Loss of *MEF2C* Expression in Osteoclasts Leads to a Sex-Specific Osteopenic Phenotype

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Abstract

Myocyte specific enhancement factor 2C (*Mef2c*) is a transcription factor studied in the development of skeletal and smooth muscles. Bone resorption studies have exhibited that reduced expression of *Mef2c* contributes to osteopetrosis and dysregulation of pathological bone remodeling. Our current study aims to determine how *Mef2c* contributes to osteoclast differentiation and to analyze the skeletal phenotype of *Mef2c*-cKO mice (*Cfms-cre;Mef2c*$^{fl/fl}$). qRT-PCR and western blot demonstrated that *Mef2c* expression is highest during the early days of osteoclast differentiation. Osteoclast genes, including c-*Fos, c-Jun, Dc-stamp, Cathepsin K*, and *Nfatc1*, had a significant reduction in expression along with a reduction in osteoclast size. Despite reduced CTX activity, female *Mef2c* cKO mice were osteopenic with decreased bone formation as determined by P1NP ELISA. There was no difference between male WT and *Mef2c*-cKO mice. Our results suggest *Mef2c* is critical for osteoclastogenesis, and its dysregulation leads to a sex-specific osteopenic phenotype.

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SECTION 1: INTRODUCTION OF CONCEPTS

Skeletal Biology Introduction

The skeleton is quite literally the foundation for human mobility, strength, and sustenance. Its complexities are the basis of pathology and physiology and exemplify the cornerstone of human health. The mere presence of certain aspects in configuration can define species and reveal mysteries of our own evolution. The classification between skeletal tissue can stem back to Aristotle who separated based on the presence of cartilage and bone.[1] After centuries of expansion and debate of taxonomy, Jean-Baptist Lamarck in 1801 introduced *animaux à vertebres* which was renamed to "Vertebrates" in 1866 by Ernst Haeckel.[2]

The human skeleton is made up of more than 200 bones. However, skeletal tissue is not limited to bones. It consists of four types of tissue: cartilage, bone, dentine, and enamel. Additionally, each tissue type can be broken down further based on their development, mineralization, and cell type.

Development of the Skeleton

The first major point of development in a human embryo is marked by gastrulation which happens around 3 weeks after fertilization and implantation. It is when a linear band of cells is reorganized into what is called the "primitive streak". Gastrulation signifies the integral stage named "lamination" where the three germ layers begin to form.[3] There is the ectoderm, the outermost layer that is superior to the mesoderm, which gives rise to the skin and nervous system, the endoderm, inferior to the mesoderm, which propagate into the linings of the digestive and respiratory system and form organs. Finally, the mesoderm, which lies between the endoderm and ectoderm, gives rise to the muscular and skeletal system.[4]

As the embryo advances its development through gastrulation, the "head to tail" orientation is established giving rise to the primitive node, this further establishes the distinct bilateral symmetry exhibited by all vertebrates. The primitive node then extends a tube in the opposite direction under the ectoderm and fuses with the endoderm to create

the notochord. The notochord is integral to the physical folding and compartmentalization of the developing embryo and molecular differentiation to create the neural tube.[3] By week ten, somitogenesis takes place which will lead to the development of the muscular and skeletal system. Somite "buds" are formed in the mesoderm.[5] As the neural tube begins to close, neural crest stem cells begin to migrate to the dorsal region of the embryo which will assist in developing the anterior region of the skull. This is the genesis of dentin of teeth, connective tissue, and bones and cartilages of the face and skull.[6,7] Somites help form the axial skeleton consisting of the skull and spinal cord while mesenchymal stem cells, also originating from the mesoderm, help form the appendicular skeleton. Mesenchymal cells either differentiate into chondrocytes, which will form cartilage, or into osteoblasts and osteocytes to directly form bone.[7]

Cartilage can also serve as an intermediate to bone formation. This process of mineralization from cartilage to bone is called endochondral ossification. Most bones develop through endochondral ossification and is tightly regulated via a plethora of transcription factors and extracellular signals. The overall schematic of this process consists of hypertrophic chondrocyte proliferation, secretion of cartilaginous matrix proteins, vascularization, growth plate development, and osteoblastogenesis.[8] Hypertrophic cartilage helps initiate vascularization which, in turn, allows osteoprogenitors and receptor activator of nuclear factor kappa-B ligand (RANKL) to begin the development of primary ossification center to generate the trabecular, sometimes referred to as "spongy", bone.[8] One of the primary methods of bone formation via endochondral ossification are osteoblasts. Osteoblasts are bone forming cells which use degraded cartilaginous matrix as a scaffold for bone. Mineralized bone is then formed and regulated via extracellular and nuclear factors.[9]

Another type of ossification is intramembranous ossification. Contrary to endochondral ossification, mesenchymal cells directly differentiate into osteoblasts and deposit bone. This mechanism is the primary method that flat bones and the bones of the skull are formed.[10]

Bones

There are four primary bone types: long bones, short bones, flat bones, and irregular bones. Examples of each include the femur, carpals in the hand, the mandible, and the coccyx at the base of the spine, respectively. Almost all bones are mineralized via endochondral ossification with the exception of flat bones which are primarily mineralized via intramembranous ossification.

