

The Regulation of Osteoclastogenesis by Bone Morphogenetic Proteins,
Twisted Gastrulation, and Histone Deacetylases

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Dedication

Dr. Huan Tran was brilliant scientist and my beloved uncle that devoted his life to discovering the unknown. When I was seven, I yearned to have an aquarium full of beautiful fish. My uncle bombarded me with library books on fish maintenance and taught me the importance of “doing your research.” Our efforts were rewarded when we successfully set up an aquatic environment full of happy fish. At the age of ten, he showed me the stars through a telescope that made it seem like they were only an arms length away. When I turned fifteen, he bought me my first ACT, GRE, and DAT preparation books, knowing that one day I would be where I am today. His passion for exploring and learning has been a thread of inspiration for me throughout my life. In 2009, Dr. Tran passed away, at the age of thirty-five, while chasing his dream of understanding how our planet Earth was formed. Throughout my graduate studies I have strived to follow in his footsteps and to this end, this dissertation is dedicated to him.

Abstract

Bone morphogenetic proteins (BMPs) are well-studied regulators of osteoblasts, and are used in a number of craniofacial and orthopedic procedures to promote localized bone formation. Studies of skeletal tissue has shed light on BMP's role as an inducer of chondrocytic and osteoblastic differentiation and function. BMPs have been used successfully in studies to: treat critical sized defects in both long and craniofacial bones; enhance fracture healing; treat non-unions and lumbar spinal fusion; and regenerate alveolar bone and portions of teeth such as dentin and pulp. However, it has been difficult to determine the optimal concentrations, appropriate temporal release, and regulation of BMPs, as both a deficiency and an excess of BMPs may lead to pathologic states. Furthermore, the cellular and molecular origin of this BMP-associated stimulation of bone resorption remains poorly understood.

The data presented in this thesis will help us better understand the modulation of osteoclastogenesis and bone resorption by the regulatory proteins BMP-2, Twisted gastrulation, and Histone deacetylase 3 and 7. The knowledge gained by studying these regulators in osteoclasts should provide important new insight into the use of BMPs in bone generation procedures, its role in pathogenesis of bone resorptive disorders, and provide a conceptual framework

for the development of successful therapies and bone regenerative strategies for diseases associated with increased bone loss and defective bone formation.

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Chapter 1

Introduction, Statement of Purpose, and Specific Aims

1.1 Introduction

Bone tissue development and homeostasis is an extremely dynamic process necessary throughout life. Bone healing requires synthesis of new bone by osteoblasts and the removal of old or damaged bone matrix by osteoclasts. Mammalian bone is under continuous remodeling due to altered serum calcium levels, changes in mechanical loading, and response to a wide range of paracrine and endocrine factors. Currently, bone morphogenetic proteins (BMPs) are used to promote healing of open fractures, bone grafts, and non-union fractures. BMPs are multi-functional growth factors involved in numerous molecular cascades and signaling pathways. Although BMPs have been widely studied as stimulators of osteoblastic bone formation, their effect on osteoclasts and their role in the pathogenesis of osteoporosis and other osteolytic conditions are not known. Osteoclasts are members of the monocyte/macrophage lineage and are formed by multiple cellular fusions from their mononuclear precursors (Vaananen and Laitala-Leinonen 2008). An important role of osteoclasts has become evident in various skeletal diseases, and specific inhibition of osteoclast function is unfolding to become a strategy to treat osteoporosis, Paget's disease, periodontitis, and many other metabolic bone diseases (Rodan and Martin 2000). The studies presented here elucidate the mechanism of bone remodeling

facilitated by: 1) BMPs; 2) a BMP antagonist called Twisted gastrulation (TWSG1); and 3) transcription regulators called histone deacetylases (HDACs).

1.1.1 Bone Remodeling

Bone is constantly being remodeled. This homeostasis is a result of the coupled activities of the osteoblasts and osteoclasts maintaining the balance between bone formation and resorption. Throughout the skeleton, bone remodeling occurs in units, called basic multicellular units (BMU) or bone remodeling units (BRU). The remodeling is classically thought as a multi-step process divided up into four distinct phases: activation/initiation, resorption, reversal, and formation (reviewed by Parfitt 1994).

Figure 1.1 illustrates the phases in a typical bone remodeling event. In the first step, the term activation is used to describe the initial event in which the surface of the resting bone is converted into a remodeling surface via recruitment of osteoclast precursors to the bone surface, differentiation, and fusion into mature osteoclasts. In the resorption phase, the osteoclasts remove mineralized and organic components of the bone matrix, which can take up to three weeks (Baron 1989). In the reversal phase, coupled bone resorption and bone formation occurs. The reversal phase can last for two weeks wherein osteoclast activity halts and osteoblast precursors are recruited to resorption lacuna. Once pre-

osteoblasts arrive and differentiate to active bone making cells the bone remodeling moves into the final formation phase. There is a deposition of osteoid and organic matrix primarily composed of type 1 collagen, which gets mineralized with newly deposited matrix. In normal homeostasis, the bone that was resorbed is equally replaced with newly generated matrix and the net gain or loss is zero.

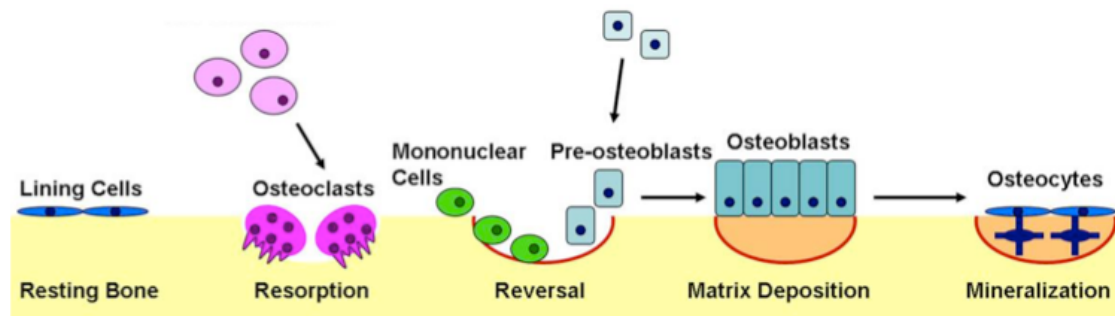


Figure 1.1: Bone Remodeling. Cartoon illustrating the phases during bone remodeling. (modified from Kapinas and Delany 2011).

1.1.2 Osteoclastogenesis

Maintenance and repair of bone requires removal of old bone by osteoclasts and synthesis of new bone matrix by osteoblasts in response to serum calcium levels, mechanical loading, and paracrine and endocrine factors. Osteoclasts are multinucleated cells derived from hematopoietic progenitors of the monocyte-macrophage lineage that resorb bone (Teitelbaum 2000). Mature

osteoclasts are actively recruited to sites of bone resorption and osteoclast differentiation is an essential part of bone remodeling which serves in the development and homeostasis of the skeleton. Several factors such as hormones, cytokines, and growth factors are involved in osteoclast differentiation and maturation (Zaidi et al. 2003; Blair and Zaidi 2006; Giannoudis et al. 2007).

Macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B (NF- κ B) (RANK) ligand (RANKL) are essential to promote osteoclastogenesis (Teitelbaum 2000). Additional osteoclastogenesis-promoting factors include Tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), and granulocyte macrophage colony-stimulating factor (GM-CSF) (Feng 2005). It is thought that the RANK interacts with TNF-associated factor (TRAF) 1, 2, 5, and 6 in the cytoplasm, which regulates the Jun N-terminal kinases (JNK) and NF- κ B pathways (Tanaka et al. 2005). This in turn regulates a variety of pathways including the member of the Ets transcription factor family, PU.1, nuclear factor of activated T-cells (Nfatc1), activator protein 1 (AP-1), and microphthalmia-associated transcription factor (MITF) to regulated osteoclast formation and function (Takayanagi 2007). Interestingly, CD44, CD47, CD9, disintegrin and metalloproteinase domain-containing protein 12 (ADAM12), Monocyte chemoattractant protein-1 (MCP-1), ATPase vacuolar, subunit 6 (ATPv6), and dendritic cell-specific seven transmembrane protein (DC-STAMP),

have been shown to aid in the fusion of monocytic-macrophage cell lines (Yagi et al. 2005; Yagi et al. 2006; Yagi et al. 2007). Osteoclast differentiation is clearly a complex cellular process.

Of particular interest, it is known that the osteoblasts, express RANKL, which binds to RANK on the osteoclast thereby promoting osteoclast differentiation. Osteoblast can act to regulate osteoclast differentiation by secreting osteoprotegerin (OPG), which binds to RANKL and prevents it from binding to RANK, thus hindering osteoclastogenesis (Simonet et al. 1997; Lacey et al. 1998). Osteoblasts are targets of most osteoclastogenic agents, such as parathyroid hormone (PTH), that exert their effect by enhancing RANKL expression and thus increasing RANKL quantity relative to OPG (Lee and Lorenzo 1999; Itonaga et al. 2004). A summary of the communication between osteoblasts necessary for osteoclastogenesis is depicted in Figure 1.2.

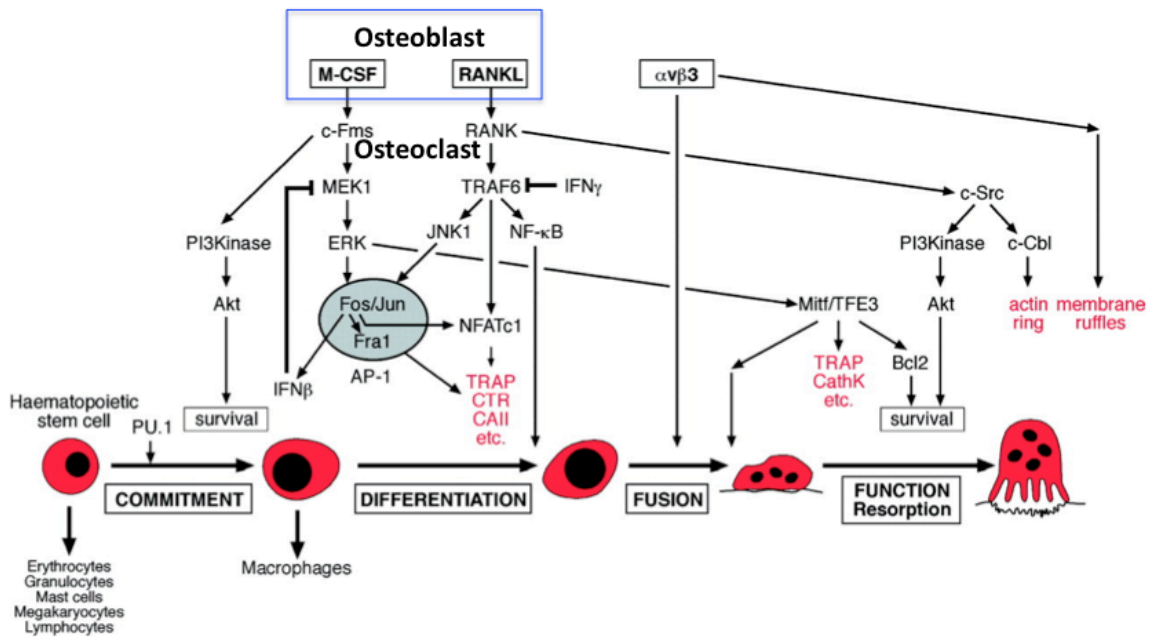


Figure 1.2: Overview of Osteoclast Differentiation and Activation. Shown here is a summary of many factors necessary for osteoclast differentiation, fusion, and functional resorption. Note signals coming from the osteoblast are key regulators for differentiation potential of the osteoclasts. Figure was modified from Wagner and Matsuo (Wagner and Matsuo 2003).

Fully differentiated, multinucleated osteoclasts are commonly characterized by both a “ruffled border” and production of a standard histochemical marker, Tartrate-resistant acid phosphatase (TRAP) enzyme. Once bound to the surface of bone, osteoclasts create an acidic environment capable of degrading the extracellular matrix environment to resorb bone (Hayman et al. 2000; Yuan et al. 2010).

1.1.3 Bone Morphogenic Proteins

Originally identified as factor(s) that induce the formation of bone and cartilages in rats, BMPs are now well established to exhibit a wide range of biological effects on various cell types (reviewed in Bleuming et al. 2007). BMPs are members of the transforming growth factor-beta (TGF β) superfamily of secreted morphogens, which binds to receptor complexes composed of Type I and Type II BMP receptors (BMPRI and BMPRII, respectively), and are mediated through Smad-dependent and Smad-independent pathways (Wozney et al. 1988).

BMPs can be further classified into several subgroups including BMP-2/4, BMP-5/6/7/8, growth and differentiation factor (GDF)-5/6/7, and BMP-9/10 (reviewed in Miyazono et al. 2010). Most of the proteins of these subgroups induce formation of bone or cartilages *in vivo*, with the exception of GDF-5/6/7, which produces more tendon-like tissue. In 2003, He and coworkers evaluated 14 types of human BMPs for their osteoinductive activity in C3H10T1/2 cells (Cheng et al. 2003). Their findings demonstrated that BMP-2, 6, and 9 plays an important role in inducing osteoblast differentiation of mesenchymal stem cells.

The TGF β family of ligands bind to two type of serine-threonine kinase receptors. In the case of BMPs, BMPRI and BMPRII are required for signal transduction. The canonical Smad-dependent pathway requires BMP ligands to

initiate signaling by binding to a portion of BMP receptors that is already complexed at the cell surface. These complexes, consisting of BMPRI and BMPRII, are called preformed complexes (PFCs). At present, PFCs are unique for the BMP receptors within the TGF β superfamily (Nohe et al. 2002). Once the BMPs are bound to the PFCs, signaling occurs through the phosphorylation of the Smad 1/5/8. Interestingly, BMPs can also activate other signaling pathways distinct from the Smad 1/5/8 pathway.

The oligomerization patterns of BMPRI and BMPRII prove to be flexible and dynamic (Nohe et al. 2002). A larger population of receptor complexes are formed after ligand binding to the high-affinity receptor BMPRI, which recruits the low-affinity receptor BMPRII, forming the active signaling complex. These complexes are called BMP-induced signaling complexes (BISCs). Intriguingly, these two distinct receptor oligomerization patterns induce different signaling pathways: PFCs signal via the canonical Smad 1/5/8 pathway and BISCs activate Smad-independent signaling via p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) (Iwasaki et al. 1999; Piek et al. 1999; Kimura et al. 2000).

Downstream of the PFC signaling, three functional classes of Smad proteins can be distinguished: receptor-regulated Smads (Smad 1/5/8 proteins), inhibitory Smads (Smad 6/7 proteins) and common mediator Smads (Smad 4

proteins) (Canalis et al. 2003). Once BMP binds to the PFCs, Smad complexes are activated, translocated into the nucleus and, in conjunction with other nuclear cofactors, help regulate the transcription of target genes (Shi and Massague 2003). This simplistic description of the intracellular signal transduction mechanism elicits a complex transcriptional and translational biological response, which among other things, regulates bone homeostasis.

Conversely, BMPs binding to BISCs activates Smad-independent pathways, such as p38 MAPK and JNK. Specifically, BMP-2 has been shown to stimulate Ras activity and downstream p38 (Canalis et al. 2003). This in turn up regulates activating transcription factor-2 (ATF-2), which further regulates various genes.

The signaling activity of BMPs is modulated by secreted molecules including Chordin, Noggin, and Twisted gastrulation (TWSG1). BMPs have been shown to bind to these molecules in the extracellular space, thereby limiting their ability to activate signaling (Canalis et al. 2003). Chordin is strongly secreted by osteoblasts and the protein contains four cysteine-rich domains that mediate the binding of Chordin to BMPs (BMP-2, 4 and 7) (Piccolo et al. 1997; Canalis et al. 2003). In adult organisms Noggin is strongly expressed in chondrocytes and osteoblasts and shows high affinity for BMP-2 and 4 (Zimmerman et al. 1996; Canalis et al. 2003). In 2002, Choe and coworkers solved the crystal structure of

Noggin binding to BMP-7 (Groppe et al. 2002). The data supports a mechanism by which Noggin inhibits BMP signaling by blocking the binding epitopes for both BMPRI and BMPRII. TWSG1 will be further discussed in sections below.

1.1.4 BMP and Osteoclast Differentiation

As previously mentioned, BMPs are a subgroup of the transforming growth factor-beta (TGF- β) superfamily of proteins (Wozney et al. 1988). Both osteoblast and osteoclast lineages express BMPs and exert their biological activities by signaling through BMPRI and BMPRII (Itoh et al. 2001; Xiao et al. 2002; Nohe et al. 2004). Binding of BMPs to BMPRII phosphorylates and activates various intracellular substrates such as receptor-regulated SMAD 1/5/8 (Lieberman et al. 2002). There have been a few studies examining the mechanisms of BMP interactions on the osteoclast lineage.

Chihara and coworkers demonstrated that BMP2 plays an important role in bone remodeling by acting as a mediator of the osteoblast-osteoclast interaction (Kanatani et al. 1995). In addition, studies demonstrated that BMP2 increased OPG expression in osteoblasts, suggesting that BMP is a negative regulator of osteoclastogenesis (Hofbauer et al. 1998). However, Behringer and coworkers showed evidence for the role of BMP in enhancing osteoclast function (Mishina et al. 2004). In these experiments, 10-month old mice harboring an

osteoblast-specific BMP receptor type 1A gene ablation showed a decrease in osteoclastic bone resorption, which suggests that loss of BMP signaling in osteoblasts leads to impairment of osteoclast function. Taken together, these studies suggest a dual role for BMPs as both an agonist and antagonist of bone formation. Interestingly, BMP signaling activity has been shown in various contexts to be regulated by extracellular proteins like TWSG1 (Larrain et al. 2001; Gaggero et al. 2005).

1.1.5 TWSG1 and Regulation of BMP Function

Initially identified in *Drosophila*, *Twsg1* encodes a 23.5 kDa secreted glycoprotein with two evolutionarily conserved cysteine-rich domains as well as a BMP binding domain (Mason et al. 1994). *Twsg1* is conserved in humans, mice, *Xenopus*, zebrafish, and chicken suggesting that it has an important preserved function. In addition to regulating craniofacial, skeletal, and salivary gland development (Petryk et al. 2004; Zakin and De Robertis 2004; Melnick et al. 2006), in 2004 Little and Mullins demonstrated that TWSG1 also promotes BMP signaling in dorsal/ventral embryonic axis patterning in the zebrafish early embryo (Little and Mullins 2004).

Mutations in TWSG1 result in a wide range of craniofacial defects and skeletal phenotypes in mice including micrognathia, agnathia, cyclopia, and other

midline facial defects (Petryk et al. 2004; Zakin and De Robertis 2004; MacKenzie et al. 2009; Billington et al. 2011). Figure 1.3 illustrates the range in craniofacial defects seen in *Twsg1* null (*Twsg1*^{-/-}) mutant mice. In 2011, Petryk and coworkers found that this variability may be contributed to differential expression of developmental genes (Billington et al. 2011). The study was able to partly rescue craniofacial defects by reducing the levels of tumor suppressor p53. The authors concluded that differential expression of transcripts causes the distinct patterns of craniofacial defects and by targeting p53, craniofacial defects may be prevented.

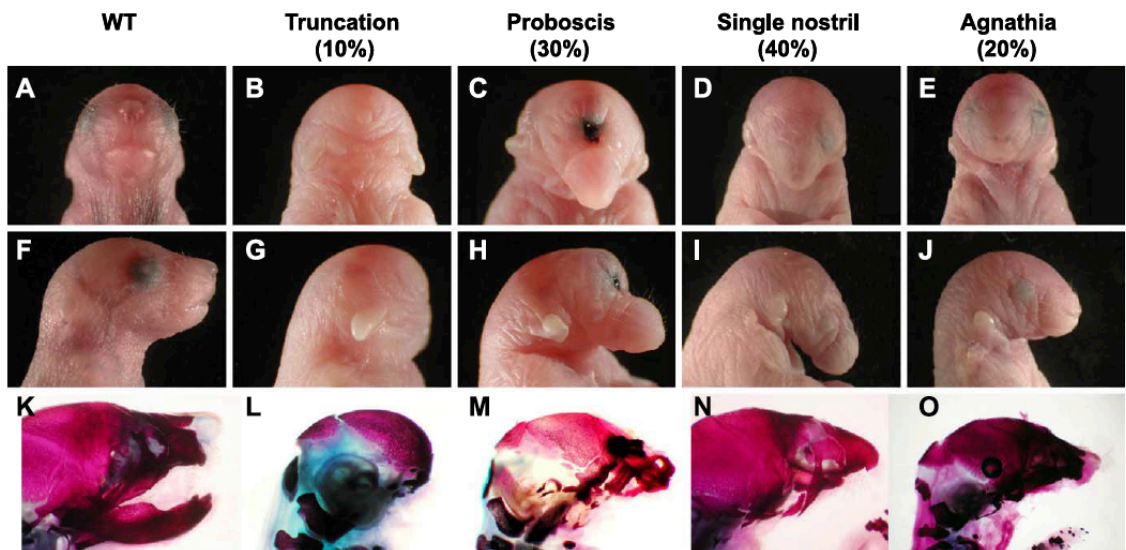


Figure 1.3: Craniofacial Defects Present in *Twsg1*^{-/-} mice. **A)** Wild type, **B)** severe anterior truncation, **C)** midline defects (cyclopia with proboscis), **D)** single nostril with agnathia (absence of mandible), **E)** agnathia alone. **F– J)** Lateral view of newborn mice shown in A– E. **L–P)** Skeletal preparations of animals shown in A–E. Figure adapted

from O'Connor and coworkers (Petryk et al. 2004).

TWSG1 is known to bind to both BMPs as well as Chordin, demonstrating TWSG1's antagonistic and agonistic capabilities. As with Noggin and Chordin, TWSG1 has been shown to block BMP-2 and BMP-4 binding to its receptor, inhibiting typical Smad 1/5/8 signaling (Chang et al. 2001). In this way, TWSG1 acts as an antagonist to BMP signaling. On the other hand, TWSG1 can act as an agonist by promoting cleavage of Chordin by BMP-1 (Petryk et al. 2004; Zakin and De Robertis 2004; Melnick et al. 2006), allowing BMPs to signal through its receptor (Sotillo Rodriguez et al. 2009).

Previously, my lab has shown that *Twsg1*^{-/-} osteoclasts exhibit increased levels of phosphorylated SMAD 1/5/8 (pSMAD 1/5/8) *in vitro*, suggesting that enhanced osteoclastogenesis in the *Twsg1*^{-/-} mice is mediated through increased BMP signaling (Sotillo Rodriguez et al. 2009). TWSG1 may, therefore, have an antagonistic role on BMP. TWSG1 is likely one of the key molecular regulators of BMP signaling in RANKL-mediated osteoclastogenesis.

1.1.6 Histone Deacetylases

HDACs exert epigenetic control of transcriptional activity by removing acetyl groups from lysine residues in histones, which condenses chromatin and

limits the accessibility of transcription factors to the DNA. They can also catalyze deacetylation of non-histone proteins, making them more stable and/or increasing their nuclear localization (Gregoretta et al. 2004). There are 18 HDACs that fall into four-classes based on structural and functional similarities. Class I HDACs (HDACs 1, 2, 3, and 8) are enzymatically active subunits of multi-protein complexes that deacetylate histones (Fischle et al. 2002; Lahm et al. 2007). Class II HDACs are subdivided into class IIa (HDACs 4, 5, 7, and 9) and class IIb (HDACs 6 and 10). Class II HDACs interact with non-histone substrates, are expressed in a tissue-specific manner, and shuttle between nuclear and cytosolic compartments in response to signaling pathway stimulation (Sengupta and Seto 2004). Class III HDACs includes the Sirtuins (Sirt 1-7) and require NADH for enzymatic activity (Backesjo et al. 2009). Class IV contains HDAC11 and is poorly understood.

Broad spectrum HDAC inhibitors (HDIs) such as trichostatin A (TSA) and sodium butyrate (NaB) block osteoclast differentiation, indicating that HDAC activity is required for osteoclastogenesis (Rahman et al. 2003; Kim et al. 2009). In 2003, Rahman and coworkers utilized a murine macrophage cell line, RAW264, and rat bone marrow cultures to show osteoclast-specific inhibition of TNF- α -induced nuclear translocation of NF κ B and soluble RANKL (sRANKL)-induced activation of p38 MAPK when exposed to TSA and NaB (Rahman et al.

2003). The authors suggest that HDAC activity is a key player in osteoclastogenesis. Recently, Lee and coworkers expanded on these findings and demonstrated that the osteoclast-specific inhibition by TSA was only effective during the early stages of osteoclastogenesis (Kim et al. 2009). Taken together, these studies validated HDACs requirement in osteoclastogenesis. However, the expression patterns and functional roles of specific HDACs in osteoclasts remain uncharacterized and the mechanisms/targets through which HDACs act in osteoclasts are still unknown.

1.1.7 Microphthalmia-associated Transcription Factor

MITF belongs to the MiT family of basic helix-loop-helix leucine zipper (bHLH-ZIP) transcription factors that regulate gene expression in a variety of cell types including melanocytes, macrophages, and osteoclasts (Hemesath et al. 1994). MITF is typically found in neural crest-derived melanocytes and brain-derived melanocytes of the pigmented retinal epithelium (Hodgkinson et al. 1993; Hughes et al. 1993). It is known that many signaling transduction pathways, cytokines, and regulators of transcription factors help to regulate MITF (Bertolotto et al. 1998; Bondurand et al. 2000; Saito et al. 2002; Elworthy et al. 2003; Sonnenblick et al. 2004). Work in the field has been focused on elucidating the regulatory network that modulates MITF in melanocytes including: Sry-related

HMG box 10 (SOX10); Paired box 3 (PAX3); Signal transducer and activator of transcription 3 (STAT3); cAMP response element binding protein (CREB); Lymphoid-enhancing factor-1 (LEF-1); Immunoglobulin transcription factor-2 (ITF2); Forkhead-box transcription factor D3 (FOXD3); and POU3F2, a POU-Homeodomain transcription factor (BRN2) (reviewed in Wan et al. 2011).

In regards to osteoclast development, in 1994 it was reported that mutations in MITF in multiple organisms result in osteopetrosis due to defective osteoclast development (Steingrimsson et al. 1994). The MITF complex integrates signals necessary for the appropriate temporal regulation of osteoclast genes such as *Cathepsin K* and *Tartrate-resistant acid ATPase (TRAP)* during differentiation. M-CSF signaling alone can regulate MITF nuclear localization and recruitment of MITF to target promoters (Bronisz et al. 2006). However, both M-CSF and RANKL are necessary for MITF to activate gene expression in osteoclasts (Sharma et al. 2007).

1.2 Statement of Purpose

1.2.1 Significance of Research

Degenerative skeletal diseases such as osteoporosis, osteolytic malignancies, and periodontitis can be attributed to increased osteoclast activity (Zaidi et al. 2003). For diseases like these, novel therapeutic strategies that target osteoclasts are needed for many craniofacial, orthopedic, and cancer applications. Incomplete understanding of the factors that regulate osteoclasts limits this development.

Bone development and homeostasis is extremely complex and is characterized by interactions of several pathways including BMP signaling. Bone healing requires synthesis of new bone by osteoblasts and the removal of old or damaged bone matrix by osteoclasts. Although BMPs have been widely studied as stimulators of osteoblastic bone formation, little to no studies have explored BMPs effect on osteoclasts and their role in the pathogenesis of osteolytic conditions (Raisz 2005).

