Analyzing Loss of HDAC4 Expression: Effect on Osteoclastogenesis

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Dedication

I dedicate this work to my mom and dad; thank you for everything!

Abstract

Histone deacetylases (HDACs) are intracellular enzymes that directly affect chromatin structure and transcription factor activity. HDACs have become an important focus in research due to their role in many fundamental processes including differentiation, growth arrest and apoptosis. It has been demonstrated that skeletal formation is regulated by HDACs in their ability to repress transcription factors required in osteoblastogenesis and osteoclastogenesis. These findings have immense clinical implications for HDACs as therapeutic agents. In this study, I seek to understand more about the role of a specific HDAC during osteoclast differentiation. It has recently been shown that HDAC7, a Class IIa HDAC, suppresses osteoclastogenesis when over-expressed and that loss of expression increases osteoclastogenesis. These effects are opposite of those reported with loss of Class I HDAC expression and broad spectrum HDAC inhibitors. My experiments are the first to examine another Class IIa HDAC, HDAC 4 and its role during osteoclastogenesis. My results demonstrate loss of HDAC4 expression leads to enhanced osteoclast differentiation and activity. Furthermore in the absence of HDAC7, loss of HDAC4 expression leads to a further enhancement of osteoclast differentiation and activity. Overall my results demonstrate a role for HDAC4 in regulating osteoclast differentiation and activity unique from the role previously demonstrated for HDAC7.

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Background

Osteoclasts

Bone homeostasis is a complex and coordinated process that consists of the synthesis of mineralized bone by osteoblasts and the resorption of bone by osteoclasts. During the remodeling phase, the amounts of bone deposition by osteoblasts will equal the amount of bone resorption by osteoclasts in the skeleton. An imbalance between osteoblasts and osteoclasts can lead to pathology such as osteoporosis, Paget's disease and rheumatoid arthritis. Osteoporosis alone is widely recognized as a growing epidemic in our elderly population. Osteoporotic fractures cause significant morbidity and mortality as well as a large financial burden to individuals and society (1). By improved understanding of the mechanism of osteoclast differentiation and function, new therapies to treat bone-related diseases can be developed.

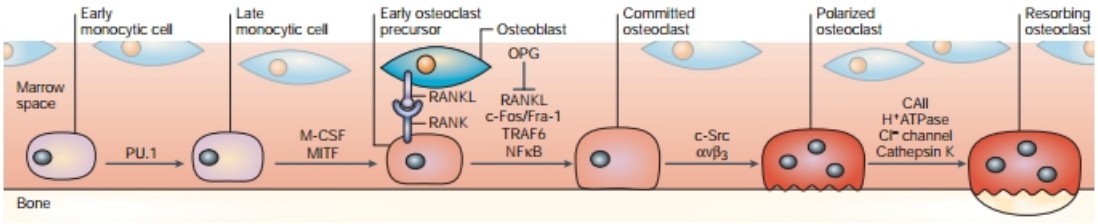

Figure 1: Regulation of osteoclast formation and function (2).

Osteoclasts are multinucleated cells that originate from monocyte/macrophage precursors. As shown in Figure 1, non-specific differentiation from a monocytic cell to an early osteoclast precursor is dependent on the PU.1 transcription factor, the MITF family of transcription factors, and the macrophage proliferation and survival cytokine (M-CSF). MITF targets genes necessary for osteoclast differentiation, including tartrate-resistant acid phosphatase (TRAP) and carbonic anhydrase II. M-CSF is expressed by osteoblasts as a membrane-soluble protein. The osteoclast precursor is then activated by the binding of RANK to its membrane-bound Receptor Activator of Nuclear Factor Kappa-B ligand (RANKL), which commits the cell to further differentiation as an osteoclast.

Until recently, the mechanism of how an osteoclast precursor is committed to its fate was not well understood. Suda et al. were the first to generate osteoclasts in vitro (3). They showed that to become an osteoclast, the cell must contact an osteoblast or a stromal cell precursor. RANKL, as mentioned above, is a member of the tumor necrosis family and is essential for osteoclast differentiation. Often expressed as a cell-surface protein by bone-marrow stromal cells, RANKL interacts with the RANK receptor on macrophages and osteoclast precursors. In mouse cells where RANK has been deleted, the mice do not form osteoclasts and are osteopetrotic, showing an increased bone mass and a lack of distinction between bone marrow space and cortex (4). Aside from playing a pivotal role in osteoclast differentiation, RANKL also stimulates resorptive activity and prolongs osteoclast survival (2, 5).

Histone Deacetylases (HDACs)

Histones are proteins that package and condense DNA into nucleosomes, their functional units. When condensed around histones, DNA is in its compact form (heterochromatin) and is not available for transcription. When histones are acetylated by histone acetyltranferases (HATs), positive charges from acetyl group cause DNA to disassociate from histones, allowing the DNA to become less tightly wound (euchromatin) and making the DNA template available for transcription. One role of histone deacetylases is to remove acetyl groups from the complex, which leads to condensation of the DNA around its histones and thereby negatively regulates gene expression.

