The Effects of Recombinant Osteoactivin on Murine Osteoclastogenesis

A Thesis

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ABSTRACT

Introduction: Healthy bone constantly remodels, in balance between osteoclast bone resorption and osteoblast bone formation. Osteoclasts differentiate in vitro from osteoclast progenitor cells in the presence of macrophage-colony stimulating factor and receptor activated nuclear factor κB-ligand, while osteoblasts differentiate in vitro from mesenchymal stem cells in the presence of ascorbic acid, β-glycerol phosphate, and dexamethasone. Imbalance between the function of osteoclasts and osteoblasts results in disease. In osteoporosis, bone resorption dominates, leading to decreased bone mass. Osteoporosis is of critical concern to an aging population, and new treatments are needed. Osteoactivin is an anabolic bone growth factor in vivo, causing increased bone mass when injected into mice. In vitro, osteoactivin has been shown to increase osteoblast differentiation and function. However, the effects of osteoactivin on osteoclasts are not as well defined. In this study, we hypothesized that recombinant osteoactivin inhibits osteoclastogenesis. In the future, osteoactivin could be a therapeutic agent for bone healing and preventing or even reversing osteoporosis.

Material and Methods: Osteoclast progenitor cells were isolated from C57BL/6 murine tibias, femurs, and humeri. Several aspects of osteoclastogenesis were tested in the presence of dose-dependent recombinant osteoactivin. First, proliferation and survival
assays, with and without macrophage-colony stimulating factor respectively, were conducted on the osteoclast progenitor cells in the presence of recombinant osteoactivin. Next, the osteoclast progenitor cells were differentiated into osteoclasts in the presence of recombinant osteoactivin. Osteoclast tartrate resistant acid phosphatase activity was measured and stained as a marker for terminally differentiated osteoclasts. Differential cell counting was performed for osteoclasts containing 3-20 and >20 nuclei. Two functional assays, Corning surface and cortical bovine bone slices, were performed as well in order to quantify osteoclast resorption activity in the presence of recombinant osteoactivin. Finally, osteoclast survival in the presence of recombinant osteoactivin was tested without receptor activated nuclear factor kB-ligand.

**Results:** Osteoclast progenitor cell proliferation and survival showed no differences from control when treated with recombinant osteoactivin. Likewise, when recombinant osteoactivin was added on the first day of osteoclast differentiation, there were negligible changes. However, when administered on day 4 or day 6 of differentiation, recombinant osteoactivin treatment resulted in fewer osteoclasts with >20 nuclei. In the Corning functional assay, recombinant osteoactivin administered on day 1 and day 4 of differentiation increased resorption, but day 6 administration decreased resorption, although not significantly. Meanwhile, in the cortical bone slice functional assay, pre-
formed osteoclasts exhibited decreased resorption with recombinant osteoactivin, although not significantly. Finally, the osteoclast survival assay with recombinant osteoactivin showed little effect at 24hrs, but an increased number of osteoclasts with >20 nuclei after 48hrs, although the results were again not significant.

**Conclusions:** These data support recombinant osteoactivin as an inhibitor of osteoclast differentiation and function, although the functional data were inconclusive. Osteoclast differentiation was clearly inhibited by recombinant osteoactivin. Further, differentiation data suggested that recombinant osteoactivin is an inhibitor of osteoclast cell-cell fusion, as evidenced by fewer terminally differentiated osteoclasts with >20 nuclei. Osteoclast functional tests were inconclusive. Recombinant osteoactivin increased osteoclast resorption of Corning surface when added on day 1 and day 4 of differentiation. However, this may be due to endogenous osteoactivin, as recombinant osteoactivin has been washed away before osteoclasts were formed. Endogenous osteoactivin appears to have been down-regulated and/or its receptors occupied due to earlier high levels of recombinant osteoactivin. Supporting this conclusion, resorption was inhibited by administration of recombinant osteoactivin on day 6 of differentiation when osteoclasts were formed, although the data was not significant. Recombinant osteoactivin also decreased pre-formed osteoclast resorption of bovine bone slices,
although not significantly. Recent data from our lab suggest osteoactivin decreases DC-STAMP expression in osteoclasts, a transmembrane protein required for osteoclast cell-cell fusion and bone resorption activity; it is possible that this protein is involved and responsible for the observed results. The osteoclast survival assay in the presence of recombinant osteoactivin suggested increased survival of large osteoclasts with >20 nuclei, although the results were not significant. Osteoclasts typically undergo apoptosis in about 3 days because their activity is so high. Perhaps, inhibition of osteoclast activity through DC-STAMP, also provides a protective effect from apoptosis. The initial hypothesis still stands: osteoclastogenesis is inhibited by recombinant osteoactivin. However, the osteoclast functional experiments must be repeated in order to achieve significance. Ultimately, this basic research could translate into novel treatments for the relatively common human ailment, osteoporosis, and improve health and quality of life for many.
DEDICATION

This thesis is dedicated to my family: Timothy Allen Khol, Ph.D., Charel Lee Khol Ph.D., Jill Elaine Khol, B.A., Adrienne Marie Khol Hammill, M.D./Ph.D., and Justin Thomas Khol, M.A. My family has always helped me, and they supported me throughout writing this thesis. Before my father passed away, he was very keen on the idea that I complete this Master of Science degree. My mother even asked me to finish my thesis as a belated birthday present for her.
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My year spent researching in Dr. Safadi’s lab was a very challenging yet rewarding experience. My colleagues made me feel accepted in this collaborative environment. I would like to thank Fouad Moussa and Gregory Sondag for their assistance with osteoclast differentiation assays and western blots. Additionally, I would like to thank Hilary Stinnett, Kimberly Novak, and Douglas (Cale) Crowder for helping me with software and mouse work. During my first six months in the lab, I was Dr. Samir Abdelmagid’s apprentice, and some of his work is highlighted in this thesis. Thomas Mbimba, an invaluable resource, always seemed to have the answer—no matter the question. Finally and most deeply, thank you Dr. Fayez F. Safadi, my mentor in so many ways.
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CHAPTER 1: INTRODUCTION

Bones, composing the endoskeleton of all mammals, are the primary organs of support. Bones also function to leverage mechanical movement through muscles, produce blood cells in the marrow, and store minerals to help maintain body homeostasis. Composed of organic type I collagen and inorganic hydroxyapatite, mineralized bone matrix is relatively resilient and lightweight. The outside of bone is known as compact bone, while the inside is called trabecular, or spongy, bone. Compact bone is hard with minimal porosity, giving bone its white appearance. Trabecular bone has much greater surface area than compact bone with high porosity and an extensive blood supply to the marrow. Long bones are so named because they are longer than they are wide, and are characteristic of the extremities. The ends of long bone are known as the epiphysis, while the shaft is referred to as the diaphysis. Between them, lies the metaphysis which contains the growth plate. Numerous cells build-up, maintain, and break-down bone in a dynamic equilibrium responsible for homeostasis in healthy bone.
1-1: Bone Cells

There are three main types of bone cells: osteoclasts (OCs), osteoblasts (OBs), and osteocytes (OSs). OCs resorb bone matrix, while OBs form bone matrix. OSs maintain the balance between OBs and OCs. When an OC resorbs bone, OBs follow soon after, regenerating the bone. OCs and OBs are short-lived cells. They exist together in functional balance, continually remodeling through healthy turn-over of bone. OSs are longer-lived cells and conduct chemical and mechanical signals, thereby regulating the bone remodeling activities of OCs and OBs.

1-1-1: Osteoclasts

Giant multi-nuclear cells, OCs arise from OC progenitor cells, or hematopoietic stem cells (HSCs). HSCs require macrophage colony stimulating factor (M-CSF) to proliferate and receptor activated nuclear factor κB ligand (RANK-L) to differentiate into OCs (Zaidi, Blair et al. 2003). Individual OCs mature on bone surface itself. An actin ring forms to seal off a resorptive compartment covered by an in-folded membrane, known as the ruffled border (Miyamoto and Suda 2003). The ruffled border releases acids and enzymes such as tartrate resistant acid phosphatase (TRAP) and cathepsin K (CATK) to dissolve bone, forming a resorptive pit, or Howship’s lacuna (Boyle, Simonet
et al. 2003). An OC tunnels for a short time, and then undergoes apoptosis. OBs follow, filling the tunnel with new bone matrix.

