Estrogen Receptor α deficiency in skeletal muscle leads to differential expression of myokines, but does not affect osteoclastogenesis

A thesis submitted to the faculty of the Graduate School at the University of Minnesota

By
Kathleen Thieu DDS

Principal Investigator: Kim Mansky PhD

In partial fulfillment of the requirements for the degree of Master of Science

May 2020

© Kathleen Thieu, 2020

Acknowledgements

Foremost, I would like to acknowledge Dr. Kim Mansky for her continued guidance and support throughout this process. She has been more than patient with me and is always willing to help and provide feedback.

I would also like to thank Dr. Massimo Costalonga for meeting with me regularly to provide feedback, for serving on my committee, and for pushing me to always think critically whether it is in literature review, research, or patient care. Also, Dr. Larry Wolff for serving on my committee and always providing encouraging words throughout residency.

I would also like to extend my gratitude to Andrew Norton for helping with cell culture and teaching me how perform various experiments in the lab. Also, Matt Wong for helping with data collection for TRAP and resorption assays. And, Corey Baumann from the Lowe Lab for setting up the contraction experiments and providing technical assistance.

I would like to thank Qi Wang for statistical analysis of the data.

Additionally, the Erwin Michael Schaffer Chair in Periodontics, University of Minnesota for the financial support of this project.

Also, to my co-residents for helping me throughout residency.

Dedications

I would like to dedicate this to my parents and sister for their continued support. I would also like to thank James Lee for putting up with me throughout this quarantine as I write my thesis from home.

Abstract

Background: Both osteoporosis and sarcopenia are musculoskeletal diseases associated with estrogen deficiency and aging. Previous studies suggest that the relationship between muscle and bone is more than purely mechanical, as there is reciprocal paracrine and endocrine signaling (Tagliaferri, Wittrant et al. 2015). Muscle can secrete myokines which can impact bone (Harry, Sandison et al. 2008, Reginster, Beaudart et al. 2016). It is unknown how muscle specific estrogen deficiency impacts bone. Understanding how estrogen deficiency impacts this muscle-bone crosstalk may lead to a clearer mechanism of how musculoskeletal diseases form.

Objective: To determine whether estrogen receptor α (ERα) signaling in muscle can lead to differential expression of myokines, which can impact osteoclastogenesis.

Methods: In order to study the crosstalk between the muscle-bone unit as a potential mechanism for osteoporosis, skeletal muscle specific estrogen receptor α deficient mice (ERαKO) (n=17) and wild type (WT) (n=7) mice were used. In the first experiment, osteoclasts from ERαKO and WT mice were evaluated for osteoclast differentiation gene expression using qRT-PCR. Tartrate resistant acid phosphatase staining (Raue, Slivka et al.) and Osteo-assays (resorption assays) were used to determine osteoclast size, number, resorption capabilities. Shapiro-Wilk test was used to evaluate normality. Non-parametric T tests were performed. In a separate experiment, extensor digitorum longus muscle was isolated and contraction experiments were performed in order to isolate myokines from ERαKO and WT mice. WT Osteoclasts were cultured with myokines from either ERαKO or WT mice

and osteoclast differentiation genes were measured by qRT-PCR. Shapiro-Wilk test was used to evaluate normality. ANOVA and Welche's T tests were performed. Finally, to determine how estrogen receptor α signaling affects the myokine profile, muscle was isolated from ERαKO and WT mice. Muscle was homogenized with zirconium oxide beads and RNA was isolated. cDNA was made using iScript. PCR array was used in order to compare myokine expression in ERαKO and WT mice.

Results: ERαKO and WT osteoclasts had no difference in size, number, resorption capability, and osteoclast differentiation gene expression. ERαKO and WT osteoclasts demonstrated no difference in expression of *Nfatc1*, *DC-stamp*, and *Cathepsin k*. There was a trend towards increased *C-Fos* expression in the osteoclasts cultured with myokines from ERαKO compared to WT ($p=0.0692$). Several myokines were downregulated in the muscle cells of ERαKO compared to WT including: *Bmp2 (*530-fold), *Osm* (226-fold), and *Hc* (66-fold).

Conclusions: ERα deficiency specific to muscle does change the myokine profile which is secreted by muscle cells. However, there is no difference in osteoclast gene expression when osteoclasts are cultured with myokines from ERαKO compared to WT. There is also no difference in size, number, and resorption pits of osteoclasts from ERαKO and WT mice. Therefore, absence of ERα signaling specific to muscle does not affect osteoclasts, as expression of osteoclast differentiation genes, osteoclast size, number, and resorption activity is unchanged. *Bmp2, Osm, Hc* are downregulated in the absence of ERα signaling in muscle cells. These genes are associated with osteoblast activity and suggest that future experiments should focus

on osteoblasts in the absence of ERα signaling in muscle in order to explore potential mechanisms for osteoporosis.

Table of Contents

Acknowledgements	i
Dedication	ii
Abstract	iii-v
Table of contents	vi
List of Tables	vii
List of Figures	viii-ix
Introduction	1
Review of the Literature	3
Rationale and Purpose	24
Material and Methods	26
Results	36
Discussion	51
Conclusion	57
Bibliography	59

List of Tables

Table 1	Myokines secreted by skeletal muscle with potential effects on bone	10
Table 2	Published Data from Lowe Lab demonstrating expression of genes in muscle in responsive to estradiol	19
Table 3	Osteoclast gene primers used for qRT PCR	29

List of Figures

		Page
Figure 1	The role of estrogen in preservation of muscle mass, muscle quality, and maintenance of muscle cells	14
Figure 2	Transcriptional regulation in osteoclasts	17
Figure 3	Pathway for osteoclast differentiation	18
Figure 4	PCR Analysis for HAS-*Cre* Mice	20
Figure 5	ß-galactosidase staining on an embryo expressing HSA-*Cre* and the LacZ reporter gene	21
Figure 6	Unpublished preliminary data from Mansky Lab on ERαKO osteoclasts	23
Figure 7	Generation of skeletal muscle specific ERαKO using a cre-lox system	26
Figure 8	Flow chart for experiment 1: osteoclast TRAP, resorption, and gene expression from ERαKO and WT mice	29
Figure 9	In vitro bath for collection of myokines	32
Figure 10	Flow chart for experiment 2: osteoclast conditioned media experiments	33
Figure 11	Flow chart for experiment 3: identification of myokines using RT^2 profiler PCR array	35
Figure 12	RT^2 profiler PCR array layout	35
Figure 13	Tartrate resistant acid phosphatase staining and Osteo Assay (Resorption activity) of osteoclasts from ERαKO and WT mice	38
Figure 14	Tartrate resistant acid phosphatase staining of osteoclasts from ERαKO and WT	39
Figure 15	Expression of genes in osteoclasts isolated from ERαKO and WT mice	40
Figure 16	Distribution of threshold cycle for housekeeping gene used (*HPRT*)	41
Figure 17	Distribution of threshold cycle for housekeeping gene used (*HPRT*) for conditioned media experiment	44
Figure 18	Gene expression of osteoclasts treated with muscle conditioned media from WT and ERαKO mice	45

Figure 19	Distribution of Cq values of housekeeping genes for PCR array	47
Figure 20	A volcano plot showing upregulated (right) and downregulated genes (left) in the muscle from ERαKO compared to WT	48
Figure 21	Heat map demonstrating cytokine PCR array from ERαKO and WT tibialis anterior muscle and associated cytokines/chemokines	49
Figure 22	Table demonstrating cytokine and chemokine genes associated with heat map	50

Introduction

Incidence of Osteoporosis and the Global Impact

Osteoporosis is a musculoskeletal disease characterized by a decrease in bone mass, while maintaining a normal ratio of mineralized to non-mineralized matrix. Because of the diminished bone mass, there is decreased torsional and compressive strength, which leads to risk of osteoporotic fractures (WHO 1994). Osteoporosis is a significant public health concern. Two hundred million women are affected by osteoporosis worldwide (International-Osteoporosis-Foundation 2017). Globally, there were approximately 9 million fractures related to osteoporosis in the year 2000 (Johnell and Kanis 2006).

In the United States, the incidence of osteoporotic fractures surpasses the combined incidence of stroke, breast cancer and heart disease (Riggs and Melton 1995). Hip fractures make up 20% of osteoporotic fractures (Kanis, Oden et al. 2012). Of the osteoporotic fractures, hip fractures demonstrate the highest mortality rate. 1 in 5 patients with hip fractures will pass away within one year. Even for patients who do survive, quality of life is diminished following hip fracture, as only one third of patients return to normal levels of function (NIH 2001, Nih Consensus Development Panel on Osteoporosis Prevention and Therapy 2001, Panel 2001, Lane 2006). Furthermore, the economic burden of osteoporosis is an astounding $849 billion annually (Bonewald, Kiel et al. 2013).

Aging and the Connection between Osteoporosis and Sarcopenia

Aging is associated with both osteoporosis and sarcopenia. Sarcopenia is age-associated, progressive loss of skeletal muscle mass and strength (Fielding, Vellas et

al. 2011, Cooper, Dere et al. 2012). After age 35, there is a significant decrease in muscle mass, strength, and power (Frontera, Hughes et al. 2000). Decrease in muscle mass associated with sarcopenia can lead to poor balance, thus increasing susceptibility to falls and fractures (Girgis, Mokbel et al. 2014). Sarcopenia is a significant predictor of hospitalization in older individuals (Zhang, Zhang et al. 2018). Individuals with osteoporosis and sarcopenia have reduced quality of life, higher risk of falls, morbidity, and loss of autonomy. While both men and women can experience osteoporosis and sarcopenia, women undergo menopause and experience more severe muscle and a bone mass loss than men(Tiidus, Lowe et al. 2013). Approximately 1-2 years before menopause, there is a decrease in ovarian production of estrogen and this production ceases during menopause. Differences in severity of osteoporosis and sarcopenia between men and women is associated with this discrepancy in estrogen levels (Armas and Recker 2012).

Disruption of Bone Homeostasis and the Mechanisms of Osteoporosis

The mechanism by which osteoporosis leads to loss of bone mass is unclear. There are many studies which propose different mechanisms for osteoporosis, and we no longer use a single paradigm to explain this musculoskeletal disease. Some evidence suggests that estrogen promotes apoptosis of osteoclasts, so when menopause occurs, osteoclasts are no longer inhibited and increased resorption occurs (Hughes, Dai et al. 1996). Also, estrogen has an inhibitory effect on osteoclasts, but this mechanism has not been well investigated. One possible mechanism is estrogen deficiency during menopause leads to a proinflammatory state and drives T-cell activation. Activated T-cells can promote osteoclastogenesis, while inhibiting

osteoblasts (Armas and Recker 2012). The coupling of osteoblast and osteoclast activity in osteoporosis is disrupted, leading to disruption in bone homeostasis.

Some studies suggest that with age, there is an increase in remodeling that occurs and impaired osteoblastic bone formation, resulting in loss of bone mass (Marie and Kassem 2011). As previously mentioned, osteoporosis and sarcopenia are coupled, and both are a result of estrogen deficiency. Because the pathophysiology for osteoporosis is no longer explained by a single mechanism and is due to an imbalance between increased osteoclastogenesis and decreased osteoblastogenesis, we plan to focus on one aspect of the paradigm, osteoclastogenesis. As previously discussed, the inhibitory function of estrogen on osteoclasts is not well defined from a mechanistic standpoint. In the present study, we explore whether estrogen deficiency in muscle cells may serve as a mechanism for osteoporosis by producing an altered myokine profile leading to increased osteoclastogenesis. The relationship between the muscle-bone unit in the context of osteoporosis will be explored.

Review of the Literature

Biomolecular Communication Between Bone and Muscle

Although muscle and bone are anatomically related, the relationship between bone and muscle is more than a purely mechanical interaction. There is evidence to suggest that there is crosstalk, or biomolecular signaling, between muscle and bone which contributes to the maintenance and function of the muscle and bone unit (Reginster, Beaudart et al. 2016). Both skeletal muscle and bone are endocrine organs which can secrete factors to regulate the function of nearby tissues. Previous studies suggest that bone and muscle communicate by paracrine and endocrine

signaling and may reciprocally coordinate growth and response to injury (Tagliaferri, Wittrant et al. 2015). The paracrine relationship between muscle and bone suggests that cross talk occurs at the muscle fiber insertion site.

One example of the cross talk between bone and muscle is demonstrated by a murine model in which intramuscular (IM) botulinum toxin A in quadriceps was used to acquire hindlimb paralysis. Significant decrease in muscle mass and bone mass was demonstrated within 21 days. The bone volume to tissue volume ratio was reduced by 43-54%. This demonstrates that muscle function is important for retaining muscle mass as well as bone mass. Therefore, the crosstalk between muscle and bone is necessary for retaining bone volume (Warner, Sanford et al. 2006). Similarly, in patients with muscular dystrophies and spinal cord injury, skeletal muscle function and mass is lost, in addition to significant bone loss. Without muscle use, there is a decrease in bone mass and bone mineral density (Bianchi, Mazzanti et al. 2003).

Muscle promotes fracture healing

Another example of the paracrine signaling between the bone-muscle unit is demonstrated by a significantly faster healing rate when muscle flaps are surrounding compound fractures (Isaacson and Brotto 2014). A murine model with tibia fracture demonstrated enhanced bone repair and healing when muscle flaps were surrounding the fracture. Histomorphometry at 28 days demonstrated that fractures covered with muscle flaps had significantly more cortical bone in the wound site than fractures covered by fasciocutaneous tissue (Utvag, Iversen et al. 2002, Harry, Sandison et al. 2008). This accelerated healing is likely due to biomolecular signaling from muscle which can encourage fracture healing.

Additionally, when skeletal muscle has undergone trauma, there is impaired fracture healing. This reiterates that healthy skeletal muscle is imperative to the bone healing process. Intact and healthy skeletal muscle is critical for bone healing because of the many cytokines and growth factors secreted by skeletal muscle(Vogt, Boorboor et al. 2005). This is the proposed reason why muscle flaps encourage healing better than skin flaps. Although skin flaps demonstrate more vascularity than muscle flaps, intact muscle secretes more cytokines and growth factors, which accelerates fracture healing (Harry, Sandison et al. 2009).

Additional studies in rats using botulinum toxin A (BXTA) to impair muscle function suggest that biomolecular signaling from muscle is critical for bone fracture healing. Quadricep muscles were treated with botulinum toxin A, leading to muscle atrophy and lack of function. For quadriceps treated with botulinum toxin A, femur fractures demonstrated inferior healing outcomes compared to saline treated quadriceps. At 8 weeks, botulinum toxin A treated quadriceps lacked mature osseous calluses and woven bone in the fracture sites, in contrast, saline treated sites demonstrated woven bone formation. This finding suggests that mechanical load and muscle function is essential for fracture healing (Hao, Ma et al. 2012).

While the majority of fracture healing is through the work of periosteum derived stem cells (PDSCs), muscle derived stem cells also contribute to fracture healing (Shah, Majeed et al. 2013). Muscle stem cells contribute to fracture callus tissue by expressing chondrocyte and osteoblast markers (Liu, Birke et al. 2011). Muscle stimulates fracture healing by increasing revascularization through the supply of osteogenic growth factors and stem cells (Liu, Schindeler et al. 2010). While the exact molecular mechanism for this improved healing rate has not been determined,

bone and musculoskeletal wound studies have shown increased prostaglandin E2 (PGE_2) secretion by osteocytes in the presence of bone fracture. Osteocytes from fractures have demonstrated greater than 1000 times PGE_2 secretion compared to muscle. Increased PGE_2 in the presence of fracture suggests a reciprocal role in biochemical signaling between muscle and bone as increased PGE_2 secretion from bone also leads to improved skeletal muscle myogenesis (Mo, Romero-Suarez et al. 2012, Isaacson and Brotto 2014).

Evidence for Bone-Muscle Crosstalk during Development

Even prior to birth, there is crosstalk occurring between muscle and bone. In developing mouse embryos, muscle force was determined to be responsible for the circumferential contour of bone. When mechanical load from muscle was absent, normal circumferential contour of bone failed to develop in utero (Sharir, Stern et al. 2011). This demonstrates that biomolecular signaling between muscle and bone is important for normal development.

From a developmental standpoint, bone and muscle have many coinciding signaling pathways. Muscle and bone development utilize similar signaling pathways which promote similar functions in both tissue types, such as GH (Growth hormone) and IGF-1 (Insulin growth factor-1). Throughout skeletal development in mice, growth hormone and IGF-1 promote differentiation of osteoblasts and myoblasts (DiGirolamo, Mukherjee et al. 2007, Mavalli, DiGirolamo et al. 2010, DiGirolamo, Kiel et al. 2013). The role of muscle in skeletogenesis has been demonstrated in several embryonic mouse models. Two "muscle-less limb" mouse models were created in $Pax3^{sp/sp}$ mice (mutants in the transcription factor Pax3, which is needed for migration of muscle stem cells into the limb buds) and $Myf5^{nlacZ/nlacZ}$:$myod^{-/-}$ double

mutants (mutant for myogenic determination genes). In the "muscle-less limb" embryonic mouse model, bone development was impaired leading to decreased bone length and reduced bone formation, as well and abnormal joint development. There was profound decrease in bone formation in scapula, humerus, ulna, and femur (Nowlan, Bourdon et al. 2010, Nowlan, Sharpe et al. 2010). This reinforces the importance of normal skeletal muscle in bone development during embryogenesis.

An essential signaling pathway in the development of bone and muscle is the Wnt pathway. In both tissues, the Wnt pathway demonstrates a similar function. Wnt signaling is essential for controlling the differentiation and function of osteoblasts (Regard, Zhong et al. 2012). Likewise, Wnt controls expression of myogenic regulatory factor (MRF), which is critical for the regulation of the myogenic lineage. The Wnt pathway promotes myogenic lineage progression, differentiation of satellite cells, and muscle fiber growth (von Maltzahn, Chang et al. 2012). During embryogenesis, a molecule secreted by bone, Indian hedgehog (Ihh), has been shown to play a critical role in skeletal muscle growth. Normally, the primary function of Ihh protein is to regulate bone growth and differentiation. $Ihh^{-/-}$ deficient embryos demonstrate reduced muscle mass compared to wild type. However, when chicken embryos were rescued by retroviral infection with Ihh, the muscle mass was restored (Bren-Mattison, Hausburg et al. 2011). Therefore, there is biomolecular signaling between bone and muscle which is important for the maintenance and function of each tissue type.