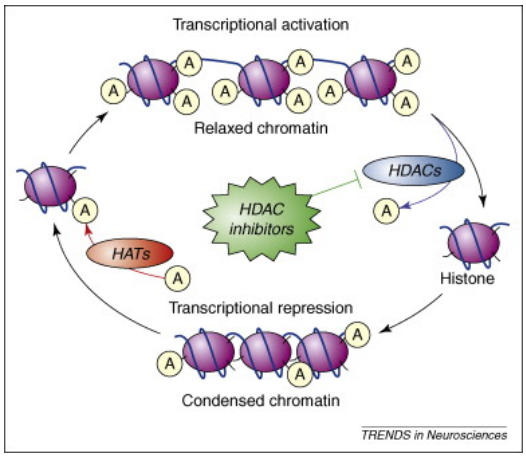

Figure 2: Illustration describing interaction between HDACs and HATs in regulating transcriptional activation (6). Acetyl functional group denoted by "A".

As described by the above mechanism, histone deacetylases (HDACs) are negative regulators of transcription and have been shown to induce specific changes in gene expression. Eighteen HDACs have thus far been identified and subdivided into two major groups. Class I HDACs (HDACs 1, 2, 3 and 8) are similar to each other in structure, are broadly expressed in most tissues, and are most often localized to cell nuclei (7). They function by removing acetyl groups in lysine residues within histones and other proteins. Class II HDACs (HDACs 4, 5, 6, 7, 9 and 10) travel between nucleus and cytoplasm when activated by specific kinase signaling pathways and are able to function independently of the deacetylase mechanism (7). Exactly how they function is not yet understood. Class II HDACs are divided groups a and b. This study focuses on Class IIa HDACs, which include 4, 5, 7, and 9 (8).

Class II HDACs demonstrate a more tissue-specific expression pattern, which suggests that they are involved in cellular differentiation and development systems. HDAC7, for example, shuttles from the nucleus to the cytoplasm during muscle cell differentiation in response to calcium signaling (CaMK)(9). It has been determined that the subcellular localization of HDAC7 is different from that of HDAC4 at various stages of muscle cell differentiation (10). Their distinct locations suggest that these HDACs complement each other, meaning their functions do not overlap during the regulation of gene expression during muscle cell development. This finding reveals an intricate system of signaling and co-repressors. It also raises questions about how gene expression is controlled in other cell types, such as in osteoclasts. This research project explores the role of class II HDACs during osteoclast differentiation.

HDACs and Regulation of Osteoclast Differentiation

There is still much to learn about the role HDACs play in osteoclast differentiation. Several studies have shown that broad-spectrum HDACs and Class I HDACs promote osteoclast differentiation, but less is known about Class II HDACs (7, 10, 11). A recent study by Jensen and Mansky et al. was one of the first to demonstrate that the *in vitro* loss of HDAC7, a class IIa HDAC, accelerated osteoclastogenesis and enhanced osteoclast formation. HDAC7 binds to and represses the transcriptional activity of MITF, which is a

transcription factor necessary for osteoclast differentiation. During osteoclastogenesis, combined stimulation of cytokines RANKL and M-CSF activate the MITF family and induce differentiation genes (12). Jensen and Mansky also examined osteoclast differentiation when HDAC7 was over-expressed. When cells were stimulated with RANKL, their differentiation into TRAP-positive multi-nucleated osteoclasts was impaired compared to controls. Expression of osteoclast marker genes reduced significantly reduced. These data demonstrate that HDAC7 acts as an inhibitor of osteoclast differentiation (12).

To further test the importance of HDAC7 expression during osteoclast differentiation, Stemig et al. showed that conditional deletion of HDAC7 in mouse osteoclasts leads to enhanced osteoclast formation, increased bone resorption and lower bone mass. In cells with reduced HDAC7 expression, the average size of TRAP-positive cells was increased. The average number of nuclei per multi-nucleated cell also increased, which decreased the total number of cells due to cell fusion (12). The phenotype of mice with HDAC7 knockout was significantly osteopenic. HDAC7 knockout in mice osteoclasts increased osteoclast activity, enhanced osteoclast function and caused progressive reduction in trabecular bone (12).

