THE EFFECT OF LSD1 AND HDAC1-2 ON OSTEOCLAST DIFFERENTIATION

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Dedication

I dedicate this thesis to my parents, Lisa and Jim and sister, Megan, who have been my source of tireless love and support throughout my 10-year academic journey at the University of Minnesota - first in the College of Biological Sciences, then in the School of Dentistry, and last but not least in the Department of Orthodontics. They were the ones who kept pushing me when I wanted to quit. Their encouragement has been the foundation of my success and I am forever grateful for the sacrifices they have made for my education.

This thesis is a tribute to their work-ethic and belief in me. I can only hope it serves as a testament to the positive impact they have had on my life.

I would also like to dedicate this thesis to my striping business, Schroeder Striping, for its success and ability to put me through school for all these years without the financial burden. For that, I am beyond grateful.

Abstract

Bone consists of several different cell types. Osteoblasts which form bone and osteoclasts which resorb bone. Bone remodeling occurs on a regular basis through coordination of osteoblast and osteoclast activity. Without it, our body's skeleton would not be able to sustain form and function. Little is currently known about the epigenetic regulation of osteoclast differentiation. Lysine specific demethylase 1 (LSD1) regulates gene expression by removing lysines on histone 4 or 9. The Mansky lab demonstrated that mice with a conditional deletion of LSD1 in macrophages/osteoclast have smaller osteoclasts due to increase in interferon-β (IFN-β) genes. LSD1 has been previously shown to interact with histone deacetylase complexes (HDACs). We hypothesized that LSD1 and HDAC1 and HDAC2 work together to regulate IFN-β regulated genes in osteoclasts. To test this hypothesis gene expression of IFN-β regulated genes was measured in the presence of HDAC inhibitors in osteoclast precursors from wild type (WT) and LSD1 deficient osteoclasts (KO). Expression of IFN-β target genes *Ifit1*, *Irf7*, and *Cxcl10* were measured by qRT-PCR. It was found that in the presence of HDAC inhibitors, IFN-β activates *Ifit1, Irf7 and Cxcl10* but is reduced compared to IFN-β alone. The regulation by HDACs 1 and 2 does not appear to be dependent on LSD1 as there is no difference in the trends between the LSD1 WT and cKO cells. More research is needed to better understand the role of epigenetic proteins that regulate osteoclast gene expression. LSD1 and HDAC inhibitor 1 and 2 have notable effects on genes that are upregulated by IFN-β, but the full understanding of these effects is not evident. This research should open the door to more elaborate studies of these epigenetic proteins to aid in better comprehension of how they regulate the bone remodeling process.

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Introduction

The human skeleton is the internal framework of the body. It is composed of over 200 bones that provide support, protection and proper function for the body's vital organs. The bones of the skeleton can be subdivided into two major categories, axial and appendicular.[7] One of the most important parts of the axial skeleton, the skull, protects our most valuable asset, the brain. The limbs form the appendicular skeleton, which provides mobility and support for the entire body.[7]

One of the most amazing aspects about bone is that it can store an immense amount of nutrients for our body's regulatory processes. Acting as a storehouse for important minerals such as calcium and phosphorus, bones are equipped to deliver nutrients into the bloodstream as well as acquire fuel from foods we eat. When these food sources are used up by bone, bone tissue regeneration can be promoted.[8]

Bone tissue regeneration, also known as bone modeling and remodeling, depends on the incorporation of these inorganic minerals into the bone through many dynamic processes.[8] Maintaining good skeletal health does not only depend on the things we can incorporate in our daily lives, such as nutrient-rich food intake and weight bearing exercise, it also relies heavily on our human biology, down to the cellular level.

Osteoclasts, osteoblasts and osteocytes are the three types of cells that play critical roles in the maintenance and regulation of bone tissue. We will take a deeper dive into what each of these

cells is capable of as well as how they fit into the dynamic process of bone remodeling. In adult humans, over 10% of the total bone content is replaced through this process in one year and about 1 million bone remodeling units (BRUs) are actively engaged in bone turnover at any given time .[9,10] This is a survival mechanism of vertebrates by which homeostasis of host tissue destruction by osteoclasts is balanced by bone formation by osteoblasts.[11] Without these mechanisms in place, skeletal pathologies and various bone diseases start to develop, such as osteoporosis.[11] In Table 1, osteoclast related bone diseases list the roles of osteoclasts in the disease, the mechanism by which this process occurs and the current therapies.[1] A common thread from the table is that excessive osteoclast production or osteoclast hyperactivity sparked by an overexpression of key regulatory factors, is the typical etiology.

Table 1. Osteoclast related bone diseases[1]

Osteoclast-related bone diseases	Osteoclast formation and function	Critical mechanisms	Current therapies and/or future targets
Osteoporosis	Excessive osteoclast formation and hyperactivated function	Estrogen deficiency, increase in RANKL levels resulting in excessive osteoclast formation and decreased bone formation	Bisphosphonates, calcitonin, estrogen replacement, SERMs, strontiumranelate, PTH peptides, RANKL antibody, sclerostin antibody
Periprosthetic osteolysis	Excessive osteoclast formation and hyperactivated function	Wear particles induce immoderate release of RANKL, resulting in excessive activation of osteoclasts	Bisphosphonates, revision surgery
Rheumatoid arthritis	Excessive osteoclast formation and hyperactivated function	Overexpression of RANKL resulting in excessive activation of osteoclasts MMP-9 and MMP-14 produced by osteoblasts	Immune inhibitors, TNF-α inhibitors, CSF-1R inhibitors, RANKL antibody
Bone tumors	Excessive osteoclast formation and hyperactivated function	Imbalance between RANKL and OPG levels in local bone tissue, resulting in excessive activation of osteoclasts	Bisphosphonates, RANKL antibody
Paget's bone disease	Excessive osteoclast formation and hyperactivated function	High-RANKL expression leading to osteoclast hyperactivity	RANKL antibody
Osteopetrosis	Impaired osteoclast formation and function	(a) Abnormality in RANKL/RANK/OPG system (b) Mutation of M-CSF factor (c) Mutation of V-ATPase subunit (d) Loss of CLC-7 chloride channels (e) Shortage of cathepsin K (f) Lack of c-Fos protein	Hematopoietic stem cell implantation

Osteoclasts are cells that are responsible for breaking down and resorbing bone tissue. They are large, multinucleated cells that are found on the surface of bone and are activated by various

intrinsic signals, such as parathyroid hormone (PTH) and interleukin-1 (IL-1), receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF).[12] Their main mechanism of action is to secrete enzymes and acids that dissolve the mineralized matrix of bone, thereby enabling the resorption of bone tissue. In contrast, osteoblasts are cells that are responsible for the formation of new bone tissue. They are found on the surface of bone and are activated by various signals, such as PTH and bone morphogenetic proteins (BMPs). Osteoblasts work by depositing a new mineralized matrix on the surface of bone, which hardens and strengthens the bone. Once osteoblasts have completed their work, they can become trapped within the mineralized matrix and differentiate into an osteocyte. They can also become a bone lining cell or undergo apoptosis.[13]

