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Bone marrow stromal cell proliferation in mice lacking NFATc1 in mature osteoclasts

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SCHOOL OF MEDICINE

Thesis

BONE MARROW STROMAL CELL PROLIFERATION IN MICE LACKING NFATC1 IN MATURE OSTEOCLASTS

by

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Boston University School of Medicine, 2013

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ABSTRACT

The transcription factor Nuclear factor of activated T cells c1 (NFATc1) is a master regulator of osteoclastogenesis. Previous knockout studies have shown that global NFATc1 deletion in younger mice leads to poor osteoclastogenesis and osteopetrosis. Here we show that the cathepsin k-cre mediated deletion of NFATc1 in mature osteoclasts leads to a phenotype with notable differences including the presence of abnormally large, multinucleate, TRAP-positive osteoclasts and the effacement of the bone marrow by stromal cells resembling a fibrotic reaction. We characterize this phenotype in a multitude of ways. We show that the fibrosis phenotype: (1) presents between age E15.5 and P5, (2) is dependent on the presence of osteoclasts and is downstream of RANK, the cell surface receptor that triggers osteoclast differentiation (3) and is not likely an osteoblast lineage-intrinsic phenotype. We hypothesized that NFATc1 in osteoclasts negatively regulates a secreted stromal lineage proliferative factor that functions in coupling. Our in-vitro assays using media conditioned by cultured wild-type or knockout osteoclasts were unable to
show differences in neither BMSC proliferation nor differentiation. Last, we identify genes that are highly upregulated in NFATc1 knock-out osteoclasts and describe those that may function in regulation of stromal-lineage differentiation or proliferation.
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Osteoclast Conditioned Media Treated \textit{Nfatc1}^{fl/\Delta} Ctsk-Cre\textsuperscript{+} BMSC Proliferation Assay

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<td>BMSC</td>
<td>Bone Marrow Stromal Cell</td>
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<td>BV/TV</td>
<td>Bone volume fraction.</td>
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<td>c-fms</td>
<td>Macrophage Colony-Stimulating Factor 1 Receptor</td>
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<td>HBSS</td>
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<td>HPRT</td>
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<td>HSC</td>
<td>Hematopoetic Stem Cell</td>
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<td>IGF</td>
<td>Insulin-like growth factor</td>
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<td>KO</td>
<td>Knock-Out</td>
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<td>M-CSF</td>
<td>Macrophage Colony Stimulating Factor</td>
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<td>MMP</td>
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<td>NDP</td>
<td>Norrie Disease Protein</td>
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<td>NFATc1</td>
<td>nuclear factor of activated T cells, cytoplasmic 1</td>
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<td>Abbreviation</td>
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<td>OCN</td>
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<td>OCP</td>
<td>Osteoclast precursor</td>
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<td>OPG</td>
<td>Osteoprotegerin</td>
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<td>OSX</td>
<td>Osterix</td>
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<td>PBL</td>
<td>Peripheral Blood Leukocytes</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>RANK</td>
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<td>Transforming Growth Factor</td>
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<td>Tgfb2</td>
<td>transforming growth factor, beta 2</td>
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<td>TNALP</td>
<td>tissue-nonspecific alkaline phosphatase</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<td>Tnfrsf22</td>
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<td>TRAP</td>
<td>tartarate-resistant acid phosphatase</td>
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<td>TRAIL</td>
<td>tumor necrosis factor (ligand) superfamily, member 10</td>
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<td>Wif1</td>
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<td>WT</td>
<td>Wild-Type</td>
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INTRODUCTION

The diversity and importance of bone functions are often overlooked. A major function of the skeletal system is to provide support for weight-bearing motor activity. Microscopic damage accrues as a result of physical activity, and is repaired by constant cycling of phases of bone degradation and bone formation (T. Negishi-Koga and Takayanagi 2009). This process is referred to as bone remodeling. Bone is, consequently, a very dynamic organ that continuously renews, replacing itself every 7-10 years (Sims and Gooi 2008). Additionally, bone houses and supports hematopoietic stem cells, is essential in regulating calcium homeostasis (Harada and Rodan 2003), and has been recently discovered to act as an endocrine organ regulating energy metabolism (Wagner and Karsenty 2001).

The delicate balance between bone resorption and bone formation is intricately regulated via a combination of chemokines, hormones, and biomechanical stimuli (T. Negishi-Koga and Takayanagi 2009). While these factors play differential roles throughout different parts of the skeletal system, it is ultimately the osteoclasts and the osteoblasts (and their precursors) that respond to these signals. The interplay between the osteoblast—the bone forming cells—and the osteoclasts—the bone resorbing cells—in their relative quantities and activities is what dictates the net effect on bone. A shift in this equilibrium determines whether the bone is in a bone forming or bone resorbing state.
The Three Cell Types of Bone – Osteoclasts, Osteoblasts and Osteocytes

The osteoclast is an incredibly specialized and unique cell. It is the only cell type capable of resorbing large quantities of bone. Its bone destructive properties make it the antithesis to the osteoblast. Osteoclasts are of hematopoietic origin and form through the fusion of mononuclear myeloid precursors (Boyle, Simonet, and Lacey 2003). Fusion and differentiation ultimately results in a multinucleated giant cell, a distinct feature on its own. These giant mature osteoclasts, in the process of differentiation, express specific machinery for the resorption of bone including the ability to actively secrete hydrochloric acid, cathepsin K and matrix metalloproteinases (MMPs), which synergize to degrade the bone matrix (Boyle, Simonet, and Lacey 2003).

The maturation of osteoclasts from their precursors is dependent on factors secreted by the osteoblast-lineage cells: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kβ ligand (RANKL) (T. Negishi-Koga and Takayanagi 2009). M-CSF interaction with its receptor on the myeloid precursor commits the cell to become an osteoclast precursor (Edwards and Mundy 2011), causing the upregulation of receptor activator of nuclear factor kβ (RANK) (Figure 1). Subsequent interaction of RANKL with RANK recruits a variety of regulators that will cause the eventual transcriptional upregulation of nuclear factor of activated T cells cytoplasmic 1 (NFATc1) (Edwards and Mundy 2011; Aliprantis et al. 2008)
NFATc1 is considered to be the master regulator of osteoclastogenesis. It is the most upregulated transcription factor induced by RANKL (Takayanagi et al. 2002) which reiterates the importance of this transcription factor for osteoclastogenesis. NFATc1 is part of a family of nuclear transcription factors that are, with the exception of NFAT5, regulated by calcineurin—a serine/threonine phosphatase activated by increasing intracellular calcium ions (T. Negishi-Koga and Takayanagi 2009). Upon RANKL stimulation, NFATc1 is dephosphorylated by calcineurin, exposing a nuclear localization (Takayanagi et al. 2002). NFATc1 then translocates into the nucleus where it cooperates with other transcription factors to activate osteoclast specific genes. Among these genes are: 1. dendritic cell-specific transmembrane protein (DC-STAMP) and the vacuolar proton pump subunit Atp6v0d2, which are necessary for fusion, 2. cathepsin K (CtsK), a protease secreted during resorption, and 3. β3 integrin, a molecule necessary for the binding and formation of the resorption lacunae (Kuroda et al. 2012). These genes, however, only represent a small proportion of genes that are targeted by NFATc1 regulation (Charles et al. 2012).

Loss of NFATc1 results in embryonic lethality (De la Pompa et al. 1998). Other methods have been employed to study the effects of NFATc1 deficiency and have demonstrated in-vivo that NFATc1 is essential in osteoclastogenesis (Winslow et al. 2006). Inducible knockouts of NFATc1 resulted in mice that exhibited severe osteopetrosis and a severe lack of tartrate-resistant acid
phosphatase-positive (TRAP-positive) multinucleated cells (Aliprantis et al. 2008).

![Diagram of Osteoclast Differentiation](image)

**Figure 1: Osteoclast Differentiation.** Binding of RANKL and M-CSF to respective receptors on the osteoclast activates NFATc1 transcription and activation to support osteoclast differentiation (Figure taken from Edwards and Mundy 2011, fig. 2)

Osteoblasts—the bone forming cells—are responsible for secreting the collagenous bone matrix and its subsequent mineralization. Osteoblasts are thus key mediators of bone mass (Zelzer and Olsen 2003). Osteoblasts arise from mesenchymal progenitors. The transition from mesenchymal stem cell to mature osteoblast is dependent on, the transcriptional control of runt-related
transcriptional factor 2 (Runx2) and downstream Osterix (Osx). Knock-out experiments have demonstrated that Runx2 is an early transcriptional regulator in osteoblast differentiation (Figure 2). Ablation of Runx2 resulted in a complete absence of osteoblasts (Kim et al. 1999). Osterix, downstream of Runx2, is responsible for the transition from pre-osteoblast to immature osteoblasts (Marie 2008). Anything affecting this transition compromises the ability of the osteoblast to lay down bone matrix, resulting in abnormal bone.

![Diagram of Mesenchymal Differentiation into Bone Cells](image)

**Figure 2: Mesenchymal Differentiation into Bone Cells.** Differentiation from mesenchymal cells into osteoprogenitor cells depends on the transcription factor Runx2. Further differentiation into osteoblasts requires the transcriptional regulator Osx (Figure Amended from Marie 2008, fig. 1).

Osteoblasts embedded in the mineralized bone matrix are subject to yet another level of differentiation, giving rise to another participant in bone remodeling—the osteocyte. As the osteoblast becomes entrapped in the osteoid, the osteoblast develops dendritic processes that extend throughout the
bone. These dendritic processes help the osteocyte fulfill its roles in regulating both mineralization and resorption (Bonewald 2011) by communication with the osteoblasts and osteoclasts.