Long bones are defined by their hollow shaft, referred to as the diaphysis, and flared ends called metaphysis. The diaphysis consists of thick, cortical bone. The density is indicative of the diaphysis metabolic activity. It is also thick to protect the marrow inside where immune cells, red blood cells, and stem cells are produced. Cortical bone, as well trabecular bone, is further made up of osteons, units whose functions can vary as vascularity, innervation, connective tissue, and osteoprogenitor cells[11]. Cortical osteons are referred to as "Haversian systems" and contribute to the function, activity, and development of the inner and outer surface of cortical bones called the outer periosteal surface and inner endosteal surface, respectively.[12] At the metaphysis, trabecular, or cancellous "spongy", bone reside. Here, a honeycomb like mesh of collagen fibers arranged in a lamellar pattern assist in bone cell production, nutrient and mineral exchange, and mechanistic "cushioning" occur.[11] Comparable to the Haversian systems, the osteons of trabecular bones are referred to simply as packets.[12] Short, flat, and irregular bones consist of different ratios of cortical and trabecular bone; however, their composition is ultimately determined by their primary function. Exemplifying this are flat bones, which provide more protection and support without contributing to much movement; therefore, they consist of more trabecular bone and are excellent areas of healing and osteoprogenitor development.[13]

Dentine and Enamel

Dentin and enamel are often overlooked regarding skeletal tissue types. However, their integral function, mechanism, and contribution to human health are unparalleled. The formation of both is intricate and begins during odontogenesis. Dentine begins to form when early mesenchymal cells interreact with epithelial cells to begin embryonic

pulp formation. This interaction leads to the development of pre-odontoblasts. Pre-odontoblasts closest to the basement membrane begin to differentiate into terminally polarized odontoblasts before other pre-odontoblasts in the pulp cavity. As the rest of the pre-odontoblasts begin to polarize, cytoskeletal proteins, microtubules, cilium, actin microfilaments, vimentin, and intermediate filaments containing nectin begin to develop into the microenvironment of the growing dentin layer. Additionally, junctional complexes and small molecules infiltrate to begin creating the extracellular matrix (ECM). As the ECM begins to mature, fibrous dentine layers consisting of collagen, phospholipids, minerals, and proteins being to mineralize and the dentine-enamel junction (DEJ) begins to be established.[14] Normal dentine is made of 40-45% hydroxyapatite and other minerals, 30% of organic matter, and 20-25% water, by volume.[15] The layer closest to the enamel and part of the dentine-enamel junction is the primary dentin layer. Closer to the pulp of the tooth there is secondary dentin, its primary function is to protect the pulp. Finally, there is a unique third layer called reparative or sclerotic dentin, here called tertiary dentin. It is formed as reactionary to external stimuli and only formed by odontoblasts that are stimulated. Stimuli can range from extensive destruction of dentin, damage to the pulp, bacterial metabolites, and toxins. Normal aging and wear contribute to the development of the tertiary layer of dentin and can incite hypersensitivity in clinical settings.[16]

Enamel is infamous for being one of the most durable tissue types in the human body due to its unrivaled dense mineralization. Enamel formation is called amelogenesis. The development of enamel can be broken down in two stages with a transition period in between each stage. The first stage is called the secretory stage in which polarizable cells called ameloblasts secrete structural enamel matrix proteins (EMPs).[17] At the DEJ, "pre-crystal" begin to form through "Tomes' process" in which exocytosing secretory vesicles deposit hydroxyapatite, carbonate, calcium, and other ions. When the first layer is formed, the ameloblast begin to move opposite of the DEJ creating rod-like structure of hydroxyapatite crystals. These complexes of secretory ameloblasts form a semipermeable membranes for intercellular diffusion of mineral ions to aid the circulation of EMPs.[18] The transition stage is short-lived and is signified by ameloblast apoptosis, resulting in

downregulation of Tomes' process and upregulation of ion transport, proteolysis, and pH homeostasis to allow the next stage to begin.[17] Much of this stage has only been revealed in the last decade due to advancements in technologies that can help quantify and visualize enamel maturation. This stage includes ameloblasts transporting mineralization and bicarbonate proteins along with morphological changes. Ameloblasts begin to change their appearance either taking on a "ruffled" (RA) or a "smooth" (SA) appearance. RA cells are characterized by distinct distal striations which limit the movement of small molecules into the enamel space; however, allowing ions to transport debris. Contrarily, SA cells allow bidirectional diffusion of small molecules into enamel space via "leaky" junctional complexes. As RA cells pump ions in and out of the enamel space, the pH is considerably lower. This allows hydroxyapatite to grow and mineralize, contributing to enamel's distinct density. The fine balance of pH balance, ions transport, and mineral incorporation enables enamel maturation stage and crystal formation and deposition.[19] This process can only happen once since the end of the maturation stage results in tooth eruption. Tooth eruption results in the apoptosis of any remaining ameloblasts.[17]

