Histone Deacetylase 3 and Histone Deacetylase 7 Have Opposite Effects on Osteoclast Differentiation

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

Amanda Marie Romsa

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN DENTISTRY

Dr. Kim Mansky, Advisor

June 2010

Acknowledgements

I owe many thanks to

Dr. Kim Mansky for her expertise, guidance and willingness to let me work in her laboratory

Dr. Raj Gopalakrishnan and Dr. John Beyer for their helpful review

The members of the Mansky/Gopalakrishnan laboratory for their assistance and direction

Dedication

This thesis is dedicated to my parents for their love and support.

Abstract

Histone deacetylases (HDACs) are negative regulators of transcription. Endochondral bone formation including chrondocyte and osteoblast maturation is regulated by HDACs. Very little is known about the role HDACs play in osteoclast differentiation. This study found that suppression of HDAC3 expression represses osteoclastogenesis, while osteoclasts suppressed for HDAC7 expression had accelerated differentiation compared to control cells.

Table of Contents

List of Tables

List of Figures

Introduction

Bone homeostasis is important for skeletal growth and bone maintenance. It involves two primary cell types (osteoblasts and osteoclasts). Osteoblasts build bone while osteoclasts resorb bone. Diseases such as osteoporosis, periodontal disease, rheumatoid arthritis, Paget's disease of bone, and cancer-associated bone disease result in pathological bone loss due to an increase in the rate of skeletal degradation by osteoclasts relative to formation by osteoblasts. The development of improved therapies preventing pathological bone loss will require a better understanding of the molecular mechanisms that control osteoclast differentiation and activity.

Osteoclast differentiation requires stringent control of gene activation and suppression in response to physiological cues. The fidelity of skeletal gene expression requires integrating a spectrum of regulatory signals that govern osteoclast differentiation. To regulate expression of osteoclast genes, it is necessary to characterize the promoter regulatory elements as well as protein/DNA and protein/protein interactions to determine the extent to which genes are transcribed. Previous and ongoing studies (Mansky lab) are interested in identifying proteins that interact with and regulate the microphthalmia transcription factor (Mitf). The goal of my project was to determine the effect on osteoclast gene expression when HDAC3 and HDAC7 expression was reduced.

Review of the Literature

Bone

Bone plays several important roles in the human body, such as providing a site for muscle attachment, maintaining mineral balance by storing calcium and phosphate ions, protecting vital organs, and playing a role in hematopoiesis. The organic part of bone is composed of 90% Type I collagen and the inorganic phase is composed of hydroxyapatite crystals (Mansky et al. 2010, Segovia-Silvestre et al., 2009).

Bone Cells

Osteoclasts and osteoblasts are the primary cells involved in bone remodeling. Osteoblasts form bone by secreting bone matrix proteins and controlling osteoclast activity. Osteoblasts are derived from mesenchymal cells and are found on bone forming surfaces.

Osteoclasts are derived from the hematopoietic stem cell lineage. Monocyte/macrophage precursors from the hematopoietic stem cell differentiate and fuse to become multinuclear osteoclasts that are found on the surface of bone resorption sites. Osteoclasts resorb bone by a process of osteoclast attachment, polarization, formation of a sealing zone at the ruffled border, resorption and apoptosis (Yavropoulou et al., 2008). The hydroxyapatite in bone is resorbed by hydrochloric acid secreted by the osteoclast at its ruffled border (Segovia-Silvestre, et al., 2009; Ishii, et al., 2008). The sealing zone acts to separate the acidic resorptive zone from the extracellular space.

Bone homeostasis involves the highly regulated coupling of osteoblast and osteoclast activity, with bone resorption preceding bone formation. Dysfunctional activity of osteoblasts and osteoclasts results in pathological bone resorption.

Osteoclast Differentiation

Osteoclast precursors require RANK ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) to bind to their respective receptor activators on the cell surface. This activation, in turn, causes osteoclast-specific genes to induce differentiation of immature bone marrow precursor cells to preosteoclasts, which will subsequently fuse to form multinucleated osteoclasts (Figure 1) (Boyle et al., 2003; Zhao et al., 2008).

