Regulation of osteoclast differentiation and activation in response to environmental stimuli
BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

REGULATION OF OSTEOCLAST DIFFERENTIATION AND ACTIVATION
IN RESPONSE TO ENVIRONMENTAL STIMULI

by

HAOMING LIU

B.A., University of California, Berkeley, 2015

Submitted in partial fulfillment of the requirements for the degree of
Master of Science

2017
Approved by

First Reader

___________________________________________________________

Elizabeth R. Whitney, Ph.D., MSPT.
Assistant Professor of Henry M. Goldman School of Dental Medicine

Second Reader

___________________________________________________________

Julia F. Charles, M.D., Ph.D.
Instructor, Harvard Medical School
ACKNOWLEDGMENTS

I especially thank Julia Charles and Joerg Ermann for their constant support, guidance and mentorship throughout the entirety of this project. I am extremely grateful for Julia Charles for her tremendous kindness and patience in teaching me about every aspect of laboratory science and clinical medicine, from technique to philosophy. Her passion and knowledge have inspired me to dive deeper in the field of rheumatology. I also thank Nicola Alesi for his advice and guidance with experiment design and data analysis, and Kelly Tsang and Jing Yan for teaching me indispensable laboratory techniques. Finally, I thank the other members of the Charles-Ermann laboratory, Belinda Beqo, Imtiyaz Hossain, and John Lee, for their help and advice throughout this project.
REGULATION OF OSTEOCLAST DIFFERENTIATION AND ACTIVATION IN RESPONSE TO ENVIRONMENTAL STIMULI

HAOMING LIU

ABSTRACT

Bone is a biomaterial composed of organic and inorganic molecules that are continuously remodeled to preserve structural integrity and allow adaptation to stress. Two major types of cells are responsible for this process: the osteoblast that synthesizes the bone and the osteoclast that resorbs the bone. A delicate balance between the function of these two cell types is required to maintain proper bone health and body homeostasis.

Three independent projects were conducted to investigate the functions of osteoclasts in response to manipulations of their environment. The differentiation and activation of osteoclasts depends largely on cell-cell communication and integration of signals such as stress and metabolic status. The canonical pathway of osteoclast differentiation is driven by receptor activator of NFkB ligand (RANKL), a cytokine produced in large part by cells of the osteoblast lineage. In inflammatory states, RANKL is also made by T cells and synovial cells in the joint. In addition to altering RANKL, inflammation may enhance osteoclast formation through various other cytokines. In project one, we examined the effect of inflammatory cytokine interleukin (IL)-X in a mouse inflammatory arthritis model and found that it is not required for osteoclast activity.
Previous studies have reported that other inflammatory cytokines, including TNF and IL-6, are able to induce osteoclast differentiation in mice, in addition to the RANKL pathway. Project two investigates whether these cytokines could have the same function in humans. In addition to inflammatory cytokines, osteoclasts have been shown to respond to extracellular stimuli such as stress and metabolic status. Factors responsible for integrating these signals, TSC2 and the mTORC1 complex, were investigated for their role in osteoclast activity, regulation of communication between osteoclasts and osteoblasts, and subsequent formation of a high bone mass phenotype.

All three projects have clinical correlations in human. Studying the effects of inflammatory cytokines could reveal mechanisms and strategies for prevention of erosions in rheumatoid arthritis and other inflammatory arthritides. Heterozygous mice for the Tsc2 gene can be used as a mouse model for diseases including tuberous sclerosis complex and Paget’s disease. Moreover, understanding the role of mTORC1 complex activity in regulating bone mass could shed light on the potential effect of long-term rapamycin treatment for patients. As demonstrated through these projects, bone is highly dynamic and regulated by numerous physiological processes.
TABLE OF CONTENTS

TITLE ................................................................................................................................. i
COPYRIGHT PAGE ............................................................................................................. ii
READER APPROVAL PAGE ............................................................................................... iii
ACKNOWLEDGMENTS ....................................................................................................... iv
ABSTRACT ......................................................................................................................... v
TABLE OF CONTENTS ..................................................................................................... vii
LIST OF TABLES ............................................................................................................... ix
LIST OF FIGURES ........................................................................................................... x
LIST OF ABBREVIATIONS ............................................................................................. xii
INTRODUCTION ............................................................................................................. 1

1.1. The inflammatory cytokine IL-X in a mouse model of inflammatory arthritis .......... 3
1.2. Human osteoclasts in rheumatoid arthritis ............................................................... 7
1.3. Absence of metabolic stimulation: Osteoclast-specific Tsc2 deletion in mice causes a high bone mass, with clinical implications for bone lesions in Tuberous Sclerosis Complex ............... 11
METHODS ...................................................................................................................... 18

2.1. The inflammatory cytokine IL-X in a mouse model of inflammatory arthritis 2.1.1 Serum Transfer Arthritis Model ......................................................................................... 18
2.2. Human osteoclast in rheumatoid arthritis ............................................................... 20
2.3. Absence of metabolic stimulation: Osteoclast-specific Tsc2 deletion in mice causes a high bone mass, with clinical implications for bone lesions in Tuberous Sclerosis Complex ............... 22
RESULTS ......................................................................................................................... 30

3.1. The inflammatory cytokine IL-X in a mouse model of inflammatory arthritis .......... 30
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cytokines used for human osteoclast cultures</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>Cytokines used for mouse osteoclast cultures</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>Plate types and functions for bone marrow and spleen osteoclast cultures</td>
<td>25</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Schematic model of the RANK-dependent pathway of osteoclastogenesis and osteoclastic bone resorption.</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Schematic of the mechanism of action of K/BxN serum transfer arthritis model (STA).</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Schematic model of the differentiation of osteoclast-like cells induced by TNFα and IL-6.</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>Schematic of TSC inhibition of mTOR signaling activity via RHEB.</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>Mouse paw erosion scoring method at the joints.</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>Micro three-point bending fixture.</td>
<td>28</td>
</tr>
<tr>
<td>7</td>
<td>IL-X-/-, and IL-XR-/- mice have more severe erosions than wild-type (WT) mice.</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>Inflammation and erosion score are highly correlated.</td>
<td>31</td>
</tr>
<tr>
<td>9</td>
<td>Human osteoclast assays in 96-well plates after TRAP staining.</td>
<td>32</td>
</tr>
<tr>
<td>10</td>
<td>Sclerotic bone lesions are found in aged Tsc2+/- mice.</td>
<td>33</td>
</tr>
<tr>
<td>11</td>
<td>mTORC1 hyperactivation in TSC deficient spleen derived osteoclasts.</td>
<td>34</td>
</tr>
</tbody>
</table>
CtskCre;Tsc2^fl/fl^ mice of both sexes demonstrate progressive increase in trabecular and cortical bone with age and increased bone strength and toughness.

Osteoclast number and activation of Tsc2ΔOC mice are unchanged in vitro.

Osteoclast-specific deletion of Tsc2 does not alter osteoclast resorption functions, as shown by osteo resorption assays.

Increased trabecular bone in 9 moth old Tsc2ΔOC mice with normal osteoclasts in vivo.

mRNA relative expression of Cathepsin K in 3 month old male Tsc2ΔOC mice.

CTX-I levels in male and female mice are unchanged in Tsc2ΔOC mice.

P1NP levels are significantly increased in male and female Tsc2ΔOC mice.

mRNA relative expression of CTHRC1 in 3 month old male Tsc2ΔOC mice.

Model showing the likely effect of osteoclast-specific Tsc2 deletion in mice.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>Angiomyolipomas</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting Cell</td>
</tr>
<tr>
<td>BMM</td>
<td>Bone Marrow Myeloid cell</td>
</tr>
<tr>
<td>BMSC</td>
<td>Bone Marrow Stromal Cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony Stimulating Factor</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>CTSK</td>
<td>Cathepsin K</td>
</tr>
<tr>
<td>CTX</td>
<td>C-terminal telopeptides</td>
</tr>
<tr>
<td>DMARD</td>
<td>Disease Modifying Antirheumatic Drug</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>FLS</td>
<td>Fibroblast-like Synovial cells</td>
</tr>
<tr>
<td>G6PI</td>
<td>Glucose-6-Phosphate Isomerase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>LAM</td>
<td>Lymphangioleiomyomatosis</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cells</td>
</tr>
</tbody>
</table>
MTOR  Mechanistic Target of Rapamycin
NF-ATC1 Nuclear Factor of Activated T-cells 1
NOD  Non-obese Diabetic
OB  Osteoblast
OC  Osteoclast
OCP  Osteoclast Precursor
OPG  Osteoprotegrin
OPN  Osteopontin
P1NP  Procollagen Type 1 Propeptides
PBMC  Peripheral Blood Mononuclear Cell
PTH  Parathyroid Hormone
RA  Rheumatoid Arthritis
RAG  Recombinase-activating Gene
RANK  Receptor Activator of NF-κB
RANKL  Receptor Activator of NF-κB Ligand
S1P  Sphingosine-1-phosphate
S6K  S6 Kinase
SPHK  Sphingosine kinase
SSR  Site-specific Recombinase
TCR  T-cell Receptor
TNF  Tumor Necrosis Factor
TNFFR  Tumor Necrosis Factor Receptor
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAF</td>
<td>TNFR-Associated Factor</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-resistant Acid Phosphatase</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous Sclerosis Complex</td>
</tr>
</tbody>
</table>
INTRODUCTION

Overview

Bone is a composite tissue composed of protein and mineral. The three main cell types residing in bone are osteoclasts (OC), which degrade bone, osteoblasts (OB) that create new bone, and osteocytes, specialized cells of the osteoblast lineage that act as mechanosensors. Bone is a highly dynamic tissue, in which degradation and deposition of new bone are continuously active, leading to a complete turnover of the skeleton occurring every 10 years. Bone remodeling is necessary for growth, damage repair (for example after a fracture), and the regulation of calcium-phosphate metabolism. The remodeling process is executed by the concerted and sequential effort of OC and OB, spatially linked in what is called the bone remodeling unit.

OC are multinucleated giant cells that differentiate from myeloid osteoclast precursors (OCP) under the influence of the cytokines: macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB (RANK) ligand (RANKL). M-CSF is a cytokine released by OBs and stromal cells, and binds to its receptor, c-Fms, on early OCP. This binding activates subsequent signaling pathways that promote the survival and differentiation of these OCPs. RANKL on OBs also binds to its receptor RANK on the expanded precursor cells, and stimulates them to commit to the OC phenotype. Osteoprotegerin (OPG) inhibits this osteoclastogenesis pathway by competing with RANK for RANKL binding. Animals that lack OPG develop osteoporosis because of accelerated OC generation. OCs degrade the bone by secreting proteolytic enzymes - cathepsin A, B, K - and acid (H+), which hydrolyze and solubilize the organic and
inorganic components of the bone. Enzyme and proton secretion is directed into the resorption lacunae which is isolated from the rest of the bone microenvironment by the osteoclasts themselves, forming a spatial barrier.