Osteoblasts

Despite the intricacies and complexities that consist of bone and skeletal biology, none would be possible without bone forming osteoblasts and bone resorbing osteoclasts. The strategic homeostatic balance between these two-cell types enables both the mechanical strength of bone but also the critical function that bone is implicated in. Dysregulation of this balance leads to pathology that is devastating to the host. High osteoblast activity can lead to osteopetrosis and rheumatoid arthritis.[20] High osteoclast activity can lead to osteopenia and osteoporosis. Osteoblasts are highly specialized cells that are mononuclear in appearance. They are derived from mesenchymal stem cells and respond to signals expressed by osteocytes for their recruitment. Osteocytes can signal osteoblast recruitment in response to microcracks, mechanical strain, hormonal cues, or even complete breaks. These signals come in the form of transcription pathways such as transforming growth factor-β (TGF-β) osterix (Osx), Runx-related transcription factor 2 (Runx-2), and Wnt pathway.[21]

Osteoclasts

The other half of integral bone remodeling is bone resorbing osteoclasts. Osteoclastogenesis is the process of osteoclasts being differentiated from hematopoietic stem cells. Osteoclasts are multinucleated, contrary to their osteoblast counterparts which are mononuclear. In addition, they are derived from monocyte/macrophages. Overactive osteoclasts can result in pathology that is detrimental to human health. Dysregulation of osteoclastogenesis can lead to osteoporosis, Paget's Disease, osteopenia, and osteosclerosis with osteolytic lesions.[22] Additionally, diseases of osteoclasts are primarily associated with post-menopausal women. In fact, more than one million women in the United States will suffer from osteoporotic fractures. The statistics reported are only of those who have been diagnosed or seeking treatments. There may be millions more who are suffering from osteoporosis or osteoclast related diseases who have not been diagnosed due to equity and access. Additionally, the growing acceptance and understanding of transgender individuals' health and utilization of hormone replacement therapy is a growing field where incidence rates and diagnostic criteria have yet to be established.[23] It is integral to investigate and elucidate the intricacies of osteoclast biology. Osteoclasts can be stimulated through a variety of mechanisms and factors.

SECTION 2: KEY REGULATORS

Macrophage Colony-Stimulating Factor (M-CSF)

Osteoclast signaling begins with macrophage colony-stimulating factor (M-CSF) being secreted from osteoblasts or bone marrow stromal cells. It is essential for osteoclastogenesis. M-CSF-deficient mice are deficient in monocytes, tissue macrophages, and osteoclasts and show osteopetrotic phenotypes.[24] M-CSF along with Receptor activator of NF-κB ligand (RANKL) are the two cytokines necessary and sufficient for osteoclast differentiation.[25]

Receptor Activator of Nuclear Factor NF-κB Ligand (RANKL)

RANK is the receptor that is recognized by RANKL and is part of the Tumor Necrosis Factor (TNF) super family. RANKL is highly expressed in osteoblasts, osteocytes, and activated T lymphocytes. It primarily binds to RANK on the surface of osteoclasts which leads to the terminal differentiation, proliferation, and activation of osteoclasts. Studies examining RANK and RANKL knockouts (KO) conclude that both RANK and RANKL KO mice have a severe osteopetrotic phenotype. It is important to mention that osteoprotegerin (OPG), which is expressed by osteoblasts and other stromal cells, is a soluble decoy receptor for RANKL. OPG capable of inhibiting RANK signaling.[26]

OPG

Osteoprotegerin (OPG) is part of the TNF receptor super family. It is also known as osteoclastogenesis inhibitory factor (OCIF) since it is a decoy receptor that binds to RANKL. Binding of OPG to RANKL downregulates osteoclast differentiation. OPG is a secreted glycoprotein synthesized by a variety of cell types including osteoblasts, lung cells, liver cells, and B lymphocytes in the bone marrow. Experiments manipulating expression of OPG has revealed that overexpression has resulted in significant osteopetrosis while knockout of OPG can cause severe loss of bone of mineralization and, in some cases, can be lethal during development.[26]

Genetic Mechanisms/Transcription Factors

While the aforementioned are key regulators of osteoclasts and osteoclastogenesis, there are still a plethora of mechanisms and transcription factors that contribute to functionality and phenotypic distinction of osteoclasts. Primarily, these factors provide signaling pathways that aid in the homeostatic balance between bone formation and bone resorption.

