Reduced HDAC7 Expression Results in Increased Osteoclastogenesis

A THESIS PROJECT SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF THE UNIVERSITY OF MINNESOTA BY

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Dedication

This thesis is dedicated to my wife Amanda for her patience and support.

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Introduction

Bone is a dynamic tissue that undergoes continuous cycles of formation and resorption [1]. These cycles allow for physiologic bone growth, repair of damaged bone, and are important for regulation of systemic calcium and phosphate levels [2]. There are two primary cell types responsible for this activity: osteoblasts, which lay down immature bone, and osteoclasts, which resorb bone. Complex physiologic signals regulate these important processes. Understanding the regulatory signals of osteoclasts has potential for clinical therapies for diseases of bone such as osteoporosis, arthritis, Paget's disease and osteolytic cancers [3, 4, 5]; as well as therapies to facilitate orthodontic tooth movement. Histone deacetylation complexes are known to be involved in the regulation of osteoclastogenesis, the differentiation of precursor cells into mature osteoclasts [6,7]. Based on their recent work, the Mansky/Gopalakrishnan lab has proposed a model for osteoclastogenesis in which the intracellular enzyme histone deacetylase 7 (HDAC7) plays a central inhibitory role [8]. The goal of this project was to further investigate this model by examining the effect of HDAC7 suppression on osteoclastogenesis using a murine HDAC7 knockout animal model.

Review of the Literature

Bone

Bone provides structural support for the body, generates cells for the blood and bone, and acts as a reservoir for minerals such as calcium and phosphate
[1,2].

Bone Cells

The primary cells of bone are osteoblasts and osteoclasts. Osteoblasts are bone-forming cells and are derived from mesenchymal stem cells. They secrete osteoid and help regulate the activity of osteoclasts. Osteoclasts are bone-resorbing cells that differentiate from monocyte precursors of hematopoietic origin [9]. Osteoclasts and osteoblasts play an essential role in skeletal development, bone remodeling and mineral homeostasis.

Excessive bone resorption by osteoclasts contributes to pathologic bone conditions such as osteoporosis. Osteoporotic fractures, especially in the elderly population, can result in significant morbidity and mortality. Understanding the mechanisms that regulate osteoclast differentiation could lead to new therapies against bone disease [3].

Osteoclast differentiation

The precise mechanisms controlling osteoclast differentiation are not fully understood. To initiate osteoclastogenesis, osteoclast precursors require macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL), which bind to c-Fms and RANK receptors respectively. c-Fms and RANK activation induces osteoclast-specific gene expression such as the transcription factors *PU.1, Mitf, c-fos* and *Nfatc1*. Combined stimulation with M-CSF and RANKL is required to induce the expression of these genes [10], which support the differentiation from immature bone marrow precursor cells to mononuclear osteoclasts. The cells then fuse together to form mature multinuclear osteoclasts containing 4-20 nuclei [9].

Dendritic cell-specific transmembrane protein (DCSTAMP) is a protein consisting of 7 transmembrane regions expressed on the surface of osteoclasts. DCSTAMP is necessary for osteoclast fusion during differentiation. Multinuclear osteoclasts are absent in DCSTAMP $^{-/-}$ cells and such mice exhibit an osteopetrotic phenotype. In DCSTAMP$^{-/-}$ osteoclasts, resorption does occur but it is less efficient suggesting that these cells are functional but defective [11,12].

Cathepsin K (*Ctsk*) is a proteinase that is produced by osteoclasts. *Ctsk* functions in an acidic environment to degrade and remove type I collagen. A defect in the *Ctsk* gene in humans and mice leads to a pycnodysostotic

phenotype where a lack of collagen breakdown results in sclerotic bone that is prone to fracture [13].

The transcription factor Mitf is of particular interest with regard to this study. Mitf belongs to the *MiT* family of basic helix-loop-helix transcription factors that regulate gene expression in a variety of cell types including osteoclasts [14]. Mitf has been shown to activate expression of several osteoclast genes, such as *bcl2, Acp5, and Ctsk* [15], and is thought to provide temporal regulation of osteoclastic gene expression during osteoclastogenesis. Mice homozygous for the *mi-Mitf* allele, a defective copy of this gene, display reduced osteoclast differentiation and an osteopetrotic phenotype [8]. In osteoclasts, M-CSF stimulation recruits Mitf to the nucleus where it joins a complex with PU.1 at promoters of genes necessary for osteoclast differentiation [10,16]. However, these promoters do not become activated until stimulation with RANKL, which suggests the existence of proteins that negatively regulate the activity of Mitf and PU.1 during osteoclast differentiation. It has recently been suggested that histone deacetylases (HDACs) may play a key role in the negative regulation of Mitf and PU.1 [8].

Histone deacetylases and their role in osteoclastogenesis

Histone deacetylases (HDACs) are intracellular proteins that regulate the function of other cellular substrates. HDACs are recognized principally for their ability to repress gene expression by manipulating the conformation of chromatin

in order to inhibit translation of specific genes. They do this by removing acetyl groups from histone core proteins at target gene promoters [17]. They are recruited by transcriptional factors to perform this function. A lesser-known function of HDACs is to regulate the activity levels of many proteins post translationally by deacetylation and deacetylation-independent means [18].