Osteocytes are the most abundant cells in bone tissue. They are formed from osteoblasts that get completely surrounded by a bony matrix called osteoid. When osteoid mineralizes, the cell is then referred to as an osteocyte. Osteocytes have long processes that extend into the surrounding matrix, which allows them to communicate with other cells and to sense changes in their environment.[14] They can be thought of as the orchestrators of the bone remodeling process because they are responsible for receiving a vast array of different signals that are communicated out to osteoblasts and osteocytes. By regulating the activity of osteoclasts and osteoblasts, they play a critical role in maintaining bone mineral homeostasis.[14] Figure 1 below shows the general remodeling process of bone, beginning with pre-osteoclasts and ending with mature osteocytes.[2]

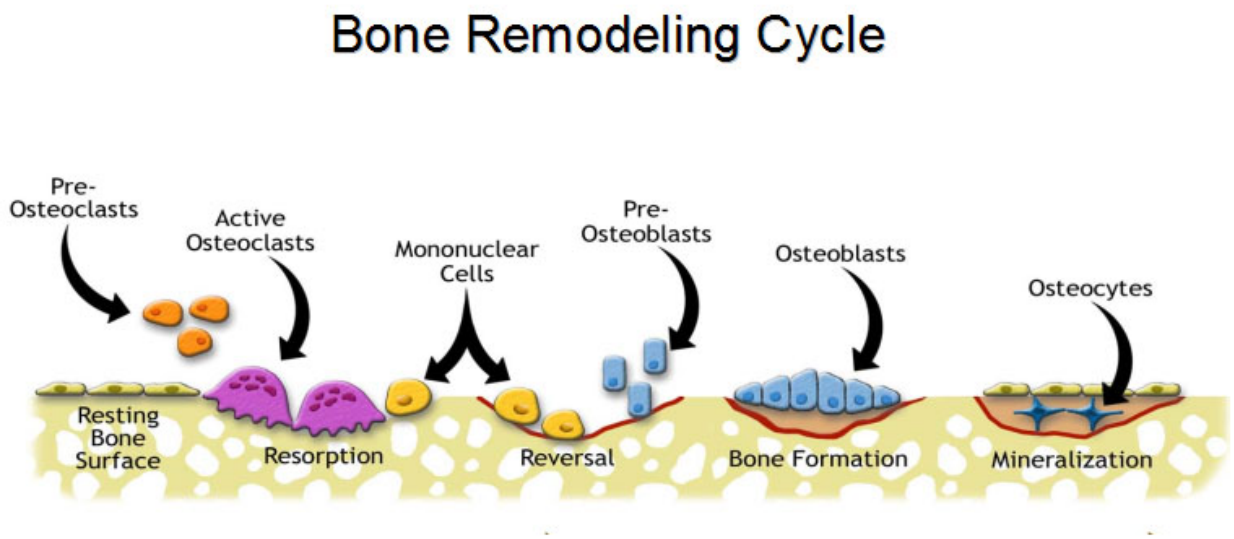

Figure 1. Bone remodeling process. There are 4 main phases depicted. Activation, resorption, reversal, and formation.[2] Once the osteoblasts are completely covered by osteoid, the matrix becomes mineralized and the cell is then referred to as an osteocyte.

Osteoclasts

Osteoclasts originate from the myeloid/monocyte lineage.[15] These monocytes travel great distances when they migrate from the bloodstream into bone tissue and then differentiate into osteoclasts. The process of osteoclast differentiation is regulated by many different signaling molecules and transcription factors; two of the most important ones being cytokines RANKL and M-CSF. These molecules bind to specific receptors on the monocyte surface, RANK and c-FMS respectively, and activate signaling pathways that lead to the expression of genes involved in osteoclast differentiation and bone resorption.[9] Figure 2 shows a schematic of how osteoclast precursor cells are regulated by various cytokines to differentiate into a multinuclear cell.[3] Signaling molecules play a role in many different biologically process. For instance, in

pathologic or inflammatory conditions, such as periodontitis and loss of alveolar bone, signaling molecules can enhance this RANKL mediated differentiation process.[16]

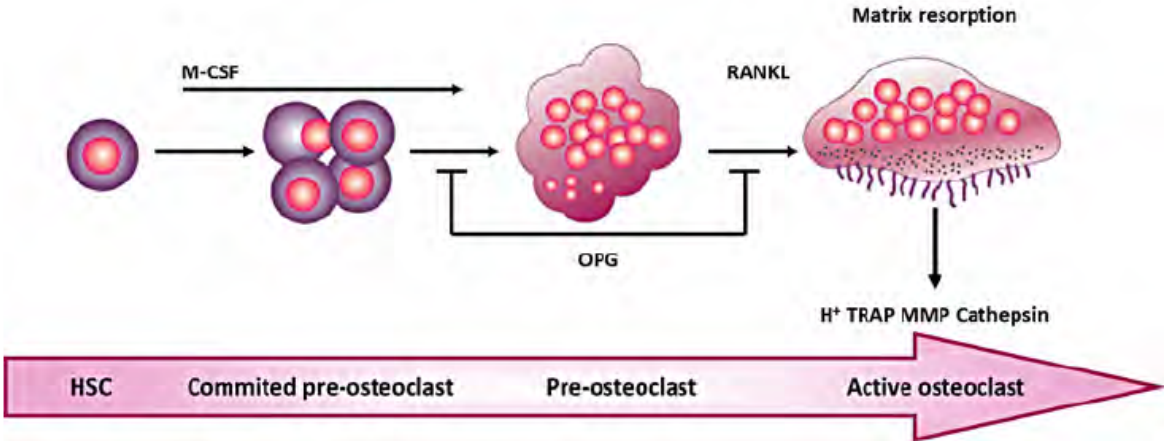

Figure 2. Key regulators in osteoclast differentiation. Osteoclast precursors can be induced to proliferate with addition of M-CSF and induced to differentiate with RANKL.[3]

Genes

Many genes are known to regulate differentiation of osteoclasts. These genes encode for proteins that further regulate this process, by binding of these proteins to the chromatin structure. One of the key regulators of osteoclast differentiation is the RANK/RANKL/OPG signaling pathway (Figure 2). This pathway involves the interaction between RANK on osteoclast precursors and its ligand, RANKL, on osteoblasts. In Figure 3 below, the action of a soluble decoy receptor osteoprotegerin (OPG) is depicted.[4] OPG is secreted by osteoblasts and the union of RANKL/RANK is competitively inhibited by it.[14] Thus, OPG inhibits osteoclastogenesis, and

therefore bone resorption. Binding of RANKL to RANK activates downstream signaling pathways, leading to the activation of transcription factors, such as nuclear factor of activated T cells (NFATc1). NFATc1 is a main driver and master regulator of osteoclast differentiation.[17] Transcription factors, such as this, regulate the expression of genes that are specific to osteoclast differentiation and function. For example, NFATc1 directly regulates the expression of genes such as *tartrate-resistant acid phosphatase* (*Acp5*), *cathepsin K* (*Ctsk*), and *matrix metalloproteinase 9* (*Mmp-9*), which are involved in bone resorption.