**The Bone Remodeling Cycle**

Bone remodeling is thought to occur in discrete phases: initiation, transition, and termination (K. Matsuo and Irie 2008). Bone resorption begins with initiation which is demarcated by the recruitment of osteoclast precursors (OCPs) and the activation and differentiation of these precursors. Once resorption has occurred, the bone environment must switch to forming new bone instead of resorbing it—a phase that is known as transition. During this transition phase, the osteoclasts are believed to undergo apoptosis while osteoblast precursors are recruited, proliferated, and differentiated. Once the bone environment equilibrates to a state of bone formation where the mature osteoblasts are actively forming new bone, the bone remodeling process is said to be in its termination phase (K. Matsuo and Irie 2008). As one would imagine, the orchestration of these phases requires highly intricate communication between the bone cells, particularly the osteoblasts and osteoclasts.

Bone remodeling relies on the coupling of bone resorption and bone formation. An integral part of this coupling phenomenon is the intercellular communication between osteoblasts, osteocytes and osteoclast lineages. Each population must signal the other at every step in the remodeling process in order to coordinate the transitions between formation and resorption. This
communication can be achieved by several means including secretion of cytokines or paracrine factors, cell-cell contact, and local factors released from the turnover of the bone matrix (N. A. Sims and Walsh 2012). Examples of each of these modes of communication are discussed below.

**Paracrine Factors.** Several aspects of paracrine communication between the osteoblasts and osteoclasts have been well studied. Among the most studied are the factors produced by osteoblast lineage cells that signal for the initiation of bone remodeling: RANKL and M-CSF. While it remains unclear what truly initiates bone remodeling, there is evidence implicating parathyroid hormone (PTH), extracellular calcium levels, and/or mechanical stress (Tatsumi et al. 2007) as possible initiators. Osteoblast lineage cells regulate osteoclastogenesis via the production of RANKL and M-CSF (Lacey et al. 1998; Lagasse and Weissman 1997) osteoclast progenitors (OCPs) express receptors for RANKL and M-CSF on the surface of their membrane—RANK and M-CSF Receptor (c-fms) respectively. RANKL drives the proliferation and fusion of the OCPs in the bone marrow to form mature osteoclasts (Boyce and Xing 2008). M-CSF is also necessary but insufficient by itself to induce the differentiation of the osteoclast precursors into osteoclasts (Yoshida et al. 1990). M-CSF appears to be essential for the survival and proliferation of the OCPs (T. Negishi-Koga and Takayanagi 2009). Deletion of M-CSF and/or RANKL expression in osteoblasts resulted in the loss of mature osteoclasts and a osteopetrosis phenotype (Yoshida et al. 1990; N. A. Sims and Walsh 2012). Furthermore,
specific deletion of RANKL in osteocytes is sufficient to cause osteopetrosis (Nakashima et al. 2011). These two factors together are critical in inducing the correct profile of genes needed to form mature osteoclasts (Boyle, Simonet, and Lacey 2003).

The RANK/RANKL system has been extensively studied. RANKL is a homotrimeric protein that is membrane bound and secreted after being cleaved by matrix metalloproteases (MMP3 or 7) or a disintegrin and metalloprotease domain (ADAM) (B. F. Boyce and Xing 2008). Both RANKL and RANK are members of the tumor necrosis factor (TNF) receptor superfamily (Boyle, Simonet, and Lacey 2003). Ablation of either RANKL expression or RANK results in an identical phenotype, osteoclast deficient osteopetrosis (Li et al. 2000), emphasizing the importance of this ligand-receptor interaction in bone remodeling. Another member of the TNF-receptor superfamily is Osteoprotegerin (OPG). OPG is a soluble decoy receptor for RANKL secreted by osteoblasts. OPG competes with RANK in binding to RANKL and, consequently, inhibits osteoclast formation and activity (Boyle, Simonet, and Lacey 2003). As expected, overexpression of OPG resulted in an osteoclast deficient osteopetrosis (Wagner and Karsenty 2001) and deletion of OPG resulted in a osteoclast rich osteoporosis (Simonet et al. 1997). The severe phenotypes observed in these in vivo studies suggest that the RANK/RANKL/OPG axis is an extremely key regulator of bone remodeling and osteoclastogenesis.
**Cell-cell signaling.** Bidirectional signaling between cell surface proteins is another mode of communication that plays a role in bone remodeling. One example of such interaction in bone cells would be the cell surface Eph/ephrin system. Cell-cell interactions via these ephrins and Eph appear to control the migration, proliferation, and differentiation of mesenchymal (Osteoblast precursor) and hematopoetic (Osteoclast precursor) stem cells (Koichi Matsuo and Otaki 2012). Eph receptors are subcategorized as members of the receptor tyrosine kinase family and are activated by ephrin ligands (Pasquale 2008). Both receptors and ligands can be further subcategorized into A and B groups and they are differentially expressed between the different bone cells and even within the same cell as bone remodeling progresses.

The aforementioned initiation phase of bone formation consists of the recruitment, activation, and differentiation of Osteoclast precursors. During early osteoclastogenesis, Osteoclast derived ephrinA2 is upregulated. Upon interaction with osteoblast EphA2, bidirectional signaling inhibits differentiation in osteoblasts while stimulating differentiation in osteoclasts (Koichi Matsuo and Otaki 2012). This specific example demonstrates how bone cells communicate directly during initiation. Interestingly, RT-PCR profiles of these osteoclasts reveal that the expression of ephrinA2 decreases as they continue in culture in RANKL and M-CSF (Irie et al. 2009). This finding further reiterates that the bone cells maintain constant communication throughout the entire remodeling process.
The Eph/ephrin system is also at work as the initiation phase draws to an end. Bidirectional signaling, specifically between osteoclast ephrinB2 and osteoblast EphB4, appears to be a negative regulator of resorption (Figure 3). Reverse signaling into osteoclasts simulated by the addition of EphB4-Fc to osteoclast cultures resulted in the inhibition of osteoclast differentiation and activity (Koichi Matsuo and Otaki 2012). Forward signaling of EphB4 into osteoblasts appeared to promote osteoblast differentiation (Koichi Matsuo and Otaki 2012). The interaction of ephrinB2 and EphB4 in this later stage of initiation helps the bone progress into the transition phase of bone remodeling.
Figure 3: Osteoclast and Osteoblast bi-directional signaling via Eph/ephrin system. Bi-directional communication between the osteoblasts and osteoclasts via the Eph/ephrin system plays a regulatory role in bone coupling. Upregulation in ephrinA2 in early osteoclasts and interaction with EphA2 in osteoblasts positively regulates osteoclast differentiation while inhibiting osteoblast differentiation, thereby attenuating bone formation and augmenting bone resorption. As osteoclasts differentiate, expression of ephrinA2 decreases while ephrinB1 increases. Subsequent interaction of ephrinB1 with EphB4 on osteoblasts inhibits osteoclast differentiation while stimulating differentiation in osteoblasts (Figure Adapted from Koichi Matsuo and Otaki 2012, fig. 3).

More recently, the cell-cell interactions of Semaphorins and Plexins have also been implicated as coupling factors that contribute to the transition phase of bone remodeling. RANKL appears to induce the expression of Semaphorin-4D (Sema4D) on osteoclasts during osteoclastogenesis (Takako Negishi-Koga et al. 2011). Sema4D is expressed exclusively on osteoclasts (Kang and Kumanogoh 2013) and interacts with Plexin-B1 receptor on the osteoblast membrane. Loss-of-function studies revealed that this specific cell-cell interaction played an important role in the suppression of bone formation. Loss of either Sema4D or Plexin-B1 resulted in increased bone mass, osteoblast surface, and bone
formation rates (Takako Negishi-Koga et al. 2011). It is believed that the Sema4D-PlexinB1 interaction has an inhibitory effect on mineralization of osteoblasts while having a stimulatory effect on osteoclast formation (Takako Negishi-Koga et al. 2011). This direct form of communication is one of the many ways the bone cells harmoniously interact to ensure proper bone remodeling.

**Factors released from the bone matrix.** During the transition phase of bone remodeling, osteoblast precursors need to be recruited and stimulated to differentiate. One initial hypothesis was that the osteoclasts direct the next steps by liberating coupling factors trapped in the bone matrix with their resorptive activity. Factors such as insulin-like growth factor (IGF) I and II and Transforming Growth Factor (TGF)-β are potential factors. Supporting this hypothesis, active TGF-β was shown to be released from the bone matrix by osteoclast resorption, and to be required for mesenchymal stem cell migration to trabecular surfaces in vivo (Tang et al. 2009). In contrast, others have found that the activity of these osteoclasts was dispensable for the activation of osteoblastic bone formation (K. Matsuo and Irie 2008). Knocking out osteoclast formation altogether via c-fos knockout resulted in reduced bone resorption as well as bone formation in mice, suggesting the presence of coupling (Grigoriadis et al. 1994). Targeted elimination of osteoclast activity without affecting osteoclast numbers via deletion of c-src or chloride-7 channel resulted in the inhibition of bone resorption while leaving bone formation unaffected (Brockstedt et al. 1996). These findings suggest that osteoclast secrete factors that recruit
osteoblast precursors and/or promotes differentiation, thus stimulating bone formation.