After the resorption phase performed by osteoclasts, mesenchymal stem cells are recruited to the site of resorption and subsequently differentiate into OBs. Mechanisms of mesenchymal stem cell recruitment are not yet fully understood, however evidence has shown that osteoclast-derived coupling factors such as collagen triple helix repeat containing 1 (CTHRC1) is able to communicate to OB. As the committed pre-OC matures into a multinucleated OC, complement component 3a (C3a) is derived from these.
multinucleated OC and acts on bone marrow stromal cells (BMSC) to stimulate osteoblastogenesis. CTHRC1 is secreted from mature active OC during bone resorption and stimulates OB differentiation as well as recruitment of BMSC or mesenchymal stem cells (MSC) to resorption lacunae. After acquiring a fully mature phenotype, OBs secrete osteoid, the organic component of bone that will be later mineralized by the incorporation of hydroxyapatite. Some OBs become incorporated within the matrix and subsequently become osteocytes, which do not produce bone matrix and function mainly to monitor bone quality and coordinate remodeling through the expression of specific membrane ligands and through the secretion of specific factors.

In this thesis, three manipulations of OC environmental stimuli were made to examine the regulation of OC differentiation and activation, and how its interaction with OB can impact the formation of bone phenotypes related to diseases such as rheumatoid arthritis, tuberous sclerosis complex, and Paget’s disease.

1.1. The inflammatory cytokine IL-X in a mouse model of inflammatory arthritis

1.1.1 Project background

Interleukin-34 (IL-X) is a cytokine induced by tumor necrosis factor (TNF) and interleukin-1B (IL-1β), two inflammatory cytokines that are highly expressed in diseases such as rheumatoid arthritis (RA). IL-X is thought to have mostly local actions, as it is not detected in the circulation. IL-X has been demonstrated to bind to two receptors: the IL-X receptor (IL-XR) and colony stimulating factor 1 receptor (CSF-1R). Signaling through CSF-1R is essential for myeloid cell proliferation and survival, including for the differentiation of osteoclasts. Mice deficient in CSF-1R are osteopetrotic and completely
lack osteoclasts\textsuperscript{13}. The primary ligand for CSF-1R is M-CSF, which is essential for OC differentiation under normal physiologic conditions\textsuperscript{3}. However, mice deficient in M-CSF (\textit{op/op} mice) develop osteoclasts with age, suggesting the presence of other ligands for CSF-1R\textsuperscript{14}.

The discovery that IL-X is also a ligand for CSF-1R suggested that once expressed, IL-X could play a role in OC differentiation. Supporting this hypothesis, IL-X is highly expressed in \textit{op/op} mice, coincident with the appearance of OCs\textsuperscript{15}. Studies have shown that IL-X stimulates the adhesion and differentiation of OC precursor cells in the presence of RANKL\textsuperscript{16}. It has further been demonstrated that IL-X can substitute for M-CSF in \textit{in vitro} OC differentiation from murine bone marrow myeloid cells and human CD14\textsuperscript{+} cells\textsuperscript{9,16} and injection of IL-X into the bone microenvironment promotes bone loss\textsuperscript{17}. Although IL-X does not appear to play a role in basal osteoclast differentiation and bone remodeling, it is possible that IL-X plays an important role in OC differentiation under inflammatory conditions (V. Chitu, R. Stanley, personal communication, September 2016). In response to inflammation, TNF-\textalpha and IL-1\beta stimulate fibroblast-like synovial cells (FLS) to express IL-X\textsuperscript{11}. 

1.1.2 K/BxN serum transfer arthritis (STA) as a model for human inflammatory arthritis

First reported by the Mathis/Benoist laboratory in 1996, the K/BxN serum-transfer arthritis (STA) is a frequently used mouse model of inflammatory arthritis\textsuperscript{18}. It was discovered by crossing T-cell receptor (TCR) transgenic KRN mice on a specific C57BL/6 background with autoimmune-prone non-obese diabetic (NOD) mice. The F1-generation K/BxN mice develop severe arthritis by the age of 4–5 weeks\textsuperscript{19}. In the adaptive immune system, antigen presenting cells (APCs) that express MHC-II molecules are able to bind to TCRs on T cells and initiate subsequent immune response\textsuperscript{20}. In K/BxN mice, there is a NOD-derived MHCII, I-Ag7, on APCs which binds to and activates T cells that express the transgenic KRN TCR, leading to B cell activation and production of immunoglobulins (IgGs)\textsuperscript{19}. The I-Ag7 molecule in K/BxN mice presents a ubiquitously expressed self-antigen, glucose-6-phosphate isomerase (G6PI), an enzyme responsible for the interconversion between d-glucose-6-phosphate and d-fructose-6-phosphate\textsuperscript{21,22}. This leads to the production of B cells producing antibodies against G6PI which causes inflammatory arthritis by mechanisms that remain unclear. Administering serum from these K/BxN mice, that contains disease-inducing IgGs, induces an inflammatory arthritis referred to as serum transfer arthritis (STA). STA is used as a model for autoimmune arthritis, such as rheumatoid arthritis (RA)\textsuperscript{23}. Transfer of serum from arthritic K/BxN mice leads to reproducible arthritis with rapid onset in mice from many other strains\textsuperscript{21}. The various strains that can develop disease after serum transfer include BALB/c, C57BL/6, and DBA/1 mice\textsuperscript{24}. The pathogenesis of STA involves a range of immune mediators
including cytokines and factors involved in pain and bone erosion, making STA and excellent mouse model for studying effector mechanisms in inflammatory arthritis\textsuperscript{21}.

1.1.3 Aims and hypothesis

The proximity of IL-X producing FLS cells to bone erosions in the joint, and the properties of IL-X discussed above, lead to the hypothesis that IL-X plays a supporting role in the erosions by inflammatory arthritis. We would expect to see that in the absence of IL-X there would be less OC activity and bone erosion in mouse with inflammatory arthritis.
1.2. Human osteoclasts in rheumatoid arthritis

1.2.1 Evidence supporting the existence of RANKL-independent osteoclastogenesis

Rheumatoid arthritis (RA) is an inflammatory disease that targets bones and causes bone erosions. Manifestations of RA include systemic bone loss and periarticular osteopenia, which is mainly due to the local release of inflammatory molecules\(^25\). In 2005, it was estimated that 1.5 million (0.6%) of US adults had RA, and this number was indicated to be on the rise\(^26\). Bone erosions in RA lead to structural damage to joints, and are mediated by osteoclasts, which are the principal cells for bone erosion and resorption\(^27\). A better understanding of the mechanisms for OC differentiation and activation will help define effective clinical approaches to inhibit bone erosions, and therefore treat patients with RA.

Although M-CSF/RANK pathway plays a vital role in physiological and pathological bone destruction, there is also evidence for RANK-independent osteoclastogenesis pathways\(^28\). It was found that mice with post-natal deletion of RANK still develop joint erosions in the setting of inflammatory arthritis. Cytokines in chronic inflammation, such as tumor necrosis factors (TNF) and interleukins (IL), are osteoclastogenic and promote severe local bone loss\(^29\). TNF is a 17-kDa cell signaling protein involved in systemic inflammation. It is produced primarily by monocytes, although it can also be produced by other cell types such as neutrophils, mast cells, and eosinophils\(^30,31\). TNF is one of the initial cytokines released in the acute phase of the immune reaction cascade in response to trauma or infection\(^31\). TNF and IL-1β, another cytokine in the acute phase response, stimulate further production of other proteins
including the pro-inflammatory cytokines IL-6 and IL-8. TNF is able to induce fever and apoptotic cell death to inhibit viral replication\textsuperscript{31,32}. Dysregulation of TNF production has been implicated in a variety of human diseases including Alzheimer's disease\textsuperscript{33}, major depression\textsuperscript{34}, and psoriasis\textsuperscript{35}. In contrast with the pro-inflammatory nature of TNF, IL-6 is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine – peptides that are derived from myocytes in response to muscular contractions\textsuperscript{31,36}. IL-6 secreted by T cells and macrophages to stimulate immune response to inflammation is an important mediator of fever. It is able to cross the blood-brain barrier and initiate the production of prostaglandin E2 (PGE2) in the hypothalamus, thereby changing the body's temperature set point\textsuperscript{37}. IL-6 has also been shown to be required for resistance against bacterium \textit{Streptococcus pneumoniae} in mice\textsuperscript{38}. In addition, OBs secrete IL-6 to stimulate OC formation\textsuperscript{39}.

Both TNF and IL-6 cytokines are elevated in RA, and a recent report suggests that they can synergistically stimulate the differentiation of OC precursors, suggesting that alternative pathways of OC formation and erosion may play a role in the setting of inflammatory arthritis. Mouse osteoclast-like cells differentiate in response to TNF and IL-6 \textit{in vitro}, as indicated by TNF/IL-6–induced OC formation from RANK\textsuperscript{-/-} synovial cultures. In this recent report, the presence of RANK-independent osteoclastogenesis pathway \textit{in vivo} was observed using the K/BxN inflammatory arthritis model, where both TNF and IL-6 were highly up-regulated. It was found that even in the absence of RANK, the mice still developed bone lesions and TRAP-positive osteoclasts in inflamed joints, indicating that RANK-independent
OC formation can occur in inflammatory conditions in vivo\textsuperscript{40}. It has also been confirmed that the osteoclastogenesis pathway by TNF and IL-6 is RANK-independent, because the OC activities were not inhibited by OPG. In terms of the downstream mechanism, it was proposed that TNF and IL-6 induce the expression of the c-Fos gene, which is essential for the transcription of \textit{Nfatc1}, the critical transcription factor for OC differentiation\textsuperscript{3}. These RANK-independent osteoclastogenesis pathways imply that cytokine blockade, with either TNFα blockers or IL-6 receptor blockers, could have additional effects on inhibiting OC formation and alleviating bone erosions in inflammatory joint diseases.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Schematic model of the differentiation of osteoclast-like cells induced by TNFα and IL-6. IL-6 binds to its receptor IL-6α on osteoclast-like cells and induces the signaling cascade that upregulates c-Fos expression at the protein level. At the same time, TNFα binds to TNFR and activates TRAFs to stimulate the activities of both c-Fos and NF-κB, leading to the expression of NF-ATc1 and subsequent OC activities. This induction is dependent on JAK pathway and the MEK/ERK pathway is likely to be involved. TNFα= TNF receptor; TRAFs= TNF receptor associated factors; NFATc1= Nuclear factor of activated T-cells cytoplasmic 1.}\end{figure}
1.2.2 Aims and hypothesis

The immediate goal of this project is to determine conditions for RANK-independent, TNF/IL6 driven OC differentiation. The ultimate goal of this project would be to examine whether the alternative pathway is enhanced in RA patients and whether it varies with disease activity. The hypothesis is that RA patients will be more sensitive to TNF/IL-6 driven osteoclastogenesis than healthy controls. If RANK-independent osteoclastogenesis is enhanced in RA patients, then it would imply that blocking either TNF or IL-6 actions could have the additional effect of inhibiting OC formation and alleviating joint destruction. We are in the process of developing an assay to test TNF/IL-6 stimulated OC differentiation and resorptive function from frozen human PBMC. The optimal conditions for TNF/IL-6–induced OC cultures will be determined, so that the presence of human osteoclasts derived from PBMC independently of RANKL can be confirmed. The precursors of these osteoclasts will also be identified to examine whether TNF/IL6-induced osteoclasts derive from the same circulating precursor as RANKL-induced osteoclasts.