The 18 human HDACs fall into four classes based on structural and biochemical characteristics. Class I HDACs include HDACs 1,2,3 and 8. They are broadly expressed and found in the cell nuclei where they associate in multiprotein complexes and deacetylate histones [19]. Class II HDACS include HDACs 4,5,6,7,9 and 10. Class II HDACs are expressed in a more tissue-restricted pattern compared to class I HDACs. Class II HDACs are further subdivided into two subclasses IIa and IIb. Class IIa HDACs, of which HDAC7 is one, can interact with corepressor proteins to bring about transcriptional repression. Class II HDACs shuttle between the nucleus and cytoplasm in response to signaling stimulation so as to modulate their inhibitory influence on gene expression [17].

Human clinical studies indicate that histone deacetylase inhibitors (HDIs) have a negative impact on bone density. Valproate, an HDI, has been used to treat bipolar disorder, epilepsy and other mood disorders since the 1960s. There are conflicting reports as to how valproate alters bone remodeling to cause bone loss in humans (i.e. whether inhibiting osteoblast activity or increasing osteoclast

activity)[20]. It is unclear if a concurrent osteogenic therapy (PTH or bisphosphonates) can attenuate HDI induced bone loss [21]. Further research needs to be done to define the mechanisms by which treatment with HDIs results in bone loss. At the molecular and cellular levels much needs to be learned about the role of specific HDACs in bone biology.

The expression of specific HDACs can be enhanced or suppressed experimentally through the use of antibodies, shRNA and knockout animal models. Broad-spectrum HDIs are available such as Trichostatin A (TSA) and sodium butyrate (NaB). These HDIs primarily target class I HDACs which function by deacetylating histones, while class II HDACs function independent of deacetylation activity [19].

Several studies have shown HDACs to be regulators of osteoclastogenesis. Non specific inhibition of HDACs with NaB and TSA have been shown to inhibit RANKL mediated osteoclastogenesis through inhibition of *c-fos* expression, NF-kB dependent transcription and/or p38 MAP kinase activity [6,14]. The Mansky/Gopalakrishnan lab demonstrated that specific suppression of HDAC3 (with shRNA) exhibits a similar effect as HDIs on osteoclast differentiation [8].

HDAC7 and its role in osteoclast differentiation

Surprisingly, it has also been shown by the Mansky/Gopalakrishnan lab that specific suppression of HDAC7 by shRNA has the effect of *enhancing* osteoclastogenesis; suppression of HDAC7 with shRNA potentiated ocsteoclastogenesis in RANKL stimulated cultures [8]. This resulted in osteoclasts that were increased in cell number, cell area and number of nuclei per cell [8]. HDAC7-suppressed osteoclasts also showed increased expression of the gene *bcl2*. *Bcl2* is an anti-apoptotic protein, which may prevent osteoclasts from undergoing apoptosis thereby allowing them a greater opportunity to grow and fuse into larger multinuclear cells. B*cl2* is one of several osteoclast genes regulated by the Mitf transcription complex [8]. HDAC7 may repress the expression of osteoclast genes, such as *bcl2,* by recruiting repressive complexes containing class I HDACs, SIN3 and CtBP to osteoclast promoters thereby blocking the activity of transcription factors, such as Mitf, at these sites [19]. RANKL may function to remove these inhibitory complexes, allowing transcription to occur. It has been shown by others that the repressive complexes containing HDAC1, SIN3 and CtBP recruited to osteoclast promoters with M-CSF stimulation are attenuated by stimulation with RANKL [22,23].

An inhibitory role for HDAC7, similar to the one mentioned above, has been noted in the differentiation of a closely related bone cell, the osteoblast. HDAC7 suppresses osteoblast differentiation by binding directly to osteoblast transcription factors such as Runx2 [24]. Runx2 is a master transcriptional

regulator of osteoblasts that functions either as a transcriptional activator or repressor depending on its interaction with other transcription factors [19]. HDAC7 represses Runx2 activity in a deacetylation-independent manner via complexing with other proteins; probably class I HDACs [25]. In osteoblasts, the influence of HDAC7 is inhibited by transiently shuttling the enzyme out of the nucleus and into the cytoplasm. Bone morphogenetic protein 2, a major stimulatory signal for osteoblastogenesis, induces the transient redistribution of HDAC7 to the cytoplasm with the help of the chaperone protein Crm1 [24]. A similar model has been proposed by the Mansky lab to explain the regulation of the inhibitory effect of HDAC7 in osteoclastogenesis.

A model for the role of HDAC7 in osteoclast differentiation

A model has been suggested by Mansky, which proposes a central role for the interaction of the transcription factor Mitf, the inhibitory enzyme HDAC7, and the cytokine RANKL, in the regulation of osteoclastogenesis. It has been proposed that in the preosteoclast, M-CSF recruits Mitf to target gene promoters to initiate the transcription of genes necessary for osteoclast differentiation. HDAC7, as an essential part of a large repressive complex, suppresses the activity of Mitf. It is further suggested that RANKL removes the inhibitory influence of HDAC7, by relocating the enzyme to the cytoplasm, allowing Mitf access to transcribe the genes necessary for osteoclastogensis to proceed (Fig1).