Muscle-Derived Factors Affecting Bone

Skeletal muscle can secrete cytokines, called myokines, which can exert autocrine, paracrine, and endocrine effects on tissues. Myokines allow muscle to

communicate with other tissues by working in a hormone-like fashion. One of the main myokines produced by muscle contraction is IL-6. When IL-6 is secreted by muscle, the classical pro-inflammatory pathway is not activated, compared to when IL-6 is secreted by macrophages, an inflammatory response is activated. The environment in which IL-6 is produced affects the overall function of the cytokine. Instead of a proinflammatory role, IL-6 produced by skeletal muscle is important for metabolism (Pedersen 2011). There are several myokines which affect bone including: IGF-1, IL-6, IL-15, IL-8, fibroblast-growth factor-2, myostatin, osteoglycin, transmembrane protein 19, and osteoactivin (Table 1) (Hamrick 2012, Cianferotti and Brandi 2014, Tagliaferri, Wittrant et al. 2015). For example, IL-8 secreted by muscle increases angiogenesis(Pedersen, Akerstrom et al. 2007). IL-15 secreted by muscle leads to increased mineralization of bone in mice (Quinn, Anderson et al. 2009).

Additionally, local growth factors secreted by muscle, such as IGF-1, FGF-2 can promote bone osteogenesis(Hamrick, McNeil et al. 2010). IGF-1 is secreted by muscle during contraction, while FGF-2 is generally released during stretching or lengthening contractions (Hamrick 2012). Also, muscle produces low molecular weight factors that are important for preventing glucocorticoid induced apoptosis in osteocytes (Jahn, Lara-Castillo et al. 2012). During exercise, skeletal muscle secretes a factor called β-aminoisobutyric acid (BAIBA) which prevents apoptosis in osteocytes by protecting against reactive oxygen species (ROS) (Kitase, Vallejo et al. 2018).

Myostatin (GDF-8) is expressed at high levels when muscle has undergone trauma. Myostatin secretion inhibits bone repair and inhibits chondrogenic differentiation of bone marrow derived stem cells. Previous studies have

demonstrated that injured muscle flaps do not promote fracture healing due to the increased expression of myostatin (Elkasrawy, Immel et al. 2012). Myostatin is a powerful negative regulator of muscle mass and bone mass. Interestingly, in myostatin knockout mouse models, there is significant muscle hypertrophy and increase in bone mineral density (Hamrick 2003, Montgomery, Pennington et al. 2005). This demonstrates the essential role of myostatin in controlling muscle mass and demonstrates how muscle mass influences bone mineral density. Therefore, future research targeting myostatins may be beneficial for treating musculoskeletal conditions.

Other factors secreted by muscle which affect the extracellular matrix remodeling in bone are: Secreted Protein Acidic and Rich in Cysteine (SPARC or Osteonectin), matrix metalloproteinase-2 (MMP-2), and Bone Morphogenic protein-1 (BMP-1). Additional research is needed to determine the mechanisms in which these factors affect bone. Evidence suggests that SPARC contributes to bone formation and mineralization, MMP-2 is involved in bone turnover, and BMP-1 may contribute to bone formation during trauma and heterotopic ossification (Jackson, Aragon et al. 2011, Hamrick 2012). Overall, there are several myokines produced by muscle which impact bone (Table 1).

Muscle-derived peptides	Factors that stimulate peptide secretion	Role(s) in bone metabolism
Growth factors		
IGF-1	Resistance exercise	Stimulates bone formation
FGF-2	Eccentric muscle contraction	Stimulates bone formation
GDF-8	Muscle damage, cachexia, atrophy	Suppresses chondrogenesis and fracture healing
Extracellular matrix molecules		
SPARC	Resistance exercise, muscle regeneration	Promotes bone mineralization
MMP-2	Resistance exercise and re-loading	Fracture callus remodeling, bone formation
BMP-1	Blast trauma to muscle	Cleaving of procollagen and possibly heterotopic ossification
Inflammatory cytokines		
IL-6	Physical activity and muscle contraction	Bone resorption and turnover
IL-7	Physical activity and muscle contraction	Bone resorption
IL-15	Resistance exercise	Increase bone mass, decrease adiposity

Table 1. Demonstrates myokines secreted by skeletal muscle with potential effects on bone. Adapted from:

Hamrick, M. W. 2012. 'The skeletal muscle secretome: an emerging player in muscle-bone crosstalk', *Bonekey Rep*, 1: 60.

Bone-Derived Factors Affecting Muscle

Although the majority of studies focus on skeletal muscle as the main component in the muscle-bone cross talk, bone also has the ability to secrete factors which can impact muscle. Bone acts as repository for calcium and calcium is imperative to the function of muscle (DiGirolamo, Kiel et al. 2013). An example of a factor secreted by bone, which demonstrates the bone-muscle cross talk, is osteocalcin. Osteocalcin is secreted by osteoblasts and binds to the *Gprc6a* receptor. In the presence of exercise or muscle contraction, there is increased production of

osteocalcin (Coiro, Volpi et al. 2012). Osteocalcin knockout mice and *Gprc6a* receptor knockout mice demonstrate significantly reduced muscle mass, which suggests that osteocalcin may be involved in maintaining muscle mass. Osteocalcin promotes muscle mass maintenance by supporting protein synthesis in myotubes. Additionally, a rescue experiment demonstrated that treatment using exogenous osteocalcin can increase muscle mass (Mera, Laue et al. 2016). Therefore, osteocalcin is a bone factor which is imperative for maintaining muscle mass. Other bone secreted factors are PGE_2 and Wnt3a, which are secreted by osteocytes in response to stress promotes myogenesis and muscle function (Mo, Romero-Suarez et al. 2012). Osteocytes also secrete FGF23 (Fibroblast growth factor 23). FGF23 has known effects on negative effects on cardiac muscle, but the exact function of FGF23 on skeletal muscle is unclear (Mirza, Larsson et al. 2009, Brotto and Bonewald 2015).

The Tie between Estrogen deficiency, Osteoporosis, and Sarcopenia

Both osteoporosis and sarcopenia are more severe in women than in men (Tiidus, Lowe et al. 2013). There is significant evidence that osteoporosis and sarcopenia are both associated with estrogen deficiency (Khosla, Oursler et al. 2012). It is unclear whether osteoporosis occurs first, or sarcopenia occurs first, but it is evident that osteoporosis and sarcopenia are often found in the same patient. One theory is that muscle declines first, leading to less skeletal loading, thus causing reduction in bone mass. Another theory is that as osteocytes age, there is decreased ability for osteocytes to produce the necessary factors required for muscle maintenance (Brotto and Bonewald 2015). Neither of these theories fully explain the mechanism behind estrogen deficiency, osteoporosis, and the relation to sarcopenia.

There is severe decrease in muscle mass and strength that women experience compared to men which is likely due to a decrease in estrogen levels after menopause (Tiidus, Lowe et al. 2013, Novotny, Warren et al. 2015). Menopause results in a decline of estradiol and progesterone production and generally occurs in women during the late 40s or early 50s (McKinlay, Bifano et al. 1985, Thomas, Renaud et al. 2001, Hansen 2018). The rate of muscle mass loss is accelerated in menopause, then slows down post menopause (Aloia, McGowan et al. 1991). Although there is a higher rate of protein synthesis post-menopause, than pre-menopause, there is still a net loss of muscle mass in post-menopause likely due to the increase in protein breakdown (Hansen 2018).

Additionally, several genes involved in catabolism, such as *Murf1* and *Foxo*$_3$, are overexpressed in the skeletal muscle of post-menopausal women, which supports the notion that the net negative muscle loss is due to increased protein breakdown (Raue, Slivka et al. 2007). Hormone replacement therapy is available to counteract some of the musculoskeletal consequences of menopause. Interestingly, women on hormone replacement therapy demonstrate significantly more muscle strength than women who were not on hormone replacement therapy (Taaffe, Sipila et al. 2005, Greising, Baltgalvis et al. 2009). Several studies also suggest that hormone replacement therapy encourages less muscle mass loss. A human transcriptome study discovered that there were transcription level changes in muscle protein metabolism during menopause and HRT appeared to reverse some of the transcriptional changes. Therefore, HRT can address some of the post-menopausal changes and prevent muscle mass loss (Pollanen, Ronkainen et al. 2007).

Estrogen Deficiency Affects Muscle Mass and Function

Estrogen is responsible for preservation of muscle mass. Estrogen is important for maintenance and function of satellite cells (muscle stem cells). In the absence of estrogen, satellite cell function is impaired (figure 1) (Collins, Laakkonen et al. 2019). Also, there is evidence that estrogen deficiency contributes to loss of muscle mass due to apoptosis in skeletal muscle (Collins, Mader et al. 2018). Estrogen is responsible for preventing skeletal muscle apoptosis by HSP (Heat shock proteins) modulation and mitochondria. There are several potential mechanisms in which estrogen deficiency can contribute to atrophy of muscle mass. Previous *in vitro* studies have demonstrated that estrogen treatment can protect murine muscle C2C12 myoblasts cells against hydrogen peroxide induced apoptosis by upregulating HSP27 (Heat shock protein 27) (Vasconsuelo, Milanesi et al. 2010).

Additionally, estrogen has been shown to affect muscle function. In a mouse model with ovarian senesce, estradiol treatment restored muscle function and contractile force, suggesting that estradiol has beneficial effects on muscle function (Greising, Carey et al. 2011). Estrogen is essential for the binding of calcium to troponin C as well as the interaction with the myosin heavy chain and actin in order to produce a force (figure 1) (Collins, Laakkonen et al. 2019). Due to the molecular interactions with myosin and actin requiring estrogen, muscle function may be impaired during estrogen deficiency. A murine study demonstrated that estradiol therapy in ovariectomized mice can restore binding of myosin to actin and repair muscle function (Moran, Nelson et al. 2007). Furthermore, the effects of estrogen on muscle were confirmed when estrogen receptor knockout mice in muscle demonstrated less muscle strength then wild type mice (Collins, Mader et al. 2018).

Overall, there is significant evidence to suggest that estrogen is responsible for supporting muscle maintenance, protection against muscle apoptosis, molecular interactions in muscle, and muscle strength.

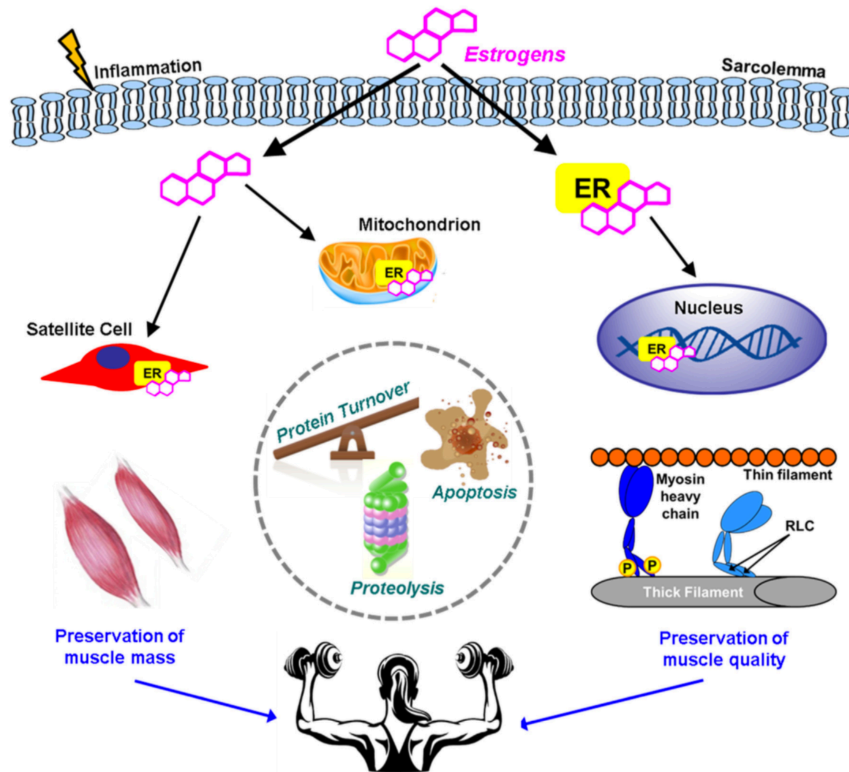

Figure 1. The role of estrogen in preservation of muscle mass, muscle quality, and maintenance of muscle cells. Estrogen is responsible for maintenance of satellite cells (muscle stem cells). Estrogen is also essential for the function of mitochondria, nuclei, and other organelles. Estrogen is needed for the binding of the myosin heavy chain to the actin thin filament in order to produce a force. Overall, estrogen is needed for preserving muscle mass and quality.

Adapted from: Collins, B. C., E. K. Laakkonen, and D. A. Lowe. 2019. 'Aging of the musculoskeletal system: How the loss of estrogen impacts muscle strength', *Bone*, 123: 137-44.

The dichotomy between osteoclasts and osteoblasts in osteoporosis

Osteoporosis is characterized by a loss of bone mass, decrease in bone strength and increased risk of fracture (WHO 1994). In osteoporosis, there is a disruption in the normal bone physiology, leading to this altered bone phenotype. In normal remodeling, bone is renewal occurs by osteoblasts and osteocytes signaling osteoclasts and allowing receptor activator nuclear factor kappa-B ligand (RANKL) to bind to RANK, which promotes osteoclastogenesis. Osteoclasts are then able to resorb small areas of bone, then mononuclear cells can then prepare the surface by depositing a thin matrix, and osteoblasts can then lay down bone collagen matrix. This matrix then undergoes mineralization and some osteoblasts are trapped within the matrix, forming a network of osteocytes (Armas and Recker 2012). In osteoporosis, the balance between building bone from osteoblasts and resorbing bone from osteoclasts is disrupted, leading to a disruption in bone homeostasis. However, it is unclear whether estrogen deficiency results in increased osteoclastogenesis or decreased osteoblastogenesis to cause the loss in bone mass seen in osteoporosis. The present study focuses on estrogen signaling in muscle and its effects on the crosstalk between the muscle-bone unit and osteoclastogenesis.

Osteoclastogenesis Pathway

Osteoclasts undergo 3 phases in order to reach maturation. These phases include proliferation, fusion, and activation. M-CSF (macrophage colony stimulating factor) and RANKL are essential for stimulating osteoclastogenesis. Exposure of the osteoclast precursor to M-CSF encourages the precursor to move to the monocyte/macrophage lineage and promotes expression of the transcription factor pu.1. RANKL exposure leads to commitment to the osteoclast lineage(Boyce 2013).

Expression of early transcription factors such as micropthalmia-associated transcription factor (mitf), cellular oncogene Fos (C-Fos), and nuclear factor kappa light chain enhancer of activated B cells (NF-kß) then allow for activation of nuclear factor of activated T- cells cytoplasmic 1 (Nfatc1), which is the major regulator of osteoclast differentiation genes (Yavropoulou and Yovos 2008, Amarasekara, Yun et al. 2018).

Nfatc1 is essential for osteoclastogenesis and activates genes such as *cathepsin K*, *dendritic cell specific transmembrane protein* (*Dc-stamp*), *osteoclast associated receptor* (*OSCAR*), and *matrix metallopeptidase-9* (*MMP-9*). Nfatc1 can also undergo auto-amplification through calcium signals. OSCAR is responsible for amplifying signals for osteoclast differentiation, but requires RANKL in order to stimulate osteoclastogenesis (Zhao, Wang et al. 2010). *Dc-stamp* promotes fusion of osteoclasts and transformation from an immature osteoclast to a mature osteoclast(Chiu and Ritchlin 2016). A mature osteoclast is formed and cathepsin k, MMP-9, along with other degradation enzymes, promote bone resorption within the Howship's lacunae (figure 2, figure 3) (Choi, Park et al. 2016).

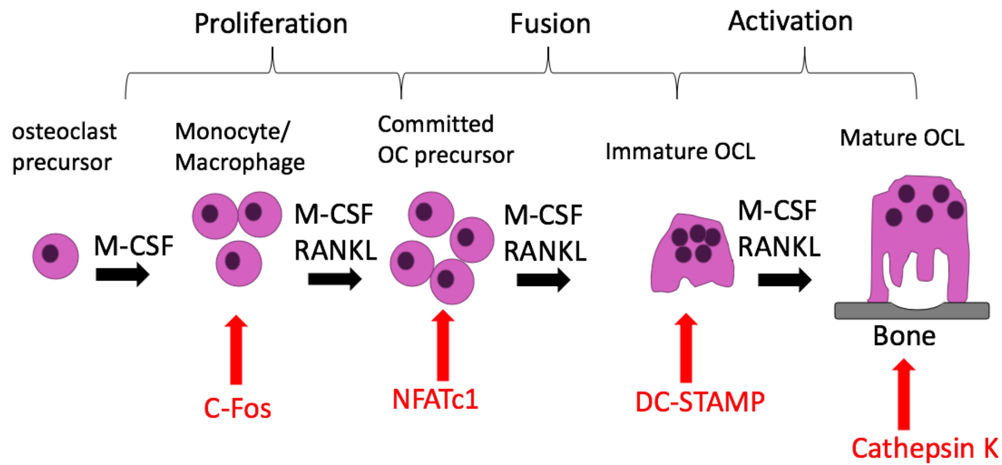

Figure 2. Transcriptional regulation in Osteoclasts. Osteoclasts undergo 3 main phases in order to reach maturation: proliferation, fusion, and activation. M-CSF encourages the osteoclast precursor to move to the monocyte/macrophage lineage and encourages expression of the transcription factor pu.1. RANKL exposure leads to commitment to the osteoclast lineage. Expression of early transcription factors such as mitf, C-Fos, and NF-kß then allows for activation of Nfatc1, which is the major regulator of osteoclast differentiation genes. Dc-stamp is activated and promotes fusion of osteoclasts and transformation from an immature osteoclast to a mature osteoclast. A mature osteoclast is formed and cathepsin k, along with other degradation enzymes, promote bone resorption within the Howship's lacunae.

