Characterizing the effect of BMP2 on osteoclast differentiation and activity

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Dedication

To my parents

Howard and Esther Hoganson

For their support and encouragement

ABSTRACT

Bone is a profoundly dynamic tissue, characterized by continuous remodeling with bone formation by osteoblasts and bone resorption by osteoclasts. Bone morphogenetic proteins (BMPs) are multifunctional cytokines belonging to the TGF-β superfamily involved in numerous molecular cascades and signaling pathways. While BMPs are well known key regulators of osteoblast biology, their role in modulating the differentiation of osteoclasts is not as well known. The goals of this study were to further characterize the role of BMP2 in osteoclast activation by evaluating the expression of BMP receptors and BMP ligands in *BMP2;LysM-Cre* cKO osteoclasts, and measure the quantity and timing of expression of BMP2 protein by osteoclasts during differentiation. Our results showed that osteoclasts maximally express BMP2 two days after addition of RANKL to *in vitro* cultures. BMP2 was also detected in media of osteoclast cultures at day 3 after RANKL addition, which correlates with the activation of SMAD1/5/8 signaling in osteoclasts. Though not significant, osteoclasts from *Bmp2;LysM-Cre* mice demonstrated a trend of increased *Rank* and *c-Fms* mRNA expression, decreased *Bmpr1a*, *Bmpr1b*, and *BmprII* mRNA expression, no change in *Bmp4* mRNA expression, and a decrease in *Bmp6* mRNA expression. These trends will be further investigated in future experiments to evaluate the significance of the role of BMP2 in their expression.

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INTRODUCTION

Bone is a profoundly dynamic tissue that undergoes continuous adaptation through bone resorption and bone formation (Jensen 2010). This homeostatic process proceeds in cycles under normal conditions, beginning in fetal skeletogenesis, maintaining bone mass and structural integrity in postnatal life, and recapitulating during fracture healing and other normal and pathologic conditions (Giannoudis 2007). Synchronization of multiple cellular components are required to keep this process coordinated (Raggatt 2010 and Sanchez-Duffhues 2015). (Figure 1).

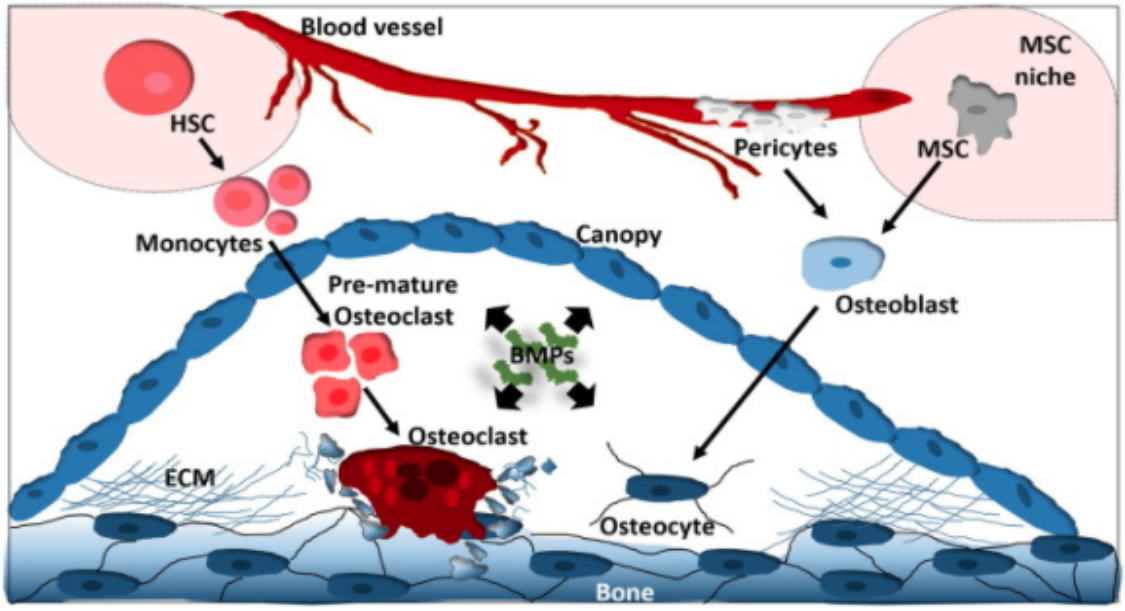

Figure 1: Basic Multicellular Unit (BMU) of bone. A canopy of mesenchymal cells facilitates the interchange of molecules between bone tissue and vasculature (Sanchez-Duffhues 2015).

Osteoblasts are specialized bone formation cells originating from pluripotent mesenchymal cells. They express osteoclastogenic factors, produce bone matrix proteins (BMPs), secrete osteoid and are responsible for bone mineralization (Raggatt 2010 and Giannoudis 2007). Osteoclasts initiate bone remodeling and are responsible for the removal of old bone matrix through resorption and maintain mineral homeostasis (Vaananen 2008). They originate from the monocyte/macrophage branch of hematopoetic stem cells and form into multinucleated cells by fusion of multiple mononuclear cells (Vaananen 2008). Two hematopoietic factors are necessary and sufficient for osteoclastogenesis; the TNF-related cytokine receptor activated NF-κB ligand (RANKL) and the polypeptide growth factor macrophage colony-stimulating factor (M-CSF), both of which are expressed by osteoblasts and osteocytes (Boyle 2003 and Vaananen 2008). Binding of M-CSF to c-FMS is required for survival and proliferation of early osteoclast precursors, while binding of RANKL to the RANK receptor on osteoclasts stimulates expression of the required genes for osteoclast differentiation, cellular fusion, and bone resorption (Jensen 2010). Osteoblasts also express osteoprotegerin (OPG), a soluble decoy receptor for RANKL, which inhibits osteoclast activation (Figure 2). The RANKL:OPG ratio produced by osteoblasts allows for close coordination of bone formation and resorption under normal physiologic conditions, and when disruptions in this homeostatic process occur pathologic bone loss or formation can result (Boyle 2003 and Jensen 2010).