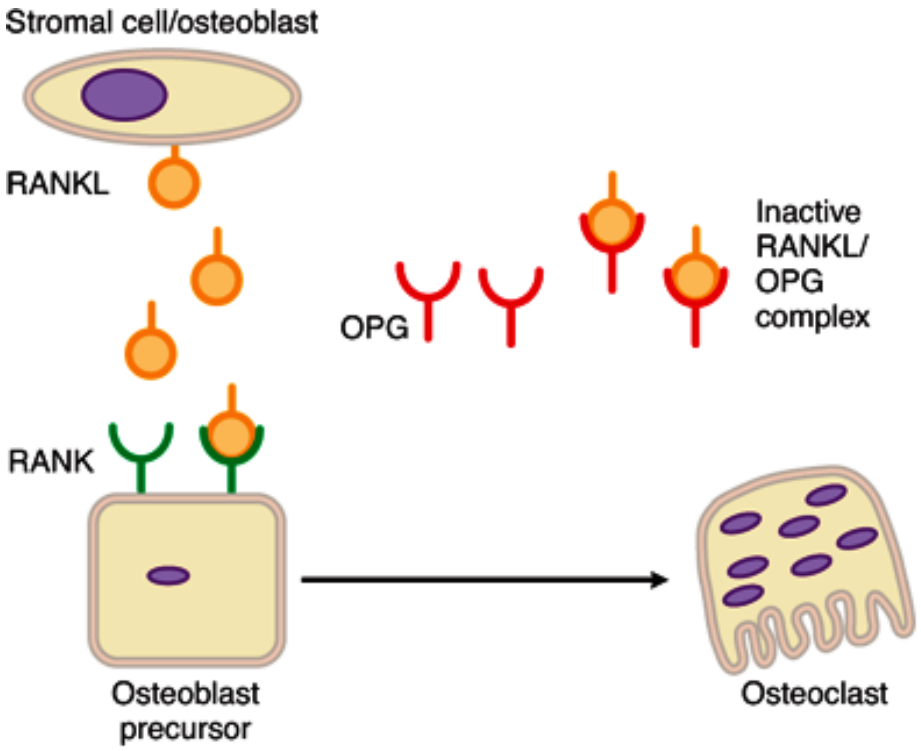

Figure 3. RANK/RANKL/OPG Pathway. The ratio of these will determine the amount of osteoclast differentiation.[4] The diagram depicts the competitive inhibitor OPG binding to RANKL to prevent osteoclastogenesis.

Transcription Factors

Transcription factors are proteins that bind to specific regions of DNA and regulate the expression of genes. Two important ones that we will touch on are C-FOS and NFATc1, both of which promote osteoclastogenesis. Transcription factors have the ability to work on their own or in tandem through complex signaling and regulatory mechanisms. For instance, NFATc1 is the master regulator of osteoclast differentiation and it is upregulated by C-FOS.[18]

Epigenetics and Osteoclasts

Epigenetics is the study of heritable changes in gene function that do not involve changes to the underlying DNA sequence; they regulate stable transcriptional programs, without causing any type of heritable alteration.[5] Epigenetic modifications, such as DNA methylation and histone modification, may influence the expression of genes and play a role in the development and function of osteoclasts.[19] One primary example of an epigenetic mechanism that is involved in osteoclast development is DNA methylation. Methylation of regions of the genome can suppress gene expression and certain genes that are important for osteoclast differentiation have been found to be methylated in monocytes. Figure 4 below shows how DNA methylation as well as histone modifications work to effect osteoclast differentiation.

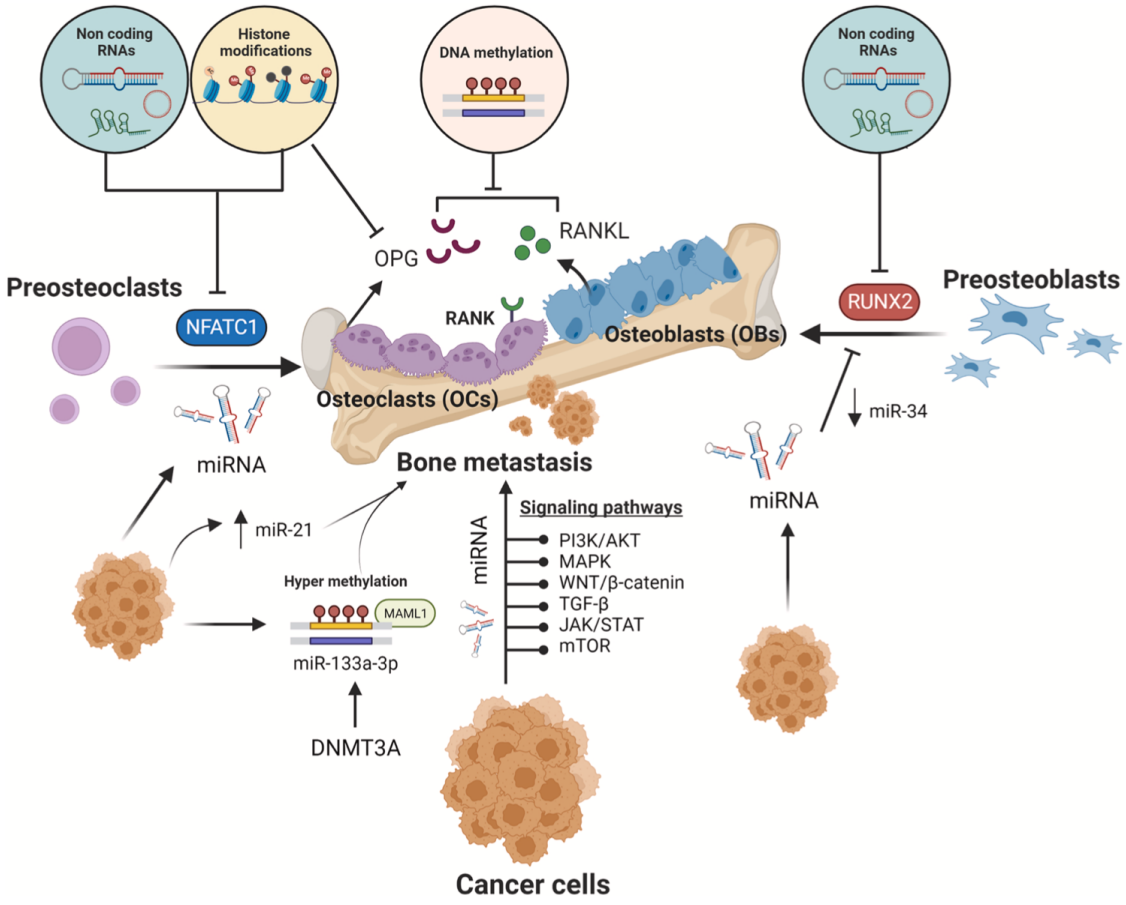

Figure 4. Epigenetic regulation of bone remodeling schematic.[5]

Histone modification provide another mechanism for epigenetic modifications to occur (Figure 4). Histones are the proteins that DNA is wrapped around to form chromatin and the manner in which these histones are modified can affect the accessibility of the DNA to the transcription machinery and hence affect the expression of genes. Acetylation, methylation, and phosphorylation are well known histone modifications that have been found to play a role in the regulation of osteoclast differentiation and function.[19] Genes such as *Nfatc1* (Figure 4) is tightly controlled by epigenetic mechanisms such as DNA methylation and histone modification during osteoclastogenesis.[20] Currently, there is research on creating drugs that regulate epigenetic

proteins, which may be used to treat regulate skeletal diseases.[21] Insights like this give real world examples of how understanding more about these epigenetic proteins will help to develop potential therapies.