In 2011, a study by Kim et al. demonstrated that RANKL induces histone deacetylase-mediated NFATc1 acetylation and stability. NFATc1 is a transcription factor known to play a role in regulating the expression of TRAP and OSCAR, both of which are osteoclast-specific downstream target genes. By inducing NFATc1 acetylation and activation, RANKL increases the transcriptional activity of NFATc1 during osteoclast differentiation. The study also showed that the presence of HDAC inhibitors during osteoclastogenesis enhanced NFATc1 acetylation by RANKL. The converse was also true; HDAC5 inhibited NFATc1 acetylation. The authors concluded that by deacetylating NFATc1, HDAC5 acts as a negative regulator of RANKL-induced osteoclast differentiation (13).

Recently, Jin and colleagues published a study that investigated HDAC9's role in bone turnover. Their research shows that during RANKL-induced osteoclastogenesis, HDAC9 expression down-regulates and remains low. TRAP expression and other osteoclast differentiation markers were increased by RANKL stimulation. HDAC9 deletion accelerated *ex vivo* osteoclast differentiation, whereas HDAC9 over-expression had the opposite effect. HDAC9 knockout mice showed significantly higher bone resorption and lower bone mass. These findings again reinforce Class II HDACs as negative regulators of osteoclast differentiation. When adult mice received HDAC9-deficient bone marrow transplants, bone resorption increased. This finding led the authors to conclude that HDAC9's effects on osteoclast differentiation are mainly due to negative regulation of PPARγ (8). Despite the studies published thus far, we have much to learn about Class IIa HDACs, their mechanism of action and their role in cellular differentiation.

Destaing and colleagues published a paper in 2005 that investigated the role of HDAC6 during osteoclastogenesis. Their primary findings demonstrate a pathway in which the transcription factor Rho affects osteoclast differentiation via microtubule acetylation and actin organization mechanisms. By injecting activated Rho in osteoclasts, microtubule acetylation decreased due to the activity of HDAC6, which was confirmed by an *in vitro* deacetylase assay. The finding that Rho and HDAC6 regulate acetylated microtubule levels in osteoclasts provide further insight into the complex role HDACs play in osteoclast maturation (14).

Due to HDACs' extensive role during cellular development and effects on skeletal physiology, better understanding of their mechanisms and function is essential. The roles HDACs play in enzymatic pathways and differentiation make them potential therapeutic agents for an enormous range of clinical conditions including cancers, HIV/AIDS, asthma, cystic fibrosis and central nervous system disorders (7). Since HDACs regulate the differentiation and repression of both osteoclasts and osteoblasts, pharmaceutical HDAC inhibitors (HDIs) are being developed and tested to target osteoporosis and other bone disorders. Yi et al. found that HDIs lead to activation of apoptotic pathways in

osteoclast precursor cells (15). HDIs are now also known as anticancer agents and selectively suppress osteoclastogenesis in vitro (16).

HDIs are being studied as therapies for inflammatory osteocyte conditions like arthritis and periodontitis (7). Some reports cite that long term use of HDAC inhibitor valproic acid as an anticonvulsant in epileptic patients caused decreased bone density and increased fracture risk (7). The findings that HDACs 9 and 7 suppress osteoclast differentiation further supports the hypothesis that long-term use of Class IIa HDAC inhibitors increases the risk of bone loss side effects (11). Research in this area is conflicting and continued research will improve our understanding of the role of specific HDACs during osteoclast differentiation. Once we can identify the roles of specific HDACs, we can engineer more sophisticated HDAC inhibitors with specified therapeutic effects and fewer deleterious side effects.

HDAC therapy may eventually become clinically relevant even in orthodontics, since the growth of craniofacial structures and orthodontic movement of teeth are regulated by the apposition, resorption and remodeling of bone. Since osteoblast and osteoclast activity dictate these processes, what we learn about HDACs may eventually affect tooth movement and bone remodeling.

Hypothesis

The hypothesis of this research project is that osteoclasts with reduced expression of HDAC 4 will have enhanced osteoclastogenesis as measured by an increase in osteoclast size and osteoclast activity.

Specific Aims

In this study, wild type osteoclasts and osteoclasts null for HDAC7 expression will be infected with 2 different lentiviruses expressing shRNAs against HDAC 4. The specific aims are first to determine if the loss of HDAC 4 expression leads to an increase in osteoclastogenesis by analyzing the size and number of osteoclasts. The second specific aim is to determine if loss of HDAC 4 expression leads to an increase in osteoclast activity in HDAC7 knockout osteoclasts. Ultimately, we anticipate that individual HDACs, as evidenced by HDAC4, have independent roles in osteoclast differentiation.

