

The Effects of Secreted Muscle Factors on Osteoclast and Osteoblast
Differentiation and Activity

A Thesis

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Dedication

To my wife,
Kelley VanLaecken
For your endless support and encouragement

Abstract

It has been well established that communication exists between osteoclasts and osteoblasts to maintain skeletal integrity. The interaction between muscle and bone is less understood. To better understand if muscle secreted factors, known as myokines, regulate osteoblast and osteoclast differentiation, we treated osteoclasts and MC3T3 (an osteoblast cell line) with control or muscle conditioned media and measured their ability to differentiate. We measured no significant change in osteoclast differentiation or activity in the presence of muscle conditioned media. While not significant, we did measure a trend towards increased mineralization of the MC3T3 cells in the presence of the muscle conditioned media. Our data suggests muscle may secrete factors that enhances the ability of osteoblasts to mineralize a matrix and promote bone formation.

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Introduction

Bone is not static, but rather a dynamic and complex organ that is constantly remodeling. Bone serves multiple roles in our body including: providing structure and support, producing blood cells, acting as a storage reservoir for minerals, and allowing mobility of our body (1, 2). Bone is comprised of three main cell types: osteoblasts, osteoclasts, and osteocytes. Osteoblasts are the bone forming cells, osteoclasts are bone resorbing cells, and osteocytes allow for bone communication. The remodeling of bone is an intricate process that requires the balance between the activity of osteoclasts, osteoblasts, and osteocytes and it is imperative that these three cells are able to work in concert to maintain the integrity of boney skeleton (1, 2).

Many factors affect the differentiation of osteoclasts and osteoblasts within the bony matrix, including muscle. It has been well established that muscle and bone are coupled mechanically, but recent research has discovered that there is also a biochemical and molecular component to the coupling of bone and muscle (3, 4). Brotto and Bonewald concluded that “the mechanical and biochemical theories of bone-muscle crosstalk are not exclusive or one more important or relevant than the other theory. These theories are most likely complimentary whereby mechanical force might prime bone and muscle for regulation and release of specific factors to exert their effects on the opposing tissue”(3). Therefore, it is necessary that we better understand the dynamic relationship between muscle and bone.

Osteoclast Differentiation and Activity

Osteoclasts are hematopoietic in origin and part of the monocyte/macrophage lineage. As the primary resorptive cell in bone, osteoclasts have the ability to resorb both organic and inorganic bone matrix (5, 6). The differentiation of osteoclasts is dependent on two key factors, M-CSF (macrophage colony stimulating factor) and RANKL (receptor activator of NF- κ B ligand) (5). M-CSF is produced by osteoblasts and regulates proliferation and differentiation of osteoclast precursor cells. In the presence of M-CSF, RANKL is able to signal and stimulate macrophage/monocyte precursors to differentiate into osteoclasts. The necessity of M-CSF and RANKL for osteoclast differentiation further demonstrates the reciprocated relationship that osteoclasts and osteoblasts have with one another (5-7).

Osteoclasts are essential for bone and calcium homeostasis. RANK receptors on osteoclasts allows them to interact with RANKL expressed on or secreted by osteoblasts and osteocytes (6). RANKL is responsible for directly regulating osteoclast differentiation (8). It has been demonstrated by multiple groups that osteoclasts are only able to differentiate when in contact with osteoblasts due to a specific molecule on the surface of osteoblasts called RANKL (as reviewed in (6)). This was further illustrated by Simonet et al. when they used the decoy receptor, osteoprotegerin (OPG), to mimic RANK and discovered that OPG prevented osteoclast formation from occurring and developed osteopetrosis (9). Conversely, when mice had excess RANKL present, they had increased osteoclast resorption and developed osteoporosis

(9). More recent studies have demonstrated that both osteoblasts and osteocytes can express RANKL and their regulation of osteoclasts is developmentally determined (10).

Bone resorption occurs when osteoclasts come into contact with bone, become polarized, and the ruffled border surrounded by the actin ring (sealing zone) forms. The sealing zone allows for the formation of a highly acidic environment within the bone. This environment allows osteoclasts to breakdown the inorganic and organic components of the bone matrix. Osteoclast polarization and sealing zone formation have been shown in part to occur due to $\alpha_v\beta_3$ integrin (6). This was demonstrated by McHugh et al. when they reported that in the absence of β_3 subunit that excess bone mass occurred in a mouse model due to the dysfunction of the mutant osteoclasts (11). Overall, osteoclasts require multiple activators to allow for differentiation and play a crucial role in the process of bone remodeling.