Lieberman and coworkers showed that overexpression of Noggin inhibited BMP-mediated growth of osteolytic prostate cancer lesions (Feeley et al. 2006). In addition, BMPs have been used successfully in studies to: treat critical sized defects in both long and craniofacial bones; enhance fracture healing; treat non-

unions and lumbar spinal fusion; and regenerate alveolar bone and portions of teeth such as dentin and pulp. However, one of the limiting factors is the difficulty in determining the optimal concentration, appropriate temporal release and regulation of BMPs, as both a deficiency and an excess of BMPs may lead to pathologic states.

The data presented in this thesis will help us better understand the regulation of osteoclastogenesis and bone resorption by BMPs, their regulatory proteins like TWSG1, and downstream transcriptional regulators like HDACs. The knowledge gained by studying these regulators might contribute to the etiology of diseases such as osteoporosis, which is a prerequisite for the development of successful therapies and bone regenerative strategies for diseases associated with increased bone loss and defective bone formation.

1.2.2 Hypothesis

My central hypothesis is that BMPs are a positive and direct regulator of osteoclast differentiation. From previous work, the data presented in this thesis, and the future studies arising from these studies, I hypothesize that osteoclastic bone remodeling can be modulated with BMPs, TWSG1, and HDACs.

1.3 Specific Aims

1.3.1 Specific Aim 1: Evaluate the Role of BMPs in Osteoclast Differentiation

I hypothesize that BMP signaling is a positive regulator of osteoclast differentiation. In this aim, I will determine the temporal requirements of exogenous BMPs on enhanced osteoclastic differentiation *in vitro*, define the profile of BMP receptor and ligand expression during osteoclast formation, and assess the effects of impaired BMP signaling upon osteoclastogenesis. The body of this work will be address in Chapter 2.

1.3.2 Specific Aim 2: Define the Effects of Overexpressing TWSG1 on Osteoclasts In Vitro and In Vivo

In the first part of this aim, I hypothesize that overexpressing TWSG1 in primary osteoclasts will inhibit osteoclastogenesis. In this aim, I will use an adenovirus delivery system to overexpress *Twsg1* in WT and *Twsg1*^{-/-} primary osteoclasts then add exogenous BMP-2 in attempts to modulate osteoclastogenesis *in vitro*. Osteoclast activity and signaling pathways will be monitored. The body of this work will be addressed in Chapter 3.

In the second part of this aim, I hypothesize that BMPs acts directly in osteoclasts to enhance osteoclastogenesis. In this aim, I will create an osteoclast-specific BMPRII null mouse by breeding floxed *BMPRII* mice with *LysM-Cre* mice and characterize the phenotype of these mice. The initial findings of these studies will be presented in Chapter 5.

1.3.3 Specific Aim 3 Characterize the Molecular Mechanisms by Which HDAC7 Regulates Osteoclast Differentiation

I hypothesize that HDAC7 regulates osteoclastogenesis by forming a multi-protein complex with MITF on the promoter of osteoclast target genes. In this aim, I will determine which regions of HDAC7 protein are essential for interaction with MITF and examine whether changes in HDAC7 expression and localization influence this interaction. The body of this work will be presented in Chapter 4.

Chapter 2

Bone Morphogenic Protein 2 Directly Enhances Differentiation of Murine Osteoclast Precursors

2.1 Overview

Previous studies found that BMPs support osteoclast formation, but it is not clear whether this is a direct effect on osteoclasts or mediated indirectly through osteoblasts. We have shown that a mouse deficient for the BMP antagonist Twisted gastrulation suggested a direct positive role for BMPs on osteoclastogenesis. In this report, we further determine the significance of BMP signaling on osteoclast formation in vitro. We find that BMP2 synergizes with suboptimal levels of RANKL to enhance in vitro differentiation of osteoclast-like cells. The enhancement by BMP2 is not a result of changes in the rate of proliferation or survival of the bone marrow derived cultures, but is accompanied by an increase in expression of genes involved in osteoclast differentiation and fusion. Treatment with BMP2 did not significantly alter expression of RANKL or OPG in our osteoclast cultures, suggesting that the enhancement of osteoclastogenesis is not mediated indirectly through osteoblasts or stromal cells. Consistent with this, we detected phosphorylated SMAD1,5,8 (p-SMAD) in the nuclei of mononuclear and multinucleated cells in osteoclast cultures. Levels of p-SMAD, BMP2 and BMP receptors increased during differentiation. RNAi suppression of Type II BMP receptor inhibited RANKL-stimulated formation of multinuclear TRAP positive cells. The BMP antagonist noggin inhibited RANKL-mediated osteoclast differentiation when added prior to day 3, while addition of

noggin on day 3 or later failed to inhibit their differentiation. Taken together, these data indicate that osteoclasts express BMP2 and BMP receptors, and that autocrine BMP signaling directly promotes the differentiation of osteoclasts-like cells.

2.2 Introduction

Bone is a highly dynamic tissue, characterized by a continuous cycle of bone formation by osteoblasts and bone resorption by osteoclasts (Sims and Gooi 2008; Henriksen et al. 2009). This cycle permits physiological bone growth, repair of damaged bone, and is important for regulation of systemic calcium and phosphate levels. Dysregulation of the cycle results in numerous pathological conditions such as osteoporosis, Paget's disease, and arthritis (Rodan and Martin 2000). It also contributes to the progression and morbidity of osteolytic cancers such as myeloma, osteosarcoma and metastatic breast, lung and prostate tumors (Guise et al. 2006; Roodman 2009).

Osteoclasts are large, multinucleated cells formed by fusion of mononuclear cells that derive from the monocyte/macrophage lineage (Vaananen and Laitala-Leinonen 2008). They produce proteases and other factors to degrade the inorganic mineral and organic protein components of bone, thereby facilitating repair and remodeling (Teitelbaum 2000). Two factors that are necessary and sufficient for osteoclast formation are M-CSF (Cecchini et al. 1997) and Receptor Activator of NF- κ B Ligand (RANKL) (Wada et al. 2006), both of which are expressed by osteoblasts. M-CSF is required for survival and proliferation of early osteoclast precursors, as the *op* mutant mouse, which lacks M-CSF, completely lacks osteoclasts (Felix et al. 1990; Wiktor-Jedrzejczak et al.

1990; Yoshida et al. 1990). Binding of RANKL, produced by osteoblasts, and the RANK receptor on osteoclasts stimulates expression of genes necessary for osteoclast differentiation, cellular fusion and bone resorption. Additionally, osteoblasts express osteoprotegerin- (OPG), a soluble decoy receptor for RANKL, which inhibits activation of RANK by RANKL. The ratio of RANKL to OPG produced by osteoblasts is a major determinant of osteoclast forming activity within the bone microenvironment and allows for close coordination between bone formation and bone resorption under normal physiological conditions. Aberrant expression of RANKL and other osteoclast promoting factors contributes to pathological bone loss associated with cancers, arthritis, and other disease states (Tanaka et al. 2005).

Bone morphogenic proteins (BMPs) are well established as key regulators of osteoblast biology (Canalis et al. 2003; Cao and Chen 2005). In addition to multiple important physiological roles in regulating bone formation, they are used clinically to promote localized bone growth and healing in a number of orthopedic and maxillofacial applications (Kirker-Head et al. 2007). BMPs are members of the TGF β family of secreted morphogens. They bind to receptor complexes composed of Type I and Type II BMP receptors (BMPR I and BMPR II). Activation of the BMP receptor complex stimulates transcriptional regulation by SMADs 1,5 and 8, and various protein kinase pathways. The signaling activity of

BMPs is modulated by secreted molecules such as Chordin, Noggin and Twisted gastrulation (*Twsg1*), which bind BMPs in the extracellular space, limiting their ability to activate signaling (Canalis et al. 2003). Although it is well established that BMPs exert multiple effects to promote osteoblast formation, the significance of BMP signaling in the osteoclast lineage is not clear. *In vitro* application of BMPs during orthopedic procedures has been found to promote a transient increase in osteoclast numbers and osteoclastic bone resorption in some instances (Toth et al. 2009). A number of studies suggested that BMPs can indirectly promote osteoclasts through enhanced expression of osteoclast-promoting factors by osteoblasts or stromal cells (Kanatani et al. 1995; Koide et al. 1999; Abe et al. 2000; Otsuka et al. 2003; Wutzl et al. 2006). However, osteoclasts express BMP receptors (Kanatani et al. 1995; Onishi et al. 1998; Kaneko et al. 2000; Itoh et al. 2001), and a growing body of literature suggests that BMPs directly influence the formation and activity of osteoclasts (Kanatani et al. 1995; Kaneko et al. 2000; Itoh et al. 2001; Wildemann et al. 2005; Okamoto et al. 2006; recently reviewed by Giannoudis et al. Giannoudis et al. 2007).

We recently characterized the bone phenotype of the *Twsg1*-deficient mouse (*Twsg1*^{-/-}), which exhibits significant osteopenia due to increased BMP signaling (Sotillo Rodriguez et al. 2009). Surprisingly, osteoblast function was not affected in these mice, nor was RANKL or OPG expression. Rather, the reduced

bone mass was attributed to increased osteoclast formation and function. The *Twsg1* gene was highly expressed in wild-type osteoclasts. *Twsg1*^{-/-} osteoclasts were significantly larger than wild-type controls, and had increased levels of phosphorylated SMAD1,5,8 (p-SMAD1,5,8) indicating excessive BMP signaling. Treatment of wild-type osteoclast cultures with recombinant BMP2 phenocopied the enlarged osteoclast phenotype. These observations indicate that the increased osteoclast formation in *Twsg1*^{-/-} mice stems from enhanced BMP signaling. In these previous studies, the presence of pSMADs in osteoclastic cultures that were not treated with exogenous BMP2 suggested that autocrine BMP signaling may be involved in osteoclast formation. Moreover, prior to day 3 we detected only a low level of pSMAD in osteoclast cultures, and we were unable to detect increased pSMAD in response to exogenous BMP prior to day 3, suggesting that BMP responsiveness may vary during osteoclast formation. The goals of the current study were to determine the temporal requirements of exogenous BMPs on enhanced osteoclastic differentiation *in vitro*, define the profile of BMP receptor and ligand expression during osteoclast formation, and to assess the effect of impaired BMP signaling upon osteoclastogenesis.

2.3 Materials and Methods

2.3.1 Cell Culture

Primary osteoclast cultures were performed according to the method described by Kobayashi et al. (Kobayashi et al. 2000). Bone marrow or spleen-derived cells were obtained from mice at one month old (bone marrow) or one week old (spleen) and cultured for three days on non-tissue culture coated dishes in osteoclast media: phenol red-free α -MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 25 units/mL penicillin, 25 μ g/mL streptomycin, 400 mM L-glutamine, and 10 ng/mL M-CSF (R&D Systems). The adherent population of cells was then replated at 2×10^4 cells per cm^2 in osteoclast medium further supplemented with RANK Ligand (RANKL) (R&D Systems), BMP2 (R&D Systems), or noggin (R&D Systems) as indicated. Murine primary calvarial osteoblasts were grown in α -MEM supplemented with 10% FBS, penicillin/streptomycin, 200 mM L-glutamine and 50 μ g/mL ascorbic acid. RAW264.7 cells were grown in DMEM supplemented with 10% FBS, 25-units/mL penicillin, 25 μ g/mL streptomycin.

2.3.2 Cell Proliferation and Survival

Cell number was determined on each day from triplicate samples using the Cell Titer 96 Aqueous One Solution Assay (Promega) according to the manufacturer's directions. The resulting data were fit to an exponential curve using the least squares method to obtain the growth rate and doubling time.

2.3.3 TRAP Staining

Cells were rinsed in PBS, fixed in 4% paraformaldehyde for 20 minutes and stained using the TRAcP5b kit (Sigma-Aldrich). Cells were photographed and analyzed using Adobe Photoshop to measure the number and size of TRAP-positive osteoclasts.

2.3.4 Quantitative Real-Time PCR

Gene expression in osteoclast cultures was assessed by real-time RT-PCR. Total RNA was isolated from cells using TRIzol reagent (Invitrogen Life Technologies) and quantified by UV spectroscopy. Reverse transcription was performed using 1 μ g of RNA and the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR was performed in triplicate using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) 2x iQ SYBR Green supermix (Bio-Rad) and normalized to L4 or GAPDH. Primer sequences used were: TRAP (Forward) 5'-CGT CTC TGC ACA GAT TGC A; (Reverse) 5'-

GAG TTG CCA CAC AGC ATC AC; NFATc1 (Forward) 5'-TCA TCC TGT CCA ACA CCA AA; (Reverse) 5'-TCA CCC TGG TGT TCT TCC TC; Cathepsin K (Forward) 5'-AGG GAA GCA AGC ACT GGA TA; (Reverse) 5'-GCT GGC TGG AAT CAC ATC TT; DC-STAMP (Forward) 5'-GGG CAC CAG TAT TTT CCT GA; (Reverse) 5'-TGG CAG GAT CCA GTA AAA GG; ATV6v0d2 (Forward) 5'-TCA GAT CTC TTC AAG GCT GTG CTG; (Reverse) 5'-GTG CCA AAT GAG TTC AGA GTG ATG; BMP2 (Forward) 5'TGG AAG TGG CCC ATT TAG AG; (Reverse) 5'-TGA CGC TTT TCT CGT TTG TG; BMP4 (Forward) 5'-CCT GGT AAC CGA ATG CTG AT; (Reverse) 5'-AGC CGG TAA AGA TCC CTC AT; L4 (Forward) 5'-CCT TCT CTG GAA CAA CCT TCT CG; (Reverse) 5'-AAG ATG ATG AAC ACC GAC CTT AGC; RANKL (Forward) 5'-CAG AAG GAA CTG CAA CAC ATT; (Reverse) TGG TAC CAA GAG GAC AGA GTG; OPG (Forward) 5'-CTG CCT GGG AAG AAG ATC AG; (Reverse) 5'-TTG TGA AGC TGT GCA GGA AC; GAPDH (Forward) 5'-TGC ACC ACC AAC TGC TTA G; (Reverse) 5'-GAT GCA GGG ATG ATG TTC.

2.3.5 Immunoblotting

Cell protein lysates were prepared in modified RIPA lysate supplemented with protease inhibitor (Roche) and phosphatase inhibitor cocktails (Sigma-Aldrich). Proteins were resolved by SDS-PAGE, transferred to PVDF membrane

(Millipore) and incubated overnight at 4°C with primary antibodies against phosphorylated SMAD1,5,8 (Cell Signaling) antibody or anti-total SMAD1,5,8 antibody (Santa Cruz), BMPR 1A (Santa Cruz), BMPR 1B (Santa Cruz), BMPR II (R&D Systems), α -tubulin (Santa Cruz) and horseradish peroxidase conjugated secondary antibodies (Santa Cruz). Immunoreactive bands were visualized using ECL Plus substrate (GE Health Systems).

2.3.6 Immunofluorescence

Cells were grown on glass coverslips and fixed in 4% paraformaldehyde for 20 minutes. They were then permeabilized in PBS/0.3% TritonX-100 for 5 minutes, blocked in immunofluorescence buffer (3% BSA, 20 mM MgCl₂, 0.3% Tween20 in PBS) for 20 minutes, and incubated with primary antibodies for 90 minutes in immunofluorescence buffer. Phosphorylated SMAD1,5,8 antibody (Santa Cruz) was used at 1:20, anti- α -tubulin (Sigma-Aldrich) was used at 1:2000. Cells were washed three times in PBS/0.1% Triton X-100, and then incubated for 30 minutes with Alexa-conjugated secondary antibodies at 1:800 (Invitrogen). After three washes, cells were stained with DAPI, washed, and mounted in 90% glycerol/ 0.4% N-propyl-gallate. Images were obtained using an Olympus Fluoview 500 confocal microscopy and processed using Adobe Photoshop.

2.3.7 Lentiviral Gene Suppression

Two lentiviral vectors encoding shRNAs against the Type II BMP receptor, or a control shRNA, together with a GFP marker, were purchased from Open Biosystems and used to produce replication defective lentivirus according to the manufacturer's protocols. Viral stocks were titrated by infection in HeLa cells and assessed by GFP fluorescence. These stocks were used to infect RAW264.7 cells and murine primary osteoclasts according to the manufacturer's recommendations. Following infection, RAW264.7 cells were subjected to puromycin selection at 4 $\mu\text{g}/\text{mL}$ for 3 days, expanded, and then treated with RANKL to stimulate osteoclast formation. Following infection, primary osteoclast cultures were stimulated with M-CSF and RANKL without prior puromycin selection, as we were unable to obtain successful differentiation of multinucleated osteoclasts following selection.

2.4 Results

2.4.1 BMP2 Synergizes with RANKL on Days 3-4 to Enhance In Vitro Differentiation of Osteoclast Precursors

We previously reported that treatment with BMP2 synergistically enhanced the *in vitro* differentiation of bone marrow cells cultured in the presence of suboptimal levels of RANKL (30 ng/mL, which is half our optimal dose) into TRAP-positive multinucleated osteoclast-like cells (Sotillo Rodriguez et al. 2009). To determine when during differentiation BMP2 acts to promote osteoclast formation, we treated bone marrow derived cultures with suboptimal levels of RANKL and added or removed BMP2 at defined intervals. After five days of culture, we performed TRAP staining to visualize differentiated osteoclast-like cells (OCLs). As shown in Fig. 1A, cells treated with suboptimal RANKL alone gave a modest number of relatively small multinuclear cells, while the continuous addition of BMP2 during the differentiation resulted in many more TRAP-positive OCLs. Treatment with BMP2 from day 0 to day 4 showed a similar positive effect on OCL formation. In contrast, when BMP2 was removed on or before day 3, we did not detect enhanced OCL formation. Similarly, although BMP2 treatment on just day 0 to day 1, day 1 to day 2, or day 2 to day 3 failed to enhance multinuclear cell formation, the induction of TRAP-positive cells was enhanced by

BMP2 treatment from day 3 to day 4 and to a lesser extent from day 4 to day 5. No TRAP-positive cells were detected in the absence of RANKL or in cultures treated with BMP2 only (data not shown). These results are quantified in Fig. 1B-C, which show that BMP2 treatments that include days 3 to 5, the average number of OCLs per field is increased by approximately 60%, and the average size of the 20 largest OCLs in each field was increased 2-3 fold. These data indicate that treatment with BMP2 between day 3 and 5, synergizes with suboptimal levels of RANKL to induce the differentiation of multinucleated OCLs.

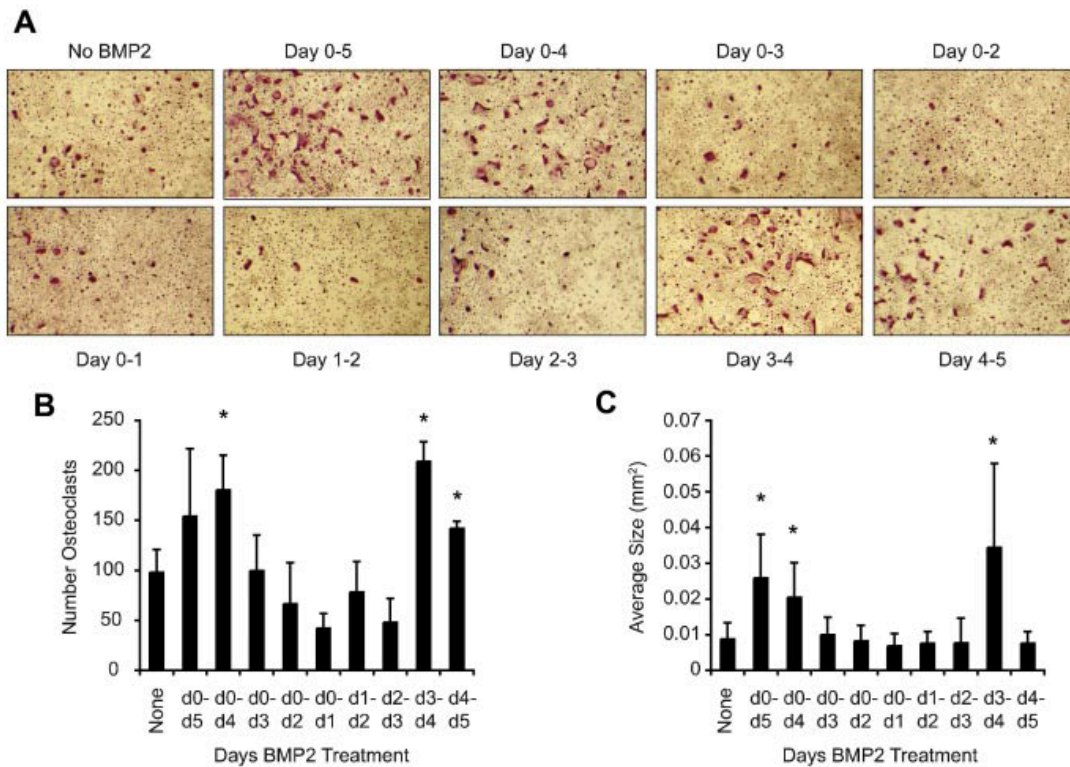


Figure 2.1: BMP2 enhances suboptimal RANKL-stimulated differentiation of osteoclast precursors. A) TRAP staining of bone marrow-derived osteoclasts

differentiated with suboptimal (30 ng/mL) RANKL and BMP2 at 30 ng/mL for five days. BMP2 was added or removed on the indicated days of the differentiation, with day 0 defined as the day RANKL was added to cells. After five days, cells were fixed and stained for TRAP. Three fields were photographed and used for quantitative analysis in B-C. Representative images are shown in A. **B)** The number of TRAP-stained cells per field; C. The average size of the 20 largest osteoclasts in each field. * $p \leq 0.05$ vs. no BMP2 treatment.

2.4.2 BMP2 Does Not Affect OCL Cell Number

To determine if the observed enhancement of OCL formation by BMP2 was mediated by increased proliferation or survival; we incubated bone marrow cultures in the presence or absence of BMP2. The cell number was measured using an MTS-based assay, and plotted to obtain the growth rate and doubling time of the cultures (Fig. 2). We detected very little difference between BMP2-treated and control cultures as control cultures treated with M-CSF and RANKL exhibited a doubling time of 68.5 hours, and cultures treated with BMP2 exhibited a doubling time of 65.8 hours. From this, we conclude that BMP2 does not enhance OCL formation by stimulating proliferation or promoting the survival of OCL precursors.

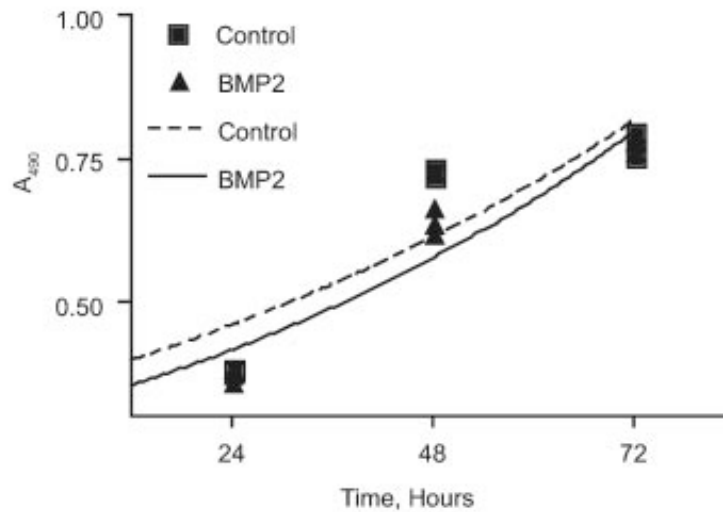


Figure 2.2: BMP2 does not affect OCL proliferation and survival. Bone marrow cultures were incubated in the presence (BMP2) or absence (control) of BMP2 for 3 days. Cell number was determined on each day from triplicate samples using the Cell Titer 96 Aqueous One Solution Assay.

2.4.3 BMP2 Enhances RANKL-Mediated Expression of Osteoclast Genes

To further determine the effect of BMP signaling on OCLs, we treated bone marrow cultures with combinations of RANKL and BMP2 and used real-time PCR to measure expression of genes involved in osteoclast differentiation. RANKL induced expression of osteoclast marker genes *NFAT-c1*, *TRAP*, *DC-STAMP*, *cathepsin K* and *ATP6v0d2*, (Fig. 3). Although BMP2 was unable to induce expression of these genes in the absence of RANKL, it enhanced

expression of all these genes in the presence of suboptimal RANKL. Treatment with the BMP-antagonist, noggin, impaired the BMP2-mediated increase in gene expression. These results further demonstrate that BMP2 enhances OCL formation in the presence of RANKL.

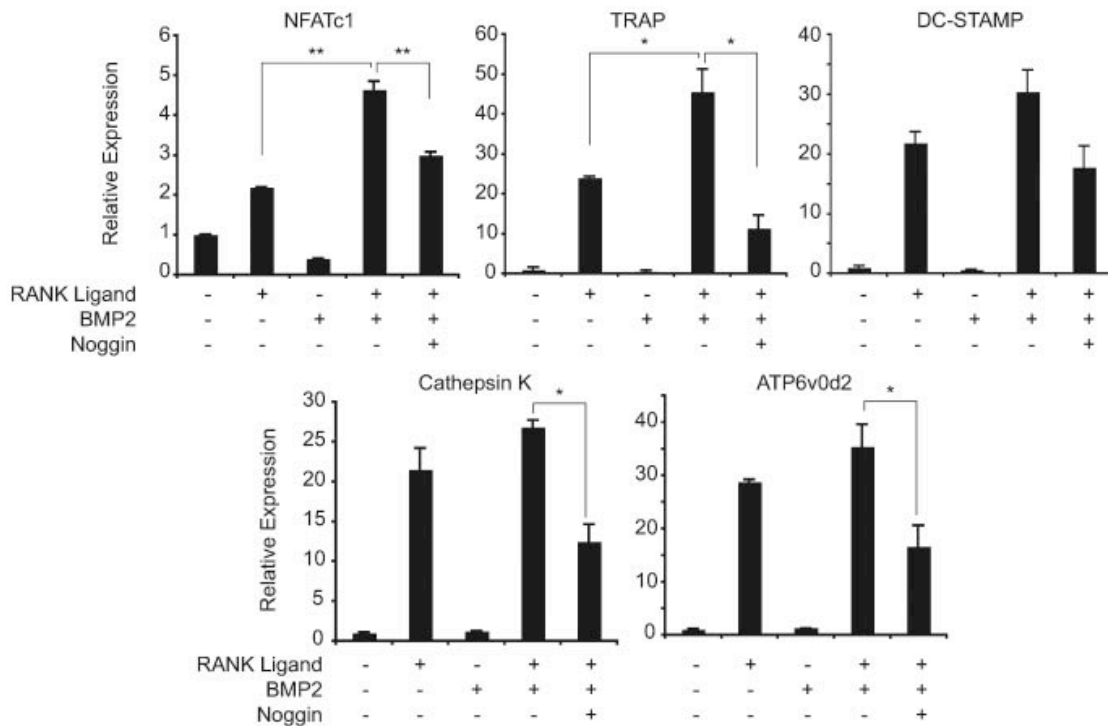


Figure 2.3: Expression of osteoclast marker genes. Bone marrow-derived osteoclast precursors were cultured in media containing 30 ng/mL RANKL, 30 ng/mL BMP2, and 1 μ g/mL noggin as indicated for three days. Gene expression was determined by real-time reverse transcription-PCR, normalized to L4 expression, and is graphed relative to the expression level in osteoclast media without RANKL or BMP2. * $p \leq 0.05$; ** $p \leq 0.005$.