The first step to approach the ultimate goal of learning about human OC activation via alternative pathway would be to develop an assay for TNF/IL-6 driven OC differentiation from frozen human peripheral blood mononuclear (PBMC) samples. We expect to find a condition in which osteoclasts will form in TNF/IL-6 cultures, though less efficiently than in RANKL cultures, while neither TNF nor IL-6 alone will induce OC formation. Next, we would test whether TNF/IL-6 are sufficient to induce osteoclastogenesis from human PBMC. The reason behind this step is that the production
of OC in the presence of TNF/IL-6 could be due to their indirect induction of RANKL, which leads to the classic pathway. To understand more about the mechanism, we would then test if the OC precursor population that responds to TNF/IL-6 is identical to that responding to RANKL. We are curious to find out about the cluster of differentiation (CD) markers present on the cells that respond to either pathways.

1.3. Absence of metabolic stimulation: Osteoclast-specific Tsc2 deletion in mice causes a high bone mass, with clinical implications for bone lesions in Tuberous Sclerosis Complex

1.3.1 Overview of the disease – Tuberous Sclerosis Complex (TSC)

Tuberous sclerosis or Tuberous Sclerosis Complex is an autosomal dominant disorder that affects multiple body systems with high penetrance and variable expression, and can cause benign and less commonly malignant lesions. Neurological manifestations are the most frequent morbidities in patients with TSC. Epilepsy is the most common symptom, affecting 8 out of 10 patients and frequently represents the first sign of the disease. Lymphangioleiomyomatosis (LAM) is the most frequent pulmonary manifestation of TSC, found in at least 40% of women with TSC and can be diagnosed based on chest computed tomography (CT) scan findings. Sclerotic bone lesions – local increase of bone density – are described in several reviews regarding the radiological aspects of TSC and are considered as abnormalities without definite clinical significance. After renal angiomyolipomas (AMLs) and cortical tubers, sclerotic bone lesions are the third most common finding on imaging in TSC patients being detected by CT. Radiographic appearance of sclerotic bone lesions is commonly associated with patchy
areas of increased bone density. The sacrum and proximal femur can also be involved, together with the classic vertebral and iliac involvement\textsuperscript{46}.

The genotype-phenotype correlation in Tuberous Sclerosis has been analyzed extensively. The probable link between TSC and a gene on chromosome 9q34 was first described in 1994. This gene was called \textit{TSC1} but linkage with this region of the genome was not found for all affected families\textsuperscript{47}. Therefore, it was theorized that there be at least one other locus associated with the disease - the locus 16p13 was then linked to Tuberous Sclerosis and the gene involved was named \textit{TSC2}\textsuperscript{48}. Individuals with Tuberous Sclerosis are heterozygous for \textit{TSC1} or \textit{TSC2}. It is well recognized that in order to develop a lesion a second somatic mutation in the other copy of the gene must occur. Loss of heterozygosity is therefore necessary for the development of the phenotype\textsuperscript{49}.

1.2.2 The Role of mTORC1 Signaling in TSC

Rapamycin was discovered as a product of a strain of bacteria called Streptomyces Hygroscopicus isolated from a soil sample from an island called Rapa Nui (Easter Island)\textsuperscript{50}. Rapamycin associates with FKB12 protein, and the resultant complex is identified to directly inhibit a single protein, which is recognized as mammalian Target of Rapamycin (mTOR)\textsuperscript{51}. The mTOR protein assembles into two different complexes: mTORC1 and mTORC2. While mTORC1 is very sensitive to rapamycin, mTORC2 is insensitive to acute rapamycin treatment but sensitive after chronic exposure\textsuperscript{52}. Less is known about the activity of mTORC2 compared to mTORC1, but mTORC2 appears to play a role in the regulation of the actin cytoskeleton\textsuperscript{53}. 
The activity of mTORC1 is regulated through a protein complex formed by TSC1 and TSC2. The loss of either protein stimulates mTOR dependent phosphorylation of its substrates\textsuperscript{54}. A key readout of mTORC1 activity is phosphorylation of S6 Kinase (S6K), a 70kD protein involved in the control of mRNA translation\textsuperscript{55}. Rapamycin decreases the activation of S6K through phosphorylation, and S6K was found to be highly phosphorylated in cells lacking the TSC1-TSC2 complex, suggesting that constitutive mTORC1 signaling might contribute to TSC pathology\textsuperscript{54}. In TSC1 or TSC2 deficient cells, in which mTORC1 is constitutively hyperactive, mRNA translation is promoted due to the increased activity of S6K. The link between TSC1-TSC2 complex and mTOR was found to be a member of the RAS superfamily called Ras-homologue enriched in brain (RHEB)\textsuperscript{56}. When the TSC1-TSC2 complex is active, TSC2 stimulates the conversion from the GTP-bound state of RHEB (active) to the GDP-bound state (inactive). The inactive form of RHEB then binds to mTORC1 and consequently inhibits its activity\textsuperscript{57}. Therefore, loss of TSC1-TSC2 triggers relieves mTORC1 inhibition, leading to aberrant mRNA translation and, through increased ribosomal biogenesis, total protein synthesis. Under normal conditions, mTORC1 can sense signals indicating the presence of nutrients, availability of ATP, hormones such as insulin, growth factors and cytokines, and therefore functions as a signal integration molecule that contributes to downstream cell growth and differentiation\textsuperscript{58}. The activity of mTORC1 is also sensitive to amino acid availability through the RAG family of proteins\textsuperscript{59}. The loss of TSC1-TSC2 complex results in an uncontrollable hyperactivation of mTORC1, regardless of the environmental status. A schematic of the
components of the pathway relevant to the pathway described above is presented in Figure 4.

**Figure 4. Schematic of TSC inhibition of mTOR signaling activity via RHEB.** TSC2 binds TSC1 to form the TSC protein complex, which integrates inputs from the cellular environment to inhibit mTORC1. The TSC protein acts as a GTPase-activating protein for Ras homology enriched in brain (RHEB), which directly stimulates mTORC1. TSC1-TSC2 complex is regulated in turn by many pathways and environmental stimuli, such as amino acids, nutrients, and stress. RAPTOR is a component of mTORC1 required for its activity.

1.2.3 Normal bone remodeling and the role of mTORC1 in bone formation

To address whether osteoclasts require *Tsc2* or mTORC1 components for normal differentiation and function *in vivo*, previous experiment in the lab has utilized both induced and conditional deletion strategies to specifically target *Tsc2* and *Raptor* in osteoclasts or their progenitors. *Mx1-Cre; Raptor*^{0/0} mice were generated - *Mx1* is expressed in myeloid precursors to encode a guanosine triphosphate (GTP)-metabolizing protein that participates in the cellular antiviral response^{61}. Bone marrow cells harvested
from Mxl-Cre; Raptor$^{fl/fl}$ mice were shown to have decreased OC formation when cultured in vitro with M-CSF and RANKL. Bone marrow cells were also harvested from Mx-1 Cre; Tsc2$^{fl/fl}$ mice and cultured in same conditions. These experiments suggest that TSC2 and mTORC1 components are necessary for the differentiation of OCPs.

The differentiation of myeloid precursors into OC not only leads to bone-resorbing activity, but is also hypothesized to stimulate bone formation through a process called OC-OB coupling. Thus changes in bone formation rate could be due to changes in OB activity or due to alterations in OB activity secondary to indirect stimulation via OC. In normal conditions, the interaction between these two key cell types in bone is regulated and balanced. However, when coupling is impaired, the balance between the activity of OB and OC is lost and bone diseases could arise as a result: osteoporosis if resorption is higher than formation, and Paget-like bone diseases if the formation is increased more than the resorption$^{1,2}$. Several potential OC-derived coupling factors, sometimes referred to as clastokines, have been identified using in vitro approaches. Among these, the factors with in vivo evidence of a role in this process are: collagen triple helix repeat containing 1 (CTHRC1), complement factor 3a (C3a) and sphingosine-1-phosphate (S1P). Evidence indicates that global deficiency of Cthrc1 decreases OB number and bone formation in vivo. Moreover, Cthrc1 is found to be strongly induced in OCs active in resorption. Deletion of Cthrc1 in OC resulted in reduced bone mass without impairing bone functions, therefore suggesting a positive role of this factor in coupling$^{6,39}$. The complement cascade component C3a is produced by OC cultures in vitro, and is observed to stimulate OB differentiation$^{40}$. Sphingosine kinase 1 (SPHK1) and its product, S1P, are induced during
OC's differentiation and S1P promotes OB differentiation \textit{in vitro}\textsuperscript{64}. A better understanding of OC-OB coupling is likely to identify therapeutic targets for improving bone health and understanding the pathogenesis of bone diseases.

Osteoporosis is a major public health problem, affecting over 9 million individuals in the US, with an additional 43 million having low bone density according to the 2004 Report of the Surgeon General. While osteoporosis is a silent disease, the clinical consequence of fragility fracture is associated with significant morbidity and mortality risks and healthcare costs\textsuperscript{65}. Although effective treatment is available, not all patients are willing to take or respond to existing therapies. Currently, less than a quarter of women with an osteoporotic fracture receive treatment and there is an unmet clinical need for alternative osteoporosis therapies\textsuperscript{66}. Thus, understanding how key cell regulatory pathways like mTORC1 affect bone formation may lead to new strategies for treating osteoporosis.

The increased activity of the mTOR pathway, responsible for the manifestations of TSC, has been widely explored in OB maturation and function but less is known about its effect in OC physiology. It has been suggested that rapamycin administration negatively impacted OCP formation, indicating a positive effect of mTORC1 in OC\textsuperscript{67}. \textit{In vivo}, micro-CT evaluation demonstrated that rapamycin ameliorates age-related bone loss by decreasing the number of OC. In a mouse model of rheumatoid arthritis, inhibition of mTORC1 by rapamycin reduced synovial OC formation and thus, protected the animal against local bone erosions and cartilage loss\textsuperscript{68}. Moreover, mTORC1 signaling was shown to be hyperactive in synovial membrane tissue samples of patients with rheumatoid arthritis, particularly in synovial OC\textsuperscript{53}. This suggests that mTORC1 hyperactivity will lead to
increased OC formation and bone loss, and that targeted inhibitors of mTORC1 could be beneficial for bone health.