Osteoblasts, Osteocytes and Bone Formation

Osteoblasts are mesenchymal in origin and responsible for bone formation. A key component to osteoblast differentiation is transcription factor CBFA1/RUNX2. RUNX2 is regulated by many factors, proteins, and hormones, such as BMPs (bone morphogenetic proteins), in order to ensure proper bone deposition by osteoblasts to maintain adequate structural support. Osteocytes are osteoblasts that are encased within bone. These cells play an important role in regulating bone turnover, undergo apoptosis, or become bone lining cells (12).

Osteocytes are able to connect with one another through long slender processes that form a network within the bone matrix (13). Osteocytes have many functions such as phosphate and mineralization homeostasis, bone homeostasis and integrity, mechanosensory functions, and regulators of bone resorption and formation (13). Osteocytes are able to sense mechanical loading through the movement of fluid and signal to other osteocytes within the bone matrix to help regulate bone homeostasis (13).

Coupling of bone resorption and formation

Resorption by osteoclasts occurs prior to bone being laid down by osteoblasts. Rutkovskiy et al. states, "Bone is constructed through 3 processes: osteogenesis, modeling, and remodeling. All of these processes are mediated by osteoblasts, which work in tight cooperation with bone-resorbing osteoclasts" (12). Osteoclasts are responsible for the modeling and remodeling of bone, and osteoblasts are responsible for osteogenesis. It has been suggested that osteoclasts release factors from the bone during resorption that signal to osteoblast precursors to come to the resorptive site to begin bone formation. The bone matrix also has certain factors present that are known to induce bone formation. These factors include BMPs, transforming growth factor (TGF- β) and insulin-like growth factor 1 (IGF-1) which are released during resorption due to osteoclasts (14). Additionally, osteoclasts have been shown to express factors such as BMPs, Wnts, semaphorins and sphingosine kinase (SPHK1) which have been shown to play a role in osteoclast-osteoblast coupling (14).

This close relationship between osteoblasts and osteoclasts and their role in bone homeostasis is also evident by RANKL reverse signaling pathway (15). In this pathway RANK contained within small extracellular vesicles are secreted by osteoclasts that can activate osteoblasts to form bone via RUNX2 activation (15, 16). When these small extracellular vesicles were blocked, bone formation did not occur and bone resorption resulted instead (15). Osteoblasts activating osteoclasts to resorb bone via RANKL expression, and osteoclasts activating osteoblasts via multiple pathways to lay down bone demonstrates the dynamic relationship that osteoclasts and osteoblasts have with one another to regulate bone homeostasis.

Overall, the balance between bone forming osteoblasts and bone resorbing osteoclasts is mediated by many factors and is crucial in order for proper repair and maintenance of the skeletal system. When an imbalance occurs in this process pathologic diseases can result, such as osteoporosis where more bone is resorbed by osteoclasts than being replaced by osteoblasts (6).

Estrogen and bone

One common reason that there is an imbalance in bone homeostasis is due to decreased levels of estrogen that occurs in postmenopausal women. Postmenopausal estrogen deficiency can have serious effects on the skeleton (17-19). Estrogen has been reported to stimulate osteoclasts to undergo apoptosis and inhibit other key factors of osteoclastogenesis (17-19). However,

decreased levels of estrogen promote osteoclast survival and can lead to increased levels of RANKL which in turn increases osteoclast differentiation and activation (19). There are different treatment options to help counteract the effect of this loss of bone homeostasis due to changes in estrogen production. Bisphosphonates are most commonly used to treat osteoporosis by promoting apoptosis of osteoclasts. However, even though there is less bone resorption with bisphosphonates, it has been found that they also limit the ability for bone repair due to the coupling of the activity between osteoclasts and osteoblasts which can lead to a compromised skeletal matrix that is more prone to fracture (6, 14).

Skeletal muscle

The largest tissue in the body is skeletal muscle and plays a crucial role in musculoskeletal movement and support (20, 21). One distinction skeletal muscle has over other types of muscle is skeletal muscle can be voluntarily controlled. Skeletal muscle is composed of multinucleated cells and is similar to osteoblasts in that it is of mesenchymal origin (20). These mesenchymal precursors lead to the formation of myoblasts. Myoblasts then fuse together to give rise to myotubes which further differentiate and eventually mature into myofibers (20). Although there is no proliferative potential in skeletal muscle, the muscle is still able to repair itself and has some regenerative capacity due to satellite cells. Satellite cells play an important role in muscle homeostasis and allow for the regenerative capacity of muscle cells. These cells are able to proliferate in response to trauma/injury and give rise to muscle cell precursors or more satellite cells (20).