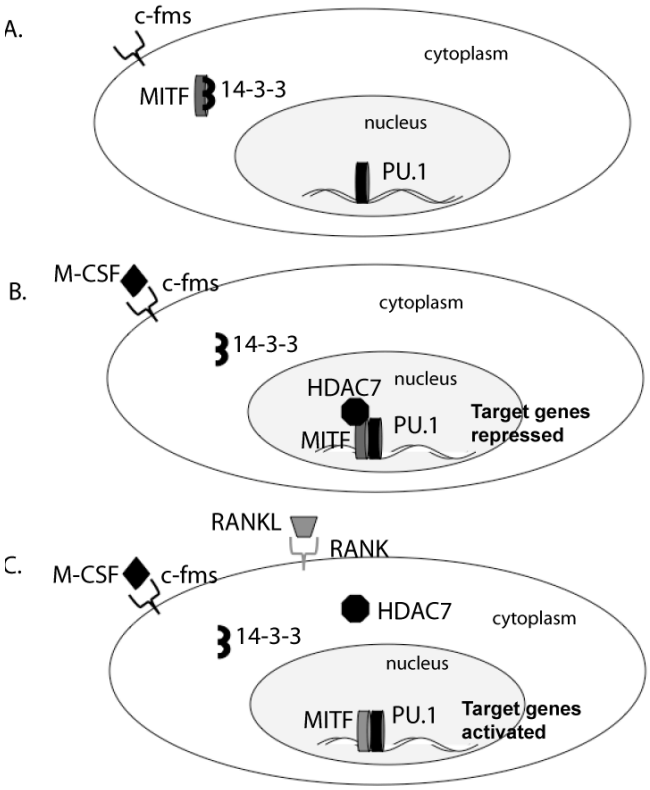

Figure 1: *Hypothesized interaction between* Mitf *and HDAC7 in osteoclast differentiation.* A. Inactive Mitf is sequestered in the cytoplasm. B. Upon stimulation with M-CSF, Mitf becomes localized in the nucleus where it complexes with other transcription factors. It is hypothesized that HDAC7 inhibits this transcription factor complex. C. It is further hypothesized that RANKL stimulation removes the inhibitory influence of HDAC7 allowing osteoclast specific genes to be transcribed.

Goal of the work

Many of the details of HDAC7's role in osteoclast differentiation have not been experimentally characterized. Much evidence is needed to verify or refute the model proposed above. The goal of this study is to further clarify the role of HDAC7 in osteoclast differentiation using an HDAC7 knockout mouse model. The effect of inhibiting HDAC7 expression in this manner has not been previously examined. This novel approach will provide valuable data with regard to the role and importance of HDAC7 in the control of osteoclast differentiation.

Hypothesis:

We propose that HDAC7 knockout osteoclast cultures will demonstrate increased levels of osteoclast differentiation compared with cultures from negative controls. The measures of osteoclast differentiation will be average number of cells, average cell area and average number of nuclei per cell. The expression of the osteoclastic marker genes *Ctsk* and *DC-STAMP* will be also measured using real-time RT-PCR.

Specific Aims:

1. To show successful suppression of HDAC7 expression using a knockout mouse model.
2. To determine the effect of the suppression of HDAC7 expression using a knockout mouse model on histologic measures of osteoclast differentiation; namely, average cell number, average cell area, and average number of nuclei per cell.
3. To determine the effect of the suppression of HDAC7 expression using a knockout mouse model on the expression of osteoclast marker genes using real-time RT-PCR; namely *Ctsk* and *DC-STAMP*.

Materials and Methods:

HDAC7 Knockout Mice

Heterozygous floxed-HDAC7 knockout mice were previously generated by Dr. Eric Olson (University of Texas Southwestern Medical Center). These mice were mated so that homozygous floxed knockout animals could be created. The resultant pups were genotyped by PCR as described by Dr. Olson [26].

Primary Osteoclast Cell Culture

Bone marrow was flushed from the femurs of homozygous floxed HDAC7 knockout mice at 1 month of age. The cells were cultured in alpha-MEM media for three days in the presence of 50 ng/ml M-CSF on non-tissue culture coated dishes. The adherent cell population containing the osteoclasts was divided into 16 samples (8 for real-time RT-PCR and 8 for histologic examination)[16,27].

On day 3, before the addition of RANKL, the cultures were infected with CMV-*Cre* or a control adenovirus for 3 hours; infection with the CMV-*Cre* adenovirus results in knockout of the floxed HDAC7 gene in exposed cultures . All samples were then cultured in separate dishes with M-CSF (30ng/ml) and RANKL (60ng/ml) for approximately 5 days or until multinuclear cells appeared in the culture.

Quantification of gene expression

Real-time RT-PCR was used to quantify the levels of mRNA of the following genes of interest: *HDAC7*, *DC-STAMP and CtsK*. On day 5, after the addition of RANKL, RNA from the cultured cells was harvested with TriZol Reagent (Invitrogen). The iScript cDNA synthesis kit (Bio-RAD) was used to make cDNA [26].