**An osteoclast derived coupling factor.** The notion that bone resorption must be tightly coupled to bone formation during transition denotes an increasingly important role for osteoclast to osteoblast communication during resorption. Osteoclasts must be able to communicate to the osteoblasts that resorption is complete and poise the mesenchymal cells for a subsequent phase of bone formation. One compelling hypothesis postulates the secretion of a “coupling factor” from the osteoclast. This coupling factor should, inferring from what must occur in the transition phase, have functions in any one or combination of the following: to recruit mesenchymal osteoblast progenitors, to stimulate the proliferation of these progenitors, or to activate the progenitors to become bone forming osteoblasts. Although some coupling mechanisms have already been identified, the complexity in the regulation of bone turnover denotes a high likelihood that there are other potential coupling factors to be identified.

We have already highlighted several notable forms of osteoclast-osteoblast communication. In these examples, the differentiation and activity of the osteoclast causes the upregulation of factors that regulated osteoblast activity. Many of these factors, like the ephrins for example, are under the control of NFATc1. These downstream genes of NFATc1 may function in coupling the activity of the osteoclast to the osteoblast.
Recent work in our lab provides support for an osteoclast derived factor promoting the proliferation of osteoblast lineage stromal cells. Deletion of key osteoclast transcription factor NFATc1 in mature osteoclasts results in a disorganized and thickened skeleton, where bone marrow has been replaced with spindle shaped osteoblast lineage cells, almost obliterating normal hematopoietic elements. In contrast to deletion of NFATc1 in osteoclast precursors, which is characterized by an osteoclast poor state, mice with deletion in NFATc1 in mature osteoclasts have numerous giant abnormal osteoclasts. Deletion of RANK in mature osteoclasts on the other hand results in mice with a classic osteoclast poor osteopetrosis without the abnormal bone marrow stromal hyperproliferation. From these observations, we hypothesize that NFATc1 in mature osteoclasts negatively regulates an osteoblast lineage proliferative factor that functions in coupling. In this thesis, we attempt to (1) characterize and define the stromal cell infiltrate in the marrows of mice deficient in NFATc1 in mature osteoclasts (using a cathepsin k-cre mediated deletion of a floxed Nfatc1 allele, henceforth referred to as Nfatc1^fl/Δ Ctsk-Cre^+^) and (2) identify this proliferative factor produced by Nfatc1^fl/Δ Ctsk-Cre^+^ osteoclasts. By elucidating the communication between osteoclasts and osteoblasts in bone coupling, we hope to illuminate novel players in bone regulation that may have a role in the pathogenesis of bone disease and that may serve as therapeutic targets to treat these diseases.
Methods

Mice

*Nfatc1*<sup>fl/fl</sup> mice were previously generated as described in Aliprantis 2008. To generate mice lacking NFATc1 in mature osteoclasts, *Nfatc1*<sup>fl/fl</sup> mice were crossed to mice expressing Cre recombinase under the control of the *Cathepsin K* promoter, which is active in mature osteoclasts. These *Ctsk-Cre* mice were a gift of Dr. Shigeaki Kato (Ref: Cell, 2007 130:811-23). Sporadic deletion of the floxed *Nfatc1* allele in the maternal line resulted in the generation of the *Nfatc1*<sup>Δ</sup> in some cases, such that the genotype of mutant mice could be either *Nfatc1*<sup>fl/fl</sup> *CtskCre*<sup>+</sup> or *Nfatc1*<sup>Δ</sup> *CtskCre*<sup>+</sup>. Genotyping was performed by PCR using the following primers to detect wild type, floxed and deleted alleles of *Nfatc1*, and the presence of *Ctsk-Cre*: *Nfatc1* (AAGGAATTACTGGGAAGCCTGGCA, AGGGACTATCATTTGGCAGGGACA, ACAGGAAACAGCTCTGTTCCACAC) CreF (GCGGTCTGGCAGTAAAAACTATC), CreR (GTGAAACAGCATTGCTGTCACTT). CD45.1 mice were purchased from Jackson. For chimera experiments, mice were irradiated (800 RAD), allowed to rest for 4 hours, followed by tail vein injection of CD45.2 donor fetal liver cells. Irradiated mice were maintained on sulfatrim to prevent infection. All animal experimentation was performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care and approved by Standing Committee on Animals at Harvard.
In-Vitro Culture of Murine Osteoclasts

Osteoclasts were cultured from hematopoetic progenitors found in bone marrow, spleen, or fetal livers. Bone marrow cells were isolated from long bones by vigorously flushing the marrow with Hanks buffered salt solution (HBSS). Splenocytes were prepared by crushing dissected spleens through a 70µm strainer followed by washing the filter with HBSS. Fetal livers were isolated from E13.5 and E15.5 fetuses and cell suspensions were prepared as with spleens (see below for additional details). The samples were pelleted by centrifugation at 1000rpm for 6-8 minutes. With spleen and bone marrow samples, red blood cell (RBC) lysis was performed using a hypotonic lysis buffer and cells were resuspended in complete α-MEM media (alpha-minimal essential media (GIBCO) supplemented with penicillin/streptomycin, 10% Fetal Bovine Serum (Hyclone)) containing either 10% CMG14-12 conditioned-medium (CMG) or 40ng/mL recombinant M-CSF (R&D) to drive myeloid cell expansion. Cells were plated onto suspension culture dishes (Corning Costar Ins) that stromal and lymphoid cells do not adhere to and expanded for three days. For osteoclast differentiation, expanded cells were lifted using either 10mM EDTA in phosphate buffered saline or non-enzymatic dissociation buffer (GIBCO). Cells were resuspended in osteoclast media (complete α-MEM containing 5ng/mL RANKL (R&D) and either 20ng/mL MCSF or 5% CMG supernatant.) and plated at density of 2x10⁴/cm². Media was replaced on day 3 and 5.

In order to assess osteoclast formation, cultures were fixed in 4% paraformaldehyde for 10 minutes, washed, and stained for tartrate resistant acid

**Fetal Liver Isolation**

E13.5 - E15.5 fetuses were isolated by caesarian-section of euthanized pregnant females. Using a stereo microscope, fetal livers were carefully removed from the fetuses and placed into complete α-MEM media. Fetal livers were homogenized by repeated pipetting and passed through a 70µm filter. Samples were pelleted by centrifugation at 1000rpm for 6-8 minutes. Fetal livers were then resuspended in freezing media (10% dimethylsulfoxide in HBSS) and stored overnight at -80⁰ C and later into liquid nitrogen.

**Generation of Osteoclast Conditioned Media**

Osteoclast conditioned media was generated by culturing expanded OCP from spleen, bone marrow or fetal livers, on 24 well plates in 0.5mL/well of either complete αMEM with 10% CMG and 5ng/mL RANKL or complete α-MEM with 20ng/mL M-CSF or 5ng/mL RANKL. At 3 and 5 days in this media fresh M-CSF and RANKL cytokines were added. Media was collected after 7 days in culture.
Osteoblast Cultures

Bone marrow stromal cells (BMSCs) were isolated from young pups, ages P5-P10. Whole hindlimbs and forelimbs were collected and pooled according to genotype. Limbs were washed with 70% Ethanol, HBSS, and placed in sterile HBSS. Limbs were then minced and digested in 1mg/mL collagenase II, and 2mg/mL dispase in α-MEM media at 37˚C for approximately 40 minutes with agitation. The enzymes were subsequently inactivated with an excess of FBS containing media. The digested limbs were then passed through a 70µm strainer and centrifuged for 5-8 minutes at 1000rpm. RBC lysis was optionally performed and cells were plated in BMSC media (α-MEM supplemented with 10% FBS, penicillin/streptomycin, L-glutamine and non-essential amino acids). For proliferation assays, cells were plated at a density of 1-3x10³/cm², while for differentiation assays cells were plated at a density of 1-1.5x10⁴/cm².

Osteoblast Proliferation Assays

AlamarBlue (Invitrogen) indicator was used to quantify number of viable cells at a given time point and used according to the manufacturer's instruction. Proliferation rates were determined by serial measurement of cell number over time. Briefly, culture media was replaced with alamar blue diluted to working concentration in culture media. Assays were incubated at 37˚C for 4 hours and fluorescence at 590nm with an excitation wavelength of 560nm was measured using a Synergy H1 fluorimeter (BioTek).
Osteoblast Differentiation Assays

BMSCs are expanded to confluence in BMSC media and lifted using 1X Trypsin-EDTA. The BMSCs are then plated at a density of $1-1.5 \times 10^4$/cm$^2$ in BMSC media and incubated at 37˚C to adhere. BMSC media was replaced by osteoblast differentiation media (BMSC media with 50µg/mL ascorbic acid and 5mM beta-glycerophosphate). To limit the formation of osteoclasts from contaminating hematopoietic elements, OPG (100ng/mL) was also added to osteoblast differentiation media.

Micro-computed tomography (µCT)

Imaging of femurs was performed using a Scanco Medical µCT 35 system with a voxel size of 7µm. Femurs were scanned in 70% ethanol using an X-ray potential, intensity, and integration time of 55kVp, 0.145mA and 600ms respectively, scanning the region from the distal femur to approximately 3mm proximal. Differentiating trabecular from cortical bone employed a semi-automated contouring approach, using a threshold of 352.3 mgHA/cm$^3$ for marrow/trabecular bone cut-off. 3D reconstruction of the entire scan was done using the manufacturer supplied software. Calculations for bone volume fraction (BV/TV) and trabecular thickness (Tb.Th) were performed on the region 0.28mm proximal to the growth plate and extending 2.1mm.
Histology

Bones were fixed in freshly prepared 4% phosphate buffered formaldehyde (PBF) for 24 hours followed by decalcification in with 3-4 changes of 0.5M EDTA over 1 month. The bones were then paraffin embedded and 10µm longitudinal sections were taken at the growth plate for H&E and TRAP staining. TRAP staining on tissue sections was performed according to published methods (Erlebacher and Derynck 1996).