Satellite cells respond to growth factors/cytokines including TGF- β , IGF-1, and even hormones such as estrogen (21). For example, TGF- β plays a role in suppressing muscle growth through the stimulation of myostatin. When loading muscles there is a downregulation in TGF- β to allow muscle growth and hypertrophy (22, 23). As previously mentioned, TGF- β is a growth factor that plays a role in bone formation. This overlap of growth factors regulating muscle and bone show the importance of understanding how these two systems function and regulate one another.

Coupling between muscle and bone

The skeleton serves many different functions for the human body. One of these functions includes acting as an attachment for muscles in order to be able to move and support the body. Muscle and bone formation and degradation are closely coupled during growth, development and aging (24). As would be expected, increased physical activity results in increased muscle mass (25). Later in life, the loss in muscle mass is correlated with loss in bone mass (26, 27). Previous studies have also shown the importance of muscle factors in regards to regulating bone mass (28, 29). It has been shown that individuals who participate in space flight, as well as patients who have been placed on bed rest experienced changes in bone markers. These findings suggest increased in bone loss is due to muscle disuse (29). The opposite effect can be seen with exercise. For example, in a study completed by Jones et al. they determined that tennis players had increased cortical thickness and hypertrophy of bone on their dominant playing arm (28). In another study completed by Jahn et al., they found

that increased amount of electrical stimulation of muscle was associated with increased production of protective factors that protected osteocytes from cell death (4). Romero-Suarez et al. and Weisleder et al. found that the loss and atrophy of muscle fibers is more accelerated with aging. This accelerated loss of fast muscle fibers could play a role in the development and progression of osteoporosis (23, 30). These studies demonstrate that bone and muscle not only interact mechanically, but also through cell-to-cell interactions such as signaling to distant target cells to preserve osteocyte viability and support bone maintenance (4).

This coupling of muscle and bone works through multiple pathways. Myokines are produced by muscle in response to muscle contraction. Myokines include peptides and cytokines that can affect functions within a cell, on nearby cells, or on distant target cells (24). In fact cell-to-cell signaling in the absence of mechanical loading was demonstrated in a study completed by Kaufman et al. In their study they demonstrated that larger pore sizes of nitrocellulose membranes placed between muscle and bone resulted in improved healing due to the fact more growth factors were allowed to diffuse across the membrane compared to smaller pore sizes (31). Also, a study completed by Zachs and Sheff demonstrates that bone formation can occur when minced muscle is applied directly on the intact bone tissue, but the same does not occur when using minced liver (30). Examples of myokines include fibroblast growth factor (FGF) and IGF-1. These factors are secreted by muscle cells and have a positive effect on the regulation of bone formation (24, 32). The coupling of bone formation,

muscle growth/development, and muscle repair can be linked to IGF-1 secretion (33, 34). Hamrick et al. found skeletal muscle has high levels of IGF-1 under normal conditions. However, when muscle injury occurred there was a decrease in production of IGF-1 leading to changes in bone due to muscle injury (32).

Hamrick et al. demonstrate that signaling between bone and muscle plays an important role in their interactions with one another and that muscle serves as a source for growth factors for bone (32). Multiple studies have demonstrated that myokines secreted by muscles can improve bone repair. In these studies, it was reported that placing healthy skeletal muscle flaps over bone fractures can improve the healing process compared to severely damaged muscle or no muscle being placed over bone fractures (24, 35). The importance of muscle/bone regulation is also demonstrated in those individuals where muscle is congenitally absent or if one becomes paralyzed. In these cases, there is a decrease in bone diameter, development in long bones, and mineralization of bone (36).

The immune system also plays a role in the functional relationship between muscle and bone formation and resorption (5). Specific immune modulators including interleukin 6 (IL-6) and interleukin 15 (IL-15) are released from muscle when exercising (24, 32). Previous studies have shown that when IL-15 is overexpressed in skeletal muscle that there is an increase in bone mass (37). One area of research that needs further investigation proposes that estrogen may differentially regulate these immune modulators which may have an effect on the skeletal system (24, 32).