C-JUN and C-FOS

Activator protein 1 (AP-1) is a transcription factor and regulator of gene expression. It is a heterodimer composed of proteins. Integral subunit proteins that enable dimerization are part of the Jun and Fos family. Jun proteins can homodimerize and are efficient in binding to DNA alone; however, Fos proteins cannot. Fos proteins can only bind to DNA when dimerized with Jun. Additionally, Jun-Fos dimers are more stable and have a higher affinity for DNA binding than Jun homodimers.[27] Specifically, C-Jun and C-Fos are the mammalian proteins that implicated in this pathway. Multiple studies have demonstrated that osteoclast differentiation is inhibited in C-Fos knockouts, and the mice are osteopetrotic. Interestingly, C-Jun knockout mice reveal early embryonic lethality; supporting the fact that C-Jun can homodimerize and without it, osteoclastogenesis is lethally dysregulated. The primary role of C-Fos and C-Jun is to upregulate the expression of RANK in osteoclast precursors to induce osteoclastogenesis.[28]

NFATC1

Nuclear factor-activated T cells c1 (NFATc1) assists in regulating multiple osteoclast specific genes. It belongs to the NFAT family and is part of the NFAT2 subfamily. There is a total of five NFAT subfamilies. All NFAT subfamilies, excluding NFAT5, are regulated by calcium signaling pathways. NFATc1 is acted upon by the aforementioned C-Fos/C-Jun pathway early in osteoclastogenesis.[28] Interestingly, NFATc1 is unique NFAT protein in that it auto amplifies. NFATc1 is consistently recruited to its own promoter and acted upon by histone acetylases (HACs) and histone deacetylases (HDACs). Downregulation is just as tightly regulated as NFATc1's upregulation. Negative regulatory mechanisms include sufficient expression of M-CSF. Primarily, downregulation relies on epigenetic modification. HDAC5 greatly inhibits NFATc1 acetylation and reduces the stability and transactivation of NFATc1, which, in turn, attenuates RANKL induced osteoclast differentiation.[29]

Cathepsin K

Further reiterating the bone resorbing phenotype exhibited by osteoclasts, cathepsin K, part of the papain-cysteine protease family, is a protease secreted by osteoclasts that

digest type-1 collagen and is the major bone degrading enzyme.[30] Studies experimenting with the overexpression of cathepsin K has revealed that highly acidic bone microenvironment and results in severe osteoporosis; conversely, and as expected, under expression results in a osteopetrotic phenotype. Cathepsin K gene expression is stimulated by RANKL and activated by Nfatc1. Additionally, to counteract the effects of cathepsin K, OPG binds to RANKL to decrease protease secretion.[31]

DC-STAMP

Dendritic cell-specific transmembrane protein (DC-STAMP) is one of the primary master regulators of osteoclastogenesis. Experiments knocking out DC-STAMP in mice models has revealed that cell to cell fusion of osteoclasts is completely abrogated.[32] Additionally, upon recovery of osteoclasts from DC-STAMP deficient mice indicated they lack multinucleation and are phenotypically osteopetrotic. The cell to cell interplay that is mediated by DC-STAMP also enhances osteoblast and osteoclast coupling, initiating fracturing healing and bone remodeling.[32]

SECTION 3: THE MEF FAMILY

MEF2 Family

The myocyte enhancer factor 2 (MEF2) family is a robust family of transcription factors that assist in sending extracellular signals to the genome. They are implicated in various cellular programs including proliferation, differentiation, and morphogenesis in a variety of cell types. The MEF2 family is housed under the MCM1, agamous, deficiens, SRF (MADS) family of transcription factors; an evolutionarily ancient and conserved genetic regulatory network.[33] Vertebrates have four genes in the MEF2 family aptly name MEF2a, b, c, and d. MEF2 is primarily a transcriptional activator; however, it relies on the recruitment and co-expression of other transcriptional factors to drive expression of its target genes. Additionally, various post-translational and transcriptional mechanisms govern its function.

The four genes that make up the vertebral MEF2 family have distinct functions determined by tissue types, cellular machinery, environment cues, and co-expression of other transcription factors. However, the highest reported expression of these gene types are associated with lymphocytes, smooth, cardiac, and striated muscle cells, epithelial tissue, and bone. In adult tissue types MEF2 proteins act as sentinels for stress-response and remodeling machinery.[34]

The MEF2 family are also integral in the control of bone development and healing. During embryonic development, MEF2 protein drive chondrocyte hypertrophy for bone vascularization, mesenchymal stem cell differentiation, and endochondral ossification.[33] However, studies implicating the four gene types with osteoclastogenesis are far and few and between; moreover, our understanding of the MEF2 family in this regard is severely underdeveloped.