Flow cytometry

BMSC isolated as above were incubated with APC conjugated anti-mouse CD45 antibody (Biolegend) at 4°C for 20 minutes, washed and CD45 cells were sorted by fluorescence activated cell sorting on a FACS Aria (BD). For chimerism studies, bone marrow and spleen were isolated as usual, peripheral blood leukocytes (PBLs) were purified on a ficoll gradient, cells were stained with allele specific anti-bodies to CD45.1 and CD45.2 (Biolegend) and analyzed by flow cytometric cell analysis on a FACS Canto (BD).

Quantitative Real-Time PCR (qRT-PCR)

RNA was isolated using a RNeasy Mini Kit (Qiagen) according to manufacturer instructions. cDNA was synthesized from the isolated RNA using a kit supplied by Applied Biosystems and a Veriti 96 Well Thermal Cylinder (Applied Biosystems) according to manufacturer instructions. FAST SYBR
Green Master Mix was used in conjunction with a Step-One Plus Real-Time PCR System (Applied Biosystems) to perform real-time quantitative PCR. Average critical temperature (Ct) values were calculated from duplicate technical replicates. Using the ∆Ct method, the quantity of mRNA was calculated relative to Hprt values. The qRT-PCR primers used in our experiments are listed in Table 1.

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<th>Reverse Primers (5’-3’)</th>
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<td>Ocn</td>
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Results

1. Phenotype of mice lacking NFATc1 in mature osteoclasts.

Genetic ablation of NFATc1 in mature osteoclasts driven by osteoclast specific promoton CathepsinK (Nfatc1\textsuperscript{fl/\textDelta} Ctsk-Cre\textsuperscript{+}) results in a distinct phenotype. These mice are smaller compared to wild-type littermates—referred to as being “runted” (Figure 4A). Similar to mutant mice lacking osteoclast activity, these Nfatc1\textsuperscript{fl/\textDelta} Ctsk-Cre\textsuperscript{+} mice are toothless (Figure 4B).

![Figures A and B]

**Figure 4: Gross Nfatc1\textsuperscript{fl/\textDelta} CtsKCre\textsuperscript{+} Phenotype.** Photographs of a Postnatal Day 14 Nfatc1\textsuperscript{fl/\textDelta} Ctsk-Cre\textsuperscript{+} mouse (seen on right in both A & B) and its wild-type littermate (seen on left in both A & B). A. Nfatc1\textsuperscript{fl/\textDelta} Ctsk-Cre\textsuperscript{+} mice are runted compared to a wild-type littermate. B. Nfatc1\textsuperscript{fl/\textDelta} Ctsk-Cre\textsuperscript{+} mice are toothless.

Micro-computed tomography (µCT) imaging was performed on femurs isolated from Nfatc1\textsuperscript{fl/\textDelta} Ctsk-Cre\textsuperscript{+} mice and a wild-type litter mate at post-natal day 14 (P14) (Figure 5). Imaging demonstrates decreased femur length, increase in trabeculae, a diminished or absent secondary center of ossification and an absence of cortical bone in mutants compared with wild-type. Although short
femur length and increased trabecular bone are consistent with defective osteoclast number or function such as may be seen in Rank\textsuperscript{fl/fl} Ctsk-Cre\textsuperscript{*} mice, defects in cortical bone and secondary center of ossification are unique to the Nfatc1\textsuperscript{fl/\Delta} Ctsk-Cre\textsuperscript{*} mutant.

![Figure 5: µCT Characterization of Nfatc1\textsuperscript{fl/\Delta} CtsK-Cre\textsuperscript{*} Phenotype.](image)

The histologic phenotype of Nfatc1\textsuperscript{fl/\Delta} Ctsk-Cre\textsuperscript{*} mice is also strikingly distinct from classic osteoclast-poor osteopetrosis. H&E sections of Nfatc1\textsuperscript{fl/\Delta}
Ctsk-Cre* femurs reveal that the marrow, normally inhabited by hematopoietic cells, is replaced by flat, spindle-shaped, fibroblast-like, stromal cells (Figure 6B, right)—a fibrotic phenotype that is not seen in other forms of osteoclast-poor osteopetrosis. In comparison, deletion of Rank in mature osteoclasts (Rank^{fl/fl} Ctsk-Cre*) results in a classic osteoclast-poor osteopetrosis, with increased trabeculae but normal hematopoietic elements present in the remaining bone marrow space (figure 6B), and absence of multinucleated osteoclasts (Figure 6C). Surprisingly, Nfatc1^{fl/∆} Ctsk-Cre* mice have abnormal giant, multinucleate, TRAP-positive osteoclasts, which are not present in osteoclast-poor osteopetrosis, suggesting that NFATc1 expression in early osteoclastogenesis is sufficient for osteoclast formation.
Figure 6: The *Nfatc1*^fl/Δ^ *Ctsk-Cre*^+^ phenotype is not a typical Osteoclast Poor Osteopetrosis. **A.** Femoral growth plate of, from right to left, wild-type, *Nfatc1*^fl/Δ^ *Ctsk-Cre*^+^ knockout, *Rank*^fl/fl^ *Ctsk-Cre*^+^ knockout. H&E 5x. **B.** 20x magnification of boxed regions in (A) reveals an abnormal, spindle shaped, stromal cell infiltrate instead of normal hematopoietic elements in the marrow in the in *Nfatc1*^fl/Δ^ *Ctsk-Cre*^+^ marrow (left) while the in *Rank*^fl/fl^ *Ctsk-Cre*^+^ (right) has normal appearing marrow. **C.** 40x magnifications of TRAP positive osteoclast lineage
cells. \( Nf\alpha c1^{fl/\Delta} Ctsk-Cre^+ \) (center) shows an example of a giant, multinucleated, TRAP-positive, abnormal osteoclast.

To determine the exact age at which marrow fibrosis presents, we performed histopathologic analysis of the femurs and humeri, respectively, of younger mice at day 5 post-natal mice (P5) and day 16.5 embryos (E16.5). The abnormal bone marrow stromal cell infiltrate was present in the P5 \( Nf\alpha c1^{fl/\Delta} Ctsk-Cre^+ \) mice but not in the E16.5 \( Nf\alpha c1^{fl/\Delta} Ctsk-Cre^+ \) mice (Figure 7). Histological analyses between these two ages will be performed in order to further pinpoint the age of onset.
Figure 7: Marrow Fibrosis Phenotype presents between Age E16.5 and P5.

A. Stromal infiltrate is present by P5. WT (left) and Nfatc1^{0/Δ} Ctsk-Cre^{+} (right) femur, H&E, 20X.

B. Nfatc1^{0/Δ} Ctsk-Cre^{+} marrow is indistinguishable from the WT littermate at E16.5. WT (left) and Nfatc1^{0/Δ} Ctsk-Cre^{+} (right) humerus, H&E, 20X.
Marrow fibrosis has been described in both conditions of high bone turnover (Weinstein 2006) and in hematological diseases (Kuter et al. 2007). Silver staining of reticulin fibers is one way of distinguishing between the two types of fibrosis, where hematological diseases tend to be positive for these fibers (Kuter et al. 2007). In order to further characterize the marrow fibrosis found in these Nfatc1\textsuperscript{fl/\Delta} Ctsk-Cre\textsuperscript{+} knockouts, we stained longitudinal sections of P14 femurs for silver binding reticulin fibers. Our positive controls, liver and spleen sections, had darkly stained and well defined fibers. The femurs in both wild-type and Nfatc1\textsuperscript{fl/\Delta} Ctsk-Cre\textsuperscript{+} mice, however, showed only a diffuse amorphous silver stain (Figure 8). The lack of well defined, silver binding reticulin fibers suggests that the marrow fibrosis seen in Nfatc1\textsuperscript{fl/\Delta} Ctsk-Cre\textsuperscript{+} mice is associated with high bone turnover rather than hematologic disease.
Figure 8: Negative Reticulin Stain in Nfatc1<sup>fl/∆</sup> Ctsk-Cre<sup>+</sup> Marrow Characterizes Fibrosis as one of High Bone Turnover. A. Reticulin stain on liver shows discrete silver stained reticulin fibers. B. No discrete positively stained fibers are detected on p15 Nfatc1<sup>fl/∆</sup> Ctsk-Cre<sup>+</sup> or C. WT femur. All images at 20X.

Another feature that distinguishes Nfatc1<sup>fl/∆</sup> Ctsk-Cre<sup>+</sup> mice from other osteoclast deficient mice is premature lethality. Nfatc1<sup>fl/∆</sup> Ctsk-Cre<sup>+</sup> die at 17 days after birth for reasons that are still unclear. Mice with a global deficiency in NFATc1 deficient experience embryonic lethality due to cardiac valve defects (De la Pompa et al. 1998). As cathepsin K is expressed in the endocardium (Lange and Yutzey 2006) we hypothesized that cathepsin K driven deletion of Nfatc1 in these mice may result in cardiac valve defects leading to premature death.
Histological analysis of the cardiac valves performed by our collaborator Dr. Yutzey, revealed that this was not the case (Figure 9). Given the replacement of bone marrow hematopoietic elements noted above, and the lack of observed extramedullary hematopoiesis (data not shown), it is possible that $Nfatc1^{fl/Δ} Ctsk$-Cre$^+$ mice die from bone marrow failure and anemia. In fact, $Nfatc1^{fl/Δ} Ctsk$-Cre$^+$ are modestly anemic prior to death (not shown), and investigation of blood counts on the usual day of death are ongoing. Alternatively, abnormal rib bones may lead to restrictive pulmonary physiology and $Nfatc1^{fl/Δ} Ctsk$-Cre$^+$ may die from respiratory compromise. This possibility is currently being pursued.
Figure 9: Premature death in Nfatc1\textsuperscript{fl/\Delta} Ctsk-Cre\textsuperscript{+} Mice is not due to Cardiac Defect. Heart valves in 4 wild-type mice (top) and 4 Nfatc1\textsuperscript{fl/\Delta} Ctsk-Cre\textsuperscript{+} mice (bottom). Valve morphology appeared normal in both genotypes (images courtesy of Katherine Yutzey).
2. Origin of Marrow Fibrosis: Osteoblast?