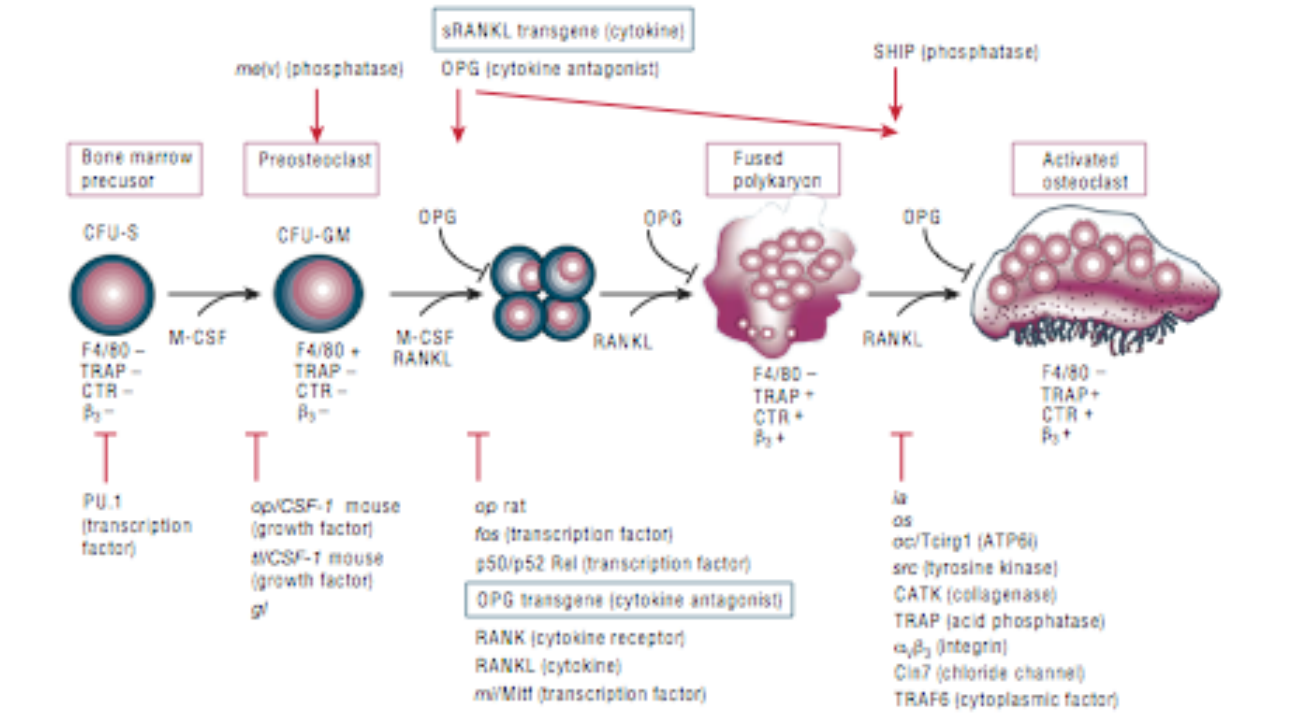

Figure 1 . Drawing detailing transcription and signaling factors involved in osteoclast differentiation (Boyle et al., 2003).

Genes Involved in Osteoclast Differentiation and Fusion

1. Cathepsin K

Cathepsin K (Ctsk) is expressed in fully differentiated osteoclasts and functions as a proteinase that degrades and removes Type I collagen. The type I collagen fragment which is normally present during collagen breakdown is not present in Ctsk knockout mice osteoclast cells in culture. In addition, a defective Ctsk in humans results in a pycnodysostotic phenotype, which is characterized by a lack of normal collagen breakdown and sclerotic bone (Segovia-Silvester et al., 2009).

2. Nuclear Factor of Activated T cells

Nuclear Factor of Activated T cells (NFAT-c1) is a transcription factor required for osteoclast differentiation and activation. It is known as the master regulator of osteoclast gene expression and helps activate subsequent genes involved in osteoclast differentiation. *NFAT-c1* $^{-/-}$ stem cells fail to develop into osteoclasts in vitro (Yavropoulou et al., 2008).

3. Dendritic Cell-specific Transmembrane Protein

Dendritic cell-specific transmembrane protein (DC-STAMP) is a gene that aids in the fusion of mononuclear osteoclast precursors into multinuclear osteoclasts. DC-STAMP is a protein comprised of seven transmembrane regions expressed on the cell surface of osteoclasts (Kukita et al., 2004; Yagi et al., 2005; Yagi et al., 2006). Mice that are *DC-STAMP* $^{-/-}$ express an osteopetrotic phenotype and contain no multinuclear osteoclasts (Yavropoulou et al., 2008; Yagi et al., 2005; Yagi et al., 2006). Resorption occurs in *DC-STAMP* $^{-/-}$ osteoclasts, but it is less efficient, suggesting that unfused

mononuclear osteoclasts are not as functional as multinuclear fused osteoclasts (Ishii et al., 2008).

Microphthalmia Transcription Factor (Mitf)

Microphthalmia transcription factor (Mitf) is a basic helix-loop-helix transcription factor that is necessary for osteoclast differentiation. Mice that are homozygous for the *Mitf (mi)* allele show an osteopetrotic phenotype and lack of osteoclast differentiation. M-CSF signaling alone can regulate Mitf nuclear localization and recruitment of Mitf to target promoters (Bronisz et al., 2006). However, Mitf does not activate gene expression by stimulation with M-CSF alone. Rather, combined stimulation with M-CSF and RANKL is required to induce expression of osteoclast differentiation genes (Sharma et al., 2007). We hypothesize that there are cellular inhibitors that regulate Mitf activation of osteoclast gene expression during M-CSF signaling.