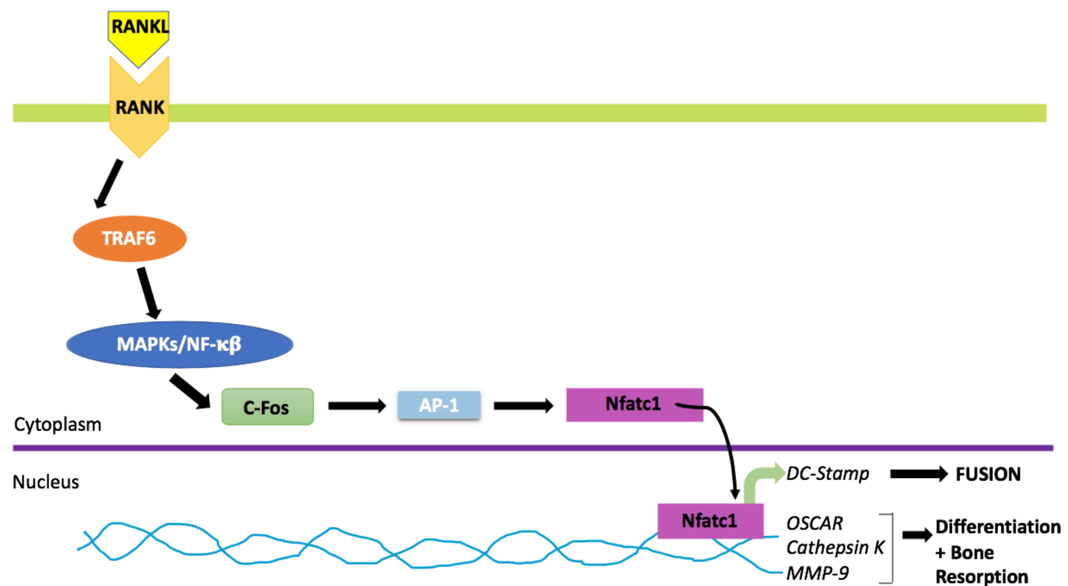

Figure 3. Pathway for osteoclast differentiation. Extracellular signaling of RANKL to RANK leads to intracellular activation of TRAF6, MAP kinases and NF-kß. C-Fos is an early transcription factor for osteoclastogenesis and allows for activation of AP-1 and Nfatc1. Nfatc1 transcription factor works in the nucleus and allows for activation of downstream osteoclast differentiations genes such as *Dc-stamp*, *OSCAR*, *cathepsin k*, and *MMP-9*.

Adapted from: Choi, B. Y., C. H. Park, Y. H. Na, H. W. Bai, J. Y. Cho and B. Y. Chung (2016). "Inhibition of RANKL-induced osteoclast differentiation through the downregulation of c-Fos and NFATc1 by Eremochloa ophiuroides (centipedegrass) extract." Mol Med Rep **13**(5): 4014-4022.

Previous Work on Muscle Specific Estrogen Deficiency and the Impact on Bone

Previous work in the Mansky/Lowe lab has demonstrated that in the absence of estrogen, there is differential expression of bone genes in muscle (Le, Novotny et al. 2018). This suggests that estradiol response in muscle may play a role in regulating genes responsible for bone maintenance. Studies on skeletal muscle in

estrogen deficient mice has demonstrated differential regulation of several cytokines. Cytokines which were found to have decreased gene expression in response to estradiol were IFN-γ, IL-15 and IL-17b, CXCL9, CXCL10, CCL5 (RANTES), CCL22, and CCL25. In contrast, CCL6 and CCL9 were upregulated in muscle in response to estradiol (Table 2) (Le, Novotny et al. 2018).

Bone genes downregulated in muscle in response to estradiol	Bone genes upregulated in muscle in response to estradiol
Interferon γ	CCL6
Interleukin 15 and 17b	CCL9 (MIP-1γ)
CXCL9 and 10	
CCL5 (RANTES)	
CCL22 and 25	

Table 2. Published data from Lowe Lab demonstrating expression of genes in muscle in response to estradiol (Le, Novotny et al. 2018). Genes included above are bone genes which demonstrate differential expression in response to estradiol.

Preliminary experiments in the Mansky lab used skmERαKO (ERαKO) mice to study the effect of muscle specific estrogen receptor α (ERα) deficiency specific on bone. To develop a mouse with *Cre* expressed in skeletal muscle, the human α-skeletal actin (HSA) promoter gene was used to express *Cre* in the HSA-*Cre* mice. The human α-skeletal actin promoter demonstrates expression restricted to skeletal muscle with limited expression on cardiac muscle (Miniou, Tiziano et al. 1999) (figure 4). In the developing embryo at 12.5 days post coitum, the specificity of HSA-*Cre* to only striated muscle is shown. This specificity is apparent, as the sternum, liver, and spinal

cord are not stained with ß-galactosidase in a mouse expressing HSA-*Cre* and the LacZ reporter gene (figure 5).

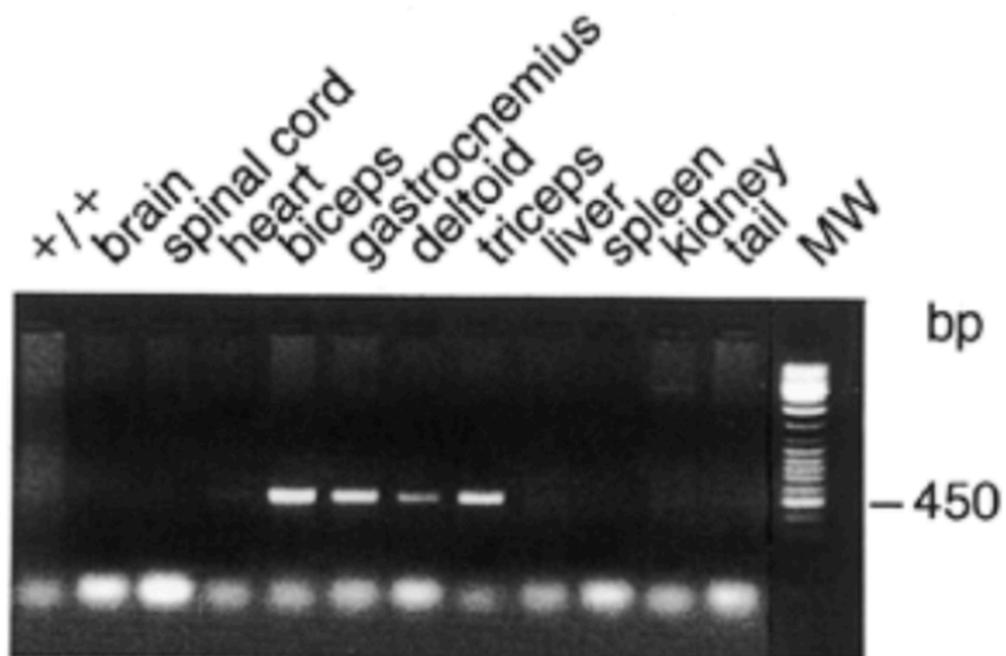

Figure 4. PCR analysis for HSA-*Cre* Mice. PCR demonstrates excision of the LoxP test gene segment by HSA-*Cre* in several tissues including brain, spinal cord, liver, spleen, kidney, tail. HSA-*Cre* is specific to skeletal muscle (biceps, gastrocnemius, deltoid, triceps) with minimal expression in cardiomyocytes.
Adapted from: Miniou, P., et al. (1999). "Gene targeting restricted to mouse striated muscle lineage." Nucleic Acids Res **27**(19): e27

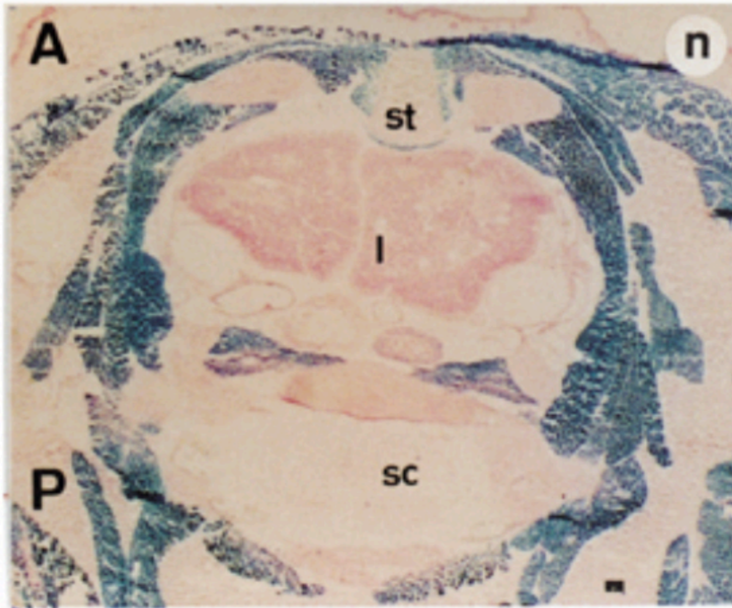

Figure 5. ß-galactosidase staining on an embryo expressing HSA-*Cre* and the LacZ reporter gene. Embryo is 12.5 days post coitum. Selective staining on myotomal muscles is demonstrated and all intercostal muscles are stained. “A” is anterior, “P” is posterior. “st” is the sternum. “l” is liver. “sc” is spinal cord. Adapted from: Miniou, P., et al. (1999). "Gene targeting restricted to mouse striated muscle lineage." Nucleic Acids Res **27**(19): e27.

In order to develop transgenic mice with ERα deficiency specific to skeletal muscle, homozygote *Esr1* (ERa) floxed females (Jackson Laboratories, Bar Harbor, ME, strain #032173) were crossed with male *HSA-cre* mice (Jackson Laboratories, Bar Harbor, ME, strain #006149). *HSA-cre* positive heterozygote *Esr1* floxed males were mated with homozygote *Esr1* floxed females to create skmERα KO mice (figure 7). skmERαKO(ERαKO) cre-lox recombinant mice have ERα deficiency specific to skeletal muscle. skmERαKO mice were used in preliminary experiments and final experiments in the present study.

The Mansky lab performed a preliminary study in which trabecular bone volume and perimeter of bone in WT and skmERαKO were measured by microCT. Although microCT was performed on only a small sample size, there was a trend toward more trabecular bone volume/tissue volume in the skmERαKO group than the WT group, suggesting that osteoporotic bone is present in skmERαKO. Additionally, skmERαKO tibias demonstrated a smaller perimeter of bone than WT mice. This increase in osteoporotic bone in skmERαKO may be due to estrogen deficiency in muscle affecting the crosstalk between muscle and bone, thus impacting the function of either osteoblasts and/or osteoclasts.

In another preliminary experiment, osteoclasts isolated from skmERαKO were smaller and demonstrated reduced demineralization of calcium phosphate by cells in skmERαKO compared to WT (figure 6). This preliminary work suggests that estrogen deficiency may contribute to reduced osteoclast activity and size. However, these findings were from a small sample size, so additional studies would need to be performed to confirm these findings. Additionally, osteoblast activity in skmERαKO and WT mice has not been determined and will require future experiments.

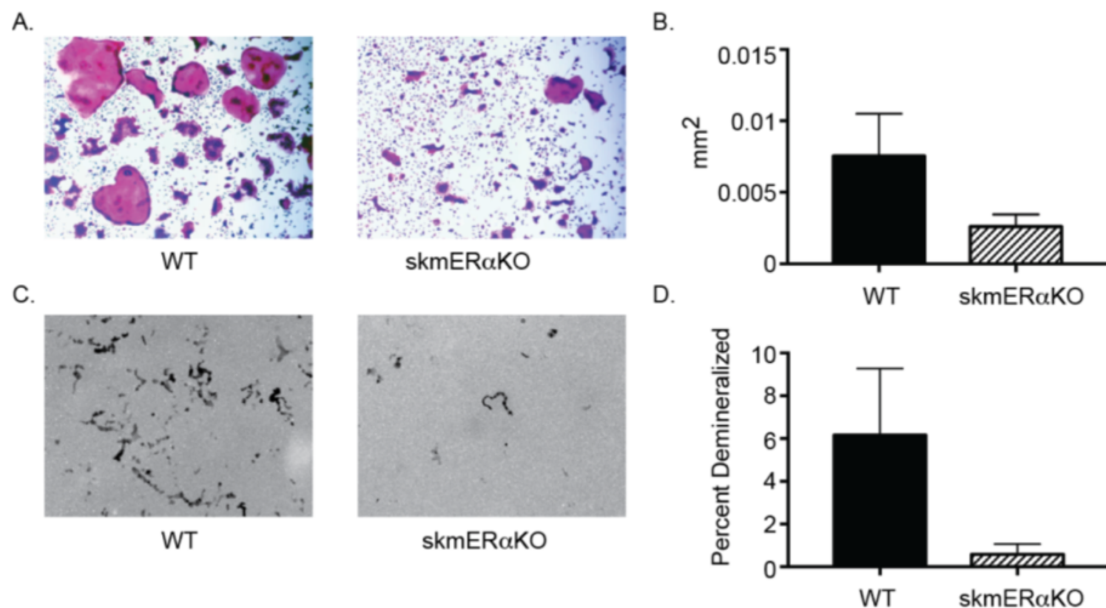

Figure 6. Unpublished preliminary data from the Mansky lab on ERαKO osteoclasts compared to WT. In the absence of estrogen signaling in muscle, osteoclasts are smaller in size and have decreased activity. A) represents TRAP staining of osteoclasts. B) represents the size of TRAP positive multinuclear osteoclasts. C) Representative images of demineralization D) percent demineralization activity of WT and skmERαKO.

Overall, these preliminary experiments suggest that estrogen deficiency leads to differential expression of bone genes by muscle. There is some unpublished preliminary data from the Mansky lab which suggests that estrogen specific deficiency in muscle may affect bone microarchitecture and size as demonstrated by microCT. Impaired osteoclast function in estrogen specific muscle deficiency has been demonstrated in a limited sample size during these preliminary experiments, so the present study will aim to increase the power of these findings. Also, the effect on osteoblasts has not been measured, however, the present study will continue to focus on osteoclasts in order to develop a better understanding of how estrogen deficiency in muscle may impact bone.

Rationale and Purpose of the Project

Although it is known that estrogen plays a role in muscle maintenance and function, it is not well understood how estrogen deficiency can impact the cross talk between bone and muscle. Because osteoporosis and sarcopenia are associated, estrogen deficiency may be responsible for altered function and myokine secretion by muscle cells, which detrimentally affects bone. Given the number of musculoskeletal diseases, it is important to understand the crosstalk between muscle bone and how estrogen deficiency can alter the molecular signaling between these tissues.

Currently, it is unclear how muscle specific estrogen deficiency impacts bone. Understanding how estrogen deficiency impacts this crosstalk may lead to a clearer understanding of how musculoskeletal diseases form. With our research, we want to test how loss of estrogen function in muscle cells, impacts the function of osteoclasts.

Overall, we hypothesize that estrogen regulates myokine expression from muscle cells and inhibits osteoclast differentiation. In estrogen deficiency, there may be differential release of myokines by muscle cells, leading to increased osteoclastogenesis. A skeletal muscle specific estrogen receptor α knockout (ERαKO) mouse model will be used to determine how osteoclast function is affected by the differential myokine production in estrogen deficiency.

With our *first specific aim*, we plan to determine effect of muscle specific ERα deficiency during development on osteoclast activity. We hypothesize that muscle specific estrogen signaling is responsible for normal muscle mass and development. This impairment in muscle will result in impaired muscle-bone cross talk, thus leading to increased osteoclast activity and size. To address this specific aim, bone marrow cells acquired directly from ERαKO and WT mice will be cultured

and analyzed to determine osteoclast activity. This will allow us to determine if muscle specific estrogen deficiency during development affects the osteoclasts.

For the *second specific aim*, we plan to determine the effects of estrogen dependent myokines on osteoclast activity. We hypothesize that estrogen-dependent myokines inhibit osteoclast activity. To address this specific aim, isolated living muscle from ERαKO and WT mice will be subjected to a series of contractions in order to produce myokines. WT osteoclasts will be cultured with conditioned media containing myokines from either ERαKO or WT. Expression of osteoclast differentiation genes will be measured.

With our *third specific aim*, we plan to determine the effects of skeletal muscle specific ERα deficiency on myokine production. We hypothesize that estrogen influences the myokine profile produced by skeletal muscle. To address the third specific aim, muscle from ERαKO and WT mice will be isolated and homogenized with zirconium oxide beads. RNA will be isolated, and cDNA will be made for ERαKO and WT mice. A PCR array will be used in order to evaluate the myokine profile of ERαKO and WT mice. PCR array will allow us to determine which genes are upregulated or downregulated in the presence or absence of estrogen. Overall, with these 3 specific aims, we plan to obtain a clearer understanding of how ERα deficiency in muscle may impact the crosstalk between the muscle-bone unit and affect osteoclasts.

Materials and methods:

Generation of Skeletal Muscle Specific ERα-knockout Mice.

Homozygote *Esr1* (ERa) floxed females (Jackson Laboratories, Bar Harbor, ME - strain #032173) were crossed with male *HSA-cre* mice (Jackson Laboratories, Bar Harbor, ME - strain #006149). *HSA-cre* positive heterozygote *Esr1* floxed males were mated with homozygote *Esr1* floxed females to create skmERα KO mice. skmERαKO (ERαKO) mice are homozygote *Esr1* floxed mice which are positive for *HSA-cre* (figure 7). ERαKO mice are lacking ERα specific to skeletal muscle. 6-month-old female mice were used in the experiments. Wild type (WT) mice are homozygote ERα floxed mice which are negative for *HSA-cre*, therefore, ERα is still expressed.