1.2.4 Aims and hypothesis

We hypothesized that the sclerotic bone lesions seen in TSC patients could be modeled using the $Tsc2^{+/-}$ mouse model, but that restricted loss of TSC2 in OC would favor OC formation and result in low bone mass. The first aim of the project was to examine skeletons of $Tsc2^{+/-}$ for sclerotic bone lesions using micro-computed tomography (micro-CT). The second aim of the project was to describe the bone phenotype in mice with deletion of $Tsc2$ gene specifically in bone marrow myeloid cells or OCPs. Mice were characterized for bone mass, bone mechanical properties, the age of onset of the phenotype, and difference between males and females. With more definite characterization of the bone phenotype, we would then examine the mechanism resulting in the observed bone phenotype.
METHODS

2.1. The inflammatory cytokine IL-X in a mouse model of inflammatory arthritis

2.1.1 Serum Transfer Arthritis Model

Experiments assessing the effect of IL-X and PTP-Z deficiency were performed by the laboratory of Dr. Vicki Kelley (Brigham & Women’s Hospital, MA, USA). C57BL/6 wild type, \( \text{Il-X}^{-/-} \), and \( \text{IL-XR}^{-/-} \) mice were obtained. For all experiments, male mice at 8–12 weeks of age were used. Mice were given intraperitoneal injections of 150μl of K/BxN sera on day 0 and 2 to induce inflammation and spontaneous arthritis. The more inflamed forepaw from each mouse was harvested on Day 10 after the injection of the serum. For assessment of correlation between inflammation and erosion severity, 14 week old male C57BL/6 mice were injected with 150μl of K/BxN sera on day 0, 2 and 7 and harvested on day 14. Both forepaws were harvested. In all experiments, inflammation was assessed by clinical scoring and by measurement of the change in forepaw and ankle thickness using calipers. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Harvard Center for Comparative Medicine.

2.1.2 Micro-computed tomography (micro-CT)

Analysis of bone erosion: Forepaws were scanned on a Scanco μCT-35 with an isotropic voxel size of 7 μm and 3-dimensional images were generated with software supplied by the manufacturer using a global threshold that set the bone/marrow cut-off at 367.8 mg HA/cm\(^3\). Periarticular erosions were scored by an observer blinded to genotype using a semi-quantitative method as previously described\(^40\). Briefly, four sites in the wrist
(ulnar epiphysis and bases of the third, fourth, and fifth metacarpals) were scored for severity of erosion using a scale of 0-3, with 3 indicating the most severe erosion.

2.1.3 Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 7. Erosion scores were analyzed using a Kruskal-Wallis test with a Dunn post-test.
2.2. Human osteoclast in rheumatoid arthritis

2.2.1 Human OC cultures

Total human peripheral blood mononuclear cells (PBMC) obtained from leukoreduction collars were frozen in 90% DMSO, 10% serum according to a standardized freezing protocol. Frozen samples were thawed and cultured in human-M-CSF (h-M-CSF, 25ng/mL) for 5 days in petri dishes (Corning, 100×10 mm) at a density of $1.2 \times 10^6$ cells/mL. This leads to the expansion of myeloid cells that can act as OCP. Expanded myeloid cells were isolated on day 6. Subsequently, cells were cultured at a density of $3.5 \times 10^6$ cells/mL with h-M-CSF (25ng/mL) plus TNF and IL-6 or either cytokine alone. H-M-CSF expanded PBMCs cultured with h-M-CSF (25ng/mL) and human-RANKL (50ng/mL) were used as positive controls. The culture media was changed every 3 days. D-MEM media was supplemented with L-glutamine (2mM), Penicillin/Streptomycin (50000units), and 10% FBS before adding cytokines for OCP expansion and differentiation.

OC differentiation was quantified after 14 days by the standard method of staining fixed cultures for tartrate-resistant acid phosphatase (TRAP) activity and counting multinucleated TRAP-positive cells. TRAP degrades skeletal phosphoproteins including osteopontin (OPN) and is essential for the bone-resorptive functions of OCs. Though used as a marker of mature OC, TRAP is expressed by other cell types including macrophages and dendritic cells\textsuperscript{69}. Since TRAP secreted from OCs into the extracellular milieu, the activity of secreted TRAP enzyme in culture supernatants was determined as a confirmatory assay\textsuperscript{70}.
Table 1. Cytokines used for human osteoclast cultures

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant Human M-CSF</td>
<td>R&amp;D System</td>
<td>25ng/mL</td>
</tr>
<tr>
<td>Recombinant Human RANKL</td>
<td>R&amp;D System</td>
<td>50ng/mL</td>
</tr>
<tr>
<td>Recombinant Human TNF</td>
<td>R&amp;D System</td>
<td>50ng/mL</td>
</tr>
<tr>
<td>Recombinant Human IL-6</td>
<td>PeproTech</td>
<td>50ng/mL</td>
</tr>
</tbody>
</table>
2.3. Absence of metabolic stimulation: Osteoclast-specific Tsc2 deletion in mice causes a high bone mass, with clinical implications for bone lesions in Tuberous Sclerosis Complex

2.3.1 Generation of Ctsk-Cre;Tsc2/fl/fl mice

Cre-loxP recombination was used to generate mice with conditional deletion of Tsc2 in mature OCs. Cre-loxP is a site-specific recombinase (SSR) technology that allows for gene knockout when triggered by an external stimulus. The Cre protein is a site-specific DNA recombinase that catalyzes the recombination of two specific loxP sites on a DNA strand. Genes in between the two loxP sites are excised out and will not be transcribed.

For the purpose of studying the effect of Tsc2 deletion in OC, we crossed mice with a “floxed” (fl) allele of the Tsc2 gene, eg where a portion of the Tsc2 gene is flanked by loxP sites to mice expressing an OC specific Cre. These mice express Cre under the control of the Cathepsin K (Ctsk) promoter. Cathepsin K is a lysosomal enzyme derived primarily from mature, active OCs. These Ctsk-Cre mice were crossed with Tsc2/fl/fl mice to generate Ctsk-Cre;Tsc2/fl/fl mice, also referred to as Tsc2ΔOC mice. In these mice, cells in which Ctsk is expressed (almost exclusively OC) will also express Cre protein and delete Tsc2. Tsc2/fl/fl mice – without the presence of Cre – were used as wild type controls. CtskCre,Tsc2/fl/fl mice with Tsc2 floxed on one allele were generated and used as heterozygous controls. All mice strains were on the C57BL/6 background. All experiments were performed in accordance with American Association for Laboratory Animal Science (AALAS) guidelines and under the supervision of the Harvard Center for comparative
medicine Institutional Animal Care and Use Committee (IACUC). *Tsc2+/-* mice were generated in by the Kwiatkowski laboratory.

### 2.3.2 ELISA for Serum CTX-I, and P1NP

Mouse serum was derived from blood samples collected by cardiac puncture using serum separation tubes (Becton Dickinson). Bone turnover markers, serum C-terminal telopeptides (CTX-I) and Procollagen type 1 propeptides (PINP), were quantified using enzyme immunoassay assays (Immunodiagnostics Systems). All ELISAs were read with Synergy H1 Hybrid Reader (Biotek).

### 2.3.3 Isolation and culture of OCP

Mice were euthanized with CO₂, and cardiac puncture or cervical dislocation were used as secondary methods to confirm death before further procedures. OC were cultured from bone marrow precursors as follows. Mouse femurs and tibia were harvested, from which bone marrow was isolated by flushing with HBSS (with Ca²⁺/Mg²⁺). The cell suspension was treated with RBC lysis buffer and resuspended in sorting media αMEM containing 10% FBS (Hyclone). Cells were cultured in petri dishes with media containing M-CSF (40ng/mL) for 3 days, then cells adherent to the dish surface were collected – due to properties of the petri dish surface, OCPs tend to stay adherent while other cell types (lymphocytes, fibroblast, etc) from the suspension stay afloat. The myeloid cell precursors were then collected and resuspended in M-CSF (20ng/mL) and RANKL (10ng/mL) at a density of 8×10⁴ cells/mL on culture plates described in Table 3. Cell culture media was changed every 3 days. Cultures were monitored for differentiation and harvested when mature OC were observed, typically day 5-7.
OC were cultured from spleen precursors as follows. Single cells suspensions were prepared by mechanical disruption and passage through a 0.22μm filters. The cell suspension was treated with RBC lysis buffer, then cultured in petri dishes in the presence of M-CSF (40ng/mL) for 5 days to expand the OCP population. Splenic myeloid progenitors were collected and plated in the same method as described above. Cytokines and culture additives for mouse OC culture media are listed in Table 2.

<table>
<thead>
<tr>
<th>Table 2. Cytokines and media used for mouse osteoclast cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
</tr>
<tr>
<td>Recombinant mouse M-CSF</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Recombinant mouse RANKL</td>
</tr>
</tbody>
</table>

The staining of an OC-specific marker tartrate-resistant acid phosphatase (TRAP) and the visualization of OC pit formation were used to quantify OC differentiation and function, both of which are standard methods in the field (3). TRAP assays were used in the method described in 2.2.1, and TRAP+ cells were manually counted. Corning Osteo Assay Surface multiple well plates were used to characterize and assay OC-mediated bone resorption. The assay wells are coated with a layer of proprietary synthetic inorganic bone mimetic, which could be digested by active OCs once they form in the culture\(^{75}\). The area of resorption pits divided by the area of the well was then calculated with Image J., and the
resulting percentage reflects the ability of cultured cells to resorb mineralized matrix and form resorption pits on the surface of the assay. Plate types and treatment for bone marrow spleen OCP cultures are listed in Table 3.

<table>
<thead>
<tr>
<th>Plate type</th>
<th>Area/well (cm²)</th>
<th>Treatment</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well (Flat bottom)</td>
<td>0.32</td>
<td>TRAP staining</td>
<td>Detection of multinucleated TRAP+ OC</td>
</tr>
<tr>
<td>24 well</td>
<td>1.9</td>
<td>TRIzol® Reagent</td>
<td>RNA extraction</td>
</tr>
<tr>
<td>12 well</td>
<td>3.8</td>
<td>TRIzol® Reagent</td>
<td>Protein extraction</td>
</tr>
<tr>
<td>Corning resorption assay</td>
<td>0.32</td>
<td>Silver nitrate, pyrogallol</td>
<td>Area of resorption by osteoclast as a measurement of activity level</td>
</tr>
</tbody>
</table>

2.3.4 Bone RNA Isolation and Real-Time PCR

TRIzol® Reagent is used to isolate RNA, DNA, and proteins from bone and OC samples (Thermofisher). Bones were homogenized in ice-cold TRIzol using bullet blender beads (Next Advance), while OCs were directly dissolved in TRIzol. RNA was isolated using a standard TRIzol protocol; quantity and quality of the RNA were measured with Synergy H1 Hybrid Reader (Biotek). Samples obtained after TRIzol extractions were further purified using the RNeasy Plus Micro Kit with on-column genomic DNA-digest
(Qiagen). Affinity Script quantitative PCR (qPCR) cDNA Synthesis Kit (Agilent Technologies) and was used to generate cDNA. Real-time PCR was performed on StepOne Plus Realtime PCR Machine ( Applied Biosystems), using Fast SYBR Green Master Mix (Thermo Fisher Scientific). Gene expression was determined relative to the housekeeping gene \textit{Hprt} (Hypoxanthine Phosphoribosyl Transferase). Fold change in gene expression between experimental and control samples was calculated by the delta-delta-Ct method.