2.4.4 BMP2-Enhanced OCL Formation is not Associated with Altered RANKL and OPG Expression

Previous studies suggested that BMPs could enhance OCL formation by an indirect mechanism that involves BMP2-altered RANKL and OPG expression in osteoblasts or bone marrow stromal cells (Kanatani et al. 1995; Onishi et al. 1998; Kaneko et al. 2000). To establish whether our observed enhancement of OCL differentiation by BMP2 is mediated indirectly through contaminating bone marrow stromal cells or osteoblasts, we compared expression of RANKL and OPG in bone marrow-derived cultures treated with suboptimal RANKL or suboptimal RANKL plus BMP2 for three days (Fig. 4). RANKL was detected in cultures of primary osteoblasts and at approximately six-fold lower levels in the OCL cultures. OPG expression in bone marrow derived cultures was 70 to 90 fold lower than primary osteoblast cultures. Treatment with BMP2 had no significant effect on expression of either RANKL or OPG in OCL cultures. These data indicate that altered RANKL or OPG expression does not account for the BMP2-mediated increase in OCL formation that we observe in our *in vitro* cultures, but are consistent with direct stimulation of OCL by BMP2.

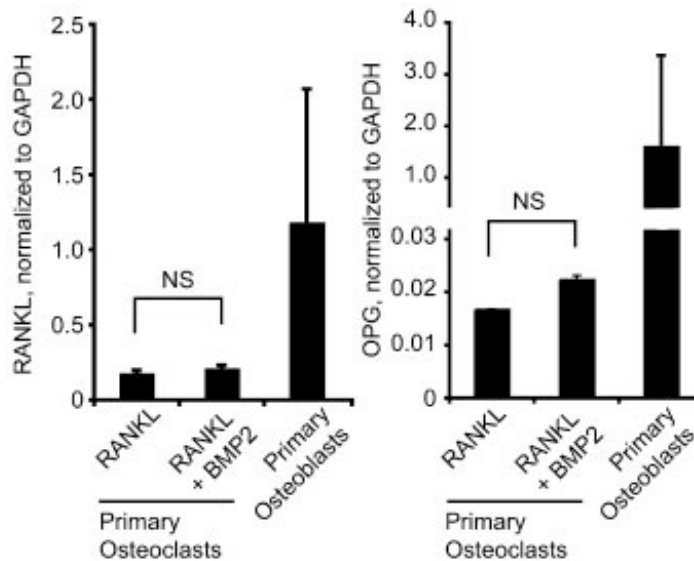


Figure 2.4: BMP2 does not affect RANKL or OPG expression in osteoclast cultures in vitro. Real-time RT-PCR was used to quantify RANKL and OPG expression in bone marrow-derived osteoclast precursor cells cultured for three days in the presence of 30 ng/mL RANKL and 30 ng/mL BMP2 as indicated, or in murine primary calvarial osteoblasts. NS, not significant.

2.4.5 BMP Signaling Becomes Activated During In Vitro OCL Differentiation

To further test whether BMP signaling acts directly on OCLs, we performed immunofluorescence against phosphorylated SMAD (p-SMAD1,5,8) on bone marrow derived OCL precursors during RANKL-stimulated differentiation. We counterstained the cells with DAPI to visualize nuclei and with

α -tubulin to reveal the overall cellular morphology. As shown in Fig. 5, weak nuclear P-SMAD staining was detected in mononucleated cells after one and three days in RANKL. Nuclear staining for P-SMAD was greatly increased in multinucleated cells after five days differentiation. No staining was observed in control slides stained without p-SMAD antibody. This increase in p-SMAD1,5,8 levels throughout OCL differentiation was confirmed by immunoblotting (Fig. 7A). The detection of p-SMAD1,5,8 in the nuclei of mononuclear and multinucleated OCL indicates that BMP signaling is activated in OCL during their differentiation.

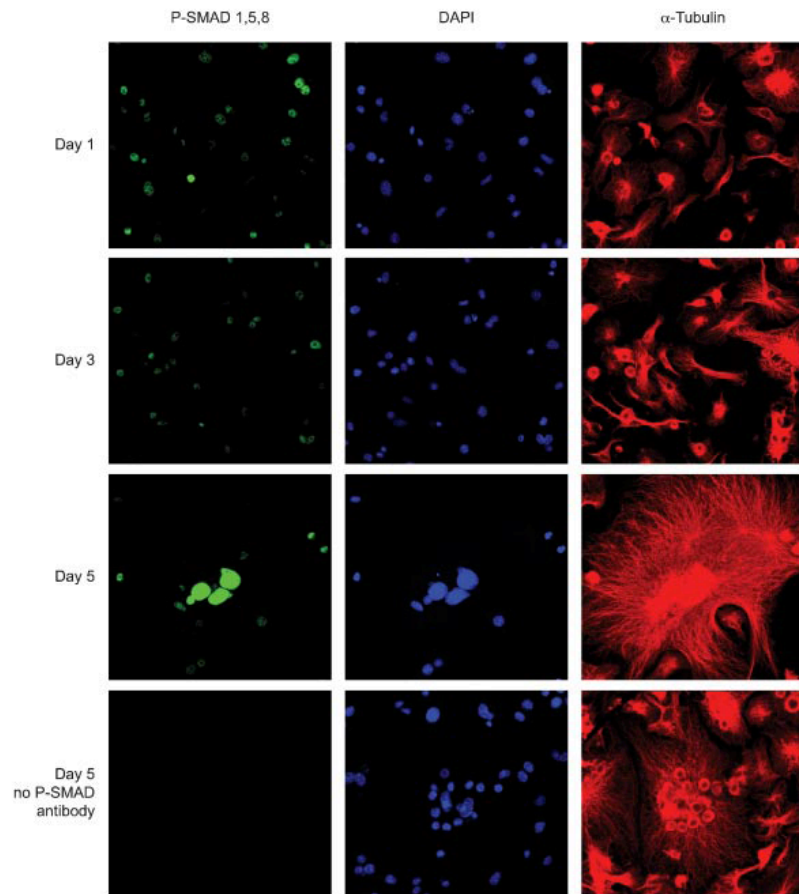


Figure 2.5: Phosphorylated SMAD1,5,8 Immunofluorescence. Osteoclasts were differentiated by RANKL for 1, 3 or 5 days and subjected to immunofluorescence staining against phosphorylated SMAD1,5,8 (green). Cells were costained against α -tubulin (red) and with DAPI (dark blue) to show cell outline and nuclei, respectively. Parallel slides from each day were stained without P-SMAD antibody as a negative control (bottom row and not shown). All images are at equal magnification and were acquired and processed identically.

2.4.6 Dynamic Expression of BMP Receptors and BMP2 Ligand in Osteoclasts

The functional BMP receptor is a heteromeric complex composed of Type I and Type II receptors (referred to as BMPRI, BMPRII and BMPRII). Binding of BMP ligands to the Type II receptors activates the Type I receptors, which phosphorylate downstream intracellular effectors including SMADs. Expression of BMP ligands and BMP receptors in osteoclasts was previously reported (Kanatani et al. 1995; Onishi et al. 1998; Anderson et al. 2000; Kaneko et al. 2000; Itoh et al. 2001; Spector et al. 2001; Zoricic et al. 2003), but whether their expression in OCLs changes during differentiation has not been described. To determine whether changes in expression of BMP receptors or BMP ligands by OCLs could account for the stage-specific activation of BMP signaling during their differentiation, we performed immunoblots against each of the BMP

receptors between day 0 and day 5 using optimal amounts of M-CSF and RANKL. Expression of BMPR 1A was first weakly detected on day 2 and increased on subsequent days (Fig. 7A). BMPR 1B was readily detected at all time points examined and showed relatively little change. BMPR II was barely detected on days 1 to 3, but increased on days 4 and 5. We measured expression of BMP2 and BMP4 ligands by real time RT-PCR. BMP2 was weakly expressed on day 1 and increased by 6 to 8-fold on subsequent days (Fig. 6B). Although BMP4 was robustly detected in several positive controls, we were unable to detect expression of BMP4 in OCL-like cell cultures at any time point examined (data not shown).

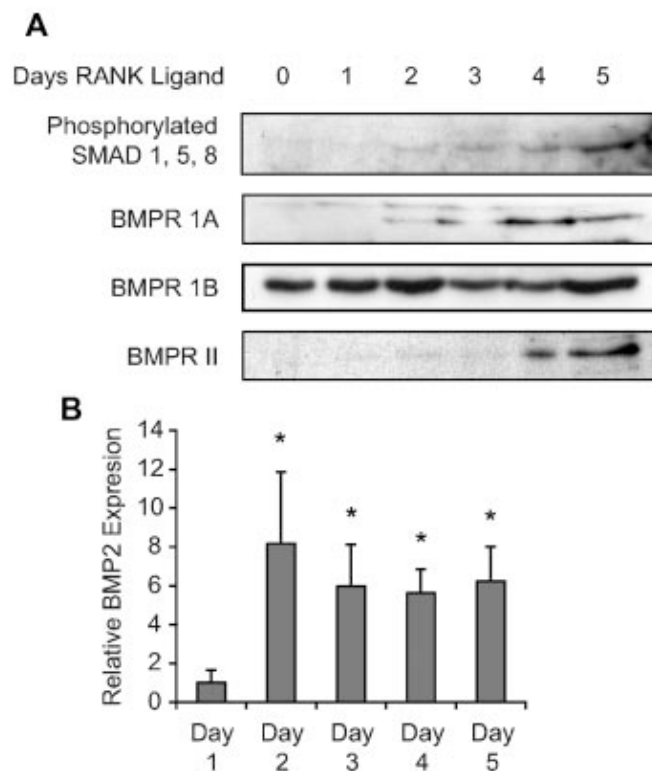


Figure 2.6: The expression of BMP receptors and BMP2 ligand increase during osteoclast differentiation. **A)** Immunoblot analysis of osteoclast precursors showing protein expression of BMP receptors and levels of phosphorylated SMAD1,5,8 on the indicated days of differentiation. **B)** Real-time PCR quantitation of BMP2 expression in osteoclast precursors on each day of differentiation. Gene expression was determined in triplicate, normalized with GAPDH and is graphed relative to expression on Day 1. * $p \leq 0.001$ vs Day 1.

2.4.7 Inhibition of BMP Signaling Inhibits OCL Formation

Based on the observation that exogenous BMP2 enhances osteoclast-like cell formation, we hypothesized that decreasing expression of BMP receptors should inhibit OCL differentiation. We made use of shRNA against the BMPR II, as this should block signaling through either type IA or type IB receptor. To ensure that any effect was due to knockdown of BMPR II rather than to any unintended target, we obtained lentiviral vectors from Open Biosystems that encode two different shRNAs against BMPR II and a control lentivirus. We began by infecting RAW264.7 cells, which are a common model for osteoclast/macrophage/ monocyte differentiation, with control shRNA or BMPR II shRNA lentivirus, subjecting them to puromycin selection, and measuring expression of BMPR II. Both western blotting (Fig. 7A) and real-time RT-PCR (Fig. 7B) indicated that expression of BMPR II is reduced by both BMPR II shRNAs,

although shRNA #2 gave a stronger reduction. Further indicating successful infection with the lentivirus and puromycin selection, we readily detected the GFP marker in almost all cells. When the control-infected RAW264.7 cells were stimulated with RANKL, they formed large, multinucleated cells as expected (Fig. 7C, yellow arrow). In contrast, treatment of the BMPR II shRNA cells with RANKL failed to stimulate formation of multinucleated cells.

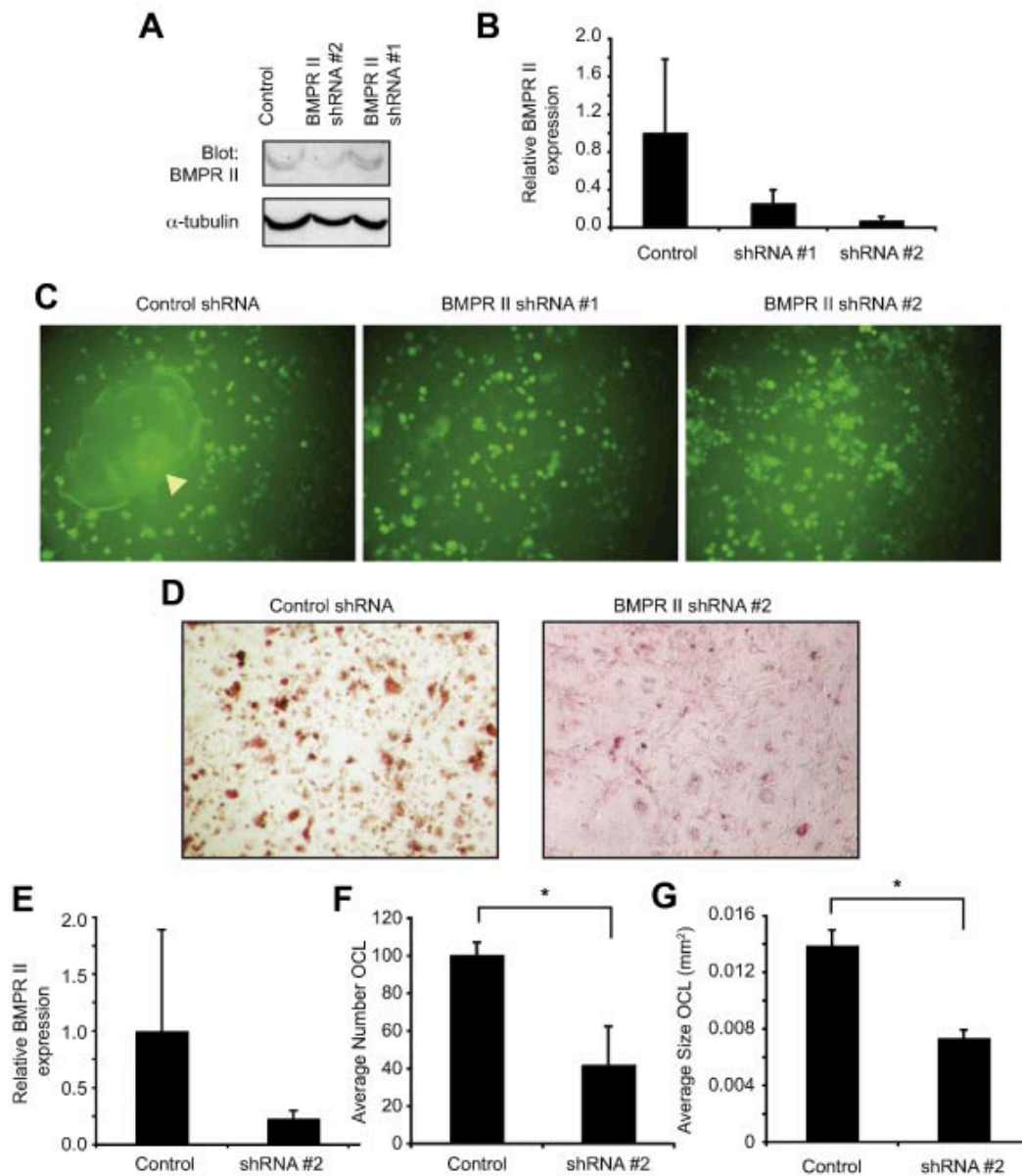


Figure 2.7: Suppression of BMPR II expression inhibits osteoclast formation. A-B)

Western blot (A) and real-time RT-PCR (B) showing expression of Type II BMP Receptor in RAW264.7 cells following infection with control shRNA or BMPR II shRNA lentiviral vectors and puromycin selection. **C)** Fluorescence micrographs showing BMPR II and control-infected RAW264.7 cells that have been stimulated with RANKL for 6 days. Note

the large multinucleated osteoclast indicated by the arrowhead in the “Control” panel. **D)** TRAP staining of Control and BMPR II shRNA -infected primary osteoclasts treated with RANKL for five days. **E)** Real time RT-PCR analysis of BMPR II expression in shRNA primary osteoclasts, plotted relative to control shRNA cells. **F-G)** Quantitative analysis showing the number (F) and size (G) of differentiated BMPR II suppressed primary osteoclasts following RANKL differentiation. *, $p \leq 0.002$.

We next infected bone marrow-derived OCLs with control lentivirus or the BMPR II shRNA #2 virus. We only used BMPRII shRNA #2 to infect primary bone marrow derived cells because both shRNAs gave a similar phenotype in RAW 264.7 cells but shRNA#2 showed higher ability to knockdown expression of BMPRII in RAW 264.7 cells. Stimulation of the control-infected cells with M-CSF and RANKL induced differentiation of multinucleated TRAP-positive OCLs (Fig. 7D). Expression of the BMPR II mRNA in these cultures was reduced by over 76% even though these cells were not subjected to puromycin selection. Quantitative analysis of these cells revealed that the differentiation of BMPR II shRNA-infected bone marrow cultures was greatly impaired relative to control-infected cells (Fig. 7E). The number of TRAP-positive OCLs was reduced from 100 to 42 cells per field (Fig. 7F) and the average size of TRAP-positive OCLs was reduced from 0.014 mm^2 to 0.007 mm^2 (Fig. 7G). These data indicate that a

reduced level of BMP receptors in bone marrow derived OCL cultures impairs their differentiation into mature, multinucleated cells.

To further confirm whether endogenous BMP signaling is necessary for OCL formation and the timing of this signaling, we differentiated OCL with optimal amount of RANKL in the presence of the BMP antagonist, noggin. Noggin was selected for these studies rather than TWSG1 to simplify interpretation. While BMP-agonist activity has been reported for TWSG1 in some circumstances (Ross et al. 2001; Oelgeschlager et al. 2003), only BMP-antagonist activity has been described for noggin. RANKL-stimulated bone marrow derived cultures were treated with noggin for defined intervals, and stained for TRAP on day 5. The average number of TRAP-positive cells was not significantly altered by any regimen of noggin treatment (not shown). OCLs treated with only RANKL exhibited an average size of 0.15 mm^2 . In agreement with our previous studies (Sotillo Rodriguez et al. 2009), treatment with noggin for the entire 5-day period reduced the average size of TRAP-positive multinucleated cells from 0.15 mm^2 to approximately 0.02 mm^2 , as did treatment from day 1 to 5 and day 2 to 5 (significantly different from RANKL-only, $p \leq 0.002$) (Fig. 8). In contrast, OCLs treated with noggin beginning on day 3 or day 4 averaged $0.10\text{--}0.11 \text{ mm}^2$ (not significantly different from RANKL-only). These observations are consistent with an endogenous BMP signal that enhances OCL formation beginning around day

3. Unexpectedly, treatment with noggin from day 0 to day 1, day 0 to day 2 and day 0 to day 3 was equally effective as noggin treatment from day 0 to day 5. These data indicate that inhibition of endogenous BMP signaling by noggin impairs the differentiation of osteoclast-like cells.

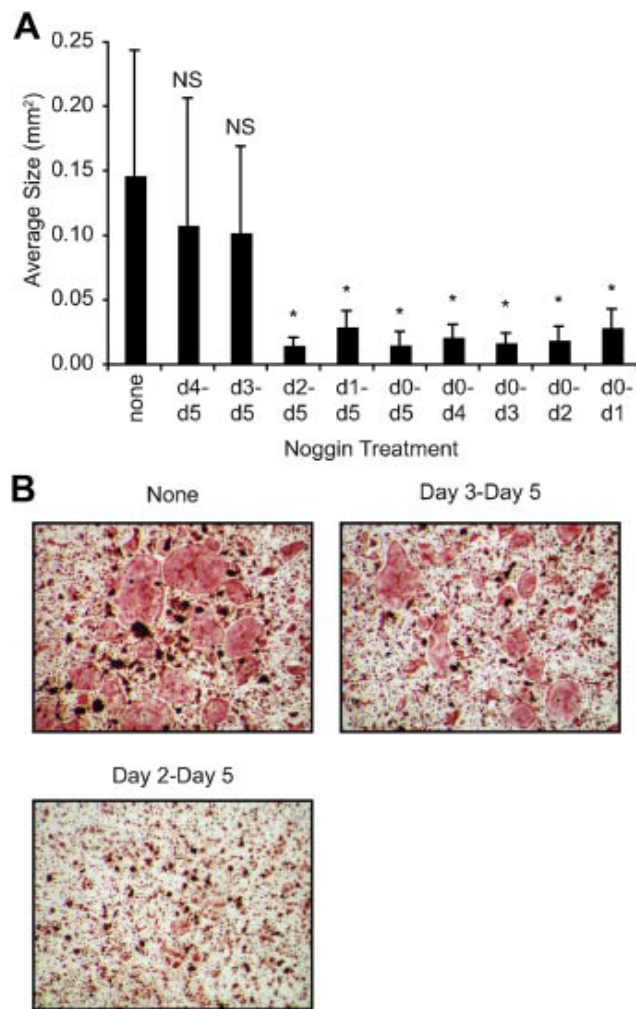


Figure 2.8: Noggin inhibits osteoclast differentiation. Spleen-derived osteoclast precursors were cultured with 60 ng/mL RANKL supplemented with 250 ng/mL noggin for the indicated days. **A)** Average size of TRAP-positive multinucleated osteoclasts. **B)**

Photographs showing TRAP stained osteoclast precursors treated with noggin for the indicated days. * $p \leq 0.001$ versus no noggin; NS, not significant versus no noggin.

2.5 Discussion

Despite their well-established functions promoting osteoblast development and function in bone formation, maintenance, and repair, the significance of BMPs during osteoclast formation remains poorly understood. In this study, we find that BMP2 directly enhances *in vitro* differentiation of murine osteoclasts in the presence of RANKL, that BMP signaling becomes activated in osteoclasts during their late stages of differentiation by an autocrine mechanism, and that inhibition of BMP signaling impairs osteoclast formation.

Emerging evidence indicates that BMPs act directly on cells of the osteoclast lineage to promote their development and/or activity. Okamoto et al. showed that increased BMP4 signaling *in vivo* stimulated both osteoclasts and osteoblasts, while the BMP antagonist noggin inhibited osteoclast formation. They also reported that exogenous BMP4 stimulated SMAD phosphorylation in macrophage cultures (Okamoto et al. 2006). In an important *in vitro* study, Itoh et al. showed that treatment of bone marrow-derived osteoclast precursors with BMP2 enhanced RANKL-mediated differentiation into mature osteoclasts (Itoh et al. 2001). Addition of a soluble form of the BMPRII receptor, which inhibits signaling through the membrane-associated receptors, attenuated osteoclast formation. Kaneko et al. showed that BMP2 directly enhanced the activity of purified rabbit mature osteoclasts (Kaneko et al. 2000). In our previous work, we

showed that mice mutant for the BMP antagonist TWSG1 exhibit osteopenia that results from increased numbers and size of osteoclasts. *Twsg1*^{-/-} osteoclasts exhibited higher levels of p-SMAD1,5,8 than wild-type osteoclasts beginning on the third day of differentiation with RANKL, and treatment of wild-type osteoclast precursors with exogenous BMP2 increased p-SMAD levels in osteoclasts only after three days pre-incubation with RANKL (Sotillo Rodriguez et al. 2009). These observations led us to hypothesize that the effect of BMPs on osteoclasts may be restricted to specific times during their differentiation.

In this report, we find that exogenous BMP2 enhanced differentiation of OCLs in the presence of suboptimal RANKL beginning on day 3, shortly before mononuclear cells begin to fuse into mature multinucleated OCLs. BMP2 did not induce OCL formation in the absence of RANKL, confirming that BMP signaling is not sufficient to induce OCL differentiation by itself. Expression of various BMPs by OCLs was noted in earlier studies (Onishi et al. 1998; Anderson et al. 2000; Itoh et al. 2001; Spector et al. 2001; Zoricic et al. 2003; Paul et al. 2009), although the expression profile of BMPs in OCLs during their differentiation was not previously reported. Our data are novel in that they define a changing profile of responsiveness to BMPs during OCL differentiation and establish that the change in responsiveness arises from increased expression of type IA and II

BMP receptors. We are also the first to demonstrate that we can detect pSMAD in the nucleus of osteoclasts.

In addition to responsiveness to exogenous BMP2, our data indicate the presence of an autocrine BMP signal during osteoclast-like cell formation, supported by expression of BMP ligands and receptor complexes, and the presence of phosphorylated SMADs in the absence of exogenous BMP ligands. Interestingly, Komarova et al. developed a mathematical model of bone remodeling that suggested autocrine regulation of osteoclasts, although they did not identify the specific regulatory factors involved (Komarova et al. 2003). The enhanced formation of *Twsg1*^{-/-} osteoclasts *in vitro* in the absence of exogenous BMPs (Sotillo Rodriguez et al. 2009) lends further support for a model involving autocrine BMP signaling by OCLs.

Several lines of evidence suggest that BMPs act primarily at late stages of osteoclast differentiation. Osteoclast-like cells exhibit a low level of BMP receptors and phosphorylated SMADs during the first two days of stimulation with RANKL, and we have noted that treatment with exogenous BMP2 during this time does not cause a detectable increase in p-SMAD levels (data not shown). The increased number of nuclei per cell described for *Twsg1*^{-/-} osteoclasts suggest that the large osteoclast size phenotype results from excessive fusion of mononuclear progenitors (Sotillo Rodriguez et al. 2009). We found that treatment

with noggin had little effect on the overall number of TRAP-positive cells, but strongly decreased the size of multinucleated osteoclast-like cells, further signifying that decreased BMP signaling impairs fusion. On the other hand, suppression of the Type II BMP receptor reduced both the number and size of TRAP-positive cells, indicative that BMP signaling plays an important role prior to fusion of TRAP-positive mononuclear precursors. The explanation for this discrepancy is not yet known. We found that BMP treatment increases expression of *DC-STAMP*, a transmembrane protein necessary for osteoclast fusion, as well as of *TRAP*, *cathepsin K* and *ATP6v0d2*, proteins involved in resorption of the bone extracellular matrix. It is not known whether increased expression of these markers is mediated directly by BMP signaling, or indirectly through increased expression of osteoclast regulatory transcription factors such as NFATc1 or through a combination of the two. Improved understanding of the precise cellular and molecular functions of BMP signaling in osteoclasts remains an important area for future investigations.

Our data from exogenous BMP2 treatment, the expression profile of BMP receptors and the progressive increase in phosphorylated SMAD levels all suggested that BMPs do not affect OCL differentiation until day 3, leading to the prediction that noggin treatment at early timepoints should not affect OCL formation. Thus, the ability of noggin treatment from day 0 until day 1 to inhibit

OCL formation equally well as noggin treatment from day 0 until day 5 was unexpected. A straightforward interpretation of these data is that BMP signaling at early timepoints is indeed necessary for OCL formation. However, work by Paine-Saunders and co-workers showed that noggin binds strongly to heparan sulfate proteoglycans (HSPGs) on the cell surface and that HSPG-bound noggin retains its BMP-antagonistic activity (Paine-Saunders et al. 2002; Irie et al. 2003). We thus speculate that when noggin is added to OCLs at early timepoints, it is retained on the cell surface and remains capable of inhibiting BMP signals at later times.

To maintain normal skeletal homeostasis, bone formation must precisely balance bone lost during resorption. Various signals from osteoclasts are thought to signal to osteoblasts to trigger the synthesis of new bone. BMPs are important positive regulators of bone formation. It is intriguing to speculate that BMPs synthesized by osteoclasts might be involved in this coupling from osteoclast activity to osteoblast activity. Indeed, a recent study found that expression of BMP6 by mature osteoclasts promotes the differentiation of mineralized osteoblasts from human mesenchymal stem cells (Pederson et al. 2008).