Briefly, our understanding of *Mef2a* and *Mef2d* and their role in osteoclast development is limited. Experiments where these two genes are knocked out have revealed that osteoclastogenesis is severely compromised. *Mef2a* and *Mef2d* have been shown to be integral monocyte differentiation into osteoclasts and lineage commitment into robust osteoclasts. Osteoclasts from *Mef2a* and/or *Mef2d* knockouts show impaired osteoclast differentiation. Interestingly, female *Mef2a*-KO mice are osteopetrotic while male *Mef2a* and *Mef2d*-KO mice of either sex had no significant skeletal phenotypic changes. This suggests a sex-specific regulation of osteoclasts by *Mef2a*. Even more interestingly, in vivo male *Mef2a* and *Mef2d* double knockout mice are osteopenic, indicating that while MEF2D is required for M-CSF and RANKL-stimulated osteoclastogenesis in vitro, osteoclasts can form in the absence of MEF2D in vivo via an unexplained RANKL-alternative pathway.[35]

Mef2c

The *Mef2c* gene is a significant factor implicated in embryonic skeletal development, osteoblast function, and bone formation. Furthermore, *Mef2c*'s role in muscular and cardiac cell types have been well studied. However, its role in osteoclasts and osteoclastogenesis is often overlooking and understudied. Recent advancements in its

understanding have revealed that *Mef2c* is a positive regulator of osteoclastogenesis and regulates bone remodeling. Additionally, it tightly regulates the expression of c-Fos and induction of NFATc1 is dependent on *Mef2c*.

The current understanding of the mechanism of *Mef2c* is under debate due to minimal review. However, the most widely accepted pathway is that *Mef2c* binds to FOS regulatory regions to induce c-FOS expression. This leads to the activation of NFATc1 and downstream osteoclastogenesis. Conditional knockout of *Mef2c* in mice resulted in increased bone mass under physiological conditions and protected mice from bone erosion by diminishing osteoclast formation. These mice become osteopetrotic and have reduced osteoclast activity.[36]

SECTION 4: LOSS OF *MEF2C* EXPRESSION IN OSTEOCLASTS

Deletion of Mef2c leads to smaller in vitro osteoclasts.

To determine the expression pattern of *Mef2c* during osteoclast differentiation, we performed a western blot using protein lysates extracted from bone marrow macrophages (BMM) from C-WT and C-KO mice. C-WT mice are wild type mice and are defined as *Mef2c-flox/flox* (*Mef2c*$^{fl/fl}$) mice. These mice were bred with *c-Fms-Cre* mice (*c-Fms-Cre*$^{+/Tg}$; to obtain *Mef2c*$^{fl/fl}$*–c-Fms-Cre*$^{+/Tg}$ (C-KO) mice. The protein lysates were cultured in M-CSF only (Day 0) or M-CSF and RANKL (Day 1 or 2) containing media. *MEF2C* is highly expressed in lysates from C-WT cells on Day 0 and Day 1. (Fig 2A). *MEF2C* expression was not detectable in lysates from C-KO mice on any of the days during differentiation (Fig 1A).

To determine if a change in osteoclast number was responsible for the osteopenic skeletal phenotype we observed by micro-CT, we cultured BMM's from female C-WT and C-KO mice. The skeletal phenotype was measured via bone volume over total volume (BV/TV), trabecular number, and trabecular thickness. We did not detect any significant difference in the number of TRAP-positive cells on day 2 or day 4 of RANKL treatment, indicating the number of osteoclasts did not differ between WT and C-KO mice (Fig 1B-C and F-G, respectively). Similarly, we did not detect any decrease in cell

number by DAPI staining of cell cultures from C-KO mice at either Day 0 or Day 2 compared to C-WT mice (Fig 1E, $p \leq 0.001$ and $p \leq 0.0001$). However, when comparing the size of osteoclasts, C-KO osteoclasts were smaller than C-WT osteoclasts after 4 days of RANKL treatment. (Fig. 1H, $p \leq 0.05$). The number of osteoclasts with 3 nuclei were significantly higher in the TRAP positive cells from the C-KO mice; however, there were no detectable cells with 20 or more nuclei as seen in the cultures from the C-WT mice (Fig 1I, $p < 0.05$). Lastly, we also determined expression of genes involved in osteoclast differentiation. We measured significant downregulation of all genes tested, suggesting that loss of *Mef2c* expression may regulate osteoclast differentiation at early stages through expression of *c-fos* and *c-jun* (Fig. 2).

