HDAC7 KO osteoclasts show increased sensitivity to RANK-L cytokine

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Dedication

To my husband, **Dr. Casey Newton**, for his support, encouragement and belief in me.

To my parents, for instilling in me the values of perseverance, faith and gratitude.

Abstract

Bone homeostasis is a balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation. Two cytokines, M-CSF and RANK-L are known to be necessary in osteoclast differentiation. Histone deacetylases (HDACs) are key regulators of gene expression, and recently have been shown to be important modulators in osteoclast differentiation. In previous research, HDAC7 has been shown to be a negative regulator of osteoclastogenesis. Osteoclasts from HDAC7 KO mice are increased in size and number. This study aims to test the hypothesis that osteoclasts from HDAC7 KO mice are hypersensitive to M-CSF and/or RANK-L. Results from my study demonstrate enhanced osteoclast differentiation in size and number of osteoclasts and resorption under varying M-CSF and RANK-L concentrations in HDAC7 KO compared to wild type osteoclasts. Mechanistically the hypersensitivity may be explained because HDAC7 KO osteoclasts had increased *C-fms* and *RANK* expression compared to wild type osteoclasts. Further research is necessary to determine if the hypersensitivity is due to increased gene expression by transcription factors MITF and PU.1.

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Introduction

Bone homeostasis is a balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation (Fig. 1). Osteoclasts are multinucleated cells differentiated from myeloid (monocyte/macrophage lineage) progenitors by mainly two cytokines: 1) M-CSF (macrophage colony stimulating factor) and 2) RANK-L (receptor activator of NF-kappa B ligand). Both M-CSF and RANK-L cytokines are necessary for osteoclast differentiation. Both M-CSF and RANK-L together activate signaling pathways/transcription factors that are necessary for osteoclast differentiation [3] (Fig. 2).