Quantitative real-time RT-PCR was performed using the MyiQ singe-color real-time PCR detection system (Bio-Rad) using 1 ul of cDNA with 2x SYBR Green supermix (Bio-Rad). SYBR green is a dye that fluoresces only when bound to double stranded DNA. This fluorescence is detected by the system, and in this way the DNA of interest can be quantified.

Primer sequences used were *Ctsk* (forward) 5'-AGG GAAGCA AGC ACT GGA TA-3', (reverse) 5'-GCT GGC TGG AAT CAC ATC TT-3'; *DC-STAMP* (forward)5'-GGG CAC CAG TAT TTT CCT GA-3', (reverse) 5'- TGG CAG GAT CCA GTA AAA GG-3'; *HDAC7* (forward) 5'-TGA AGA ATG GCT TTG CTG TG-3', (reverse) 5'-CAC TGG GGT CCT GGT AGA AA-3'; *L4* (forward) 5'-CCTTCTCTGGAACAACCTTCTCG-3' (reverse) 5'-AAGATGATGAACACCGACCTTAGC-3' . Parameters used for all real-time RT-PCR reactions consisted of the following cycles: 95 degrees for 3 minutes, 94 degrees for 15 seconds, 60 degrees for 30 seconds, 72 degrees for 30 seconds, repeat 2 through 4 for 45 times, 12 degrees for infinity.

The genes of interest were normalized to L4, the housekeeping gene. L4 is a ribosomal gene and as such occurs in similar amounts in each cell. L4 provides a standard against which the process can be calibrated to compensate for variations in the synthesis of cDNA. The relative expression for each gene of interest was reported as the mean of each experimental group +/- confidence intervals based on duplicate independent RNA samples. The fold difference between groups for each measure was also reported. All experiments were repeated twice.

Western Blotting

HDAC7 antibody was purchased from Abcam. On day 5, after the addition of RANKL, the osteoclast cells were harvested in Nonidet P-40 lysis buffer (20mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40 and protease and phosphatase inhibitors). Extracts were resuspended in sample buffer and resolved by SDS-PAGE. The resolved proteins were transferred to PVDF membrane, blocked, and blotted in primary antibody overnight at 4 degrees Celsius. On the next day the blot was incubated with horseradish peroxidase (anti-rabbit, GE Healthcare) for 2 hrs at room temperature. Antibody binding was detected using the ECL system (GE Healthcare)[8].

Quantification of Osteoclast Differentiation

Size and number of cells

Histological examination of the cultures was conducted to quantify the degree of osteoclast differentiation. TRAP staining was performed. Cells were rinsed in PBS, fixed in 4% paraformaldehyde for 20 minutes and stained using the leukocyte acid phosphatase kit (Sigma-Aldrich, 387-A). Cells were photographed and analyzed using Image-J software. The number and area of TRAP-positive osteoclasts per field at 10X magnification were recorded and averaged [8].

Counting Nuclei

After cells were TRAP-stained and photographed, cells were stained with DAPI (Molecular probes) for 5 minutes at room temperature. Cells were photographed at 10X magnification and counted using Image J software [8].

Statistical Analysis

All experiments were run in duplicate and the results expressed (graphically) as group means +/- confidence intervals. Student's t-tests were used to compare means between groups for each measure; $p < 0.05$ indicates significance. For ease of interpretation, fold increases were also reported. This was done for the data concerning gene expression, cell counts, cellular area and nuclei counts. All statistical analysis was performed using Prism 4 (Graphpad software, Inc. San Diego,CA).

Results:

Specific Aim 1: To show successful suppression of HDAC7 expression using a knockout mouse model.

The first aim was to demonstrate the successful attenuation of HDAC7 expression in the CMV-*Cre* infected HDAC7 floxed osteoclasts. Loss of HDAC7 expression was measured with real-time RT-PCR and western blot. Expression of *HDAC7* was measured in mature osteoclasts from HDAC7 floxed mice that had been exposed to CMV-*Cre* or a control adenovirus. To measure gene expression, HDAC7 mRNA expression was compared to L4, the internal housekeeping gene. Cultures exposed to CMV-*Cre* showed reduced expression of *HDAC7* as compared with negative controls; however, the reduction was not significant (4 fold decrease). Western blot analysis of HDAC7 protein showed less HDAC7 protein expression in CMV-*Cre* infected cultures (Fig 2).

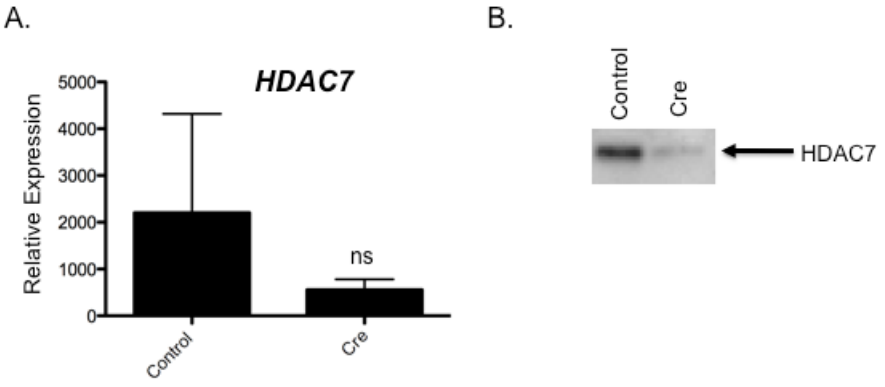

Figure 2: *Expression of HDAC7 in control and Cre infected osteoclasts from HDAC7 floxed mice.* A. Real-time RT-PCR expression of HDAC7. B. Western blot of HDAC7 expression in Cre or control infected osteoclasts.