Overall, muscle plays a crucial component to bone maintenance and repair. Stein et al stated “Muscle is perhaps the most crucial factor in the physiological process of fracture healing.”(38) Having a better understanding of this muscle-bone interaction and how these processes work will allow us to develop new drugs that target specific factors/components of this system, while limiting the effects associated with normal bone remodeling. Our research will take a closer look at how secreted muscle factors affect the differentiation of osteoclasts and osteoblasts.

Material and Methods

Mice and Ethics

Bone marrow macrophages were obtained from 6 week old female C57Bl/6 mice. All mice were housed in the animal care unit of the University of Minnesota and maintained according to the guidelines set by the Institutional Animal Care (IACUC) and the Committee of the Office for the Protection of Research Subjects. All experimental protocols were approved by IACUC (protocol 180636053A) at the University of Minnesota. Euthanasia was performed by CO₂ inhalation.

Collection of muscle conditioned media

Extensor digitorum longus muscles from C57Bl/6 6 week old female mice were dissected and placed in an *in vitro* bath of Krebs-Ringer bicarbonate buffer.

Muscle was subjected to a series of controlled and quantifiable contractions as standard in the Lowe lab (39). Media surrounding the muscle (conditioned media; 1 mL) was collected and frozen at -80°C until added to osteoclast or MC3T3 cell cultures.

Harvesting of Bone Marrow Macrophages and Osteoclast Differentiation

Bone marrow was harvested from tibiae and femora of mice. The muscle tissue was removed, tibiae and femora were dissected and the ends of the bone were cut. Bone marrow was flushed, red blood cells were lysed, and remaining cells were plated and cultured overnight in osteoclast media. Osteoclast media was composed of phenol red-free alpha-MEM (Gibco) containing 5% fetal bovine serum (Atlanta Biologicals), 25 units/mL penicillin/streptomycin (Invitrogen), 400 mM L-Glutamine (Invitrogen) in the presence of 1% CMG 14-12 culture supernatant containing M-CSF. Non-adherent cell populations were separated, counted and re-plated in 12-well plates in osteoclast media supplemented with culture supernatant 1% CMG 14-12 culture supernatant containing M-CSF. Cells were refed two days later with 1% CMG 14-12 culture supernatant, RANKL (10 ng/ml) with either no media, 5% or 2.5% control or 5% or 2.5% muscle stimulated media. Control media contains the Krebs buffer alone and the injured media (muscle stimulated media) contains the krebs buffer and the muscle stimulated media. The cells were then fed every other day for the next 4 days.

Tartrate Resistant Acid Phosphatase (TRAP) Stain

Osteoclasts were fixed with 4% paraformaldehyde (PFA), washed with PBS, and then stained for tartrate resistant acid phosphatase (TRAP) expression with 5 mg tartrate, Naphthol AS-MX phosphate, 0.5 mL M, M-Dimethyl formamide, 50 mL acetic acid buffer (1 mL acetic acid, 6.8 g sodium acetate trihydrate, 11.5 g sodium tartrate in 1 L water) and 25 mg Fast Violet LB salt. Using light microscopy the cells were then observed and photographed at 4X magnification. Osteoclast number and size were quantitated using the computer program NIH Image J.

Demineralization assay

Primary bone marrow cells were plated on Corning Osteoassay surface plates. Cells were fed as described above to allow for osteoclast differentiation. The media was completely removed once osteoclast differentiation was observed and 100 μ L/ well of 10% bleach was added and allowed to incubate at room temperature for 5 minutes. The bleach solution was then aspirated and the wells were washed twice with 150 μ L of dH₂O. The plate was then allowed to air dry completely at room temperature for 3–5 hours. The wells were observed at 4x magnification for the formation of resorption pits and images were captured with light microscopy. Images were measured and analyzed using NIH ImageJ.

Conditioned media treatment of MC3T3 cells

MC3T3 cells were treated with 5% or 2.5% conditioned media from control or injured media (muscle stimulated media). MC3T3 cells were obtained from

ATCC and maintained under recommended conditions. The cells were fed with ascorbic acid, alpha-MEM, and 5% or 2.5% conditioned media for 11 days. On the 11th day, cells were fed with β -glycerophosphate and Von Kossa staining was performed the next day.