Methods and Experimental Design

Harvesting of bone marrow and primary osteoclasts

Primary bone marrow macrophages were harvested from the femurs and tibiae of wild type mice or *HDAC7*$^{fl/fl}$*;LysM-Cre* mice. The ends of the bones were cut and the marrow was flushed. Red blood cells were lysed from the flushed bone marrow tissue with red blood cell lysis buffer, and the remaining cells were plated on 100 mM plates and cultured overnight in osteoclast media (phenol red-free alpha-MEM (Gibco) with 5% heat-inactivated fetal bovine serum (Hyclone), 25 units/mL penicillin/streptomycin (Invitrogen), 400 mM L-Glutamine (Invitrogen), and supplemented with 1% CMG 14-12 conditioned media containing M-CSF). The non-adherent cell population, including osteoclast precursor cells, was then separated and re-plated at approximately 100,000 cells per 24 well in the presence of 1% CMG 14-12. Typically tissue culture dishes with 24 wells were used in each round of the experiment. One tray was designation for TRAP staining and the other, a calcium phosphate coated plate, was used to analyze resorption.

Lentiviral Transfection

Lentiviral vectors encoding short hairpin RNA (shRNAs) against HDAC 4 (Open Biosystems) or a control shRNA, were used to produce replication defective lentivirus according to the manufacturer's protocols. Twenty-four hours after plating, lentivirus was added to the cells at 37°C in the presence of 1% CMG 14-12 conditioned media. Two

distinct lentiviruses were used in this study: each virus was added to 3 wells on each plate specific for reducing expression of HDAC4. Two distinct viruses were used to target HDAC4 to account for any off target effects of the shRNA. The same pattern for addition of virus was used in each round of experiment and is shown in Table 1. A control virus expressing scrambled shRNA (no effect on HDACs) was added to 3 wells to demonstrate the effect of virus infection on the osteoclasts. Three wells had no virus added to them, which proved the viability and successful differentiation of the osteoclasts.

Table 1 - Experiment Design*

WT Control shRNA	WT Control shRNA	WT Control shRNA	KO Control shRNA	KO Control shRNA	KO Control shRNA
WT HDAC4 shRNA #1	WT HDAC4 shRNA #1	WT HDAC4 shRNA #1	KO HDAC4 shRNA #1	KO HDAC4 shRNA #1	KO HDAC4 shRNA #1
WT HDAC4 shRNA #2	WT HDAC4 shRNA #2	WT HDAC4 shRNA #2	KO HDAC4 shRNA #2	KO HDAC4 shRNA #2	KO HDAC4 shRNA #2
WT NV	WT NV	WT NV	KO NV	KO NV	KO NV

*WT = wild type cells; KO = HDAC7 knockout cells, NV = no virus

The next day lentivirus was removed from the wells and cells were fed with 1% CMG 14-12 conditioned media and RANKL. Cells were fed every other day with RANKL and 1% CMG 14-12 conditioned media for 6 days.

TRAP Strain

Primary osteoclasts were fixed with 4% paraformaldehyde (PFA) and washed with PBS. The cells were then stained for tartrate resistant acid phosphatase (TRAP) expression with 5 mg tartrate, 0.5 mL Napthol AS-MX phosphate, M-Dimethyl formamide, 50 mL acetic acid buffer (1 mL acetic acid, 6.8 g sodium acetate trihydrate, 11.5 g sodium tartrate in 1 L water) and 25 mg Fast Violet LB salt. The cells were incubated for a minimum of 10 minutes and then captured with light microscopy. The measurements were analyzed using NIH Image J version 1.49.

Resorption Plates

The resorption plates used in this experiment were coated with calcium phosphate substrate to mimic bone matrix. To prepare the cells on these plates, the media was aspirated and 100 uL of 10% bleach was added to each well and incubated at room temperature for 5 minutes. The cells were then washed twice with H_2O, allowed to air-dry and then photographed with light microscopy. The measurements were analyzed using NIH Image J version 1.49.

Statistical Analysis

All experiments were completed three or more times. The results are expressed as a mean ±standard deviation. The computer program Prism version 5 will be used to calculate the student *t*-test and ANOVA analyses with a Tukey multiple comparison test.

Results

As discussed above, Mansky and Jensen *et al.* were among the first to establish that an individual HDAC specifically inhibits osteoclast differentiation. Their reports demonstrate that HDAC7 activity inhibits osteoclast differentiation by interactions with MITF. With this role of HDAC7 established, it raises questions regarding other HDACs in the Class IIa family. To address this question, we designed the present study to investigate the role of HDAC4 in osteoclastogenesis. By making use of lentiviral vectors that encode shRNAs against HDAC4, we tested the differentiation ability of osteoclasts expressing decreased HDAC4 expression. To ensure that any effects were due specifically to HDAC4 knockdown, two distinct shRNAs were compared against HDAC4. Infection of mouse bone marrow cells in culture with either HDAC4 shRNA #1 or #2 led to reduced HDAC4 mRNA expression by approximately 50% (Fig. 3) compared to the control shRNA.