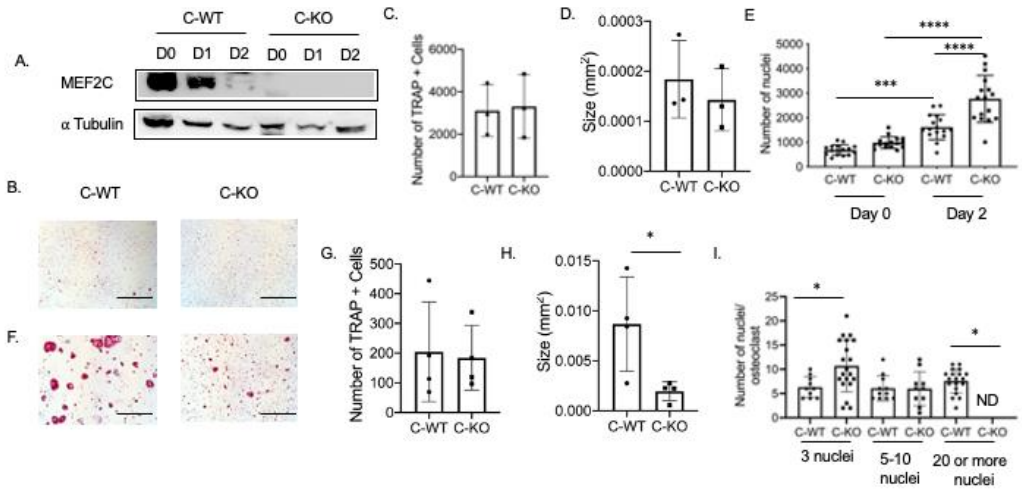

Figure 1. *In vitro* Osteoclasts from female C-KO mice are smaller than C-WT osteoclasts (A) Western blot of osteoclast lysates at day 0 (M-CSF only) or day 1 or 2 (M-CSF and RANKL) (B) Representative TRAP images at day2 (C) average number and (D) size of TRAP+ cells (E) number of nuclei at Day 0 and Day 2 (F) representative TRAP images at day 4. (G) average number and (H) size of TRAP+ cells (I) number nuclei/osteoclast at day 4. Scale bar = 0.5 mm * $P \leq 0.05$, ***$P < 0.001$ and ****$P \leq 0.0001$

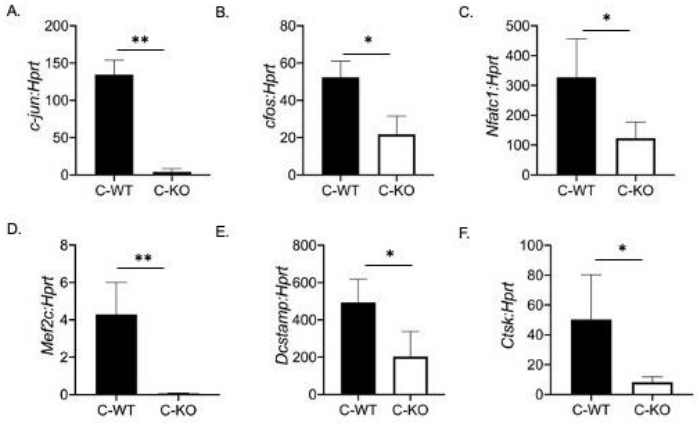

Figure 2. Osteoclasts from C-KO mice have reduced osteoclast gene expression. BMMs were isolated from C-WT and C-KO mice and cultured in M-CSF and RANKL for 2 days. RNA was isolated to measure gene expression by qRT-PCR. Relative expression of osteoclast marker genes normalized to WT controls against *Hprt* (A) *c-jun*, (B) *c-Fos*, (C) *Nfatc1*, (D) *Mef2c*, (E) *Dc-stamp*, and (F) *Cathepsin K* (*Ctsk)*. Bars show means ± SD. * $P \leq 0.05$, ** $P \leq 0.01$ C-WT vs C-KO.

In vivo osteoclasts are decreased in female C-KO mice.

Our *in vitro* phenotype of smaller osteoclasts did not explain the *in vivo* osteopenic skeletal phenotype we observed for our female C-KO mice. To determine *in vivo* osteoclast number and activity, we determined tartrate-resistant acid phosphatase (TRAP) and type 1 collagen (CTX) from the serum of our male and female C-WT and C-KO mice. Female C-KO mice had reductions in serum markers for osteoclast number (Fig 3A, $p \leq 0.05$) and activity (Fig. 3E, $p \leq 0.05$) and number and size of osteoclasts per bone surface by histology (Fig. 3B-D, $p \leq 0.05$). This *in vivo* data agreed with our *in vitro* data that loss of *MEF2C* expression leads to impaired osteoclast differentiation.