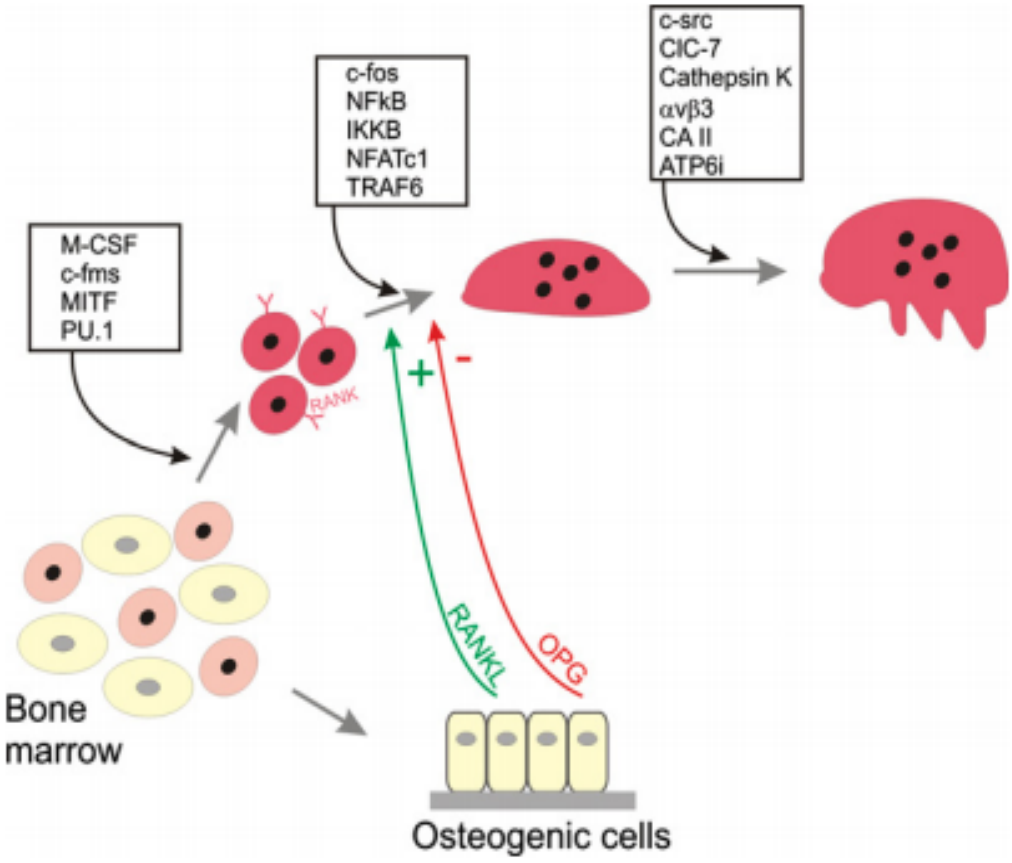

Figure 2. Schematic of stimulation of myeloid cells via M-CSF and RANKL into osteoclasts by osteoblasts (Vaananen 2008).

Both osteoblasts and osteoclasts are subject to paracrine and autocrine regulation by several cytokines and growth factors (Roderiquez 2009). Bone morphogenetic proteins (BMPs) are multifunctional cytokines belonging to the TGF-β superfamily involved in numerous molecular cascades and signaling pathways. In bone tissue, osteoprogenitor cells, osteoblasts, chondrocytes, platelets, and endothelial cells produce BMPs, which are stored in the extracellular matrix. During bone repair and remodeling, BMPs are released, triggering a cascade of events via time dependent signaling, resulting in

chondrogenesis, osteogenesis, and angiogenesis (Carreira 2014). While BMPs are well known key regulators of osteoblast biology, their role in modulating the differentiation of osteoclasts is not as well known.

As reviewed by James et al., in 2002, the FDA approved the first clinical application of recombinant human BMP2 in anterior lumbar interbody fusions because of its osteogenic properties, and use spread to dentistry in 2007 for oral and maxillofacial reconstructions (James 2016). It was believed to be near perfect in achieving bone growth and healing without adverse side effects. But with the rise in use of BMP2, an increasing side effect profile became evident (James 2016). BMP2 delivered by hydrogel in alveolar cleft grafts resulted in severe gingival swelling when compared to autologous bone from the iliac crest, resulting in early closing of the study (Neovius 2013). BMP-based therapy has since been shown to result in increased bone resorption, suggesting BMPs potentiate osteoclast differentiation and activation (Sánchez-Duffhues 2015). This study aims to further understand BMP2 signaling components in osteoclasts.

REVIEW OF THE LITERATURE

BMP signaling is mediated through a heteromeric receptor complex, made up of serine/threonine kinase type I and type II receptors on the cell surface, that transduce intracellular signals via SMAD (canonical signaling pathway) or mitogen-activated protein kinase (MAPK) (noncanonical signaling pathway)

cascades (Cao 2005 and Wu 2016). Both pathways have been shown to be activated in osteoclasts (Broege 2013). Three type I receptors have been shown to bind BMP ligands; type IA and IB BMP receptors and type IA activin receptor. Three type II receptors have also been identified; type II BMP receptor (BMPR II) and type II and IIB activin receptors. BMPR 1A, 1B and II are specific for BMPs, whereas the activin receptors are also signaling receptors for activins. Upon binding of the BMP ligand, type I and type II receptors form a heterotetrameric-activated receptor complex that transduces a signal into the cytoplasm to regulate expression of genes, many of which are responsible for maintaining bone homeostasis (Cao 2005 and Sánchez-Duffhues 2015). BMP activity is antagonized by extracellular secreted molecules such as CHORDIN, NOGGIN, and Twisted Gastrulation (TWSG1), and intracellular antagonists including SMAD6, SMAD7, SMURF1 and SMURF2. (Carreira 2014). (Figure 3).