Histone Deacetylases

Histone deacetylases (HDACs) are a group of enzymes responsible for broadly promoting transcriptional repression and silencing by removing acetyl groups from histone core proteins at target gene promoters, resulting in a less transcriptionally active state (Sengupta, et al., 2004). HDACs are divided into four classes: Class I HDACs (HDAC 1, 2, 3, and 8) are found in the cell nuclei; Class II HDACs shuttle between the nucleus and cytoplasm and exhibit tissue restricted expression patterns. The restricted expression pattern suggests Class II HDACs are involved in cellular differentiation and developmental processes (Fischle, 2002). These HDACs are further divided into Class IIa (HDAC 4,5,7,9) and Class IIb (HDAC 6 and 10). Class IIa HDACs contain functional

deacetylase catalytic domains; however, they have not been shown to function by directly deacetylating histones. Instead, Class IIa HDACs recruit repressor complexes that contain Class I HDACs and co-repressor proteins (Figure 2). Deacetylation of chromatin is most likely conducted by the class I HDACs. Class III HDACs include the NAD+-dependent sirtuin deacetylases and Class IV HDACs include HDAC11.

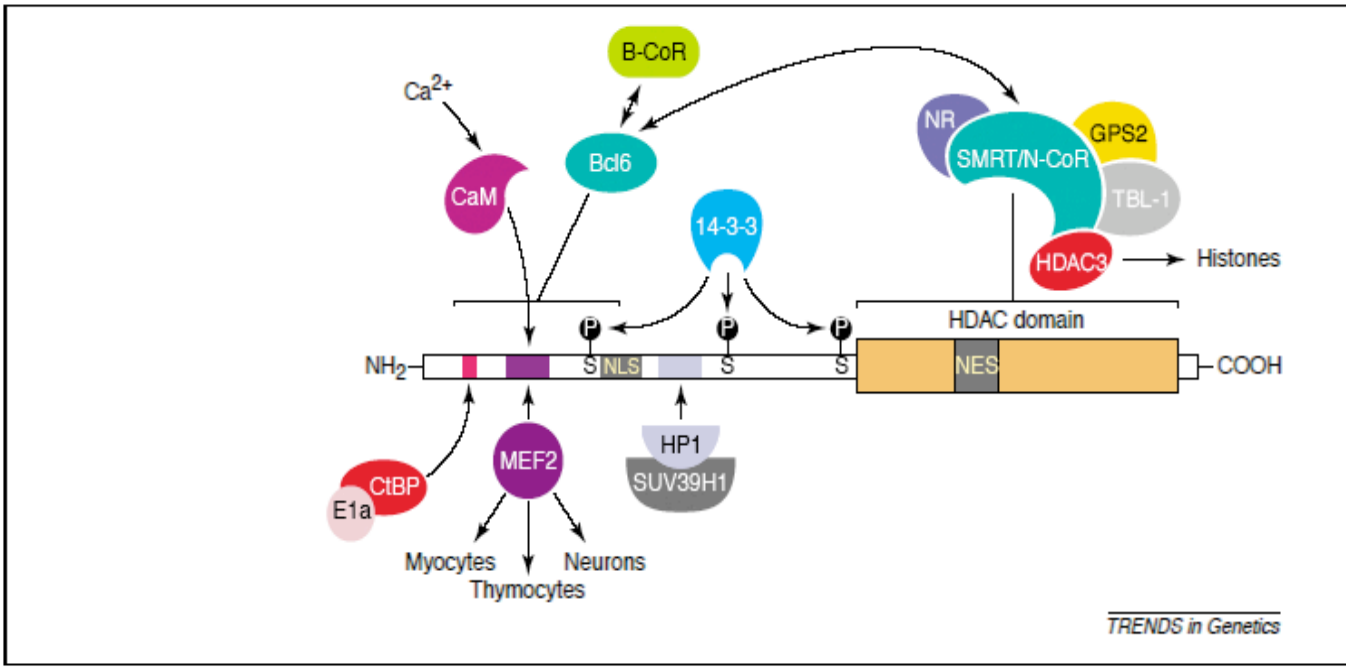

Figure 2. Binding partners of HDAC7. Drawing detailing proteins that have been identified that interact with HDAC7 (Verdin, et al. 2003).