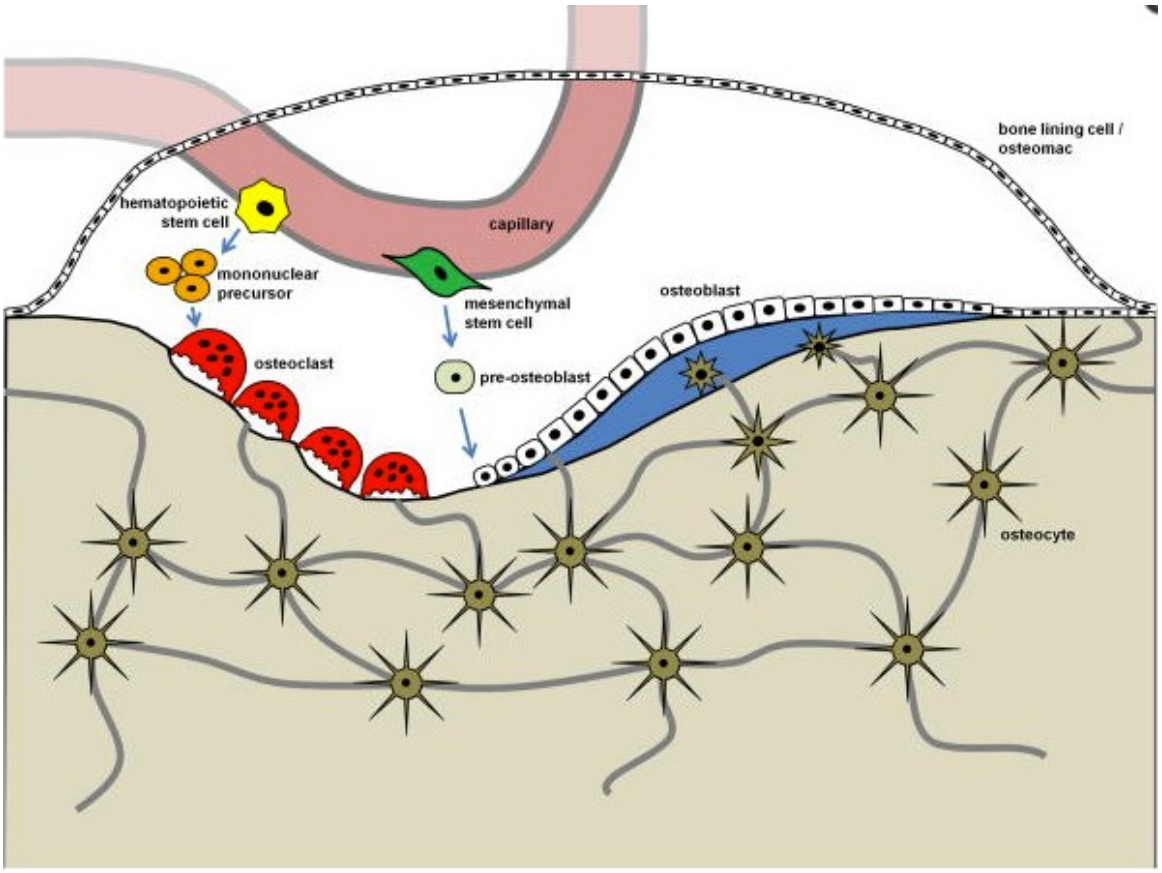

Fig. 1. Bone remodeling. [5].

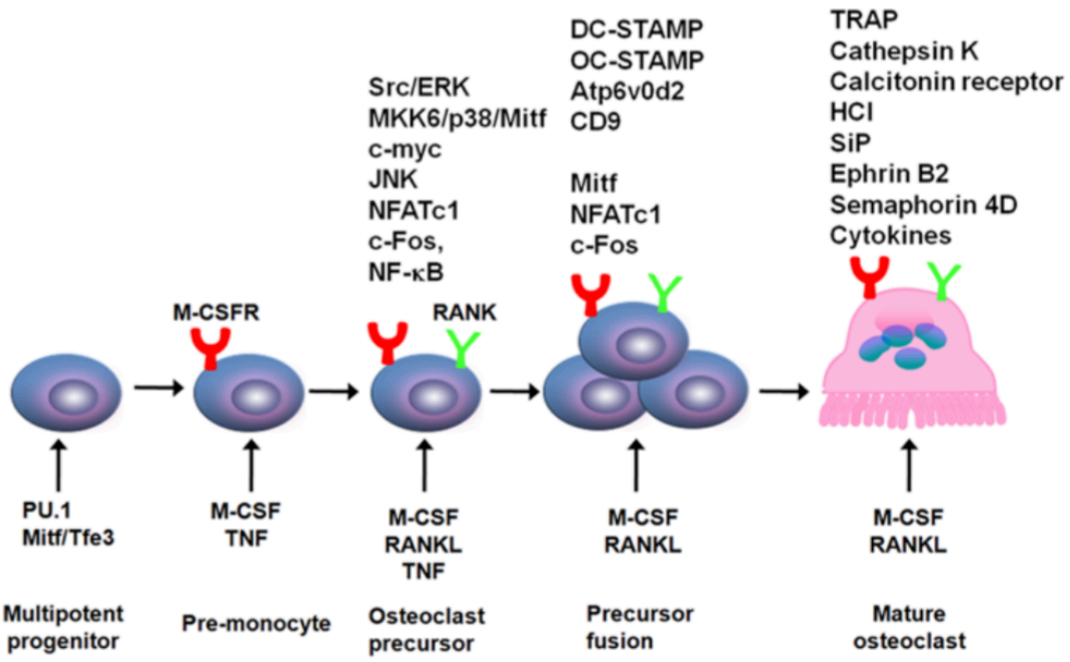

Fig. 2. Schematic of osteoclast differentiation.

Osteoclast differentiation is supported by osteoblasts and stromal cells via cell-cell interactions [12]. The main role of M-CSF is to provide proliferation and survival signals during osteoclastogenesis [13]. M-CSF cytokine binds to cell-surface C-FMS receptor, which results in autophosphorylation by receptor C-FMS kinase resulting in a cascade of intracellular signals. [20]. The main role of RANK-L is to provide differentiation signals and activate multiple pathways during osteoclastogenesis [13]. Osteoblasts produce RANK-L in response to bone resorbing factors. RANK-L activates signal transduction pathways, turning on transcription factors NF-kB, c-Fos, nuclear factor of activated T-cells (NFATc1), and microphthalmia transcription factor (MITF) [13]. The binding of RANK-L to the RANK receptor on the cell surface of osteoclast precursors leads to the recruitment of TRAF6, which is a member of the TNF receptor-associated factor family proteins, and leads to NF-kB activation and nuclear translocation.