![Figure 1. Scanning electron micrograph of an osteoclast resorbing bone.](http://www.brsoc.org.uk/gallery/#myGallery-picture(5))

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1-1-2: Osteoblasts

Mono-nuclear cuboidal cells, OBs arise from mesenchymal stem cells (MSCs). MSCs can also differentiate into cartilage cells, connective-tissue cells, fat cells, muscle cells, and nerve cells. In vitro, dexamethasone, ascorbic acid, and β-glycerol phosphate
induce MSCs to differentiate into OBs (Reger, Tucker et al. 2008). OBs, smaller than the giant OC, act together in concert to produce extracellular organic matrix, and then mineralize it. First, the osteoid, or organic matrix, is synthesized of collagen. Then, the osteoid becomes bone through mineralization, matrix calcification with hydroxyapatite ($\text{Ca}_{10}\text{[PO}_4\text{]}_6\text{[OH]}_2$). OBs often become encased within the mineralized bone matrix they have formed, and some of the entrapped OBs shrink into OSs, the most abundant bone cell (Noble 2008).

![Figure 2. Osteoblasts actively synthesizing osteoid. (Hunt)](http://en.wikipedia.org/wiki/Osteoblast#mediaviewer/File:Bony_Nidus_2.jpg)
1-1-3: Osteocytes

Although popularly considered quiescent cells within the mineralized bone matrix, OSs can communicate with OCs and OBs directly through gap junctions or indirectly through paracrine signaling to regulate bone maintenance. OSs express RANK-L, providing a basis for the OS role in OC-mediated bone resorption, yet they also have the innate capacity to both form and resorb bone (Schaffler, Cheung et al. 2014). Living an average of 25 years, OSs are considered the mature residents of bone (Knothe Tate, Adamson et al. 2004), and key regulators of bone mass (Dallas and Bonewald 2010). Eventually, OSs die, and are resorbed by OCs along with the matrix surrounding them.

Figure 3. Osteocyte. [Link to Wikimedia](http://commons.wikimedia.org/wiki/file:osteocyte_2.jpg) (Wikimedia)
1-2: Osteoclast Differentiation

Osteoclastogenesis is a critical step in bone homeostasis. OC differentiation involves chemical messengers which signal HSCs to differentiate. Until recently, OC-OB co-cultures provided the only means of studying OCs in vitro. The discovery of the RANK signaling pathway has made it possible to differentiate OCs directly. RANK-L is also known by the names of: osteoclast differentiation factor, osteoprotegerin ligand, and tumor necrosis factor-related activation-induced cytokine (Burgess, Qian et al. 1999). M-CSF and RANK-L stimulate genes in HSCs which express OC characteristic proteins (Zaidi, Blair et al. 2003). Cell-cell fusion of mono-nuclear OCs is an essential part of OC differentiation, necessary for fully functional multi-nuclear OCs (Iwasaki, Ninomiya et al. 2008). Dendritic cell specific transmembrane protein (DC-STAMP) and osteoclast-stimulating transmembrane protein (OC-STAMP) are both stimulated by RANK-L and required for OC cell-cell fusion and bone resorption activity (Miyamoto 2011).
Figure 4. Osteoclastogenesis. Bone marrow-derived monocytes/hematopoietic stem cells arise in the long bones. Macrophage-colony stimulating factor stimulates hematopoietic stem cells to become osteoclast progenitor cells which are differentiated into osteoclasts with the addition of receptor activator of NF-κB ligand. Reprinted by permission from Macmillan Publishers Ltd. on behalf of Nature (Boyle, Simonet et al. 2003).

1-2-1: Bone Resorption

The primary function of OCs is bone resorption. OC polarize to attach to bone, forming the actin sealing ring and ruffled border. The OC vitronectin receptor, αvβ3,
induces OC polarization, crucial for bone resorption (Chambers 2011). The resulting resorptive compartment is filled with acids, TRAP, and CATK; all of which are expressed by OC genes induced by RANK-L (Boyle, Simonet et al. 2003). Acids proceed to dissolve the crystalline hydroxyapatite, followed by CATK resorption of the organic matrix (Phan, Xu et al. 2004).

**Figure 5. Schematic diagram of an osteoclast attaching to bone surface.** A multi-nuclear osteoclast attaches to bone with an actin ring sealing the resorptive compartment. Acid, cathepsin K, and tartrate resistant acid phosphatase from the ruffled border resorb bone, forming a resorptive pit, or Howship’s Lacuna. Reprinted by permission from Macmillan Publishers Ltd. on behalf of Nature (Boyle, Simonet et al. 2003).
1-3: Osteoblast Differentiation

Pluripotent MSCs can differentiate into several cell types (Zomorodian and Baghaban Eslaminejad 2012). *In vitro*, OB differentiation from MSCs proceeds in three distinct stages: proliferation (days 1-4), matrix maturation (days 5-14), and mineralization and matrix deposition (days 15-28) (Birmingham, Niebur et al. 2012). Various osteogenic factors supplement the OB differentiation medium *in vitro*: dexamethasone induces cell proliferation and differentiation, ascorbic acid is a co-factor for the production of collagen in the extracellular matrix, and β-glycerol phosphate supports matrix mineralization (Zomorodian and Baghaban Eslaminejad 2012).

![Mesenchymal stem cells develop into osteoblasts](image)

**Figure 6. Osteoclast progenitor cells differentiate into osteoblasts.** MSCs are fibroblastic, meaning spindle shaped cells which produce collagen (Brammer 2011). © 2011 Brammer, Oh, Frandsen, & Jin. CC-BY-NC-SA 3.0 license
1-3-1: *Bone Formation*

The primary function of OBs is bone formation. Pre-OBs first locate to a newly formed OC resorption cavity. Early in OB differentiation (day 5-14), collagen type I matrix is laid down, forming osteoid (Franz-Odendaal, Hall et al. 2006). Alkaline phosphatase is an enzyme characteristic of pre-OBs and OBs (Phan, Xu et al. 2004), while terminally differentiated OBs express high levels of osteocalcin and osteopontin, coinciding with calcium and phosphate deposition (Zomorodian and Baghaban Eslaminejad 2012). The collagen type I matrix serves as a scaffold for the mineralization process which follows (Birmingham, Niebur et al. 2012). OBs do not work alone in bone remodeling; cadherins, connexins, and integrins support cell-cell adhesion, cell-cell communication, and cell-matrix interactions, respectively (Marie 2002).

1-4: *Bone Remodeling*

Bone is best known for its role in skeletal support. However, bone is not static, but rather constantly remodels throughout our life-spans, completely reforming the adult skeleton every 10yrs (Manolagas 2000). Healthy bones exist in this dynamic equilibrium where OC bone resorption equals OB bone formation. Imbalance between these two physiological processes results in disease, such as osteoporosis or osteopetrosis. In osteoporosis, bone resorption predominates leading to decreased bone
mass. In osteopetrosis, bone formation predominates leading to increased bone mass. Disruption of bone cell communication can lead to metabolic bone disease (Phan, Xu et al. 2004). Bone cell homeostasis is also perturbed by aging in both sexes, reducing OB activities and stimulating OC activity (Hill 1998). Not surprisingly then, the incidence of osteoporotic fractures increases every year as the population ages (Ettinger 2003). Pathological bone cell conditions can be therapeutically treated by deliberately altering the balance between bone resorption and bone formation. Treatment for osteoporosis currently involves potent anti-catabolic drugs (Marie and Kassem 2011).

**Figure 7. Bone remodeling.** Bones are constantly remodeling as osteoclasts break down bone and osteoblast form new bone. Osteocytes stimulate osteoclast differentiation, releasing the differentiation factor receptor activator of NF-κB ligand. Reprinted by permission of Elsevier. (Nakashima, Hayashi et al. 2012)
1-4-1: Osteoporosis

Osteoporosis is a human disease that results from an imbalance in bone cell differentiation and function. This disease is characterized by bone loss, defined as “a value for bone density or bone mineral content that is more than 2.5 standard deviations below the young adult mean value”, while osteopenia is characterized by bone mass 1.1 to 2.4 standard deviations below the young adult average (WHO 1994). Women and men both show more evidence of osteoporosis with increasing age, with its accompanying bone fragility and elevated risk of bone fracture (Marie and Kassem 2011). Low calcium and vitamin D intake can be the cause, but post-menopausal women still lose bone at higher rates than men when estrogen levels are reduced (Ettinger 2003). The search is underway for treatments to reverse these effects either by anabolic factors that stimulate OBs, inhibit OCs, or both.