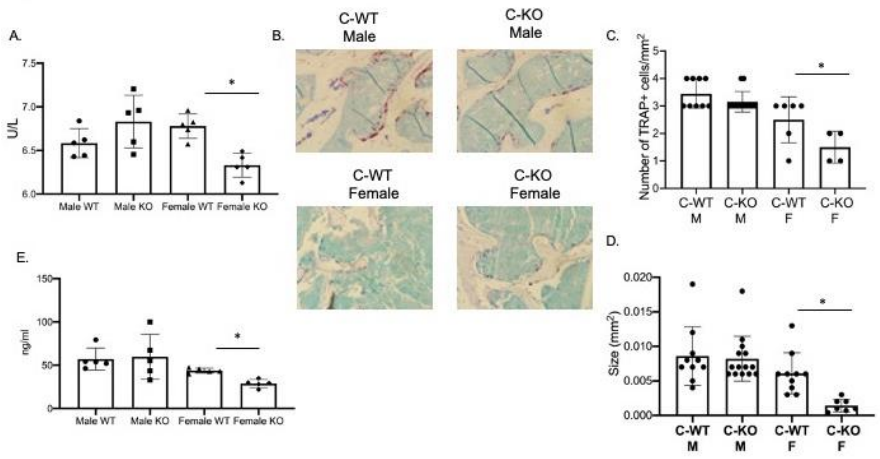

Figure 3. ***In vivo*** **osteoclasts are decreased in female C-KO mice**. Three-month-old C-WT and C-KO mice serum bone biomarkers were analyzed by ELISA. (A) TRAP ELISA (B) representative images of TRAP-stained sections of trabecular bone (C) number (D) size of *in vivo* osteoclasts and (E) CTX ELISA. Bars show means ± SD**.** Scale bar = 10 mm * $P \leq 0.05$ C-WT vs C-KO

Female C-KO mice have decreased bone formation

As the osteoclast phenotype did not explain the skeletal phenotype, we determined bone formation by ELISA analysis of the serum. P1NP, a marker of bone formation, was significantly decreased in the female C-KO mice (Fig 4A). With the decrease in P1NP, we measured cortical thickness by micro-CT. Micro-CT analysis revealed a significant decrease in cross-sectional thickness in female C-KO mice (Fig 4B and Sup Fig 1). We did not measure significant changes in any other cortical parameters (data not shown). Besides *MEF2C*'s expression in macrophages and osteoclast, *MEF2C* is also expressed in osteoblasts and osteocytes. We performed immunohistochemistry with bone sections from C-WT and C-KO mice which exemplified no changes in *MEF2C* expression in osteoblasts and osteocytes from C-KO mice. (Sup Fig 2).

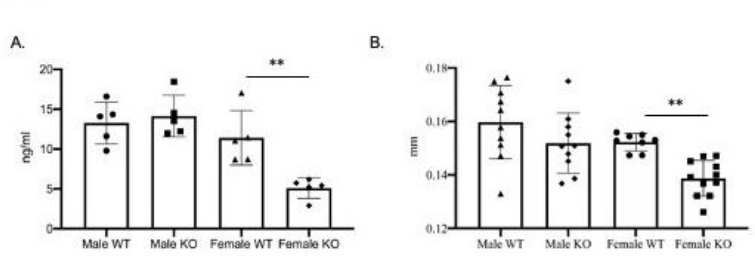

Figure 4. **Bone formation is decreased in female C-KO mice**. Three-month-old C-WT and C-KO mice were analyzed for cortical bone parameters by micro-CT. (A) ELISA analysis of P1NP as a marker of bone formation (B) Comparison of cortical thickness. ** $P \leq 0.01$ C-WT vs C-KO

SECTION 5: DISCUSSION OF PROJECT DATA

Mef2c and the MEF2 family have been extensively studied in myocytes and neural cells. Additionally, MEF2 factors are evolutionarily conserved and function collaboratively through various transcription factors and networks[34]. Few studies have investigated the implication of *Mef2c* and bone resorption despite its significance in bone healing and bone mineral density[37]. Here we demonstrate that female *Mef2c* cKO mice (*Cfms-cre;Mef2c*$^{fl/fl}$) are osteopenic compared to their wild-type (WT) littermates.
We begin by characterizing the skeletal phenotype of female and male *Mef2c*-cKO mice. The skeletal phenotype for C-KO females was significantly different from female C-WT and from their male counterparts. A previous study examining C-KO mice demonstrated an osteopetrotic phenotype of male C-KO mice; however, in our study, the C-KO females were osteopenic compared to their WT littermates[37]. Differences between the phenotype measured in the two studies may be due to the differences in the Cre-expressing mice used (*Mx1-Cre* for Fujii et al. and *Cfms-Cre* in our study). *Mx1-Cre* is an inducible system and targets hemopoietic cells, while *C-fms-Cre* targets macrophages, osteoclasts, and dendritic cells.

Surprisingly, we did not measure a significant change in number but in size of osteoclasts from female C-WT and C-KO mice, with the size of osteoclasts being significantly different on day 4. We were unable to identify osteoclasts with 20 or more nuclei in cultures from C-KO mice. This data along with no significant change in number of TRAP positive cells suggests that C-KO osteoclasts are not changed in their ability to form TRAP positive cells but in their ability to fuse into large multinuclear

cells. Furthermore, serum amounts for tartrate-resistant acid phosphatase (TRAP) and type 1 collagen (CTX), used to assess osteoclasts activity, were significantly lower. Given this *in vitro* phenotype, we would have anticipated having an osteopetrotic skeletal phenotype. However, *P1NP*, the N-terminal propeptide of type 1 collagen and a marker of bone formation, was reduced in our female C-KO mice compared to female C-WT mice. Our data suggest the osteopenic skeletal phenotype we determined in the female C-KO mice may be due to a decrease in bone formation.