2.3.5 Micro-CT

After serial fixation in 4\% (g/100 mL) paraformaldehyde and 70\% (vol/vol) ethanol, femurs were scanned using a \textit{μCT} 35 (Scanco Medical AG). Scans were conducted in 70\% ethanol using a voxel size of 7 \textmu m, X-ray tube potential of 55 kVp, intensity of 0.145 Ma, and integration time of 600 ms. A region beginning 0.35 mm proximal to the growth plate and extending 1 mm proximally was selected for trabecular bone analysis. A second region 0.6 mm in length and centered at the midpoint of the femur was used to calculate diaphysis parameters. The region of interest was thresholded using a global threshold that set the bone/marrow cutoff at 352.3 mg HA/cm$^3$ for trabecular bone and 589.4 mg HA/cm$^3$ for cortical bone. 3D microstructural properties of bone were calculated using software supplied by the manufacturer. Bone length was measured using the scout view feature.

Skeletal surveys were performed using whole body micro-CT scan on 12 month-old female Tsc2+/- mice. Sclerotic lesions were identified as localized increase in signal intensity on the scan.
2.3.6 Bone biomechanical testing

In collaboration with Shefelbine laboratory, biomechanical properties of \textit{CtskCre;Tsc2}^{fl/fl} bone were assessed. Three point-bending experiments were performed on an Instron test machine (Instron 5966) to compute the properties of \textit{CtskCre;Tsc2}^{fl/fl} femurs in a post-processing analysis. 8 bones from male mice were tested (5 mutant and 3 control) in a displacement control mode and a displacement rate of 0.01 mm/s. Load was monitored by a 50 N load cell. Each femur was loaded in the anterior-posterior orientation on a micro three-point bending set-up (plunger radius 0.25 mm and a span length 6 mm as shown in Figure 6). The end of the test was marked with 40% load drop in each case. A custom MATLAB code (Matlab, MathWorks) was used to analyze the load-displacement data for each sample and compute the bending stiffness (N/mm), yield load (N), ultimate load (N), and fracture energy (mJ) for every specimen.
2.3.7 Cell lysates and Immunoblot

For immunoblot analyses cells were washed with ice-cold PBS and lysed on ice in 1x RIPA buffer (Cell Signaling Technology) with phosphatase and protease inhibitors (Sigma). Lysates were normalized to protein concentration, denatured with loading buffer, and resolved by electrophoresis on a 4-12% Bis-Tris gel (Invitrogen). Proteins were transferred to PVDF membranes, blocked in 5% BSA or nonfat milk for 1h probed with primary antibodies (1:1000) in 1x TBST buffer (Cell Signaling Technology) overnight at 4°C. Membranes were washed with TBST and incubated with secondary antibody (1:10000) for 1h at room temperature and chemiluminescence was visualized using Super Signal (Pierce) and the SynGene G-BOX gel documenting system. The following antibodies were used: phospho-p70S6K (Thr 389) (Cell Signaling Technology), Actin
(Cell Signaling Technology), TS6K (Cell Signaling Technology), and Cathepsin K (abcam).

2.3.8 Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 7. Statistical significance was determined by Student’s t-test.
RESULTS

3.1. The inflammatory cytokine IL-X in a mouse model of inflammatory arthritis

As discussed above, IL-X is produced by FLS near the joints and can act as a ligand for OC-promoting CSF-1R, an essential receptor for myeloid cell proliferation and survival. We anticipated that mice deficient in IL-X would develop less severe arthritis when induced with serum from K/BxN mice. However, in contrast to our hypothesis, forepaw erosions in mice deficient for either *Il-X* or its receptor *Il-XR* had higher erosions score than wild-type (*WT*) mice (Figure 7). The difference between *Il-X*−/− mice and WT mice was highly statistically significant (p=0.0054 for *Il-X*−/− vs WT), as was the difference between the *Il-xr*−/− mice and WT (p=0.0498). Thus, deficiency in either IL-X or IL-XR results in more severe erosions in the forepaw, which is reflected by the images with more evident perforations and cortical erosions on ulnar bone and metacarpals.
Figure 7. IL-\(X^{-}\), and IL-XR\(^{-}\) mice have more severe erosions than wild-type (WT) mice. The erosion scores are graphed with each point representing one paw (upper panel). Mean with SEM is plotted in the graph and significance determined by Kruskall Wallace test with a Dunn post-test. Representative 3D reconstruction images for each genotype are shown (bottom panel). For consistency, all left paws were chosen to represent the scores.

Figure 8. Inflammation and erosion score are highly correlated. Delta paw thickness for each individual paw as a measure of inflammation is graphed versus erosion score for that paw for \(n=20\) paws. Increased thickness indicates increased inflammation. The Pearson correlation coefficient, \(r\), is 0.8633, \(p<0.0001\).

As \(IL-X^{-}\) and \(IL-XR^{-}\) mice developed more severe inflammation than wild-type control mice in this experiment, we asked whether severity of inflammation correlated with erosion score. We compared the change in paw thickness from day 0 to day 14 of STA for each of 20 paws from C57BL/6 strain WT mice and compared this to the erosion score for each individual paw at day 14. We find that erosion score is highly correlated to inflammation as measured by the change, or delta, in paw thickness, as demonstrated in Figure 8.
3.2. Human osteoclasts in rheumatoid arthritis

The work of testing out the ideal protocol for human OC culture is in progress. The cultures have been suboptimal with some TRAP+ cells but lack of mature active OC after 14 days of culture.

Protocol adjustments to improve differentiation include changing the OCP density and plate types. Since myeloid cells committed to OC lineage were more adherent to glass surfaces than other cells types, glass chambers were used for cultures as an additional step of purification. However, the result was similar than to 96-well plates shown in Figure 9 above. Potential reasons for lack of differentiation and future directions for further experiments are discussed in section 4.2.

![Figure 9. Human osteoclast assays in 96-well plates after TRAP staining. A. myeloid cells were cultured in media with MCSF (25ng/mL) only as control. Clusters (pointed by arrow) are due to unknown reasons. B. myeloid cells cultured in MCSF (25ng/mL) and RANKL (50ng/mL) shows little differentiation into osteoclasts. However, several round, multinucleated cells were suspected to be early osteoclasts. Spindle-shaped macrophages can be seen dispersed throughout the well. Clusters of cells are more prevalent than in control wells.](image)
3.3. Absence of metabolic stimulation: Osteoclast-specific Tsc2 deletion in mice causes a high bone mass, with clinical implications for bone lesions in Tuberous Sclerosis Complex

3.3.1 Mice heterozygous for Tsc2 develop sclerotic bone lesions

Sclerotic bone lesions are characterized by a local increase in bone mineral density. Sclerotic lesions were identified in 12 month old Tsc2 +/- mice in the pelvis and vertebrae, which are also the most common location of lesions in TSC patients. Lesions were identified in 4/4 Tsc2 +/- mice and 0/2 Tsc2 +/+ littermates. Example lesions are shown in Figure 10.

Figure 10. Sclerotic bone lesions are found in aged Tsc2 +/- mice. 12 month old Tsc2 +/- and Tsc2 +/- littermates were imaged by microCT, pixel size 15μm. Resulting 2D images were reviewed by 2 independent reviewers blinded to genotype. Representative images of lesions (white arrows) and corresponding anatomic position of controls are shown.
3.3.2 Deletion of Tsc2 results in hyperactivation of mTORC1 in OCs

Bone marrow and splenocytes from CtskCre;Tsc2\(^{fl/fl}\) (Tsc2ΔOC) mice were harvested and cultured in the presence of M-CSF and RANKL, with cultures in M-CSF only as controls. Differentiation were estimated by visualization using light microscopy. Once differentiation was observed, usually after 6 days of cultures, cells were harvested using 4% paraformaldehyde. Wildtype and Tsc2ΔOC mice have similar degrees of commitment to the OC lineage, as demonstrated by their similar expression level of Cathepsin-K, a marker of OC maturation. As shown in Figure 11, western blot analysis of S6K phosphorylation in Tsc2ΔOC spleen derived OC lysates demonstrates a 2-fold increase in S6K phosphorylation level compared to a littermate control. Thus, deletion of Tsc2 in OCs results in the expected increase in mTORC1 activity.

Figure 11. mTORC1 hyperactivation in TSC deficient spleen derived osteoclasts. A. Western blot showing increased phosphorylated S6K (pS6K) and total S6K (tS6K) in spleen monocyte derived osteoclasts in Tsc2ΔOC and Tsc2fl/fl controls. Cathepsin K expression confirmed the osteoclast lineage commitment in both genotypes; actin loading control. B. Increased pS6K/tS6K ratio is consistent with increased mTORC1 activity in Tsc2ΔOC osteoclasts. Densitometry of the immunoblot bands was evaluated with ImageJ.
3.3.3 *CtskCre; Tsc2* Null mice have a high bone mass phenotype and increased resistance to fracture