LSD1

Lysine Specific demethylase 1 (LSD1) is a protein and transcriptional regulator that belongs to the family of enzymes known as histone demethylases.[22] Methylation or demethylation of histones can drastically affect the way DNA is packaged, making it more or less accessible to the transcriptional machinery.

As a powerful regulator of gene expression, LSD1 removes methylation groups from lysine 4 and 9 on histone 3 (H3) proteins. Depending on which methyl group is removed, genes are either activated or repressed. As a homodimeric protein, it contains two identical subunits and is composed of several domains. It has a FAD binding domain, an LSD1 domain and the SWIRM domain; the most important being the FAD binding domain at the N-terminal, which is what allows for its demethylation activity.[22]

LSD1 has been shown to play a role in the regulation of IFN-β genes in the context of osteoclast differentiation. IFN-β is a type of cytokine signaling protein that is produced by many cell types in response to viral or bacterial infection and it has been implicated in the negative regulation of osteoclast differentiation and bone resorption. It regulates inflammation after injury and therefore has anti-inflammatory effects. Ultimately, when IFN-β is high, osteoclast differentiation is low.

Through bulk RNA sequencing analysis comparing osteoclast precursors from LSD1-WT and LSD1 deficient, the Mansky lab determined that genes regulated by IFN-β were upregulated in cells from LSD1 deficient mice. This data suggests that LSD1 promotes osteoclast differentiation by down regulating genes involved in IFN-β pathway.[22,23]

IFN-β inhibits osteoclast differentiation by blocking the activity of the transcription factor C-FOS and by inducing the expression of the anti-osteoclastogenic cytokine IFN-gamma. It has been shown that IFN-β treatment reduces bone resorption in vitro and in vivo, suggesting that IFN-β has a protective effect on bone loss.[24,25]

LSD1 has been implicated in the development of periodontal disease, which is an inflammatory process that affects the tissues that surround and support the teeth.[26] Gingival tissues, periodontal ligament tissues, and alveolar bone can all be affected by periodontal disease and this can ultimately lead to bone loss, excessive tooth mobility and eventual edentulism. The osteogenic differentiation in human periodontal ligament stem cells (hPDLSC) has been shown to be delayed in patients with periodontitis.[26,30] hPDLSC's are essentially major mesenchymal stem cells that are derived from the oral cavity and they are responsible for things such as mediation of tissue repair, antigen construction and osteogenic differentiation of teeth that suffer from periodontal disease.[31] Histone demethylases, such as LSD1 have also been shown to be implicated in the realm of periodontitis by directly altering genes that are involved in periodontal growth and inflammation.[32] While we know how LSD1 works mechanistically, understanding

how this epigenetic protein works to regulate osteoclast differentiation and activity in these inflammatory conditions is still a poorly understood area of research.

HDACs

There are 18 different histone deacetylases (HDACs) within the human genome. HDACs are a family of enzymes that remove acetyl groups from histones, leading to a more condensed chromatin structure and reduced gene expression.[27] This research will be looking specifically at class I HDACs, HDAC1 and 2. HDACs play a role in bone development and skeletal maintenance and they have been shown to have roles in the ossification process. Prior research has found intramembranous bony defects associated with class I HDAC expression, using a mouse model.[28]

Recent studies from the Mansky lab as well as from others demonstrate that HDAC 1 and 2 and LSD1 interact with each other to regulate gene expression. Specifically, HDAC1 and HDAC2 have been shown to physically interact with LSD1 to form a complex that regulates gene expression through histone modification.[28,29] Although, the exact mechanisms by which HDACs and LSD1 regulated gene expression is not well understood and more research is needed to fully elucidate the molecular mechanisms underlying the interactions between these enzymes and their role in gene regulation. Figure 4 shows LSD1 and HDAC1's role in gene expression.[6]

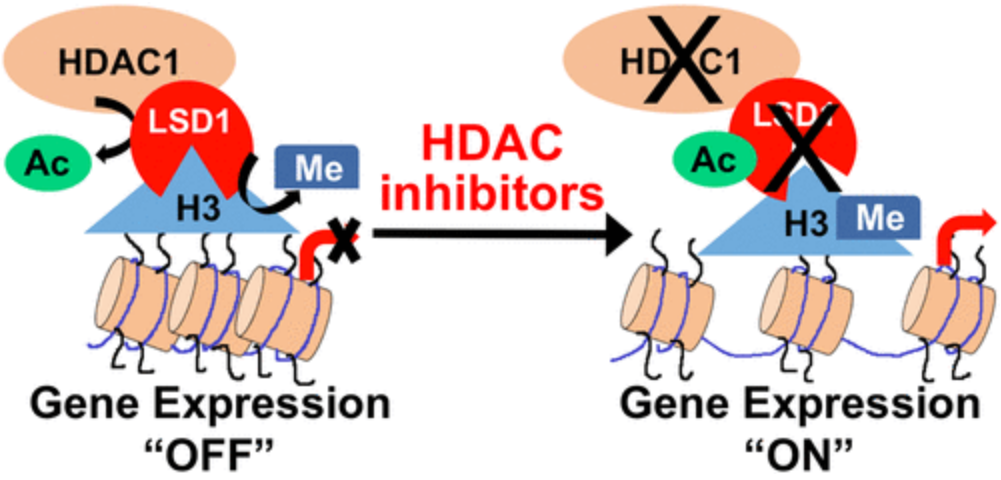

Figure 5. LSD1 and HDAC1's role in gene expression. Expression is turned on with knockout of LSD1 and introduction of HDAC inhibitors.[6]

Specific Aims

1) To measure gene expression of IFN-β regulated genes in the presence of HDAC inhibitors.
2) To measure gene expression of IFN-β regulated genes in the absence of LSD1 and the presence of HDAC inhibitors.

Hypothesis

Based on past research from the Mansky Lab demonstrating that LSD1 and HDAC1 and 2 can interact during osteoclast differentiation and that LSD1 deficient cells had an upregulation of IFN-β regulated genes, it was hypothesized that LSD1 and HDAC1 and 2 regulate IFN-β regulated genes in osteoclasts.

Material and Methods

Ethics

The use and care of mice used in this study were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee, IACUC protocol number 2104-39006A. Euthanasia was performed by CO2 inhalation.

Harvest and culture of primary osteoclasts from long bone marrow/Treatment with HDACi

Primary bone marrow macrophages were harvested from the femurs and tibiae of 2-month-old C57Bl/6 mice. Femurs and tibiae were dissected and adherent tissue was removed. The ends of the bones were cut, and the marrow was flushed from the inner compartments. Red blood cells were lysed from the flushed bone marrow tissue with RBC lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH7.4) and the remaining cells were plated and cultured overnight in 100 mm tissue culture dishes (TPP, MidSci) in osteoclast media (phenol red-free alpha-MEM (Gibco) with 5% fetal bovine serum (Atlanta Biologicals), 25 units/mL penicillin/streptomycin (Invitrogen), 400 mM L-Glutamine (Invitrogen), and supplemented with 1.5% CMG 14–12 supernatant (culture supernatant containing M-CSF, Dr. Sunao Takeshita, Nagoya City University, Nagoya, Japan). 24 hours later the non-adherent cell population, including osteoclast precursor cells, was then separated from the adherent cells and re-plated in 24-well plates (TPP, MidSci) at 2 x 10 4 cells/cm 2 in osteoclast media supplemented with 1.5% CMG 14–12 culture supernatant. Two days later cells were re-fed with 1.5% CMG 14–12 culture supernatant and 5 ng/mL RANKL (R and D Systems) to stimulate osteoclast differentiation.