Given the expression pattern of *MEF2C* during osteoclast differentiation, we hypothesize that changes in *MEF2C* expression may change secreted factors from either macrophages or preosteoclasts that regulate bone formation[26, 38-42]. Changes in secreted factors by macrophages or osteoclasts in C-KO mice may explain the osteopenic phenotype that we measured. The role of macrophages in regulating bone formation has been well studied demonstrating that macrophages are able to regulate bone marrow mesenchymal stem cells (BMSCs), osteoblasts and osteocyte activity[43]. Osteal macrophages, or osteomacs, are a specialized group of macrophages that support bone homeostasis[39]. The role of osteoclasts in regulating osteoblast activity has also been well studied. Osteoclasts have been shown to regulate osteoblast activity through soluble and membrane factors[44]. Recent studies with human biopsies treated with a single dose of Densomab, a humanized monoclonal antibody against RANKL, suggest osteoclasts' resorption exposes proteins in the extracellular matrix (ECM) that regulate bone formation[45]. Understanding the mechanism by which *MEF2C* expression in macrophages, preosteoclasts, and osteoclasts regulates bone formation is an interesting question but beyond the scope of this study.

Similar to our study with MEF2A, only female C-KO mice had a significant skeletal phenotype compared to their C-WT and male littermates[35]. Multiple studies have determined that estrogen is the primary hormone regulator of the skeleton in both men and women[46,47,48]. In previous studies looking at the role of MEF2 and class IIA HDACs in cardiac cells where estrogen has a cardioprotective effect, estrogen receptor alpha was shown to be a direct target of MEF2; however, the study did not specify which MEF2 factor[49]. We observe an osteopenic phenotype in our C-KO female but not male mice.

This study and our previous study of MEF2A and MEF2D in osteoclasts suggests that in osteoclasts, expression and or activity of the estrogen receptor may be regulated by MEF2. Further studies will be needed to elucidate the potential role of the MEF2 family of transcription factors and estrogen receptor expression in osteoclast precursors.

In summary, female *Mef2c* cKO mice (*Cfms-cre;Mef2c*$^{fl/fl}$) display an osteopenic phenotype compared to their wild-type (WT) littermates. As exemplified through our *in vitro* and *in vivo* data, traditional bone resorption markers between *Mef2c* cKO mice and WT littermates during osteoclast differentiation are significantly downregulated. Our study further suggests that *MEF2C* may regulate osteoclast and osteoblast coupling. In combination our data suggests that MEF2 family of transcription factors play a significant role in regulating osteoclast differentiation.

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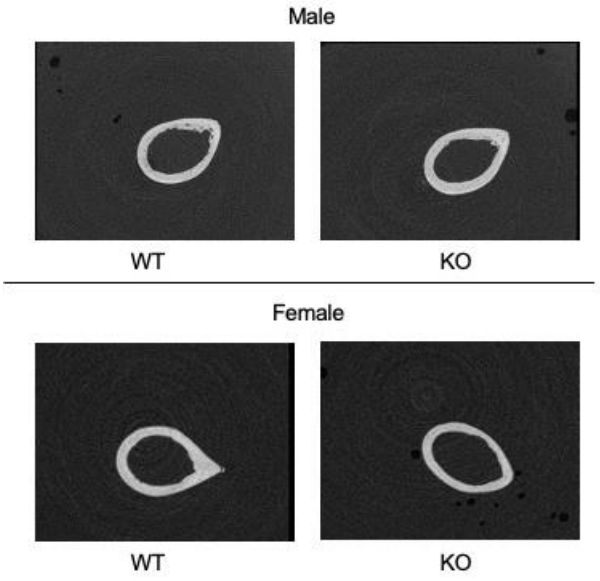
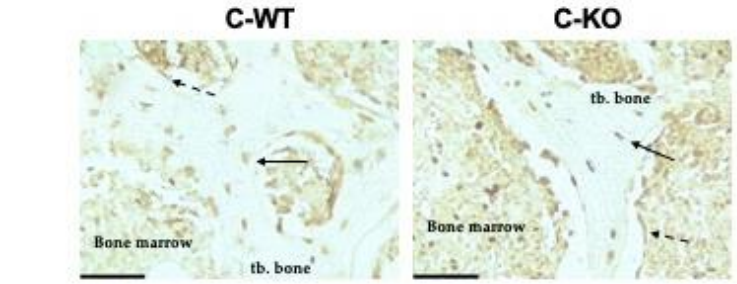
Appendices



Supplemental Figure 1. **Micro-CT of cortical bone.** Micro-CT analysis revealed a significant decrease in cross-sectional thickness in female C-KO mice compared to WT mice.



Supplemental Figure 2. **Osteoblasts and Osteocytes are unaffected by knockout.** immunohistochemistry with bone sections from C-WT and C-KO mice exemplify no changes in *MEF2C* expression in osteoblasts and osteocytes from C-KO mice.