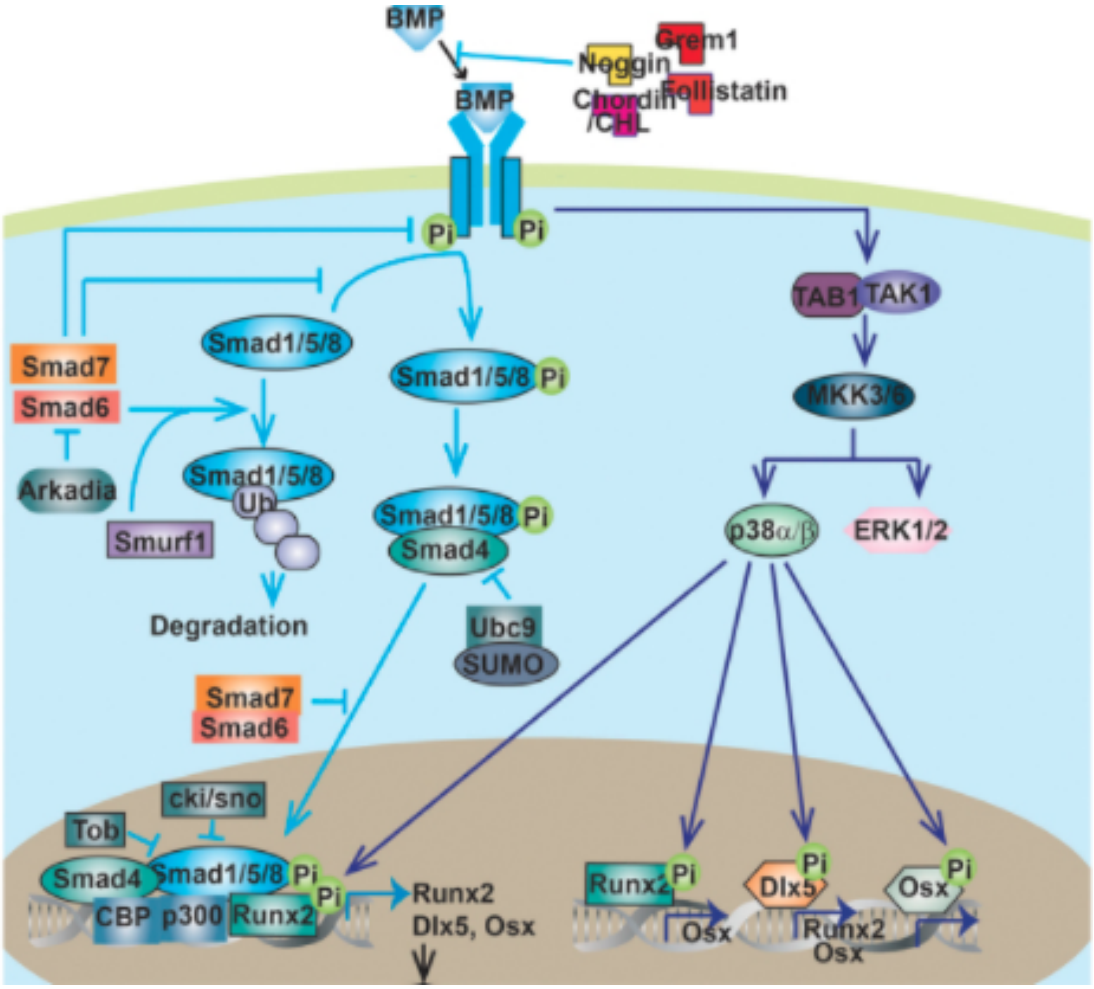

Figure 3. BMP signaling via the canonical (left) and noncanonical (right) pathways. Binding of BMPs to the heterotetrameric receptor complex induces phosphorylation of SMADs 1,5,8 enabling them to form a trimeic complex with SMAD 4 and be retained in the nucleus to regulate gene expression (Wu 2016).

In vitro and *in vivo* studies have demonstrated that BMP2 induces osteoclast activity both indirectly and directly (Giannoudis 2007, James 2016, Jensen 2010, and Kanatani 1995). Indirectly, BMPs promote osteoclasts through enhanced expression of osteoclast promoting factors by osteoblasts or stromal cells (Jensen 2010). Kanatani was first to provide evidence that BMP2 stimulates osteoclastic bone resorption and stimulates osteoclast-like cell formation in the

presence and absence of stromal cells *in vitro*. (Kanatani 1995). Multiple reports have since indicated that osteoclasts express BMP receptors and ligands, and BMPs stimulate osteoclast differentiation directly *in vitro* (Garimella 2008, Roderiquez 2009, Itoh 2001, Kanatani 1995, Kaneko 2000, Jensen 2010). Osteoclast differentiation is enhanced by BMPs when supported by M-CSF and RANKL, and BMP2 can directly stimulate pit formation in the absence of exogenous RANKL (Kaneko 2000, Roderiquez 2009, Jensen 2010). Rodriguez et al. demonstrated that mice deficient of *Twsg1* (*Twsg1*$^{-/-}$) a known antagonist of BMP, exhibited significant osteopenia due to increased BMP signaling and had no effect on osteoblast function or RANKL or OPG expression. They concluded that the reduced bone mass was due to increased osteoclast formation and function by demonstrating that TWSG1 deficient osteoclasts were significantly larger than controls, and had increased levels of phosphorylated SMAD1, 5, 8 indicating excessive BMP signaling. Enhanced osteoclastogenesis was reversed by dose-dependent exposure to NOGGIN, a known BMP antagonist, further demonstrating that these observations stemmed from enhanced BMP signaling. (Rodriquez 2009, Jensen 2010).

Continued research in the Mansky/Gophalakrishnan lab has shown that conditional knockout of BMPRII in cells of the myeloid lineage including osteoclasts (*BmprII*$^{fl/fl}$*;LysM-Cre)* results in an osteopetrotic phenotype when compared to wild-type mice due to decreased osteoclast activity. Bone marrow macrophages (BMMs) isolated from knockout mice were significantly inhibited in

ability to differentiate into mature osteoclasts in the presence of M-CSF and RANKL and reduced expression of several genes critical for osteoclast differentiation and fusion were observed. They also investigated the temporal relationship between the canonical and noncanonical BMP signaling pathway and found that BMPs transduce their signals through the noncanonical pathway in early, pre-fusion, stages of maturation, and switch to the canonical pathway, but it is still unclear how BMP signaling is transduced exclusively through either pathway (Broege 2013).

