confidence in treatment modalities involving BMPs will be gained when the exact role that BMPs play on osteoclasts is elucidated. An awareness of the potential to induce not only osteoblast activation, but also osteoclast activation is necessary when considering procedures that the FDA has approved for the use of recombinant human BMP (rhBMP). So far, these include “lumbar spinal fusion, long bone nonunion fractures, and sinus and alveolar ridge augmentation” [2]. Several reports advocate using rhBMP2 in spinal surgeries to successfully induce bone formation, while other reports list ectopic bone formation, and osteoclast activation and bone resorption as negative

sequelae [27]. Of more significance to dentistry, periodontal disease, facial trauma, and alveolar bone atrophy in edentulous sites all may contribute to an overall lack of bone that complicates restorative dental procedures. Some groups have advocated the use of rhBMP2 either in conjunction with or potentially as a substitute for other bone graft materials. rhBMP2 has also been studied for use in the aid of osseointegration of implants and for ridge augmentation, often with encouraging results. In addition, forces applied to teeth during orthodontic tooth movement induce local changes in bone. The altered levels of secreted proteins cause bone remodeling around the teeth, ultimately leading to more esthetically pleasing and functional tooth alignment. The ideal concentration of rhBMP2 and the carrier method are questions of further study before BMP treatment becomes a predictable and universal practice in dentistry [28,29]

These results indicate a role for direct action of BMP2 on osteoclasts, as evidenced by the increase in osteoclast gene expression after treatment with BMP2. An upregulation of NFATc1, Acp5, ATP6v0d2, Ctsk, and DCSTAMP genes is measurable by RT-PCR quantification of WT osteoclast mRNA in the presence of RANKL and BMP2.

Summary

Osteoblasts and osteoclasts are the two primary cell types involved in the dynamic process of bone remodeling. Under normal conditions, osteoblasts and osteoclasts are coupled, whereas the highly regulated process of bone remodeling

is disrupted under pathological conditions. Evidence of the supportive role that BMPs play on osteoblasts is widely accepted in the study of skeletal biology, whereas the interaction between BMPs and osteoclasts remains to be determined. Several proteins inhibit BMPs from binding to their receptors, including Noggin and the less understood, Twisted Gastrulation. *Twsg1*$^{-/-}$ mice are smaller, have less cortical and trabecular bone, and have larger, more fused osteoclasts than WT mice. Previous experiments performed in the Petryk/Gopalakrishnan/Mansky lab support that the osteopenic phenotype in *Twsg1*$^{-/-}$ mice result from increased osteoclastogenesis, and not reduced osteoblast function. Also, this increased osteoclast activation and function has been shown to be a direct result of increased osteoclast activation, and not indirectly dependent on an increased RANKL-induced osteoclastogenesis. In this study, WT osteoclasts were treated with M-CSF, RANKL, BMP2, RANKL + BMP2, and RANKL + BMP2 + Noggin in triplicate cultures. RT-PCR was used to evaluate gene expression levels in order to quantify BMPs effect on target gene expression levels. The addition of BMP2 to RANKL always induced an upregulation of Cathepsin K, Nfatc1, Acp5, DCSTAMP, and ATP6v6d02 gene expression, whereas the addition of Noggin always resulted in a down-regulation of all of the five target gene expression levels. These results demonstrate a trend that supports a direct mechanism of action on osteoclast activation. Statistically significant upregulation in gene expression levels of Nfatc1 and DCStamp was noted when comparing RANKL vs RANKL + BMP2 treatment groups. DCStamp is a gene important in the fusion of mononuclear osteoclasts to become multinuclear osteoclasts, which supports the

previous findings of larger osteoclasts with increased nuclei found in *Twsg1*$^{-/-}$ mice. Further studies are necessary to confirm the direct role of BMPs on osteoclast activation, including clinical trials that will help identify the role therapeutic BMP treatments will have in treating pathologic bone conditions.

Conclusion

1. Real-time PCR results show that treatment of WT osteoclasts with RANKL induced an upregulation of all of the five genes of interest, whereas BMP2 alone did not increase gene expression for any of the five genes.
2. The RANKL+BMP2 treatment group did produce enhanced expression of all five genes of interest beyond that of RANKL alone. Average fold increases in gene expression levels comparing RANKL+BMP2 treated osteoclasts to RANKL treated osteoclasts are as follows: Ctsk (1.74 fold), Nfatc1 (3.54 fold), Acp5 (1.95 fold), DCSTAMP (2.13 fold), and Atp6vOd2 (1.58 fold). Nfatc1 and DCSTAMP had statistically significant p-values of 0.0036 and 0.0152, respectively using the unpaired t-test.
3. Unpaired t-tests were also calculated to determine the change in gene expression levels between RANKL+BMP2 versus RANKL+BMP2+Noggin treatment groups. Nfatc1 (p=0.0448)) and DCSTAMP (p=0.0121) were statistically significant. Average fold decreases in gene expression comparing RANKL + BMP2 versus RANKL + BMP2 + Noggin treatment groups are as follows: Ctsk (1.91 fold), Nfatc1 (2.27 fold), Acp5 (2.84 fold), DCSTAMP (2.49 fold), and Atp6v0d2 (1.62 fold).

4. These results are consistent with some of the data previously collected in the Petryk/Gopalakrishnan/Mansky lab in which osteoclasts are larger and contain more nuclei in *Twsg1*$^{-/-}$ mice compared to WT mice. DCSTAMP is a gene that regulates fusion. It is possible that the augmentation of RANKL by BMP2 increases gene expression involved in fusion as seen in the results of this study. Nfatc1 is a master regulator for osteoclast gene expression and is known to activate other key genes involved in osteoclast differentiation
5. These results indicate a role for direct action of BMP2 on osteoclasts, as evidenced by the increase in osteoclast gene expression after treatment with BMP2.

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