2.1. Loss of NFATc1 in Osteoblast-lineage cells does not cause Fibrosis Phenotype.

One hypothesis that may explain the marrow fibrosis phenotype is that Cathepsin K expression in cells of the osteoblast or other mesenchymal/stromal cells results in NFATc1 deletion in the $Nfatc1^{fl/\Delta}$ Ctsk-Cre$^+$ mice, resulting in stromal cell intrinsic effects on proliferation or differentiation. In fact, cathepsin K expression in osteocytes, cells of the osteoblast lineage, has been demonstrated (Bonewald 2011). Thus, we tested the possibility that the marrow fibrosis is an osteoblast-lineage intrinsic phenotype by deleting NFATc1 in osteoblasts. In prior work from the lab, an osteoblast specific promotor, osterix (Osx), was used to drive Cre-mediated deletion of NFATc1 and no gross differences bone morphology were observed in these mice (Figure 10). Secondly, genotyping of stromal cells purified and cultured from $Nfatc1^{fl/\Delta}$ Ctsk-Cre$^+$ mice did not show deletion of the floxed allele. These results substantiate the notion that the proliferation of stromal cells is more likely secondary to the deletion of NFATc1 in mature osteoclasts.
As the stromal cell infiltrate in \( \text{Nfatc1}^{\text{fl/\Delta}} \) \( \text{Ctsk-Cre}^+ \) mice occasionally demonstrated invasion into the hypertrophic chondrocyte zone (data not shown), we hypothesized that the stromal cell phenotype could arise from malignant transformation. Thus, karyotyping was performed on stromal cells cultured from \( \text{Nfatc1}^{\text{fl/\Delta}} \) \( \text{Ctsk-Cre}^+ \) bone. No clonal abnormalities were evident (data not shown), arguing against malignant transformation arising in stromal cells of \( \text{Nfatc1}^{\text{fl/\Delta}} \) \( \text{Ctsk-Cre}^+ \) mice.

### 2.2. Osteoblast differentiation from \( \text{Nfatc1}^{\text{fl/\Delta}} \) \( \text{Ctsk-Cre}^+ \) BMSC is normal

Although we were unable to detect deletion of the floxed \( \text{Nfatc1} \) allele by genotyping of \( \text{Nfatc1}^{\text{fl/\Delta}} \) \( \text{Ctsk-Cre}^+ \) stromal cells, it remains possible that this genetic background results in stromal cell intrinsic defects in osteoblast differentiation. Ctsk-Cre mediated deletion of NFATc1 in a minority of stromal...
cells, below the threshold of detection by PCR genotyping, could inhibit the ability of the osteoblasts to differentiate, causing the progenitors to accumulate as a consequence. To test this hypothesis, we sort purified CD45⁻ non-hematopoietic cells from bone digests of $Nfatc^{fl/\Delta}\ Ctsk-Cre^+$ and WT littermate controls and expanded them in BMSC media. Sort-purified BMSCs were cultured in osteoblast differentiation media for 21 days. Osteoblast differentiation was assessed by staining for alkaline phosphatase (a marker of osteoblast differentiation) and by von kossa staining to assess formation of mineralized nodules (a marker of mature osteoblast function). Alkaline phosphatase and von kossa staining at 7, 14, and 21 of differentiation showed no apparent differences between BMSCs isolated from WT mice and those isolated from $Nfatc^{fl/\Delta}\ Ctsk-Cre^+$ mice (Figure 11). These results are representative of two independent experiments.

**Figure 11: Osteoblast Differentiation from WT and $Nfatc^{fl/\Delta}\ Ctsk-Cre^+$ BMSC is indistinguishable.** Osteoblast cultures stained for alkaline phosphatase activity (blue) and for mineral deposition by von kossa staining (black) at 7, 14, and 21 days is shown.

In addition to the alkaline phosphatase and von kossa staining, we also performed quantitative real time PCR on these samples to measure the
expression of various known osteoblast markers during differentiation (Figure 12). We specifically measured the expression levels of tissue-nonspecific alkaline phosphatase (TNALP), runt related transcription factor 2 (Runx2), osterix (OSX), and osteocalcin (OCN) and normalized their expression to the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT). We found that expression levels of all the targets in the \textit{Nfatc1}^{flo\Delta} \textit{Ctsk-Cre}^{+} BMSCs matched and even surpassed the levels in WT BMSCs. Altogether, this data suggests that there does not appear to be an intrinsic defect in the ability of \textit{Nfatc1}^{flo\Delta} \textit{Ctsk-Cre}^{+} BMSCs to differentiate into osteoblasts.
Figure 12: Expression of Osteoblast Differentiation Markers on Differentiated WT vs Nfatc1\textsuperscript{fl/\Delta} Ctsk-Cre\textsuperscript{+} Sort Purified CD45- BMSCs. Quantitative RT-PCR was performed to measure expression levels of known osteoblast differentiation markers on samples of sort purified CD45- BMSCs that were in osteoblast differentiation media for either 0, 7, 14, or 21 days. All expression levels were normalized to HPRT expression levels.
3. Marrow Fibrosis is Secondary to Loss of NFATc1 in Mature Osteoclasts

3.1. RANK and Osteoclasts are Required for the Fibrosis Phenotype

Given that NFATc1 is activated by RANKL signaling (Takayanagi et al. 2002), we hypothesized that RANK stimulation in the absence of NFATc1 in mature osteoclasts may lead to the production of a factor (which we will refer to as Factor X) that induces BMSC proliferation. In the absence of RANK signaling then, and thus the absence of osteoclasts, Factor X will not be produced and the bone phenotype should look like that of Rank\textsuperscript{fl/fl} Ctsk-Cre\textsuperscript{+} mice—the classic osteoclast-poor osteopetrosis with no stromal cell proliferation. To test this hypothesis we generated osteoclast specific NFATc1 and RANK double knockouts to examine histologically. We crossed Nfatc1\textsuperscript{fl/fl} Ctsk-Cre\textsuperscript{+} mice to Rank\textsuperscript{fl/fl} Ctsk-Cre mice to generate Nfatc1\textsuperscript{fl/fl} Rank\textsuperscript{fl/fl} Ctsk-Cre double mutant mice.

To fully characterize the dependence of the NFATc1 phenotype on RANK, we also examined Nfatc1\textsuperscript{fl/Δ} Ctsk-Cre\textsuperscript{+} heterozygous for RANK (Figure 13). Histological analyses of the femurs of P16 mice demonstrated that RANK heterozygosity did not rescue the Nfatc1\textsuperscript{fl/Δ} Ctsk-Cre\textsuperscript{+} phenotype. The mice still demonstrate the same phenotype: they are runted, toothless, have increased bone volume, and have the trademark proliferation of BMSCs in the marrow.
Figure 13: Rank Heterozygosity does not Rescue Nfatc1^{fl/Δ} Ctsk-Cre^{+} Phenotype. Images growth plate (left) and marrow (right) of femurs from P15 mice. A. Wild-type control Nfatc1^{fl/+} Rank^{fl/+} Ctsk-Cre^{+}. B. Nfatc1^{fl/Δ} Rank^{fl/+} Ctsk-Cre^{+}, and C. Nfatc1^{fl/Δ} Rank^{fl/+} Ctsk-Cre^{+}. H&E, 5x (right panels) and 20x (left panels).
A total of approximately 130 pups were screened before a single osteoclast specific NFATc1/RANK double knockout was identified. This number falls far below the expected mendelian ratio, suggesting the possibility of embryonic or early post-natal loss of these double knock-outs. Saggital sections of the femurs of this P14 double-knockout and its littermates were stained for H&E (Figure 14). As expected, the Nfatc1$^{fl/\Delta}$ Rank$^{fl/fl}$ Ctsk-Cre, double osteoclast specific knockouts looked very similar to Rank$^{fl/fl}$ Ctsk-Cre, exhibiting the classical osteoclast-poor osteopetrosis with normal hematopoietic marrow elements. TRAP staining confirmed that Nfatc1$^{fl/\Delta}$ RANK$^{fl/fl}$ Ctsk-Cre, like RANK$^{fl/fl}$ Ctsk-Cre, have an absence of TRAP-positive osteoclasts (Figure 14C). μCT analysis was performed and the bone phenotype of the Nfatc1$^{fl/\Delta}$ RANK$^{fl/fl}$ Ctsk-Cre double knock-out was compared to a wild-type littermate and age matched single osteoclast-specific knock outs (Figure 15). The Nfatc1$^{fl/\Delta}$ RANK$^{fl/fl}$ Ctsk-Cre double knock-out was most similar to the RANK$^{fl/fl}$ Ctsk-Cre. Collectively, these data suggest that RANK signaling is required to for expression of this NFATc1 dependent phenotype, possibly because RANK signaling is required for osteoclast formation.
Figure 14: *Nfatc1*<sup>fl/∆</sup> *Rank*<sup>fl/fl</sup> *Ctsk-Cre*<sup>+</sup> Double Mutants Resemble *Rank*<sup>fl/fl</sup> *Ctsk-Cre*<sup>+</sup> Single Mutants Histologically. H&E staining of femur growth plates of P14 mice shown at 5X, 10X and 20X magnifications (A&B). A. Wild-type control *Nfatc1*<sup>fl/+</sup> *Rank*<sup>fl/+</sup> *Ctsk-Cre*<sup>+</sup>. B. *Nfatc1*<sup>fl/∆</sup> *Rank*<sup>fl/fl</sup> *Ctsk-Cre*<sup>+</sup> double mutant with the classic osteoclast poor osteopetrosis. Higher magnification reveals normal hematopoietic elements in the bone marrow. C. TRAP staining of the WT control reveals the presence of many TRAP positive osteoclasts (left).
while similar TRAP staining in $\text{Nfatc1}^{\text{fl/\Delta}}$ $\text{Rank}^{\text{fl/fl}}$ $\text{Ctsk-Cre}^+$ Double Mutants revealed a complete absence of TRAP positive osteoclasts (right).