Other mouse models expressing a conditional deletion of BMPR1A in mature osteoclasts (*Bmpr1a*$^{fl/fl}$*;Ctsk-Cre*) demonstrated increased bone resorption suggesting activation of BMPR1A negatively regulates osteoclast activity (Okamoto 2011). Additionally, mice with a deletion of *Bmpr1a* in osteoclasts measured increase bone formation suggesting that BMPR1A signaling in osteoclasts regulates osteoclast and osteoblast coupling (Okamoto 2011). Recently, *Bmpr1a* was conditionally deleted from osteoclasts with *LysM-Cre* which would decrease BMPR1A expression in all myeloid cells. The osteoclasts from these mice were decreased in their ability to form multinuclear TRAP positive cells with decreased expression of SMAD1, 5, 8. These findings are similar to BMPRII conditional knockouts and confirm the significance of BMP signaling in osteoclast differentiation.

More recently in unpublished studies the Mansky/Gopalakrishnan lab demonstrate that osteoclasts from mice conditionally deleted for BMP signaling

proteins SMAD1 and SMAD5 are osteopenic with increased osteoclast activity (KMansky, personal communication). Surprisingly these mice also had increased cortical thickness and osteoblast activity (KMansky, personal communication). To account for the change in osteoblast activity in mice that were null for BMP signaling in osteoclasts, gene expression of known coupling factors between osteoclasts and osteoblasts was measured. It was determined that SMAD1/5 conditional knockout mice had increased expression of *Wnt1a*, *Sphk1* and *Gja1* (KMansky, personal communication). This data suggests that BMP signaling may negatively regulate osteoclast and osteoblast coupling and supports the hypothesis that through direct and indirect osteoclast induction, BMP2 plays an important role in bone remodeling by acting as a mediator of osteoblast-osteoclast interaction. (Kanatani 1995).

A scientifically complete explanation of the role of BMPs on bone formation and resorption has yet to be proposed or supported (Giannoudis 2007). To better understand the role of BMP2 on osteoclast activation, we evaluated the expression pattern of components of the BMP signaling pathway in *Bmp2;LysM-Cre* cKO osteoclasts and determine the relative timing of BMP2 expression during osteoclast differentiation.

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

1. BMP2 expression in osteoclasts will peak after day 2 of osteoclast differentiation.

2. Expression of components of the BMP signaling pathway will be decreased in *Bmp2;LysM-Cre* cKO osteoclasts.

Specific Aims

1. Measure quantitative and temporal expression of BMP2 by osteoclasts. Quantitative real-time PCR (RT-PCR) has previously demonstrated that *Bmp2* mRNA is expressed by osteoclasts and expression peaks at day 2 in culture (Jensen 2010). An ELISA was used to measure the quantity of BMP2 protein expressed in cell lysates and culture supernatant by wild type osteoclasts during differentiation.

2. Determine if BMP2 regulates expression of BMP signaling components in osteoclasts by performing quantitative RT-PCR to measure expression of BMP receptors (*Bmpr1a*, *Bmpr1b*, and *BmprII*), and BMP ligands (*Bmp4* and *Bmp6*), and osteoclast differentiation receptors (*Rank and c-Fms*) which have been previously shown to be expressed by osteoclasts (Jensen 2010).

MATERIALS AND METHODS

Study Design

This is an experimental study in which the investigator controls the independent variables. The independent variables for this study include the time of culture in M-CSF and RANKL and the populations of wild type and *Bmp2;LysM-Cre cKO* BMMs. Outcome variables include RNA expression levels of BMP receptors and

BMP ligands and protein expression of BMP2.

Cell culture

BMMs were harvested from the femurs and tibiae of wild type and *Bmp2;LysM-Cre* cKO mice. BMMs were cultured in M-CSF alone as an undifferentiated control, or M-CSF with RANKL for 24, 48, 72 and 96 hours.

ELISA

Wild type BMMs were cultured in M-CSF alone (Day 0) or 24, 48, 72 and 96 hours (Day 1-4) in M-CSF and RANKL. Cell lysates and culture supernatant were separated and an ELISA was performed following the manufacturer's protocol for Quantikine ELISA BMP2 Immunoassay (R&DSystems). Cell lysates and culture supernatant of *Bmp2;LysM-Cre cKO* were used as a control to demonstrate that the knockouts do not express detectable BMP2. Optical density was read at 450 nm.

Quantitative real-time PCR

Because BMP2 expression peaked at 48 hours after RANKL stimulation in our ELISA experiments, RNA from WT and *Bmp2;LysM-Cre* cKO BMM cultures were harvested at Day 2 using Trizol reagent (Ambion, Life Technologies) and quantified using UV spectroscopy. cDNA was prepared using 1 μg of RNA and the iScript cDNA synthesis kit (Biorad) per manufacturer's protocol. Quantitative

RT-PCR was performed using the MyiQ Single Color Real-Time PCR Detection System (Biorad) with primers to detect *Bmpr1a* (For-GCTCATCGAGACCTGAAGAG and Rev-GTCTGGAGGCTGGATTATGG), *Bmpr1b* (For-AGCACAGATGGGTACTGCTTC and Rev-TCTAGTCCTAGACATCCAGAGGTG), *BmprII* (For-TGGCAGTGAGGTCACTCAAG and Rev-TTGCGTTCATTCTGCATAGC), *Bmp4* (For- CCTGGTAACCGAATGCTGAT and Rev-AGCCGGTAAAGATCCCTCAT) , *Bmp6* (For-CACAGTCCTCTTCTTCGGGC and Rev-CTTTTGCATCTCCCGCTTCT), *c-Fms* (For- TGCTAAAGTCCACGGCTCAT and Rev- TCGGAGAAAGTTGAGATGGTGT), and *Rank* (For-CCAGGACAGGGCTGATGAGAA and Rev-TGGCTGACATACACCACGATGA). Experimental genes were normalized to *Hprt* (For-GAGGAGTCCTGTTGATGTTGCCAG and Rev-GGCTGGCCTATAGGCTCATAGTGC).