More and more individuals are being diagnosed with osteoporosis as the population ages. The National Osteoporosis Foundation estimates that about 54 million Americans meet the criteria for osteoporosis or osteopenia. The estimated cost of treating 2 million osteoporotic bone fractures per year is almost $20 billion, with increased risk of mortality associated. By 2025, this number is projected to rise to 3 million fractures per year, or over $25 billion (NOF). Accompanying morbidity and
mortality rates are high. Almost 10% of patients with bone-loss related fractures need to enter nursing homes due to permanent disability, while the mortality rate for hip fractures approaches 20% (Christodoulou and Cooper 2003).

1-4-2: Osteopetrosis

Osteopetrosis, rarer than osteoporosis, also results from an imbalance in bone cell differentiation and function. This disease is characterized by OC dysfunction leading to decreased resorption and increased bone mass, although OB abnormalities have also been identified (Lazner, Gowen et al. 1999). Strangely, the increased bone mass in osteopetrosis is often associated with increased risk of bone fractures, as bone remodeling is inhibited (Stark and Savarirayan 2009). Animal models have provided insights into bone remodeling defects. In the osteopetrotic rat mutant, osteoactivin (OA) was a novel factor discovered to be highly overexpressed (Safadi, Xu et al. 2001).

1-5: Osteoactivin

Studies have shown that OA enhances OB differentiation and matrix mineralization (Abdelmagid, Barbe et al. 2008). There are two isoforms of OA: transmembranous and secreted (Abdelmagid, Barbe et al. 2007). Osteoactivin is a 572 amino acid protein, also known by the name of transmembrane glycoprotein non-metastatic melanoma protein B, or GPNMB (Safadi, Xu et al. 2001). Our lab has shown
rOA increases OB differentiation and function in vitro (Abdelmagid, Barbe et al. 2008). Further study revealed OA is temporally and spatially overexpressed in a rat fracture model compared to normal bone (Abdelmagid, Barbe et al. 2010).

While data has been generated to support OA effects on OBs, investigation into the other side of this physiological process has, so far, not been settled. Recently, OA was found to also be highly expressed by terminally differentiating OCs (Sheng, Wergedal et al. 2008). OA also plays a key role in OC differentiation and function.

**Figure 8. Primary structure of osteoactivin protein.** The osteoactivin protein has three main parts: the (ECD) extracellular domain, the (TMD) transmembrane domain, and the (CD) cytoplasmic domain. Amino acid position is depicted by numbers. Other abbreviations are: (SP) signal peptide, (RGD) integrin binding domain, (PKD) polycystic kidney disease domain, (PRRD) proline rich repeat domain, and (LL) dileucine sorting sequence. (Abdelmagid, Barbe et al. 2008)
1-6: Significance

The effects of OA on OCs are not well defined. This thesis examines the role of OA in osteoclastogenesis. The current dogma would suggest that OA is a positive regulator of osteoclastogenesis. OCs differentiated in the presence of anti-OA antibodies inhibited OC differentiation and function in vitro (Sheng, Wergedal et al. 2008). Further, overexpression of OA in transgenic mouse OCs stimulated OC resorption, resulting in bone loss in vivo (Sheng, Wergedal et al. 2012). However, preliminary data from our lab suggests that OA is actually a negative regulator of osteoclastogenesis.

Our lab has taken different approaches to testing the effects of OA on osteoclastogenesis. OCs from wild type DBA/2J-Gpnmb+/SjJ and OA mutant DBA/2J mice were analyzed for in vitro differences in osteoclastogenesis. DBA/2J mice have a nonsense mutation in the OA/GPNMB gene (Anderson, Smith et al. 2001), the only known difference from wild type (Anderson, Smith et al. 2002). Studies in our lab suggested that this loss of function mutation promotes OC differentiation, but inhibits OC functional activity (Abdelmagid, 2014 unpublished observations).

Other studies of OA in our lab suggest OA is an anabolic bone factor in vivo, causing increased bone mass in C57BL/6 mice when injected (Stinnett, 2013 unpublished
observations). OA could work to increase bone production in two ways: increasing OB function and decreasing OC function.

Characterizing OC differentiation and function in C57BL/6 mice is quite important, as these are the most commonly used rodents in laboratory experiments (JAX). Coincidently, Fig. 8 shows mouse OA is more similar to human OA than is the rat. This study on C57BL/6 could help define OA as an anabolic bone growth factor. A better understanding of the effects of OA on OCs, and how rOA might work to inhibit OC differentiation and/or function could lead to new therapeutic strategies to inhibit OC function in patients with significant bone loss in vivo.

1-7: Specific Aims

Our lab has found OA to be an anabolic bone growth factor. There are three ways OA could stimulate bone growth: increase OB function, decrease OC function, or both. Our lab has already demonstrated that OA increases OB differentiation and function. A question remains, “Does OA also decrease OC differentiation and/or function?”

**Hypothesis: Osteoclastogenesis is inhibited by recombinant osteoactivin.**
**Aim 1:** To demonstrate whether rOA inhibits OC progenitor cell proliferation and survival. If rOA is a negative regulator of OC progenitor cell proliferation and survival, this affects measurements of differentiation and function.

**Aim 2:** To demonstrate whether rOA inhibits OC differentiation. If fewer OCs terminally differentiate, this affects measurements of function.

**Aim 3:** To demonstrate whether rOA inhibits OC function. This aspect will indicate whether rOA reduces OC activity, and would strongly support that rOA is a negative regulator of OC function.
CHAPTER 2:  
THE EFFECTS OF RECOMBINANT OSTEOACTIVIN  
ON MURINE OSTEOCLASTOGENESIS

2-1: Abstract

In this study, C57BL/6 mouse osteoclasts are tested for the effects of rOA on differentiation, function, and survival. Osteoclast progenitor cell proliferation and survival assays (24hrs, 48hrs, and 72hrs) with rOA (0.1 mM, 0.5 mM, and 1.0 mM) yielded no change from control. Osteoclasts were then differentiated in the presence of dose-dependent rOA (10 ng/mL, 50 ng/mL, and 100 ng/mL) given on day 1, day 4, or day 6. Day 1 administration mimicked osteoclast progenitor cell data and results were not changed from control. However, given on day 4 or day 6, dose-dependent rOA had the effect of inhibiting osteoclast cell-cell fusion, as evident by fewer large osteoclasts (>20 nuclei) and increased number of small osteoclasts (3-20 nuclei). Next, osteoclast function was tested in the presence of 50 ng/mL of rOA. In the first test, OCs were differentiated on Corning osteoassay surface with time-dependent (day 1, day 4, or day 6) rOA administration. Administration on day 1 or day 4 exhibited increased osteoclast resorption, but this was likely due to down-regulation of endogenous osteoactivin and/or blocking of its receptors due to earlier high concentrations of rOA. Osteoclasts were not yet formed; they usually appear on day 7. However, osteoclasts were present
after the critical day 6 administration of rOA, and resorption was inhibited, although not significantly. These data must be qualified, as OC differentiation for these time points was reduced. The definitive test for osteoclast function \textit{in vitro} uses cortical bovine bone slices. In this bone slice experiment, pre-formed osteoclasts were tested, yielding inhibited resorption with 50 ng/mL of rOA, although not significantly. Finally, osteoclast survival in the presence of rOA was tested with RANK-L but without M-CSF. After 48hrs with 50 ng/mL of rOA, a greater number of large osteoclasts (>20 nuclei) survived, although not significantly. Inhibited OC cell-cell fusion and activity could be due to decreased DC-STAMP gene expression.

\textbf{2-2: Introduction}

In 2001, the Safadi lab discovered that OA was 3- to 4-fold overexpressed in osteopetrotic rats and cloned the gene for this protein (Safadi, Xu et al. 2001). OA was first thought to be exclusive to OBs, and early work centered around this assumption. In 2008, OA was found to be highly expressed in OCs as well, and many sought to characterize its effects on OC differentiation and function (Sheng, Wergedal et al. 2008). (DBA-2J) Our preliminary data indicate that OA inhibits OC differentiation and function, despite contrary claims in the literature. In this study, the hypothesis that rOA
inhibits C57BL/6 OC differentiation and function was tested in vitro. rOA is already known to stimulate OB differentiation and function.