To evaluate if loss of *Tsc2* results in a bone phenotype, femoral bones from cohorts of male and female mice at 3 months of age were analyzed by micro-CT. Parameters that were examined included bone volume fraction (BV/TV), trabecular bone marrow density, trabecular number and spacing, cortical thickness and porosity, and periosteal area. Increased trabecular bone mass and cortical thickness is seen in both males and females by 3 months of age, with a suggestion that the phenotype may be stronger in females (Figure 12. A-I). All the bone parameters examined by micro-CT are significantly increased in 3 month-old *Tsc2ΔOC* mice compared to a pooled control group of wild type and heterozygous mice (Figure 12. A-C). For trabecular bones, BV/TV (0.3732 ± 0.03488 vs 0.1100 ± 0.01001; p < 0.0001) and trabecular bone marrow density (BMD, 969.6 ± 8.077 mg of HA/ cm³ vs 896.4 ± 5.492 mg of HA/ cm³; p < 0.0001) are significantly increased. Consistent with this, trabecular number is also increased (5.687 ± 0.3008/mm vs 4.377 ± 0.2036/mm; p = 0.0009), along with trabecular thickness (0.08575 ± 0.004971/mm vs 0.04244 ± 0.001003/mm; p < 0.0001) and decreased trabecular spacing (0.1681 ± 0.008263/mm vs 0.2320 ± 0.01158/mm; p = 0.0003). For cortical bones, cortical thickness (0.2418 ± 0.006798 mm vs 0.1944 ± 0.003965 mm; p < 0.0001) and periosteal area (1.344 ± 0.05185 mm² vs 1.041 ± 0.02862 mm²; p < 0.0001) were both significantly increased. Cortical porosity (0.09158 ± 0.003486 vs 0.1032 ± 0.001873; p = 0.0043) and cortical BMD (1182 ± 3.540 mg of HA/cm³ vs 1207 ± 4.208 mg of HA/cm³; p = 0.0001) are decreased, and cortical BMD did not show any difference at 3 month old.
Interestingly, mice appear to continue to accrue bone with aging, as bone mass and cortical thickness are increased even further in a cohort of 9 month-old male mice (Figure 12. D-F). Trabecular and cortical bone parameters of the right femur of 9 month-old mice were evaluated using micro-computed tomographic imaging (micro-CT). All parameters
were significantly increased in Tsc2ΔOC mice compared to Tsc2fl/fl (wild type). Examining cortical bone, we found that at 9 months of age Tsc2ΔOC mice have increased cortical thickness (0.2702 ± 0.01125mm vs 0.2130 ± 0.01277mm; p<0.02), periosteal circumference (15.40 ± 0.8008 mm vs 9.430 ± 0.2006 mm; p<0.01), and periosteal area (2.843 ± 0.1219 mm2 vs 2.151 ± 0.03554 mm2; p<0.01). Cortical porosity was unchanged (0.07769 ± 0.005264 vs 0.07928 ± 0.005511; p= 0.8696). Trabecular bone is also dramatically increased in Tsc2ΔOC mice, as measured by BV/TV (bone volume/ tissue volume) (0.4981 ± 0.04740 mm2 vs 0.1397 ± 0.01306 mm2; p=0.0007) and trabecular BMD (bone mineral density) (961.9 ± 9.300 vs 892.9 ± 6.340; p=0007). Other trabecular parameters were altered consistent with the observed increase in BV/TV, with an increase in trabecular number (7.675 ± 0.8254 vs 3.930 ± 0.1093, p=0.01) and trabecular thickness (0.09413 ± 0.004678 vs 0.05095 ± 0.0008694 mm, p<0.0001), and decrease in trabecular spacing (0.1541 ± 0.01738 vs 0.2417 ± 0.007311 mm; p=0.01). The progressive increase from 3mo old to 9mo old indicates that the increase in bone formation and accretion of bone mass is likely to be progressive throughout the lifetime of the animal.

In addition to micro-CT, biomechanical evaluation of the mutant bones using the three-point bending tests demonstrated have increased work to fracture for CtskCre;Tsc2fl/fl femurs, suggesting that increased mTORC1 in Ctsk-expressing cells drives formation of good quality bone (Figure 12. G-I). Mechanical properties of male Tsc2fl/fl and Tsc2ΔOC samples were calculated and analyzed by Shefelbine lab. Compare the results between Tsc2fl/fl and Tsc2ΔOC specimens, student t-test was used in each case. All measures were significantly different between the groups and higher in Tsc2ΔOC samples (except for the
elastic to plastic energy ratio). Stiffness, yield load and the ultimate load is increased in
*Tsc2ΔOC* bones and fracture energy is also much higher in *Tsc2ΔOC* samples compared to
*Tsc2*fl/fl indicating that *Tsc2ΔOC* samples are stronger and fracture toughness is increased
in these bones.

### 3.3.4 *Tsc2* deficiency in OCs has no effect on differentiation or function

In order to understand the paradoxical increase in bone mass in mutant mice, further
analysis was done to determine whether OC or OB functions were altered by the deletion
of *Tsc2* or the hyperactivity of mTORC1. Loss of *Tsc2* in OCs had no significant effect on
OC differentiation *in vitro*, since the bone marrow myeloid (BMM) precursor cells were
able to differentiate into mature OCs, as assessed by TRAP+ staining. Moreover, the
number of *Tsc2ΔOC* derived OCs was comparable to control derived OCs. Similar results
were obtained from cultures of splenocyte-derived OCs ([Figure 13](#)). To further evaluate
the function of the *TSC2* deficient OCs *in vitro*, the secretion of TRAP in the cultured
media by bone marrow derived OCs and spleen derived OCs from both *CtskCre*⁻/⁻;*Tsc2*fl/fl
and *Tsc2ΔOC* mice was measured. The media was stained for TRAP and the absorbance
was measured at 405nm. No significant differences were observed between the absorbance
of culture media of *Tsc2ΔOC* bone marrow derived OCs and control (2.906 ± 0.4061 vs
3.108 ± 0.7515, p= 0.8095), as well as in *Tsc2ΔOC* spleen derived OCs and control (1.851
± 0.4970 vs 2.441 ± 0.4525, p= 0.4756).
Figure 13. Osteoclast number and activation of Tsc2ΔOC mice are unchanged in vitro. A. Osteoclast differentiation from Tsc2ΔOC splenic monocytes as assessed by TRAP+ multinucleated cells (MNC). B. TRAP secretion in bone marrow and spleen derived osteoclasts. C. To measure TRAP activity, p-NPP was added to cell culture supernatant and absorbance was measured at 405nm. WT group n=3 vs Tsc2Δosteoclasts n=2. Each symbol represents the assay absorbance value of cells derived from one animal; significance was determined by Student’s t-test. Mean and Standard Deviation for each group are represented in the graphs.
In vitro, OC resorption activity was also examined by plating BMM and splenocytes on Corning Osteo Resorption assays. There was no significant difference between the areas of resorption from wildtype controls and \( Tsc2\Delta OC \) mice. Three experiments were performed, giving the same results (Figure 14). This again suggests that defective OC resorptive function is unlikely to explain the high bone mass phenotype in \( Tsc2\Delta OC \) mice. Other possible explanations would include increased OB activities induced either directly by unanticipated Cre expression and \( Tsc2 \) deletion in an OB population or indirectly by specific deletion of \( Tsc2 \) in \( Ctsk \)-expressing cells.

![Image of in vitro OC resorption activity](image)

- \( A. \)
- \( B. \)

<table>
<thead>
<tr>
<th>Relative % area</th>
<th>BMM</th>
<th>Splenocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tsc2 ( ^{+/+} )</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>CtskCre;Tsc2 ( ^{0/0} )</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>
**In vivo** OC differentiation was assessed in femur histological sections to determine if Tsc2 deficiency altered. Nine month-old male Tsc2ΔOC mice appear to have normal OCs, despite the TSC2 deletion, as assessed by TRAP staining. Despite an apparently normal formation of OCs, an increase in bone deposition is clearly visible in the femur histology of Tsc2ΔOC mice, with a decrease in bone marrow space, in both epiphysis and diaphysis (Figure 15). Moreover, relative mRNA expression of Cathepsin K as a marker of mature OC in whole bone was found to be slightly but significantly increased (2 fold) in 3 month old Tsc2ΔOC male mice. This is consistent with the **in vivo** and **in vitro** TRAP+ staining showing the formation of mature OCs, and further confirms that TSC2 loss in mature OCs does not inhibit OC formation or ability of synthesize at least some of the lysosomal enzymes necessary for the bone resorption activity (Figure 16).

---

**Figure 14.** Osteoclast-specific deletion of Tsc2 does not alter osteoclast resorption functions, as shown by osteo resorption assays. Osteoclast activity from 3mo male and female splenocytes were cultured on Corning resorption assays for their digestive activity. A. Transparent resorption pits are visible post-treatment with silver nitrate and pyrogallol, with brown areas indicating incomplete resorption and black areas without any osteoclast activity. BMM Osteo Assays have similar results (not shown). B. Resorption areas of activated osteoclast in vitro is unchanged in Tsc2ΔOC mice compared to the wild type. Each dot represents one animal; data is representative of three individual experiments. Resorbed areas are visualized using light microscopy and measured using Image J; significance was determined by Student’s t-test. Mean and standard error of mean for each group are represented in the graphs.
Although OCs formed in vitro in Tsc2ΔOC mice, it is possible that Tsc2ΔOC OCs do not resorb normally, which would explain the high bone mass phenotype in the mutants. To evaluate the in vivo activity of OCs in Tsc2ΔOC mice, CTX-I level was measured as a
reflection of OC activity. CTX-I is the C-terminal telopeptide sequence of type I collagen, the predominant form of collagen found in bone. This telopeptide is cleaved by the activity of OCs during bone resorption and released into the serum. Its serum levels are proportional to OC activity at the time blood sample is drawn. CTX-I was normal in 9 month old male Tsc2ΔOC mice (30.05 ± 11.10 ng/ml vs 74.83 ± 40.39 ng/ml, p<0.0001) compared to WT + Heterozygous and decreased but not significantly in Tsc2ΔOC females compared to WT + Heterozygous (33.04 ± 7.591 ng/ml vs 25.29 ± 2.242 ng/ml, p=0.23). Therefore, defective OC resorptive function is unlikely to explain the high bone mass phenotype in Tsc2ΔOC mice.

The serum level of CTX-I was measured in serum of 3 month old mice as well (Figure 17). As seen for the 9 month-old female cohort, serum CTX-I was found comparable between Tsc2ΔOC mice and the control group in both females (15.35 ng/ml vs 22.86 ng/ml, p= 0.3504) and males (14.9 ng/ml vs 14.49 ng/ml, p= 0.9226).