Statistical Analysis

All experiments were completed in duplicate and performed three times. Data was evaluated as the mean ± standard deviation. Student unpaired T-test or two-way ANOVA analysis followed by a Tukeys multiple comparison test was used to compare data. $P < 0.05$ indicated significance. Statistical analysis was performed using Prism 7 software for Mac OSX.

RESULTS

BMP2 protein expression is time dependent

Research by the Mansky lab as well as others had demonstrated that BMP2 can directly enhance RANKL mediated osteoclast differentiation (Jensen 2010 and Rodriguez 2009). However, it had not been determined if osteoclasts express BMP2, how much or at what time point during differentiation. Previous research had shown that wild type osteoclasts express *Bmp2* RNA and that its expression peaks at day 2 in culture with M-CSF and RANKL (Jensen 2010). To demonstrate that osteoclasts express and secrete BMP2 protein, an ELISA was performed to measure BMP2 expression from osteoclast lysates and cell culture supernatant. Results demonstrate that initial and peak BMP2 protein expression in osteoclasts occurs after 2 days of RANKL stimulation in cell lysates, decreasing at days 3 and 4. Secreted BMP2 protein was measured in the culture supernatant on day 3 after RANKL stimulation. No secreted form of BMP2 protein was detected on any other day of osteoclast differentiation. Additionally, cellular or secreted BMP2 from osteoclasts cultured from *Bmp2;LysM-Cre* mice was not detected.

	Mean BMP2 concentration (pg/mL)	Standard Deviation	Minimum (pg/mL)	Maximum (pg/mL)
WT BMM Lysate				
D0	**0**		**0**	**0**
D1	**0**		**0**	**0**
D2	**366.25**	**321.73**	**138.75**	**593.75**
D3	**51.25**	**261.63**	**0**	**236.25**
D4	**151.875**	**175.89**	**27.5**	**276.25**
WT BMM Supernatant				
D0	**0**		**0**	**0**
D1	**0**		**0**	**0**
D2	**0**		**0**	**0**
D3	**58.75**	**63.63**	**13.75**	**103.75**
D4	**0**		**0**	**0**

Table 1: ELISA results of BMP2 expression in lysate and supernatant of osteoclasts incubated with M-CSF only (D0) or with M-CSF and RANKL for 1 to 4 days (D1-D4).

c-Fms and Rank expression in BMP2cKO osteoclasts

Unpublished studies from the Mansky lab have demonstrated that osteoclasts from *Bmp2;LysM-Cre* are more numerous but smaller compared to their wild type littermates (Fig. 4).

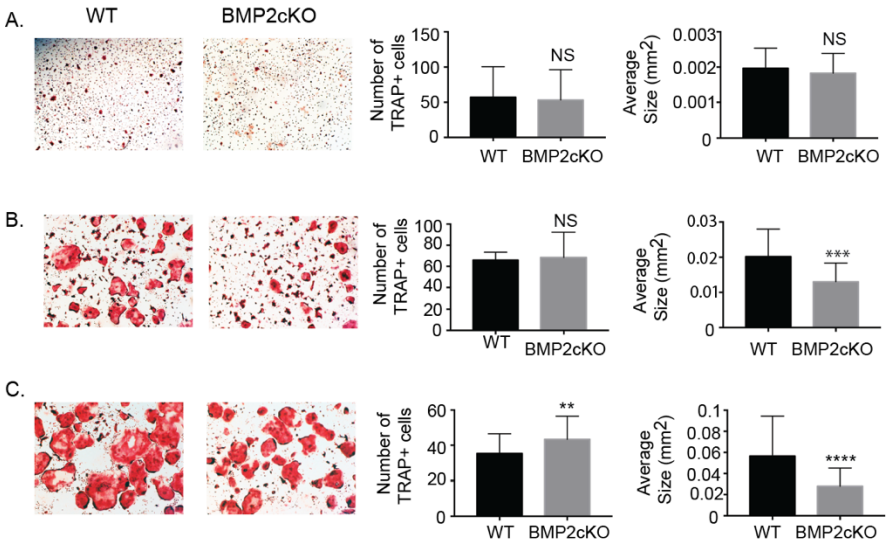

Figure 4. Osteoclasts from BMP2 null osteoclasts are smaller than WT osteoclasts. BMMs were flushed from WT or BMP2 cKO mice. BMMs were stimulated with M-CSF and RANKL for indicated days. (A) TRAP stained images of BMMs differentiated with M-CSF and RANKL for 2 days (B) 3 days or (C) 4 days. Samples were compared using T-test ** $p<0.01$ vs. WT,*** $p<0.001$ vs. WT, **** $p<0.0001$ vs. WT

To understand the mechanism(s) by which BMP2 expressed by osteoclasts regulate osteoclast differentiation and activity, RT-qPCR was performed to measure expression levels of *c-Fms* and *Rank* expression receptor, for M-CSF and RANKL respectively. As shown in Table 1, BMP2 protein expression peaked on day 2 of osteoclast differentiation; therefore, all qRT-PCR experiments were performed on osteoclasts from WT and *BMP2;LysM-Cre* cKO mice cultured with M-CSF and RANKL for 48 hours. As shown in Figure 5, there was an increase in *c-Fms* and *Rank* expression in osteoclasts from *Bmp2;LysM-Cre* mice; however, the increase measured was not significant.