NF-kB then activates c-Fos, which induces *Nfatc1* transcription (Fig 3- 4). Induction of *Nfatc1* is thought to be a hallmark event in determining the cell fate of osteoclasts [9]. RANK-L activates p38 mitogen activated protein kinase (MAPK), which in turn phosphorylates MITF. MITF forms a complex with PU.1 and is required for terminal differentiation of osteoclasts [12]. MITF regulates osteoclastogenesis by upregulating expression of genes related to osteoclast activity such as TRAP (tartrate- resistant acid phosphatase), and regulates osteoclast differentiation/development via regulation of cell fusion [15]. Transcription factor, PU.1, stimulates the expression of *c-fms* and therefore is essential for the hematopoietic stem cell's commitment to the myeloid lineage [22, 23]. Studies suggest *Rank* expression is regulated by PU.1 in cooperation with MITF and may represent one of the key events in osteoclast differentiation [22, 23] (Fig. 5). Dysfunction of any of the above factors in osteoclasts will result in arrest of osteoclast differentiation, leading to osteopetrosis [9, 14, 22,].

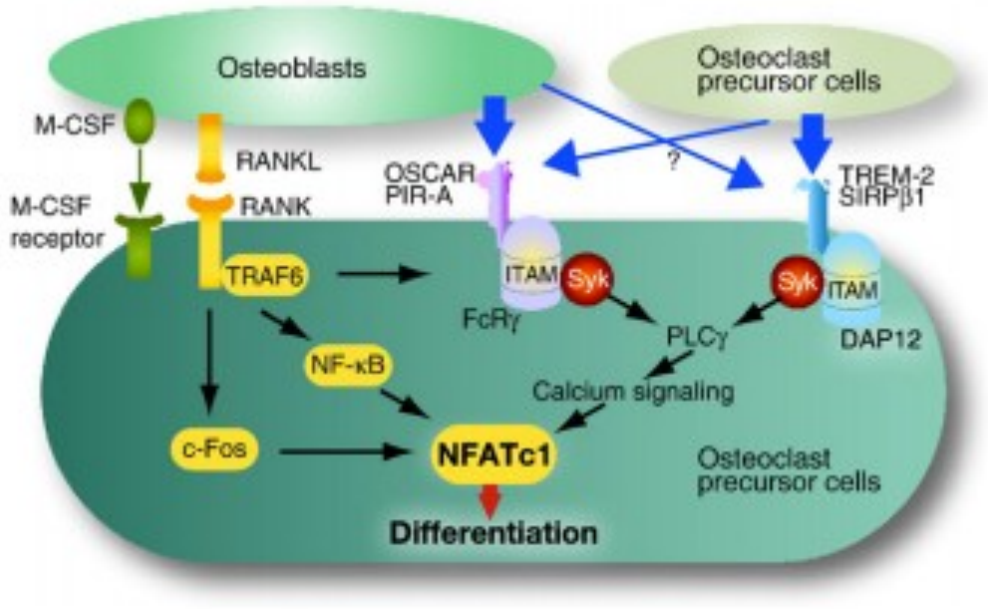

Fig. 3. RANK-L signals in osteoclastogenesis. [9]

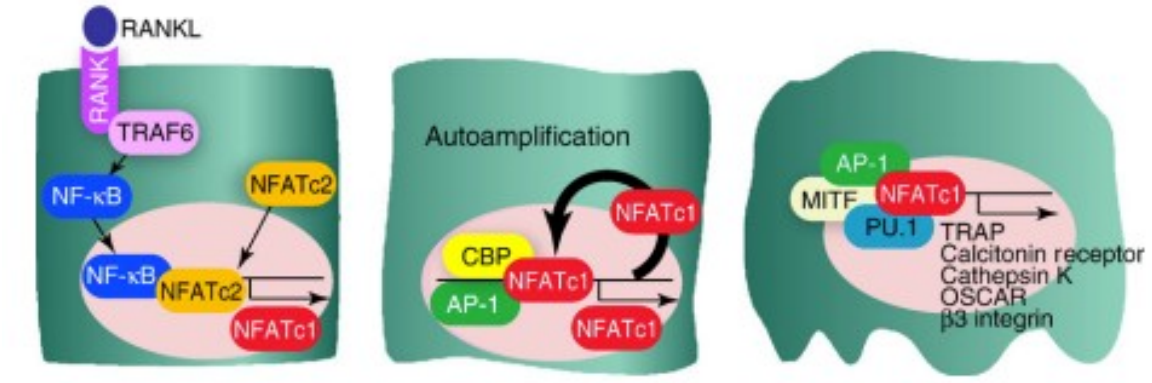

Fig. 4. Osteoclast differentiation governed by NFATc1 [9]