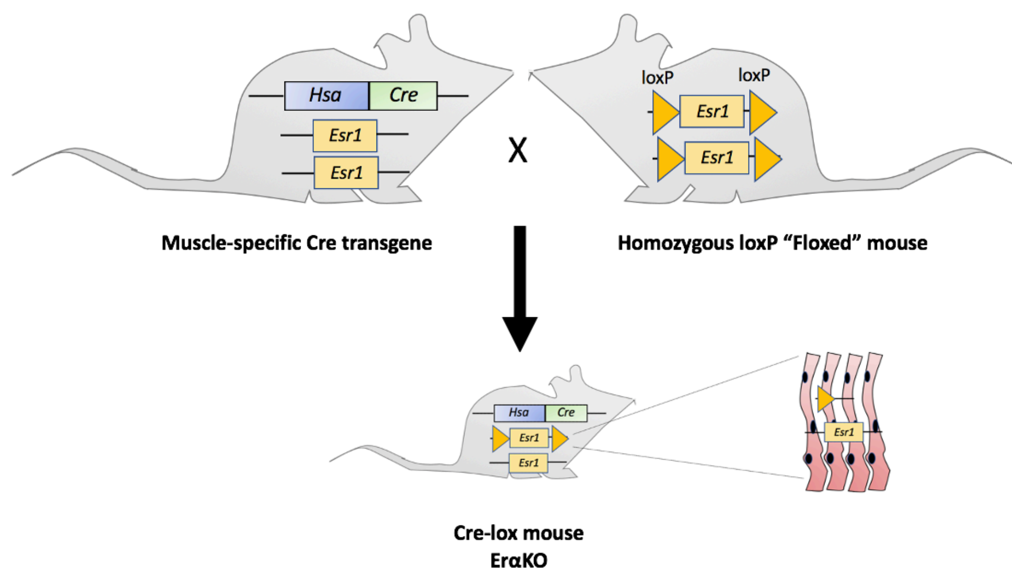

Figure 7. Generation of skeletal muscle specific ERαKO using a cre-lox system. ERαKO mice are generated by mating male *HSA-cre* mice with Homozygote *Esr1* (ERα) floxed females. ERαKO mice are homozygote *Esr1* floxed mice which are positive for *HSA-cre*. ERα deficiency is specific to skeletal muscle.

Experiment 1: Osteoclast TRAP, resorption, and gene expression from ERαKO and WT mice

Osteoclast gene expression of osteoclasts isolated from ERαKO and WT mice

Femurs and tibias were harvested from 17 ERαKO and 7 WT mice. Bone marrow cells were flushed from femurs and tibias using α-Minimum essential media (αMEM) with 5% FBS, penicillin-streptomycin, and L-glutamine (OCL Media). Red blood cells were removed via lysis from flushed bone marrow cells. Cells remaining were grown in OCL media with CMG 14-12 supernatant (Dr. Sunao Takeshita, Nagoya City University, Nagoya, Japan) containing M-CSF at 37°C overnight. Non-adherent cells were removed, and cells were counted using a hemocytometer. Cells were split into 24 well plates and cultured in OCL media with CMG 14-12 for 48 hours. Cells were fed every other day with OCL media with CMG 14-12 and 10ng of RANKL. At day 4, cells were harvested for RNA or fixed for TRAP staining or resorption assay. Sequence for experiment 1 is demonstrated in figure 8.

RNA isolation for Real Time qPCR of Osteoclast differentiation genes

Day 4 osteoclasts were harvested in TRIzol reagent (Life Technologies, Carlsbad, California). RNA was isolated from TRIzol samples according to manufacturer's instructions. Isolated RNA was resuspended in 20ul of DEPC H_2O. Samples were analyzed by spectrophotometry in order to determine concentration and purity. cDNA was made using iScript reaction mix (Bio-Rad, Hercules, California) according to manufacturer's instructions. Relative expression of osteoclast differentiation genes was determined by qRT-PCR. SYBR green master mix (Bio-Rad, Hercules,

California) was used with primers for *Nfatc1*, *Dc-stamp*, *C-Fos*, *Cathepsin K*, and *Hprt* in order to set up qRT-PCR (see Table 2). Samples were run on Bio-Rad CFX Connect for one 3 minute cycle at 95° C, then 40 cycles alternating between 15 second at 95° C, 30 seconds at 58° C, and 30 seconds at 72° C.

Osteoclast TRAP Staining, Resorption, and Imaging

Osteoclast differentiation and activity was quantified by TRAP staining. Osteoclast demineralization was quantitated using Osteo-Assay plates (Corning, Corning, NY) for both WT and ERαKO groups. Osteo-Assay plates contained a synthetic inorganic crystal calcium phosphate surface which mimics bone. The Osteo-Assay plates are used to measure resorption pits from osteoclasts. Cells were plated into 24-well plates. Cells were TRAP stained at day 2 and day 4. Osteoclasts were counted under a bright field microscope at 4x magnification. Average size and number of TRAP positive cells was determined for each TRAP plate using FUJI/ImageJ. Number of demineralization areas and size of resorption pits from Osteo Assay plates were counted at day 4 using FUJI/ImageJ.

Statistical Analysis

Relative expression of *Nfatc1*, *Dc-stamp*, *C-Fos*, *Cathepsin K* to *Hprt* was calculated using Cq values. Normalization tests were performed on Prism and data was determined to be non-parametric. Unpaired T tests were performed to compare WT and ERαKO mice for relative expression of each gene. Non-parametric paired T tests were used to compare the same group at day 2 and day 4. Results from TRAP staining and resorption assay were plotted on Prism. Normalization tests were performed on Prism and TRAP staining and resorption data was determined to be non-parametric.

Non-parametric unpaired T tests were performed to compare WT and ERαKO mice TRAP and resorption quantities. Non-parametric paired T tests were used to compare the same group at day 2 and day 4.

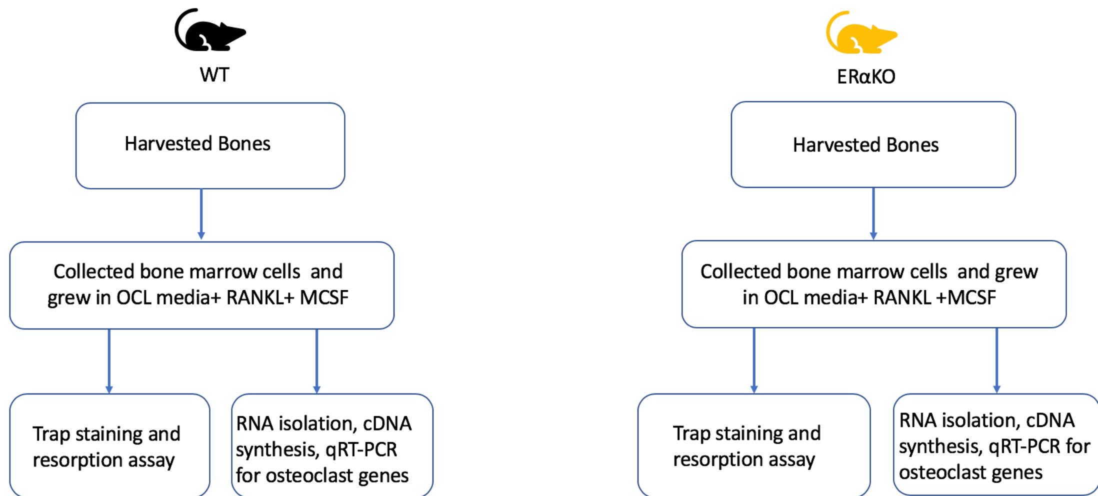

Figure 8. Flow chart for Experiment 1: Osteoclast TRAP, resorption, and gene expression from ERαKO and WT mice.

Table 3. Osteoclast gene primers used for qRT PCR.

Gene Primer	5' to 3' sequence Forward Primer	5' to 3' sequence Reverse Primer
Cathepsin K	AGG GAA GCA AGC ACT GGA TA	GCT GGC TGG AAT CAC ATC TT
C-Fos	CGA AGC GGA GAC AGA TCA ACT T	TCC AGT TTT TCC TTC TCT TTC AGC AGA
DC-STAMP	GGG CAC CAG TAT TTT CCT GA	TGG CAG GAT CCA GTA AAA GG
Nfatc1	TCA TCC TGT CCA ACA CCA AA	TCA CCC TGG TGT TCT TCC TC
HPRT	GAG GAG TCC TGT TGA TGT TGC CAG	GGC TGG CCT ATA GGC TCA TAG TGC

Experiment 2: Osteoclast conditioned media experiments

Isotonic contractions and myokine secretion

Extensor digitorum longus muscles from four groups of female mice were dissected and placed in an *in vitro* bath containing krebs media (figure 9). Krebs media contains 1.8 g/L D-Glucose, 0.0468 g/L magnesium chloride [Anhydrous], 0.34 g/L potassium chloride served, 7.0 g/L sodium chloride, 0.1g/L sodium phosphate dibasic [Anhydrous], and 0.18g/L sodium phosphate monobasic [Anhydrous].The two groups of female mice were 1) ERαKO and 2) Wild type. Live extensor digitorum longus muscle underwent controlled contraction experiments as previously described (Collins, Mader et al. 2018). Myokines secreted from the live muscle contractions were collected in the *in vitro* bath.

Conditioned media experiments with osteoclasts

2.5% conditioned media from the muscle from WT and ERαKO was used to treat bone marrow macrophages. Based on unpublished preliminary experiments, we determined that 2.5% conditioned media compared to 5% and 10% provided a pronounced response in WT osteoclast gene expression. Bone marrow cells were flushed from femur and tibia of WT mice using OCL media. Cells were grown in OCL media with CMG 14-12 supernatant (Dr. Sunao Takeshita, Nagoya City University, Nagoya, Japan) containing M-CSF for 1 day. Then non-adherent cells were removed and re-plated in duplicate. Cells were grown in OCL media and CMG14-12 for 2 more days at 37° C. Osteoclasts were then grown in OCL media, CMG14-12, and RANKL with either 2.5% conditioned media, Krebs media for four

days. On day 4, cells were harvested as previously described for qRT-PCR. Sequence for experiment 2 is demonstrated in figure 10.

Osteoclast differentiation in the presence of conditioned media

RNA was isolated from osteoclasts treated with conditioned media using TRIzol reagent. cDNA was created using iScript (Bio-Rad, Hercules, California). Relative expression of osteoclast differentiation genes was determined by quantitative Real Time PCR. SYBR green (Bio-Rad, Hercules, California) master mix was used with primers for *Nfatc1*, *Dc-stamp*, *C-Fos*, *Cathepsin K*, and *Hprt* (See table 2). Samples were run on Bio-Rad CFX Connect for one 3 minute cycle at 95° C, then 40 cycles alternating between 15 second at 95° C, 30 seconds at 58° C, and 30 seconds at 72° C.

Statistical Analysis

Relative expression of *Nfatc1*, *Dc-stamp*, *C-Fos*, *Cathepsin K* to *Hprt* was calculated using Cq values. Osteoclast genes were normalized to *HPRT* using comparative analysis, then fold change relative to Krebs was calculated. Krebs media served as the negative control. Shapiro-Wilk normalization tests were performed on Prism, and data was determined to be non-parametric. ANOVA was used to compare WT, ERαKO, and krebs for *Nfatc1*, *Dc-stamp*, *C-Fos*, *Cathepsin K.* Welche's T test was performed to compare WT and ERαKO mice for *C-Fos* only, as there was no difference for *Nfatc1*, *Dc-stamp, and cathepsin k* with ANOVA.

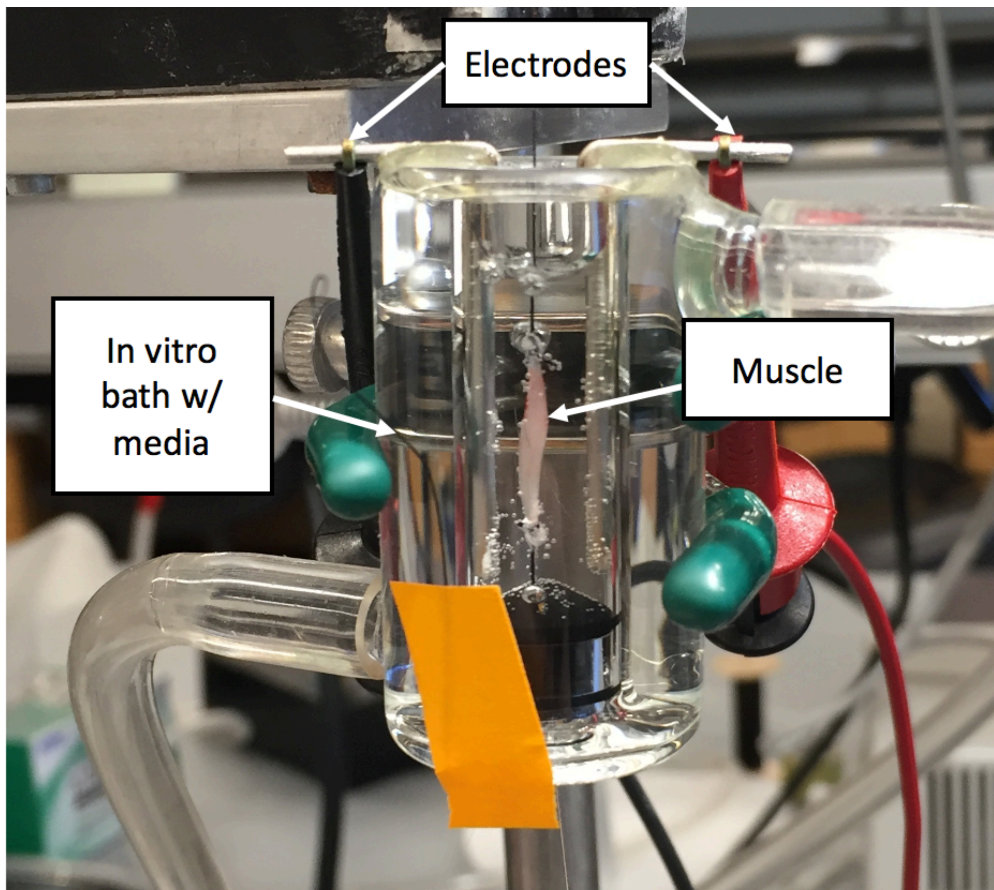

Figure 9. In vitro bath for collection of myokines. Extensor digitorum longus muscle was connected to electrodes in order to stimulate muscle contraction and production of myokines. Secreted myokines were collected in the in vitro bath containing Krebs media.

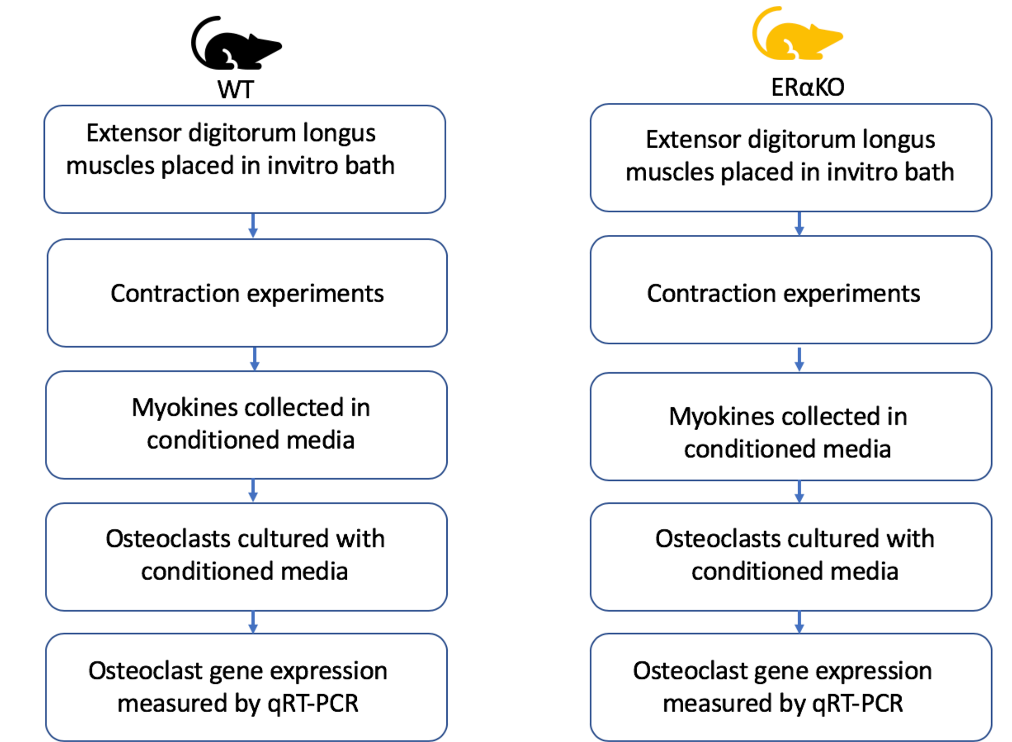

Figure 10. Flow chart for Experiment 2: Osteoclast conditioned media experiments.

Experiment 3: Identification of myokines using RT^2 Profiler PCR Array

Isolation of muscle, homogenization, mRNA isolation, and cDNA synthesis.

Tibialis anterior muscles from ERαKO and WT mice were isolated. Tibialis anterior muscle was placed in a safe lock 1.5 ml Eppendorf tube with 1.0mm and 2.0mm Zirconium oxide beads and 300 ul of TRIzol. Tibialis anterior muscle was homogenized in the Bullet Blender at speed 12 for 5 minutes. RNA isolation was performed using TRIzol per manufacturer's instructions. Samples were reconstituted in 20 ul of DEPC water. Following RNA isolation, samples were measured in the spectrophotometer for purity and concentration. The iScript cDNA synthesis kit was used to create cDNA.

RT^2 Profiler PCR array

Myokines of the WT and ERαKO groups were identified by the RT^2 Profiler PCR Array—Mouse Cytokines and Chemokines (Qiagen Cat. no. 330231 PAMM-150ZA). The RT^2 Profiler PCR array measures the expression of 84 chemokines and cytokines. The PCR plate was laid out as shown (Figure 12). A master mix containing 1350 ul SYBR Green, 102ul cDNA (5100 ng), and 1248 RNase-free water was made. 25ul of the master mix was placed into each well of the 96 well RT^2 Profile array. The prepared array was placed in the BioRad CFX Connect. Samples were run for one 10minute cycle at 95° C, then 40 cycles alternating between 15 seconds at 95° C and 1 minute at 60° C. ERαKO and WT data was analyzed on Gene Globe. Housekeeping mRNA expression of genes *ß-actin* (*Actb*), *ß-2 microglobulin* (*B2m*), *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*), *ß*-glucuronidase (*Gusb*), and *heat shock protein 90 alpha class B member 1*(*Hsp90ab1*) were analyzed. Sequence for experiment 3 is

demonstrated in figure 11. Analysis was performed using Gene Globe and all test genes were compared to *B2m* and *Hsp90ab1*.