Figure 17. CTX-I levels in male and female mice are unchanged in Tsc2ΔOC mice. A. Serum CTX-I measured with competitive ELISA in 3 month old Tsc2fl/fl and CtskCre;Tsc2fl/fl male (Tsc2fl/fl n=7 and Tsc2ΔOC n=5)and female (Tsc2fl/fl n=7 and Tsc2ΔOC n=6). B. Serum CTX-I measured with competitive ELISA in 9 month old Tsc2fl/fl and CtskCre;Tsc2fl/fl mice male (Tsc2fl/fl n=5 and Tsc2ΔOC n=5)and female (Tsc2fl/fl n=6 and Tsc2ΔOC n=11). There was no significant difference between the genotypes in either sex or age group. Each symbol represents one animal; significance was determined by Student’s t-test. Mean and standard error of mean for each group are represented in the graphs.
3.3.5 Osteoblast activity is increased in *Tsc2ΔOC* mice

The function of OBs, the cells responsible for the deposition of bone matrix, was then examined as the next step to understand the cell type responsible for high bone mass phenotype by measuring serum P1NP levels. P1NP is the N-terminal propeptide of procollagen 1. In bone, collagen is synthesized by OBs in the form of procollagen, and the propeptide is removed by specific proteinases before the individual collagen molecules can assemble into the triple helix that forms the collagen fibril. The propeptide can be found in the circulation and its concentration in the serum reflects the synthesis rate of collagen type I and consequently OB activity. Therefore, the concentration of P1NP in both 9 month old male and female mice were measured. The serum concentration of procollagen type I N propeptide (P1NP), a measure of OB activity, was elevated in *Tsc2ΔOC* male (55.61 ± 10.31 ng/ml vs 82.48 ± 21.83 ng/ml, p<0.0001) and female (57.24 ± 16.38 ng/ml vs 100.74 ± 10.81 ng/ml, p=0.04) mice compared to *CtskCre*−/−;*Tsc2*fl/fl and *CtskCre;Tsc2*fl/fl mice, suggesting that loss of *Tsc2* in OCs stimulates bone formation by OBs ([Figure 18](#)). Serum P1NP levels in the 3 month-old cohort is also highly elevated in mutant compared to control mice, similar to what was seen in the 9 month-old cohort. Bone formation was increased as measured by P1NP in both females (66.64 ng/ml vs 165.5 ng/ml, p= 0.002) and males (71.83 ng/ml vs 192.9 ng/ml, p= 0.004). Therefore, the high bone mass phenotype – even at early stage of life – is the result of a hyperactivation of the OBs, even though the loss of *Tsc2* function is specific to Cathepsin- K expressing OCs. These results, in combination
with the OC activity data, lead to the hypothesis that alteration in the coupling of OC activity to OB function is responsible for the increased bone formation in \( Tsc2\Delta OC \) mice.

3.3.6 Coupling factor CTHRC1 is increased in \( Tsc2\Delta OC \) mice

A panel of putative OC-OB coupling factors including CTHRC1, SPHK1, and SPHK2 was examined. These factors have been suggested to increase bone formation, although the exact mechanisms and cell sources are unknown. Expression of \( Cthrc1 \) was found to be significantly elevated in bones from 9-month-old \( Ctsk\text{Cre};Tsc2^{fl/fl} \) mice, while the other factors did not show significant difference from \( Tsc2^{fl/fl} \) mice. With this preliminary result, we next focused on \( Cthrc1 \) expression as a possible factor explaining the increase in bone formation in mutant mice. The experiment was repeated with a cohort of 3-month-old male mice. Bones from three pairs of littermates were harvested, and their bone CTHRC1 and mRNA levels were examined. The level of \( Cthrc1 \) mRNA was found
to be increased 9.6 fold (Tsc2\textsuperscript{fl/fl} relative mRNA expression 1.101 ± 0.2127 and Tsc2\textDeltaOC 9.646 ± 1.731 n=6, p= 0.0016) as demonstrated in Figure 19, panel C.

As shown in Figure 19, bone expression of CTHRC1 is increased at both the mRNA and protein level in CtskCre;Tsc2\textsuperscript{fl/fl} compared to Tsc2\textsuperscript{fl/fl} controls. The data presented here lead to our central hypothesis for future analysis: loss of Tsc2 in OCs and the concomitant increase in mTORC1 activity result in a high bone mass (HBM) phenotype due to CTHRC1-mediated stimulation of bone formation. The approaches laid out in future directions (section 4.3.6) will allow us to address whether the observed HBM originates from the OC or an alternative Ctsk-expressing cell. Questions regarding the biological mechanism of the candidate anabolic factor CTHRC1 and \textit{in vitro} OB differentiation and will also be addressed.
Figure 20. Model showing the likely effect of osteoclast-specific Tsc2 deletion in mice. Top panel: in normal osteoclast (Tsc2fl/fl), mTOR activity leads to normal level of CTHRC1 production and osteoblast differentiation. Bottom panel: The hypothesis of this project is that in Tsc2ΔOC mice, the absence of inhibition of TSC2 complex on mTOR indirectly promotes the expression of CTHRC1. Subsequently, osteoblast differentiation and activation are also increased, leading to increased bone formation and high bone mass phenotype with strong bones observed.
DISCUSSION

4.1. The inflammatory cytokine IL-X in a mouse model of inflammatory arthritis

Contrary to our hypothesis, it was found that bone erosion in an inflammatory arthritis model is more severe when IL-X is absence than when IL-X is present. Therefore, it is safe to conclude that IL-X is not required for the generation of OCs in inflammatory arthritis. The higher erosion scores in $IL-X^{-/-}$ and $IL-XR^{-/-}$ groups could be explained by the more severe inflammation observed in these groups, as we found that erosion score positively correlates with inflammation severity. The erosion scores observed in wild type mice in this experiment were lower than what we have previously reported$^{40}$. This could be due to the differences in the protocol for inducing STA – forepaws were harvested on day 10 instead of day 14, allowing less time for erosions to develop. Variation in the potency of K/BxN serum used could also lead to the different degrees of inflammation. Further analysis would include scanning and scoring of the other forepaw from each mouse. Histologic analysis could also be conducted to detect and quantify OC formation in mutant mice compared to wild-type. This work demonstrates that although IL-X is induced in the synovium in inflammatory arthritis and can activate the cFms receptor, it does not play a significant role in OC formation in inflammatory arthritis.
4.2. Human osteoclasts in rheumatoid arthritis

The ultimate goal of this study is to examine whether an alternative pathway induced by TNF and IL-6 is enhanced in RA patients and whether it varies with disease activity. Whole human PBMC were used to isolate OCP and OC cultures because it best represents the cell population for osteogenesis in physiological conditions. Once the ideal conditions for the classic M-CSF/RANKL pathway using with whole PBMC as precursors, we will further to test the ability of varying concentrations of TNF/IL-6 to drive osteoclastogenesis in vitro. However, our cultures to date have been suboptimal. One possible explanation is that we are using frozen PBMC rather than fresh, which could hypothetically yield better differentiation. PBMC may have lost differentiation ability and/or viability due to the freeze and thaw process after harvest.

Once culture conditions are optimized for human OC cultures, the ability of TNF/IL-6 OC cultures to resorb bone will be tested by culturing cells on commercially available calcium phosphate monolayers for 20 days, followed by von Kossa staining of remaining substrate, as previously described\(^\text{15}\). Resorption area will then be quantified using Image J. As a confirmatory assay, TNF/IL-6 OCs will be cultured on dentin and resorption pits visualized with fluorescently labeled wheat germ agglutinin\(^7\). In both cases, cells will be cultured in parallel with RANKL as a positive control and M-CSF alone as a negative control. However, the frequency of OCPs may be too low in total PBMC for efficient OC differentiation. In this case, CD14\(^+\) peripheral monocytes will be isolated by positive selection using CD14 antibody labeled magnetic microbeads (MACS\(^\circledR\) microbeads). Equal density of CD14\(^+\) cells will be plated as described above and OC
formation and resorption activity assayed.

In addition to testing fresh PBMC, OCP could be more effectively isolated and expanded by using fluorescence activated cell sorting (FACS) to enrich for various myeloid precursor populations. In this case, specific populations isolated according to cell surface markers would be isolated and cultured for osteoclastogenesis instead of whole PBMC. As the next step of the project, cells will be obtained from leukoreduction collars, which are used to deplete donated blood for transfusion of all lymphocytes. Subsequently, the cells will be stained with panels of monocyte markers and isolated by flow cytometric cell sorting using the FACS Aria in the Human Immunology Core associated with the Rheumatology Division at Brigham and Women’s Hospital. Equal cell numbers from each population will be plated in M-CSF with either TNF/IL-6 or RANKL and OC formation assessed. Evidence from previous work in the lab and others strongly suggests that human OCPs reside in the CD14+ monocyte population\textsuperscript{29,30}. Thus, PBMC will be gated on CD14+ to identify monocytes, and CD14+ cells will be further subdivided into those that are cFMS- RANKL-, cFMS+ RANKL+, and cFMS+ RANKL-. The presence of cFMS is crucial for osteoclastogenesis as it is the receptor for M-CSF\textsuperscript{80}. The ability of these three populations to differentiate into OCs will be compared to total CD14+ cells. Additional markers that can be examined include CX3CR1, which has been reported to identify both bone marrow and circulating OCPs in mouse, and CD16, a marker of inflammatory monocytes that has been reported to enrich OCPs in psoriatic arthritis\textsuperscript{15,64,78}.

To test whether TNF/IL-6 are sufficient to induce osteoclastogenesis and therefore are independent of M-CSF/RANKL pathway, cell cultures with PBMC will be repeated in
the presence of OPG, a decoy receptor for RANKL that blocks its activity\(^5\). As an additional control, blocking antibodies of TNF and IL-6 and/or the Jak inhibitor tofacitinib that blocks IL-6 signaling will be used to demonstrate that OC differentiation requires both TNF and IL-6\(^81\).

4.3. Absence of metabolic stimulation: Osteoclast-specific Tsc2 deletion in mice causes a high bone mass, with clinical implications for bone lesions in Tuberous Sclerosis Complex

4.3.1 Previous studies and experiments

While the effect of mTORC1 activity modulation in OC has been examined in some detail, little was known about the role of mTORC1 in OC \textit{in vivo}. In one report, OC formation was inhibited by deletion of either \textit{mTor} or \textit{Raptor} in hematopoietic precursors or \textit{ex vivo} by rapamycin treatment, suggesting that mTORC1 activity is required for OC differentiation\(^67\). Consistent with this, rapamycin treatment of aged rats decreased OC number and bone resorption as measured by serum TRAP5b\(^68\). In contrast, others have reported that rapamycin treatment of adult mice increased serum TRAP5b, suggesting that rapamycin enhanced OC bone resorption\(^82\).

4.3.2 Tsc2\(^+/-\) mice as a model for sclerotic bone lesions in Tuberous Sclerosis

The skeleton is frequently involved in TSC, with the most common finding being sclerotic bone lesions seen in 88-100% of TSC patients\(^45\). The sclerotic bone lesions are seen most prevalent in vertebral bodies, pedicles and posterior elements, as well as in ribs, sternum, proximal femora, and humerus\(^83\). Although frequently asymptomatic and poorly documented, these lesions can cause back pain and other clinical manifestations\(^84,85\).
Preliminary observations suggest a worsening of the sclerotic lesions with time\textsuperscript{81}. Ossification was also observed within a cortical tuber, thus raising the question if mTORC1 hyperactivity in specific unknown cells can stimulate ossification through the secretion of specific factors, even outside the normal bone tissue\textsuperscript{87}. Little is known about common bone diseases in TSC, including whether patients with TSC are more or less likely to develop common bone diseases such as osteoporosis and fragility fractures.