Day 0 (just M-CSF) or day 2 (M-CSF + RANKL) cells were stimulated with IFN-β (Biotechne, 1.2 x10^5 U/mg) plus or minus HDAC inhibitors (Romidepsin and Tinostamustin, Selleck chemicals). Romidepsin was used at a concentration of 2.5nM and Tinostamustin was added at a concentration of 5nM.

RNA extraction and analysis

RNA was isolated from cells plated in triplicate using Trizol reagent (Ambion, Life Technologies) and quantified using UV spectroscopy. 1 μg of RNA was used to synthesize cDNA with the iScript cDNA synthesis kit (Bio-Rad) as per the manufacturer's protocol. Quantitative real-time PCR (RT-qPCR) was performed using the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad). Each 20 μl reaction contained 1 ul of cDNA, 10 μl of iTaq Universal SYBR Green Supermix, and 25 μM forward and reverse primers. The PCR conditions were as follows: 95°C for 3 minutes, and the 40 cycles of 94°C for 15 seconds, 58°C for 30 seconds and 72°C for 30 seconds, followed by melting curve analysis (95°C for 5 sec, 65°C for 5 sec and then 65°C to 95°C with 0.5°C increase every 5 seconds). Experimental genes were normalized to *Hprt*. All measurements were performed in triplicate and analyzed using the ΔΔCT method.

Real-time qRT-PCR

RNA was harvested from cells plated in triplicate using Trizol Reagent (Ambion, Life Technologies) and quantified using UV spectroscopy. cDNA was then prepared from 1 μg RNA using the iScript cDNA Synthesis Kit (Bio-Rad) as per the manufacturer's protocol. Quantitative

real-time PCR was performed in duplicate using the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad). Each 20 µl reaction contained 1 µl cDNA, 10 µl iTaq Universal Sybr Green Supermix and 500 nM forward and reverse primers. The PCR conditions were as follows: 95°C for 3 minutes, and the 40 cycles of 94°C for 15 seconds, 56°C for 30 seconds and 72°C for 30 seconds, followed by melting curve analysis (95°C for 5 sec, 65°C for 5 sec and then 65°C to 95°C with 0.5°C increase every 5 seconds). Primer sequences are listed in Table 2.

Table 2: Primers used for qRT-PCR

Gene and primer direction	**Primer name 5′-3′ sequence**
Ifit qPCR F	CAACTGAGGACATCCCGAAACA
Ifit qPCR R	ATGTGGGCCTCAGTTTCAAAGT
Irf7 qPCR F	GAGCGAAGAGAGCGAAGAGG
Irf7 qPCR R	GCCCCACAGTAGATCCAAGC
Cxcl10 qPCR F	ATTGCTGCCGTCATTTTCTG
Cxcl10 qPCR R	ATCCTCACTGGCCCGTCAT

Statistical Analysis

Graph Pad Prism Version 9 was used for the analysis of this data. One-way ANOVA followed by A Tukey multiple comparison test were conducted. All results were expressed in the form of significance using a p-value <0.05. All experiments were performed in triplicate.

Results

Target genes *Ifit1*, *Irf7* and *Cxcl10* were identified by bulk RNA-sequencing of day 0 and day 2 osteoclasts from Lsd1cKO mice. While these genes had been shown to be regulated by IFN-β in other cell types, it was necessary to confirm whether these genes were indeed regulated by IFN-β in osteoclasts. Each of these genes was tested using day 0 (M-CSF only) and osteoclast

precursors (2 days M-CSF and RANKL). As shown in Figure 6, treatment of day 0 or day 2 osteoclast precursors with IFN-β for 3 hours resulted in a significant increase in expression of *Ifit, Irf7 and Cxcl10* compared to mock (M-CSF and RANKL only), which was the control. From Figure 6, A-F shows statistical significance with $p<0.05$.

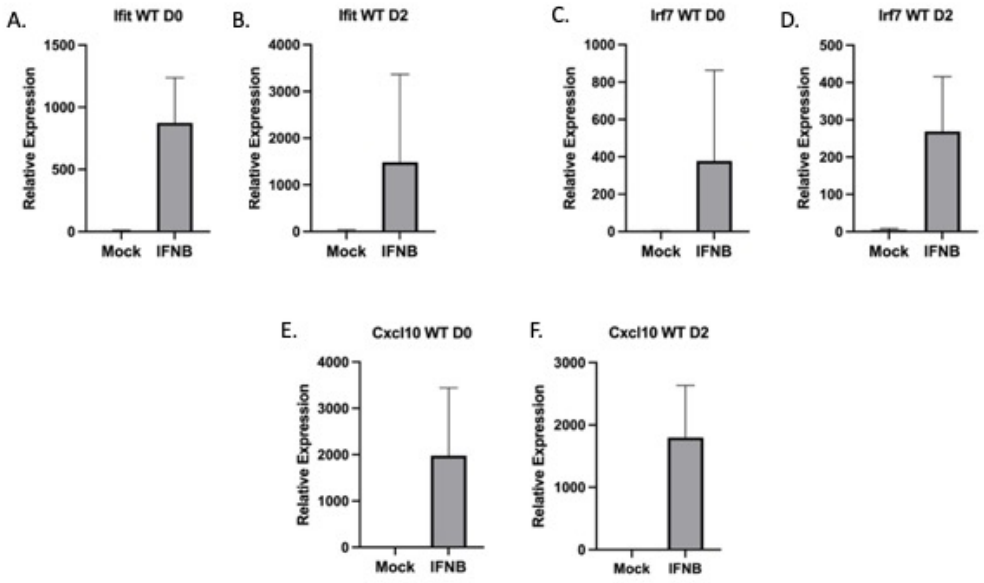

Figure 6. Gene Expression of *Ifit1, Irf7 and Cxcl10* in the presence of IFN-β in WT cells.

Next we measured gene expression of these same three genes using WT day 0 and day 2 cells in the presence and absence of HDAC inhibitors and in the presence and absence of IFN-β stimulation. HDAC inhibitors romidepsin and tinostamustine (EDO-S101) were used at a concentration that is selective for inhibition of HDAC1 and 2. From Figure 7, it is evident that in the presence of HDAC inhibitors, IFN-β activates *Ifit1, Irf7 and Cxcl10* but is reduced compared to IFN-β alone. Figure 8 shows this reduction in expression comparing IFN-β alone to IFN-β

plus HDAC inhibitors. Additionally from Figure 7, treatment with HDAC inhibitors alone did not increase expression of *Ifit1, Irf7 and Cxcl10* compared to mock in a statistically significant way.