Collectively, our data suggest a model in which BMP2 directly enhances osteoclast-like cell formation. The important role of osteoclasts to the pathogenesis of diseases including osteoporosis, periodontal disease and

cancer-associated bone disease, manifests the need for understanding the factors that regulate osteoclast formation and activity. Moreover, because BMPs are used clinically to promote localized bone growth and regeneration, further elucidation of BMP biological effects will also enable refinement of therapeutic applications.¹

¹ We thank members of the Petryk laboratory for insightful comments and assistance during this project, and Chee Sohn for technical assistance. We acknowledge the use of a confocal microscope made available through National Center for Research Shared Instrumentation Grant 1 S10 RR16851. This work was supported by grants from the National Institutes of Health Minn CResT T32-DE007288 to L.P., Musculoskeletal Research Training Grant T32-AR050938 to C.J.B, and R01-AR48147 to J.J.W, and R01-DE016601 to A.P.

Chapter 3

Bone Morphogenic Protein 2 Signaling in Osteoclasts is Negatively Regulated by the BMP Antagonist, Twisted Gastrulation

3.1 Overview

Bone morphogenetic proteins (BMPs) have been shown to regulate both osteoblasts and osteoclasts. We have previously shown that BMP2 can directly enhance RANKL-mediated osteoclast differentiation by increasing the size and number of osteoclasts. In our previously published study of mice deficient for the BMP antagonist Twisted gastrulation (*Twsg1*), we showed that the *Twsg1*^{-/-} mice were osteopenic and that this could be explained by an enhancement of osteoclast formation and activity. In vitro studies using osteoclasts isolated from *Twsg1*^{-/-} mice showed increased levels of phosphorylated Smad (pSmad) 1, 5, and 8. We also showed that BMP antagonist Noggin could inhibit osteoclast differentiation and reverse the osteoclast phenotype in *Twsg1*^{-/-} mice. In the present study using an adenoviral vector we demonstrate that overexpressing TWSG1 in primary osteoclasts decreases the size and number of multinuclear TRAP-positive osteoclasts, expression of osteoclast genes and resorption ability. Overexpression of TWSG1 did not affect osteoclast proliferation or apoptosis. However, overexpression of TWSG1 decreased the levels of pSmad 1, 5, and 8 in osteoclasts. Addition of exogenous BMP2 to osteoclasts overexpressing TWSG1 rescued the size, gene expression, and levels of pSmad 1, 5, and 8 compared to cultures infected with a control virus. Finally, TWSG1 overexpression in osteoclasts isolated from the *Twsg1*^{-/-} mice rescued size of the

osteoclasts while further addition of exogenous BMP2 reversed the effect of TWSG1 overexpression and increased the size of the osteoclasts similar to control virus infected cells. Here we demonstrate that overexpressing TWSG1 in osteoclasts via an RGD adenoviral vector results in inhibition of osteoclastogenesis and may provide a potential therapy for inhibiting osteoclast activity in a localized manner.

3.2 Introduction

Mammalian bone is under continuous remodeling in response to altered serum calcium levels, changes in mechanical loading, structural damage and a wide range of paracrine and endocrine factors. Osteoclasts are derived from the monocyte/macrophage lineage and are formed by multiple cellular fusions from their mononuclear precursors (Vaananen and Laitala-Leinonen 2008). They initiate bone remodeling and resorb old bone. Altered osteoclast activity is an important component of various skeletal diseases. Thus, specific inhibition of osteoclast function has become a major strategy to treat osteolytic bone diseases such as osteoporosis, metastatic bone disease, arthritis, and other metabolic bone diseases (Rodan and Martin 2000).

Bone morphogenetic proteins (BMPs) are multi-functional growth factors involved in numerous molecular cascades and signaling pathways. In addition to well-characterized actions on osteoblasts, BMPs are essential for osteoclast differentiation, but their role in the latter process remains incompletely characterized. Reports from our lab and others indicate that osteoclasts express BMP receptors and their ligands, BMP2 and BMP7 (Kanatani et al. 1995; Kaneko et al. 2000; Itoh et al. 2001; Jensen et al. 2010). Treatment of osteoclasts with exogenous BMP2 directly enhances RANKL-stimulated differentiation of osteoclast precursors in vitro and stimulates survival and resorptive activity of

mature osteoclasts. (Kanatani et al. 1995; Kaneko et al. 2000; Itoh et al. 2001; Jensen et al. 2010). Conversely, shRNA knockdown of BMPRII inhibited osteoclast formation (Jensen et al. 2010). BMPs exert their biological activities by signaling through type I and II serine/threonine kinase transmembrane receptors (Itoh et al. 2001; Xiao et al. 2002; Nohe et al. 2004; Cao and Chen 2005). The signaling activity of BMPs is inhibited by secreted molecules such as Chordin (CHRD), Noggin, and Twisted gastrulation (TWSG1), which physically interact with BMPs and limit their ability to activate BMP receptors and downstream signaling pathways (Canalis et al. 2003).

Recently, we described a *Twsg1*-deficient (*Twsg1*^{-/-}) mouse which exhibited an osteopenic phenotype that was attributed to increased osteoclast formation and function (Sotillo Rodriguez et al. 2009). Osteoblast function was not affected in these mice, however osteoclasts from *Twsg1*^{-/-} mice were significantly larger and increased in number compared to osteoclasts from WT mice both in vitro and in vivo. Interestingly the enhanced osteoclastogenesis phenotype seen in the *Twsg1*^{-/-} mice could be recapitulated in osteoclasts from wild type mice following treatment with exogenous recombinant BMP2 and suboptimal amounts of RANKL (Sotillo Rodriguez et al. 2009). Binding of BMPs to their receptor complex leads to phosphorylation of specific intracellular substrates such as receptor-regulated SMAD 1,5 and 8, whereas SMAD 2 and 3

mediate signaling for TGF-betas and the activins (Lieberman et al. 2002). *Twsg1*^{-/-} mice derived osteoclasts had increased levels of phosphorylated SMAD (pSMAD) 1,5 and 8 consistent with increased BMP signaling due to loss of an antagonist (Sotillo Rodriguez et al. 2009). Collectively, these data suggest that TWSG1 attenuates BMP signaling in osteoclasts thereby functioning to limit osteoclast activity and bone remodeling.

The goal of the current study is to further characterize the role of TWSG1 in osteoclastogenesis. We hypothesized that TWSG1 regulates osteoclastogenesis by inhibiting BMP signaling. Overexpressing TWSG1 in WT osteoclast precursors by adenovirus (Ad) transduction significantly reduced the size and activity of the resulting osteoclasts. Addition of increasing amounts of exogenous BMP2 to TWSG1 overexpressing osteoclasts restored their size confirming the opposing activities of TWSG1 and BMP signaling in osteoclasts. Finally, we were able to rescue the increased size phenotype of *Twsg1*^{-/-} osteoclasts by infecting them with the TWSG1 expressing Ad.

3.3 Materials & Methods

3.3.1 Mice

Generation and genotyping of *Twsg1*^{-/-} mice were previously described (Petryk et al. 2004). The use and care of the mice in this study was approved by the University of Minnesota Institutional Animal Care and Use Committee.

3.3.2 Primary osteoclast cultures

Femoral and tibial bone marrow cells were collected from 4-week-old wild-type (WT) or *Twsg1*^{-/-} mice in the 129SvEv background. The tibiae and femora were removed and dissected free of adhering tissues. The bone ends were removed and the marrow cavities flushed by slowly injecting media through one end using a 25-gauge needle. The flushed bone marrow cells were cultured for 3 days on non-tissue culture coated dishes in phenol red-free α -MEM supplemented with 5% heat-inactivated fetal bovine serum, 25 units/mL penicillin, 400 mM L-glutamine, and 10 ng/mL M-CSF (R&D Systems). The adherent cell population, containing the committed osteoclast precursors, were re-plated at 2×10^4 cells/cm² in osteoclast media further supplemented with 60 ng/mL RANKL (R&D Systems) and/or BMP2 (R&D Systems) as indicated. Osteoclast resorption assay was performed on dentine discs as previously described (Sotillo Rodriguez

et al. 2009).

3.3.3 Construction of adenovirus expressing TWSG1

The Ad type 5 (Ad5) vector expressing full length Twsg1 cDNA (Ad-T) contains the CMV promoter-driven TWSG1 transgene cassette inserted in place of the deleted E1 region of a common Ad5 vector. The full-length TWSG1 cDNA expressed in pCMV-Tag 4A vector (NotI and BamHI sites) was cloned into pShuttleCMV plasmid (Davydova et al. 2004) using the NotI and BglIII sites. The resultant plasmid, pShuttleCMV-TWSG1, was linearized with PmeI digestion and subsequently co-transformed into *E. coli* BJ5183 with the RGD fiber-modified Ad backbone plasmid (RGDAdEasy). After selection of recombinants, the recombinant DNA was linearized with PacI digestion and transfected into 911 cells to generate Ad-T. The virus was propagated in 293 cells, dialyzed in phosphate-buffered saline (PBS) with 10% glycerol, and stored at -80°C. Titering was performed with a plaque-forming assay using 911 cells (pfu/mL) and optical density-based measurement (Vp/mL). An identical replication-incompetent CMV promoter-driven luciferase expression vector (Ad-C) was used as a control vector. Detailed information about procedures taken to construct the RGD fiber-modified Ad5 backbone plasmid (RGDAdEasy) and Ad-C control vector is available upon request.

3.3.4 Adenovirus Infection of Osteoclasts

Primary bone marrow cells that had been in M-CSF for 3 days on non tissue culture coated plates were plated in 12-well plate (2.5×10^5 cells/well). Twenty four hours after plating the osteoclasts, the cells were incubated with 10 multiplicity of infection (MOI) of either Ad-T or Ad-C for 3 h at 37°C in the presence of M-CSF and RANKL. After 3 hours, the adenovirus was removed from the osteoclasts and the cells were refed with M-CSF, RANKL and where indicated BMP2 in fresh α -MEM medium. MOI was calculated using the pfu/mL measurement. On day 3 post-infection, total mRNA was extracted for use in determining gene expression by real time RT-PCR or protein was extracted for Western blots. Luciferase activity was determined with the Luciferase Assay System (Promega) 48 hours post infection. Experiments were performed in triplicate and normalized to protein concentration (BioRad).

3.3.5 TRAP Staining

Seven days post infection primary osteoclasts were fixed in 4% paraformaldehyde (PFA). Fixed cells were washed by PBS and stained with acid phosphoric reagents with tartrate (5 mg Naphthol AS-MX phosphate, 0.5 mL N, N-Dimethyl formamide, 50 mL acetic acid buffer (1 mL acetic acid, 6.8 g sodium

acetate trihydrate, 11.5 g sodium tartarate in 1 L water), and 25 mg Fast Violet LB salt. Triplicate samples were used.

3.3.6 Real time RT-PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) and quantitated by UV spectroscopy. cDNA was synthesized using 2 μ g of RNA and the iScript cDNA Synthesis kit (Bio-Rad). PCR reactions, data quantification, and analysis were performed using MyiQ Single-Color real time PCR Detection System (Bio-Rad). Values were normalized to *L4* mRNA. Primer sequences used were NFATc1 (Forward) 5'-TCA TCC TGT CCA ACA CCA AA; (Reverse) 5'-TCA CCC TGG TGT TCT TCC TC; Cathepsin K (Forward) 5'-AGG GAA GCA AGC ACT GGA TA; (Reverse) 5'-GCT GGC TGG AAT CAC ATC TT; DC-STAMP (Forward) 5'-GGG CAC CAG TAT TTT CCT GA; (Reverse) 5'-TGG CAG GAT CCA GTA AAA GG; Twsg1 (Forward) 5'-ATG AAG TCA CAC TAT ATT; (Reverse) 5'-CTG AAT GAG GCA TTT GCT CA. All measurements were performed in triplicate and analyzed using the $2^{-\Delta\Delta Ct}$ method.

3.3.7 Immunofluorescence

To visualize expression of TWSG1 in osteoclasts, cells were grown on glass cover slips and fixed in 4% PFA for 20 minutes. The cells were then

permeabilized in PBS/0.3% Triton X-100 for 5 minutes, blocked in immunofluorescence buffer (3% BSA, 20 mM MgCl₂, 0.3% Tween-20 in PBS) for 20 minutes, and incubated with primary antibodies for 90 minutes in immunofluorescence buffer. Anti-mouse TSG antibody (R&D Systems) was used at 1:200. Cells were washed three times in PBS/0.1% Triton X-100, and then incubated for 30 minutes with Alexa-conjugated secondary antibodies at 1:800 (Invitrogen). After three washes, cells were stained with DAPI, washed, and mounted in 90% glycerol/0.4% N-propyl-gallate. Images were obtained using an Olympus Fluoview 500 confocal microscope.

3.3.8 Immunoblotting

Cell protein lysates were prepared in modified RIPA lysate supplemented with protease inhibitor (Roche) and phosphatase inhibitor cocktails (Sigma-Aldrich). Crude lysates were sonicated and cleared by centrifugation at 12,000 rpm at 4°C. Proteins were resolved by SDS-PAGE, transferred to PDVF membrane (Millipore) and incubated overnight at 4°C with primary antibodies against pSMAD 1,5 and 8 (Cell Signaling), SMAD 5 (Cell Signaling), Caspase-3 (Cell Signaling), α -tubulin (Cell Signaling) and appropriate horseradish peroxidase conjugated secondary antibodies (Santa Cruz). Immunoreactive bands were visualized using ECL Plus substrate (GE Health Systems).

3.3.9 Proliferation Assay

Primary osteoclast cells were plated at 3×10^4 cells/well in 48-well plate and cultured in α -MEM containing 10% FBS and 50 ng/ml M-CSF (R&D Systems). Cells were stimulated with 60 ng/ml RANKL (R&D Systems) and infected with either Ad-C or Ad-T. Each condition was performed in triplicate. Each day osteoclast proliferation was assessed by CellTiter 96 Aqueous One Solution Cell Proliferation kit (Promega) according to the manufacturer's instructions. Absorbance was recorded at 490 nm.

3.3.10 Statistical Analysis

All experiments were run in triplicates and results are expressed as mean \pm SD. One way ANOVA with a Tukey post-hoc test was used to compare data; $p < 0.05$ indicates significance.

3.4 Results

3.4.1 Expression of *TWSG1* in Osteoclasts

We previously showed that *TWSG1* is expressed in differentiated osteoclasts (Sotillo Rodriguez et al. 2009). To further define *Twsg1* expression throughout osteoclast differentiation, we performed real time RT-PCR to measure *Twsg1* mRNA expression in osteoclast precursors undergoing RANKL-stimulated differentiation. *Twsg1* mRNA was present in osteoclasts at day 0 and increased as they matured (Fig. 1A). Immunofluorescence against *TWSG1* confirmed the presence of *TWSG1* protein located throughout the cytoplasm during all stages of osteoclast differentiation (Fig. 1B).

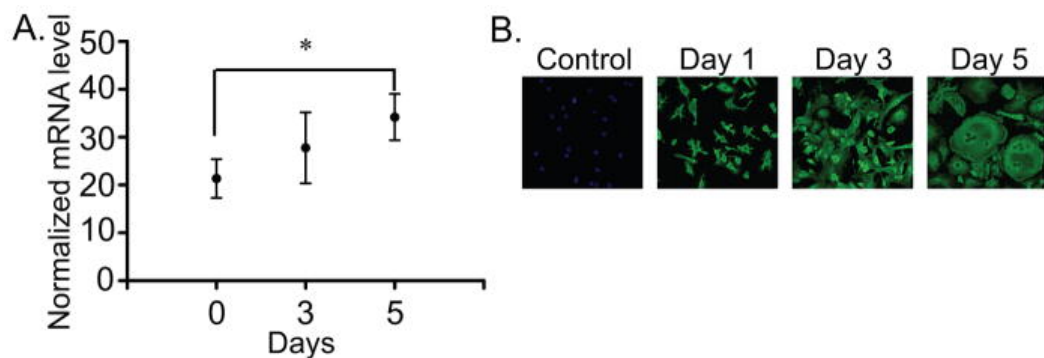


Figure 3.1: Expression of *Twsg1* during osteoclast differentiation. A) Expression profile of *Twsg1* mRNA from M-CSF (day 0) and M-CSF+RANKL treated osteoclast cultures (day 3 and 5). $*p \leq 0.01$ Day 0 compared to Day 5. **B)** *TWSG1*

immunofluorescence staining (green) of 1, 3 and 5 day osteoclast cultures. Cells were co-stained with DAPI (dark blue) to show cell nuclei. Parallel slides from each day were stained without TWSG antibody as a negative control (control).

3.4.2 Construction of Adenovirus Expressing Full Length

TWSG1

We previously showed that osteoclasts from mice null for *Twsg1* were larger in size than osteoclasts from WT mice (Sotillo Rodriguez et al. 2009). To determine the phenotype of osteoclasts when TWSG1 is overexpressed, we generated an Ad that expresses full length TWSG1.

The efficiency of Ad-mediated gene transfer largely depends on the biology virus-cell interaction: the initial high-affinity binding of Ad to the primary coxsackie-adenovirus receptor (CAR) and the interaction between Arg-Gly-Asp (RGD) motifs in the Ad penton base protein and the integrin molecules on the cell surface. To obtain the most effective transduction in osteoclasts, we sought to determine which adenoviral vectors equipped with different genetically modified fibers would overcome low transduction efficiency of CAR deficient cells (Davydova et al. 2004). We infected bone marrow cultures with either 100 or 1000 Vp/cell of fiber-unmodified Ad (Unmod Ad), RGD Ad which has a RGD-4C peptide inserted in the H1 loop of the Ad type 5 (Ad5) fiber knob, CpK Ad which

has a polylysine motif inserted in the Ad5 fiber gene, or 5/3 Ad which expresses a chimeric fiber composed of the Ad5 shaft and the Ad type 3 knob. All of these Ads express luciferase reporter gene and are identical except for their genetically modified fibers (Davydova et al. 2004). We found that bone marrow cultures infected with the RGD or the CpK modified adenovirus at either 100 (black bars) or all the modified adenovirus at 1000 Vp/cell (white bars) expressed greater luciferase activity compared to either the unmodified (100 or 1000 Vp/cell) or the other modified Ad constructs (100 Vp/cell, Figure 2A). It has been reported that RGD modification allows the Ad to use the cellular integrins, thus remarkably increasing the viral infectivity in many cells (Davydova et al. 2004). Based on this report and our observation, we further constructed the TWSG1 expressing Ad5 armed with RGD-modified fiber and applied them to bone marrow cultures.

3.4.3 Characterization of TWSG1 Expressing Ad

We confirmed that *Twsg1* mRNA expression increased in osteoclasts proportionally to increasing MOI (Fig. 2B). Because TWSG1 has been previously shown to function as a BMP antagonist in mammalian systems, we determined whether BMP signaling was reduced by overexpression of TWSG1 in osteoclasts. We found reduced levels of pSMAD 1, 5, and 8 in TWSG1 overexpressing osteoclasts compared to Ad-C infected osteoclasts (compare

lanes Ad-C to Ad-T, Fig. 2C). These observations indicated that infection of osteoclasts with Ad-T results in increased TWSG1 expression and decreased BMP signaling as expected.

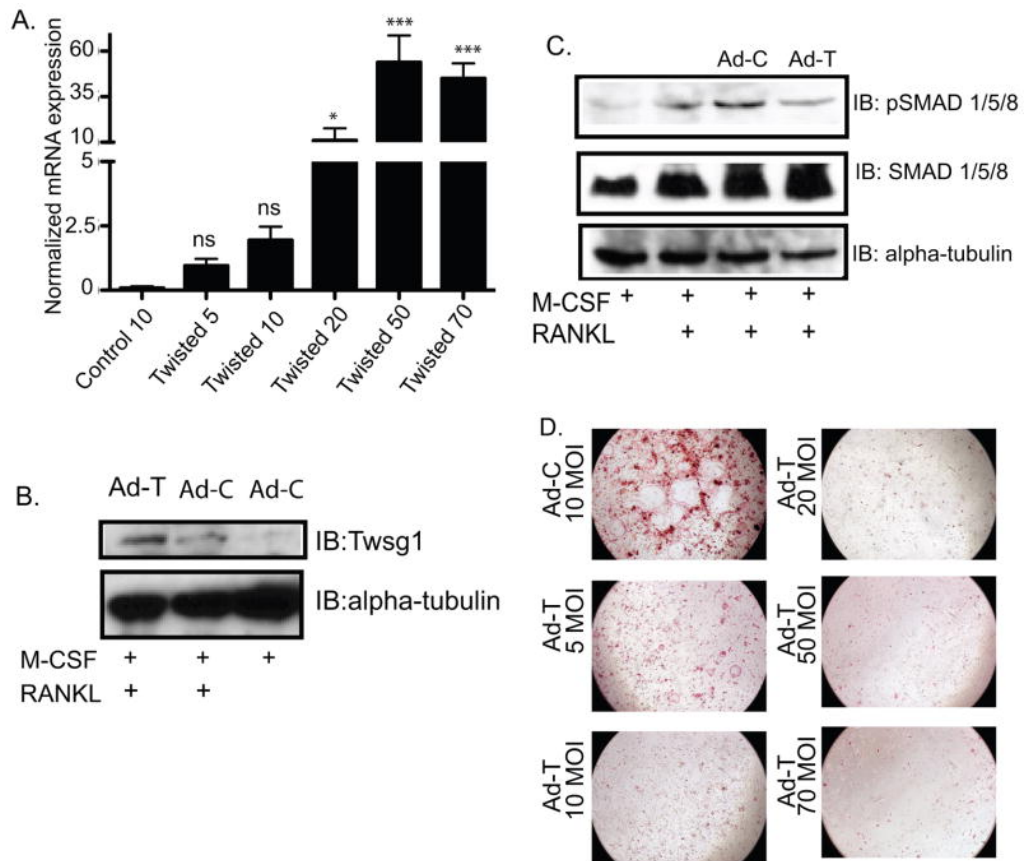


Figure 3.2: Characterization of TWSG1 expressing adenovirus. **A)** Bone marrow cultures infected with fiber-modified Ad at 100 (black bars) or 1000 (white bars) Vp/mL. Luciferase reporter activity is presented as relative luciferase units (RLU) and the results of three experiments each performed in duplicate are presented. * $p \leq 0.01$ unmod compared to RGD or CpK; *** $p \leq 0.0001$ unmod compared to RGD, CpK or 5/3, ns=not significant. **B)** Real time RT-PCR analysis of *Twsg1* expression from osteoclasts infected

with Ad-T at increasing MOIs. Osteoclasts were harvested after incubation with RANKL for 3 days. * $p \leq 0.01$ control compared to Twisted 20, *** $p \leq 0.0001$ control compared to Twisted 50 or 70, ns=not significant. **C)** Immunoblot analysis of osteoclast precursors showing levels of phosphorylated SMAD 1, 5 and 8 and SMAD 5 as a loading control in Ad-C or Ad-T infected osteoclasts. Osteoclasts were harvested after 4 days in the presence of RANKL. **D)** TRAP staining of 7 day osteoclast cultures infected with the indicated MOI of Ad-T.

3.4.4 TWSG1 Inhibits Osteoclastogenesis

We began our characterization of the effect of TWSG1 overexpression on osteoclastogenesis by infecting osteoclast precursors with increasing amounts of the adenoviral vectors, stimulating differentiation by treatment with M-CSF and RANKL, and assessing the resulting multinucleated osteoclasts by TRAP staining. Osteoclasts infected with Ad-T at an MOI of 5 were notably smaller than Ad-C infected cells at the same MOI. Increasing the MOI of Ad-T to 10 gave a strong decrease in OCL size that was not further reduced at higher levels of infection (Fig. 2D). Because a MOI of 10 was the lowest level to give the maximal effect, this level of infection was used in all subsequent experiments.

Quantitative analysis indicated that a MOI of 10, TWSG1-overexpressing osteoclast cultures (Fig. 3A) displayed a significant reduction in the number of TRAP-positive multinucleated cells (Fig. 3B). Further these cells had an average

size of approximately 0.003mm^2 while Ad-C infected cells were significantly larger, averaging 0.015mm^2 (Fig. 3B). For determination of osteoclast size and number only TRAP-positive osteoclasts containing 3 or more nuclei were measured. Ad-T infected osteoclasts also exhibited significantly less resorptive ability when plated on dentine substrates (Fig. 3C). These observations demonstrate that TWSG1 overexpression inhibits osteoclast formation and function.

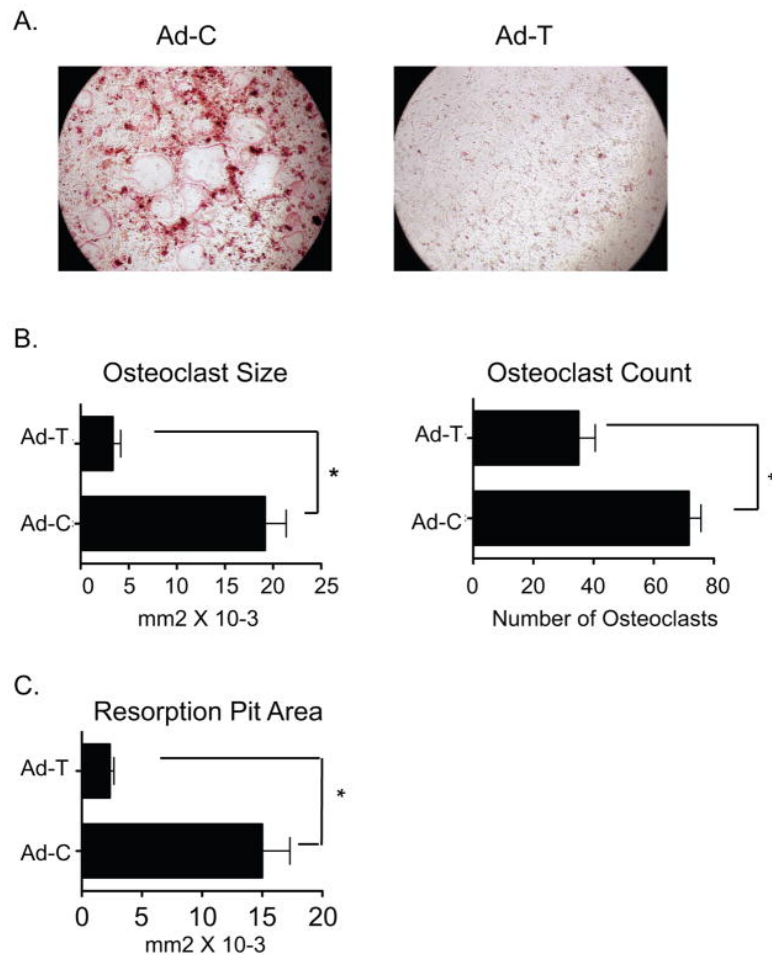


Figure 3.3: TWSG1 overexpression inhibits osteoclastogenesis. A) TRAP staining

of 7 day osteoclast cultures infected with Ad-C or Ad-T at 10 MOI. Note that these are larger images of the cells shown in Figure 2D. * $p < 0.05$ compared to Ad-C **B)** Histomorphometric analysis of TRAP-stained osteoclasts. * $p < 0.05$ compared to Ad-C. TRAP positive osteoclasts containing 3 or more nuclei were counted. **C)** Quantification of resorption pit area of toluidine blue stained dentine discs. Osteoclasts were incubated in the presence of RANKL for 10 days. * $p < 0.05$ compared to Ad-C.