Specific Aim 2: To determine the effect of the suppression of HDAC7 expression using a knockout mouse model on histologic measures of osteoclast differentiation; specifically, average osteoclast number, average osteoclast size, and the average number of nuclei per osteoclast.

Cultures were photographed at 10X magnification and the number of cells per field was recorded and averaged for each group. The *HDAC7* suppressed cultures showed a significantly lower average number of osteoclasts per field ($p= 0.0087$; Fig 3).

Cultures were photographed at 10X magnification and Image J software was used to analyze the area of the osteoclasts. The cells in the *HDAC7* suppressed cultures had a significantly larger average area compared to the negative controls (Fold increase= 6, $p< 0.0001$; Fig 3).

The cultures were then subjected to DAPI staining so that the nuclei could be better visualized. The nuclei per cell were counted and averaged. There was a significantly larger average number of nuclei per cell in the *HDAC7* suppressed cultures as compared to the negative controls (fold increase= 4.8, $p<0.0001$; Fig 3).

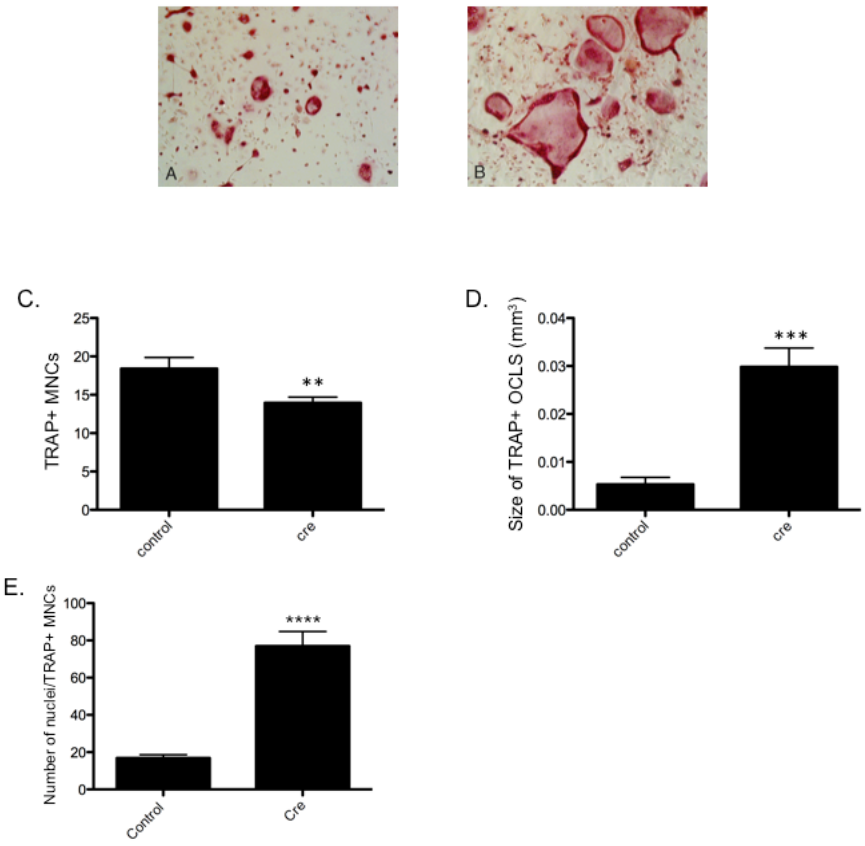

Figure 3: *Average number of cells, cellular area and number of nuclei per cell in control and Cre infected osteoclasts from HDAC7 floxed mice*. Images of TRAP stained osteoclasts from HDAC7 floxed mice infected with (A) control or (B) Cre expressing adenoviral vector. (C) Images were photographed and the number of TRAP positive multinuclear cells were counted (D) and size was measured. (E) Average number of nuclei was also measured.

Specific Aim 3: To determine the effect of the suppression of *HDAC7* expression using a knockout mouse model on the expression of osteoclast marker genes using real-time RT-PCR; namely, *DC-STAMP* and *Ctsk*.

The third aim was to determine the effect of the attenuation of HDAC7 expression on osteoclastic gene expression. Gene expression was measured for mature osteoclasts obtained from floxed HDAC7 mice that had been exposed to CMV-*Cre* adenovirus and those that had been exposed to CMV-control

adenovirus, a negative control. The osteoclast target genes measured by real-time RT-PCR were *DCSTAMP* and *Ctsk*. Their expression was compared to L4, the internal housekeeping gene.

The *HDAC7* suppressed cell cultures showed significantly increased expression of DCSTAMP as compared to the negative controls (10 fold increase, $p<0.0024$; Fig 4A).