The involvement of HDACs in osteoblast activity has been well documented (Schroeder et al., 2004; Jensen et al., 2008; Kang et al., 2005) For example, over-expression of HDAC 3 or 7 in MC3T31 preosteoblasts inhibits matrix mineralization, suggesting that these HDACs play an important role in osteoblast differentiation (Jensen et al., 2008; Schroeder et al., 2004). However, not much is known about the role HDACs

have in osteoclast differentiation. General HDAC inhibitors such as trichostatin A (TSA) and sodium butyrate (NaB) have been shown to inhibit osteoclast differentiation by affecting NF-κB and p38 MAP kinase signaling (Rahman et al., 2003). Another general HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), currently under clinical trials for its ability to suppress tumor cell proliferation, was shown to inhibit osteoclast differentiation by enhancing apoptosis of osteoclasts (Takada et al., 2006). To date there have been no studies performed that have studied individual HDACs and their role in osteoclast differentiation. My study will examine the effects of HDAC3 and HDAC7 shRNA on osteoclast differentiation.

Hypothesis

The hypothesis of my project is that osteoclasts with reduced expression of HDAC3 or HDAC7 will increase osteoclast gene expression leading to an increase in osteoclast differentiation.

Specific Aims

1. To determine the effect of HDAC3 silencing on osteoclast gene expression.
2. To determine the effect of HDAC7 silencing on osteoclast gene expression.

Materials and Methods

Primary Osteoclast Cell Culture and Lentiviral Infection of Osteoclasts

Osteoclasts were isolated by briefly flushing bone marrow from femurs of WT mice. The cells were cultured in alpha-MEM media for three days in the presence of 50ng/ml M-CSF (macrophage-colony stimulating factor) on non-tissue culture dishes. M-CSF selects for osteoclasts by promoting survival and proliferation of osteoclast precursors.

The adherent cell population containing primary osteoclasts was infected with lentiviral vectors (Open Biosystems) that encoded shRNAs against HDAC7, HDAC3, or a control shRNA. Following infection, the cultures were stimulated with M-CSF and RANKL for three days.

Quantification of Gene Expression

RNA from cultured osteoclasts was extracted using TriZol Reagent (Invitrogen) and quantified by UV spectroscopy. cDNA was generated using reverse transcriptase polymerase chain reaction using iScript cDNA synthesis kit (Bio-Rad). This cDNA was then used to measure osteoclast gene expression (Cathepsin-K, Nfatc1, and DC-STAMP) by quantitative real-time polymerase chain reaction (RT-PCR) using MyIQ Single-Color Real-Time PCR Detection System (Bio-Rad).

RT-PCR is a tool that allows relatively quick and precise quantification of mRNA levels where genes of interest are amplified and measured in real time. Nucleotides, forward and reverse primers specific for the target gene, osteoclast cDNA, and SYBRGreen (Bio-Rad IQ SYBR Green Supermix) make up the reaction mixture for each

sample. This system detects SYBR Green, which fluoresces only when bound to double stranded DNA. RT-PCR using cDNA from each osteoclast treatment group was run in duplicate for each of the three genes of interest and the internal standard housekeeping gene, the *L4* gene. *L4* is a ribosomal gene that is expressed at similar levels in undifferentiated and differentiated osteoclasts and therefore serves as a control that all other genes are compared against.

Threshold cycle (Ct) values were gathered after each RT-PCR run from the MyIQ software and a series of calculations comparing each target gene to the reference gene L4 were computed using Microsoft Excel. These calculations led to the Average Expression and Fold Change values that are entered into GraphPad for statistical analysis.

Statistical Analysis

All experiments were run in triplicate and results are expressed as mean ± SD. Statistical analysis included Student's t-tests to measure significance between HDAC7 shRNA and control shRNA treatment groups and between HDAC3 shRNA and control shRNA treatment groups. Significance is indicated by $p < 0.05$.

Results

Non-selective HDAC inhibitors such as TSA and NaB have been shown to inhibit osteoclast differentiation. However, the significance of individual HDACs on osteoclast differentiation has not been previously reported. In light of HDACs importance in regulating osteoblast differentiation, we chose to suppress expression of HDAC3 and HDAC7 and determine the effect on osteoclast gene expression.

Suppression of HDAC3 inhibits osteoclast differentiation – Prior studies in Mansky lab measured HDAC3 expression in control and HDAC3 shRNA infected murine bone marrow cultures. To ensure that any effects were due to specific knockdown of HDAC3, two distinct shRNAs against HDAC3 were compared. Infection of cultures with either HDAC3 shRNA reduced HDAC3 expression by approximately 50% compared to the control shRNA (Fig. 3A). A reduction in HDAC3 protein levels was seen when RAW 264.7 cells were infected (Fig. 3B). While control shRNA cultures readily formed TRAP- positive osteoclasts upon stimulation with RANKL, osteoclast formation was strongly reduced in cultures infected with either HDAC3 shRNA (Fig 3C). Quantitative analysis of these cultures indicated that both the number and size of TRAP-positive cells was significantly reduced in HDAC3-shRNA cultures compared to the controls (Fig 3D-E).