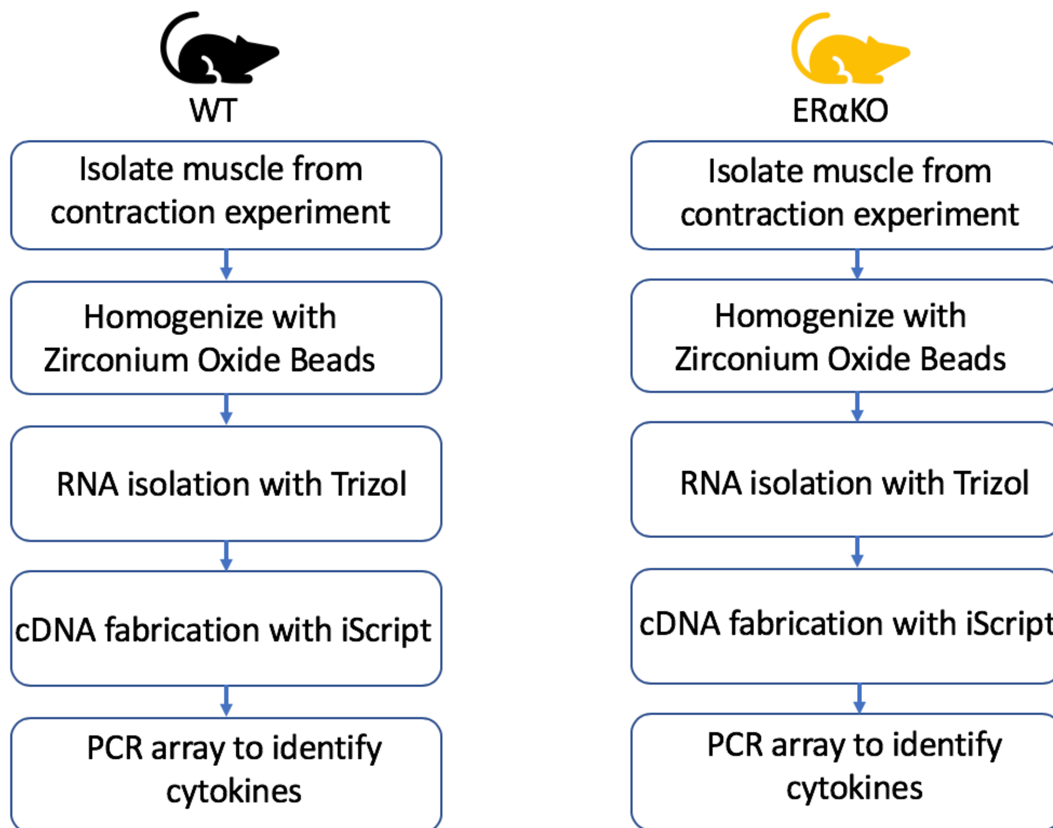

Figure 11. Flow chart for Experiment 3: Identification of myokines using RT² Profiler PCR Array.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Adipoq	Bmp2	Bmp4	Bmp6	Bmp7	Ccl1	Ccl11	Ccl12	Ccl17	Ccl19	Ccl2	Ccl20
B	Ccl22	Ccl24	Ccl3	Ccl4	Ccl5	Ccl7	Cd40lg	Cd70	Cntf	Csf1	Csf2	Csf3
C	Ctf1	Cx3cl1	Cxcl1	Cxcl10	Cxcl11	Cxcl12	Cxcl13	Cxcl16	Cxcl3	Cxcl5	Cxcl9	Fasl
D	Gpi1	Hc	Ifna2	Ifng	Il10	Il11	Il12a	Il12b	Il13	Il15	Il16	Il17a
E	Il17f	Il18	Il1a	Il1b	Il1rn	Il2	Il21	Il22	Il23a	Il24	Il27	Il3
F	Il4	Il5	Il6	Il7	Il9	Lif	Lta	Ltb	Mif	Mstn	Nodal	Osm
G	Pf4	Ppbp	Spp1	Tgfb2	Thpo	Tnf	Tnfrsf11b	Tnfsf10	Tnfsf11	Tnfsf13b	Vegfa	Xcl1
H	Actb	B2m	Gapdh	Gusb	Hsp90ab1	MGDC	RTC	RTC	RTC	PPC	PPC	PPC

Figure 12. RT² Profiler PCR array layout

Results

Osteoclasts from ERαKO and WT demonstrate no difference in cell count, size, and resorption

As previously mentioned, postmenopausal women demonstrate both sarcopenia and osteoporosis. Osteoporosis and sarcopenia have been shown to be due to loss of estrogen. Estrogen is essential for muscle function and retaining muscle mass (Collins, Laakkonen et al. 2019). Although it is known that estrogen deficiency impairs muscle function and activity, it is unclear whether loss of bone may be due to changes in cytokines released by muscle. To measure the effect of muscle specific ERα deficiency on osteoclasts, bone marrow macrophages (BMMs) from ERαKO and WT mice were cultured in M-CSF and RANKL to stimulate osteoclast differentiation. We evaluated osteoclast gene expression, size, number, and resorption activity at both day 2 and day 4 after RANKL treatment. We measured no difference in average cell count between the osteoclasts from ERαKO and WT mice at day 2 and day 4 (figure 13a). Compared to day 2, the average cell count at day 4 is significantly lower both the ERαKO and WT ($p<0.0001$and $p=0.0016$, respectively) (figure 13a,14). At day 4, osteoclasts are significantly larger in size than at day 2 in both ERαKO and WT groups ($p<0.0001$ and $p= 0.0132$, respectively) (figure 13b, 14). However, there is no difference in average size of osteoclasts when comparing ERαKO and WT mice (Figure 13b, 14). Osteo assays to measure resorption revealed no significant difference in number of pits and size of demineralized area from ERαKO and WT cells at day 4 (figure 13c, d).

Osteoclasts from ERαKO and WT demonstrate no difference in C-Fos, Nfatc1, Dc-stamp, and Cathepsin k

To determine if osteoclast gene expression was changed in osteoclasts from ERαKO mice, qRT-PCR was done. All genes of interest were compared to the housekeeping gene, *HPRT*. Stability of HPRT as a reference gene was verified. HPRT demonstrated minimal deviation and was deemed as appropriate for use as a reference gene (figure 16). Osteoclasts from ERαKO and WT mice demonstrate no difference in *C-Fos* expression (figure 15a). There was a significant decrease in *C-Fos* relative expression when comparing day 2 ERαKO to day 4 ERαKO ($p=0.0017$). *C-Fos* relative expression remains unchanged from day 2 to day 4 in WT.

There was a trend toward increased *Nfatc1* relative expression in ERαKO compared to WT at day 2, however, this was not statistically significant ($p=0.1107$) (figure 15b). There was no difference between *Nfatc1* expression in ERαKO and WT mice at Day 4. There was a significant decrease in *Nfatc1* expression at day 4 compared to day 2 in the ERαKO group ($p<0.0001$). In contrast, WT demonstrate no change in *Nfatc1* relative expression to *HPRT* from day 2 to day 4 (figure 15b).

There is no difference in expression of *Dc-stamp* between the osteoclasts of ERαKO and WT mice (figure 15c). WT demonstrates significant increase in relative expression of *Dc-stamp* from day 2 to day 4 ($p=0.0112$). ERαKO demonstrates a similar trend in *Dc-stamp* relative expression, but this was not significant ($p=0.1771$) (figure 15c). Also, there was no difference in *Cathepsin k* expression in ERαKO and WT osteoclasts at day 2 and day 4 (figure 15d).

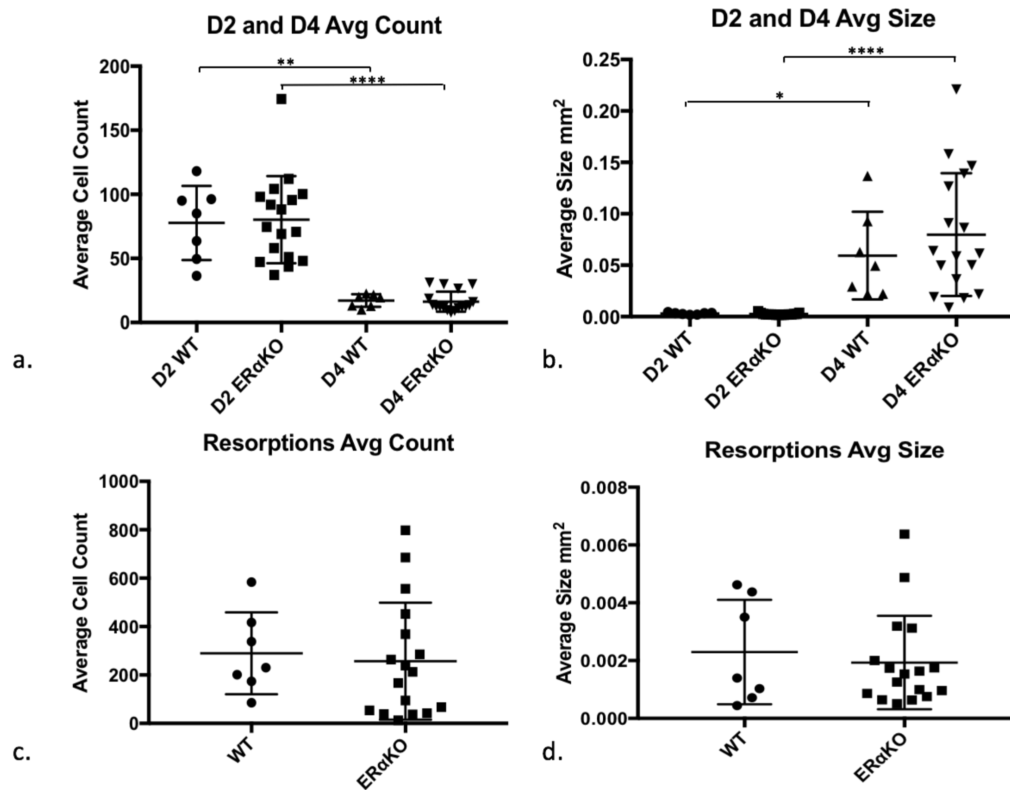

Figure 13. Tartrate resistant acid phosphatase staining (TRAP) and Osteo Assay (Resorption activity) of osteoclasts from ERαKO and WT mice. a) Average cell count of osteoclasts at D2 (day 2) and D4 (day 4) in ERαKO ($p<0.001$) and WT mice ($p=0.016$). b) Average size of osteoclasts at D2 and D4 in ERαKO ($p<0.0001$) and WT ($p=0.0132$) c) Average pit number at day 4 between WT and ERαKO ($p=0.4939$). d) Average pit size at day 4 between WT and ERαKO ($p=0.8525$).

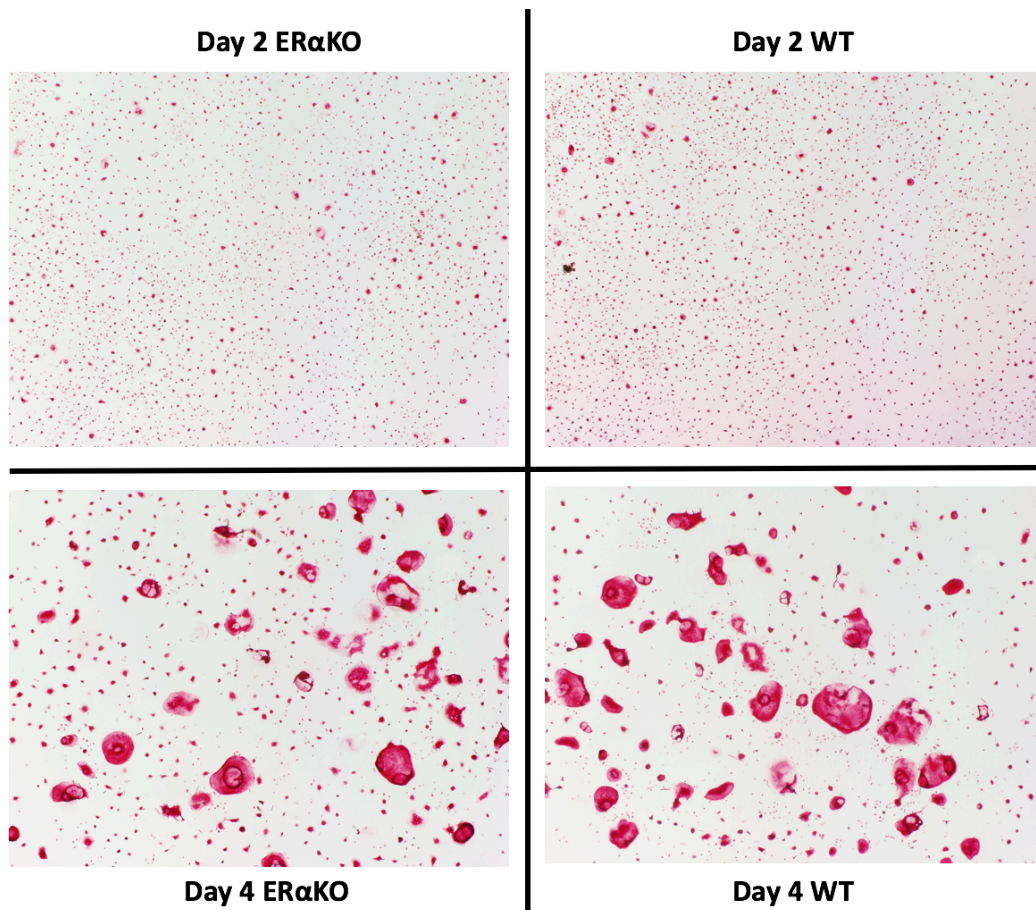

Figure 14. Tartrate resistant acid phosphatase staining (TRAP) of osteoclasts from ERαKO and WT. Representative images of each group are included at Day 2 and Day 4. ERαKO and WT mice demonstrate no difference in size and number of TRAP stained osteoclasts at each time point.

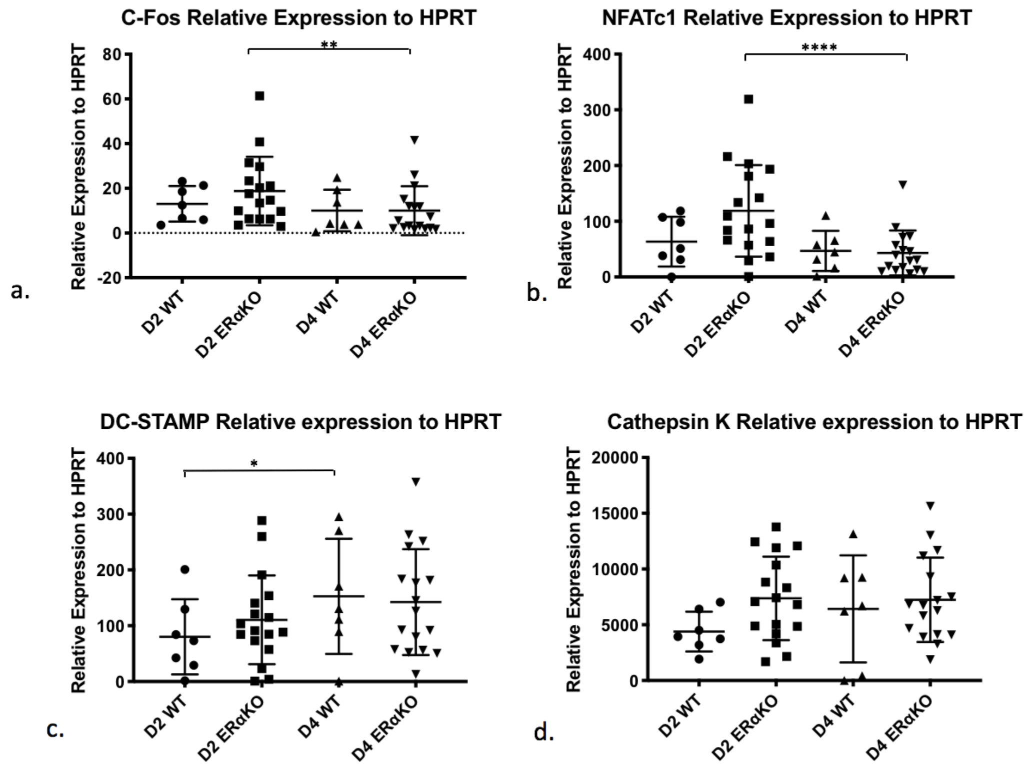

Figure 15. Expression of genes in osteoclasts isolated from ERαKO and WT mice. Osteoclasts from the long bones of mice were isolated. qRT-PCR was used to determine expression of osteoclast differentiation genes from ERαKO and WT cultures. a) *C-Fos* b) *Nfatc1*. c) *Dc-stamp* d) *Cathepsin k*. All genes were normalized to HPRT levels.

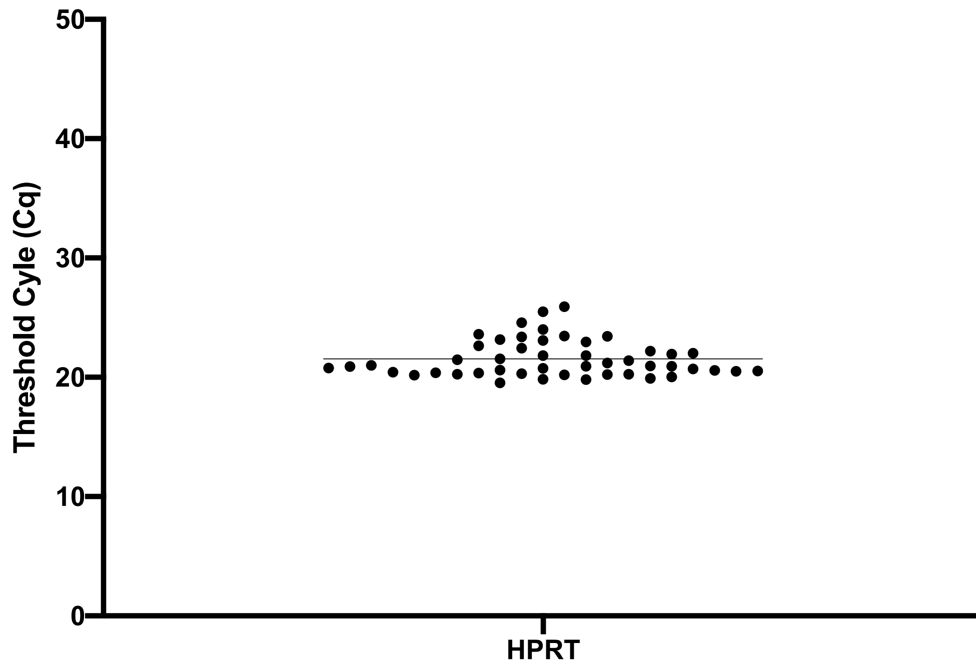

Figure 16. Distribution of threshold cycle for housekeeping gene used (*HPRT*).