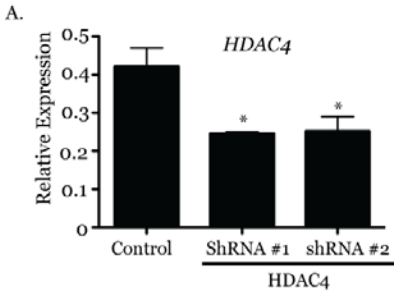

Figure 3. qPCR comparing expression of HDAC4 in osteoclasts infected with HDAC4 shRNA lentiviruses. Data shown are the mean + SD. Expression of HDAC4 is graphed relative to GAPDH. Samples were compared using one-way ANOVA * $p<0.05$

Suppression of HDAC4 augments osteoclasts differentiation

We then infected wild type mouse cells with two distinct shRNA viruses. Control shRNA cultures formed TRAP-positive osteoclasts upon stimulation with RANKL as expected. When compared with controls, osteoclasts showed enhanced differentiation in cells infected with either shRNA against HDAC4 (Fig. 4). Quantitative analysis of these cultures indicated that the number of cells increased significantly when compared to controls in both HDAC4-shRNA cultures (approximately 2.5 fold, Fig. 4B). Further analysis revealed that the size of TRAP-positive cells increased significantly in HDAC4-shRNA #2 cultures compared to controls (3 fold, Fig. 4C).

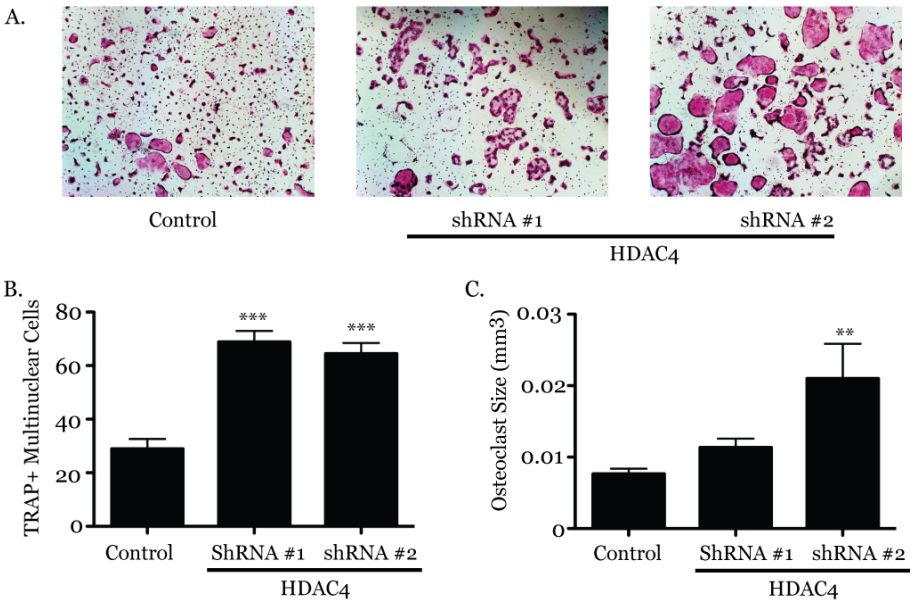

Figure 4: Suppression of HDAC4 augments osteoclast differentiation (A) Representative image of TRAP stained osteoclast cultures infected with control or HDAC4-shRNA expressing lentiviruses (B-C) Histomorphic analysis of TRAP-stained osteoclasts, *$p \leq 0.05$ vs. control shRNA WT cells infected with shRNA #1 and #2

Suppression of HDAC4 increases resorption

We also infected wild type mouse cells with HDAC4 shRNA viruses #1 and #2 and plated the cells on calcium phosphate substrate-coated trays. To quantify osteoclast activity, we compared three different values: total pit number per well, the average pit size, and the total percent absorbed per well. In shRNA #2 infected cells, the average pit size (approximately 2 fold) and the percent area absorbed (3 fold) increased significantly when compared with controls (Fig. 5). ShRNA #1 infected cells did not show significant differences in any of the three categories measured compared to control infected cells.