3.4.5 Twsg1 Inhibits Osteoclast Formation Day 1 and 2

We have previously shown that addition of exogenous BMP2 to osteoclast cultures on the third day after the addition of RANKL enhances formation of TRAP positive cells (Jensen et al. 2010). To determine the effects of overexpressing Twsg1 in osteoclasts at different times after addition of RANKL, infections were performed on osteoclasts at different days of differentiation and then the cultures were TRAP stained. TRAP analysis shows that there was a significant difference in size and number of osteoclasts when virus was added on day 1 or day 2 after the addition of RANKL compared to cells infected with either the control virus or when virus was added on day 3 after the addition of RANKL (Figure 4). However, there was no significant change in the number or size of TRAP positive osteoclasts compared to the osteoclasts infected with the control virus when virus was added 3 days after the addition of RANKL (Figure 4B, compare Ad-C and Ad-T d3).

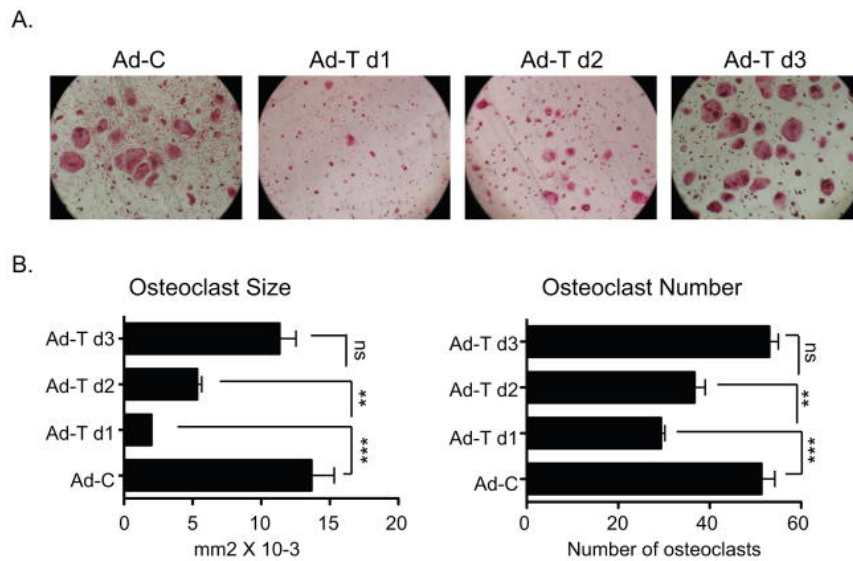


Figure 3.4: TWSG1 inhibits osteoclastogenesis on day 1-2 after RANKL addition.

A) TRAP staining of 7 day osteoclast cultures infected with either Ad-C or Ad-T at 1 day (d1), 2 days (d2) or 3 days (d3) after the addition of RANKL to the cultures. **B)** Histomorphometric analysis of TRAP-stained osteoclasts. TRAP stained osteoclasts with 3 or more nuclei were counted. ** $p \leq 0.01$ Ad-C compared to Ad-Td2 and *** $p \leq 0.0001$ Ad-C compared to Ad-d1, ns=not significant.

3.4.6 TWSG1 does not Alter Apoptosis of Proliferation of

Osteoclasts

One possible explanation for the impaired osteoclast formation in TWSG1 overexpressing cultures is that TWSG1 either induced cell death or interfered with cell proliferation. Cleavage of caspase-3 is a crucial step of programmed cell death (Porter and Janicke 1999). We observed an increase of cleaved caspase-3

in cells treated with RANKL (Fig. 5A), which is consistent with earlier work demonstrating that RANKL can induce apoptosis (Bharti et al. 2004). Importantly, no difference was observed in the amount of cleaved caspase-3 between osteoclasts infected with Ad-C or Ad-T (Fig. 5A, compare Ad-C+RANKL and Ad-T+RANKL). This suggests that Ad-T does not induce more apoptosis than that observed in cells infected with the Ad-C and the decrease in formation of TRAP-positive multinuclear cells seen in Figure 3 is not due to the osteoclasts undergoing apoptosis.

We next tested whether the effects of TWSG1 overexpression could result from decreased proliferation of osteoclast precursors. Osteoclast cultures were treated with M-CSF alone, M-CSF+RANKL, M-CSF+RANKL+Ad-C, or M-CSF+RANKL+ Ad-T. Cell number was determined using an MTS-based assay after 24, 48, 72, and 98 hours. We observed a slight decrease in proliferation when RANKL was added to the cells, which is consistent with work done by others demonstrating that RANKL can suppress cell propagation (Bharti et al. 2004). Interestingly, infecting osteoclasts with Ad-T does not interfere with osteoclast proliferation when compared to Ad-C (Fig. 5B). These results suggest that TWSG1 overexpression does not inhibit osteoclast formation by affecting proliferation.

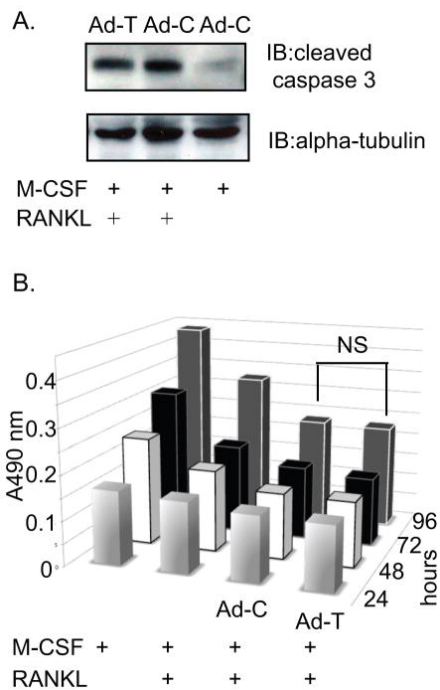


Figure 3.5: Overexpressing TWSG1 does not affect osteoclast apoptosis or proliferation. **A)** Immunoblot analysis of cleaved caspase-3 levels and α -tubulin as a loading control from Ad-C or Ad-T infected osteoclast cultures. Osteoclast cultures were incubated in the presence of RANKL for 3 days. **B)** Bone marrow cultures were infected with Ad-C or Ad-T adenovirus and treated as indicated. Cell number was determined on each day from triplicate samples using the Cell Titer 96 Aqueous One Solution Assay. ns=not significant.

3.4.7 TWSG1 Reduces Osteoclast Gene Expression

Real time RT-PCR validated the enhanced expression of *Twsg1* (approximately 5 fold increase compared to Ad-C+RANKL) in infected cultures

but also revealed a marked reduction in osteoclast markers (Fig. 6, compare Ad-C+RANKL and Ad-T+RANKL): *Nfatc1* (2 fold decrease compared to Ad-C+RANKL, $p<0.05$), *Cathepsin K* (*Ctsk*, 2 fold decrease compared to Ad-C+RANKL, $p<0.05$), and *DC-STAMP* (2.5 fold decrease compared to Ad-C+RANKL, $p<0.05$). These data suggest that TWSG1 is affecting osteoclast differentiation by changing gene expression.

3.4.8 BMP2 Rescues TWSG1 Overexpressing Osteoclasts

If TWSG1 inhibits osteoclast formation by inhibiting BMP signaling, we predicted that addition of BMP2 should rescue the osteoclast phenotype of TWSG1 overexpression. Osteoclast cultures were infected with Ad-C or Ad-T, followed by treatment with RANKL and either 20 ng/mL (low dose) or 80 ng/mL (high dose) of BMP2. As was previously described, TWSG1 overexpression reduced pSMAD 1, 5, and 8 levels. However, addition of increasing amounts of BMP2 increased pSMAD 1, 5, and 8 levels (Fig. 7A, compare Ad-T, Ad-T+20- or Ad-T+80 ng/mL BMP2). When cells were treated in this manner and stimulated to undergo differentiation with RANKL, BMP2 restored both the number and size of multinucleated TRAP-positive osteoclasts (Fig. 7B). Furthermore, BMP2 reversed the TWSG1-mediated repression of osteoclast markers, *Nfatc1*, *Ctsk*, and *DC-STAMP* in a dose dependent manner, but had no effect on *Twsg1* expression

(Fig. 7C, compare 3 with 4-5). These results further indicate that TWSG1 inhibits osteoclast differentiation by inhibiting BMP signaling.

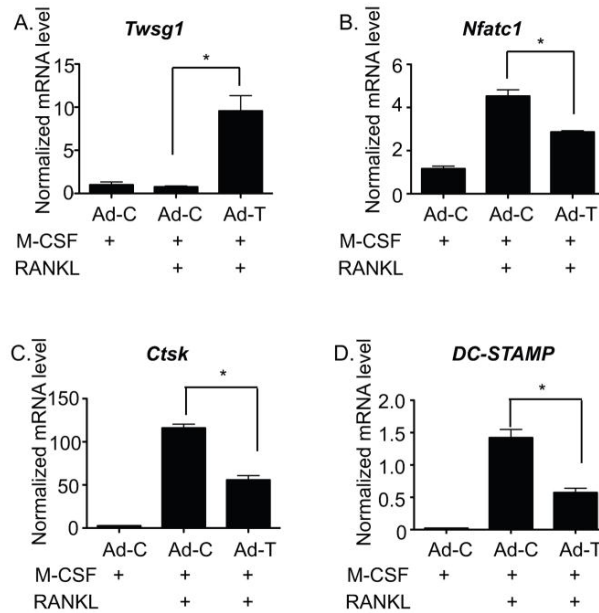


Figure 3.6: Regulation of osteoclast gene expression and activity by TWSG1.

Expression profile of **A) *Twsg1***, **B) *Nfat-c1***, **C) *Ctsk***, and **D) *DC-STAMP*** mRNA from osteoclast cultures infected with Ad-C or Ad-T. Osteoclast cultures were incubated in the presence of RANKL for 3 days. * $p \leq 0.05$ compared to Ad-C.

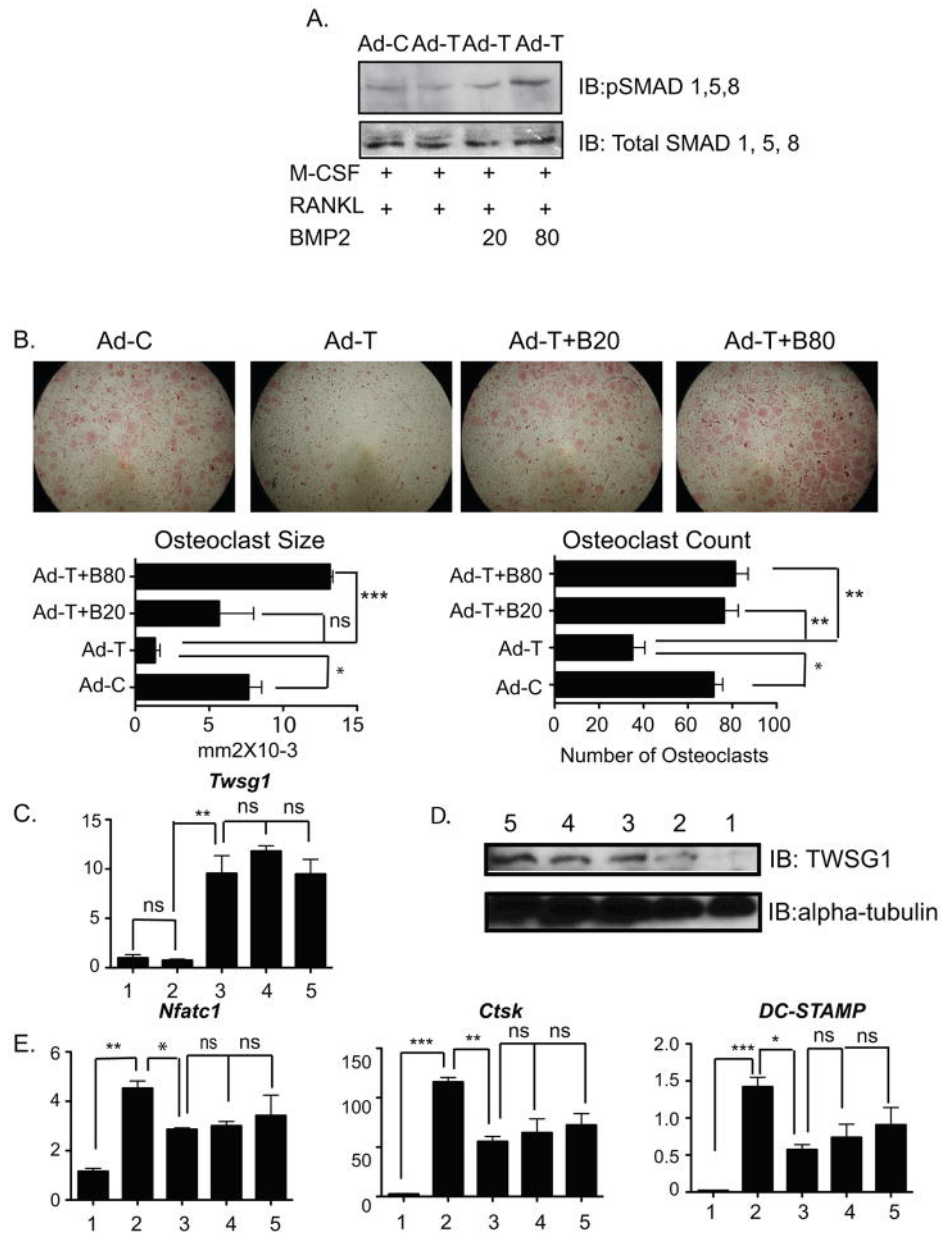


Figure 3.7: BMP2 reverses the effects of TWSG1 overexpression. A) Immunoblot analysis of osteoclast precursors showing protein expression of levels of pSMAD 1, 5 and 8 in Ad-C or Ad-T osteoclasts and treated with BMP2 where indicated. Osteoclasts were incubated in the presence of RANKL and BMP2 for 3 days. **B)** TRAP staining and

histomorphometry of 7 day osteoclast cultures infected with Ad-C or Ad-T. Where indicated in the figure osteoclasts were also treated with either 20 or 80 ng/mL of recombinant BMP2. TRAP positive osteoclasts with 3 or more nuclei were counted. * $p < 0.05$ compared to Ad-C, ns = not significant . **C)** Expression profile of *Twsg1*, *Nfat-c1*, *CtskK*, and *DC-STAMP* mRNA from osteoclast cultures infected with Ad-C (1), Ad-C + RANKL (2), Ad-T +RANKL (3), Ad-T + RANKL+ BMP2 (20 ng/mL,4) or Ad-T+RANKL+BMP2 (80 ng/mL,5). Osteoclasts were incubated in the presence of RANKL and BMP2 for 3 days. * $p < 0.05$ compared to Ad-C, ns=not significant.

3.4.9 TWSG2 Overexpression Rescues the In Vitro Osteoclast

Phenotype from Twsg1 Null Mice

Next, we determined the effect of TWSG1 overexpression in *Twsg1*^{-/-} primary osteoclasts. TWSG1 overexpression rescued the *Twsg1*-knockout phenotype by restoring the infected osteoclasts to both size and number similar to that of WT cells (Fig. 8). Further, the rescue of *Twsg1*^{-/-} osteoclasts by TWSG1 overexpression was reversed by addition of increasing amounts of BMP2, which progressively increased both osteoclast size and number. These results confirm that the phenotype of *Twsg1*^{-/-} osteoclasts arises due to up-regulated BMP signaling and indicate that the balance between pro- and anti-BMP activities regulates osteoclast size.

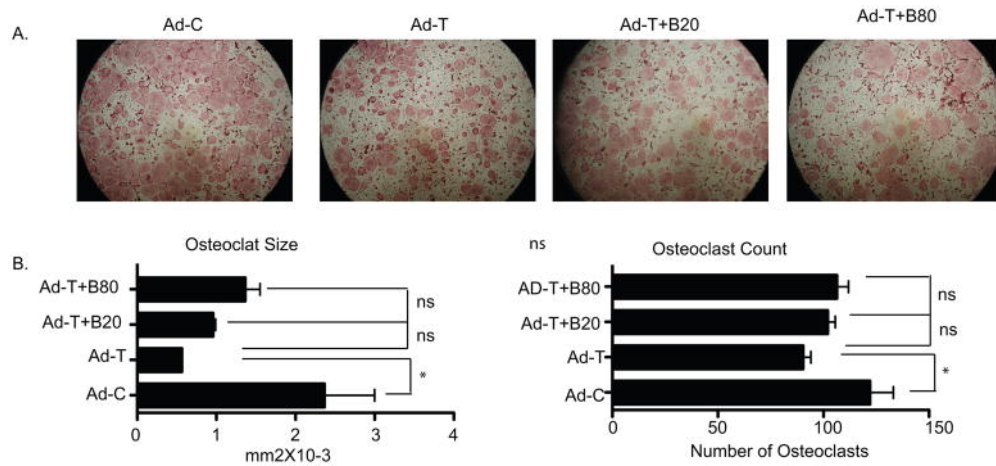


Figure 3.8: Antagonistic activity of TWSG1 overexpression and BMP2 on *Twsg1*^{-/-} osteoclasts. TRAP staining and histomorphometry of 7 day osteoclast cultures from *Twsg1*^{-/-} mice infected with Ad-C or Ad-T. Where indicated in the figure osteoclasts were also treated with either 20 or 80 ng/mL of recombinant BMP2. TRAP positive osteoclasts with 3 or more nuclei were counted. * $p < 0.05$ compared to Ad-C, ns=not significant.

3.5 Discussion

We previously showed that osteoclasts from mice deficient for *Twsg1* expression were more numerous and larger than osteoclasts from their WT littermates. In the current report we present experiments that confirm the inhibitory role of TWSG1 in osteoclast differentiation. We show that TWSG1 is expressed in osteoclasts at all stages during differentiation both by real time PCR and immunofluorescence. Overexpression of TWSG1 significantly decreased both the size and number of multinuclear TRAP-positive cells and inhibited pSMAD 1, 5, and 8 signaling. Conversely, addition of exogenous BMP2 rescued the diminished osteoclast phenotype following TWSG1 overexpression. These results are consistent with our previous data in which treatment of osteoclasts with Noggin inhibited their differentiation (Sotillo Rodriguez et al. 2009; Jensen et al. 2010). Thus experiments using either Noggin or TWSG1 indicate that decreased BMP signaling impairs osteoclast fusion. The hypothesis that expression of TWSG1 impairs osteoclast fusion was supported by data showing that proliferation and apoptosis were not different in osteoclasts infected with Ad-T compared to Ad-C (Fig. 5). The proliferation and apoptosis data indicate that the number of osteoclast precursors was not increased or decreased but that the reduced multinuclear TRAP positive osteoclasts was due to less fusion of the precursors. Taken together our data further supports the hypothesis that TWSG1

negatively regulates osteoclastogenesis by disrupting pro-osteoclastic BMP signaling.

In our present study we show that TWSG1 overexpression inhibits RANKL-induced osteoclast differentiation. Consistent with this, we previously showed that BMP2 treatment of osteoclasts enhanced RANKL-stimulated osteoclast differentiation, although BMP2 did not support differentiation in the absence of RANKL (Sotillo Rodriguez et al. 2009; Jensen et al. 2010). The precise mechanism by which BMP signaling regulates osteoclasts remains unknown; from our data and other studies we speculate that BMP signals may influence responses to RANKL signaling. In addition to classical SMAD signaling pathways, BMPs activate mitogen activated protein kinase (MAPK) pathways ERK, JNK, and p38 in some cases acting through the TAK1/TAB1 pathway. RANKL has also been shown to activate JNK and p38 MAPK through TAK1. Thus we speculate that BMPs may regulate osteoclast fusion through SMAD and MAPK signaling pathways.

TWSG1 in mammalian systems has been shown to act as a BMP antagonist. When we infected osteoclasts after 3 days in the presence of RANKL, we were not able to inhibit osteoclast differentiation by overexpression of TWSG1. We hypothesize that BMP signaling is critical during an early stage of osteoclast differentiation. We were able to rescue the osteoclast phenotype

following overexpression of TWSG1 by adding increasing amounts of BMP2. We also report that overexpression of TWSG1, rescues the in vitro osteoclast phenotype in *Twsg1*^{-/-} mice. As further confirmation of TWSG1s' role in regulating BMP signaling, we were again able to reverse the reduction in size seen with TWSG1 infection. These results suggest that BMP-signaling directly promotes the differentiation of mature osteoclasts and further clarifies a novel anti-resorptive function for TWSG1 by modulating BMP-signaling.

TWSG1 has been shown in other invertebrate and vertebrate systems to act as a BMP antagonist by forming a ternary complex with CHRDL1, a cysteine rich protein, and BMPs. Other cysteine rich (CR) proteins have been shown to modulate TGF- β /BMP signaling and these CR proteins may also modulate TWSG1's interaction with BMPs (Larrain et al. 2001; Oelgeschlager et al. 2003). It is not known if the mechanism by which TWSG1 inhibits BMP signaling during osteoclast differentiation is through its interaction with CHRDL1 or other CR proteins.

Due to their ability to inhibit bone resorption, bisphosphonates are widely used effectively to treat widespread osteolytic bone diseases such as osteoporosis and metastatic bone disease. However, bisphosphonates are less desirable for treatment of localized osteolytic diseases such as periodontitis. Therefore, local delivery of genes that can inhibit osteoclast function may provide

an effective alternate. Gene therapies rely on the delivery of foreign DNA into cells. This has led to the engineering of efficient delivery systems, such as adenoviruses, retroviruses, or lentiviruses into cells. Traditional viral systems, including retrovirus and lentivirus systems, have limited therapeutic use as their DNA integrates into the host genome, which may cause unwanted side effects. Using a robust adenoviral delivery systems to administer TWSG1 transiently, locally, and non-evasively to the cells or tissues may prove to be an efficient and clinically relevant anti-resorptive tool to combat local aberrant bone loss. Specifically, the use of an RGD modified adenovirus system, like the one used in this report, has additional advantages that can allow for lower vector dose to achieve similar therapeutic effects while preventing inflammatory responses to the vector. Here we demonstrate that overexpressing TWSG1 to osteoclasts in vitro via an RGD adenoviral construct results in an inhibition of osteoclast formation.

The balance between bone formation and resorption at sites of bone remodeling is regulated by autocrine and paracrine factors such as BMPs. In addition to multiple important physiological roles in regulating bone formation, BMPs are used clinically to promote localized bone growth and healing in a number of orthopedic and maxillofacial applications (Kirker-Head et al. 2007). In vitro application of BMPs during orthopedic procedures has been found to

promote a transient increase in osteoclast numbers and osteoclastic bone resorption in some instances (Toth et al. 2009). Our data provide a mechanistic explanation for this clinical observation and suggest rational strategies to minimize inappropriate activation of osteoclasts in this context and in other pathological skeletal conditions.²

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Chapter 4

HDAC3 and HDAC7 Have Opposite Effects on Osteoclast Differentiation

4.1 Overview

Histone deacetylases (HDACs) are negative regulators of transcription. Endochondral bone formation including chondrocyte and osteoblast maturation is regulated by HDACs. Very little is known about the role HDACs play in osteoclast differentiation. It has been previously reported that HDAC inhibitors (HDIs), trichostatin A and sodium butyrate, suppress osteoclast differentiation through multiple mechanisms. In this study, we report that suppression of HDAC3 expression similar to HDIs inhibits osteoclast differentiation while osteoclasts suppressed for HDAC7 expression had accelerated differentiation compared to control cells. Mitf a transcription factor is necessary for osteoclast differentiation. We demonstrate that Mitf and HDAC7 interact in RAW 264 cells and osteoclasts. Mitf's transcriptional activity is repressed by HDAC7. Lastly, we show that either the amino or carboxy terminus of HDAC7 is sufficient for transcriptional repression and that HDAC7's repression is insensitive to TSA, indicating that HDAC7 represses Mitf at least in part by deacetylation-independent mechanism.

4.2 Introduction

Formation of the skeleton is a complex process involving coordinated function of osteoclasts and osteoblasts. Subsequently, bone homeostasis is maintained by osteoblasts, which synthesize mineralized bone, and osteoclasts, highly specialized multinuclear cells that resorb bone. Many skeletal diseases such as osteoporosis, Paget's disease of bone, rheumatoid arthritis, and bone metastases arise from an imbalance in the relative activities of osteoblasts and osteoclasts.

Histone deacetylases are best known for promoting transcriptional repression and silencing through the removal of acetyl groups from histone core proteins at target gene promoters, resulting in a less transcriptionally active state (Sengupta and Seto 2004). They also catalyze deacetylation of non-histone substrates, thereby regulating the stability or activity of their substrates (Gregoretta et al. 2004). The eighteen human HDACs fall into four classes based on structural and biochemical characteristics. Class I HDACs include HDACs 1, 2, 3, and 8 while class II HDACs include 4, 5, 6, 7, 9 and 10. Class II HDACs are further subdivided into two subclasses, IIa and IIb. Class IIa HDACS (HDAC 4, 5, 7 and 9) can also interact with additional non-deacetylase co-repressor proteins to bring about transcriptional repression (Sengupta and Seto 2004). Broad-spectrum HDAC inhibitors (HDIs) such as trichostatin A (TSA) and sodium

butyrate (NaB) are thought to have an effect on cells by inhibiting of HDACs. TSA is generally considered a nonspecific HDAC inhibitor, as it has similar K_i for all isoforms examined (Drummond et al. 2005) and NaB has been shown to inhibit all class I and II HDACs.

A number of HDACs regulate chondrocyte and osteoblast differentiation and activity through interactions with transcription factors such as Runx2, Smads, Twist and pRb (Luo et al. 1998; Westendorf et al. 2002; Schroeder et al. 2004; Kang et al. 2005; Jeon et al. 2006; Lee et al. 2006; Westendorf 2006; Hayashi et al. 2007). Interactions between HDACs and Runx2 inhibit Runx2's activity thereby suppressing osteoblast differentiation (Schroeder et al. 2004; Jensen et al. 2008), while HDIs accelerate osteoblastic differentiation (Schroeder et al. 2004).

Very little is known about how HDACs regulate osteoclast gene expression and differentiation. HDIs TSA and NaB have been shown to inhibit receptor activator of NF- κ B ligand (RANKL)-mediated osteoclast differentiation due to inhibition of c-fos expression, NF- κ B dependent transcription and p38 MAP kinase activity (Rahman et al. 2003; Kim et al. 2009). HDAC1 is recruited to the promoters of osteoclasts genes by STAT3 and Eos/Mitf/Pu.1 complex (Hu et al. 2007; Kim et al. 2007). Hu et al. demonstrated that co-repressors CtBP, HDAC1 and Sin3A were present on Ctsk and Acp5 promoters when osteoclast

precursors were stimulated with macrophage colony stimulating factor (M-CSF) but their levels were significantly reduced following 3 days combined stimulation with M-CSF and RANKL (Hu et al. 2007). The cytokines RANKL (Wada et al. 2006) and M-CSF are necessary and sufficient for osteoclast differentiation. The combination of these two factors activates transcription factors such as Nfatc1, Mitf, PU.1 and c-fos (Matsuo et al. 2000; Weilbaecher et al. 2001; Mansky et al. 2002; Takayanagi et al. 2002; Boyle et al. 2003), which are necessary for osteoclast differentiation. Mitf belongs to the MiT family of basic helix-loop-helix transcription factors that regulate gene expression in a variety of cell types including melanocytes, macrophages and osteoclasts (Hemesath et al. 1994). The MiT family includes Mitf, Tfe3, Tfeb and Tfec (Hershey and Fisher 2004; Steingrimsson et al. 2004). The importance of Mitf in osteoclast differentiation is confirmed by the lack of osteoclast differentiation and the resulting osteopetrotic phenotype observed in mice homozygous for the Mitf (mi) allele. Similar osteopetrotic phenotype is also noted in the double mutants homozygous for the Mitf (vga) hypomorphic allele and the Tfe3 null allele (Steingrimsson et al. 1994; Steingrimsson et al. 2002; Hershey and Fisher 2004). Recent results indicate that the Mitf complex integrates signals necessary for the appropriate temporal regulation of osteoclast genes such as Ctsk and Acp5 during differentiation. M-CSF signaling alone can regulate Mitf nuclear localization and recruitment of Mitf

to target promoters (Bronisz et al. 2006). However, Mitf does not activate gene expression with stimulation with M-CSF alone. Rather, combined stimulation with M-CSF and RANKL is required to induce expression of osteoclast differentiation genes (Sharma et al. 2007).