Figure 15: $\text{Nfatc1}^{\text{fl/\Delta}}$ $\text{Rank}^{\text{fl/fl}}$ $\text{Ctsk-Cre}^+$ Double Mutant Most Resembles $\text{Rank}^{\text{fl/fl}}$ $\text{Ctsk-Cre}^+$ Single Mutant in $\mu$CT Imaging. $\mu$CT scans of P15 femurs from (from left to right): $\text{Nfatc1}^{\text{fl/\Delta}}$ $\text{Rank}^{\text{fl/fl}}$ $\text{Ctsk-Cre}^+$ double mutant, $\text{Nfatc1}^{\text{fl/+}}$ $\text{Rank}^{\text{fl/+}}$ $\text{Ctsk-Cre}^+$ wild-type littermate, $\text{Nfatc1}^{\text{fl/\Delta}}$ $\text{Ctsk-Cre}^+$ single mutant, and $\text{Rank}^{\text{fl/fl}}$ $\text{Ctsk-Cre}^+$ Single Mutant.
3.2. Loss of NFATc1 in Hematopoietic Stem Cells is Insufficient to Produce Marrow Fibrosis in Fetal Liver Chimeras

BMSCs and osteoblasts are of mesenchymal origin while osteoclasts are of hematopoietic origin. The results found in the previous section, along with the phenotype of the $Nfatc1^{fl/\Delta}$ Rank$^{fl/fl}$ Ctsk-Cre$^+$, suggest that osteoclasts, and thus hematopoietic cells, are required for expression of the phenotype. To determine if hematopoietic cells are sufficient for expression of this proliferative stromal cell phenotype, we generated fetal liver chimeras. Adult CD45.1 hosts were lethally irradiated to eliminate the hematopoietic cell pool. Fetal liver cells, a rich source of hematopoietic progenitors, from either wild-type or $Nfatc1^{fl/\Delta}$ Ctsk-Cre$^+$ CD45.2 donors were subsequently injected into the hosts to reconstitute the hematopoietic cell population. If the reconstituted hematopoietic cell population induces the marrow fibrosis phenotype, then we would have strong evidence that loss of NFATc1 in hematopoietic cells is sufficient to produce the marrow fibrosis phenotype. Mice were examined at 8 weeks, with a larger cohort planned to be analyzed 24 weeks post-transfer for differences in histology and µCT analyses. Flow cytometry was also performed on the spleens, bone marrow, and peripheral blood leukocytes to verify and quantify the chimerism.

Flow cytometric analysis using antibody stains specific for CD45.1 (Host) and CD45.2 (Donor) revealed that at 8 weeks greater than 90% of cells in blood, spleen, and bone marrow were donor derived (data not shown). At 8 weeks, µCT analysis showed that hosts receiving hematopoietic cells from $Nfatc1^{fl/\Delta}$ Ctsk-
*Cre* donor had a slightly increased ratio of bone volume fraction (BV/TV) and trabecular thickness (Tb. Th.) compared with the host receiving WT cells (Figure 16 A and B). This result is consistent with poor osteoclast formation due to NFATc1 deletion.

![Figure 16: µCT analysis of Week 8 Chimeric Mice. A. µCT scans of the femurs of CD45.1 WT hosts 8 weeks after reconstitution of hematopoietic stem cell pool using CD45.2 fetal of the indicated genotypes. B. Calculated bone parameters.](image)

<table>
<thead>
<tr>
<th></th>
<th><em>Nfatc1</em>fl/∆ <em>Ctsk-Cre</em> donor</th>
<th><em>Nfatc1</em>fl/∆ <em>Ctsk-Cre</em> donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV</td>
<td>3.5%</td>
<td>4.94%</td>
</tr>
<tr>
<td>Tb. Th.</td>
<td>0.1775</td>
<td>0.182</td>
</tr>
</tbody>
</table>

Histological analysis was performed on the femurs of these week 8 chimeras (Figure 17). Although the marrow elements appeared relatively normal (Figure 17 A&B), we observed the trademark abnormal, giant, multinucleated, TRAP-positive osteoclasts (Figure 17C) in the recipient of *Nfatc1*fl/∆ *Ctsk-Cre* donor cells, suggesting that the osteoclast specific phenotype is transferred as
would be expected. Although results from our 24 week post-transfer cohort are still outstanding, our initial results suggest that hematopoietic cells are not sufficient to produce the fibrosis phenotype in an adult. If the 24 week results similarly do not demonstrate transfer of the phenotype, it is possible that the rapid bone turnover and high osteoclast activity seen in neonatal mice is required to drive the phenotype. We thus plan to repeat these transfers on newborn mice where bone turnover is very high, to see if the phenotype can be transferred under these conditions.
Figure 17: Fetal liver chimeras do not develop the \textit{Nfatc1}^{fl/Δ} \textit{Ctsk-Cre}^+ phenotype at 8 weeks post transfer. Femur histology on recipients of A. \textit{Nfatc1}^{fl/Δ} \textit{Ctsk-Cre}^+ fetal liver cells or B. WT fetal liver cells revealed no significant differences in histology or morphology, H&E, 10x. C, D. 40X TRAP stained sections show demonstrate large abnormal appearing osteoclasts in the recipients of \textit{Nfatc1}^{fl/Δ} \textit{Ctsk-Cre}^+ fetal liver. E. Osteoclasts in recipients of WT fetal liver are small and flat.
4. **Identification of an Osteoclast Derived Osteoblastic Proliferative Factor.**

4.1. **Osteoclast Conditioned Media treated BMSC Proliferation Assays**

The proliferative stromal cell seen in \( Nfatc1^{fl/\Delta} \) \( Ctsk-Cre^+ \) mice was also previously noted in a different mouse model by Ohishi et al. Ohishi studied mice with constitutively activated parathyroid hormone (PTH) or parathyroid hormone related protein (PTHrP) receptor (Ohishi et al. 2009) and identified an expansion of fibroblastoid cells in the bone marrow that was similar to the phenotype observed in our \( Nfatc1^{fl/\Delta} \) \( Ctsk-Cre^+ \) mice. Ohishi additionally found that elimination of osteoclasts altogether by administration OPG eliminated the marrow fibrosis, while simply limiting the activity and number of osteoclasts via bisphosphonates only resulted in a partial reduction of the marrow fibrosis (Ohishi et al. 2009). This observation suggested that the marrow fibrosis was dependent on the presence osteoclasts in addition to their resorptive activity. In other words, the osteoclasts themselves—more so than their resorptive activity—are somehow signaling stromal cells to proliferate. The notion that the osteoclasts are signaling the stromal cells or osteoblast precursors to proliferate, again, evokes the concept of coupling. Characterizing this marrow fibrosis and its underlying causes would thus deepen our understanding of osteoclast-osteoblast coupling mechanisms.

Our studies of \( Nfatc1^{fl/\Delta} \) \( Ctsk-Cre^+ \) osteoblast differentiation and the rescue of the fibrotic phenotype by RANK deficiency suggests that the stromal proliferation seen in \( Nfatc1^{fl/\Delta} \) \( Ctsk-Cre^+ \) mice is a secondary effect of deletion of
NFATc1 in osteoclasts and not an osteoblast intrinsic phenotype. We hypothesize that NFATc1 in osteoclasts negatively regulates an osteoblast-lineage proliferative factor that functions in coupling. In other words, osteoclasts produce a factor, which we refer to as Factor X, that stimulates osteoblast lineage cells to proliferate and that this factor is negatively regulated by NFATc1 (Figure 18).

**Figure 18: Proposed Hypothesis on Osteoclast Production of an Osteoblast Lineage Proliferative Factor.** Deletion of NFATc1 in mature osteoclasts removes negative regulation normally imposed on the production or secretion of a factor that stimulates proliferation on osteoblast-lineage cells (figure courtesy of Antonios Aliprantis).

We attempted to develop an assay to identify Factor X on osteoblast lineage cells. We differentiated osteoclasts from either $Nfatc1^{fl/\Delta}$ $Ctsk-Cre^+$ or wild-type hematopoietic progenitors in-vitro and collected the media that they were cultured in. We hypothesized that this media, which we called conditioned media, would contain factors that are secreted by the osteoclasts, including Factor X. We then cultured BMSCs with varying concentrations of conditioned
media and assayed proliferation by serial AlamarBlue assays as a measure of cell number.