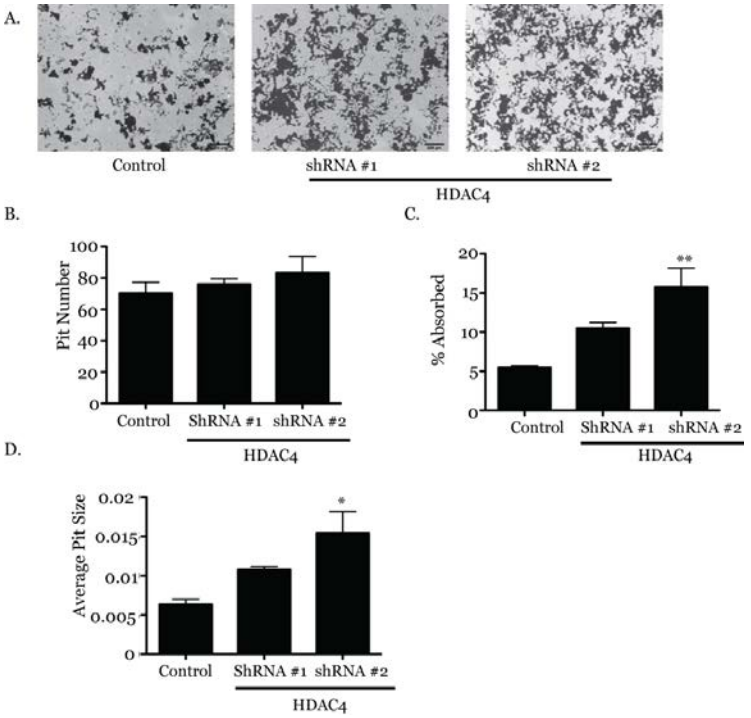

Figure 5: Suppression of HDAC4 increases resorption (A) Representative image of calcium phosphate well infected with control or HDAC4-shRNA expressing lentiviruses (B-D) Quantification of resorptive activity, *$p \leq 0.05$ vs. control shRNA WT cells infected with shRNA #1 and #2

Previous studies demonstrated that multiple class IIa HDACs are expressed in muscle cells and that they complement each other in the regulation of muscle cell gene expression. To add further evidence that class IIa HDACs function independently in osteoclasts, we repeated the experiments described above in *HDAC7$^{fl/fl}$; LysM-Cre* mice (i.e. osteoclasts null for HDAC7 expression). We found that osteoclasts null for HDAC7 infected with shRNA against HDAC4 had increased differentiation as visualized via

TRAP-staining (Fig. 6). Quantitative analysis of these cultures indicated that both the number (increased 2.5 fold) and the size (increased 4.5 fold) of the TRAP-positive cells increased significantly in HDAC4-shRNA #1 and #2 cultures compared to controls (Fig. 6B-C).

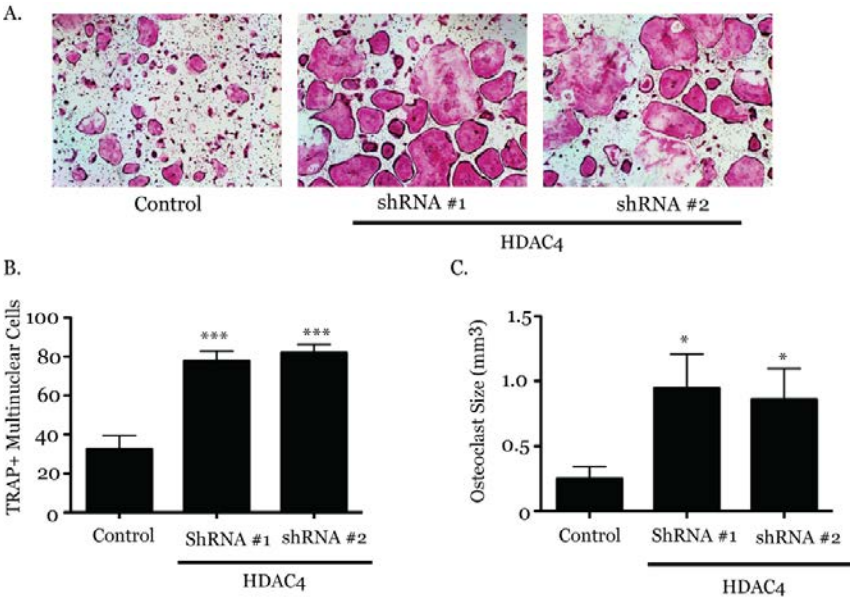

Figure 6: Suppression of HDAC4 augments osteoclast differentiation in HDAC7 KO mice (A) Representative image of TRAP stained osteoclast cultures from HDAC7 null mice infected with control or HDAC4-shRNA expressing lentiviruses (B-C) Histomorphic analysis of TRAP-stained osteoclasts, *p ≤ 0.05 vs. control shRNA WT cells infected with shRNA #1 and #2

As in the first round of experiments, we infected osteoclasts from HDAC7 null mice with shRNA viruses #1 and #2 and plated the cells on calcium phosphate substrate-coated wells. To quantify osteoclast differentiation, we used the previously described methods. In shRNA #2 infected cells, the average pit number increased approximately 1.5 fold and the percent resorbed increased approximately 3 fold when compared with controls (Fig. 7B-C). ShRNA #1 infected cells did not show significant differences in any of the three categories measured compared to controls (Fig. 7D).