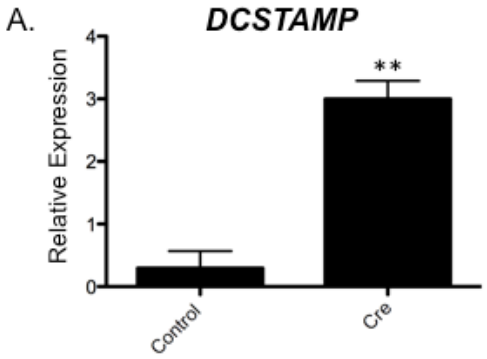

Figure 4A: Expression of *DCSTAMP in control and Cre infected Osteoclasts from HDAC7 floxed mice*.

The *HDAC7* suppressed cell cultures also showed increased expression of *Ctsk*; however, this effect was not significant (5.5 fold increase; Fig 4B).

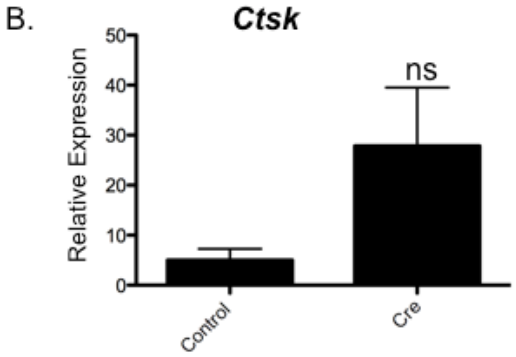

Figure 4B: Expression of *Cathepsin K in control and Cre infected osteoclasts from HDAC7 floxed mice.*

Discussion:

The results of the western blot and real-time RT-PCR showed that the expression of *HDAC7* was suppressed in the osteoclast cultures exposed to CMV-*Cre* adenovirus. A 4-fold decrease was seen although this effect was not significant. This result suggests that the experimental manipulation was at least partially successful. It is not clear why this effect was not significant, although it was noted that the variance was high for the control group making significance harder to achieve. It is expected that repetition of this experiment with a larger sample size would achieve significance for this effect.

The results of the real-time RT-PCR data showed a significant increase in the expression of the osteoclast marker gene *DC-STAMP* in the *HDAC7* suppressed cultures. *DC-STAMP* expression was increased 10 fold. The expression of the osteoclast marker gene *Ctsk* was increased 5.5 fold in the *HDAC7* suppressed cultures, although this effect was not significant. These results suggest that suppression of *HDAC7* expression increased osteoclast differentiation. This finding was expected and is in line with a single previous study in which *HDAC7* expression was attenuated in osteoclasts via shRNA administration [8]. The current study supports the theory that HDAC7 is a negative regulator of osteoclastogenesis and is only the second known study to demonstrate this effect.

It is not clear why the increase in *Ctsk* was not significant in the current study. Previous work in which *HDAC7* expression was attenuated via shRNA resulted in a significant increase in the expression of both *DC-STAMP* and *Ctsk.* This difference could be due to the fact that *HDAC7* expression was suppressed via different experimental manipulations in the two studies. Perhaps, attenuation with a knockout model allows partial function of HDAC7 to occur while use of shRNA does not. It was also noted that the variance in the HDAC7 suppressed group was large with respect to *Ctsk* expression suggesting the introduction of random error that has the potential to be eliminated in future experiments. High variability is a commonly encountered problem when working with primary cell cultures.

The results also showed that the *HDAC7* suppressed cultures demonstrated a significant increase in osteoclast size as compared to the negative controls. This result was expected and agrees with previous work [8]. Further, the results showed a significant increase in the average number of nuclei per cell in the *HDAC7* attenuated cultures as compared to the negative controls. This result was also expected and agrees with previous work [8]. Unexpectedly, the results also showed a significant decrease in the average number of osteoclasts per field. This result is in contrast to previous work, which has shown an increase in the number of osteoclasts in *HDAC7* suppressed cultures. It seems unlikely that the decrease noted in the current study resulted from increased apoptosis because the images from the *HDAC7* suppressed cultures show a high density of

cells. Furthermore, it has been shown previously that suppression of *HDAC7* increases the expression of *bcl2*, an anti-apoptotic protein [28]. A more likely explanation for the observed effect is that the osteoclasts in the *HDAC7* suppressed cultures became so large that fewer cells were able to fit per microscopic field of view. Taken together, the above results suggest more than just a general upregulation in osteoclastogenesis; they suggest also that an increase in the fusion of osteoclasts occurred in the *HDAC7* suppressed cultures.

Little is known about the mechanism by which *HDAC7* suppression might potentiate osteoclastogenesis in general including an effect at the fusion stage. It is known that DCSTAMP is required for osteoclast fusion and that *HDAC7* attenuation increases DCSTAMP expression, as was observed in the current study. The Mansky/Gopalakrishnan lab has hypothesized a model in which RANKL disrupts the HDAC7-mediated suppression of Mitf and PU.1 (Fig 1). This model might be refined to include an inhibitory role for HDAC7 at multiple time points during osteoclast differentiation including at the fusion stage. HDAC7 may inhibit PU.1 at the preosteoclast stage to attenuate osteoclast lineage commitment by inhibiting the expression of RANK on the cell surface. Later, at the mononuclear osteoclast stage, HDAC7 might inhibit the expression of *DC-STAMP* thus reducing osteoclast fusion. RANKL is known to stimulate the developing osteoclast at multiple stages of development, each time initiating a different developmental process. It would seem reasonable then that a single inhibitory signal, HDAC7, could influence the osteoclast differently at multiple

points along its developmental path. In this way it might be seen that HDAC7 could assert its most powerful influence on the attenuation of osteoclast fusion.