Cq values were plotted for *HPRT* in WT and ERαKO osteoclast gene expression. Mean Cq value for *HPRT* is 21.55 (SD 1.54).

Osteoclasts cultured with myokines from ERαKO demonstrate increased C-Fos expression while NFATc1, Dc-stamp, and Cathepsin K demonstrate no difference

Previous studies have suggested that the connection between bone and muscle is more than purely mechanical interaction (Brotto and Bonewald 2015). There is evidence to suggest that there is reciprocal biochemical signaling between muscle and bone, which influences the function of each. We presume that muscle specific estrogen deficiency leads to differential expression of myokines compared to WT. With this experiment, we aimed to determine the differential effect of myokines from ERαKO and WT mice on WT osteoclasts. In this experiment, osteoclasts were cultured with conditioned media containing myokines from either *ERαKO* or WT muscle. To determine the stability of the housekeeping gene (HPRT), real time qPCR was used to evaluate expression of HPRT of the osteoclasts cultured with to myokines from ERαKO and WT. Figure 17 demonstrates the Cq values for the housekeeping gene used in the osteoclast conditioned media experiments. This figure demonstrates the stability of *HPRT* and validates its use as a reference gene for this experiment. The mean Cq value for *HPRT* is 21.54 with a standard deviation of 0.038 (Figure 17).

Figure 18 demonstrates osteoclast gene expression in WT osteoclasts cultured with conditioned media from muscle of WT or ERαKO mice. Osteoclast genes were normalized to HPRT using comparative analysis, then fold change relative to Krebs was calculated. Krebs media served as the negative control. Krebs media contains 1.8 g/L D-Glucose, 0.0468 g/L magnesium chloride [Anhydrous], 0.34 g/L potassium chloride served, 7.0 g/L sodium chloride, 0.1g/L sodium phosphate dibasic [Anhydrous], and 0.18g/L sodium phosphate monobasic [Anhydrous].

Based on our hypothesis, we expected to find an increase in osteoclast differentiation gene expression in the osteoclasts cultured with myokines from

ERαKO compared to WT. This is because we anticipate that presence of estrogen activity on muscle cells plays an inhibitory role of on osteoclastogenesis. Our findings demonstrate no difference between osteoclasts cultured with ERαKO and WT myokines for all osteoclast differentiation genes (*C-Fos, Nfatc1, Dc-stamp, cathepsin k*). There is a trend towards higher expression of *C-Fos* in the ERαKO-conditioned media osteoclast group compared to the WT (p=0.0692). The ERαKO-conditioned media osteoclast group demonstrates 45.9-fold change in *C-Fos* relative to Krebs, while WT demonstrates 20.7-fold change (SD 1.15 and 12.21, respectively) (figure 18a). There is a 38-fold change in *Nfatc1* relative to Krebs in the WT conditioned media compared to a 62.7-fold change in ERαKO (SD 24.08 and 5.46, respectively) (figure 18b). For *Dc-stamp*, there was an 89.9-fold change relative to Krebs in the ERαKO conditioned media group (SD 18.63), compared to a 63.87-fold change in the WT (SD 44.2) (Fig 18c). ERαKO conditioned media group demonstrated a 33-fold change in *Cathepsin k* relative to Krebs (SD 5.07), similarly, WT demonstrated a 31.1-fold change (SD 18.09) (Figure 18d).

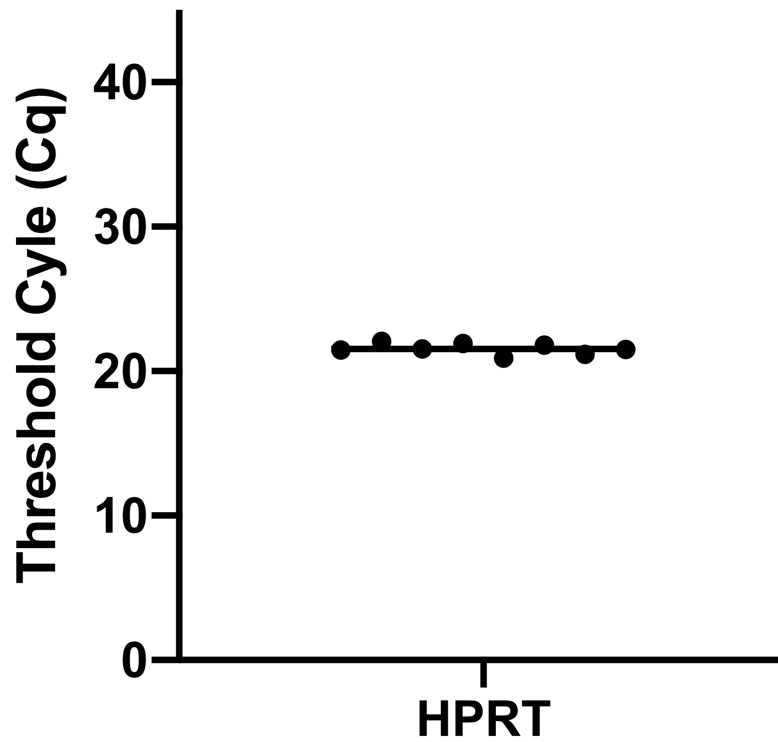

Figure 17. Distribution of threshold cycle for housekeeping gene used (*HPRT*) for conditioned media experiment. Cq values were plotted for *HPRT* in osteoclast conditioned media experiment. The mean Cq value for *HPRT* is 21.54 with a standard deviation of 0.038.

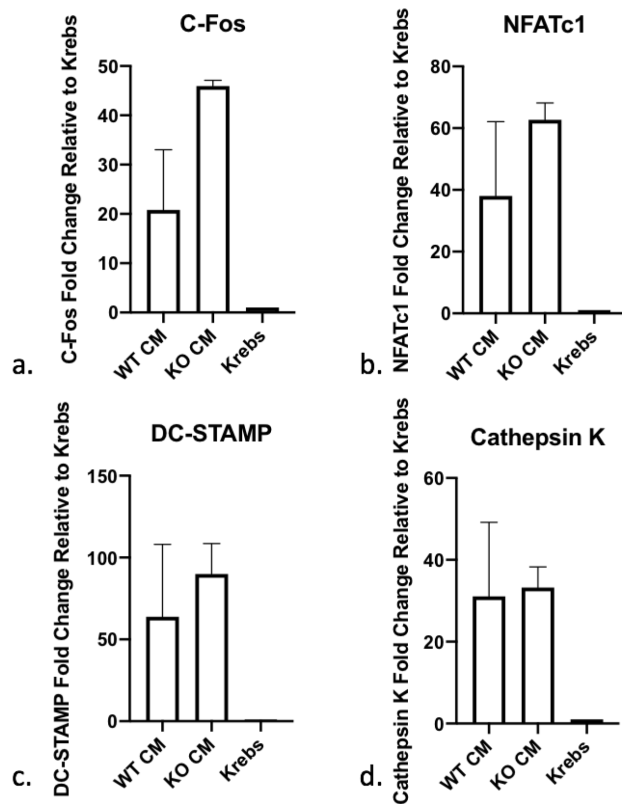

Figure 18. Gene expression of osteoclasts treated with muscle conditioned media from WT and ERαKO mice. Conditioned media (CM) from either WT (n=3) or ERαKO (n=3) muscle were cultured with osteoclasts. qRT-PCR was used to determine expression of genes in osteoclasts grown in the presence of conditioned media from ERαKO and WT mice. Relative expression of *Nfatc1*, *Dc-stamp*, *C-Fos*, *Cathepsin K* to *Hprt* was calculated using Cq values. Osteoclast genes were normalized to *HPRT* using comparative analysis, then fold change relative to Krebs was calculated. Krebs is set equal to 1. Expression is shown relative to Krebs. Krebs serves as the negative control. ANOVA was performed for WT, ERαKO, and krebs for each gene. Welche's T test was performed for *C-Fos* to compare WT and ERαKO (p=0.692). a) *C-Fos* b). *Nfatc1* c) *Dc-stamp* D) *Cathepsin k*

Estrogen deficiency in muscle leads to down regulation of bone morphogenic protein-2, Oncostatin M, and hemolytic complement

While estrogen deficiency was previously shown to affect muscle mass and function, it is unclear how estrogen deficiency specific to muscle can alter myokine expression. It is also unclear which myokines are affected. To determine if estrogen deficiency in muscle leads to differential production of myokines, the RT^2 Profiler PCR array was used on ERαKO and WT muscle extracts. RT^2 Profiler PCR array demonstrated several differences in cytokine and chemokine expression in ERαKO and WT muscle. Eighty-four test genes were evaluated by PCR array. Test genes were selected through evaluation of existing literature for cytokines and chemokines involved in signaling between muscle and bone(Cianferotti and Brandi 2014, Brotto and Bonewald 2015, Tagliaferri, Wittrant et al. 2015). PCR array selected from Qiagen contained the majority of genes of interest for bone-muscle signaling. Several housekeeping genes were evaluated (*Actb*, *B2m*, *Gapdh*, *Gusb,* and *Hsp90ab1*). *B2m* and *Hsp90ab1* demonstrated the smallest standard deviation in Cq values (1.10 and 1.17, respectively), compared to *Actb*, *Gapdh*, and *Gusb* (figure 19). Therefore, *B2m* and *Hsp90ab1* were identified as appropriate reference genes for PCR array analysis. All 84 test genes on the PCR array were compared to *B2m* and *Hsp90ab1*.

For PCR array data analysis, a 2-fold change cutoff criterion was used to determine if the gene was upregulated or down regulated. Of the 84 test genes analyzed, 3 genes were downregulated in the muscle from ERαKO relative to the WT ($p>0.05$). There were 10 genes out of 84 test genes which were upregulated in the muscle from ERαKO relative to the WT ($p>0.05$) (figure 20).

Several genes appear to be down regulated in the ERαKO compared to the WT. *Bmp2* demonstrates 530-fold down regulation compared to WT. *Hc*

demonstrates 66-fold down regulation in ERαKO compared to WT. *Osm* shows 226-fold down regulation compared to WT. Genes which demonstrate at least 2-fold up regulation in the ERαKO compared to WT are *Cxcl11, Fasl, Ifna2, Ifng, Il2, Il22, Il5, Il7, Tnfsf11, Vegfa (*figure 21, 22).

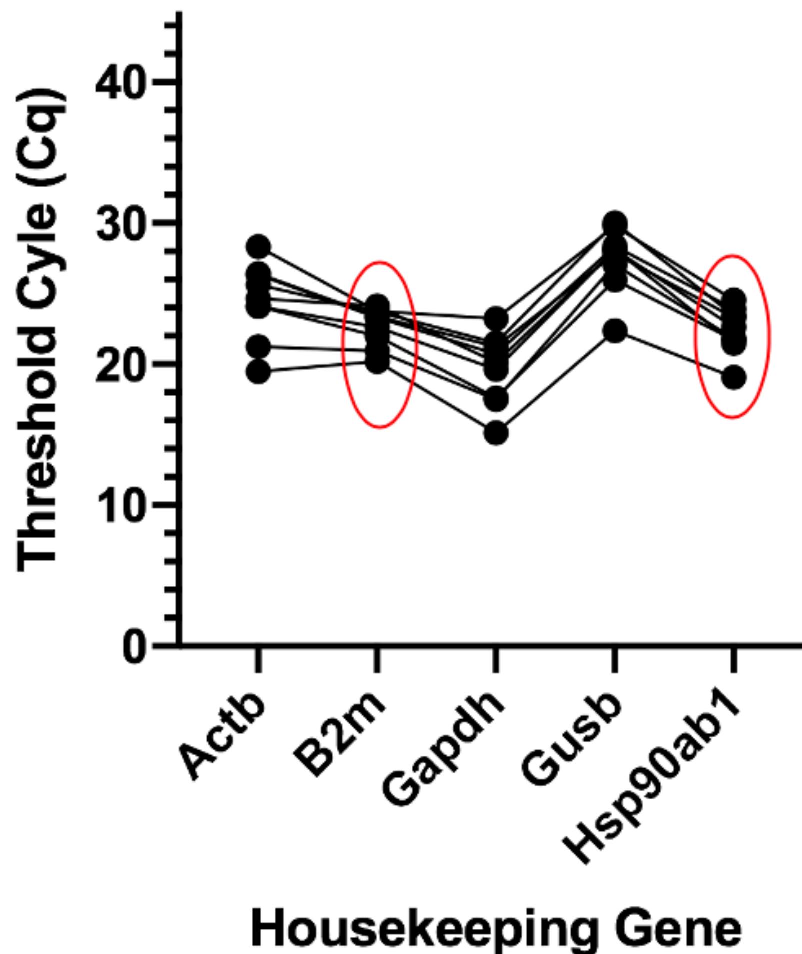

Figure 19. Distribution of Cq values of housekeeping genes for PCR array. *B2m and Hsp90ab1* demonstrated the smallest deviation are most appropriate for use as reference genes in the PCR Array. *Actb, Gapdh*, and *Gusb* demonstrated greater spread, and it was determined that they were not appropriate for use as reference genes.

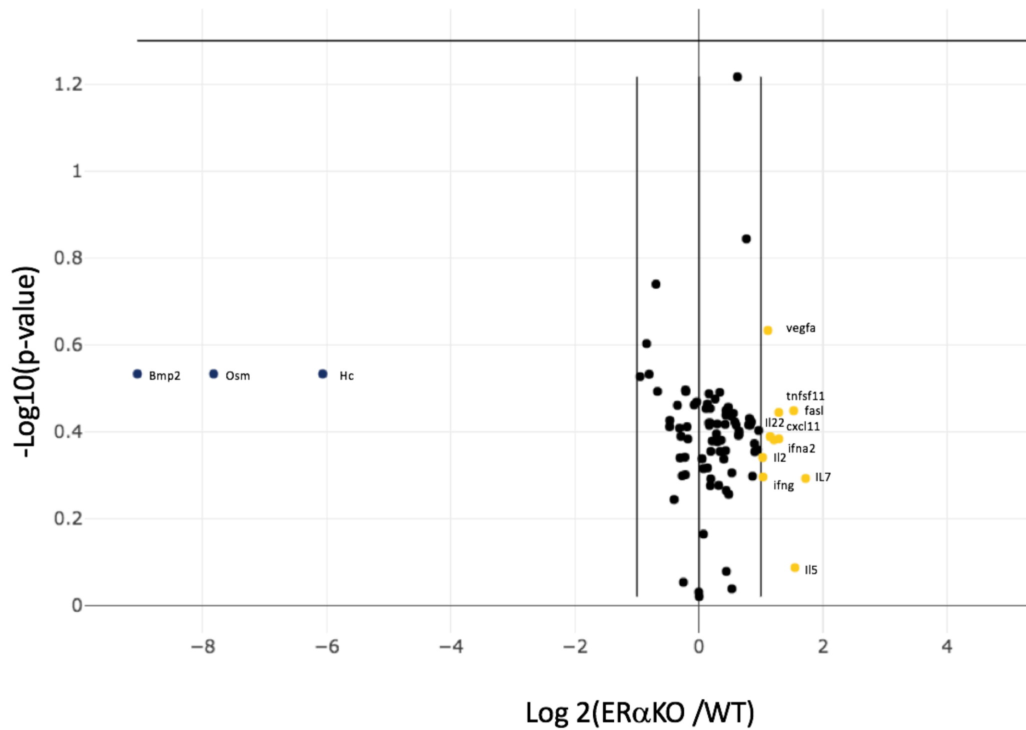

Figure 20. A volcano plot showing upregulated (right) and downregulated genes (left) in the muscle from ERαKO compared to WT. The x-axis represents the log2 fold change. The graph used a 2-fold cutoff to determine of the gene was differentially expressed or not. The y-axis is -Log10(p-value) of the student's T test p values. All p-values >0.05. The yellow dots on the right represent highly upregulated genes. The navy-blue dots on the left represent the highly downregulated genes.

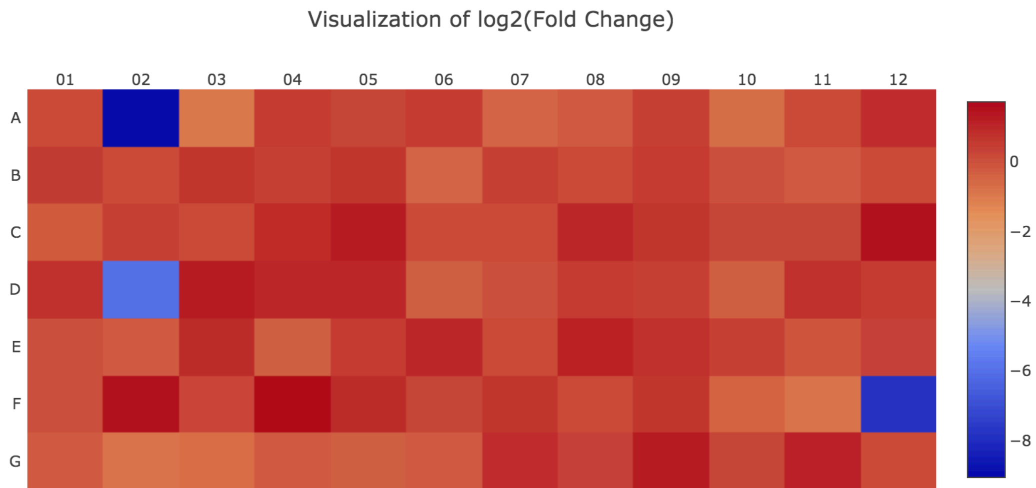

Figure 21. Heat map demonstrating cytokine PCR array from ERαKO and WT tibialis anterior muscle and associated cytokines/chemokines. Log2 (fold change) is calculated with WT housekeeping genes (*B2m* and *Hsp90ab1*) as the reference. Genes associated with figure 21 are shown in figure 22. Downregulated genes (demonstrated in blue) are *Bmp2*, *Hc*, and *Osm*. Genes demonstrated in dark red have at least 2-fold up regulation in the ERαKO compared to WT are *Cxcl11, Fasl, Ifna2, Ifng, Il2, Il22, Il5, Il7, Tnfsf11, and Vegfa*.