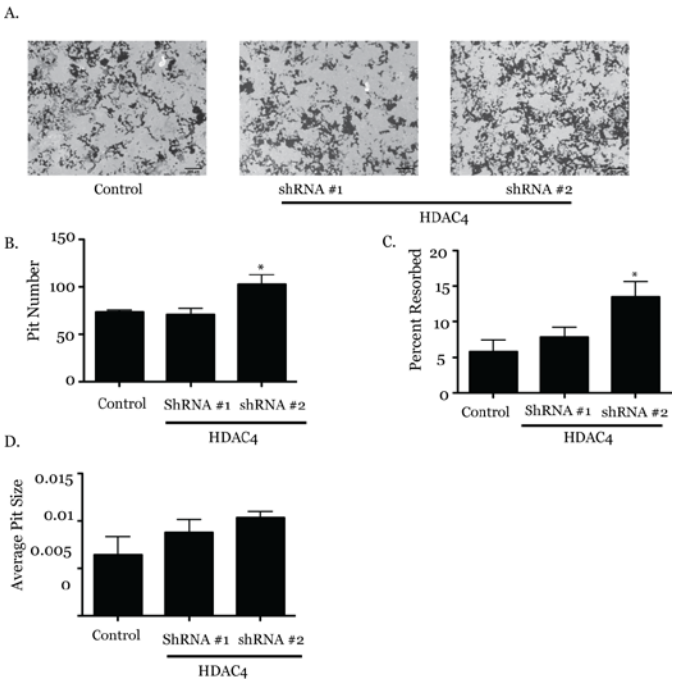

Figure 7: Suppression of HDAC4 in HDAC7 KO phenotype increases resorption
(A) Representative image of resorption plates infected with control or HDAC4-shRNA expressing lentiviruses
(B-D) Quantification of resorptive activity, *$p \leq 0.05$ vs. control shRNA HDAC7 KO cells infected with ShRNA #1 and #2

Discussion

HDAC4's Role in Osteoclastogenesis

The current study is the first to investigate the role of HDAC4 in osteoclastogenesis. As predicted, the current study demonstrates that cells deficient in HDAC4 produced larger and more numerous osteoclasts. The results also show that osteoclasts with reduced HDAC4 expression resorbed more calcium/phosphate compared to control cells. These results corroborate the findings of the Mansky lab regarding HDAC7, supporting the rather new research finding that Class IIa HDACs are negative regulators of osteoclastogenesis (12). Because HDAC4 and HDAC7 are in the same class of HDAC proteins, we believe that they function by similar mechanisms. We can therefore infer that HDAC4, like HDAC7, may inhibit osteoclast differentiation through a deacetylase-independent mechanism.

HDAC4's Role in Osteoclastogenesis in HDAC7 deficient mice

Additionally, this study found that the inhibition of HDAC4 in HDAC7-deficient mice further enhanced both the size and number of osteoclasts. These results demonstrate that

these HDACs have separate functions and do not compensate for each other throughout the osteoclastogenesis pathway. In both wild type and HDAC7 null osteoclasts, both the number and size of osteoclasts increased in the absence of HDAC4 expression. These findings mimic the results observed in the osteoclasts null for HDAC7 (12). The increase in number of osteoclasts suggests that the loss of HDAC4 may result in an increase in the proliferation of osteoclast precursors. Alternatively, the loss of HDAC4 expression in osteoclasts may decrease apoptosis of osteoclast precursors. We can attribute the increase in number and size of multinuclear osteoclasts to a possible increase in proliferation, decrease in apoptosis or changes in expression of genes necessary for osteoclast fusion.

Histone Deacetylase Inhibitors

There is a growing field of research centered around Histone Deacetylase Inhibitors (HDIs). HDIs, which affect gene expression by increasing histone acetylation, regulate chromatin structure and transcription by blocking the action of HDACs. As mentioned above, we know that HDIs have a potentially therapeutic effect against cancer by inducing cell differentiation, inhibiting cell proliferation, and inducing apoptosis of tumor cells in cultures and animal models. Additionally, the development of HDIs as therapeutic agents is underway for the treatment of blood malignancies (11).

A study that investigated the role of two broad-spectrum HDI's found that they had a novel action in inhibiting osteoclast differentiation. Specifically, Rahman et al. found that trichostatin A (TSA) and sodium butyrate (NaB) modulate osteoclast-specific signals by reducing mRNA expression of cathepsin K and calcitonin receptor and inhibiting the formation of prosteoclast-like cells in rat bone marrow cultures. Surprisingly, while these two HDIs inhibited differentiation into osteoclasts, they did not affect the development of macrophages (11). Both trichostatin A and sodium butyrate have been shown to induce differentiation of some leukemia cells, and since they are now being used as therapies for leukemia and lymphomas, it is essential to further understand their role in primary bone marrow cell development (11) (Figure 8).