Future directions for further study could investigate the possibility of different effects for *HDAC7* suppression at different points during osteoclast differentiation. To test this hypothesis osteoclast cultures at different stages of differentiation could be infected with an adenoviral vector overexpressing HDAC7 to determine which genes are changed in expression following this manipulation. Alternatively, the introduction of shRNA (in wild type cultures) or *Cre* adenovirus (in HDAC7 floxed cultures) could suppress HDAC7 activity at similar time points.

Future studies could also focus on examining the cellular location of HDAC7 at different points during osteoclast differentiation. This would allow confirmation of the hypothesized shuttling of HDAC7 to the cytoplasm to disinhibit the transcription factors Mitf and PU.1. A similar scenario has been proposed for osteoblast differentiation where BMP2 stimulation transiently relocates HDAC7 from the nucleus to the cytoplasmic compartment thereby allowing Runx2-dependent transcription to occur [29]. Additionally, a constitutively nuclear or cytoplasmic HDAC7 could be overexpressed in osteoclasts to determine if both forms of HDAC7 inhibit osteoclast differentiation and activity [21].

Future directions in the Mansky/Gopalakrishnan lab have focused on mating the *HDAC7* floxed mice to a mouse that expresses *Cre* in the myeloid lineage only. The result is mice that have HDAC7 knocked out only in myeloid–derived cells. Osteoclasts originate from the myeloid lineage. This manipulation provides an in vivo model of *HDAC7* suppression that reduces the possibility that the phenotypic effects observed are due to the suppression of *HDAC7* in non osteoclasts. Initial analysis of the mice expressing *Cre* and homozygous for the floxed *HDAC7* allele reveals animals that are osteopenic compared to their wild type littermates. When the osteoclasts from these mice are cultured, the cells are found to be larger in size compared to osteoclasts from the wild type littermates. This data supports the results of the current study [21].

Understanding the regulatory signals of osteoclasts has potential for clinical therapies for diseases of bone such as osteoporosis, arthritis, Paget's disease and osteolytic cancers [3, 4, 5], as well as, therapies to facilitate orthodontic tooth movement. For example, the potentiation of osteoclastogenesis in the alveolar bone of an orthodontic patient might result in enhanced bone resorption and faster orthodontic tooth movement. This would be particularly useful for patients whose teeth respond slowly to traditional orthodontic forces. Furthermore, if potentiated osteoclastogenesis could be targeted precisely, such as via local injection, it might also offer a means by which the anchorage values of specific teeth could be altered. This might allow the use of simpler biomechanics to achieve certain tooth movements which might be otherwise be

impossible, entail complex biomechanics, or demand a high level of patient cooperation.

Conclusion:

This study has provided further evidence that HDAC7 plays an important inhibitory role in osteoclast differentiation. Murine HDAC7 knockout osteoclast cultures demonstrated increased expression of the osteoclast marker genes *DCSTAMP* and *Ctsk*. The osteoclasts from these cultures were found to be larger in size and containing of more nuclei than negative controls suggesting that an increase in osteoclastogenesis, including an increase in osteoclast fusion, was induced.

These results support the model put forth by the Mansky/Gopalakrishnan lab in which HDAC7 is a key suppressor of osteoclastogenesis acting primarily through inhibition of the Mitf transcription complex. The current results suggest that this model must be refined to account for a significant influence of HDAC7 on the process of osteoclast fusion. Understanding the mechanisms by which HDAC7 interacts with various transcription factors at different stages of osteoclast development will be an important area of future study. HDAC7's influence on osteoclast fusion and the mechanisms by which this influence is suppressed is of particular interest. This work holds the promise of potential therapies for the treatment of pathological bone states and the facilitation of orthodontic tooth movement (8).

REFERENCES

[1] Henriksen K, Neutzsk-Wulff AV, Bonewald LF, Karsdal MA. Local communication on and within bone controls bone remodeling. Bone 2009; 44:1026-1033.

[2] Jensen E, Pham L, Billington C, Espe K, Carlson A, Westendorf J, Petryk A, Gopalakrishnan R, Mansky K. Bone morphogenic protein 2 directly enhances differentiation of murine osteoclast precursors. Journal of Cellular Biochemistry 2010; 109:672-682.

[3] Allen J, Fotsch C, Babij P. Emerging targets in osteoporosis disease modifications. Journal of Medicinal Chemistry 2010; 53:4332-4353.

[4] Rodan G, Martin T. Therapeutic approaches to bone diseases. Science 2000; 289:1508-1514.

[5] Roodman G. Pathogenesis of myeloma bone diseases. Leukemia 2009; 23:435-441.

[6] Rahman M, Kukita A, Kukita T, Shobuike T, Nakamura T, Koshani O. Two histone deacetylase inhibitors, trichostatin A and sodium butyrate, suppress differentiation into osteoclasts but not into macrophages. Blood 2003; 101:3451-3459.