In my study, quantitative real time RT-PCR analysis demonstrated that expression of osteoclast marker genes *NFAT-c1* (4 fold reduction, $p \leq 0.0098$), *Ctsk* (2.5 fold reduction, $p \leq 0.0395$) and *DC-STAMP* (1.8 fold reduction, $p \leq 0.0136$) were reduced in the HDAC3 shRNA cells (Fig. 3F). These data indicate that reduction of HDAC3 expression impairs osteoclastogenesis, thus suggesting that HDAC3 activity is necessary for osteoclast formation.

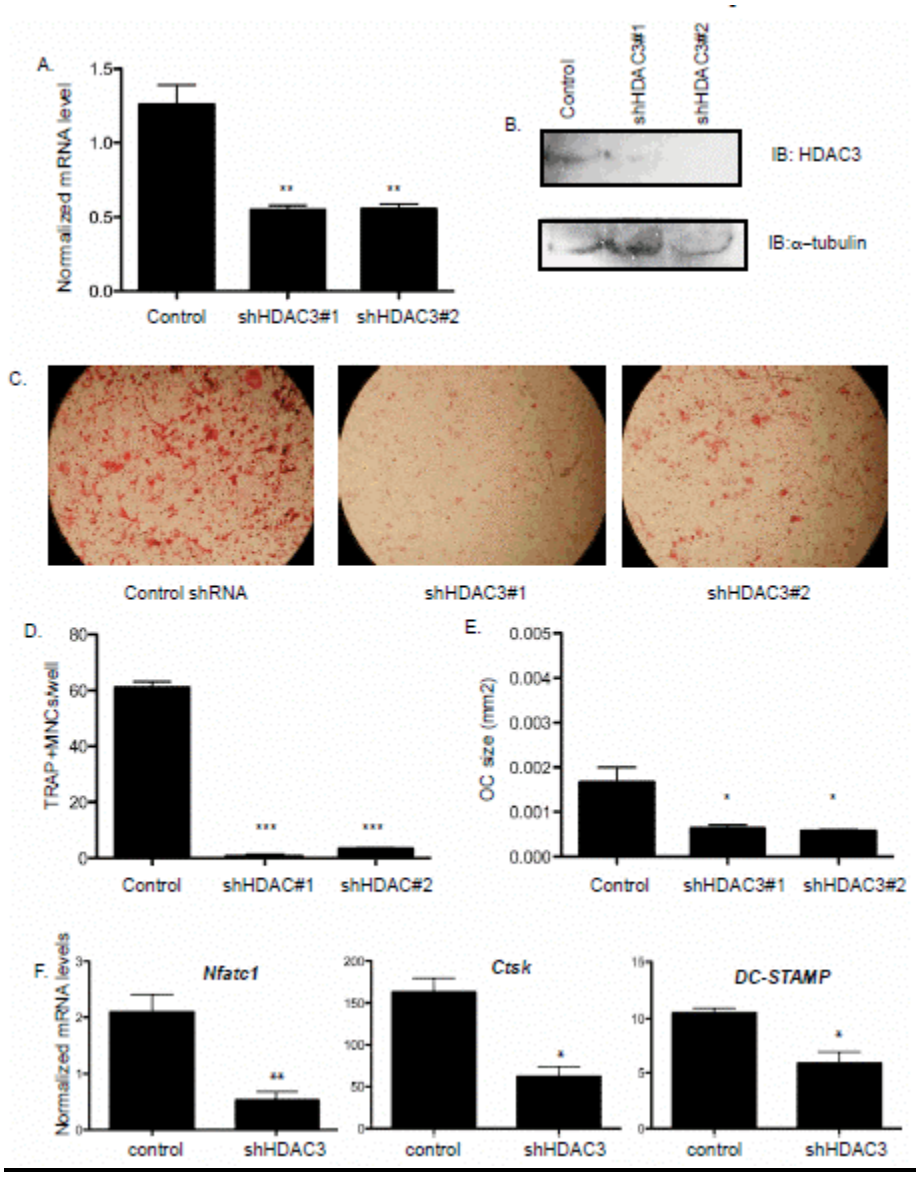

Figure 3. Suppression of HDAC3 inhibits osteoclast differentiation. (A) Real time RT-PCR of bone marrow, **p< 0.007 vs. control shRNA (B) Western blot of RAW 264.7 lysates showing expression of HDAC3 (C) TRAP staining of osteoclast cultures (D) and (E) Histomorphometric analysis of TRAP stained osteoclasts, ***p<0.0001 and *p< 0.05 vs. control shRNA (F) expression profile of *Nfat-c1, Ctsk* and *DC-STAMP*, *p<0.05 and **p<0.005 vs. control shRNA following infection with control shRNA or HDAC3 shRNA lentiviral vectors.