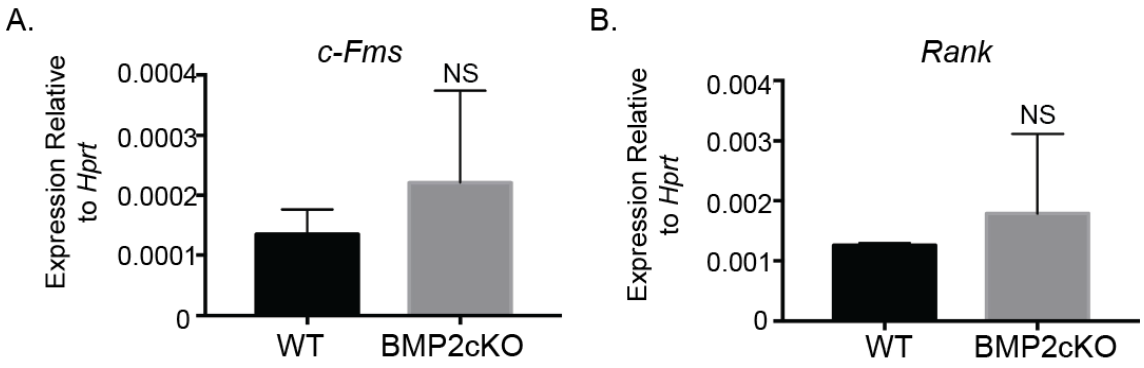

Figure 5: Expression of *c-Fms* and *Rank* in WT and *Bmp2;LysM-Cre* cKO osteoclasts. qRT-PCR comparing expression of osteoclast genes from WT and *Bmp2;LysM-Cre* mice. (A) *c-Fms*, and (B) *Rank*. Data shown are the mean+SD of three independent experiments in which gene expression was measured from three wells of each genotype, with each PCR reaction performed in duplicate. Expression of each gene is graphed relative to *Hprt*. Samples were compared using unpaired student T-test.

Expression of BMP receptors in BMP2 null osteoclasts

The Mansky lab had previously demonstrated that osteoclasts express BMP receptors (Jensen 2010) and that expression of *BmprII* is necessary for osteoclast differentiation and activity as mice that are deficient for *BmprII* expression in myeloid cells are osteopetrotic (Broege 2013). Additionally, Li et al. conditionally deleted *Bmpr1a* from osteoclasts with *LysM-Cre* which would decrease BMPR1a expression in all myeloid cells. The osteoclasts from these mice were decreased in their ability to form multinuclear TRAP positive cells with decreased expression of SMAD1, 5, 8 (Li et al. 2017). To determine if expression of BMP2 by osteoclasts regulates expression of BMP receptors, expression of BMP receptors *BmprII*, *Bmpr1a* and *Bmpr1b* was measured by qRT-PCR. As shown in Figure 6, there was a trend of decreased expression of

BMP receptors *Bmpr1a*, *Bmpr1b*, and *BmprII*. This data suggests BMP2 expression may at least in part regulate expression of BMP receptors.

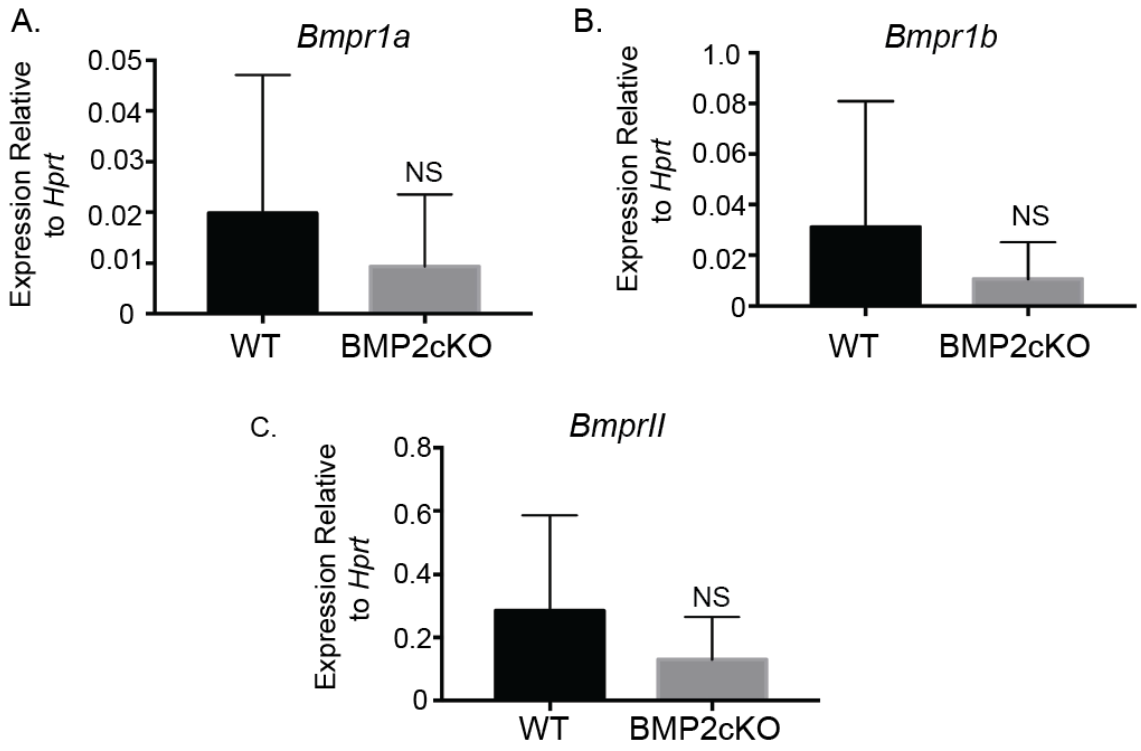

Figure 6: Expression of BMP receptors by *Bmp2;LysM-Cre* cKO and WT osteoclasts. qRT-PCR comparing expression of osteoclast genes from WT and *Bmp2;LysM-Cre* mice. (A) *Bmpr1a* (B) *Bmpr1b and (C) BmprII*. Data shown are the mean+SD of three independent experiments in which gene expression was measured from three wells of each genotype, with each PCR reaction performed in duplicate. Expression of each gene is graphed relative to *Hprt*. Samples were compared using unpaired student T-test.

BMP Ligand Expression in BMP2cKO osteoclasts

Osteoclasts have been shown to express BMP ligands (Garimella 2008), so lastly, changes in expression of BMP ligands BMP4 and BMP6 in osteoclasts in the absence of BMP2 expression were evaluated. As shown in Figure 7, *Bmp4* had no change in expression, whereas *Bmp6* trended toward decreased

expression in the *BMP2;LysM-Cre* cKO cultures but as seen with the other genes measured in this study, the results were not significant.