In the current work we demonstrate that suppression of HDAC3 by shRNA closely mirrors the inhibitory effect of HDIs on osteoclast formation. Unexpectedly, we show that suppression of HDAC7 has the opposite effect, enhancing osteoclast formation. Further experiments support a model in which HDAC7 inhibits osteoclast differentiation by repressing Mitf activity. Lastly we show that repression of Mitf by HDAC7 is deacetylation-independent.

4.3 Materials and Methods

4.3.1 Cell Culture, Luciferase Assays and Transfections

Osteoclasts were isolated from bone marrow of mice as previously described. Bone marrow was flushed from femurs and the resulting cells were cultured for 3 days in the presence of 50 ng/mL of M-CSF on non-tissue culture coated dishes. The adherent cell population, containing the osteoclasts, was cultured for the indicated time and amounts of M-CSF and RANKL. RAW 264.7 c4 cells were grown in DMEM supplemented with 10% FBS, 25-U/ml penicillin, 25 mg/mL streptomycin. RAW264.7 c4 are a cell clone derived in Dr. Ian Cassidy's lab at the University of Queensland from commercially available RAW 264.7 cells (American Type Culture Collection, Manassas, VA) that requires both M-CSF and RANKL for efficient differentiation into osteoclast-like cells, but not for growth or survival. These cells were a gift obtained from Dr. A.Ian Cassady and Dr. David Hume. The conditions for differentiating RAW 264.7 c4 cells into osteoclasts-like cells were previously described (Bronisz et al. 2006; Meadows et al. 2007). Differentiation of RAW 264.7c4 and osteoclasts were optimized using 10 ng/mL of M-CSF and 60 ng/mL of RANKL (R &D Systems). NIH 3T3 and 293T cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum, 2% l-glutamine and 0.5% penicillin/streptomycin

(Invitrogen). NIH 3T3 and 293T cells were transiently transfected by Lipofectamine Plus Reagent (Invitrogen) according to the instructions of the manufacturer. The luciferase activities were measured using 5X Cell Lysis Buffer (Promega) according to the instruction of the manufacturer.

4.3.2 Antibodies and Chemicals

Polyclonal Mitf antibody was generated by 21st Century Biochemicals (Marlboro, Mass) using a peptide containing mouse Mitf amino acids 85-96 as an immunogen. HDAC7 antibody (clone A7), myc (clone 9E10), gal4 DNA binding domain (clone RK5C1) and actin (clone I-19) were purchased from Santa Cruz Biotechnology and HDAC3 (clone 7G6C5), histone H3 (9715) were purchased from Cell Signaling and acetylated-histone H3 (06-599) was purchased from Upstate/Millipore. M-CSF and RANKL were purchased from R & D Systems (Minneapolis, MN) and used at 10 ng/mL (M-CSF) or 60 ng/mL (RANKL). Trichostatin A (TSA) was used at 20 nM (Sigma).

4.3.3 Immunoprecipitation and Western Blotting

Twenty-four hours after transfection, the 293T were harvested in NP-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40 and protease and phosphatase inhibitors). Extracts were incubated with target

antibody and Protein A/G beads (Pierce) overnight at 40C. Next day immunoprecipitates were washed three times in lysis buffer. The bound proteins were resuspended in sample buffer and resolved by SDS-PAGE. The resolved proteins were transferred to PVDF membrane, blocked and blotted in primary antibody overnight at 40C. The next day the blot was incubated with horseradish peroxidase anti-rabbit or anti-mouse (GE Healthcare) for one hour at room temperature. Antibody binding was detected using ECL system (GE Healthcare). Plasmids- FLAG Mitf was previously described (Bronisz et al. 2006.) FLAG HDAC7 and FLAG HDAC7 1-478 and 472-912 were previously described (Jensen et al. 2008). pMI-Mitf contains Mitf amino acid residues 1-185 cloned into an expression plasmid fusing the Gal4 DNA binding domain to the amino terminus of Mitf.

4.3.4 RNA Isolation and Real Time PCR

RNA was extracted using TRIzol (Invitrogen). 2 µg of purified total RNA was reverse transcribed by iScript cDNA Synthesis kit (Bio-Rad). Quantitative real-time RT-PCR was performed using MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) using 1 µL of the cDNA with 2X iQ SYBR Green supermix (Bio-Rad). Osteoclast genes were normalized to L4 RNA. Primer sequences used were NFATc1 (Forward) 5'-TCA TCC TGT CCA ACA CCA AA;

(Reverse) 5'-TCA CCC TGG TGT TCT TCC TC; Cathepsin K (Forward) 5'-AGG GAA GCA AGC ACT GGA TA; (Reverse) 5'-GCT GGC TGG AAT CAC ATC TT; DC-STAMP (Forward) 5'-GGG CAC CAG TAT TTT CCT GA; (Reverse) 5'-TGG CAG GAT CCA GTA AAA GG; HDAC3 (Forward) 5'-CTG GCT TCT GCT ATG TCA AC; (Reverse) 5'-ACA TAT TCA ACG CAT TCC CCA; HDAC7 (Forward) 5'-TGA AGA ATG GCT TTG CTG TG; (Reverse) 5'-CAC TGG GGT CCT GGT AGA AA. All measurements were performed in triplicate and analyzed using the $2^{-\Delta\Delta Ct}$ method.

4.3.5 Lentiviral Infection on Osteoclasts

Lentiviral vectors encoding shRNAs against HDAC7 (Jensen et al. 2008) or, HDAC3 (Open Biosystems) or a control shRNA, were used to produce replication defective lentivirus according to the manufacturer's protocols. Viral stocks were titrated by infection in HeLa cells. Following infection, primary osteoclast cultures were stimulated with M-CSF and RANKL.

4.3.6 TRAP Staining

Cells were rinsed in PBS, fixed in 4% paraformaldehyde for 20 min and stained using the TRAcP5b kit (Sigma–Aldrich). Cells were photographed and

analyzed using Adobe Photoshop to measure the number and size of TRAP-positive osteoclasts.

4.3.7 Counting Nuclei

After cells were TRAP stained and photographed, cells were stained with DAPI (Molecular Probes) for 5 minutes at room temperature. Cell were rinsed and stained with Vybrant cell-labelling dye (Molecular Probes) for 15 minutes at 37°C. Cells were photographed at 10X magnification and counted using Adobe Photoshop.

4.3.8 Statistical Analysis

All experiments were run in triplicates and results are expressed as mean \pm SD. Each experiment was done at least three times and the error bars represent independent experiments. Student's t-tests or a 1 way ANOVA analysis followed by a Tukey's multiple comparison test were used to compare data; $p < 0.05$ indicates significance.

4.4 Results

4.4.1 Suppression of HDAC3 inhibits osteoclast differentiation

Previous reports indicated that the non-selective HDAC inhibitors TSA and NaB inhibit osteoclast formation (Rahman et al. 2003). However, these data do not indicate the significance of individual HDACs to osteoclastogenesis. In light of its importance in regulating osteoblastic differentiation (Schroeder et al. 2004; Razidlo et al. 2010), we asked how HDAC3 affects osteoclast formation. To this end, we made use of lentiviral vectors encoding shRNAs against HDAC3 or a control shRNA. To ensure that any effects were due to specific knockdown of HDAC3, we compared two distinct shRNAs against HDAC3. Infection of murine bone marrow cultures with either HDAC3 shRNA reduced HDAC3 mRNA expression by approximately 50% compared to the control shRNA (Fig. 1A). We observed a similar reduction in HDAC3 protein levels when we infected RAW 264.7 cells (Fig. 1B). While control shRNA cultures readily formed TRAP-positive osteoclasts upon stimulation with RANKL, osteoclast formation was strongly reduced in cultures infected with either HDAC3 shRNA (Fig 1C). Quantitative analysis of these cultures indicated that both the number and size of TRAP-positive cells was significantly reduced in HDAC3-shRNA cultures compared to the controls (Fig 1D-E). Similarly, quantitative RT-PCR analysis revealed that

expression of osteoclast marker genes NFAT-c1, cathepsin K (Ctsk) and DC-STAMP were reduced in the HDAC3 shRNA cells (Fig. 1F). These data indicate that reduction of HDAC3 expression, like treatment with HDIs, impairs osteoclastogenesis and suggest that HDAC3 activity is necessary for osteoclast formation.

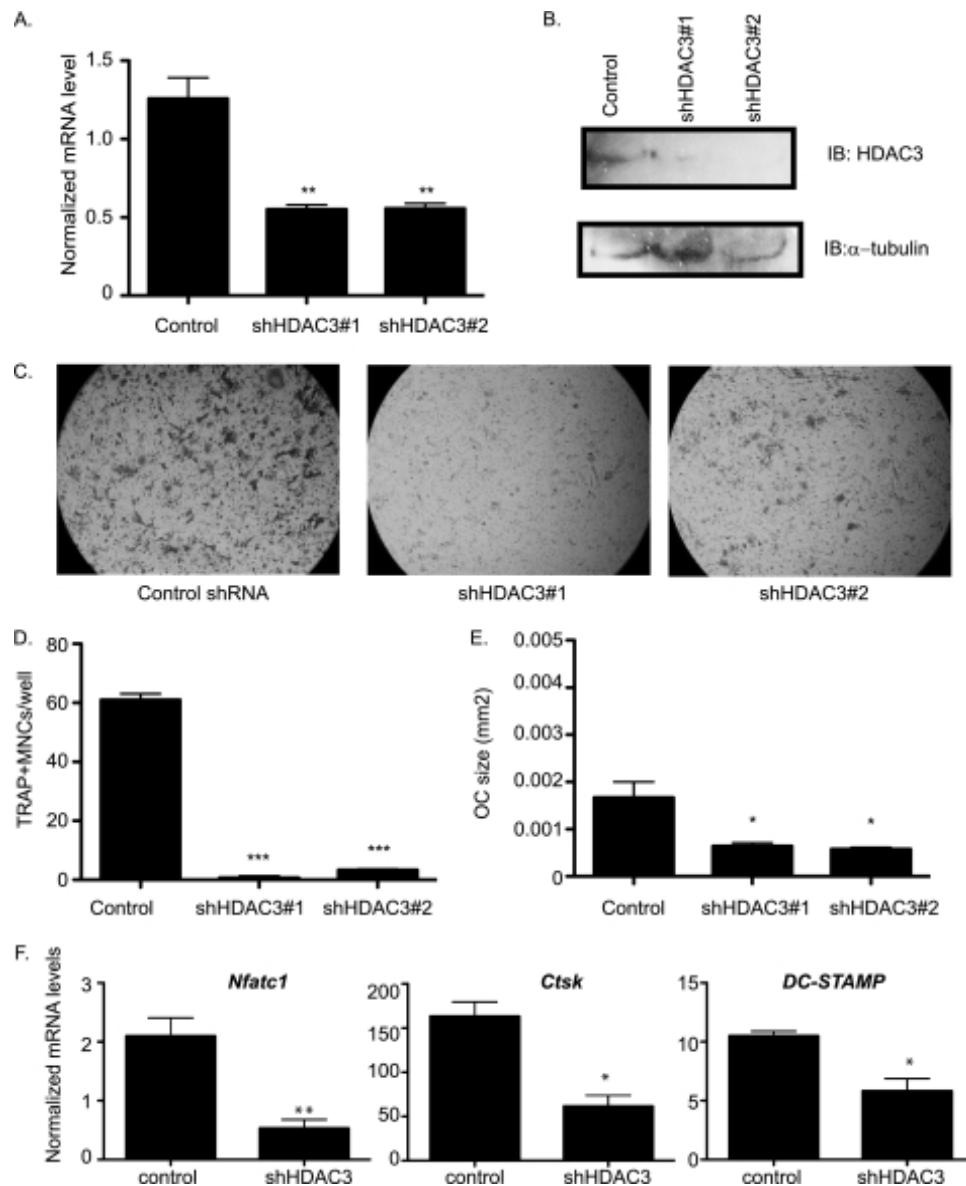


Figure 4.1: Suppression of HDAC3 inhibits osteoclast differentiation. **A)** Real time RT-PCR of bone marrow. **, $p \leq 0.007$ vs. control shRNA. **B)** Western blot (IB) of RAW 264.7 lysates showing expression of HDAC3. **C)** TRAP staining of osteoclast cultures. **D)** and **E)** Histomorphometric analysis of TRAP stained osteoclasts (OC). MNCs, multinucleated cells. ***, $p \leq 0.0001$ and *, $p \leq 0.05$ vs. control shRNA. **F)** expression profile of *Nfat-c1*, *Ctsk* and *DC-STAMP*. *, $p \leq 0.05$ and **, $p \leq 0.005$ vs. control shRNA following infection with control shRNA or HDAC3 shRNA lentiviral vectors.

4.4.2 HDAC7 activity inhibits osteoclast differentiation

We next examined the effect of suppressing expression of the Class II deacetylase, HDAC7. Infection of bone marrow cultures with the previously described HDAC7 shRNA vector (Jensen et al. 2008) or a HDAC7 shRNA purchased from Open Biosystems reduced HDAC7 mRNA levels by 16 or 10-fold respectively compared to the control vector (Fig. 2A), and HDAC7 protein levels showed a clear reduction (Fig. 2B). In contrast to the HDAC3-suppressed cells, osteoclast differentiation in HDAC7 suppressed cultures was enhanced compared to controls (Fig. 2C). The average size of TRAP-positive multinucleated osteoclasts in HDAC7-suppressed cells was increased 5.6 and 5.8-fold (Fig. 2D), and the mean number of TRAP-positive multinucleated cells per well showed a 1.3 and 2.3-fold increase (Fig. 2E). Real time RT-PCR demonstrated that HDAC7-suppressed osteoclasts showed increased expression

of osteoclast markers: *Nfat-c1* (3.5 fold increase, $p < 0.009$), *Ctsk* (5.5 fold increase, $p < 0.02$) and *DC-STAMP* (12 fold increase, $p < 0.008$). These results reveal that HDAC7 suppression enhances osteoclast formation, thus suggesting that HDAC7 activity inhibits osteoclastogenesis.

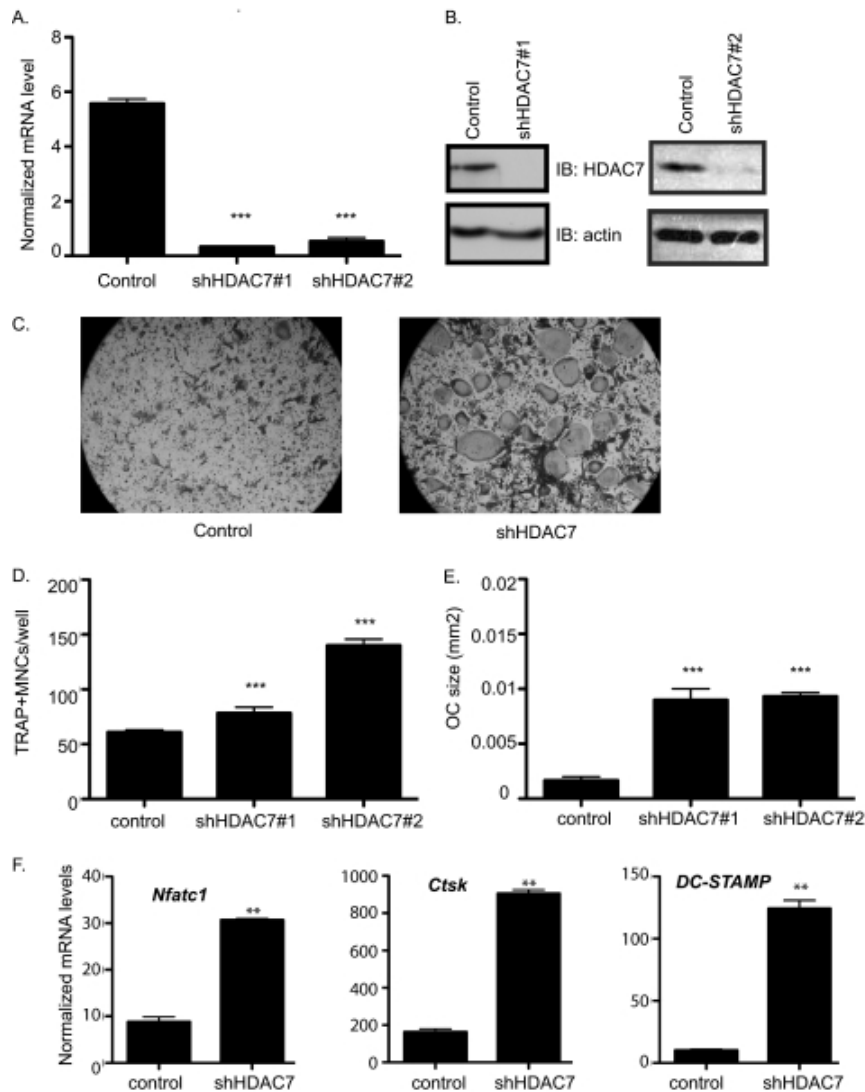


Figure 4.2: Accelerated osteoclast differentiation in HDAC7-suppressed osteoclasts. **A)** real-time RT-PCR of bone marrow. ***, $p \leq 0.0001$ versus control

shRNA. **B)** Western blot (*IB*) of osteoclast lysates showing expression of HDAC7. **C)** TRAP staining of osteoclast cultures infected with control or HDAC7 shRNA-expressing lentiviruses. **D) and E)** histomorphometric analysis of TRAP-stained osteoclasts (*OC*). *MNCs*, multinucleated cells. *******, $p \leq 0.0001$ versus control shRNA. **F)** expression profile of *Nfat-c1*, *Ctsk*, and *DC-STAMP*. ******, $p \leq 0.001$ versus control shRNA following infection with control shRNA or HDAC7 shRNA lentiviral vectors.

To further test the hypothesis that HDAC7 inhibits osteoclastogenesis, we made use of a lentiviral vector to overexpress myc-tagged HDAC7. Immunoblotting detected strong expression of a myc-tagged protein of 115 kDa, the predicted molecular weight of HDAC7 (Fig. 3A). When these cultures were stimulated with RANKL, their differentiation into TRAP-positive multinucleated osteoclasts was impaired compared to control cells (Fig. 3B). Overexpression of myc HDAC7 reduced number of TRAP positive cells 17 fold (Fig. 3C) and reduced the size of the osteoclasts 1.75-fold (Fig. 3D). Expression of osteoclast marker genes NFAT-c1, *Ctsk* and DC-STAMP were significantly reduced (Fig. 3F). Taken together these data demonstrate that HDAC7 acts as an inhibitor of osteoclast differentiation.

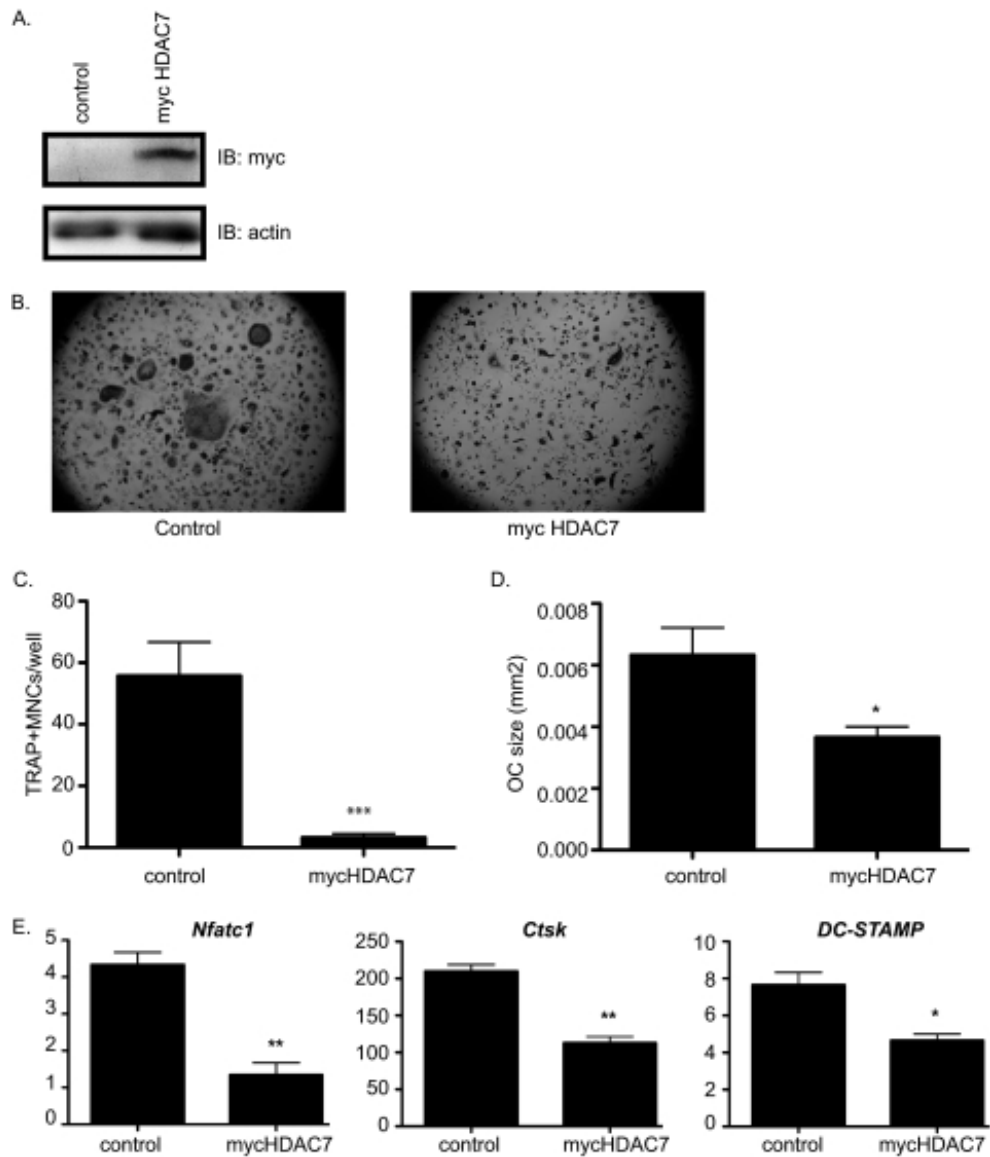


Figure 4.3: Overexpression of myc-HDAC7 inhibits osteoclast differentiation. A) Western blot (IB) of osteoclast lysates. **B)** TRAP-stained images. **C)** and **D)**, histomorphometric analysis of TRAP-stained osteoclasts (OC). MNCs, multinucleated cells. ***, $p \leq 0.005$ and *, $p \leq 0.05$. **E)** expression profile of *Nfat-c1*, *Ctsk*, and *DC-STAMP* of osteoclast infected with control or Myc-HDAC7-expressing lentivirus. **, $p \leq$

0.005 and *, $p \leq 0.05$ versus control-infected cells.

4.4.3 Overexpression of HDAC7 prevents fusion of osteoclasts precursors

We next wanted to determine if the enhanced osteoclast differentiation we detected with suppressed HDAC7 expression was due to increased fusion of osteoclasts precursors. When we compared nuclei per cell, we detected a 1.8-fold increase in nuclei per cell in the osteoclasts expressing the shRNA against HDAC7 (Fig. 4A) while nuclei per cell was not statistically different in the mycHDAC7 overexpressing osteoclasts compared to the control infected cells (Fig. 4A). As shown in Fig. 4B we detected significantly more total nuclei (1.7 fold increase, $p < 0.009$) in osteoclasts expressing the shRNA against HDAC7 compared to the control infected osteoclasts. We detected no difference in the total nuclei in the mycHDAC7 overexpressing osteoclasts compared to the control infected cells (Fig. 4B). The increase in total number of nuclei that we detect in shHDAC7 expressing osteoclasts suggests that HDAC7 functions to decrease proliferation or increase apoptosis in osteoclasts. Mitf has been shown to activate the bcl2 promoter in melanocytes and osteoclasts (McGill et al. 2002). We analyzed expression of bcl2 and detected a 3.75 increase in bcl2 expression in the HDAC7 suppressed cells compared to control infected cells (Fig. 4C). We

detected no significant increase in *bcl2* expression in the myc HDAC7 overexpressing cells compared to the control infected cells (Fig. 4C).

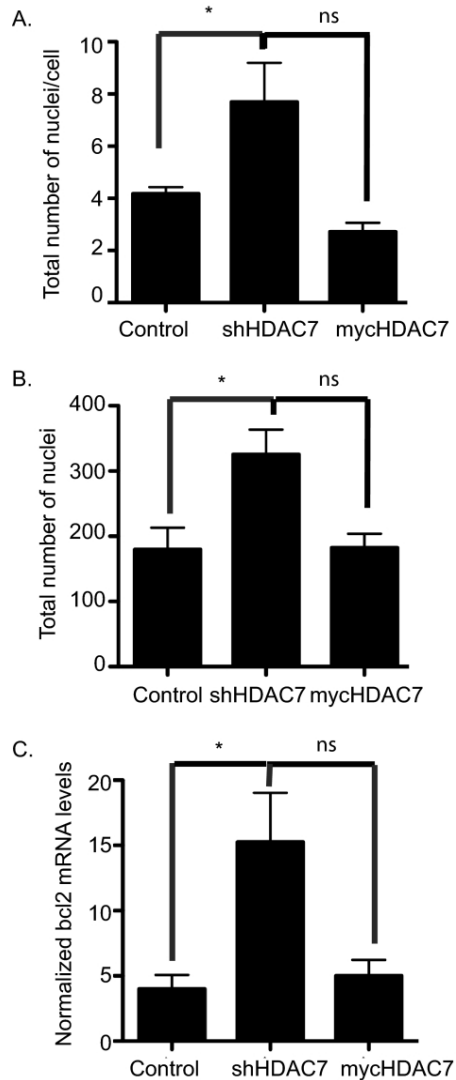


Figure 4.4: HDAC7 expression inhibits cell-cell fusion. Day 5 osteoclast cultures were stained with DAPI and Vybrant cell-labeling dye, and histomorphometry was performed to assess the total number of nuclei per multinucleated cell **A**) and the total number of nuclei **B**). *, $p < 0.01$ versus control-infected cells, ns = not significant when

compared with control-infected cells. **C)** expression profile of *bcl2* in osteoclasts infected with control, shHDAC7-, or Myc-HDAC7-overexpressing lentivirus. *, $p < 0.01$ versus control-infected cells, *ns* = not significant when compared with control-infected cells.