Mineralization Assay

Von Kossa Stain was used to evaluate mineralization. Cells were incubated with varying percentages of ethanol for 5 minutes at a time at 37⁰ Celsius. The cells were then washed with water twice, and a silver nitrate solution (1.25g silver nitrate and 25mL water) was added into each well. The plates were covered with aluminum foil and incubated for 1 hour at 37⁰ Celsius. Finally, the plates were washed with water two times and the last wash was left on overnight. The cells were photographed and using NIH Image J mineralization was quantitated.

Statistical Analysis

All experiments were completed in duplicate and performed at least twice. Student's t-test or 1-way ANOVA analysis followed by a Tukey's multiple comparison test were used to compare data; $p < 0.05$ indicates significance. Statistical analysis was performed using Prism 8 for Mac OSX.

RESULTS:

Secreted muscle factors did not affect osteoclast differentiation

Data from other research groups have suggested that muscles release factors that regulate bone; however, these studies have primarily focused on muscle-osteocyte regulation (as reviewed in (3)). To continue to understand the molecular interaction between muscle and bone, we wanted to ask if muscle secretes factors regulate osteoclasts and/or osteoblasts. In order to determine if secreted muscle factors played a role in regulating osteoclast differentiation, we treated bone marrow macrophages with M-CSF and RANKL to stimulate osteoclast differentiation (untreated) or control or injured conditioned muscle media. The cells were fed every other day for the next four days. The TRAP positive stained osteoclasts were observed using light microscopy, photographed and size and number were quantitated using NIH Image J. As seen in figure 1, we found no statistically significant difference between the control and injured group when comparing either the number of osteoclasts (Fig 1B). There was a size increase with injured media treated osteoclast cultures; however, there was no significant difference between control and injured condition media treated osteoclast cultures suggesting the media itself caused an enhancement of osteoclast differentiation (Fig 1C).

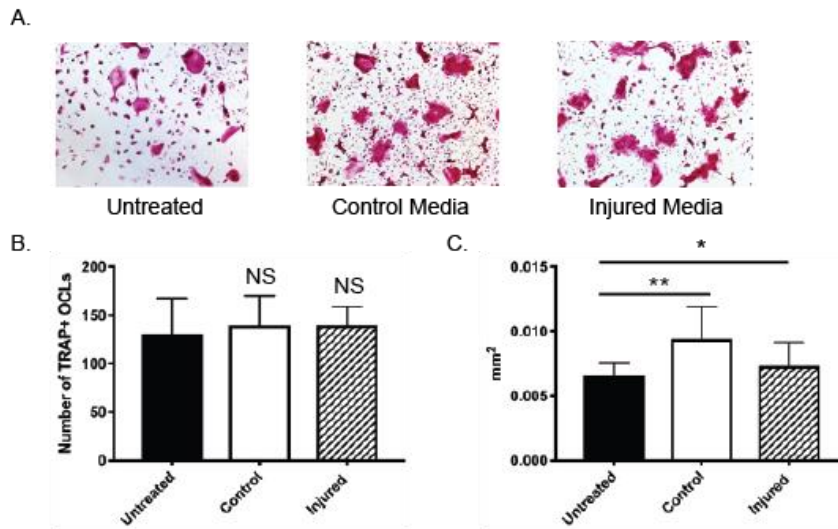


Figure 1. Myokines do not affect osteoclast differentiation. Bone marrow macrophages were stimulated to differentiate into osteoclasts with no conditioned media (untreated), 2.5% control or injured muscle media. Osteoclasts were TRAP stained, photographed and quantitated using NIH Image J. (A) Representative TRAP images of treated osteoclasts (B) number of TRAP positive osteoclasts and (C) size of osteoclasts. * $p < 0.05$ and ** $p < 0.01$ comparing control and injured media to untreated cultures. NS is not significant

Secreted muscle factors did not affect osteoclast demineralization

Next we asked if myokines affected or regulated osteoclast activity by plating bone marrow macrophages on a calcium phosphate surface (Osteoassay plates) and treating with M-CSF, RANKL and control or injured muscle conditioned media. Once differentiation occurred 10% bleach was added, aspirated, and the wells were washed dH₂O. The areas of demineralization were

photographed under light microscopy and NIH Image J was used to quantify the number, size, and area of demineralization. As seen in Figure 2 there was a trend towards a higher number of pits and a higher percent area of demineralization compared to the untreated group; however, there was no significant difference between the groups with any of the measurements.