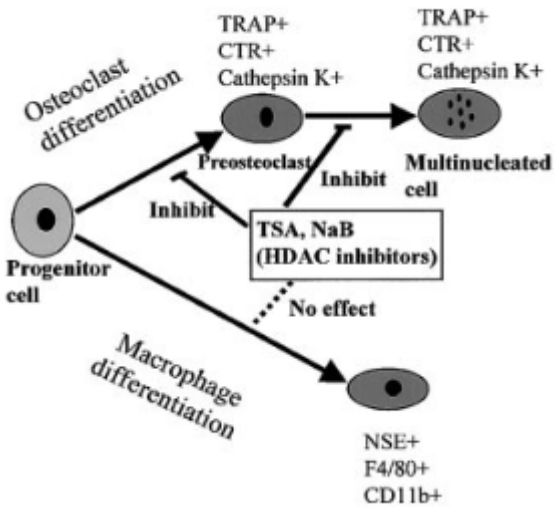

Figure 8: Effect of HDAC Inhibitors on the Differentiation into osteoclasts and macrophages. HDAC inhibitors suppressed the processes of osteoclast differentiation, POC formation, as well as MNC formation, whereas they did not affect macrophage differentiation (11).

The study referenced above, and others since, show that the HDIs TSA and NaB directly inhibit osteoclastogenesis. Jensen and Mansky demonstrated that the inhibition of HDAC3 had similar inhibitory effects on osteoclast formation. When applied *in vitro*, HDAC3 behaved as a histone deacetylase inhibitor. In the same study, Jensen and Mansky showed that HDAC7 had the opposite effect. When HDAC7 was inhibited, osteoclastogenesis increased. "The contrast between HDAC7 suppression and HDAC3 suppression with HDI treatments on osteoclast formation suggest that HDAC7's effects in osteoclasts are mediated through distinct mechanisms that do not require deacetylase activity (17)." These findings emphasize the need to isolate specific functions of HDACs in order to develop more advanced and specific therapeutic agents.

Future Research

Based on the results of this study, HDAC4 negatively regulates osteoclast differentiation. Because the experiments used cultured cells in vitro, we cannot conclusively demonstrate HDAC4's role in regulating osteoclast differentiation. To prove HDAC4's role, it is necessary to engineer a mouse species null for HDAC4 expression in osteoclasts, as was done with HDAC7. Although these studies are beyond the scope of this project, they are currently underway in the Mansky lab.

These experiments demonstrate that HDAC4 plays a role in osteoclast differentiation, however the mechanism by which HDAC4 inhibits osteoclast differentiation is not yet clear. Preliminary studies in the Mansky lab have shown that HDAC4 expresses early in the process of osteoclast differentiation (Figure 9). This data leads us to believe that HDAC4 interacts with transcription factors expressed early during differentiation, such as c-fos, to regulate osteoclast differentiation.

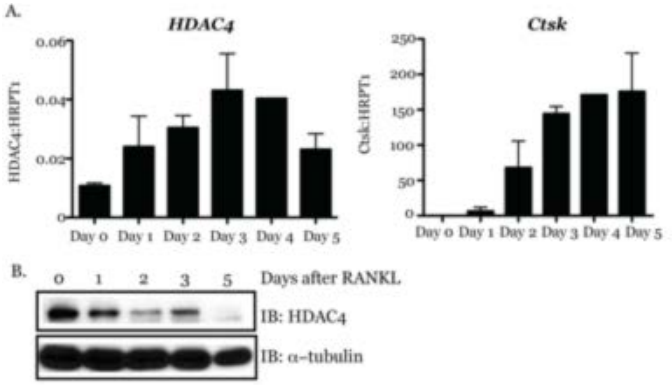

Figure 9. HDAC4 expression in osteoclasts. Bone marrow macrophages were either cultured in either M-CSF (day 0) or M-CSF and RANKL (day 1-5) and RNA and protein was collected. (A) qPCR was done to measure expression of *HDAC4* or *cathepsin K (Ctsk*). (B) Western blot of HDAC4 or α-tubulin protein expression.

Many studies are currently analyzing HDACs' role in various tissues and cell types, but there is still much to learn. Due to the incredibly complex role of HDACs in gene expression, we probably will never understand every pathway and role they play. However, the more we learn, the more we are able to hone therapies that target cells we want to eliminate and protect cells that we need to preserve.

Eventually, it is possible that HDACs and their proven role in osteoclastogenesis will be useful in accelerating or modifying orthodontic therapy. In orthodontics, tooth movement is accomplished by resorbing bone in front of the tooth (in the direction of movement) and laying down new bone after the movement has been achieved. Understanding HDACs' role in bone apposition and resorption more fully may allow clinicians to control tooth movement at the cellular level. In other words, by applying HDAC inhibitors locally and intraorally, we may be able to control the speed and amount of bone resorption desired in specific areas in the oral cavity. These would have potential

implications in tooth movement, post-orthognathic surgical healing, the need for alveolar ridge grafting, suture remodeling and much more.

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