2-3: Materials and Methods

2-3-1: C57 Black6 Mice

C57BL/6 mice were acquired from Jackson Laboratory. The mouse colony was housed and maintained at Northeast Ohio Medical University according to the guidelines of the Institutional Animal Care and Use Committee, protocol #11-034.

2-3-2: Isolation of Osteoclast Progenitor Cells

Using sterile techniques, tibias, femurs and humori were isolated from 6 to 8 week-old, male C57BL/6 mice (Kominsky, Abdelmagid et al. 2008). First, the mice were euthanized using CO₂. Surgical scissors were employed to cut the skin away close to the hips, while forceps were used to peel back the skin over the legs. The exposed flesh was then scraped from the bone with a razor blade. The feet were removed with surgical scissors, and the fibulas were scraped off as well. Finally, the femurs and tibias were the cut near the growth plate and placed in α-MEM (Corning/Cellgro) + 1% penicillin/streptomycin (P/S) (Fisher Scientific). Humeri were removed similarly, cutting the skin away, and discarding the radii and ulnas. Then, the remaining flesh was
scraped away with a razor blade. Finally, the humeri were cut near the growth plate, and placed in α-MEM as before.

Next, bone marrow mononuclear cells (BM-MNCs) were isolated from the bone marrow. Bone ends were cut off with a razor blade, and the bone marrow was flushed into a 50 mL tube with a 10 mL syringe and 21-gauge needle containing α-MEM + 10% fetal bovine serum (FBS) (Lee BioSolutions) +1.0% (P/S) + 0.1% amphotericin (EMD Millipore); (α-MEM CM). BM-MNCs were washed by shaking and then centrifuged (Eppendorf Centrifuge 5810 R) at 1200 RPM for 12 minutes at 4°C. The cell pellet was re-suspended in α-MEM CM. Finally, the BM-MNCs were cultured in a 10 cm dish, and stored overnight at 37°C in a 5% CO₂ incubator (Eppendorf Galaxy 170 S).

The next day, the non-adherent cells, bone marrow-derived monocytes/hematopoietic stem cells (BMM/HSCs), were separated with α-MEM CM rinse and strained through a 40 μm nylon mesh cell strainer (Fisher Scientific). For counting, a 25 μL sample of these cells was diluted (1:1) with 25 μL of 4.0% acetic acid to rupture red blood cells, and then further diluted (1:4) with 200 μL trypan blue (MP Biomedicals). A hemocytometer was then used to count the cells: the cell counts from the four corners were averaged and multiplied by a dilution factor of 10X (25 μL : 250
μL), and then multiplied by 10⁴. BMM/HSCs are typically plated at 10⁵ cells/200 μL/well in a 96-well plate.

2-3-3: Recombinant Osteoactivin Treatment

The effects of exogenous rOA (R & D) titration (0.1 μM, 0.5 μM, and 1.0 μM) on BMM/HSCs proliferation were studied over a time-course (24hrs, 48hrs, and 72hrs). Next, the effects of exogenous rOA titration (0.1 μM, 0.5 μM, and 1.0 μM) on BMM/HSCs survival were studied over that same time-course (24hrs, 48hrs, and 72hrs). Then, the effects of exogenous rOA titration (10 ng/mL, 50 ng/mL, and 100 ng/mL; 100 ng/mL = 1.24 μM) on OC differentiation were studied at various time points of differentiation (day 1, day 4, and day 6). Next, the effects of exogenous rOA (50 ng/mL) on OC function were tested at various time points of differentiation (day 1, day 4, or day 6) by differentiating OCs on osteologic disks (Corning). Also, OC function was tested by differentiating OCs normally, and then transferring the OCs to bovine bone slices. Finally, the effects of exogenous rOA (50 ng/mL) on OC survival were studied over a time-course (24hrs and 48hrs).
2-3-4: Osteoclast Progenitor Cell Proliferation and Survival Assays

BMM/HSCs were isolated as previously described (Section 2-3-2). However, for
the survival assay, BMM/HSCs were re-suspended in α-MEM + 5.0% FBS + 1.0% P/S
+0.1% amphotericin. Three 96-well plates were used for each of the proliferation and
survival assays (24hrs, 48hrs, and 72 hrs). Proliferation assays were conducted with the
addition of 20 ng/mL of M-CSF (R&D), while the survival assay was conducted without
M-CSF. Both proliferation and survival used a cell concentration of \(10^5\) cells/well. There
were six replicates of each rOA concentration (no treatment control, 0.1 \(\mu\)M, 0.5 \(\mu\)M, and
1.0 \(\mu\)M). The plates were then incubated (24hrs, 48hrs, and 72hrs) at 37°C in a 5% CO₂
incubator.

Proliferation was measured with a CyQUANT NF Cell Proliferation assay
(Invitrogen), measuring DNA incorporation. For the proliferation assay, plates were
terminated at 24hrs, 48hrs, and 72hrs. In order to quantify BMMs/HSCs proliferation,
the media was drawn off, and 100 \(\mu\)L of dye binding solution was added to each well +
one blank. Dye binding solution contains: 20% 1X HBSS (Invitrogen), and 0.2% Dye
Delivery Reagent (Invitrogen) in dH₂O. HBSS was in 5X solution, and needed to be
diluted to 1X prior to use. Plates were incubated in the dark at 37°C for 2 hours. Finally,
fluorescent intensity was read on a BioTek Synergy H4 microplate reader (BioTek) at
~485 nm excitation and ~528 nm emission. The data was analyzed using Gen5 software (BioTek).

Survival was measured using an MTT assay, mitochondrial activity assay. For the survival assay, plates were terminated at time points as before. In order to quantify BMMs/HSCs survival, the media was drawn off, and 100 µL of alamar blue dye solution was added to each well + one blank. Alamar blue dye solution contains: 90% 1X HBSS and 10% 1X alamar blue. Alamar blue (Invitrogen) comes in 10X, while HBSS come in 5X. Both needed to be diluted to 1X prior to use. Plates were then incubated in the dark at 37°C for 4 hours. Finally, fluorescent intensity was read on a BioTek Synergy H4 microplate reader at ~570 nm excitation and ~585 nm emission. The data was analyzed using Gen5 software.

2-3-5: Osteoclast Differentiation

On day 0, BMM/HSCs were isolated as previously described (Section 2-3-2). On day 1, the cells were diluted in α-MEM CM (5X10⁵ cells/mL), and 20 ng/ml of M-CSF was added to the cell mixture. Four independent concentrations of rOA (control, 10 ng/mL rOA, 50 ng/mL rOA, and 100 ng/mL) were assessed at three time points (day 1, day 4, or day 6). 10⁵ cells at 200 µL/well were added to 96-well plates, and incubated for 3 days at 37°C in a 5% CO₂ incubator. At day 4, BMM/HSCs were the differentiated by
changing the media with 200 μL/well α-MEM CM + 20 ng/mL of M-CSF + 20 ng/mL of RANK-L (R&D). On day 6, the media was changed again with 200 μl/well α-MEM CM + 20 ng/mL of M-CSF + 20 ng/mL of RANK-L. Large multinucleated OCs usually appeared by day 7 or day 8, and OC differentiation was terminated. OCs were then fixed to prepare for TRAP activity assay and staining.