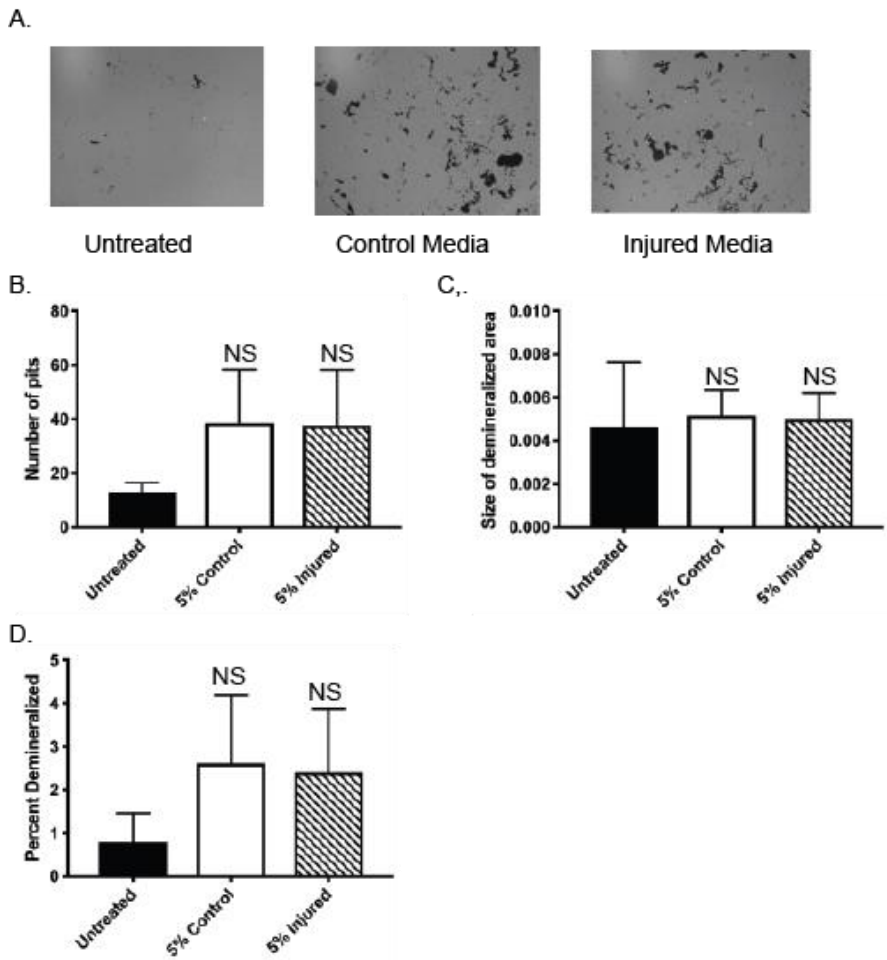


Figure 2. Myokines do not affect osteoclast activity. Bone marrow macrophages were isolated and treated with M-CSF, RANKL and conditioned muscle media. Bone marrow macrophages were cultured Osteoassay plates until areas of demineralization appeared. Plates were treated and areas of

demineralization were quantitated using NIH Image J. (A) Representative images of demineralization (B) Number of pits, (C) size of demineralization areas and (D) percent of demineralization on plates. NS is not significant

Secreted muscle factors increased the number of mineralized cells in osteoblasts

Lastly, we wanted to ask if muscle secreted factors regulate the ability of osteoblasts to mineralize a matrix. In order to determine if secreted muscle factors have an effect on osteoblast mineralization, we treated the osteoblast cell line, MC3T3, with conditioned media from muscle. For controls, MC3T3 cells were fed with and without ascorbic acid (ascorbic acid stimulates differentiation) and MC3T3 cells were treated with 2.5% and 5% conditioned media from control or muscle stimulated media. We found that the number of mineralized nodules produce by osteoblasts significantly increased in the 5% control and injured groups (Figure 3B). In Figure 3C we can see that there was a significant increase in the size in the mineralized nodules between the 2.5% control and the 2.5% injured. However there is no significance between the control and injured conditioned media treated groups.