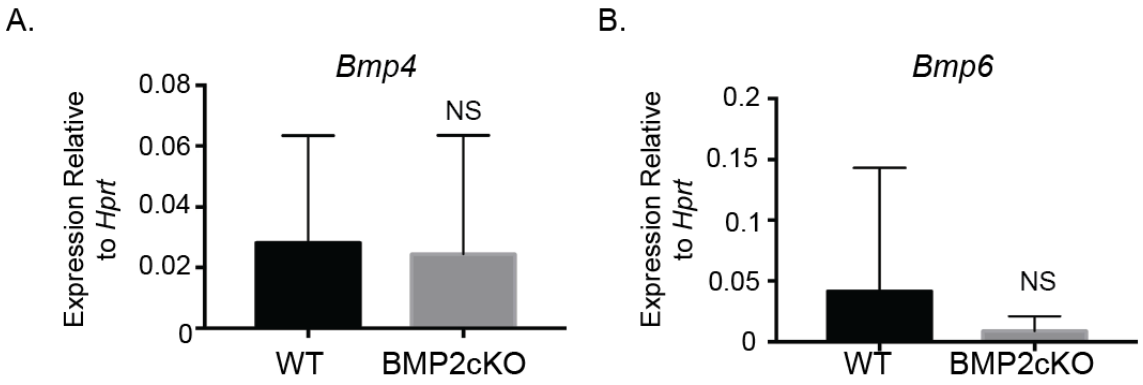

Figure 7: Expression of *Bmp4* and *6* in *BMP2;LysM-Cre* cKO and WT osteoclasts. qRT-PCR comparing expression of osteoclast genes from WT and *Bmp2;LysM-Cre* mice. (A) *Bmp4* and (B) *Bmp6.* Data shown are the mean+SD of three independent experiments in which gene expression was measured from three wells of each genotype, with each PCR reaction performed in duplicate. Expression of each gene is graphed relative to *Hprt*. Samples were compared using unpaired student T-test.

DISCUSSION

Previously it has been shown that BMPs can enhance osteoclast differentiation. The mechanism(s) by which BMPs regulate osteoclast differentiation are not completely understood. Previous research in the Mansky/Gopalakrishnan lab as well as others has demonstrated that BMP signaling is necessary for osteoclast differentiation (Rodriguez 2009, Broege 2013, Okamoto 2011).

My results demonstrate that osteoclasts maximally express BMP2 two days after addition of RANKL to *in vitro* cultures. Also BMP2 is detected in media of osteoclast cultures around day 3 after RANKL addition. The detection of BMP2

at day 3 of osteoclast differentiation correlates with the activation of SMAD signaling in osteoclasts (Jensen 2010). My BMP2 expression data and previous data from the Mansky/Gopalakrishnan lab suggests the BMP noncanonical signaling pathway may be activated by another BMP ligand other than BMP2 since no BMP2 protein was detected in the media until day 3 of osteoclast differentiation (Table 1).

To understand more fully the role of BMP signaling during osteoclast differentiation, the Mansky/Gopalakrishnan lab created mice that were null for BMP2 expression in cells of the myeloid lineage including osteoclasts. In unpublished data they demonstrate that osteoclasts from *Bmp2;LysM-Cre* mice are smaller but more numerous compared to wild type littermates. This, along with the BMP2 expression data presented in the results, suggests that BMP2 is maximally expressed at the time of osteoclast fusion. While BMP2 does not appear to be necessary for early stages of osteoclast differentiation it does appear to be necessary for enhancing osteoclast size. Lastly loss of BMP2 expression may explain the increase, though not significant, in expression of *Rank and c-Fms* observed in my results. As osteoclasts fail to fully mature (ie increase in size) in the absence of BMP2, osteoclasts may upregulate *Rank* and *c-Fms* to try and compensate.

My results showed no change in *Bmp4* mRNA expression in the absence of BMP2, which agrees with previous literature (Tsuji 2006). In a previous study looking at limb formation in conditional deletion of BMP2, BMP4, or BMP7 in

mice, all deletions resulted in normal skeletogenesis. Postnatally, BMP2 had frequent fractures that failed to heal. However, when BMP2 and BMP4 were concomitantly knocked out, mice had extremely malformed limbs and a severe impairment of osteogenesis, which was not observed in BMP-2/-7 mice (Wu 2016). It is possible that BMP2 and BMP4 have distinct signaling pathways to avoid severe malformation and lethality potential. In the future it will need to be determined if BMP4 has a role in osteoclast differentiation.

Though not significant, my results show a trend of downregulation of *Bmp6* in the absence of BMP2. It has been suggested that BMP6 is a coupling factor between osteoclasts and osteoblasts. Osteoclasts recruit osteoprogenitors to sites of bone resorption via secretion of S1P and BMP6, and stimulate differentiation of osteoblasts via Wnt10b and BMP6 secretion (Pederson 2008). *Bmp2;LysM-Cre* mice have reduced bone formation as measured by P1NP activity (KMansky, personal communication), which may be a result of downregulation of BMP6. This would suggest that BMP6 regulation is dependent upon BMP2 expression.

As previously demonstrated in the literature, BMP receptors play an important role in osteoclastogenesis and osteoclast function (Li et al 2017, Broege 2013). My results though not significant suggest that BMP2 plays a role in the regulation of expression of *Bmpr1a*, *Bmpr1b*, and *BmprII*. It could be that other factors including other BMP ligands or RANKL regulate BMP receptor expression along with BMP2. This hypothesis will be addressed in future

experiments.

The more we understand BMP2's role in osteoclastogenesis, the more we can understand its possible therapeutic targets and clinic usefulness. In orthodontics, tooth movement occurs by bone resorption in front of the tooth, in the direction of tooth movement, and bone apposition occurs behind the tooth, when the desired tooth movement is achieved. Application of BMPs or BMP inhibitors may allow us in the future to control the balance of bone remodeling at a cellular and local level, ultimately affecting tooth movement, post-surgical healing, the need for bone grafting, and suture remodeling.

CONCLUSION

1. Osteoclasts maximally express BMP2 two days after addition of RANKL to *in vitro* cultures.
2. BMP2 is detected in media of osteoclast cultures at day 3 after RANKL addition, which correlates with the activation of SMAD1, 5, 8 signaling in osteoclasts.
3. Though not significant, osteoclasts from *Bmp2;LysM-Cre* mice demonstrate a trend of increased *Rank* and *c-Fms* expression. This may be a compensatory mechanism due to failure of complete maturation of osteoclasts as a result of loss of BMP2.
4. *Bmp4* mRNA expression in osteoclasts from *Bmp2;LysM-Cre* mice does not change in the absence of BMP2 which is consistent with previous literature.