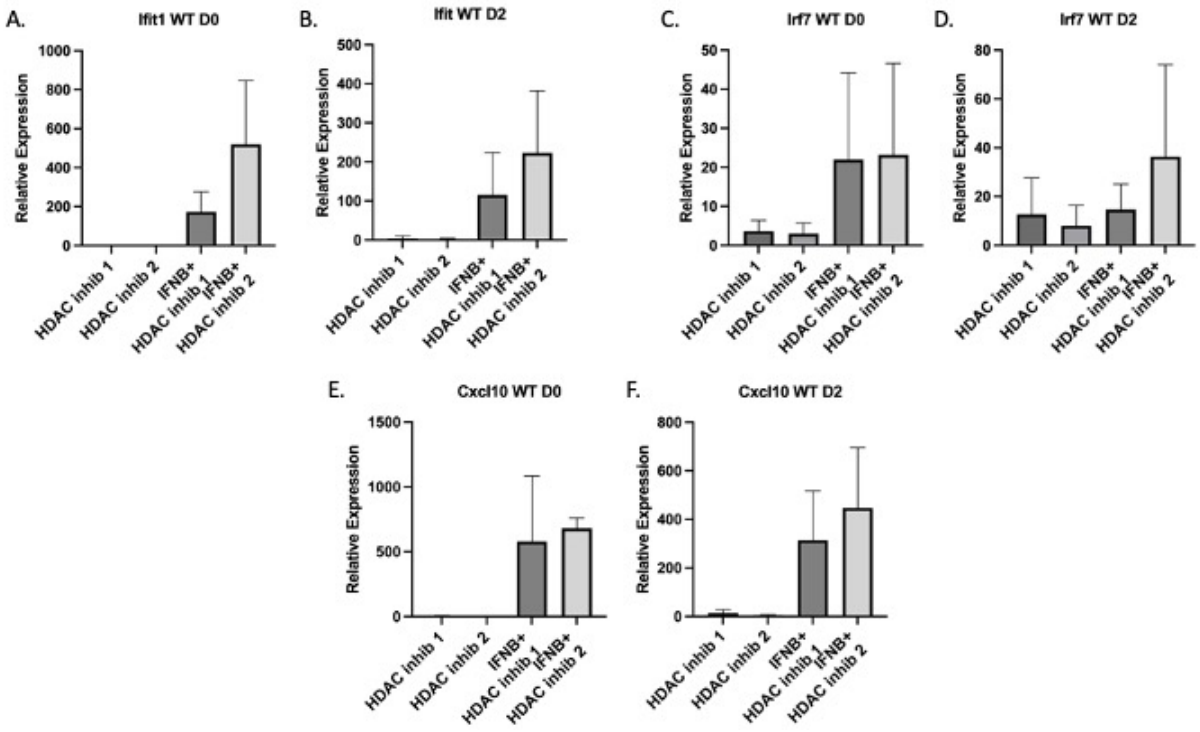

Figure 7. Gene Expression of *Ifit1, Irf7 and Cxcl10* in the presence of HDACi's in WT cells.

Comparing expression in the presence of IFN-β versus IFN-β plus the HDAC inhibitors, there is a significant drop in gene expression, which was not expected based on our hypothesis. This finding held up across in WT cells at both day 0 and day 2. Figure 8 shows these findings and the decrease in gene expression between IFN-β and IFN-β plus the HDAC inhibitors for both HDACi1 and 2 were statistically significant with $p<0.05$.

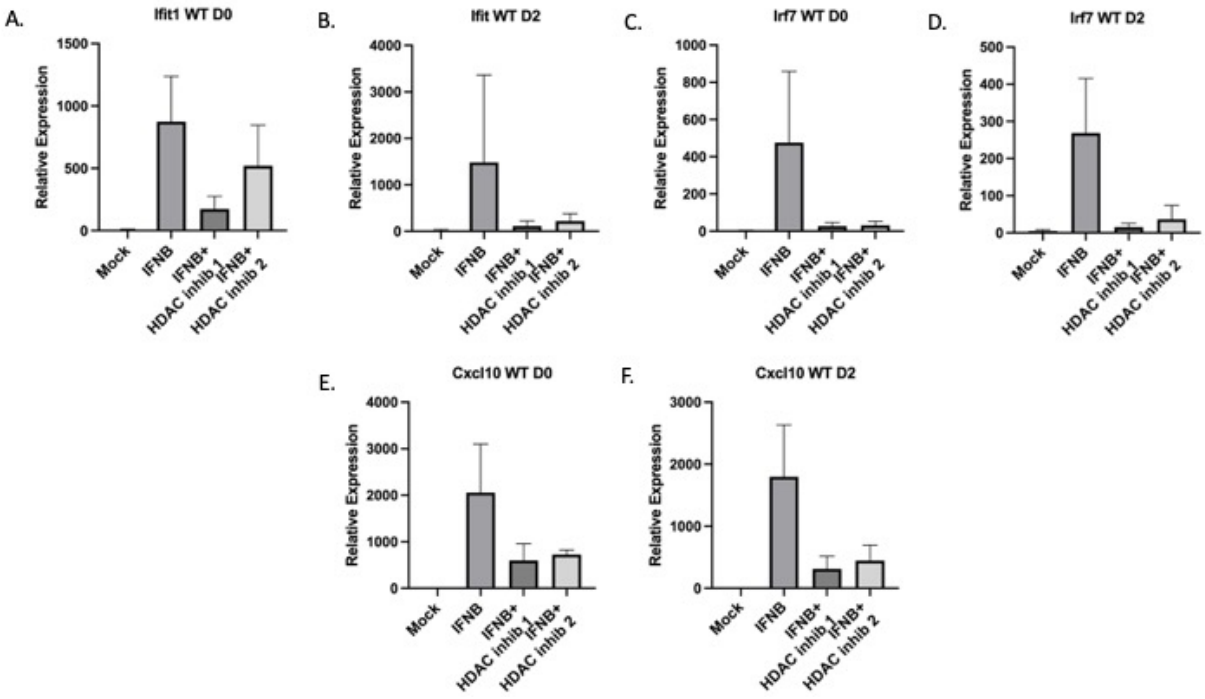

Figure 8. Gene Expression of *Ifit1, Irf7 and Cxcl10* in the presence of IFN-β versus IFN-β plus HDACi's for WT cells.

To determine if LSD1 was required for HDAC inhibition of IFN-β regulated genes, LSD1 deficient osteoclasts were treated with HDAC inhibitors to evaluate their effect on gene expression. Figure 9 shows the results of these experiments. Comparing Figure 9 to Figure 8, the KO cells seem to show a greater difference when comparing gene expression in the presence of IFN-β versus IFN-β plus HDACi's. The expression in the presence of HDACi's was significantly lower, which was not expected, based on our hypothesis.