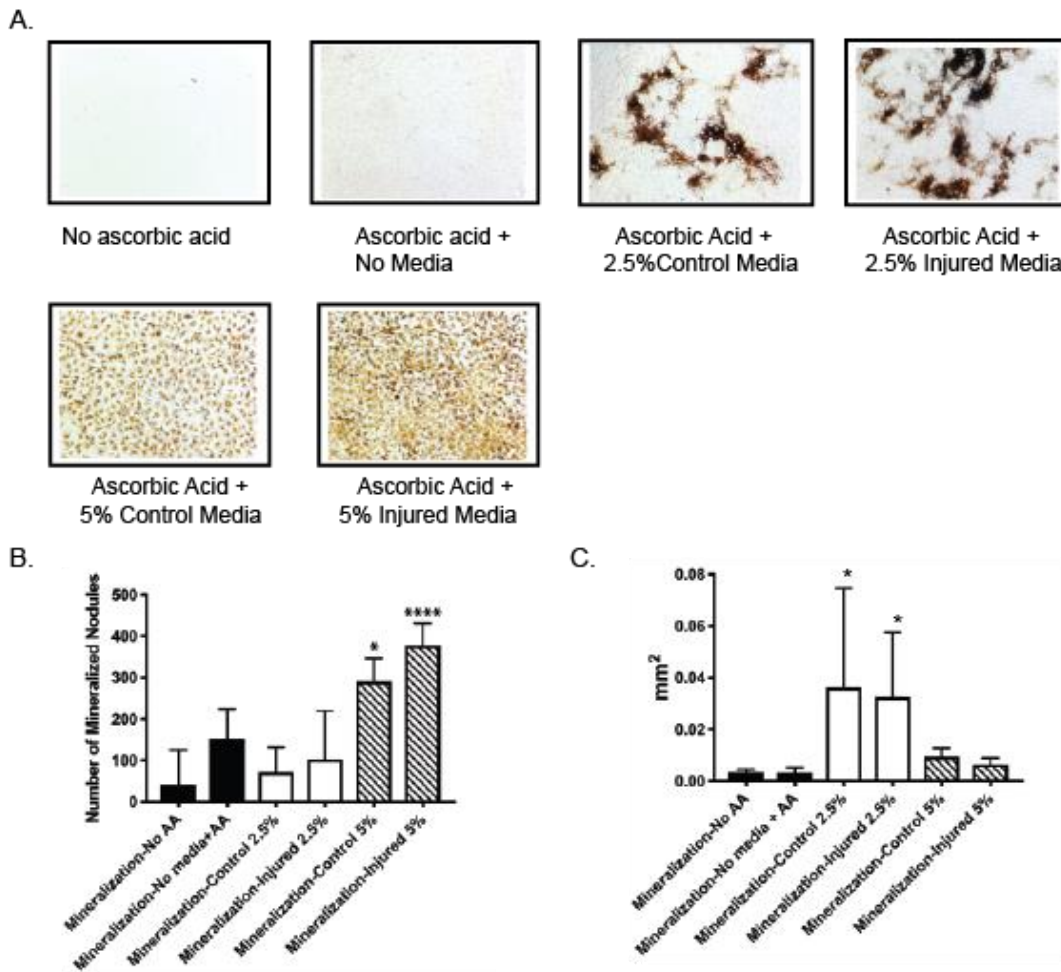


Figure 3. Muscle secreted factors regulate osteoblast mineralization.

MC3T3 cells were plated and treated with ascorbic acid and muscle conditioned media. Cells were given b-glycerophosphate to stimulate mineralization.

Mineralization was visualized and photographed. NIH Image J was used to

quantitate size and number of mineralization areas. (A) Representative images

of mineralization (B) Number of mineralization areas and (C) Size of

mineralization areas. * $p < 0.05$ 5% control compared to untreated, 2.5% control

and injured treated cells compared to control cells, **** $p < 0.0001$ 5% injured

Discussion

As previously discussed, it has been established that bone cell differentiation and activity can be regulated by secreted myokines from muscle cells (3). In this study we show that secreted muscle factors do not affect osteoclast differentiation or demineralization in the short-term, but these factors do trend to affecting osteoblast mineralization. While my results with the MC3T3 were not significant between the control and injured muscle media, the trend that we measured was increased mineralization with the MC3T3 treated with injured muscle media. These results are similar to preliminary experiments completed by the Mansky Lab which found a significant increase in osteoblast mineralization with the injured conditioned media. It is not altogether clear why I did not measure a significant change in osteoblast activity with the injured conditioned media. It could be that the MC3T3 cells have been passaged too many times and have lost their ability to mineralize a matrix. Originally the Mansky lab performed these experiments with different condition media. The control conditioned media appears to stimulate osteoblast mineralization compared to untreated cells. It could be, while unlikely, that there are some differences in the control media that the Mansky lab is currently using from the Lowe lab.