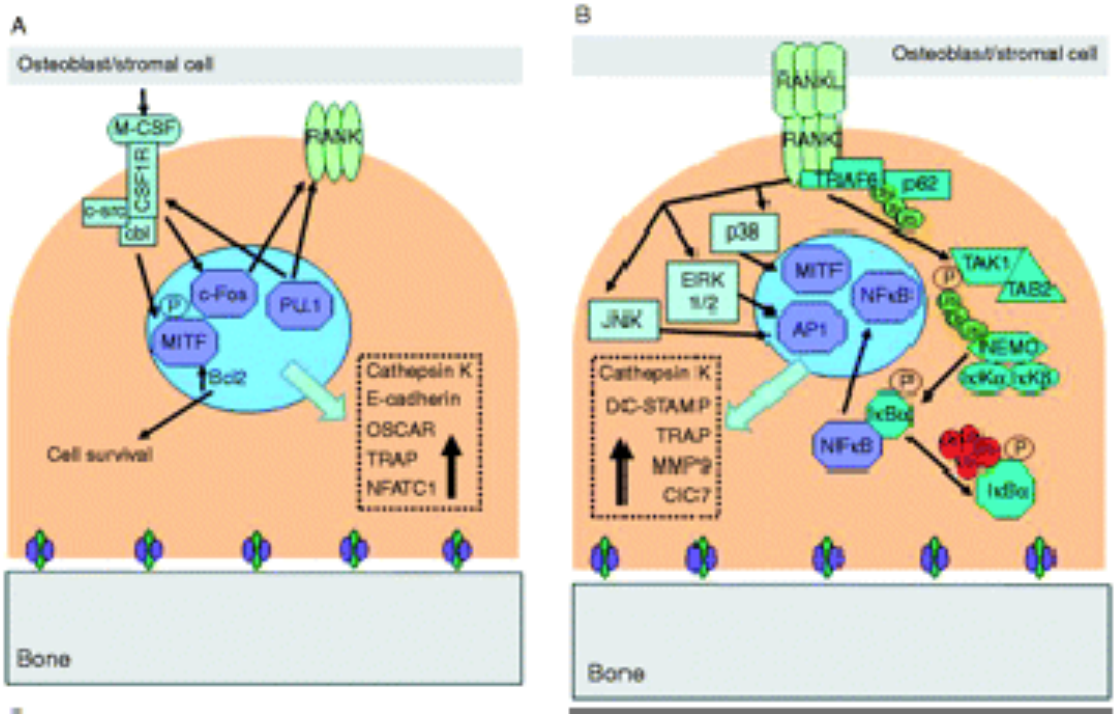

Fig. 5. Commitment of cells to the myeloid lineage [23].

Transcription regulation in eukaryotes is influenced by the manner in which DNA is packaged. DNA is packaged into chromatin, which is a highly organized and dynamic protein-DNA complex. The fundamental subunit of chromatin is the nucleosome, which is composed of an octomer of four core histones [2]. The compact, inaccessible DNA is made available to DNA binding proteins or transcription factors via modification of the

nucleosome (methylation, phosphorylation, and acetylation).

The level of the nucleosome is where it is thought that some histone deacetylases (HDACs) assert their activity. HDACs are important regulators of many biological processes including endochondral/intramembranous ossification and the dynamic balance of bone remodeling [1]. There are 18 HDACs in the human genome, which are classified into 4 groups. HDAC 7 is in group IIa [5]. The majority of the research regarding the mechanism of action of HDACs is based on group I, which has established their mechanism of action as a deacetylase. Less research has been completed to understand the mechanisms by which class II HDACs regulate transcription; however, what is known is that their mechanism of action is not completely dependent on their deacetylase activity [2]. The developmental and physiological functions of HDACs revealed in mouse gene deletion studies have guided research that explores HDAC inhibition as a therapeutic modality to address common pathologies of the bone such as osteoporosis, Paget's disease, rheumatoid arthritis, and cancer metastasis [4, 17, 18].