4.4.4 HDAC7 binds to Mitf in osteoclasts

The Mitf transcription factor is one of the essential regulators of osteoclast formation. While treatment with M-CSF recruits Mitf to target gene promoters, co-stimulation with M-CSF and RANKL is necessary for efficient activation of Mitf-dependent transcription. This suggests that corepressors prior to stimulation with RANKL might inhibit Mitf targets. Based on its effect as an inhibitor of osteoclast differentiation, we hypothesized that HDAC7 might interact with Mitf. We immunoprecipitated lysates from RAW 264.7 c4 a stable subclone of the commercially available RAW 264.7 cells or primary osteoclasts with an antibody against Mitf or an IgG control and examined the lysates by immunoblotting against HDAC7. We detected strong interaction between Mitf and HDAC7 in RAW 264.7 c4 cells (Fig. 5A). A similar interaction between Mitf and HDAC7 was detected in primary osteoclasts, which were just stimulated with M-CSF (Fig. 5B). We did not detect any HDAC7 when cell lysates were immunoprecipitated with the control IgG antibody (left lane, Fig. 5). These data indicate that HDAC7 and Mitf can interact in RAW 264.7 c4 and osteoclasts.

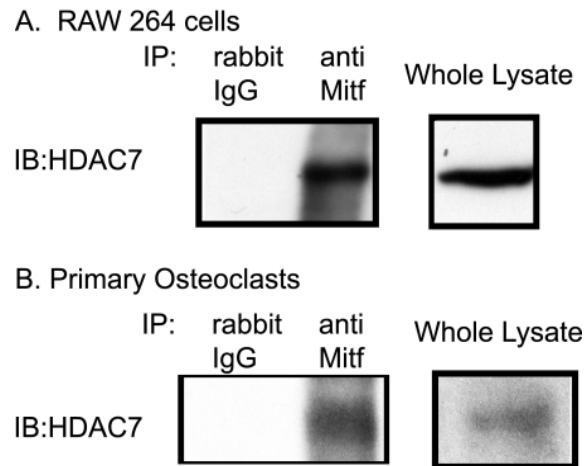


Figure 4.5: HDAC7 interacts with Mitf. A) and B) representative Western blots (*IB*) of Mitf immunoprecipitates (*IP*) from RAW 264.7 c4 cells (A) and primary mouse osteoclasts (B) immunoblotted against HDAC7 (Abcam). Osteoclasts or RAW 264.7 c4 cells were stimulated with M-CSF (10 ng/ml) for 2 days.

4.4.5 HDAC7 represses Mitf-dependent transcription

We previously showed that the amino terminus of Mitf contains the domains necessary for activation of osteoclast genes (Mansky et al. 2002). To further characterize the interaction between Mitf and HDAC7 and to determine whether the Mitf amino terminus is sufficient for functional interaction between HDAC7 and Mitf, we cloned Mitf amino acid residues 1-185, which encompass Mitf's transactivation domains, into an expression plasmid fusing the Gal4 DNA binding domain to the amino terminus of Mitf (pMI-Mitf). To further map the interaction between Mitf and HDAC7, we overexpressed pMI-Mitf and FLAG-

HDAC7 in 293T cells. Cell lysates were immunoprecipitated with an antibody that recognizes FLAG-HDAC7 and analyzed by Western blot for the presence of pMI-Mitf. We could only detect the amino terminus of Mitf in the presence of the FLAG-HDAC7 (Fig. 6A). In NIH3T3 cells, the resulting pMI-Mitf activated the gal4-TK luciferase reporter nearly 100,000-fold, while expression of HDAC7 alone had no effect on the reporter (Fig. 6B). We decided to study the effect of HDAC7 on Mitf's activation of the gal-TK luciferase reporter in NIH 3T3 cells because NIH 3T3 cells do not express endogenous Mitf like RAW 246.7 cells, and NIH 3T3 cells are easier to transfect than osteoclast precursors. Co-transfection of pMI-Mitf with increasing amounts of HDAC7 gave a dose-dependent significant repression of the Mitf activity (Figure 6B, compare pMI-Mitf to pMI-Mitf+HDAC7).

To map the interaction between HDAC7 and Mitf, we overexpressed HDAC7 1-478 and HDAC7 472-912 and pMI-Mitf in 293T cells. Cell lysates were immunoprecipitated with antibody that recognizes either HDAC7 1-478 or HDAC7 472-912 and analyzed by Western blot for the presence of pMI-Mitf. We could only detect the amino terminus of Mitf in the presence of HDAC7 1-478 (Fig. 6C). To determine what region or regions of HDAC7 mediate Mitf repression, we transfected NIH3T3 cells with gal4-TK luciferase reporter, pMI-Mitf and either HDAC7 1-478 or HDAC 472-912, which encode the amino-terminal domain and

the carboxy-terminal deacetylase domain of HDAC7 (Fig. 6D). Both the amino terminal and carboxy terminal portions of HDAC7 gave strong and significant repression of Mitf activity.

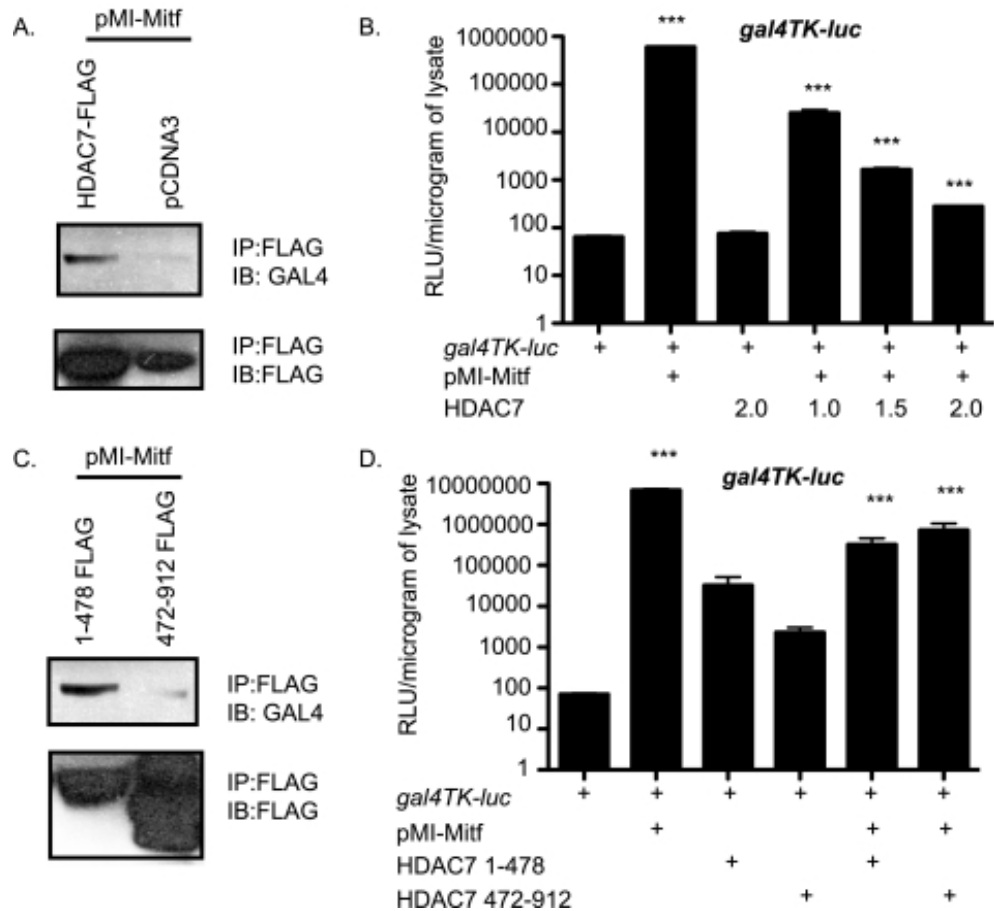


Figure 4.6: Amino terminus of Mitf is sufficient for HDAC7 repression. **A)** 293T cells were transfected with FLAG-HDAC7 and pMI-Mitf. Lysates were immunoprecipitated (*IP*) with FLAG antibody and immunoblotted (*IB*) with an antibody that recognizes the Gal4 DNA binding domain. **B)** NIH 3T3 cells were transiently transfected with 5× Gal4-TK-luciferase reporter construct and/or pMI-Mitf and full-length HDAC7. Reporter activity is presented as relative luciferase units (*RLU*), and the results

of three experiments each performed in duplicate are presented. *******, $p \leq 0.0001$ versus *gal4-TK-luc* or *gal4-TK-luc+Mitf*. **C)** 293T cells were transfected with FLAG-HDAC7 1–478 or 472–912 and pMI-Mitf. Lysates were immunoprecipitated with FLAG antibody and immunoblotted with an antibody that recognizes the Gal4 DNA binding domain. **D)** NIH 3T3 cells were transiently transfected with 5× Gal4-TK-luciferase reporter construct and/or pMI-Mitf and either HDAC7 1–478 or HDAC7 472–912. Reporter activity is presented as relative luciferase units, and the results of three experiments each performed in duplicate are presented. *******, $p \leq 0.0001$ versus *gal4-TK-luc* or *gal4-TK-luc+Mitf*.

4.4.6 Repression by HDAC7 does not require deacetylation

HDAC7 has been shown to repress transcription through deacetylation-dependent and –independent mechanisms. If HDAC7 is repressing Mitf activity through a deacetylation independent mechanism then HDAC7 should be able to repress Mitf’s activation in the presence of the broad spectrum HDAC inhibitor TSA. When we repeated the transfections shown in Fig. 7A in the presence of TSA, we found that HDAC7 represses Mitf’s activation of the *gal4-TK luciferase* similarly regardless of the presence of TSA (Fig. 7B). Moreover, the repressive activities of 1-478 and 472-912 HDAC7 fragments were also unaffected by TSA. To verify the activity of TSA in these experiments, parallel cultures were treated with TSA or DMSO vehicle and subjected to immunoblotting against total histone

H3 and acetylated histone H3. Figure 7C shows that overnight treatment with TSA increased the level of acetylated histone H3, consistent with reduced HDAC activity in the TSA-treated cells. These data indicate that multiple domains of HDAC7 are involved in repression of Mitf and that neither deacetylase catalytic activity nor the HDAC7 deacetylase domain is required for functional repression of Mitf.

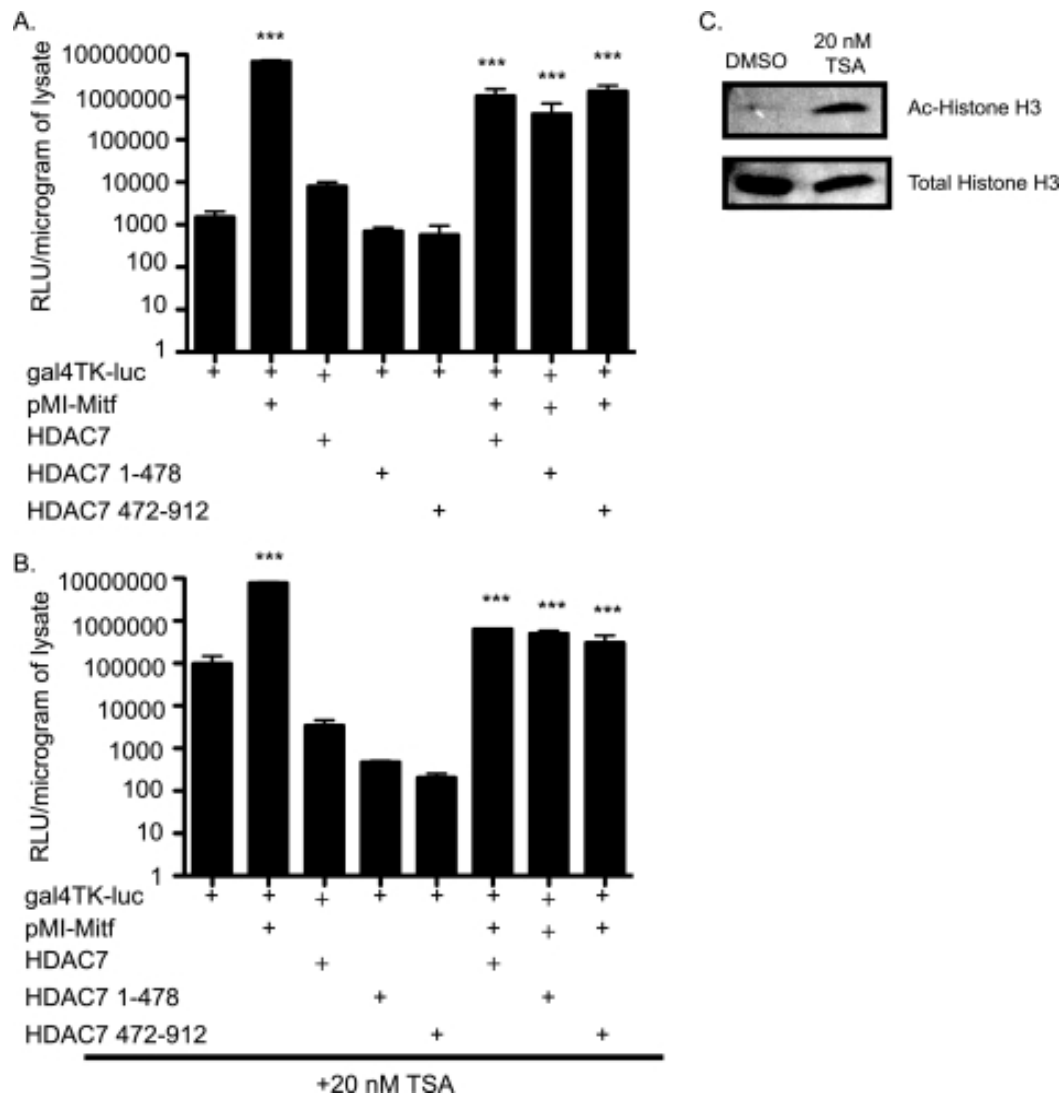


Figure 4.7: HDAC7 represses Mitf in a deacetylation independent mechanism. A) NIH 3T3 cells were co-transfected with Gal4TK-luc, pMI-Mitf, and/or FLAG-HDAC7 1–428 or 422–912. Cells were treated with DMSO. Reporter activity is presented as relative luciferase units (*RLU*), and the results of three experiments each performed in duplicate are presented. **B)** repression of Mitf by HDAC7 is insensitive to TSA. Transfections were performed as described in *A*, and the cells were treated with 20 nm TSA for 16–20 h before harvest. ***, $p \leq 0.0001$ versus *gal4-TK-luc* or *gal4-TK-luc+Mitf*. **C)** increased levels of acetylated histone H3 after TSA treatment. NIH 3T3 cells were treated with TSA in parallel to the experiment shown in *B*. Protein extracts were subjected to SDS-PAGE and immunoblotted against total and acetylated histone H3.

4.5 Discussion

Several studies have shown that histone deacetylase inhibitors (HDIs) TSA and NaB directly inhibit osteoclastogenesis (Rahman et al. 2003; Kim et al. 2009). The current study is among the first to identify the HDACs to be involved in this inhibitory effect on osteoclast formation. We show that inhibition of HDAC3 mirrors the effects of TSA and NaB. Thus, HDAC3 activity is necessary for osteoclast formation. Targets of HDAC3 in osteoclasts are not yet known, although HDIs inhibit RANKL-induction of p38 activity and expression of c-fos and NFAT-c1 (Rahman et al. 2003). Yi et al. found that HDIs lead to activation of apoptotic pathways in osteoclast precursors (Yi et al. 2007). HDIs are being investigated as potential therapeutic agents for a variety of cancers, HIV/AIDS, asthma and central nervous system disorders (Routy 2005; Gottesfeld and Pandolfo 2009; Halili et al. 2009; Tan et al. 2010). Thus understanding their impact on skeletal physiology is important and potentially clinically relevant. HDIs accelerate osteoblastic differentiation both in vitro and in vivo (Schroeder et al. 2004; Jensen et al. 2008). This observation, together with the discovery that HDIs or suppression of HDAC3 inhibit osteoclast formation might lead to the prediction that HDIs may lead to increased bone mass. However, studies have suggested that long-term administration of the histone deacetylase inhibitor valproic acid (VPA) for treatment of epilepsy may actually be associated with

decreased bone mass (Vestergaard et al. 2004). The cellular and physiological explanation for this effect remains unclear.

Our current studies reveal an unexpected function for HDAC7 in osteoclasts that are distinct from HDAC3. Suppression of HDAC7 enhanced osteoclast formation, while overexpression of HDAC7 impairs osteoclast formation, indicating that HDAC7 represses osteoclast differentiation. Analyzing total nuclei of osteoclasts expressing the shRNA against HDAC7 indicates that suppression of HDAC7 expression leads to an increase in total nuclei compared to control infected cells. Furthermore we were able to detect an increase in *bcl2* mRNA expression in HDAC7 suppressed cells. *Mitf* has been shown to activate the *bcl2* promoter in melanocytes and osteoclasts (McGill et al. 2002). We have hypothesized that loss of HDAC7 would lead to more *Mitf* activation of the *bcl2* promoter, a prosurvival factor and may account for the increase in total number of nuclei that we detected in shHDAC7 expressing osteoclasts.

M-CSF stimulation recruits *Mitf* to the nucleus and localizes *Mitf* in a complex with PU.1 to osteoclast promoters (Sharma et al. 2007). However, M-CSF stimulation alone is not sufficient to activate gene expression. We were able to detect an interaction between endogenous *Mitf* and HDAC7 when the osteoclasts were stimulated with M-CSF only. It is the combination of M-CSF and RANKL signaling that leads phosphorylated, activated *Mitf*, recruitment of RNA

polymerase II and robust gene expression (Sharma et al. 2007). Based on our ability to detect an interaction between *Mitf* and HDAC7 in osteoclasts, we hypothesize a model in which treatment with RANKL disrupts the interaction between *Mitf* and HDAC7, permitting *Mitf*-mediated transcriptional activation. Studies are currently underway to test this hypothesis by using ChIP analysis of *Mitf* target genes during osteoclast differentiation. This is similar to the scenario proposed for osteoblast differentiation where BMP2 stimulation transiently relocates HDAC7 from the nucleus to the cytoplasmic compartment, thereby permitting Runx2-dependent transcription. (Jensen et al. 2008). We have not detected a change in HDAC7 subcellular localization following RANKL treatment (unpublished observation). RANKL stimulation leads to phosphorylation of *Mitf* on specific residues (Mansky et al. 2002), suggesting possible mechanisms by which the *Mitf*-HDAC7 interaction might be regulated. However, we cannot rule out that HDAC7 also interacts with other transcription factors involved in osteoclast differentiation like PU.1 or NFAT-c1. It also remains unclear whether the altered expression of any particular gene in the HDAC7 suppressed cells reflects regulation by HDAC7 directly or indirectly.

The contrast between HDAC7 suppression and HDAC3 suppression or HDI treatments on osteoclast formation suggest that HDAC7's effects in osteoclasts are mediated through distinct mechanisms that do not require

deacetylase activity. In further support of this, we found that repression of the gal4-TK promoter by HDAC7 is insensitive to treatment with TSA, and showed that the HDAC7 deacetylase domain is dispensable for repression. The amino terminal of class IIa HDACs repress transcription by a deacetylase-independent mechanism(s), which include interactions with CtBP and HP1 (Dressel et al. 2001). The carboxy-terminal portion of HDAC7 also possessed robust repressive activity, which was insensitive to TSA in our assays. This is similar to the physical and repressive interactions between HDAC7 and Runx2, which also appear to involve multiple regions of HDAC7 (Jensen et al. 2008). As with Mitf, HDAC7 repression of Runx2 is insensitive to TSA and can be mediated by the amino-terminal domain (Jensen et al. 2008). HDAC7 and other Class IIa HDACs can regulate transcription by recruiting large repressive complexes containing Class I HDACs, Sin3 and SMRT/N-CoR (Kao et al. 2000; Li et al. 2000; Fischle et al. 2001; Guenther et al. 2001; Fischle et al. 2002). The HDAC3-interaction domain of HDAC7 was mapped to amino acids 438-912 (Fischle et al. 2001), which are included in the carboxy-terminal construct we used. Hu et al. have demonstrated by ChIP assay that HDAC1, Sin3A and CtBP are recruited to the Ctsk and Acp5 promoters during M-CSF stimulation but their association is decreased with RANKL signaling (Hu et al. 2007). It is possible that one mechanism used by HDAC7 during M-CSF stimulation of osteoclasts HDAC7 is based on recruitment

of such repressive complexes. This recruitment of repressive complexes could explain why the carboxy terminus of HDAC7 inhibits Mitf activation even when we detect no interaction between the C-terminus of HDAC7 and Mitf by co-immunoprecipitation (Fig. 6).

This study establishes HDAC7 as a novel Mitf repressor. Understanding the mechanisms by which HDAC7 represses Mitf function, their significance in the context of the larger transcription complex (c-fos/Mitf/PU.1/Nfatc1) at osteoclast promoters and the regulatory inputs that coordinate their activity will be significant areas for future studies to increase our understanding of skeletal development, maintenance and pathological states.³

³ We acknowledge A. Ian Cassady for the gift of the RAW 264.7 c4 cells. MinnCResT T32 DE007288 to L.P. from the National Institute of Dental and Craniofacial Research, R03 DE020117 to E.J. and R01 AR056642 to R.G.

Chapter 5

Future Efforts Understanding Regulators of Osteoclastogenesis, Summary, and Conclusions

5.1 Future Efforts

5.1.1 Introduction

Novel therapeutic strategies that target osteoclasts are needed for many craniofacial, orthopedic, and cancer applications. Incomplete understanding of the factors that regulate osteoclasts limits this development. As mentioned previously, BMPs are used to promote healing of open fractures, bone grafts, and non-union fractures. Although BMPs have been widely studied as stimulators of osteoblastic bone formation, little to no studies have explored the effects of BMPs on osteoclasts or the role BMPs play in the pathogenesis of osteolytic conditions.

In Chapter 2, I confirmed that osteoclasts express BMP receptors as well as BMP-2 (Jensen et al. 2010). Increasing levels of phosphorylated Smad 1/5/8 were detected during osteoclast differentiation, which indicated that the BMP/Smad pathway was activated in osteoclasts (Jensen et al. 2010). Further, BMPs can also regulate non-Smad signaling pathways such as the MAP kinases (Nohe et al. 2002; Hassel et al. 2003; Nohe et al. 2004), which are known to be important in osteoclast differentiation (Hotokezaka et al. 2002; Lee et al. 2002; Li et al. 2003). At present, the molecular mechanisms by which BMP signaling enhances osteoclast formation are poorly understood. From Chapter 3 and previously published work, mice null for the expression of a known BMP

antagonist, TWSG1, showed osteopenia that resulted from increased numbers, size, and activity of osteoclasts (Pham et al. 2011). *Twsg1*^{-/-} osteoclasts exhibited increased levels of Smad 1/5/8 *in vitro*, suggesting that the enhanced osteoclastogenesis in *Twsg1*^{-/-} mice is mediated through increased BMP signaling. Further, exogenous BMP2 can directly enhance RANK Ligand (RANKL)-mediated formation of wild-type osteoclasts (Sotillo Rodriguez et al. 2009).

The next steps towards our goal of understanding the molecular signals that regulate osteoclasts will be to confirm the requirement for BMP signaling in osteoclasts and to determine the molecular mechanisms by which BMP signaling regulates their differentiation. I hypothesize that BMPs function in an osteoclast-specific manner, utilizing the BMPRIIs to enhance osteoclastogenesis. In attempts to validate this hypothesis, I have created an osteoclast-specific BMPRII conditional knockout mouse by breeding floxed BMPRII mice with *LysM-Cre* mice and have performed initial characterizations to determine the effect on osteoclast formation and skeletal physiology. These studies are important to further establish the consequences of BMP signaling on osteoclast biology and bone resorption.

5.1.2 BMPRII Conditional Knockout

To characterize a mouse model that is null for *BMPRII* expression specifically in osteoclasts, a mouse model was generated utilizing CRE-mediated recombination technology. I have obtained the floxed-*BMPRII* mice previously generated by Dr. Hideyuki Beppu (Beppu et al. 2005) from Dr. Mike O'Connor (University of Minnesota, Minneapolis, MN). Expression of the CRE recombinase mediated deletion of exons 4 and 5 (transmembrane and portion of the kinase domain) of the *BMPRII* gene, resulting in null expression of *BMPRII* (Beppu et al. 2005). See Figure 5.1A for a diagram of the targeting vector constructed. To generate a mouse model that shows osteoclast-specific deletion of *BMPRII*, a *LysM-Cre* mice was chosen (Clausen et al. 1999). *LysM-Cre* targets the myloid cell lineage and a diagram depicting all the cell lines affected is shown in Figure 5.1B. All lines of mice were in the C57BL/6 background; *BMPRII* floxed mice were mated with *LysM-Cre* mice to generate a *BMPRII* conditional knockout used the remaining studies.

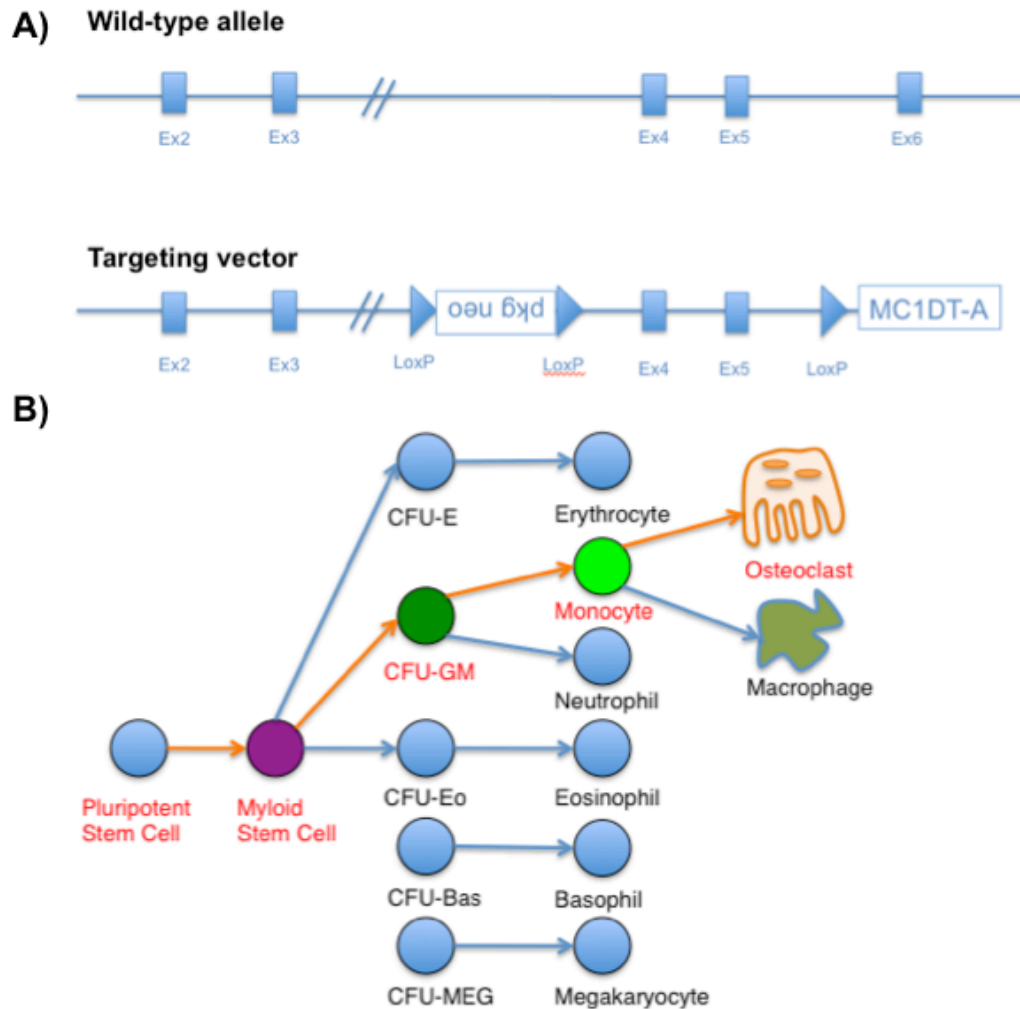


Figure 5.1: Generation of BMPRII conditional null model system. **A)** Molecular structure of *BMPRII* floxed allele. Top: Wild-type Bottom: Targeting vector. PGK-NEO cassette flanked by loxP sites (triangles) was inserted to *ClaI* site in reverse direction. A MC1DT-A cassette was attached to the 3'-end of the targeting construct for negative selection. Figure adapted from Li and coworkers (Beppu et al. 2005). **B)** LysM-CRE targets the myeloid cell lineage at the level of the myeloid stem cell.