We hypothesize that although secreted muscle factors had no effect on osteoclast differentiation and demineralization in the short-term there could be an effect in the long-term. Previous studies have shown that the immediate result of loading muscles results in hypertrophy of the muscle and in result cause an increase in bone formation. During this bone formative state there is an increase

in osteoblast activity and little osteoclast activity occurring. However, once osteoblasts begin formation and signal osteoclasts for bone remodeling, we hypothesize that there could be an increase in osteoclast differentiation and demineralization. In future studies osteoclast differentiation in the presence of secreted muscle factors should be evaluated not only in the short-term, but also the long-term to account for this suspected increase in osteoclast function. By allowing time for osteoblasts to differentiate and signal to osteoclasts, we can evaluate if there is an indirect effect that secreted muscle factors have on osteoclast differentiation and demineralization. We could use condition media from osteoblasts treated with muscle conditioned media in osteoclast cultures to determine if there is any change in osteoclast differentiation or activity.

Along with further evaluating osteoclast differentiation/demineralization, it would be beneficial to further evaluate osteoblast differentiation/mineralization. We found that secreted muscle factors increase osteoblast mineralization, but we are not sure what specifically is causing this increase. By determining what specific chemokines/cytokines are released from secreted muscle factors that result in increased osteoblast mineralization, specific drugs could be developed that target these specific factors/components of this system, while limiting unwanted side effects.

Another surprising result we found was the number of mineralized cells seen in the control with ascorbic acid. Although our photos in Fig 3 show that little mineralization had occurred, we found a high count of mineralized cells using our NIH image J software. This is due to the contrast of the pictures not

being adequately adjusted prior to quantitating data. The darker areas in the photo that are not due to demineralization were still included as if mineralization occurred. We will look to correct this feature in NIH J imaging software in future experiments.

As previously discussed, muscle and bone are interrelated through different chemical signals to allow regulation and differentiation to occur (3, 32). With the average age for menopause in women being 51 years old, most women live one-third of their lives in an estrogen deficient state (40). Furthermore, musculoskeletal disorders such as sarcopenia and osteoporosis are one of the most common conditions that affect the elderly (41). Therefore, understanding the musculoskeletal impact of estrogen deficiency is crucial. It is hypothesized that estrogen currently facilitates its effect on muscle through estrogen receptor ER-alpha (19). Changes in inflammatory cytokines/chemokines from skeletal muscle in estrogen deficient mice has been measured by Lowe's Lab. They found that in the absence of estrogen several cytokines were differentially regulated (42). These cytokines have been shown in other experiments to affect activity of osteoblasts and/or osteoclasts. By blocking ER alpha receptor in muscle, tissue can selectively be evaluated. We hypothesize in future studies that loss of estrogen in one cell (muscle cell) will have a detrimental effect on the function of other cells (bone cells) and without estrogen muscle differentially secretes myokines that have a detrimental effect on bone cells.

Another interesting topic regarding osteoporosis due to deficient estrogen, is in the field of orthodontics. It is common in the field of orthodontics to be

treating adult patients that are actively taking bisphosphonates (43). Often times, bisphosphonates are being used to counteract the effects of osteoporosis by limiting osteoclast function. Bisphosphonates target high turnover areas, which occur in the mouth during orthodontic tooth movement (44). Due to the inhibited function of osteoclasts seen when taking bisphosphonates, there is slower tooth movement and impaired bone healing (43). This can have a significant impact on orthodontic treatment. Bisphosphonates currently work by promoting osteoclast apoptosis, but has been found to have side effects such as: atrial fibrillation, osteonecrosis of the jaw, and over suppression of bone remodeling that limits the ability for skeletal repair. Bisphosphonates also have a long skeletal half-life, and the drug can remain in the body almost a decade after the initially dose (44). By better understanding the process of muscle and bone differentiation and how these processes effect one another, we can potentially develop targeted drugs to modulate these processes. This could allow us to eliminate bisphosphonate treatment with more site specific treatment and limit unwanted side effects, such as slower tooth movement and impaired healing of bone. Eliminating these side effects could allow for more efficient and predictable treatment in the field of orthodontics.

In summary, future directions for this study would be to evaluate how secreted muscle factors effect osteoclast differentiation in the long-term and if there is an indirect effect, evaluate what specific chemokines/cytokines result in the increased osteoblast mineralization in the presence of secreted muscle

factors, and evaluate how secreted muscle factors affect osteoclast and osteoblast differentiation in the presence and absence of estrogen.

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