HDAC inhibition therapies have the potential to be used in conjunction with orthodontics in that orthodontic tooth movement and craniofacial growth and development are regulated by apposition, resorption and remodeling of the bone. Application of HDAC inhibitors (HDACi) in the future may be used to modulate osteoblast/osteoclast activity in a specific and controlled manner.

Review of the Literature

The effect of gene deletions on osteoclastogenesis has been evaluated in the literature not only to understand mechanisms of action and gene transcription as a whole, but also to develop new therapeutic strategies for bone diseases [2]. HDAC inhibitors

have been shown to induce specific changes in gene expression [2]. These processes include growth arrest, differentiation, cytotoxicity and induction of apoptosis. More specifically, identifying key modulators of bone resorption may reveal new therapeutic targets for prevention/treatment of bone diseases thus, research in HDAC inhibition as a drug therapy has shown increasing interest. Currently in humans, HDACi have been used to treat epilepsy, bipolar disorder, and cancer [5], however studies indicate HDACi may also be used as antitumor agents (preventing vascularization during tumor metastasis), and as anti-inflammatory agents due to their effects on cell death [16, 11].

Limited progress has been made in finding therapies for the bone loss associated with inflammatory diseases such as rheumatoid arthritis and periodontitis; however, studies are investigating HDACi and their response to repress bone loss in these diseases [19]. HDACi have been shown in mouse models and in vitro to repress bone resorption in osteoclasts and may show promise to be used in combination with current anti-inflammatory treatments [19]. A large challenge with HDACi is their lack of specificity. An important example is the dichotomous effect of inhibiting class I HDACs (of which most of the HDACi research has been developed) versus class II HDACs. HDAC3 (class I HDACs) suppression inhibits osteoclast differentiation whereas HDAC7 (class II HDACs) suppression actually enhances osteoclast differentiation [19]. In general, HDACi are tolerated well; however, their widespread effects in a variety of cells and tissues highlight the need for continued research to develop specific therapeutic targets.

As was previously mentioned, HDAC7's mechanism of transcription regulation is unlike class I HDACs. Jensen et al. studied HDAC3 and HDAC7 effects on osteoclast differentiation. HDAC3 belongs to class I and acts via its deacetylase activity whereas

HDAC7 can regulate transcription independent of its deacetylase activity. Furthermore, the study showed HDAC7 activity inhibits osteoclast gene expression and is thus a negative regulator of osteoclast differentiation. Conversely, HDAC7 suppression had the opposite effect of HDAC3 in that HDAC7 suppression enhanced osteoclast formation [3]. Previous research on HDAC7 knock-out (HDAC7 KO) mice demonstrate that HDAC7 KO mice have osteoclasts which are larger in size, increased nuclei number, and show enhanced bone resorption [3,6]. In addition, Jensen et al demonstrated that HDAC7 regulates osteoclast differentiation by negatively regulating the activity of the transcription factor MITF. It is through the loss of HDAC7's negative regulation of MITF that differentiation of HDAC7 KO osteoclasts are enhanced. Lastly, in unpublished data from the Mansky lab, they have demonstrated that HDAC7 can interact with and regulate PU.1 activity, the binding partner of MITF.

Previous studies by Wu et. al and Takeshita et al demonstrated that osteoclasts null for negative regulators PECAM or SHIP are hypersensitive to varying M-CSF and RANK-L concentrations [7,8]. These studies guided this study to determine if HDAC7 null osteoclasts are hypersensitive to M-CSF and RANKL. In support of this hypothesis, PU.1 has been demonstrated to regulate *c-fms* expression, and MITF and PU.1 have been shown to regulate expression of *Rank* [21, 22, 23]. As stated above, both MITF and PU.1 have been shown to be targets of HDAC7 in osteoclasts. Given the above facts, I decided to determine if HDAC7 KO osteoclasts are hypersensitive to M-CSF and RANK- L.

Hypothesis and Specific Aims

Hypothesis

The following hypothesis is based on the review of current literature and on the

studies mentioned in the introduction. My hypothesis is that osteoclasts from HDAC7 KO mice will be hypersensitive to the cytokines, M-CSF and RANK-L compared to osteoclasts expressing HDAC7 due to increased expression of C-FMS and RANK, the receptors for M-CSF and RANK-L respectively.