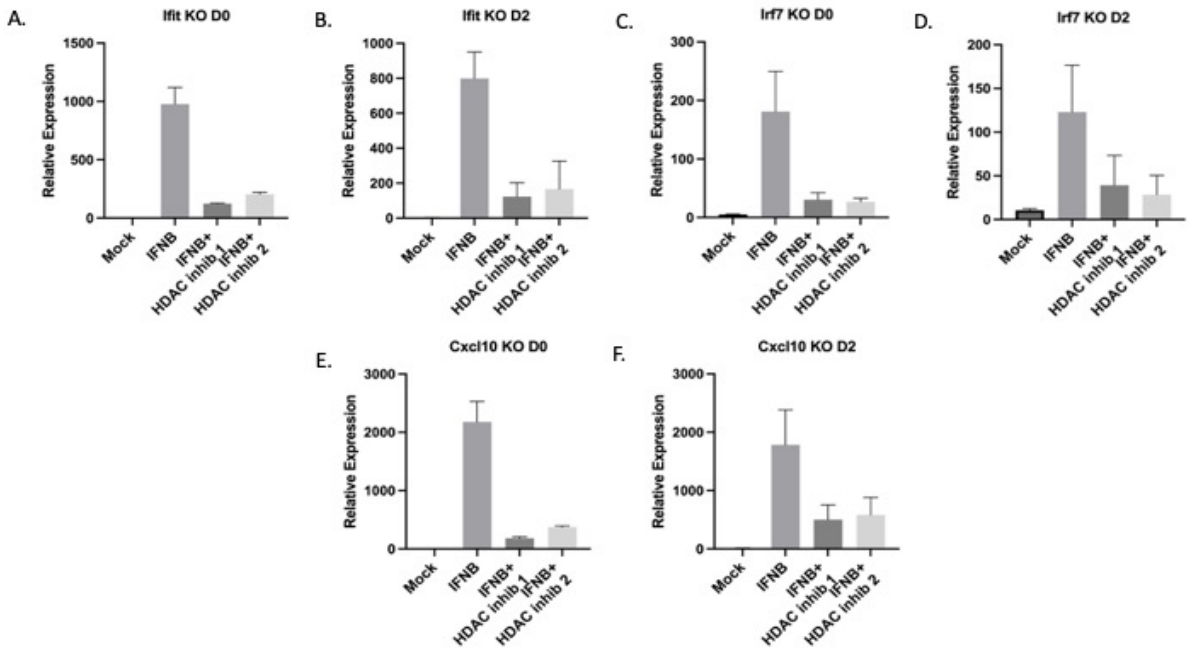

Figure 9. Gene Expression of *Ifit1, Irf7 and Cxcl10* in the presence of IFN-β versus IFN-B plus HDACi's for KO cells.

Discussion

Ifit1, *Irf7*, and *Cxcl10* are genes that are associated with antiviral immunity and are typically upregulated in response to viral infections. IFN-β is a cytokine that is produced by cells in response to viral infection and it has been shown to inhibit osteoclast differentiation and bone resorption through the regulation of c-FOS. The induction of interferon stimulated genes (ISGs) in osteoclast precursors may represent a mechanism by which the immune system regulates bone homeostasis during viral infections. Ours is the first study to demonstrate that these genes are upregulated by IFN-β stimulation of osteoclast precursors.

As one can see, osteoclast differentiation is an extremely nuanced process and regulatory control from transcription factors allow osteoclasts to respond to environmental cues. Epigenetic proteins allow for this nuanced regulation as changes in epigenetics are not permanent, but reversible. Besides the role of osteoclasts in maintaining bone homeostasis along with osteoblasts and osteocytes, osteoclasts also become active during pathological conditions such periodontal disease or rheumatoid arthritis.

Based on prior work completed in the Mansky lab, it was demonstrated that LSD1 interacts with HDAC1 and 2 by co-immunoprecipitation. This is the first reported case demonstrating that LSD1, HDAC1 and 2 interact during osteoclast differentiation. In other cell types it has been shown that LSD1, HDAC1 and HDAC2 interact to regulate gene expression. To test if LSD1, HDAC1 and HDAC2 regulated IFN-β genes, I treated day 0 and day 2 osteoclast precursors with

HDAC inhibitors plus and minus IFN-β stimulation. While I did determine an increase in IFN-β genes in the presence of HDAC inhibitors, it did not reach levels seen with IFN-β treatment alone.

Based on my hypothesis that LSD1 and HDAC1 and 2 regulate IFN-β regulated genes in osteoclasts, our data suggests that HDAC1 and 2 positively regulate the expression of IFN-β genes. The regulation by HDACs 1 and 2 does not appear to be dependent on LSD1 as there is no difference in the trends between the LSD1 WT and cKO cells, therefore my hypothesis is not supported. Figures 8 and 9 show that HDAC inhibitor 1 and 2 have effects on the expression of the genes of interest, but this effect is negative, when compared to IFN-β alone for both WT and KO. This observed decrease in the expression of *Ifit1, Irf7 and Cxcl10* in response to HDAC inhibitors 1 and 2 in the presence of IFN-β was unexpected given that IFN-β induces the expression of these genes. If my hypothesis was correct, treatment with inhibitors against HDAC 1 and 2 should further induce this upregulation.

There could be several explanations for this result. One possibility is that the HDAC inhibitors used in the experiment may have off-target effects that interfere with the expression of IFN-β gene expression. HDAC inhibitors are not perfectly selective and can have pleiotropic effects on gene expression. In essence, HDAC inhibitors can have wide ranging effects at the systemic and cellular level; this is the reason why they are currently used to treat a variety of disorders from things like hematopoietic malignancies to psychiatric disorders.[33] By impacting many different cellular pathways, HDAC inhibitors can create unexpected changes in gene expression. To test

the role of HDAC 1 and 2 without the side effects of inhibitors the Mansky lab needs to create conditional knockout mice in myeloid cells for HDAC1 or 2.

Another possibility is that the combination of IFN-β and HDAC inhibitors could have caused a negative feedback loop that led to a decrease in the expression of the genes of interest. This could have limited the duration and amplitude of the response that we were measuring. Negative feedback networks have been identified in BMP-9 osteogenic signaling, which provides insight into the complexities of these epigenetic proteins. [34] It is clear that interferon signaling pathways are complex and the induction of ISGs is subject to complex regulatory mechanisms. Further experimentation and analysis would be necessary to determine the underlying mechanisms responsible for the observed decrease in gene expression and to understand the implications of this result for the biological activity of the HDAC inhibitors and IFN-β.

Conclusion

Osteoclastic differentiation is an extremely nuanced process and much research is still needed to better understand the role of epigenetic proteins that regulate osteoclast gene expression. LSD1 and HDAC inhibitor 1 and 2 have notable effects on genes that are upregulated by IFN-β, but the full understanding of these effects is not evident. This research should open the door to more elaborate studies of these epigenetic proteins to aid in better comprehension of how they operate in the bone remodeling process. It is the hope that by fully understanding these mechanisms, future drug therapies to repress the negative effects of bone related diseases such as periodontitis may be developed with more precision.