Suppression of HDAC7 enhances osteoclast differentiation - Prior studies in Mansky lab measured HDAC7 expression in control and HDAC7 shRNA infected murine bone marrow cultures. The lentivirus encoding the shRNA against HDAC7 has been previously shown to suppress HDAC7 expression in osteoblasts (Jensen, 2008). Infection of cultures with HDAC7 shRNA vector reduced HDAC7 mRNA levels by 16-fold compared to the control vector (Fig. 4A). In addition, HDAC7 protein was reduced (Fig. 4B). In contrast to the HDAC3-suppressed cells, osteoclast differentiation in HDAC7 suppressed cultures was enhanced compared to controls (Fig. 4C). The average size of TRAP- positive multinucleated osteoclasts in HDAC7- suppressed cells was increased 9-fold (Fig. 4D), and the mean number of TRAP-positive multinucleated cells per well increased 1.5-fold (Fig. 4E)

In my study, quantitative real time RT-PCR showed that expression of osteoclast marker genes *NFAT-c1* (3 fold increase, $p \leq 0.009$), *Ctsk* (1.8 fold increase, $p \leq 0.0257$) and *DC-STAMP* (1.7 fold increase, $p \leq 0.0088$) were increased in the HDAC7 shRNA cells (Fig. 4F). These results reveal that HDAC7 suppression enhances osteoclast formation, thus suggesting that HDAC7 activity inhibits osteoclastogenesis.