Figure 9. **Time-line of osteoclast differentiation.** OC from BM-MNCs is typically a 7-8 day procedure. Day 0, six to eight week-old C57BL/6 mice were sacrificed and BM-MNCs collected and cultured overnight in α-MEM CM. Day 1, the non-adherent BMM/HSCs were collected and plated in 96-well plates in α-MEM CM and 20ng/mL M-CSF to prime the osteoclast progenitor cells. Day 4, macrophages were differentiated into OCs with a media change of α-MEM CM + 20 ng/mL of M-CSF + 20 ng/mL of RANK-L. Day 6, OC differentiation was continued with another media change of α-MEM CM + 20 ng/mL of M-CSF + 20 ng/mL of RANK-L. Day 7-8, OC differentiation was terminated, and the cells were fixed to prepare for TRAP activity assay and staining.
2-3-6: TRAP Activity Assay and TRAP Staining

The TRAP activity assay measures a key marker for OC differentiation, so TRAP staining allows the visualization of violet-stained terminally differentiated OCs (>3 nuclei). Several solutions must be prepared before beginning the procedure. TRAP buffer (pH 5.2) can be stored for future use: 20 mL of 3 M sodium acetate buffer (Fisher Scientific) + 6.0 g of sodium tartrate (Fisher-Scientific), and filled with dH₂O to 500 mL. TRAP substrate solution must be prepared fresh for each assay: 10 mL of TRAP buffer + 10.0 mg of pNpp (Sigma). TRAP staining solution is photosensitive, so a 15 mL tube was prepared and covered with foil. Next, 1 mg of naphthol AS-MX phosphate (Amresco) was added to the tube. The naphthol was dissolved in 150 μL of N,N-dimethyl Formamide (Fisher Scientific), and then 10 mL of TRAP buffer was added. Finally 5 mg of fast red violet dye (Sigma) was added. TRAP staining solution was kept wrapped in foil until ready for use.

OCs were differentiated as previously described (Section 2-3-5). On day 8, the media in the plate was aspirated, and then 100 μL of 10% formalin (Fisher Scientific) was added to each well in order to fix the OCs. The OCs were allowed to fix for 30 minutes. After incubation, the formalin was aspirated, and the wells were washed with 200 μL of dH₂O. Next, 100 μL of (1:1) methanol (Acros Organics) : acetone (Fisher Scientific) was
added. After 3 minutes, the 1:1 methanol : acetone, was aspirated and the wells were allowed to dry. Then, 150 μL of TRAP substrate solution was added to each well + one blank, and the plate was then wrapped in foil and stored for at 37°C for one hour. After incubation, 100 μL of the TRAP substrate solution from each well in the first plate was added to corresponding wells in a new second plate. Then, 50 μL of 1N NaOH was added to each corresponding well in the second plate + the blank. The remaining TRAP substrate solution was aspirated from the first plate, and each well was washed with 200 μL of dH2O. Then, 100 μL of TRAP staining solution was added to each well of the first plate, and the plate was placed in the dark at 37°C and checked every 20 minutes to assess staining. The absorbance of the second plate was read using a BioTek Synergy H4 microplate reader at 405 nm. Data was analyzed using Gen5 software. When the OCs in the first plate were sufficiently stained, each well was washed 2X with dH2O to remove any remaining dye crystals. In order to preserve the stained OCs, 50 μL of 60% glycerol (Fisher Scientific) + 40% PBS was added to each well.

Only terminally differentiated OCs (>3 nuclei) will stain for TRAP. These OCs will appear as violet, and be set off from the background. TRAP staining facilitates the differential counting of OCs according to nuclei count. OCs with 3-20 and >20 nuclei
were counted separately, with the total OC number being simply the two counts added together.

![Image](image108x319)

**Figure 10. Osteoclasts stained for TRAP.** TRAP-positive violet C57BL/6 osteoclasts were differentially counted according to the number of nuclei. Data were collected for osteoclasts with 3-20 and >20 nuclei using a live image Nikon Ti Eclipse inverted microscope and automatic object capture of NIS-Elements software (Nikon). (Example: 3-20 nuclei as * and >20 nuclei as **) (Scale: 100 μm)

2-3-7: **Osteoclast Function: Osteologic Disks**

Osteologic disks utilize an osteoassay surface made of crystalline calcium phosphate. The Corning plate was designed to mimic the bone environment *in vivo.*
OCs were differentiated on the osteologic surface and treated with rOA. Differentiation was terminated on day 7. OCs were removed with bleach solution. The calcium phosphate surface was then stained with Von Kossa for contrast. Total pit area was then quantified.

In the Corning osteoassay, OCs were differentiated as previously described (Section 2-3-5) on osteologic disks with no treatment control or with 50 ng/ml of rOA added (day 1, day 4, or day 6). After 7 days, OCs were removed with bleach, and Von Kossa staining was performed to enhance the contrast of pit area. Total pit area was quantified as a percent threshold using stitched image on Nikon Ti Eclipse microscope with automatic object capture using NIS-Elements software (Nikon).

For Von Kossa staining, a 5% silver nitrate staining solution was prepared by adding 500 mg of Silver Nitrate (Fisher Scientific) to 10 mL of dH2O, and 100 µL was added to each well. Then, the plate was placed under UV light for one hour with the lid off to develop the stain. Next, the silver nitrate was decanted and rinsed with dH2O. Next, 5% sodium carbonate developing solution was prepared by adding 500 mg of sodium carbonate (Acros Organics) to 10 mL of 10% formalin. 100 µL was added to each well. The plate was developed for 5 minutes. The sodium carbonate darkened the silver
nitrate staining. Then, the sodium carbonate was decanted and the osteologic disks were rinsed with dH₂O, and allowed to dry.

2-3-8: Osteoclast Function: Bone Slices

Cortical bovine bone slices represent the truest test for resorption as a measure of OC function. On day 0, BMM/HSCs were isolated as previously described (Section 2-3-2), and 8 wells of two 6-well plates were coated 1mL/well with 15 µg/mL of collagen type I (BD Sciences) in 0.02 N acetic acid. On day 1, the remaining collagen was drawn off, and BMM/HSCs (2.5 million/well) were cultured in 5 wells of a 6-well plate, 3 mL/well of α-MEM CM + 20 ng/mL of M-CSF. On day 4, media was changed with 3 mL/well α-MEM CM + 20 ng/mL of M-CSF + 40 ng/mL of RANK-L. On day 6, media was changed again with 3 mL/well α-MEM CM + 20 ng/mL of M-CSF + 40 ng/mL of RANK-L. On day 8, OCs were lifted off of the 6-well plate with 0.02% collagenase B (Roche Diagnostics) in cell dissociation buffer (Gibco), or 2 mg/mL. Media was aspirated and then collagenase (1 mL/well) was added to each well for 30 minutes. The OCs were then gently aspirated, and centrifuged at 500 RPM for 5 minutes at 4°C. The supernatant was drawn off, and the OCs were re-suspended in α-MEM CM. 200µL of cells were added to each of 5 wells in a 96-well plate containing bone slices. There were two control wells. Additionally, there were three treatment wells: three wells of bone
slices + 50 ng/mL of rOA. On day 10, media was changed with α-MEM CM + 50 ng/mL of rOA. On day 11, the experiment was terminated. Media was aspirated, and PBS was added to the wells.

The OCs were removed from the bone slices with gentle rubbing. Next, the disks were stained with 1% toluidine blue (Electron Microscopy Sciences). The OCs excavated resorption pits in the bone slices, and the toluidine blue stains residual acids from OC resorption. The disks were placed in 1% toluidine blue for 10 seconds, and then washed in dH₂O for 30 seconds. The slices were then dried, and the resorption was quantified. Total pit area was quantified as a percent threshold using stitched image on Nikon Ti Eclipse microscope with automatic object capture using NIS-Elements software.

2-3-9: Osteoclast Survival

OCs were differentiated as previously described (Section 2-3-5) in three 96-well plates. A survival assay was conducted on the three plates with the day 8 plate as baseline. Day 9 and day 10 plates represented 24hrs and 48hrs of rOA treatment respectively. On day 8, the first plate was terminated, and a TRAP activity assay and staining were performed. The day 9 and day 10 plates were also changed (α-MEM CM + 20 ng/ml of M-CSF +/- 50 ng/ml of rOA) at that time on day 8. The 24hr and 48hr plates
were terminated similarly on day 9 and day 10, respectively. Finally, the OCs were
differentially counted according to the number of nuclei (3-20 and >20).