References

1. Bi H, Chen X, Gao S, et al. Key Triggers of Osteoclast-Related Diseases and Available Strategies for Targeted Therapies: A Review. *Front Med*. 2017;4:234. doi:10.3389/fmed.2017.00234
2. Describe the process of bone remodeling - OrthopaedicsOne Clerkship - OrthopaedicsOne. Accessed April 30, 2023. https://www.orthopaedicsone.com/display/Clerkship/Describe+the+process+of+bone+remodeling
3. Fig. 5. Osteoclast differentiation, maturation and key factors... ResearchGate. Accessed April 30, 2023. https://www.researchgate.net/figure/Osteoclast-differentiation-maturation-and-key-factors-involved-Fully-mature-osteoclasts_fig4_224830042
4. Osteoprotegerin holds potential as a therapeutic agent for bone disorders. McGraw Hill's AccessScience. doi:10.1036/1097-8542.BR0412161
5. Sharma G, Sultana A, Abdullah KM, et al. Epigenetic regulation of bone remodeling and bone metastasis. *Semin Cell Dev Biol*. Published online November 2022:S1084952122002944. doi:10.1016/j.semcdb.2022.11.002
6. Nalawansha DA, Pflum MKH. LSD1 Substrate Binding and Gene Expression Are Affected by HDAC1-Mediated Deacetylation. *ACS Chem Biol*. 2017;12(1):254-264. doi:10.1021/acschembio.6b00776
7. McKinley M, O'Loughlin V, Harris R. *Human Anatomy*. Fifth. McGraw-Hill Education
8. Hong MH, Lee JH, Jung HS, Shin H, Shin H. Biomineralization of bone tissue: calcium phosphate-based inorganics in collagen fibrillar organic matrices. *Biomater Res*.

2022;26(1):42. doi:10.1186/s40824-022-00288-0

9. Asagiri M, Takayanagi H. The molecular understanding of osteoclast differentiation. *Bone*. 2007;40(2):251-264. doi:10.1016/j.bone.2006.09.023
10. Alliston T, Derynck R. Interfering with bone remodelling. *Nature*. 2002;416(6882):686-687. doi:10.1038/416686a
11. Karsenty G, Ferron M. The contribution of bone to whole-organism physiology. *Nature*. 2012;481(7381):314-320. doi:10.1038/nature10763
12. Veis DJ, O'Brien CA. Osteoclasts, Master Sculptors of Bone. *Annu Rev Pathol Mech Dis*. 2023;18(1):1160148605. doi:10.1146/annurev-pathmechdis-031521-040919
13. Neve A, Corrado A, Cantatore FP. Osteoblast physiology in normal and pathological conditions. *Cell Tissue Res*. 2011;343(2):289-302. doi:10.1007/s00441-010-1086-1
14. Tresguerres FGF, Torres J, López-Quiles J, Hernández G, Vega JA, Tresguerres IF. The osteocyte: A multifunctional cell within the bone. *Ann Anat - Anat Anz*. 2020;227:151422. doi:10.1016/j.aanat.2019.151422
15. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature*. 2003;423(6937):337-342. doi:10.1038/nature01658
16. Tsukasaki M. RANKL and osteoimmunology in periodontitis. *J Bone Miner Metab*. 2021;39(1):82-90. doi:10.1007/s00774-020-01165-3
17. Yao Z, Getting SJ, Locke IC. Regulation of TNF-Induced Osteoclast Differentiation. *Cells*. 2021;11(1):132. doi:10.3390/cells11010132
18. Huang H, Chang EJ, Ryu J, Lee ZH, Lee Y, Kim HH. Induction of c-Fos and NFATc1 during RANKL-stimulated osteoclast differentiation is mediated by the p38 signaling pathway. *Biochem Biophys Res Commun*. 2006;351(1):99-105.

doi:10.1016/j.bbrc.2006.10.011

19. Astleford K, Campbell E, Norton A, Mansky KC. Epigenetic Regulators Involved in Osteoclast Differentiation. *Int J Mol Sci*. 2020;21(19):7080. doi:10.3390/ijms21197080
20. Kurotaki D, Yoshida H, Tamura T. Epigenetic and transcriptional regulation of osteoclast differentiation. *Bone*. 2020;138:115471. doi:10.1016/j.bone.2020.115471
21. Shao C, Liu Y, Zhao Y, et al. DNA methyltransferases inhibitor azacitidine improves the skeletal phenotype of mild osteogenesis imperfecta by reversing the impaired osteogenesis and excessive osteoclastogenesis. *Bone*. 2023;170:116706. doi:10.1016/j.bone.2023.116706
22. Maiques-Diaz A, Somervaille TC. LSD1: biologic roles and therapeutic targeting. *Epigenomics*. 2016;8(8):1103-1116. doi:10.2217/epi-2016-0009
23. Lv L, Ge W, Liu Y, et al. Lysine-specific demethylase 1 inhibitor rescues the osteogenic ability of mesenchymal stem cells under osteoporotic conditions by modulating H3K4 methylation. *Bone Res*. 2016;4(1):16037. doi:10.1038/boneres.2016.37
24. Place DE, Malireddi RKS, Kim J, Vogel P, Yamamoto M, Kanneganti TD. Osteoclast fusion and bone loss are restricted by interferon inducible guanylate binding proteins. *Nat Commun*. 2021;12(1):496. doi:10.1038/s41467-020-20807-8
25. Weinstock-Guttman B, Hong J, Santos R, et al. Interferon-β modulates bone-associated cytokines and osteoclast precursor activity in multiple sclerosis patients. *Mult Scler J*. 2006;12(5):541-550. doi:10.1177/1352458506070605
26. Shao Q, Liu S, Zou C, Ai Y. Effect of LSD1 on osteogenic differentiation of human periodontal ligament stem cells in periodontitis. *Oral Dis*. 2023;29(3):1137-1148. doi:10.1111/odi.14066
27. Faulkner B, Astleford K, Mansky K. Regulation of Osteoclast Differentiation and Skeletal

Maintenance by Histone Deacetylases. *Molecules*. 2019;24(7):1355. doi:10.3390/molecules24071355

28. Singh N, Gupta M, Trivedi CM, Singh MK, Li L, Epstein JA. Murine craniofacial development requires Hdac3-mediated repression of Msx gene expression. *Dev Biol*. 2013;377(2):333-344. doi:10.1016/j.ydbio.2013.03.008
29. Haberland M, Mokalled MH, Montgomery RL, Olson EN. Epigenetic control of skull morphogenesis by histone deacetylase 8. *Genes Dev*. 2009;23(14):1625-1630. doi:10.1101/gad.1809209
30. Wu S, Liu W, Zhou L. MiR-590-3p regulates osteogenic differentiation of human mesenchymal stem cells by regulating APC gene. *Biochem Biophys Res Commun*. 2016;478(4):1582-1587. doi:10.1016/j.bbrc.2016.08.160
31. Turksen K, ed. *Cell Biology and Translational Medicine, Volume 5: Stem Cells: Translational Science to Therapy*. Vol 1144. Springer International Publishing; 2019. doi:10.1007/978-3-030-17589-4
32. Francis M, Pandya M, Gopinathan G, et al. Histone Methylation Mechanisms Modulate the Inflammatory Response of Periodontal Ligament Progenitors. *Stem Cells Dev*. 2019;28(15):1015-1025. doi:10.1089/scd.2019.0125
33. Hull EE, Montgomery MR, Leyva KJ. HDAC Inhibitors as Epigenetic Regulators of the Immune System: Impacts on Cancer Therapy and Inflammatory Diseases. *BioMed Res Int*. 2016;2016:1-15. doi:10.1155/2016/8797206
34. Hu N, Wang C, Liang X, et al. Inhibition of Histone Deacetylases Potentiates BMP9-Induced Osteogenic Signaling in Mouse Mesenchymal Stem Cells. *Cell Physiol Biochem*. 2013;32(2):486-498. doi:10.1159/000354453