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	Adipoq 1.12	Bmp2 -530.34	Bmp4 -1.93	Bmp6 1.36	Bmp7 1.23	Ccl1 1.44	Ccl11 -1.39	Ccl12 -1.14	Ccl17 1.35	Ccl19 -1.59	Ccl2 1.14	Ccl20 1.77
B	Ccl22 1.49	Ccl24 1.08	Ccl3 1.56	Ccl4 1.35	Ccl5 1.56	Ccl7 -1.39	Cd40lg 1.32	Cd70 1.14	Cntf 1.44	Csf1 -1.03	Csf2 -1.16	Csf3 1.13
C	Ctf1 -1.13	Cx3cl1 1.28	Cxcl1 1.10	Cxcl10 1.82	Cxcl11 2.31	Cxcl12 1.14	Cxcl13 1.14	Cxcl16 1.95	Cxcl3 1.55	Cxcl5 1.23	Cxcl9 1.25	Fasl 2.88
D	Gpi1 1.70	Hc -66.87	Ifna2 2.44	Ifng 2.04	Il10 1.92	Il11 -1.27	Il12a 1.03	Il12b 1.47	Il13 1.35	Il15 -1.23	Il16 1.76	Il17a 1.39
E	Il17f 1.05	Il18 -1.17	Il1a 1.87	Il1b -1.24	Il1rn 1.39	Il2 2.04	Il21 1.12	Il22 2.22	Il23a 1.74	Il24 1.34	Il27 -1.06	Il3 1.26
F	Il4 1.05	Il5 2.92	Il6 1.16	Il7 3.29	Il9 1.86	Lif 1.20	Lta 1.51	Ltb 1.12	Mif 1.54	Mstn -1.32	Nodal -1.75	Osm -226.22
G	Pf4 -1.19	Ppbp -1.79	Spp1 -1.62	Tgfb2 -1.17	Thpo -1.22	Tnf -1.16	Tnfrsf11b 1.80	Tnfsf10 1.26	Tnfsf11 2.44	Tnfsf13b 1.22	Vegfa 2.16	Xcl1 1.10

Figure 22. Table demonstrating cytokine and chemokine genes associated with heat map. Fold regulation for ERαKO compared to WT is noted for each gene. Blue boxes represent genes that demonstrate at least a 2-fold down regulation in ERαKO compared to WT. Red boxes represent genes that demonstrate at least a 2-fold upregulation in ERαKO compared to WT.

Discussion

Osteoporosis leads to loss of bone mass and increased risk of falls and fractures. This poses a significant health concern that affects millions worldwide (WHO 1994, International-Osteoporosis-Foundation 2017). Many studies suggest that muscle-cross talk is important for the maintenance and function of bone (Warner, Sanford et al. 2006, Tagliaferri, Wittrant et al. 2015, Reginster, Beaudart et al. 2016). It is unclear how muscle specific estrogen deficiency impacts bone and may serve as a potential mechanism for osteoporosis. Understanding how estrogen deficiency impacts the crosstalk in the bone-muscle unit may lead to a clearer understanding of how musculoskeletal diseases form.

The present study assesses how estrogen deficiency specific to muscle may affect the biochemical signaling between muscle and bone. For our overall hypothesis, we hypothesized that estrogen regulates myokine expression and inhibits osteoclast differentiation. For example, in the absence of estrogen signaling on muscle, myokine secretion may be altered, thus leading to increased osteoclast activity. To explore this question, the present study included three experiments to address the overall hypothesis.

Previous studies have demonstrated that the interaction between muscle and bone is more than purely mechanical (Warner, Sanford et al. 2006, Tagliaferri, Wittrant et al. 2015, Reginster, Beaudart et al. 2016). There is paracrine and endocrine signaling between muscle and bone, which reciprocally coordinates bone and muscle growth and maintenance. Even during development, muscle appears to affect formation of bone, as demonstrated in a "muscle-less limb" embryonic mouse model which had impaired bone development (Nowlan, Sharpe et al. 2010). To determine if muscle specific ERα deficiency during development will affect

osteoclasts, osteoclasts were harvested from the long bones of ERαKO and WT mice. Osteoclast differentiation gene expression, size, number, and resorption pits were evaluated. The present study determined that the absence of ERα signaling specific to muscle cells during development does not affect osteoclast differentiation as *C-Fos*, *Nfatc1*, *DC-stamp*, and *cathepsin k* expression was unchanged. Additionally, osteoclast size, number, and resorption pits did not differ in the absence of ERα signaling on muscle. These findings suggest that estrogen deficiency in muscle does not affect osteoclast activity.

One explanation is that estrogen deficiency does alter muscle function and myokine secretion, but there are redundancies in myokine function. For example, if there are redundancies in function of the myokines produced, this could prevent changes from occurring in osteoclasts. Therefore, there would be no difference in osteoclast size, resorption, or differentiation genes. Another possible explanation is that isolating the osteoclasts in vitro, away from muscle, may lead to loss of the signal irrespective of what the influence of muscle may be.

Also, it is possible that ERα deficiency specific to muscle during development does not affect osteoclasts, but instead affects osteoblast function. An alternative mechanism for osteoporosis is, estrogen signaling is responsible for driving osteoblastogenesis. Therefore, in the absence of ERα signaling on muscle, an alternative myokine profile is produced and there is a disruption in bone homeostasis due to reduction of osteoblastogenesis. This suggests that additional studies on osteoblasts would need to be performed.

Previous studies have confirmed the cross talk between muscle and bone through myokines. Muscle has been shown to secrete many factors that can influence bone such as IGF-1, IL-6, IL-15, IL-8, fibroblast-growth factor-2, myostatin,

osteoglycin, transmembrane protein 19, and osteoactivin (Cianferotti and Brandi 2014, Tagliaferri, Wittrant et al. 2015). To evaluate whether myokine secretion from muscle can truly affect osteoclasts, conditioned media from muscles of ERαKO and WT mice were tested. Conditioned media from muscle of ERαKO and WT mice were cultured with WT osteoclasts to determine if differential myokine expression due to estrogen deficiency affects osteoclasts.

There was no difference in osteoclast gene expression of *Nfatc1*, *DC-stamp*, and *cathepsin K* in the WT osteoclasts cultured with myokines from ERαKO compared to wild type mice. However, osteoclasts cultured with myokines from ERαKO demonstrated a trend of higher expression of *C-Fos* compared to WT (p=0.0692). *C-Fos* is an early transcription factor for osteoclast differentiation (figure 2, figure 3). *C-Fos* is responsible for the transition from osteoclast precursors into osteoclasts (Boyce, Yamashita et al. 2005). Our findings suggest that ERα deficiency specific to muscle may lead to an altered myokine profile which affects early precursors of osteoclast differentiation, leading to increased *C-Fos* expression. However, the promotion of increased osteoclast differentiation is not continued in the intermediate and late stages of osteoclast differentiation, as *Nfatc1*, *DC-stamp*, and *cathepsin K* expression demonstrate no difference between ERαKO and WT. *Nfatc1* is the master regulator of terminal differentiation of osteoclasts and is responsible for activating downstream genes such as *DC-stamp* and *cathepsin K (Choi, Park et al. 2016)*. Overall, these findings suggest that there is a trend of increased expression of *C-Fos*, an early transcription factor of osteoclast differentiation, when muscle cells cannot respond to estrogen. However, these signals are not maintained, leading to no difference in downstream expression of osteoclast differentiation. This suggests that

in the absence of estrogen signaling in muscle, the end result of osteoclast differentiation is not affected.

Finally, a PCR array was used in the present study in order to determine if ERα deficiency in muscle alters the myokine profile. The PCR array allowed us to analyze 84 myokine genes to determine if there is differential expression due to estrogen signaling in myocytes. The expression of several genes were found to be down regulated including *Bmp2*, *Osm*, and *Hc*. *Bmp2 (*Bone morphogenic protein 2) displays potent osteogenic potential and plays a significant role in endochondral bone development. Previous studies with *Bmp2* limb-specific knockouts have demonstrated spontaneous fractures and inability to initiate fracture healing (Wang, Green et al. 2014). *Bmp2* plays a critical role in regulating osteoblast differentiation. *Bmp2* recruits and activates SMADs, which results in activation of essential genes involved in osteoblast differentiation, such as *Runx2* and *Osterix* (Ryoo, Lee et al. 2006). In the present study, *Bmp2* was downregulated by 530-fold in the ERαKO compared to wild type. This suggests that in the absence of estrogen signaling on muscle, down regulation of *Bmp2* occurs, which may promote decreased osteoblast differentiation. In the context of osteoporosis, perhaps osteoblast differentiation is diminished due to an altered myokine profile in the absence of estrogen signaling on muscle, which leads to an altered bone phenotype with diminished bone mass. Therefore, future studies should evaluate osteoblasts in the ERαKO model to determine if altered biochemical signaling from the muscle-bone unit is affecting osteoblasts and contributing to the bone phenotype in osteoporosis.

Oncostatin M (*Osm)* was downregulated by 226-fold in the ERαKO compared to WT. Oncostatin M is a pleiotropic cytokine which demonstrates anabolic effects on bone, but can also indirectly promote osteoclastogenesis. The primary role of *Osm is*

promoting osteoblast differentiation by pushing mesenchymal stem cell (MSC) commitment toward mineralizing osteoblasts. *Osm* can also directly promote osteoblast mineralization and differentiation (Sims and Quinn 2014). Previous studies on Oncostatin M receptor (OSMR) null mice demonstrated impaired osteoblast differentiation and increased adipogenesis (Walker, McGregor et al. 2010). Therefore, *Osm* plays a significant role in osteoblast differentiation and inhibiting adipogenesis. In the present study, *Osm* was downregulated by 226-fold in the ERαKO compared to wildtype. This decrease in expression of *Osm* would prevent mesenchymal stem cell (MSC) commitment towards osteoblasts. This suggests that osteoblastogenesis may also be down regulated in the absence of ERα signaling in muscle. ERα signaling in muscle is essential for the production of myokines which promote osteoblastogenesis, such as *Osm* and *Bmp2*. In the absence of ERα signaling in muscle, such as in the case of osteoporosis, the decrease in bone mass is likely due to diminished expression of genes such as *Osm* and *Bmp2*, leading to reduced osteoblastogenesis.

The present study also demonstrated 66-fold down regulation of *Hc* (hemolytic complement) in ERαKO compared to WT. *Hc* encodes precursors for C5 in the complement pathway. *Hc* encodes a precursor protein, which is modified to generate C5 alpha chain, C5 beta chain, C5a anaphylatoxin, and C5b (NCBI 2020). Previous studies have demonstrated that *Hc* may play a role in bone repair. In *Hc* deficient (C5-/- knockout) mice, there was a significant reduction in bone fracture repair after femur osteotomy compared to wild type mice (Ehrnthaller, Huber-Lang et al. 2013). This suggests that C5 is involved in the bone remodeling and fracture repair process. Previous studies have demonstrated that C5a is responsible for inducing osteoblast migration, inducing an inflammatory response of osteoblasts, and can also alter the release of IL-6 from osteoblasts (Pobanz, Reinhardt et al. 2000, Huber-Lang,

Kovtun et al. 2013). This may be one mechanism in which C5 is important for fracture healing. In the context of the present study, *Hc* (C5) is down regulated in the absence of estrogen signaling on muscle, which may prevent regular osteoblast migration and function. Therefore, this may suggest that estrogen signaling in muscle cells is responsible for *Hc* expression and maintenance of osteoblast migration and function.

It is possible that in osteoporosis, *Bmp2, Osm*, and *Hc* are downregulated in the absence of estrogen signaling on muscle. The downregulation of these anabolic genes leads to reduced osteoblastogenesis, a disruption in bone homeostasis, and overall loss of bone mass. In general, these down regulated genes (*Bmp2*, *Osm*, and *Hc*) suggest that osteoblasts should be evaluated in future experiments with ErαKO mice in order to determine how estrogen signaling on muscle may alter myokine production and impact osteoblastogenesis.

Additionally, there were several genes which were upregulated by two to three-fold in the ERαKO compared to WT. These genes include: *Cxcl11, Fasl, Ifna2, Ifng, Il2, Il22, Il5, Il7, Tnfsf11, and Vegfa*. Given that the level of upregulation is minimal between ERαKO compared to WT, additional studies may need to be performed in order to verify the upregulation of these genes.

There are several limitations of the study. The present study includes in vitro experiments, which may not fully represent what is occurring in vivo. There are additional cellular signals in vivo which are not accounted for in an in vitro experiment. Another factor to consider is the architectural proximity of the bone and muscle unit. Several studies have cited endocrine and paracrine cross talk between muscle and bone, however, it is unclear as to the exact proximity required for this communication. The in vitro model does not account for architectural differences that

may be present in the in vivo. Additionally, the potency of estrogen may be diminished in a cell culture setting, therefore, the effects that are present in vivo may not be seen in vitro. Even though there are drawbacks with in vitro studies, we can appreciate that the present study does demonstrate some effects of muscle specific ERα deficiency on myokine production and effects on early osteoclastogenesis through *C-Fos* expression.

For future directions, there should be additional studies to explore the effect of myokines from ERαKO and WT mice on osteoblasts. This would allow us to understand if lack of estrogen signaling on muscle leads to an altered myokine profile, thus affecting the osteoblast function and altering bone homeostasis. This may be a potential underlying mechanism in osteoporosis. Additionally, in vivo experiments would provide more information about the underlying mechanism, as all biochemical signals and architecture would be preserved. Therefore, there would be benefit in repeating the experiments in vivo and exploring both osteoclast and osteoblast function.

Conclusion

Overall, the ERαKO murine model allowed for skeletal muscle cell specific deletion of ERα in order to study the role of estrogen signaling on muscle-bone cross talk. ERα deficiency in skeletal muscle cells during development does not appear to affect the osteoclast phenotype. Evaluation of biomolecular signaling between the muscle-bone unit was performed by culturing WT osteoclasts with either myokines from ERαKO or WT mice. This revealed that the myokine profile in the absence of ERα signaling may only have minimal effect on the early phase of osteoclastogenesis through *C-Fos*. However, in the absence of ERα signaling, myokine expression in

myocytes does not affect the end result of osteoclastogenesis, as there was no difference in expression of terminal osteoclastogenesis genes. Consequently, ERα signaling in muscle appears to have no influence on the end result of osteoclastogenesis.

Identification of myokines by PCR array revealed downregulation of several genes in ERαKO including *Bmp2*, *Osm*, and *Hc*. Previous studies suggest that these are anabolic genes and may play a role in osteoblastogenesis. Therefore, ERα signaling in muscle may be responsible for secretion of myokines, which promote osteoblastogenesis. Future studies should evaluate if myokines secreted from ERα deficient muscle influences osteoblasts. This may reveal potential mechanisms behind the disruption in bone homeostasis observed in osteoporosis.

Bibliography

Aloia, J. F., D. M. McGowan, A. N. Vaswani, P. Ross and S. H. Cohn (1991). "Relationship of menopause to skeletal and muscle mass." Am J Clin Nutr **53**(6): 1378-1383.

Amarasekara, D. S., H. Yun, S. Kim, N. Lee, H. Kim and J. Rho (2018). "Regulation of Osteoclast Differentiation by Cytokine Networks." Immune Netw **18**(1): e8.

Armas, L. A. and R. R. Recker (2012). "Pathophysiology of osteoporosis: new mechanistic insights." Endocrinol Metab Clin North Am **41**(3): 475-486.

Bianchi, M. L., A. Mazzanti, E. Galbiati, S. Saraifoger, A. Dubini, F. Cornelio and L. Morandi (2003). "Bone mineral density and bone metabolism in Duchenne muscular dystrophy." Osteoporos Int **14**(9): 761-767.

Bonewald, L. F., D. P. Kiel, T. L. Clemens, K. Esser, E. S. Orwoll, R. J. O'Keefe and R. A. Fielding (2013). "Forum on bone and skeletal muscle interactions: summary of the proceedings of an ASBMR workshop." J Bone Miner Res **28**(9): 1857-1865.

Boyce, B. F. (2013). "Advances in the regulation of osteoclasts and osteoclast functions." J Dent Res **92**(10): 860-867.

Boyce, B. F., T. Yamashita, Z. Yao, Q. Zhang, F. Li and L. Xing (2005). "Roles for NF-kappaB and c-Fos in osteoclasts." J Bone Miner Metab **23 Suppl**: 11-15.

Bren-Mattison, Y., M. Hausburg and B. B. Olwin (2011). "Growth of limb muscle is dependent on skeletal-derived Indian hedgehog." Dev Biol **356**(2): 486-495.

Brotto, M. and L. Bonewald (2015). "Bone and muscle: Interactions beyond mechanical." Bone **80**: 109-114.

Chiu, Y. H. and C. T. Ritchlin (2016). "DC-STAMP: A Key Regulator in Osteoclast Differentiation." J Cell Physiol **231**(11): 2402-2407.

Choi, B. Y., C. H. Park, Y. H. Na, H. W. Bai, J. Y. Cho and B. Y. Chung (2016). "Inhibition of RANKL-induced osteoclast differentiation through the downregulation

of c-Fos and NFATc1 by Eremochloa ophiuroides (centipedegrass) extract." Mol Med Rep **13**(5): 4014-4022.

Cianferotti, L. and M. L. Brandi (2014). "Muscle-bone interactions: basic and clinical aspects." Endocrine **45**(2): 165-177.

Coiro, V., R. Volpi, S. Cataldo, M. G. Magotti, M. L. Maffei, C. Giumelli, A. Araldi, L. Volpi and P. Chiodera (2012). "Effect of physiological exercise on osteocalcin levels in subjects with adrenal incidentaloma." J Endocrinol Invest **35**(4): 357-358.