2-3-10: Statistical Analysis

Quantitative data was generated by the experiments. Differences between
individual groups were analyzed for statistical significance using Prism 5 software
(GraphPad). For comparisons between two group means, an unpaired t-test was
performed. Any difference with a probability value less than 0.05 was considered
statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Group means and +/-
standard errors of the mean were plotted in graphs. In-vitro experiments on OC
progenitor proliferation and survival, as well as OC differentiation were repeated 2
times with at least 6 replicates per experiment. OC function experiments on Corning
surface were repeated 2 times with 2 replicates per treatment, while a single bovine bone
slice experiment was performed with 2 controls and 3 replicates of each treatment. OC
survival was also only tested once, with 6 replicates per treatment.
2-4: Results

2-4-1: Dose-dependent Effects of Recombinant Osteoactivin Treatment on Osteoclast Progenitor Cell Proliferation or Survival

BMM/HSC proliferation and survival are important controls for an accurate description of OC differentiation and/or function. Data must be qualified if rOA affects BMM/HSC proliferation and/or survival. In these assays, BMM/HSC proliferation and survival (24hrs, 48hrs, and 72hrs) treatments with rOA (no treatment control, 0.1 μM, 0.5 μM, and 1.0 μM) were found to be similar.
Figure 11. **Graphs of osteoclast progenitor cell proliferation and survival with dose-dependent rOA treatment.** Proliferation of C57BL/6 hematopoietic stem cells was measured over 3 days. (A) $10^5$ cells/well were cultured in 10% FBS with 0.1 μM-1.0 μM recombinant osteoactivin with 20 ng/mL of MCSF and terminated at 24hrs, 48hrs, and 72 hrs. CyQuant Dye was added to bind DNA, and the fluorescence was measured using a BioTek Synergy H4 microplate reader with excitation ~485nm and emission at 530 nm, and read with Gen5 software (Biotek). Survival of C57BL/6 hematopoietic stem cells was also measured over 3 days. (B) $10^5$ cells/well were cultured in 5% FBS with 0.1 μM-1.0 μM recombinant osteoactivin without MCSF, and terminated at 24hrs, 48hrs, and 72 hrs. A mitochondrial activity assay (MTT) was performed, and the absorbance was measured using a Biotek Synergy H4 microplate reader at ~540 nm, and read with Gen5 software (Biotek).
2-4-2: Dose-Dependent Effects of Recombinant Osteoactivin Treatment on Osteoclast Differentiation

C57BL/6 OC differentiation was tested with rOA dose titration (no treatment control, 10 ng/mL, 50 ng/mL, or 100 ng/mL) only on day 1. Media was changed on day 1, day 4, and day 6, and the experiment was terminated on day 7. OCs were then stained for TRAP (violet). OC images for all treatments appeared similar.
Figure 12. **Osteoclasts differentiated with dose-dependent rOA treatment on day 1.**

On day 1, rOA treatments (no treatment control, 10 ng/mL, 50 ng/mL, or 100 ng/mL) were added to normally differentiating (M-CSF and RANK-L) C57BL/6 osteoclasts. Terminated on day 7, osteoclasts were stained for TRAP. Later, the osteoclasts were visualized using a live image Nikon Ti Eclipse inverted microscope. Images were stitched with NIS-Elements software (Nikon). The treatments include: (A) no treatment control, (B) 10 ng/mL of rOA, (C) 50 ng/mL of rOA, or (D) 100 ng/mL of rOA. (Scale: 1000 μm) Zoom to show osteoclast detail: (E) no treatment control, (F) 10 ng/mL of rOA, (G) 50 ng/mL of rOA, or (H) 100 ng/mL of rOA. (Scale: 500 μm)

OC differentiation was not significantly affected by dose-dependent administration of rOA on day 1. TRAP activity, total OC count, OC count (3-20 nuclei),
and OC count (>20 nuclei) were similar for all rOA treatments introduced on day 1.

These data are consistent with the osteoclast progenitor cell proliferation assay.

Figure 13. **Graphs of TRAP activity and osteoclast numbers after differentiation with dose-dependent rOA treatment on day 1.** On day 1, rOA dose titration (no treatment control, 10 ng/mL, 50 ng/mL, or 100 ng/mL) was added to normally differentiating (M-CSF and RANK-L) C57BL/6 osteoclasts. Terminated on day 7, (A) osteoclast TRAP activity was measured using a Biotek Synergy H4 microplate reader at an absorbance at 405 nm, and read with Gen5 software (Biotek). The osteoclasts were then stained for TRAP, and (B) the total number of osteoclasts was counted using a live image Nikon Ti Eclipse inverted microscope and NIS-Elements software (Nikon). Similarly, (C) the OCs with 3-20 nuclei were counted, and (D) the OCs with >20 nuclei were also counted.
C57BL/6 OC differentiation was again tested with rOA dose titration (no treatment control, 10 ng/mL, 50 ng/mL, or 100 ng/mL) only on day 4. Media was changed on day 1, day 4, and day 6, and the experiment was terminated on day 7. OCs were then stained for TRAP. Compared to control, OC images of the rOA treatments (50 ng/mL and 100 ng/mL) introduced on day 4 exhibit a dramatic reduction in the number of large OCs (>20 nuclei), suggesting that rOA treatment inhibits OC cell-cell fusion.
Figure 14. **Osteoclasts differentiated with dose-dependent rOA treatment on day 4.** On day 4, rOA treatments (no treatment control, 10 ng/mL, 50 ng/mL, or 100 ng/mL) were added to normally differentiating (M-CSF and RANK-L) C57BL/6 osteoclasts. Terminated on day 7, osteoclasts were stained for TRAP. Later, the osteoclasts were visualized using a live image Nikon Ti Eclipse inverted microscope. Images were stitched with NIS-Elements software (Nikon). The treatments include: (A) no treatment control, (B) 10 ng/mL of rOA, (C) 50 ng/mL of rOA, or (D) 100 ng/mL of rOA. (Scale: 1000 µm) Zoom to show osteoclast detail: (E) no treatment control, (F) 10 ng/mL of rOA, (G) 50 ng/mL of rOA, or (H) 100 ng/mL of rOA. (Scale: 500 µm)

OC differentiation was inhibited by dose-dependent administration of rOA introduced on day 4. Compared to control, TRAP activity for rOA added on day 4 was significantly decreased at 100 ng/mL ($p=0.0268$). However, although total OC count and
OC count (3-20 nuclei) for rOA added on day 4 were significantly increased from control for 10ng/mL, OC count (>20 nuclei) for rOA added on day 4 was very highly significantly decreased for 50ng/mL and 100ng/mL (p<0.0001 and p=0.0008, respectively).

Figure 15. Graphs of TRAP activity and osteoclast numbers after differentiation with dose-dependent rOA treatment on day 4. On day 4, rOA treatments (no treatment control, 10 ng/mL, 50 ng/mL, or 100 ng/mL) were added to normally differentiating (M-CSF and RANK-L) C57BL/6 osteoclasts. Terminated on day 7, (A) osteoclast TRAP activity was measured using a Biotek Synergy H4 microplate reader at an absorbance at 405nm, and read with Gen5 software (Biotek). The osteoclasts were then stained for TRAP, and (B) the total number of osteoclasts was counted using a live image Nikon Ti Eclipse inverted microscope and NIS-Elements software (Nikon). Similarly, (C) the OCs with 3-20 nuclei were counted, and (D) the OCs with >20 nuclei were also counted. (A-D) *p < 0.05, **p < 0.001, ***p < 0.001.
Finally, C57BL/6 OC differentiation was tested with rOA dose titration (no treatment control, 10 ng/mL, 50 ng/mL, or 100 ng/mL) only on day 6. Media was changed on day 1, day 4, and day 6, and the experiment was terminated on day 7. OCs were then stained for TRAP. Compared to control, OC images of the rOA treatments (50 ng/mL and 100 ng/mL) introduced on day 6 exhibit fewer large OCs (>20 nuclei).
Figure 16. **Osteoclasts differentiated with dose-dependent rOA treatment on day 6.**

On day 6, rOA treatments (no treatment control, 10 ng/mL, 50 ng/mL, or 100 ng/mL) were added to normally differentiating (M-CSF and RANK-L) C57BL/6 osteoclasts. Terminated on day 7, osteoclasts were stained for TRAP. Later, the osteoclasts were visualized using a live image Nikon Ti Eclipse inverted microscope. Images were stitched with NIS-Elements software (Nikon). The treatments include: (A) no treatment control, (B) 10 ng/mL of rOA, (C) 50 ng/mL of rOA, or (D) 100 ng/mL of rOA. (Scale: 1000 μm) Zoom to show osteoclast detail: (E) no treatment control, (F) 10 ng/mL of rOA, (G) 50 ng/mL of rOA, or (H) 100 ng/mL of rOA. (Scale: 500 μm)