Osteoclasts were generated from livers isolated from E13.5 or E15.5 fetuses and remained in culture for 5-7 days, after which the media was collected and pooled according to genotype (WT and Nfatc1^{fl/Δ} Ctsk-Cre^{+}). BMSCs were plated onto 96 well plates at densities of 1000 cells/well and were allowed to adhere overnight in normal BMSC media. The BMSCs were subsequently changed into BMSC media with 75%, 50% or 25%, and 0% concentrations of the conditioned media from WT or Nfatc1^{fl/Δ} Ctsk-Cre^{+} osteoclasts. Proliferation was measured at several time points using AlamarBlue reagent and fluorescence spectrophotometry. We found no significant differences in proliferation between BMSCs growing in either media at any of the concentrations (Figure 19). To determine whether the initial plating density may have interfered and obscured any differences in proliferation, a similar assay was performed using an initial density of 200 cells per well. Plating at this density also failed to show any significant differences in proliferation of BMSCs (Figure 19B).
Hematopoietic contaminants from the BMSC isolation may have developed into osteoclasts upon introduction to RANKL and M-CSF rich conditioned media. As a consequence, these contaminating osteoclasts may have produced factors that interfered with our results. In order to minimize this confounding factor, a similar conditioned media proliferation assay was performed on BMSCs isolated from Nfatc1^{fl/Δ} Ctsk-Cre^{+} mice. The hematopoietic stem cells in these mice lose the ability to form osteoclasts and would thus...
interfere minimally. Similar proliferation experiments using these \textit{Nfatc1}^{fl/\Delta} Ctsk-Cre$^+$ BMSCs still were unable to demonstrate significant differences in proliferation with treatment of the different conditioned media (Figure 20).

\textbf{Figure 20: Osteoclast Conditioned Media Treated \textit{Nfatc1}^{fl/\Delta} Ctsk-Cre$^+$ BMSC Proliferation Assay.} BMSCs from \textit{Nfatc1}^{fl/\Delta} Ctsk-Cre$^+$ mice were treated in either \textit{Nfatc1}^{fl/\Delta} Ctsk-Cre$^+$ osteoclast conditioned media or WT osteoclast conditioned media and then assayed for proliferation using AlamarBlue over the
course of 144 hours. No differences in proliferation were observed between the two treatment groups at any concentration.

The lack of significant differences in proliferation of BMSCs with treatment of either of the conditioned media may be due to a multitude of factors. The presence of osteoclasts from the contaminating hematopoietic elements from the BMSC isolation may overwhelm any effect produced by the conditioned media. We plan to address this in future experiments by adding OPG to the BMSC cultures to inhibit the formation of osteoclasts. Another possibility may be that the osteoclasts that we culture in vitro are inherently different from the primary osteoclasts in vivo. The osteoclasts in culture, for example, have a much shorter life-span than do the osteoclasts in vivo. Methods for isolating primary osteoclasts, however, are highly limited at the moment and we are planning to further investigating this. Co-culturing these osteoclasts with osteoblasts or with whole limbs to emulate in vivo conditions may yield better results. Yet another possibility may be that proliferative factor is only released from osteoclasts after apoptosis (K. Matsuo and Irie 2008). Further experimentation using different approaches to generate osteoclast conditioned media will be necessary to identify the factor which couples Nfatc1^/∆ Ctsk-Cre^ osteoclasts to stromal cell proliferation.
4.2. Candidate coupling factors identified by Microarray Analysis of *Nfatc1*-deficient Osteoclasts

The hyperproliferation of bone marrow stromal cells is unique feature of NFATc1 deficiency in mature osteoclasts. Thus, we hypothesize that the putative coupling factor is regulated by NFATc1. Utilizing existing microarray data comparing the transcriptome of osteoclasts generated from precursors lacking NFATc1 to WT osteoclasts, we identified genes upregulated in the absence of NFATc1 and reviewed their function to identify possible candidate proliferative factors. In previous work from the lab, microarray transcriptional profiling was done on duplicate samples of bone marrow derived osteoclasts generated from polyI:C treated *Nfatc1*^{fl/fl} *MxCre* mice, which lack NFATc1 in osteoclast precursors, and *Nfatc1*^{fl/fl} *MxCre* controls (Aliprantis et al. 2008; Charles et al. 2012). Using this available data, we identified transcripts that were upregulated by at least two-fold in the absence of NFATc1. This list was further reduced and condensed to reflect genes that either 1) were identified in the literature as being secreted, 2) have important functions in maintenance, proliferation, or differentiation of bone cells; or 3. were known to be critical to bone homeostasis. Table 2 below summarizes candidate coupling factors identified by this approach, their expression levels in the *Nfatc1*^{fl/fl} *MxCre*^+ vs. the *Nfatc1*^{fl/fl} *MxCre*, the false discovery rate (FDR), and their cellular location as listed by Mouse Genome Informatics (give website address).
<table>
<thead>
<tr>
<th>Gene</th>
<th>KO vs WT Expression</th>
<th>Cellular Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tgfb2</td>
<td>3.4462892</td>
<td>Extracellular Matrix, Extracellular Region, Secretory Granule</td>
</tr>
<tr>
<td>Fgf13</td>
<td>2.2899338</td>
<td>Cytoplasm, growth cone, cell projection,</td>
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<tr>
<td>Inhbb</td>
<td>2.2806169</td>
<td>Extracellular region, perinuclear region of cytoplasm.</td>
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<td>Tnfrsf22</td>
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<td>Extracellular</td>
</tr>
<tr>
<td>Ndp</td>
<td>1.9958691</td>
<td>Extracellular, Extracellular Matrix</td>
</tr>
</tbody>
</table>

4.2.1. A Description of Candidate Coupling Factors Identified by Microarray Analysis

Transforming Growth Factor Beta-2 (Tgfb2)

TGF-β is known to have an important role in the modulation of bone formation. Both TGF-β1 and β2 are variably expressed in bone. The effects of TGF-β1 and TGF-β2 on cell death have been shown to also be highly variable. In osteoblasts, TGF-β1 was shown to decrease activated apoptosis. In bone-marrow derived osteoprogenitor cells, however, TGF-β signaling was shown to promote apoptosis (Dufour, Holy, and Marie 2008). Dufou, Holy, and Marie showed in their 2008 study that TGF-β2 prevents osteoblast apoptosis via Alpha5 Beta1-integrin, Pi3k/Akt signaling cascade and downstream Bcl-2 and phospho-Bad survival proteins. These findings coupled with the highly variable
yet poorly understood nature of TGF-β on bone make Tgfb2 a feasible candidate in our search for a proliferative factor to explain *Nfatc1*°/Δ *Ctsk-Cre* + phenotype.

**Fibroblast Growth Factor 13 (FGF13)**

Though little is known about FGF13 other than its potential role in regulating functions of cells in the bulge and basal region of the epidermis (Kawano et al. 2004), other members of the same FGF family have been found to have active roles in bone regulation. Endogeneous FGF has been shown to control chondrogenesis and osteoblastogenesis via recruitment of chondroblasts and cells of osteoblast lineage to sites of endochondral bone formation (Miraoui and Marie 2010). FGF2, specifically, has been demonstrated to create an increased pool of osteoprogenitors and has been found to be critically important in the anabolic effects of PTH on bone (Hurley et al. 2006). Sabbieti et al. has additionally shown that the exported FGF2 isoform is a critical determinant of bone mass via modulation of the Wnt signaling pathway (Sabbieti et al. 2009). While FGF13 itself does not appear to be secreted (Wu et al. 2012), another member of the FGF family, FGF18, was shown to be an essential autocrine positive regulator of the osteogenic differentiation program in murine mesenchymal stem cells (Hamidouche et al. 2010). Knock-out studies of receptors for FGF have demonstrated a decrease in proliferation of osteoprogenitor cells in FGF-receptor deficient mice, a feature of direct relevance in our search for a proliferative factor in our *Nfatc1*°/Δ *Ctsk-Cre* + phenotype.
Further studies of the specific function of FGF13 and its relation to other family members may elucidate more about its potential role in bone homeostasis.

Tumor Necrosis Factor Receptor Super Family 22 (Tnfrsf22)

Tnfrsf22 appears to be a duplicated gene of Tnfrh1 that encodes decoy receptors of the tumor necrosis factor superfamily. Being a member of the TNF superfamily suggests the possibility that Tnfrsf22 may have features similar or analogous to other members of the superfamily. One of these features is the ability to bind TRAIL (Schneider et al. 2003), a ligand that has been implicated in causing the apoptosis of osteoblasts (Mori et al. 2009), and may thus have a role regulating the proliferation of osteoblast-lineage cells.

Wnt Inhibitory Factor 1 (Wif1)

Wif1 is one of three extracellular modulators of the Wnt signaling pathway identified so far. The roles of these extracellular modulators appear to be negative (Han and Lin 2005). The Wnt pathway has a crucial role in the pathway for the commitment of mesenchymal progenitors to the osteoblast lineage and for bone homeostasis (Galli et al. 2012). The fact that Wif is a known extracellular modulator of the Wnt pathway, which is integral to bone homeostasis, makes Wif1 a candidate for further study.

Norrie Disease Protein (NDP)
Studies on NDP have been quite limited. Current literature has only elucidated the role of NDP as an essential player in retinal neuron development & capillary development in the brain & ear. NDP is thought to act through the Wnt/B-Catenin pathway (Braunger and Tamm 2012), however, and thus may have as yet undiscovered function in bone.