Collins, B. C., E. K. Laakkonen and D. A. Lowe (2019). "Aging of the musculoskeletal system: How the loss of estrogen impacts muscle strength." Bone **123**: 137-144.

Collins, B. C., T. L. Mader, C. A. Cabelka, M. R. Inigo, E. E. Spangenburg and D. A. Lowe (2018). "Deletion of estrogen receptor alpha in skeletal muscle results in impaired contractility in female mice." J Appl Physiol (1985) **124**(4): 980-992.

Cooper, C., W. Dere, W. Evans, J. A. Kanis, R. Rizzoli, A. A. Sayer, C. C. Sieber, J. M. Kaufman, G. Abellan van Kan, S. Boonen, J. Adachi, B. Mitlak, Y. Tsouderos, Y. Rolland and J. Y. Reginster (2012). "Frailty and sarcopenia: definitions and outcome parameters." Osteoporos Int **23**(7): 1839-1848.

DiGirolamo, D. J., D. P. Kiel and K. A. Esser (2013). "Bone and skeletal muscle: neighbors with close ties." J Bone Miner Res **28**(7): 1509-1518.

DiGirolamo, D. J., A. Mukherjee, K. Fulzele, Y. Gan, X. Cao, S. J. Frank and T. L. Clemens (2007). "Mode of growth hormone action in osteoblasts." J Biol Chem **282**(43): 31666-31674.

Ehrnthaller, C., M. Huber-Lang, P. Nilsson, R. Bindl, S. Redeker, S. Recknagel, A. Rapp, T. Mollnes, M. Amling, F. Gebhard and A. Ignatius (2013). "Complement C3 and C5 deficiency affects fracture healing." PLoS One **8**(11): e81341.

Elkasrawy, M., D. Immel, X. Wen, X. Liu, L. F. Liang and M. W. Hamrick (2012). "Immunolocalization of myostatin (GDF-8) following musculoskeletal injury and the

effects of exogenous myostatin on muscle and bone healing." J Histochem Cytochem **60**(1): 22-30.

Fielding, R. A., B. Vellas, W. J. Evans, S. Bhasin, J. E. Morley, A. B. Newman, G. Abellan van Kan, S. Andrieu, J. Bauer, D. Breuille, T. Cederholm, J. Chandler, C. De Meynard, L. Donini, T. Harris, A. Kannt, F. Keime Guibert, G. Onder, D. Papanicolaou, Y. Rolland, D. Rooks, C. Sieber, E. Souhami, S. Verlaan and M. Zamboni (2011). "Sarcopenia: an undiagnosed condition in older adults. Current consensus definition: prevalence, etiology, and consequences. International working group on sarcopenia." J Am Med Dir Assoc **12**(4): 249-256.

Frontera, W. R., V. A. Hughes, R. A. Fielding, M. A. Fiatarone, W. J. Evans and R. Roubenoff (2000). "Aging of skeletal muscle: a 12-yr longitudinal study." J Appl Physiol (1985) **88**(4): 1321-1326.

Girgis, C. M., N. Mokbel and D. J. Digirolamo (2014). "Therapies for musculoskeletal disease: can we treat two birds with one stone?" Curr Osteoporos Rep **12**(2): 142-153.

Greising, S. M., K. A. Baltgalvis, D. A. Lowe and G. L. Warren (2009). "Hormone therapy and skeletal muscle strength: a meta-analysis." J Gerontol A Biol Sci Med Sci **64**(10): 1071-1081.

Greising, S. M., R. S. Carey, J. E. Blackford, L. E. Dalton, A. M. Kosir and D. A. Lowe (2011). "Estradiol treatment, physical activity, and muscle function in ovarian-senescent mice." Exp Gerontol **46**(8): 685-693.

Hamrick, M. W. (2003). "Increased bone mineral density in the femora of GDF8 knockout mice." Anat Rec A Discov Mol Cell Evol Biol **272**(1): 388-391.

Hamrick, M. W. (2012). "The skeletal muscle secretome: an emerging player in muscle-bone crosstalk." Bonekey Rep **1**: 60.

Hamrick, M. W., P. L. McNeil and S. L. Patterson (2010). "Role of muscle-derived growth factors in bone formation." J Musculoskelet Neuronal Interact **10**(1): 64-70.

Hansen, M. (2018). "Female hormones: do they influence muscle and tendon protein metabolism?" Proc Nutr Soc **77**(1): 32-41.

Hao, Y., Y. Ma, X. Wang, F. Jin and S. Ge (2012). "Short-term muscle atrophy caused by botulinum toxin-A local injection impairs fracture healing in the rat femur." J Orthop Res **30**(4): 574-580.

Harry, L. E., A. Sandison, E. M. Paleolog, U. Hansen, M. F. Pearse and J. Nanchahal (2008). "Comparison of the healing of open tibial fractures covered with either muscle or fasciocutaneous tissue in a murine model." J Orthop Res **26**(9): 1238-1244.

Harry, L. E., A. Sandison, M. F. Pearse, E. M. Paleolog and J. Nanchahal (2009). "Comparison of the vascularity of fasciocutaneous tissue and muscle for coverage of open tibial fractures." Plast Reconstr Surg **124**(4): 1211-1219.

Huber-Lang, M., A. Kovtun and A. Ignatius (2013). "The role of complement in trauma and fracture healing." Semin Immunol **25**(1): 73-78.

Hughes, D. E., A. Dai, J. C. Tiffee, H. H. Li, G. R. Mundy and B. F. Boyce (1996). "Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta." Nat Med **2**(10): 1132-1136.

International-Osteoporosis-Foundation. (2017). "Osteoporosis Facts and Statistics " Retrieved April 15th, 2020, from https://www.iofbonehealth.org/facts-and-statistics/calcium-studies-map.

Isaacson, J. and M. Brotto (2014). "Physiology of Mechanotransduction: How Do Muscle and Bone "Talk" to One Another?" Clin Rev Bone Miner Metab **12**(2): 77-85.

Jackson, W. M., A. B. Aragon, J. Onodera, S. M. Koehler, Y. Ji, J. D. Bulken-Hoover, J. A. Vogler, R. S. Tuan and L. J. Nesti (2011). "Cytokine expression in muscle following traumatic injury." J Orthop Res **29**(10): 1613-1620.

Jahn, K., N. Lara-Castillo, L. Brotto, C. L. Mo, M. L. Johnson, M. Brotto and L. F. Bonewald (2012). "Skeletal muscle secreted factors prevent glucocorticoid-induced osteocyte apoptosis through activation of beta-catenin." Eur Cell Mater **24**: 197-209; discussion 209-110.

Johnell, O. and J. A. Kanis (2006). "An estimate of the worldwide prevalence and disability associated with osteoporotic fractures." Osteoporos Int **17**(12): 1726-1733.

Kanis, J. A., A. Oden, E. V. McCloskey, H. Johansson, D. A. Wahl, C. Cooper, I. O. F. W. G. o. Epidemiology and L. Quality of (2012). "A systematic review of hip fracture incidence and probability of fracture worldwide." Osteoporos Int **23**(9): 2239-2256.

Khosla, S., M. J. Oursler and D. G. Monroe (2012). "Estrogen and the skeleton." Trends Endocrinol Metab **23**(11): 576-581.

Kitase, Y., J. A. Vallejo, W. Gutheil, H. Vemula, K. Jahn, J. Yi, J. Zhou, M. Brotto and L. F. Bonewald (2018). "beta-aminoisobutyric Acid, l-BAIBA, Is a Muscle-Derived Osteocyte Survival Factor." Cell Rep **22**(6): 1531-1544.

Lane, N. E. (2006). "Epidemiology, etiology, and diagnosis of osteoporosis." Am J Obstet Gynecol **194**(2 Suppl): S3-11.

Le, G., S. A. Novotny, T. L. Mader, S. M. Greising, S. S. K. Chan, M. Kyba, D. A. Lowe and G. L. Warren (2018). "A moderate oestradiol level enhances neutrophil number and activity in muscle after traumatic injury but strength recovery is accelerated." J Physiol **596**(19): 4665-4680.

Liu, R., O. Birke, A. Morse, L. Peacock, K. Mikulec, D. G. Little and A. Schindeler (2011). "Myogenic progenitors contribute to open but not closed fracture repair." BMC Musculoskelet Disord **12**: 288.

Liu, R., A. Schindeler and D. G. Little (2010). "The potential role of muscle in bone repair." J Musculoskelet Neuronal Interact **10**(1): 71-76.

Marie, P. J. and M. Kassem (2011). "Osteoblasts in osteoporosis: past, emerging, and future anabolic targets." Eur J Endocrinol **165**(1): 1-10.

Mavalli, M. D., D. J. DiGirolamo, Y. Fan, R. C. Riddle, K. S. Campbell, T. van Groen, S. J. Frank, M. A. Sperling, K. A. Esser, M. M. Bamman and T. L. Clemens (2010). "Distinct growth hormone receptor signaling modes regulate skeletal muscle development and insulin sensitivity in mice." J Clin Invest **120**(11): 4007-4020.

McKinlay, S. M., N. L. Bifano and J. B. McKinlay (1985). "Smoking and age at menopause in women." Ann Intern Med **103**(3): 350-356.

Mera, P., K. Laue, J. Wei, J. M. Berger and G. Karsenty (2016). "Osteocalcin is necessary and sufficient to maintain muscle mass in older mice." Mol Metab **5**(10): 1042-1047.

Miniou, P., D. Tiziano, T. Frugier, N. Roblot, M. Le Meur and J. Melki (1999). "Gene targeting restricted to mouse striated muscle lineage." Nucleic Acids Res **27**(19): e27.

Mirza, M. A., A. Larsson, L. Lind and T. E. Larsson (2009). "Circulating fibroblast growth factor-23 is associated with vascular dysfunction in the community." Atherosclerosis **205**(2): 385-390.

Mo, C., S. Romero-Suarez, L. Bonewald, M. Johnson and M. Brotto (2012). "Prostaglandin E2: from clinical applications to its potential role in bone- muscle crosstalk and myogenic differentiation." Recent Pat Biotechnol **6**(3): 223-229.

Montgomery, E., C. Pennington, C. M. Isales and M. W. Hamrick (2005). "Muscle-bone interactions in dystrophin-deficient and myostatin-deficient mice." Anat Rec A Discov Mol Cell Evol Biol **286**(1): 814-822.

Moran, A. L., S. A. Nelson, R. M. Landisch, G. L. Warren and D. A. Lowe (2007). "Estradiol replacement reverses ovariectomy-induced muscle contractile and myosin dysfunction in mature female mice." J Appl Physiol (1985) **102**(4): 1387-1393.

NCBI. (2020, 05/05/2020). "Hc Hemolytic Complement[Mus musculus (house mouse)]."   Retrieved 05/15, 2020, from https://www.ncbi.nlm.nih.gov/gene/15139.

NIH (2001). "Osteoporosis prevention, diagnosis, and therapy " JAMA **285**(6): 785-795.

Nih Consensus Development Panel on Osteoporosis Prevention, D. and Therapy (2001). "Osteoporosis prevention, diagnosis, and therapy." JAMA **285**(6): 785-795.

Novotny, S. A., G. L. Warren and M. W. Hamrick (2015). "Aging and the muscle-bone relationship." Physiology (Bethesda) **30**(1): 8-16.

Nowlan, N. C., C. Bourdon, G. Dumas, S. Tajbakhsh, P. J. Prendergast and P. Murphy (2010). "Developing bones are differentially affected by compromised skeletal muscle formation." Bone **46**(5): 1275-1285.

Nowlan, N. C., J. Sharpe, K. A. Roddy, P. J. Prendergast and P. Murphy (2010). "Mechanobiology of embryonic skeletal development: Insights from animal models." Birth Defects Res C Embryo Today **90**(3): 203-213.

Panel, N. C. D. (2001). "Osteoporosis prevention, diagnosis, and therapy." JAMA **285**(6): 785-795.

Pedersen, B. K. (2011). "Muscles and their myokines." J Exp Biol **214**(Pt 2): 337-346.

Pedersen, B. K., T. C. Akerstrom, A. R. Nielsen and C. P. Fischer (2007). "Role of myokines in exercise and metabolism." J Appl Physiol (1985) **103**(3): 1093-1098.

Pobanz, J. M., R. A. Reinhardt, S. Koka and S. D. Sanderson (2000). "C5a modulation of interleukin-1 beta-induced interleukin-6 production by human osteoblast-like cells." J Periodontal Res **35**(3): 137-145.

Pollanen, E., P. H. Ronkainen, H. Suominen, T. Takala, S. Koskinen, J. Puolakka, S. Sipila and V. Kovanen (2007). "Muscular transcriptome in postmenopausal women with or without hormone replacement." Rejuvenation Res **10**(4): 485-500.

Quinn, L. S., B. G. Anderson, L. Strait-Bodey, A. M. Stroud and J. M. Argiles (2009). "Oversecretion of interleukin-15 from skeletal muscle reduces adiposity." Am J Physiol Endocrinol Metab **296**(1): E191-202.

Raue, U., D. Slivka, B. Jemiolo, C. Hollon and S. Trappe (2007). "Proteolytic gene expression differs at rest and after resistance exercise between young and old women." J Gerontol A Biol Sci Med Sci **62**(12): 1407-1412.

Regard, J. B., Z. Zhong, B. O. Williams and Y. Yang (2012). "Wnt signaling in bone development and disease: making stronger bone with Wnts." Cold Spring Harb Perspect Biol **4**(12).

Reginster, J. Y., C. Beaudart, F. Buckinx and O. Bruyere (2016). "Osteoporosis and sarcopenia: two diseases or one?" Curr Opin Clin Nutr Metab Care **19**(1): 31-36.

Riggs, B. L. and L. J. Melton, 3rd (1995). "The worldwide problem of osteoporosis: insights afforded by epidemiology." Bone **17**(5 Suppl): 505S-511S.

Ryoo, H. M., M. H. Lee and Y. J. Kim (2006). "Critical molecular switches involved in BMP-2-induced osteogenic differentiation of mesenchymal cells." Gene **366**(1): 51-57.

Shah, K., Z. Majeed, J. Jonason and R. J. O'Keefe (2013). "The role of muscle in bone repair: the cells, signals, and tissue responses to injury." Curr Osteoporos Rep **11**(2): 130-135.

Sharir, A., T. Stern, C. Rot, R. Shahar and E. Zelzer (2011). "Muscle force regulates bone shaping for optimal load-bearing capacity during embryogenesis." Development **138**(15): 3247-3259.

Sims, N. A. and J. M. Quinn (2014). "Osteoimmunology: oncostatin M as a pleiotropic regulator of bone formation and resorption in health and disease." Bonekey Rep **3**: 527.

Taaffe, D. R., S. Sipila, S. Cheng, J. Puolakka, J. Toivanen and H. Suominen (2005). "The effect of hormone replacement therapy and/or exercise on skeletal muscle attenuation in postmenopausal women: a yearlong intervention." Clin Physiol Funct Imaging **25**(5): 297-304.

Tagliaferri, C., Y. Wittrant, M. J. Davicco, S. Walrand and V. Coxam (2015). "Muscle and bone, two interconnected tissues." Ageing Res Rev **21**: 55-70.

Thomas, F., F. Renaud, E. Benefice, T. de Meeus and J. F. Guegan (2001). "International variability of ages at menarche and menopause: patterns and main determinants." Hum Biol **73**(2): 271-290.

Tiidus, P. M., D. A. Lowe and M. Brown (2013). "Estrogen replacement and skeletal muscle: mechanisms and population health." J Appl Physiol (1985) **115**(5): 569-578.

Utvag, S. E., K. B. Iversen, O. Grundnes and O. Reikeras (2002). "Poor muscle coverage delays fracture healing in rats." Acta Orthop Scand **73**(4): 471-474.

Vasconsuelo, A., L. Milanesi and R. Boland (2010). "Participation of HSP27 in the antiapoptotic action of 17beta-estradiol in skeletal muscle cells." Cell Stress Chaperones **15**(2): 183-192.

Vogt, P. M., P. Boorboor, B. Vaske, E. Topsakal, M. Schneider and T. Muehlberger (2005). "Significant angiogenic potential is present in the microenvironment of muscle flaps in humans." J Reconstr Microsurg **21**(8): 517-523.

von Maltzahn, J., N. C. Chang, C. F. Bentzinger and M. A. Rudnicki (2012). "Wnt signaling in myogenesis." Trends Cell Biol **22**(11): 602-609.

Walker, E. C., N. E. McGregor, I. J. Poulton, M. Solano, S. Pompolo, T. J. Fernandes, M. J. Constable, G. C. Nicholson, J. G. Zhang, N. A. Nicola, M. T. Gillespie, T. J. Martin and N. A. Sims (2010). "Oncostatin M promotes bone formation independently of resorption when signaling through leukemia inhibitory factor receptor in mice." J Clin Invest **120**(2): 582-592.

Wang, R. N., J. Green, Z. Wang, Y. Deng, M. Qiao, M. Peabody, Q. Zhang, J. Ye, Z. Yan, S. Denduluri, O. Idowu, M. Li, C. Shen, A. Hu, R. C. Haydon, R. Kang, J. Mok, M. J. Lee, H. L. Luu and L. L. Shi (2014). "Bone Morphogenetic Protein (BMP) signaling in development and human diseases." Genes Dis **1**(1): 87-105.

Warner, S. E., D. A. Sanford, B. A. Becker, S. D. Bain, S. Srinivasan and T. S. Gross (2006). "Botox induced muscle paralysis rapidly degrades bone." Bone **38**(2): 257-264.

WHO (1994). Assessment of fracture of risk and its application to screening for postmenopausal osteoporosis: report of a WHO study group [meeting held in Rome from 22 to 25 June 1992]. . WHO technical report series World Health Organization

Yavropoulou, M. P. and J. G. Yovos (2008). "Osteoclastogenesis--current knowledge and future perspectives." J Musculoskelet Neuronal Interact **8**(3): 204-216.

Zhang, X., W. Zhang, C. Wang, W. Tao, Q. Dou and Y. Yang (2018). "Sarcopenia as a predictor of hospitalization among older people: a systematic review and meta-analysis." BMC Geriatr **18**(1): 188.

Zhao, Q., X. Wang, Y. Liu, A. He and R. Jia (2010). "NFATc1: functions in osteoclasts." Int J Biochem Cell Biol **42**(5): 576-579.