OC differentiation was variably affected by dose-dependent administration of rOA on day 6. Compared to the control, TRAP activity on day 6 was not significantly affected. Total OC count for rOA added on day 6 was highly significantly increased
from control for 100 ng/mL ($p=0.0093$), and OC count (3-20 nuclei) was significantly increased for 50 ng/mL and highly significantly for 100 ng/mL ($p=0.0027$). Meanwhile, OC count (>20 nuclei) for rOA added on day 6 was highly significantly decreased compared to control for 10 ng/mL, 50 ng/mL, and 100 ng/mL ($p=0.0060$, $p=0.0029$, and $p=0.0011$, respectively).
Figure 17. **Graphs of TRAP activity and osteoclast numbers after differentiation with dose-dependent rOA treatment on day 6.** On day 6, rOA treatments (no treatment control, 10 ng/mL, 50 ng/mL, or 100 ng/mL) were added to normally differentiating (M-CSF and RANK-L) C57BL/6 osteoclasts. Terminated on day 7, (A) osteoclast TRAP activity was measured using a Biotek Synergy H4 microplate reader at an absorbance at 405 nm, and read with Gen5 software (Biotek). The osteoclasts were then stained for TRAP, and (B) the total number of osteoclasts was counted using a live image Nikon Ti Eclipse inverted microscope and NIS-Elements software (Nikon). Similarly, (C) the OCs with 3-20 nuclei were counted, and (D) the OCs with >20 nuclei were counted. (A-D) *p < 0.05, **p < 0.001, ***p < 0.001.
2-4-3: Time-dependent Effects of Recombinant Osteoactivin Treatment on Osteoclast Function: Osteologic Disks

Function was tested by differentiating C57BL/6 OCs on osteologic disks with 50 ng/mL of rOA given on different days of differentiation (no treatment control, day 1, day 4, or day 6). Media was changed on day 1, day 4, and day 6, and the experiment was terminated on day 7. The OCs were then removed, and the osteologic disks were stained with Von Kossa.

![Image of osteoclast resorption of osteologic disks](image)

**Figure 18: Osteoclast resorption of osteologic disks.** Fourteen osteologic disks resorbed by osteoclasts differentiated normally (M-CSF and RANK-L) in the presence of 50 ng/mL of rOA administered at different time points. Resorption disks were Von Kossa stained to increase contrast of resorption. A live image Nikon Ti Eclipse inverted microscope was used to visualize resorption, and images were stitched with NIS-Elements software (Nikon).
Compared to control, resorption was visually increased with day 1 and day 4 administration of rOA (lighter), but reduced with rOA added on day 6 (darker).
Figure 19. **Osteologic disk resorption by osteoclasts differentiated with time-dependent 50 ng/mL treatment of rOA.** C57BL/6 OCs were differentiated normally (M-CSF and RANK-L) on osteological disks in the presence of 50 ng/mL of rOA administered (no treatment control, on day 1, on day 4, or on day 6). The disks then stained with Von Kossa to increase the contrast of resorption. A live image Nikon Ti Eclipse inverted microscope was used to visualize resorption, and images were stitched with NIS-Elements software (Nikon): (A) no treatment control, (B) rOA was added on day 1, (C) rOA was added on day 4, or (D) rOA was added on day 6. (Scale: 1000 μm) Zoom to show resorption detail: (E) no treatment control, (F) day 1 (G) day 4, or (H) day 6. (Scale: 500 μm)

OC resorption of osteologic disks was affected by administration of 50 ng/mL of rOA at different time points of differentiation (no treatment control, day 1, day 4, and...
day 6). Compared to control, average OC resorption was increased with day 1 addition of rOA, and significantly increased with day 4 administration. Alternatively, administering rOA on day 6 decreased OC resorption, although not significantly.

Figure 20. **Graph of osteologic disk resorption after osteoclast differentiation with time-dependent 50 ng/mL treatment of rOA.** C57BL/6 Osteoclasts were differentiated normally (M-CSF and RANK-L) on osteological disks in the presence of 50ng/mL of rOA (no treatment control, on day 1, on day 4, or on day 6). The disks were then stained with Von Kossa to increase the contrast of resorption. Total pit area was quantified as a percent threshold using stitched images on Nikon Ti Eclipse inverted microscope with automatic object capture using NIS-Elements software (Nikon). *p < 0.05, **p < 0.001, ***p < 0.001.
2-4-4: Effects of Soluble and Matrix Recombinant Osteoactivin Treatment on Osteoclast Function: Bone Slices

Finally, C57BL/6 OC function was tested using the gold standard assay with bone slices. OCs were differentiated normally for 7 days, and then transferred onto dentine disks for 3 days. The OCs were treated with rOA (no treatment control or 50 ng/mL of rOA). The experiment was terminated on day 10, and OCs were removed from the bone slices. Next, the bone slices were stained with toluidine blue for resorption. Treatments with rOA exhibit less resorption
Figure 21. Bone slice resorption by osteoclasts with 50 ng/mL soluble or 1 μg/mL matrix treatment of rOA. C57BL/6 osteoclasts were differentiated normally (M-CSF and RANK-L) through day 7, and then transferred to bone slices with no treatment control or 50 ng/mL of rOA. On day 10, the experiment was terminated. The osteoclasts were removed, and the resorption pits were stained with toluidine blue. A live image Nikon Ti Eclipse inverted microscope was used to visualize resorption, and the images were stitched with NIS-Elements software (Nikon): (A) no treatment control or (B) 50 ng/mL of soluble rOA. (Scale: 1000 μm) Zoom to show resorption detail: (C) no treatment control or (D) 50 ng/mL of rOA. (Scale: 250 μm)

Compared to control, OC resorption of bone slices was decreased with rOA treatment, although not significantly. Average resorption was decreased with both 50 ng/mL of soluble rOA and 1 μg/mL of matrix coated rOA.
Figure 22. Graph of bone slice resorption by osteoclasts with 50 ng/mL of rOA. C57BL/6 osteoclast were differentiated normally (M-CSF and RANK-L) through day 7, and then transferred onto bone slices with no treatment control or 50 ng/mL of rOA. On day 10, the experiment was terminated. The osteoclasts were removed, and slice resorption was stained with toluidine blue. Total pit area was quantified as a percent threshold using stitched images on Nikon Ti Eclipse microscope with automatic object capture using NIS-Elements software (Nikon).

2-4-5: Osteoclast Survival with Recombinant Osteoactivin Treatment

C57BL/6 OCs were differentiated normally for 8 days, and then tested for survival (no RANK-L). OCs were terminated on day 8, day 9, or day 10. Day 9 represented 24hrs of treatment with rOA (no treatment control or 50 ng/mL), while day
10 represented 48hrs of treatment with rOA (control or 50 ng/mL). After termination, OCs were stained for TRAP. Control and treatment appeared similar after 24hrs, but treatment exhibited marked increase in the number of large OCs (>20 nuclei) over control after 48hrs.
Figure 23. **Osteoclast survival with 50 ng/mL treatment of rOA.** C57BL/6 OCs were differentiated normally (M-CSF and RANK-L) through day 8. A survival assay with M-CSF, but without RANK-L was performed with or without the addition of 50 ng/mL of rOA on day 8. The experiments were terminated on day 8, day 9 (24 hrs), or day 10 (48 hrs). Osteoclasts were visualized using a live image Nikon Ti Eclipse inverted microscope. Images were stitched with NIS-Elements software (Nikon) showing: (A) no treatment control on day 8, (B) no treatment control (24 hrs), (C) no treatment control (48hrs), (D) treatment with 50 ng/mL of rOA (24hrs), and (E) treatment with 50 ng/mL of rOA (48hrs). (Scale: 1000 μm) Zoom to show osteoclast detail: (F) no treatment control on day 8, (G) no treatment control (24hrs), (H) no treatment control (48hrs), (I) 50 ng/mL of rOA (24hrs), and (J) 50 ng/mL of rOA (48hrs). (Scale: 500 μm)

OC survival (24 hrs) was not affected, for the most part, by the administration of 50 ng/mL of rOA. Compared to control (24hrs), TRAP activity, total OC count, OC
count (3-20 nuclei), and OC count (>20 nuclei) were very similar to treatment with 50 ng/mL of rOA. However, on day 10 (48hrs.) treatment with 50 ng/mL of rOA demonstrated increased TRAP activity, total OC count, and OC count (>20 nuclei) compared to control, although not significantly.