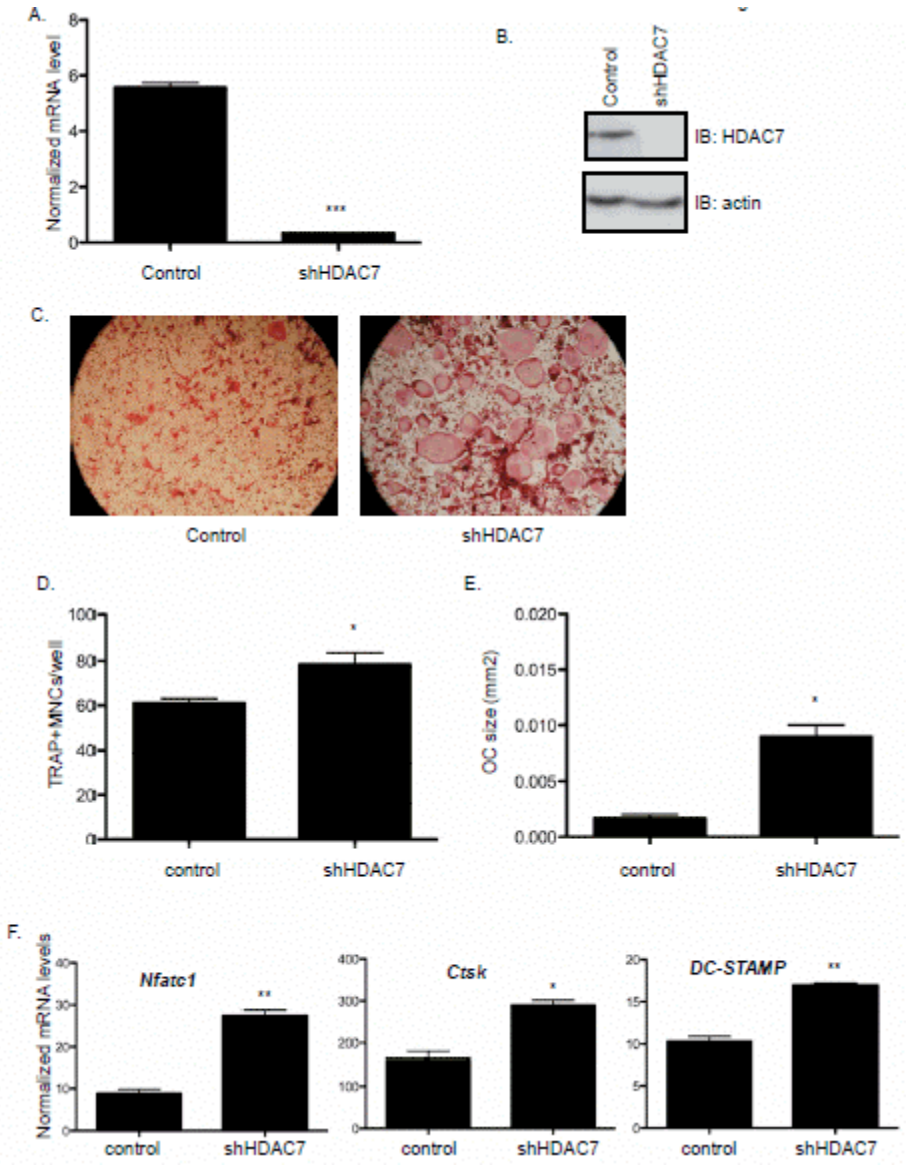

Figure 4. Accelerated osteoclast differentiation in HDAC7-suppressed osteoclasts. (A) Real time RT-PCR of bone marrow, ***p<0.0001 vs. control shRNA (B) Western blot of osteoclast lysates showing expression of HDAC7 (C) TRAP staining of osteoclast cultures infected with control or HDAC7 shRNA expressing lentiviruses. (D) and (E) Histomorphometric analysis of TRAP-stained osteoclasts, *p< 0.05 vs. control shRNA and (F) expression profile of *Nfat-c1, Ctsk* and *DC-STAMP*, *p<0.05 and **p< 0.005 vs. control shRNA following infection with control shRNA or HDAC7 shRNA lentiviral vectors.

Discussion

Suppression of HDAC3 had the opposite effect on osteoclastogenesis than was hypothesized. One theory for this is that HDAC3 suppression acts like the HDAC inhibitors TSA and NaB because HDAC3 only works by deactelyting proteins. One hypothesis was that the cells with suppressed HDAC3 expression underwent apoptosis as was seen with the osteoclasts treated with HDAC inhibitors TSA and NaB. This hypothesis would have to be furthered verified with additional experiments.

As was hypothesized, reduced expression of HDAC7 resulted in increased osteoclast gene expression. The panel of osteoclast genes analyzed indicates that different stages of osteoclast differentiation are affected, including fusion and resorption. In agreement with increased osteoclast gene expression, experiments in Dr. Mansky's lab found that knocking down HDAC7 resulted in an increased size and number of osteoclasts. Additionally, the Mansky lab has demonstrated that overexpression of HDAC7 decreases osteoclast differentiation (Jensen et al., 2010).

Mitf is necessary for osteoclast differentiation. It has been shown that HDAC7 and Mitf interact in RAW 264.7 cells and primary osteoclasts during M-CSF signaling (Jensen et al., 2010). Dr. Mansky's lab has shown that the deacetylation domain of HDAC7 is not required for HDAC7's ability to repress Mitf activation (Jensen et al., 2010). HDAC7 has been shown to recruit class I HDACs such as HDAC3 to promoters and it could be that this is one of the mechanisms to explain how HDAC7 is repressing Mitf 's activity. Since HDAC7's activity is not dependent on its deacetylation activity this may explain why suppression of HDAC7 versus HDAC3 expression lead to different phenotypes in osteoclasts. Although an interaction between Mitf and HDAC7 was detected, it cannot be ruled out that HDAC7 can interact with other transcription factors

such as PU.1, which is also necessary for osteoclast differentiation. It remains unclear whether the altered expression of any particular gene in the HDAC7 suppressed cells reflects regulation by HDAC7 directly or indirectly. ChIP assays will need to be done to determine if a Mitf-HDAC7 complex directly regulates these genes. These data indicate that HDAC7 is an important regulator of osteoclast differentiation (Figure 5).

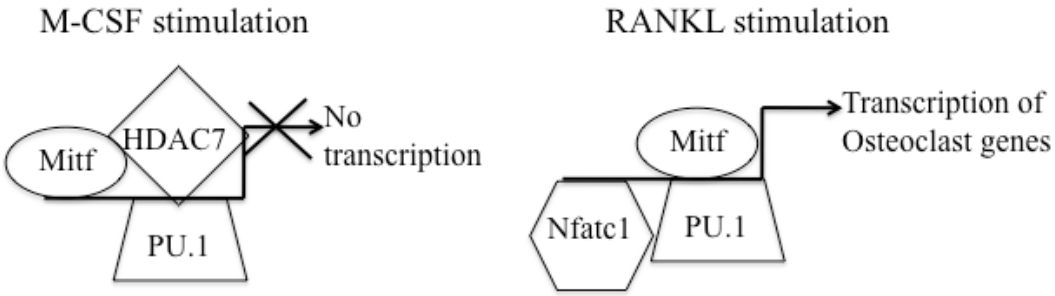

Figure 5. Model of the interaction between HDAC7 and Mitf. Upon M-CSF stimulation of osteoclasts, Mitf and HDAC7 interact on osteoclast promoters and prevent Mitf from activating genes necessary for osteoclast differentiation. With RANKL stimulation of osteoclast precursors, Mitf and HDAC7 can no longer interact and Mitf in complex with PU.1 and Nfatc1 activate genes necessary for osteoclast differentiation.