4.2.2. Validation of Candidates by qRT-PCR

We next verified that candidate NFATc1 regulated coupling factors identified above are also induced in mature osteoclasts lacking NFATc1, and thus are valid potential candidates for a coupling factor made by Nfatc1fl/Δ Ctsk-Cre+ osteoclasts. Using primers specific for the genes in Table 1, we examined expression levels of each candidate in Nfatc1fl/Δ Ctsk-Cre+ and wild type control osteoclast cultures by RT-PCR. RNA was prepared from osteoclasts cultured from either Nfatc1fl/Δ Ctsk-Cre+ or WT fetal livers, n=3 for each genotype. As an additional control, fetal liver derived Nfatc1fl/Δ Ctsk-Cre+ or WT macrophages were differentiated in the presence of MCSF. To verify osteoclast differentiation and that Nfatc1 was deleted in the Nfatc1fl/Δ Ctsk-Cre+ cultures, we performed a RT-PCR using Calcitonin Receptor, a marker of mature osteoclasts, and primers to the floxed exon of Nfatc1. We normalized all the expression levels relative to HPRT (Figure 21). Although none of the candidates showed significant differences in expression between wild-type and Nfatc1fl/Δ Ctsk-Cre+ osteoclasts, the trend suggests that Tgfb2, Fgf13, and Tnfrsf22 are induced in the absence of Nfatc1 in mature osteoclasts. We plan to repeat this experiment with additional
osteoclast cultures. Once verified, we plan to purchase these factors in their active forms and design experiments to evaluate their effects on BMSC proliferation or differentiation.
Figure 21: Validation of Microarray Candidates using Quantitative RT-PCR. RT-PCR results showing: the expression of known osteoclast markers Calcitonin Receptor and NFATc1exon3, the expression of candidate genes TGFb2 (Transforming Growth Factor-β2), FGF13 (Fibroblast Growth factor 13), Tnfrsf22 (Tumor Necrosis Factor Receptor Super Family 22), and Inhbb (Inhibinbb). Samples included day 6 WT OC, Nfatc1^fl/∆ Ctsk-Cre^ osteoclast (KO), WT Macrophages, and Nfatc1^fl/∆ Ctsk-Cre^ Macrophages. All expression values are normalized to HPRT.
Discussion

We set out to characterize and elucidate an underlying mechanism of the unexpected phenotype of bone marrow effacement by stromal cell proliferation accompanied by giant abnormal osteoclasts observed in \( \text{Nfatc1}^{\text{fl/\Delta}} \text{Ctsk-Cre}^+ \) mice. While our understanding of this phenotype has been furthered on several fronts, many questions are left unanswered. However, investigating the mechanism by which loss of NFATc1 in mature osteoclasts results in stromal cell proliferation may provide new insights into osteoclast-osteoblast cross talk.

One of the striking features we have noted in the µCT scans of our \( \text{Nfatc1}^{\text{fl/\Delta}} \text{Ctsk-Cre}^+ \) femurs is the absence of cortical bone. This implies that there may be a role for osteoclasts in the initial formation of cortical bone, a process developmentally distinct from trabecular bone formation. This is an intriguing possibility that warrants further study.

The osteoblast–specific deletion of \( \text{Nfatc1} \) provides evidence against the hypothesis that the fibrosis phenotype is of mesenchymal origin. It must be noted, however, that \( \text{Nfatc1} \) was deleted using \( \text{Osterix-Cre} \). \( \text{Osterix} \) is expressed late in the osteoblast lineage and deletion of \( \text{Nfatc1} \) earlier in the osteoblast lineages may provide stronger evidence to dispute this hypothesis. We are currently planning to use \( \text{Prx1-Cre} \), one of the earliest possible osteoblast-lineage specific promotors, to drive deletion of \( \text{Nfatc1} \).

The identification and characterization of the stromal cell infiltrate in \( \text{Nfatc1}^{\text{fl/\Delta}} \text{Ctsk-Cre}^+ \) has proven to be a difficult task to accomplish. We have, so
far, relied mainly on the morphology of these cells and their ability to differentiate into osteoblasts in vitro to identify these cells as osteoblast lineage cells.

To verify that the marrow fibrosis phenotype is of hematopoietic origin, we attempted to see if the reconstitution of an adult WT mouse’s hematopoietic system with \( Nfatc1^{fl/\Delta} \ Ctsk-Cre^{+} \) HSC’s would also transfer the fibrotic phenotype. The failure for this to occur, as mentioned previously, may be due to the possible dependence of the fibrosis phenotype on a state of high bone turnover, which is not met in adult mice. We plan to repeat the experiment on neonatal pups to see if the marrow fibrosis phenotype is indeed dependent on a state of high bone turnover.

Failure of the conditioned media assays to demonstrate activity in BMSCs proliferation or differentiation assays may be due to a plethora of reasons. Based on the short lifespan of in vitro osteoclasts compared with in vivo osteoclasts, we believe that the in vitro conditions used in our experiments may not sufficiently represent the in vivo conditions. We thus plan to address this by performing osteoclast and osteoblast co-cultures for conditioned media, or by using whole limb cultures to provide an environment rich in all the in-vivo factors.

Microarray analysis is a powerful tool in comparing expression profiles and in pinpointing specific genes that are upregulated under a given condition. Using results from the microarray analysis, we identified candidate coupling factors that were upregulated in osteoclasts in the absence of \( Nfatc1 \), were present in extracellular regions or on the membrane surface and that had functions that
may regulate proliferation, differentiation, or apoptosis of osteoblast-lineage cells. We then verified that these genes are expressed in osteoclasts, and found trends toward increased expression in Nfatc1\textsuperscript{fl/\Delta} Ctsk-Cre\textsuperscript{+} for Tgfb2, Fgf13, and Tnfrsf22. The microarray results analyzed to identify candidates in this paper were from in NFATc1\textsuperscript{fl/fl} MxCre\textsuperscript{+} vs. the NFATc1\textsuperscript{fl/fl} MxCre\textsuperscript{-} Osteoclasts. We, however, are more interested in the expression profiles of mature osteoclasts—the NFATc1\textsuperscript{fl/fl} CtsKCre\textsuperscript{+} osteoclasts. We thus have plans to perform a similar microarray analysis using RNA from Nfatc1\textsuperscript{fl/\Delta} Ctsk-Cre\textsuperscript{+} osteoclasts. In parallel we plan to pursue the candidates listed in Table 1 by further examining whether they are upregulated in Nfatc1\textsuperscript{fl/\Delta} Ctsk-Cre\textsuperscript{+} and assaying their effects on BMSC proliferation or differentiation. This last step is, of course, contingent on the availability of these factors in their active form.

The bone marrow stromal cell proliferation seen in our Nfatc1\textsuperscript{fl/\Delta} Ctsk-Cre\textsuperscript{+} mice resembles the same fibrotic stroma seen in the bone marrows of human patients with fibrous dysplasia. These patients also manifest an effacement of hematopoietic elements in their bone marrow by hyperproliferating BMSCs (Riminucci et al. 1999). Furthermore, fibrous dysplasia appears to be dependent on the presence of osteoclasts as treatment with denosumab, an antibody to RANKL, reduced the expansion of fibrous dysplasia dramatically (A. M. Boyce et al. 2012). The analogous features of fibrous dysplasia and the NFATc1fl/fl, CtskCre phenotype delineates a possible path for translational research.
We believe that the prospects for this project are promising. Current understanding of osteoclast to osteoblast cross-talk is not very well developed and pursuit of a “coupling factor” is highly warranted given the potential implications finding such a factor would bring to our understanding of skeletal biology both in homeostasis and disease. Discovery of a “coupling” factor would not only deepen our understanding for bone remodeling, but would open up many avenues to understanding the pathogenesis of human skeletal diseases and possible therapeutic targets.
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Research Experience:

Graduate Thesis Research
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09/2012-Present
  • Investigated cellular cross-talk between osteoblasts and osteoclasts in order to identify a factor that couples bone resorption to bone formation.
  • Planned a series of in-vitro experiments that primarily examined how ablation of NFATc1 and RANKL in osteoclasts affected osteoblast

Research Skills:

Animals:
  • Basic mouse care and husbandry.
  • Intraperitoneal Injections.
  • Dissection and harvesting of tissues and organs. Particularly limbs, spleen, livers, and fetuses.

Basic Lab Techniques:
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• Basic histology.

Tissue Culture:
• Basic cell culture technique.
• Primary osteoblast isolation.
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• Shadowed cardiologist and director of Interclinic, Dr. Manual Cid Martinez.
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• Observed daily a broad range of basic clinical techniques and medical procedures including ECGs, sonograms, echocardiography, and X-rays.

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• Shadowed doctors performing sleep studies.
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• Researched hazardous materials and properly maintained database of Material Safety Data Sheets (MSDS).
• Assisted in performing annual laboratory inspections.
• Researched, produced, and updated protocol signs for proper setup, usage, and maintenance of biological safety cabinets in accordance with national standards.
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- Teach 4-6 students a week in a one-on-one environment.
- Recruited and trained other instructors.
- Designed a general curriculum and plan for the entire program, which now consists of over 20 students in total.
- Coordinate weekly meetings with other instructors to discuss and innovate lesson plans, resolve issues, and plan for future growth

**Languages:**
- English (Native Speaker)
- Vietnamese (Native Speaker)
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**Software:**
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- Programming Skills: Web-design/HTML

**Hobbies & Interests:**
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- Classical & Jazz music.
- Tennis & Running.
- Astronomy

**References:**
References are available upon request.