Future research will need to look at whether BMP4 has a role in osteoclast differentiation.

5. *Bmp6* mRNA expression in osteoclasts from *Bmp2;LysM-Cre* mice had a trend of down regulation, though not significant. BMP6 regulation may be dependent upon BMP2 expression but will need to be investigated further with future experiments.

6. *Bmpr1a*, *Bmpr1b*, and *BmprII* mRNA expression in osteoclasts from *Bmp2;LysM-Cre* mice had a trend of down regulation, though not significant. BMP2 may play a role in the regulation of expression of BMPR1A, BMPR1A, and BMPRII but will need to be investigated in future experiments.

REFERENCES

Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and Activation. *Nature.* 2003;423(May):337-342.

Broege A, Pham L, Jensen ED, et al. Bone Morphogenetic Proteins Signal Via SMAD and Mitogen-activated Protein (MAP) Kinase Pathways at Distinct Times during Osteoclastogenesis. *J Biol Chem*. 2013;288(52):37230-37240.

Canalis E, Economides AN, Gazzerro E. Bone morphogenetic proteins, their antagonists, and the skeleton. *Endocr Rev.* 2003:24:218–235.

Cao X, Chen D. The BMP signaling and in vivo bone formation. *Gene.* 2005: 357:1–8.

Carreira AC, Lojudice FH, Halcsik E, Navarro RD, Sogayar MC, Granjeiro JM. Bone morphogenetic proteins: facts, challenges, and future perspectives. *J Dent Res*. 2014;93(4):335-345.

Garimella R, Tague SE, Zhang J, et al. Expression and Synthesis of Bone Morphogenetic Proteins by Osteoclasts : A Possible Path to Anabolic Bone Remodeling. *The Journal of Histochemistry & Cytochemistry.* 2008;56(6):569-577.

Giannoudis P V., Kanakaris NK, Einhorn TA. Interaction of bone morphogenetic proteins with cells of the osteoclast lineage: Review of the existing evidence. *Osteoporos Int*. 2007;18(12):1565-1581.

Itoh K, Udagawa N, Katagiri T, Iemura S, Ueno N, Yasuda H, Higashio K, Quinn JM, Gillespie MT, Martin TJ, Suda T, Takahashi N. Bone morphogenetic protein 2 stimulates osteoclast differentiation and survival supported by receptor activator of nuclear factor-kappaB ligand. *Endocrinology.* 2001:142(8): 3656–3662.

James AW, LaChaud G, Shen J, et al. A Review of the Clinical Side Effects of Bone Morphogenetic Protein-2. *Tissue Eng Part B Rev*. 2016;22(4): 284-297.

Jensen ED, Pham L, Billington CJ, et al. Bone morphogenic protein 2 directly enhances differentiation of murine osteoclast precursors. *J Cell Biochem*. 2010;109(4):672-682.

Kanatani M, Sugimoto T, Kaji H et al. Stimulatory effect of bone morphogenetic protein-2 on osteoclast-like cell formation and bone-resorbing activity. *J Bone Miner Res.* 1995:10:1681–1690

Kaneko H, Arakawa T, Mano H, Kaneda T, Ogasawara A, Nakagawa M, Toyama Y, Yabe Y, Kumegawa M, Hakeda Y. Direct stimulation of osteoclastic bone resorption by bone morphogenetic protein (BMP)-2 and expression of BMP receptors in mature osteoclasts. *Bone*. 2000:27:479–486.

Li et al. Pharmacologic Calcitriol Inhibits Osteoclast Lineage Commitment via the BMP-Smad1 and IkappaB-NF-kappaB Pathways. J Bone Miner Res. 2017:32:1406

Neovius E, Lemberger M, Docherty Skogh A, Hilborn J, Engstrand T. Alveolar bone healing accompanied by severe swelling in cleft children treated with bone morphogenetic protein-2 delivered by hydrogel. *J Plast Reconstr Aesthetic Surg*. 2013;66(1):37-42.

Okamoto M, Murai J, Yoshikawa H, Tsumaki N. Bone Morphogenetic Proteins in Bone Stimulate Osteoclasts and Osteoblasts During Bone Development. *J Bone Miner Res*. 2006;21(7):1022-1033.

Pederson L, Ruan M, Westendorf JJ, Khosla S, Oursler MJ. Regulation of bone formation by osteoclasts involves Wnt / BMP signaling and the chemokine sphingosine-1-phosphate. *PNAS*. 2008;105(52):20764-20769.

Raggatt LJ, Partridge NC. Cellular and Molecular Mechanisms of Bone Remodeling. *J Biol Chem.* 2010:285(33):25103-25108.

Rodriguez JS, Mansky KC, Jensen ED, Carlson AE, Schwarz T, Pham L, Mackenzie B, Prasad H, Rohrer MD, Petryk A, Gopalakrishnan R. Enhanced osteoclastogenesis causes osteopenia in Twisted gastrulation-deficient mice through increased BMP signaling. *J Bone Miner Res.* 2009:24:1917–1926.

Sánchez-duffhues G, Hiepen C, Knaus P. Bone morphogenetic protein signaling in bone homeostasis. *Bone*. 2018;80(2015):43-59.

Shi C, Iura A, Terajima M, Liu F, Lyons K, Pan H. Deletion of BMP receptor type IB decreased bone mass in association with compromised osteoblastic

differentiation of bone marrow mesenchymal progenitors. *Nat Sci Reports*. 2016;(March):1-13.

Tsuji K, Bandyopadhyay A, Harfe BD, et al. BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. *Nat Genet*. 2006;38(12):1424-1429.

Vaananen HK, Laitala-Leinonen T. Osteoclast lineage and function. *Arch Biochem Biophys*. 2008:473:132-138.

Wu M, Chen G, Li Y-P. TGF-β and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. *Bone Research*. 2016;4:1-21.