This study, along with findings from Mansky lab, suggests that HDAC7 may be a novel Mitf repressor. Understanding the mechanisms by which HDAC7 represses Mitf function, their significance in the context of the larger transcription complex (c-fos/Mitf/PU.1/Nfatc1) at osteoclast promoters and the regulatory inputs that coordinate their activity will be significant areas for future studies to increase our understanding of skeletal development, maintenance and pathological states.

Conclusions

1. Suppression of HDAC3 expression impairs osteoclastogenesis, suggesting that HDAC3 activity is necessary for osteoclast formation.

2. Suppression of HDAC7 expression enhances osteoclastogenesis, suggesting that HDAC7 activity inhibits osteoclast formation.

References

Boyle W, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature. 2003;423:337-342.

Bronisz, A., Sharma, S. M., Hu, R., Godlewski, J., Tzivion, G., Mansky, K. C., and Ostrowski, M. C. (2006) Mol Biol Cell 17, 3897-3906.

Fischle, W., Dequiedt, F., Hendzel, M.J., Guenter, M.G., Lazar, M.A., Voelter, W., and Verdin, E. (2002) Enzymatic Activity Associated with Class II HDACs is Dependent on a Multiprotein Complex Containing HDAC3 and SMRT/N-CoR Molecular Cell 9:45-57.

Ishii M, Saeki Y. Osteoclast cell fusion: mechanisms and molecules. Modern Rheumatology. 2008;18:220-227.

Jensen ED, Schroeder TM, Bailey J, Gopalakrishnan R, Westendorf JJ. 2008. Histone deacetylase 7 associates with Runx2 and represses its activity during osteoblast maturation in a deacetylation-independent manner. J Bone Miner Res 23(3):361-372.

Jensen, ED, Pham, L., Kaiser, B., Romsa, A., Schwarz, T., Gopalakrishnan, R. and Mansky, K.C. (2010) HDAC3 and HDAC7 have opposite effects on osteoclast differentiation. submitted for review.

Kang, JS, Alliston, T. Delston, R., and Derynck, R. (2005) Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. EMBO J 24:2543-2555.

Kukita T, Wada N, Kukita A, Kakimoto T, Sandra F, Toh K, Nagata K, Iijima T, Horiuchi M, Matsusaki H, Hieshima K, Yoshie O, Nomiyama H. RANKL-induced DC-STAMP is Essential for Osteoclastogenesis. Journal of Experimental Medicine. 2004;200: 941-946.

Mansky K, Gopalakrishnan R, personal communication 2010.

Rahman MM, Kukita A, Kukita T, Shobuike T, Nakamura T, Kohashi O. 2003. Two histone deacetylase inhibitors, trichostatin A and sodium butyrate, suppress differentiation into osteoclasts but not into macrophages. Blood 101(9):3451-3459.

Schroeder TM, Kahler RA, Li X, Westendorf JJ. 2004. Histone deacetylase 3 interacts with runx2 to repress the osteocalcin promoter and regulate osteoblast differentiation. J Biol Chem 279(40):41998-42007.

Sengupta, N., and Seto, E. (2004) Journal of Cellular Biochemistry 93, 57-67.

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

Sharma, S. M., Bronisz, A., Hu, R., Patel, K., Mansky, K. C., Sif, S., and Ostrowski, M. C. (2007) J Biol Chem 282, 15921-15929.

Takada, Y., Gillenwater, A., Ichikawa, H. and Aggarwal, B.B. (2006) J Biol Chem 281, 5612-5622.

Verdin, E., Dequiedt, F., and Kasler, H.G. (2003) Trends in Genetics 19, 286-293.

Yagi M, Miyamoto T, Sawatani 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.

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

Yavropoulou MP, Yovos JG. Osteoclastogenesis – Current knowledge and future perspectives. Journal of Musculoskeletal and Neuronal Interactions. 2008;8:204-216.

Zhao, Z, Shao, J, Chen W, Li YP. Osteoclast differentiation and gene regulation. Frontiers in Bioscience. 2007;12:2519-2529.

Appendix I. Average Gene Expression

	control	shHDAC3	shHDAC7
Nfatc1	8.85	0.533	27.336
Cathepsin K	163.275	61.51	289.035
DC-STAMP	10.3	5.833	17.0

Appendix II. Analysis of changes in gene expression changes for osteoclasts suppressed for HDAC3

	Average Fold Change	t-test-p-value	Significance
Nfatc1	-16.6	0.0098	Yes
Cathepsin K	-2.65	0.0379	Yes
DC-STAMP	-1.76	0.0136	Yes

Appendix III. Analysis of changes in gene expression changes for osteoclasts suppressed for HDAC7

	Average Fold Change	t-test-p-value	Significance
Nfact1	+3.08	0.0090	Yes
Cathepsin K	+1.77	0.0251	Yes
DC-STAMP	+1.65	0.0088	Yes