**Figure 24**: Graphs of TRAP activity and osteoclast numbers of osteoclast survival with 50 ng/mL treatment of rOA. C57BL/6 OCs were differentiated normally (M-CSF and RANK-L) through day 8. A survival assay with M-CSF but without RANK-L was conducted with or without the addition of 50 ng/mL rOA on day 8. The experiments were terminated on day 8, day 9 (24 hrs), or day 10 (48 hrs). (A) TRAP assays were performed on the three time points using a Biotek Synergy H4 microplate reader at an absorbance at 405 nm, and read with Gen5 software (Biotek). Then, the OCs were stained for TRAP, and differential counts were performed: (B) total OC counts, (C) OC count with 3-20 nuclei, and (D) OC count with >20 nuclei.
2-5: Discussion

These data support rOA as an anabolic bone growth factor in C57BL/6 mice, not only through stimulating OB bone formation, but also by inhibiting OC bone resorption. Data analysis suggests both OC differentiation and OC function were inhibited by adding rOA. Endogenous OA levels must be considered in these experiments, as OCs naturally express OA. Adding rOA represents an increase far beyond native OA levels.

Understanding the effects of rOA added on day 1, day 4, and day 6 of OC differentiation was straightforward. OC differentiation was evidently inhibited by the administration of rOA. Day 4 and day 6 administration of rOA clearly demonstrated reductions in the number of larger OCs and increased the number of smaller OCs. This led to the observation that OC cell-cell fusion appears to be inhibited by rOA. Reduced DC-STAMP gene expression has been linked to OA (Abdelmagid, 2014 unpublished observations), and DC-STAMP is essential for OC differentiation and activity (Miyamoto 2006).

The effects of adding rOA to a Corning surface functional assay is harder to explain with certainty. Day 1 and day 4 exhibited marked increases in function, but this is counter-intuitive, as differentiation data showed fewer large OCs with day 4 and day 6 administration of rOA. Fewer large OCs should not demonstrate increased resorption.
The reason for these results lies in the nature of the assay. This assay was performed in vitro. In an in vivo experiment, OA levels would be constant, but media was changed on day 1, day 4, and day 6 of this in vitro experiment. This aspect must be considered, as there were no OCs present before day 6 of resorption. Only day 6 data represents OC resorption in the presence of rOA, and resorption was inhibited, although not significantly.

A logical explanation for rOA stimulating OC resorption with day 1 and day 4 administration is that endogenous OA was down-regulated and/or its receptors were blocked by earlier stimulation with high levels of rOA. The increased resorption, then, was likely actually due to decreased endogenous OA and/or OA receptor signaling. This explanation is consistent with rOA inhibiting OC resorption with day 6 administration.

Bone slice resorption data also supported rOA inhibition of OC function, although results were not significant. In this experiment, OCs were tested for resorption with rOA administration. Pre-formed OCs were transferred onto bone slices, and resorption was decreased for rOA administration, although not significantly. Reduced DC-STAMP gene expression is also consistent with decreased OC resorption activity (Miyamoto 2006).
OC survival data also showed rOA effects on OCs. Compared to control, 48hrs of rOA treatment demonstrated an increased number of larger OCs (>20 nuclei), although not significantly. Increased OC survival with rOA treatment suggests rOA treatment effects on OC function will be magnified. Perhaps, the previously demonstrated inhibition of OC cell-cell fusion with rOA provides a protective effect from apoptosis for larger OCs. OCs have a half-life of 3 days because their activity is so high (Ishii and Saeki 2008). Increased survival could be due to less OC activity, which is consistent with reduced levels of DC-STAMP gene expression (Miyamoto 2006).

Taken together, these data could suggest reduced DC-STAMP gene expression, which inhibits OC differentiation and resorption, yet increases OC survival. The mechanism appears to stem from decreased OC cell-cell fusion, as demonstrated by significant reductions in the number of larger OCs when differentiated with rOA. OC resorption and survival data was much less convincing, but perhaps significance could be found with more experimental replicates.
CHAPTER 3: CONCLUSIONS AND FUTURE STUDIES

Aim 1: To demonstrate whether rOA inhibits OC progenitor cell proliferation and survival.

Results confirm that rOA does not affect the proliferation or survival of OC progenitor cells. There was little to no difference between the means for no treatment control and 0.1-1 μM treatment with rOA, and the standard errors were negligible. We can conclude that, because rOA treatment does not affect OC proliferation and survival, measurements of differentiation and function do not have to be corrected.

Aim 2: To demonstrate whether rOA inhibits OC differentiation.

Results confirm that rOA inhibits the differentiation of OCs. Administration of rOA on day 1 had little effect, similar to proliferation data for OC progenitor cells, as conditions were similar. However, there were very highly significant decreases in the mean number of large OCs for 50 ng/mL and 100 ng/mL administered on day 4 of differentiation, compared to no treatment control. With 10 ng/mL, 50 ng/mL, and 100 ng/mL of rOA administered on day 6 of OC differentiation, there were highly significant decreases in the mean number of large OCs, compared to no treatment. This data supports the conclusion that rOA inhibits OC cell-cell fusion, suggesting the possibility
that DC-STAMP gene expression is inhibited by rOA. Measurements of OC function must take into account fewer large osteoclasts differentiate in the presence of rOA.

**Aim 3:** To demonstrate whether rOA inhibits OC function.

These results were inconclusive. The function experiments will need to be repeated with more replicates in order to achieve significance, and perhaps significance will not be reached. The treatment resorption means for both OC function experiments, Corning surface and bovine bone slices, suggested a decrease in resorption over no treatment control, but uncertainty remains.

The Corning surface OC resorption assays were confounded by method errors. Administration of rOA treatment on day 1 or day 4 both- actually indicated an increase in resorption over no treatment control. However, OCs did not form until day 7, and these treatments reflect other confounding variables, such as endogenous OA levels. As the rOA was washed off before OCs formed and rOA effects of OC differentiation indicate contrary results, the explanation seems to lie in down-regulated endogenous OA and/or reduced OA receptor signaling. This could be due to rOA over-stimulation earlier in differentiation. This logic is consistent with the OC resorption mean for day 6 rOA treatment which was less than no treatment control, but not significantly. These results do not strongly support that rOA is a negative regulator of OC function.
The bone slice OC resorption assay also had reduced resorption means for rOA treatment over no treatment control, but the differences were not significant. This experiment was not confounded by the effects of rOA on OC differentiation. Pre-formed OCs were transferred to the bone slices before rOA was administered. These results, as well, do not strongly support that rOA is a negative regulator of OC function.

**Hypothesis: Osteoclastogenesis is inhibited by recombinant osteoactivin**

This initial hypothesis was not disproven by any of the findings of this research, and still stands. OC differentiation was clearly inhibited by treatment with rOA. The functional data suggested rOA treatment inhibits OC function, but was much less conclusive. Repeating the OC function experiments is crucial to achieving significance, and the future direction of this research.

Several future experiments are logical extensions of this work. A dose-dependent resorption assay with rOA treatment for day 1, day 4, and day 6 might support this study. If resorption responds to rOA in a dose-dependent manner, demonstrating increasing resorption for day 1 and day 4 and decreasing resorption on day 6, further investigation would be merited. Beyond repeating the function experiments, ELISA could confirm down-regulation of endogenous OA in OCs after
treatment with rOA earlier in differentiation. It will be necessary to control exogenous rOA to ensure accurate measurement of endogenous OA levels. This could be accomplished easily with frequent washing, or more definitively with tagging rOA to differentiate between OA sources. Finally, OA receptor studies might confirm a down-regulation of OA receptor signaling with administration of rOA earlier in differentiation, as it is possible that rOA occupies the OA receptors for an extended period of time without stimulation. Our lab recently identified CD44 as the possible OC OA receptor. The next step would be to characterize this receptor.

Ultimately, this basic research could translate into novel treatment for the relatively common human ailment, osteoporosis, and improve health and quality of life for many. In the future, OA could be a therapeutic agent for bone healing and preventing or even reversing osteoporosis.
References