Despite the fact that the vast majority of patients with TSC present with dense bone lesions, only few mouse models with deficient for TSC1 or TSC2 in bone cells have been generated (J.F. Charles, personal communication, November 2016). Generation of mouse models with OC-specific \textit{Tsc2} deletion would allow further investigation of the role of OCs in the development of the bone lesions in TSC. As previously reported, the bone lesions in TSC consists of focal areas of dense bone formation, therefore our model recapitulates the lesions observed in TSC patients. \textit{Tsc2} +/- mice are currently the best model to study TSC, since they have the same gene mutation as human patients. It is speculated that the dense bone lesions in TSC patients emerge from a second hit mutation in OBs and/or in OCs at various stage of development. Reflecting the unknown impact of TSC heterozygosity on the skeleton, the \textit{Tsc2} +/- mouse is a reasonable pre-clinical model for skeletal lesions in TSC. This model can be used to assess the histology of these lesions and whether they result in bone deformity or focal areas of weakness. \textit{TSC2} +/- mice can also be used to assess how bones respond to long-term administration of rapamycin or analogs that are used to treat TSC patients.
4.3.3 OC specific loss of Tsc2 causes a high bone mass phenotype

In our mouse model, OC-specific Tsc2 deletion led to the increase in trabecular bone BV/TV and cortical bone density. The increased resistance to fracture in the mutant mice further indicate that the bones formed have good quality. The high bone mass phenotype is prominent in almost all parameters in micro-CT imaging analysis and develops progressively with age. The strong, resitant bones in Tsc2ΔOC mice could be due to a decrease in OC activity, an increase in OB activity, or the combination of the two. Both branches were examined in our study.

We find that the loss of Tsc2 does not decrease the generation of OCs, either in vitro or in vivo. In fact, the level of OC activity could even be increased, as shown by the increase in mRNA expression of Cathepsin K from femurs harvested from 3 month old male Tsc2ΔOC mice compared to control. This increase is hard to explain because the expression of resorption enzymes and the number of OCs is comparable between Tsc2-deficient and wild-type OCs. Nevertheless, it further confirms that Tsc2-deficient OCs are fully capable of attaining a mature active phenotype. The increase in bone deposition and strength, assessed by femur histology, micro-CT scan, and biomechanical testing, was then surprising given the unchanged OC number and function in Tsc2ΔOC mice in vitro and in vivo.

Because of the normal activities of OC, we examined whether alteration in OB activity could contribute to the increased bone formation and potentially be the key to explaining the high bone mass phenotype. Genetic manipulation of the TSC complex-mTORC1 in mice using conditional deletion of Tsc1, Tsc2 or components of mTORC1 has
revealed essential functions of these proteins in bone health. A variety of Cre drivers have been used to genetically manipulate the TSC complex-mTORC1 pathway at various stages of OB differentiation. Since normal bone mineralization requires mTORC1 activity in osteoprogenitor cells that give rise to OB, deletion of either mTor or Raptor in these precursor cells leads to diminished ossification and short limbs\textsuperscript{88}. However, increased mTORC1 activity can be detrimental in more mature OB. Previous studies suggest that OB specific deletion of either Tsc1 or Tsc2 results in poor bone quality and impaired mineralization, despite of the high bone mass phenotype. The cellular mechanism for the poor quality seen with OB-specific Tsc1 or Tsc2 deletion is the reduced maturation of mutant OB. Moreover, this phenotype can be rescued by OB-specific deletion of one mTor allele or rapamycin treatment, indicating that decrease in OB function is due to an increase in mTORC1 activity\textsuperscript{88,89}.

In contrast to previous findings, our study has found an increased level of OB activity due to Tcs2 deletion in Ctsk-expressing cells in mice. P1NP – the bone formation marker – was measured in the same plasma samples in which CTX-I was measured. An increase in the level of the marker of bone deposition was found in both male and female Tsc2\textsuperscript{ΔOC} mice at 3 months and 9 months of age. Interestingly, others have examined the serum bone markers by ELISA in a cohort of female patients with TSC (Nico Alesi, M.D., February 2017, personal communication). No difference was found in CTX-1 and P1NP levels when comparing the patients a control group of age matched females. However, it is worth noting that even if almost 100% of the patients have 4 or more bone dense lesions, as reported by Avila et al\textsuperscript{45}, the lesions are unlikely to sufficiently increase the circulating
P1NP levels to an extent detectable by ELISA. Moreover, both levels of P1NP and CTX-1 fluctuate enormously throughout one’s life in human. Bone formation markers are therefore not the most reliable tool to reflect *in vivo* OB functions in human.

An increase in bone formation marker level in our mouse model confirms that the high bone mass phenotype in *Tsc2ΔOC* mice could be explained by an indirect effect of the mutation on OB activity. Two likely explanations include an increase in the number and differentiation of OBs and the intrinsic ability of mature OB to form bones. To address this, we plan to culture OB lineage cells digested off of the bone surface *in vitro* and examine both OB colony forming units (CFU) and matrix formation. CFU count indicates the number of OB precursor cells present in the bone, since OB precursors tend to differentiate in clusters of colonies. On the other hand, matrix formation reveals the intrinsic bone-forming ability of functional mature OB. A reproducible mouse OB *in vitro* culture protocol has been developed in the lab, along with staining methods that would allow for the quantification of both parameters. With the hypothesis that the high bone mass phenotype is due to OC-OB interactions, we might expect that OC-specific deletion of *Tsc2* leads to an increase in differentiation of OB from precursors, rather than the functions of mature OB. Therefore, we would expect an increase in CFU in *Tsc2ΔOC* mice. Alternatively, if OC-specific deletion of *Tsc2* alters the function of mature OB, there may be no difference in CFU assays. We do not anticipate any cell-intrinsic effects on OB from OC-specific deletion of *Tsc2*, and thus we do not expect a significant difference in matrix formation ability between *Tsc2ΔOC* mice and wild type controls.
4.3.4. Stimulation of osteoblast activity by OC-derived CTHRC1

The increase of OB activity in a mouse model in which *Tsc2* is deleted in OCs (or other *Ctsk*-secreting cells) could be explained by alteration of OC-OB coupling. It has been suggested that OCs produce soluble or cell surface factors that can stimulate OB function. Several of these coupling factors have been identified based on *in vitro* studies, out of which SPHK1, SPHK2 and CTHRC1 were chosen by previous experimenters to be examined. In the panel of these factors, there was a huge increase in the expression of *Cthrc1* mRNA extracted from femurs of 9 months male *Tsc2ΔOC* mice compared to wildtype control, whereas the levels of SPHK1 and SPHK2 did not show significant changes (data not shown). Confirming our hypothesis, mRNA extracted from 3 month-old *Tsc2ΔOC* femurs also demonstrated increased *Cthrc1* expression in mutants compared to controls. Although this increase is more modest compared to the 9 month old difference, it is nevertheless significant in a large cohort of animals. The increase in *Cthrc1* fold change is also in line with the progressive increase in bone mass shown by microCT.

Although CTHRC1 has been proposed to be secreted by OCs and promote the activity of OB\textsuperscript{62}, the exact cell source of this molecule and its mechanism of action are still being investigated. A recent article indicates that CTHRC1 is produced by osteocytes – cells derived from OBs embedded in bones – to increase bone formation by inhibiting the activity of OCs\textsuperscript{90}. Moreover, evidence shows that osteocytes are able to express Cathepsin K in female mice during lactation\textsuperscript{91}. Whether the same is true for non-lactating mice is unclear. However, if this is the case, then the high bone mass phenotype could be explained by *Tsc2* deletion in osteocytes and the resultant elevation of osteocyte-derived CTHRC1.
To determine the cellular origin of CTHRC1, mice with osteocyte-specific \( Tsc2 \) deletion could be generated and examined. We would anticipate the presence of high bone mass phenotype despite of the unaffected OCs. Radiation chimeras could also be generated, since osteocyte precursors are mesenchymal and will not be erased by lethal radiation, while OCPs are hematopoietic and will be affected by the procedure.

### 4.3.6. Conclusion and future directions

In conclusion, our study on bone lesions of TSC is the first to establish an \textit{in vivo} mouse model of TSC2 loss in mature OCs. The \( Tsc2^{\Delta OC} \) mouse model was generated to study mTORC1 as a regulator of OC formation and resorptive function. We found instead that it could be an indirect regulator of CTHRC1, an OC-OB coupling factor that promotes OB activity. Further experiments will be needed to clarify the nature of this regulation. Observations from this study have several implications for the advancement of the knowledge of TSC as a disease, in which the extraordinary presence of bone lesions has just begun to be recognized by the scientific community. Moreover, the recognition of mTORC1 as one of the actors involved in the induction of bone formation by OCs may suggest potential new avenues of therapy of bone diseases such as osteoporosis.

One aim in the future would be to further characterize sclerotic bone lesions histologically and identify their microscopic structures. Identifying the cell type that is responsible for this clinical manifestation would prompt investigation of human TSC patients, whose bone health is not well known. The skeletal impact of \( Tsc2 \) heterozygosity could be characterized by assessing the mass and strength of these bones with lesions. The effects of chronic mTORC1 inhibition on bone mass and strength can also be determined
with clinically relevant doses of rapamycin in immature and adult mice. Based on our data suggesting a role for mTORC1 activity in stimulating OC-derived factor(s) promoting bone formation and published data showing a requirement for mTORC1 in OB and OC differentiation, we anticipate that long-term treatment with rapamycin will cause adynamic bone. These bones may not be different in quantity but will have poorer biomechanical properties. In growing animals, where rapid bone formation is needed to attain peak adult bone mass, we anticipate a decrease in peak bone mass.

4.4 Concluding thoughts: environmental inputs are vital for osteoclastogenesis and healthy bone

Healthy bone is essential for everyday activity and functions in human. Through discussions on the role of inflammatory cytokines in the alternative pathways of osteoclastogenesis and the effect of metabolic regulators on bone formation, it is evident that the bone is a dynamic structure in which key players communicate with one another and interact with other systems of the body. The environmental inputs for bone remodeling can be regarded as the combination of stimulation via cytokines and the integration of other cellular signals. Studies on these stimuli and their regulatory effects on OC and/or OB are crucial for understanding disease pathology. New findings such as IL-X as a ligand of CSF-1R and the presence of alternative osteoclastogenesis pathways in human sparked further investigation into the mechanism of OC differentiation and activation, particularly in rheumatoid arthritis. The observation of a prominent bone phenotype and bone lesions in mutant mice led to the identification of a new animal model for TSC. An increase in coupling factors due to mTORC1 hyperactivity in OC offered a new gateway to examine
the clinical effect of rapamycin and its analogs in the disease, while also inspired further research in other cell types and interactions. Despite of the complexity and intricacy of its environmental stimuli, efforts are being made to gain insights of osteoclastogenesis to provide effective therapeutics and allow more people to have healthier, stronger bones.
REFERENCES


   doi:10.1177/0022034513500306


52. Loewith, R. *et al.* Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Molecular Cell* 10, 457–468 (2002).


67


88. Martin, S. K. et al. NVP-BEZ235, a dual pan class I PI3 kinase and mTOR inhibitor, promotes osteogenic differentiation in human mesenchymal stromal cells. *Journal of*