[7] Kim H, Ha H, Lee J, Jung K, Yang D, Woo K, Lee Z. Trichostatin A inhibits osteoclastogenesis and bone resorption by suppressing the induction of c-Fos by RANKL. European Journal of Pharmacology 2009; 623:22-29.

[8] Pham L, Kaiser B, Romsa A, Schwarz T, Gopalakrishnan R, Jensen E, Mansky K. HDAC3 and HDAC7 have opposite effects on osteoclast differentiation. The Journal of Biological Chemistry 2010; 14:12056-12065.

[9] Vaananaen H, Laitala-Leinonen T. Osteoclast lineage and function. Archives of Biochemistry and Biophysics 2008; 473:132-138.

[10] Sharma S, Bronisz A, Hu R, Patel K, Mansky K, Sif S, Ostrowski M. MITF and PU.1 recruit p38 MAPK and NFATc1 to target genes during osteoclast differentiation. Journal of Biological Chemistry 2007; 282:15921-15929.

[11] Yagi M, Miyamoto T, Toyama Y, Suda T. Role of DC-STAMP in cellular fusion of osteoclasts and macrophage giant cells. Journal of Bone Mineral Metabolism 2006; 24:355-358.

[12] Yagi M, Miyamoto T, Swatani Y, Iwamoto K, Hosogane N, Fujita N, Morita K, Ninomiya K, Suzuki T, Miyamoto K, Oike Y, Takeya M, Toyama Y, Suda T. DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. The Journal of Experimental Medicine 2005; 202:345-351.

[13] Segovia-Silvestre T, Neutzsky-Wulff A, Sorensen MG, Christiansen C, Bollerslev J, Karsdal M, Henriksen K. Advances in osteoclast biology resulting from the study of osteopetrotic mutations. Human Genetics 2009; 124:561-577.

[14] Hemesath T, Steingrimsson E, McGill E, Hansen M, Vaught J, Hodgkinson C, Arnheiter H, Copeland N, Jenkins N. Microphthalmia, a critical factor in melanocyte development, defines a discrete transcription factor family. Genes and Development 1994; 8:2770-2780.

[15] McGill G, Horstmann M, Widlund H, Du J, Motyckova G, Nishimura E, Lin Y, Ramaswamy S, Avery W, Ding H, Jordan S, Jackson I, Korsmeyer S, Golub T, Fisher D. Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. Cell 2002; 109:707-718.

[16] Bronisz A, Sharma S, Hu R, Godlewski J, Tzivion G, Mansky K, Ostrowski M. Microphthalmia-associated transcription factor interactions with 14-3-3 modulate differentiation of committed myeloid precursors. Molecular Biology of the Cell 2006; 17:3897-3906.

[17] Sengupta N, Seto E. Regulation of histone deacetylase activity. Journal Cellular Biochemistry 2004; 93:57-67.

[18] Gregoretti I, Lee Y, Goodson H. Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. Journal of Molecular Biology 2004; 338:17-31.

[19) Verdin E, Dequiedt F, Kasler H. Class II histone deacetylases: versatile regulators. Trends in Genetics 2003; 19(5):286-293.

[20] Schroeder T, Westendorf J. Histone deacetylase inhibitors promote osteoblast maturation. Journal of Bone and Mineral Research 2005; 20:2254-2263.

[21] Mansky K, personal communication.

[22] Kim K, Lee J, Kim J, Jin H, Zhou B, Lee S, kim N. Protein inhibitor of activated STAT 3 modulates osteoclastogenesis by down-regulation of NFATc1 and osteoclast-associated receptor. Journal of Immunology 2007; 178:5588-5594.

[23] Hu R, Sharma S, Bronisz A, Srinivasan R, Sankar U, Ostrowskin M. Eos, MITF, and PU.1 recruit corepressors to osteoclast-specific genes in committed myeloid progenitors. Molecular and Cellular Biology; 27:4018-4027.

[24] Westendorf J. Transcriptional co-repressors of Runx2. Journal of Cellular Biochemistry 2006; 98:54-64.

[25] Gregory P, Wagner K, Horz W. Histone acetylation and chromatin remodeling. Experimental Cell Research 2001; 265:195-202.

[26] Chang S, Young B, Li S, Qi X, Richard J, Olson E. Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10. Cell 2006; 126:321-334.

[27] Meadows N, Sharma S, Faulkner G, Ostrowski M, Hume D, Cassady A. The expression of Clcn7 and Ostm1 in osteoclasts is coregulated by microphthalmia transcription factor. Journal of Biological Chemistry 2007; 282:1891-1904.

[28] McGill G, Horstmann M, Widlund H, Du J, Motyckova G, Nishimura E, Lin Y, Ramaswamy S, Avery W, Ding H, Jordan S, Jackson I, Korsmeyer S, Golub T, Fisher D. Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. Cell 2002; 109:707-718.

[29] Jensen E, Schroeder T, Baily J, Gopalakrishnan R, westendorf J. Histone deacetylase 7 associates with Runx2 and represses its activity during osteoblast maturation in a deacetylation-independent manner. Journal Bone Miner Res 2008; 23:361-372.