5.1.3 Reduced BMPRII Suppress Osteoclast Formation In Vitro

To preliminary test if loss of BMPRII has an effect on osteoclast differentiation, I infected osteoclasts with an adenoviral vector expressing Cre recombinase (Ad-Cre). Both osteoclast number and size were reduced in the osteoclasts from the BMPRII floxed mice compared to WT infected with the Ad-Cre (Figure 5.2A-B). Quantitative PCR analysis confirmed that the floxed-BMPRII osteoclasts expressed less *BMPRII*, *TRAP*, and *Cathepsin K* mRNA compared to WT cells (Figure 5.2C). With the use of this *BMPRII^{fl/fl}* mouse model system, these results further validate that loss of *BMPRII* expression impairs osteoclast differentiations *in vitro*. It is evident that BMP signaling directly promotes the differentiation of osteoclasts.

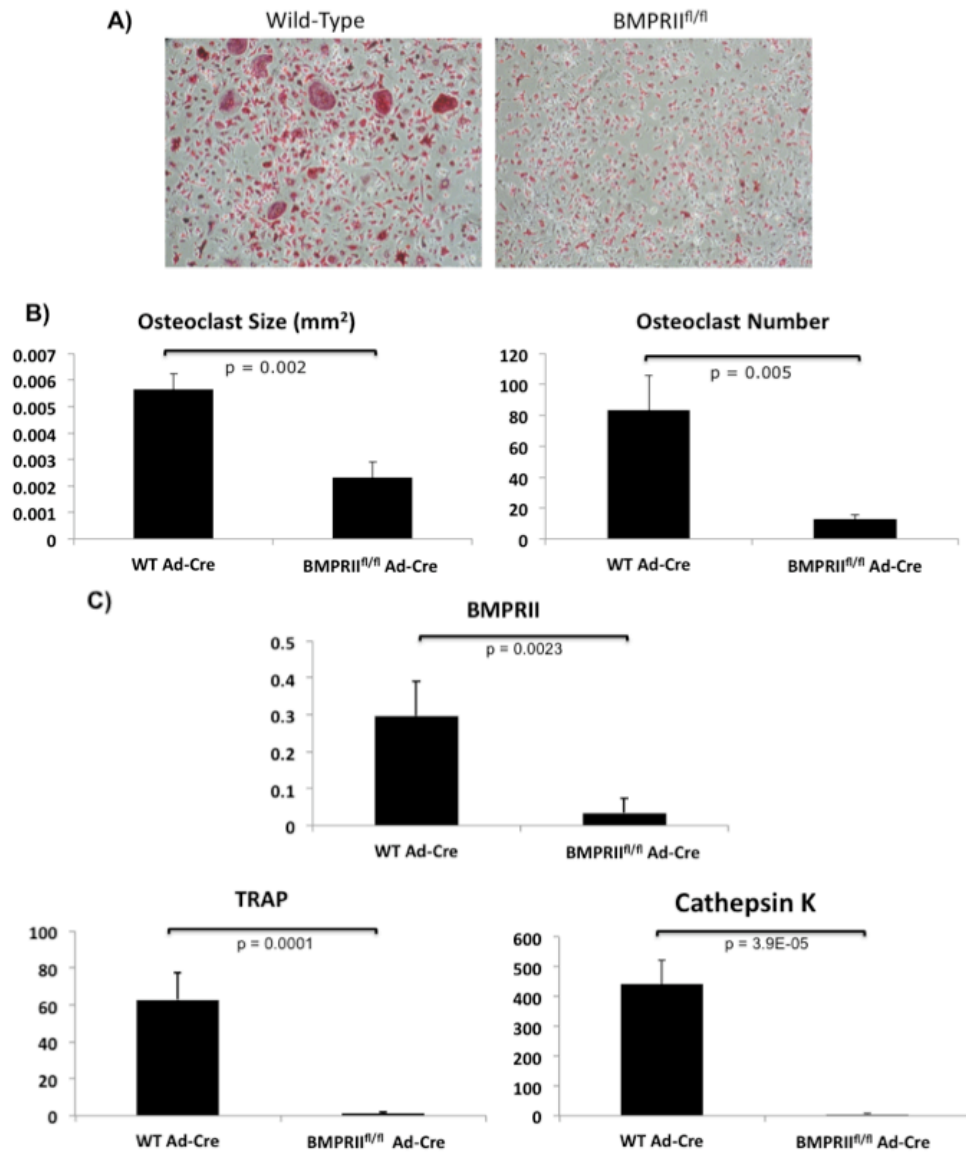


Figure 5.2: Reducing BMPRII suppresses osteoclastogenesis. Primary osteoclasts from wild-type and *BMPRII^{fl/fl}* mice were transduced with Ad-Cre. **A)** TRAP stained osteoclasts. On the Left: wild-type osteoclasts transduced with Ad-Cre; on the right: *BMPRII^{fl/fl}* osteoclasts transduced with Ad-Cre. **B)** Quantitation of osteoclast size and

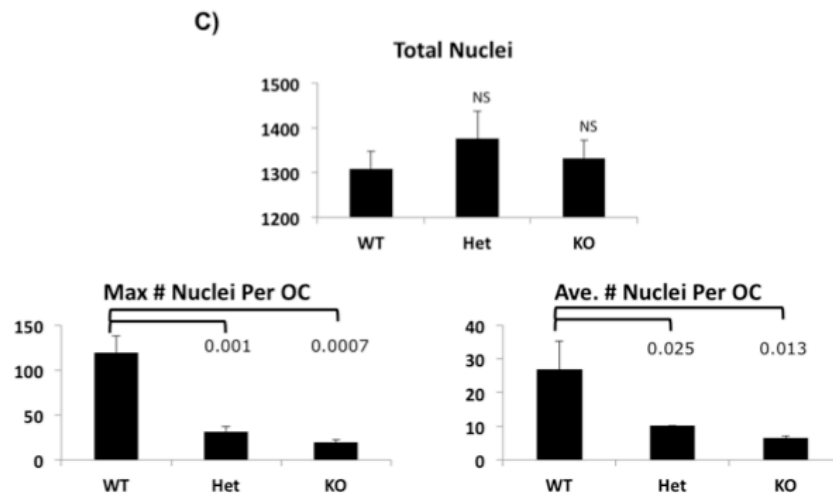
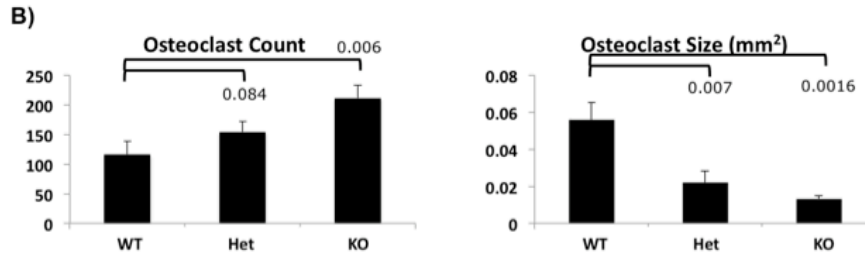
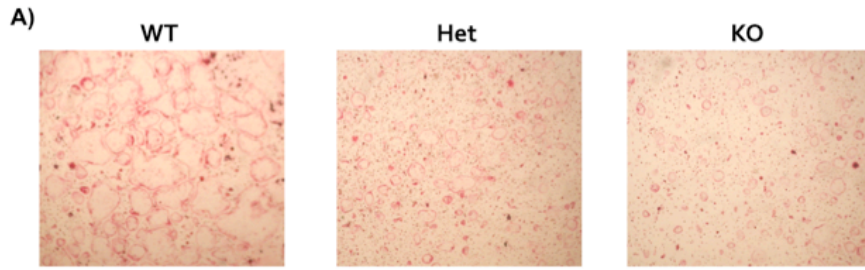
number using ImageJ software. **C)** Quantitative PCR of *BMPRII*, *TRAP*, and *Cathepsin K* on wild-type and *BMPRII^{f/f}* transduced osteoclast. Data are mean \pm SD.

5.1.4 Reduced BMPRII Suppress Osteoclast Formation In Vivo

My preliminary studies indicated that knocking down expression of the BMPRII with the use of Ad-Cre in osteoclasts inhibited osteoclasts differentiation *in vitro*. Next, I investigated the role of BMP signaling in *in vivo* osteoclast differentiation. Osteoclasts were flushed from bone marrow of appropriate genotypes of the wild-type (WT), BMPRII heterozygous (Het), and BMPRII conditional knockout mice (KO). Equal number of cells were plated and stimulated with M-CSF and RANKL. To measure osteoclast formation TRAP staining was performed on the cells after four days in differentiation media. The number, size, and nuclei analysis was determined as shown in Figure 5.3A-C. It appears that suppressed levels of BMPRII in osteoclasts lead to the reduced osteoclast size. Although I observed an increase in the number of total osteoclasts in the KO osteoclasts, the nuclei analysis revealed that the average number of nuclei in the KO or Het osteoclasts were significantly reduced compared to WT. This suggests an overall reduction in osteoclast activity with reduced levels of BMPRII.

Consistent with these findings, Figure 5.3D shows the gene expression profile of osteoclast differentiation markers (*Nfatc1*, *TRAP*, *Cathepsin K*, *OCstamp*, and *ATPv6*). Significantly reduced levels of the differentiation markers were observed in the Het and KO osteoclasts compared to WT. These findings support the hypothesis that BMPRII is required for osteoclastogenesis *in vivo*.

To further validate the *in vivo* requirement for BMPRII in osteoclastogenesis, Micro-CT analysis was performed and standard trabecular measurements were obtained (Figure 5.3E-F). As seen by the reconstruction of bone structures and the bone volume fraction obtained from the micro-CT data, there is an increase in trabecular bone formation in KO mice at both three month and six month time points compared to WT mice. This finding further emphasizes the importance of BMPRII for bone resorption, *in vivo*.



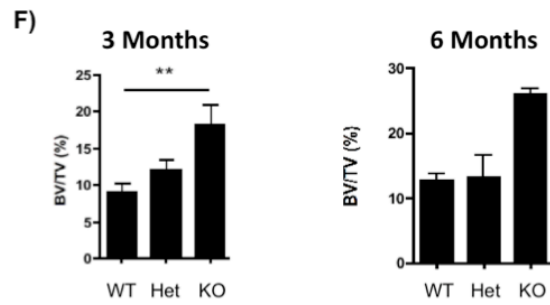
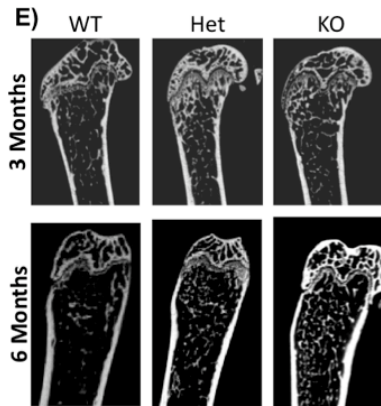
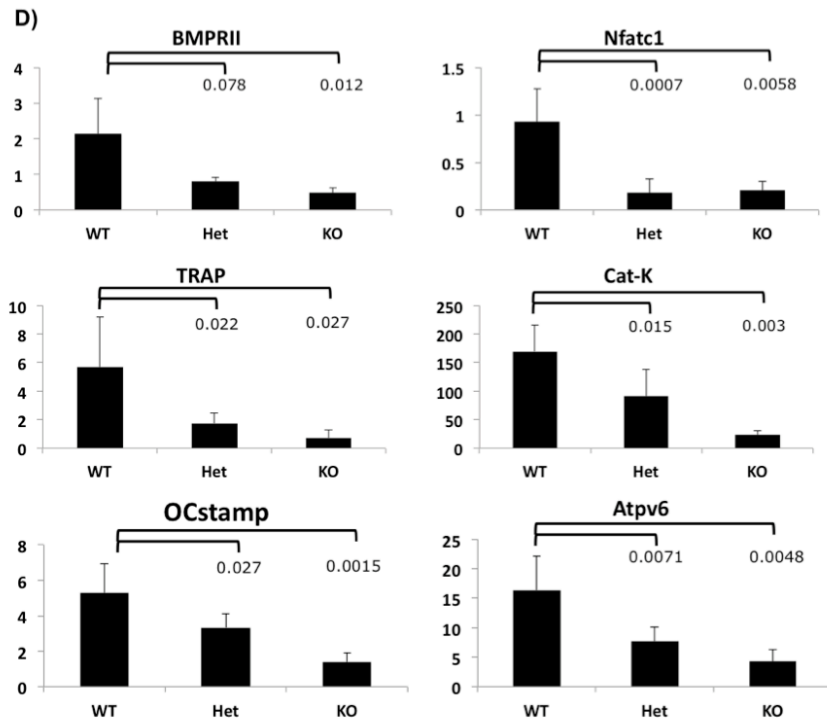


Figure 5.3: Analysis of *BMPRII* conditional knockout mice reveals a decrease in osteoclastogenesis. For Figures A-D, Primary osteoclasts were isolated from bone marrow and cultured in differentiation media for 4 days before being processed. **A)** TRAP stained osteoclasts from wild-type (WT), heterozygous (Het), and conditional knockout mice (KO). **B)** Quantitation of osteoclast size and number using ImageJ software. **C)** Nuclei analysis. Cells were fixed and stained with DAPI and nuclei were counted to determine total nuclei, maximum number of nuclei per osteoclast, and average number of nuclei per osteoclast. **D)** Quantitative PCR for *BMPRII*, *Nfatc1*, *TRAP*, *Cathepsin K*, *OCstamp*, and *ATPv6*. **E)** Reconstructions of bone structure from μ CT. Cortical bone and trabecular bone in femoral head are shown. Trabecular bone was dramatically increased in KO. **F)** Trabecular bone measurements by μ CT. Data are mean \pm SD; n = 8 (control); n = 10 (cKO). Significant differences are indicated between WT and KO (Student's t-test, **p < 0.005; *p < 0.05). BVF, bone volume fraction (%); BV, trabecular bone volume (mm³); TV, total volume selected for analysis (mm³). For all graphs, p values are listed above bars. NS, not significant.

5.1.5 MAPK Signaling Pathway is Reduced in *BMPRII*

Conditional Knockout Mice

The previous results demonstrate the requirement for *BMPRII* both *in vitro* and *in vivo* when using an osteoclast-specific knockout mouse model system. To gain insight into the molecular mechanism downstream of the BMP and BMP

receptor activation events, Western blot analysis of wild-type (WT), BMPRII heterozygous (Het), and BMPRII conditional knockout (KO) osteoclasts for BMPRII, pSMAD 1/5/8, phospho-p38, and phospho-ERK, were performed on cells that were cultured in differentiated media for four days (Figure 5.4A). By Quantitative PCR analysis, I validated the reduction of *BMPRII* mRNA in both the Het and KO osteoclasts (Figure 5.3.D). However, I was unable to detect a difference in BMPRII protein levels by Western blot analysis (Figure 5.4A, top panel). A plausible explanation might be that although the mRNA expression levels are reduced, the low levels may not result in an apparent decrease in protein concentrations. As this is speculative, the reasons behind this observation remain unclear.

Surprisingly, no changes in pSmad 1/5/8 expression were observed, but rather a decrease in phosphorylated p38 and phosphorylated ERK (Figure 5.4.A). This suggests that the conditional BMPRII knockout osteoclasts, which eliminates Exon 4 and 5, can still aid in signaling through the PFCs, but has seemingly lost its ability to signal through the BISCs. In addition, phosphorylated Protein Kinase D (PKD) levels were reduced in the Het (BMPRII +/-) and KO (BMPRII -/-) osteoclasts (Figure 5.4B). Decreased phosphorylated PKD levels may coincide with reduced osteoclastogenesis via a decrease in BMP activity. Finally, I probed *Twsg1*^{-/-} osteoclasts for PKD levels, and observed an increase in phosphorylated

PKD in these cells. Increased phosphorylated PKD levels may be consistent with enhanced osteoclastogenesis via an increase in BMP activity.

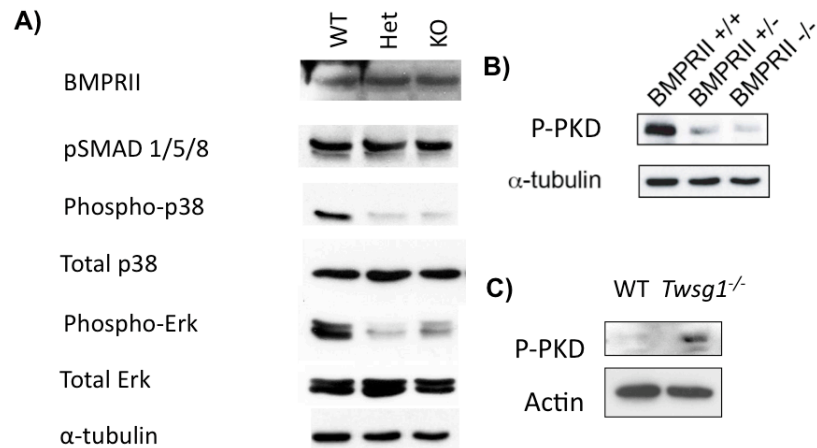


Figure 5.4: Signaling pathways affected in *BMPRII* conditional knockout mice. A) Western blot analysis of wild-type (WT), *BMPRII* heterozygous (Het), and *BMPRII* conditional knockout (KO) osteoclasts for BMPRII, pSMAD 1/5/8, phospho-p38, total p38, phospho-ERK, total ERK, and α -tubulin. **B)** Western blot analysis of wild-type (*BMPRII* +/+), *BMPRII* heterozygous (*BMPRII* +/-), and *BMPRII* conditional knockout (*BMPRII* -/-) osteoclasts for phospho-PKD (P-PKD) and α -tubulin. **C)** Western blot analysis of wild-type (WT) and *Twsg1* knockout (*Twsg1*^{-/-}) osteoclasts for phospho-PKD (P-PKD) and Actin.

Although these are only initial studies, it appears that a model proposed in Figure 5.5 may explain observations seen while investigating the BMPRII conditional knockouts. I hypothesize that during normal osteoclastogenesis, BMPs can signal through either PFCs or BISCs. However, in our BMPRII KO model, signaling seemingly occurs through the PFC but not the BISC complex. PKDs appear to be down regulated with this lack of BISC signaling. Furthermore, p38 and HDACs may be regulated downstream of PKD, as similar findings have been observed in osteoblasts (Jensen et al. 2009). Additional studies are necessary to truly understand the osteoclast-specific molecular puzzle sparked by BMPs.

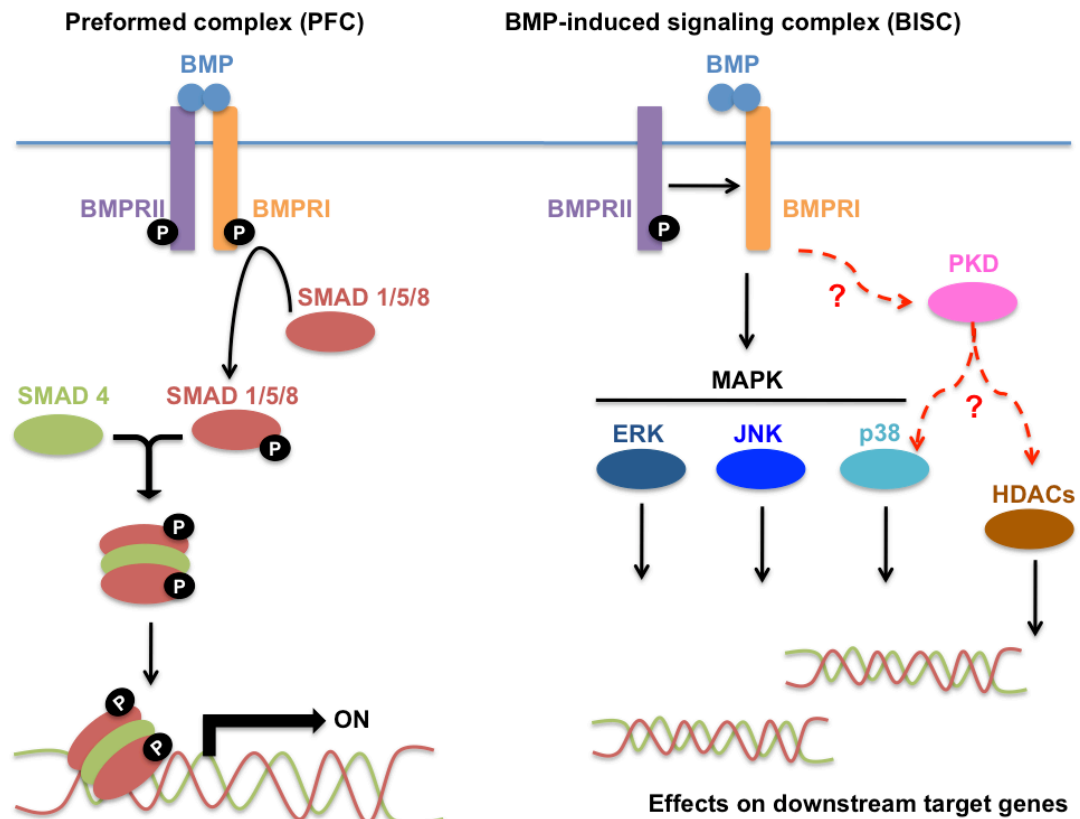


Figure 5.5: A proposed model illustrating the signaling pathways used by BMPs to regulate gene expression during osteoclastogenesis. BMPs bind to its receptor complex. When BMPs bind to the preformed complexes (PFCs), it initiates the Smad pathways by phosphorylation of Smad 1/5/8. Phosphorylated Smad 1/5/8 results in the regulation of specific target genes associated with osteoclastogenesis. Alternatively, signaling occurs through the Smad-independent pathway through a BMP-2-induced oligomerization of the BMP receptors. The activation of this complex results in the induction of downstream target genes for osteoclast formation through p38 MAPK. Proposed by the red dotted arrows is the hypothesis that BMPs stimulate PKD that, work

through p38 or HDACs to modulate osteoclastogenesis, similarly to what has been shown with PKD's role in osteoblastogenesis (Jensen et al. 2009).

5.2 Summary

5.2.1 Chapter 2: BMP-2 Enhancement of Murine Osteoclast Differentiation

BMPs are well-studied regulators of osteoblasts, and are used in a number of craniofacial and orthopedic procedures to promote localized bone formation. In various cases where recombinant BMP-2 is used to enhance bone formation, a transient increase in osteoclastic bone resorption has been reported. The cellular and molecular origin of this BMP-associated stimulation of bone resorption remains unknown. The goals of the study presented in Chapter 2 were to refine the transient requirements of exogenous BMP-2 on enhanced osteoclastogenesis *in vitro*, determine the gene expression profile for BMP receptors and ligands throughout osteoclastic differentiation, and evaluate the effects on osteoclastogenesis under sub-optimal levels of BMP signaling (Jensen et al. 2010). The data presented suggests that recombinant BMP-2 was sufficient at enhancing osteoclastogenesis in murine primary osteoclasts. In addition, the expression of both BMP receptors and BMP-2 ligand increased during normal osteoclast differentiation. Finally, reducing BMP signaling, using lentiviral shRNA constructs against BMPRII, significantly reduced osteoclast size and number. Taken together, this study was pivotal in linking BMPs as regulators of

osteoclastogenesis. These experiments provide important new insights into the use of BMPs in bone generation procedures, its role in pathogenesis of bone resorptive disorders, and provide a conceptual framework for the development of novel therapies targeting osteoclasts.

5.2.2 Chapter 3: Modulating Osteoclastogenesis using BMP-2 and TWSG1

TWSG1 is a crucial extracellular regulator of BMP signaling, having both an anti-BMP and pro-BMP functions. *Twsg1*^{-/-} mice models display severe osteopenia that may be attributed to enhanced osteoclastogenesis. Our previous work suggests that disrupting TWSG1 enhances osteoclastogenesis through increased BMP signaling. Investigating the mechanism(s) by which BMPs and *Twsg1* control osteoclast function will better our understanding of physiological regulation of bone remodeling and pathogenesis of diseases that result from altered bone remodeling. To that end, the goals of the study presented in Chapter 3 were to overexpress TWSG1 in murine primary osteoclasts to further elucidate its role in osteoclast formation (Pham et al. 2011). The data presented suggests that overexpression of TWSG1 could be accomplished by utilizing an adenovirus delivery system. I showed that overexpressing TWSG1 could suppress osteoclastogenesis in both wild-type and *Twsg1*^{-/-} cells. Furthermore,

rescue of the TWSG1 suppressed osteoclasts was achieved with recombinant BMP-2. This work provides a novel approach to modulating osteoclastogenesis *in vitro* and suggests that TWSG1 could be used a potential anti-resorptive agent.

5.2.3 Chapter 4: The Effects of HDAC3 and HDAC7 on Osteoclastogenesis

Among other things, HDACs are negative regulators of transcription. Very little is known about the role HDACs play in osteoclast differentiation. Previous work has demonstrated that modulators of HDAC activity including TSA and NaB are sufficient to suppress osteoclast differentiation through multiple mechanisms. The goals of the study presented in Chapter 4 were to determine if HDACs could be used as a novel regulator of osteoclastogenesis *in vitro* (Pham et al. 2011). The data presented suggests suppression of HDAC3 by shRNA closely mirrors the inhibitory effect of HDIs on osteoclast formation. Interestingly, suppression of HDAC7 has the opposite effect, enhancing osteoclast activity. This indicates that HDAC7 represses osteoclast differentiation. HDAC7 was shown to interact with MITF, which establishes HDAC7 as a novel MITF repressor necessary for osteoclastogenesis.

To further evaluate the cellular and molecular origin of BMP-associated stimulation of bone resorption, next, I sought out to investigate the osteoclast-

specific role of BMP signaling on osteoclast formation by generating an osteoclast-specific BMPRII conditional null mouse. Preliminary results are in Section 5.1: Future Efforts. It is still to be determined if the role of PKD, MITF, and HDACs in controlling osteoclastic gene expression parallel that of osteoblastic differentiation (Jensen et al. 2009).

5.3 Conclusions

A plethora of studies in the skeletal tissue has shed light on BMP's role as an inducer of chondrocytic and osteoblastic differentiation and function. However, BMPs, regulators of BMPs, and downstream pathways leading to osteoclastic bone resorption are poorly understood. The transduction of BMP signaling is very specific and tightly regulated at each step of the signaling cascades towards bone development and homeostasis. Defects in the BMP pathway give rise to developmental disorders and dysfunctions in humans, such as bone diseases, cartilage diseases, and cancer. The studies presented in this thesis have elucidated some of the mechanisms of bone remodeling facilitated by BMP-2, TWSG1, and HDAC3 and 7. The knowledge gained will provide further details on how BMPs function in specific cell types, which should give important new insight into the use of BMPs in bone generation procedures, its role in pathogenesis of bone resorptive disorders, and provide a conceptual framework for the development of novel therapies targeting osteoclasts.

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