Specific Aims

The specific aims of this study are:

1) Determine if differentiation and activity of HDAC7 KO osteoclasts are hypersensitive to M-CSF compared to wild-type (WT) osteoclasts.

2) Determine if differentiation and activity of HDAC7 KO osteoclasts are hypersensitive to RANK-L as compared to WT osteoclasts.

As will be further discussed in the methods, specific aims 1 and 2 will be addressed by varying amounts of M-CSF while holding the amount of RANK-L constant and vice versa, varying amounts of RANK-L while holding the amount of M-CSF constant. The specific aims will be achieved by answering the following questions: Will osteoclasts be larger in size? (Evaluated by TRAP stain and Image J software), Will there be increased number of osteoclasts? (Evaluated by TRAP stain and Image J software), and will there be increased resorption? (Evaluated by Image J software in percentage of area resorbed, average size of resorption pit and number of pits).

3) Determine if the hypersensitivity is due to increased *C-fms* and *Rank* receptors. This aim will be determined by performing qPCR to measure receptor expression.

Materials and Methods

Study Design

This study is an experimental study where the investigator controls the

independent variables. The independent variables are M-CSF and RANK-L, which will be varied by the investigator. The outcome variables are osteoclast differentiation and bone resorption. These outcome variables will depend on the investigator controlled independent variables. The control will be the wild-type (WT) osteoclasts response to varying concentrations of M-CSF and varying concentrations of RANK-L. Confounders of this study will be human error in measurement of concentrations where consistency/accuracy will be very important, the variability of resorption plate calcium/phosphate coating, and variability in cell response to varying concentrations. The population of this study will be at least three WT mice and at least three HDAC7 KO mice. The experiment will be performed at least three times (in triplicate, independent experiments).

Harvesting of bone marrow

Bone marrow macrophages will be harvested from the femurs and tibia of 4 week old WT and HDAC7 KO mice. Femur and tibia will be dissected, the ends cut off and the marrow space flushed with media. The red blood cells will be lysed with RBC lysis buffer and the remaining cells will be plated on 100 mm plates and cultured overnight in osteoclast media and 1% CMG 14-12 conditioned media containing M-CSF. The non-adherent cell population including osteoclast precursor cells will be separated and re-plated on tissue culture dishes and calcium hydroxide plates in osteoclast media supplemented with 1% CMG 14-12 conditioned media. There will be 18 wells per plate with approximately 100,000 cells/well. Number of cells was determined based on other published studies, based on experimental conditions to show adequate differentiation and show measurable changes to number of osteoclasts, size of osteoclasts and to measure

resorption. Two days later one set of differentiation and resorption plates will be fed with varying recombinant M-CSF (R and D systems) and constant RANK-L (20 ng/ml) and the other set of differentiation and resorption plates will be fed with varying amount of RANK-L and constant recombinant M-CSF (10 ng/ml). Cell differentiation and resorption will be observed and captured with light microscopy and the measurements will be analyzed using NIH Image J software.

TRAP Stain

Osteoclasts will be fixed with 4% paraformaldehyde and washed with PBS. The cells with be stained for tartrate resistant acid phosphatase (TRAP) expression with 2.5 ml tartrate, 250 ul Naphthol AS-MX phosphate, 12.5 ml Acetate buffer, 25 ul Triton X- 100, 9.8 ml deionized water, and 7.5 mg Fast Red Violet LB salt. Cells will then be observed and captured with light microscopy and the measurements will be analyzed using NIH Image J software.

Resorption Assay

Media from resorption plate wells will be aspirated and 100 ul/well of 10% bleach solution will be added. Plates will be incubated in bleach solution for 5 minutes at room temperature. Bleach will be aspirated from each well and the wells will be washed twice with 150 ul/well of deionized water. Plates will be allowed to air dry at room temperature before being analyzed for the formation of resorption pits. Resorption pits will be observed and captured with light microscopy and the measurements will be analyzed using NIH image J software (version 1.49).

PCR

RNA was harvested from wild type and HDAC7 KO cells at different times

during osteoclast differentiation using Trizol Reagent (Ambion, Life Technologies) and quantified using UV spectroscopy. Three populations of WT and HDAC7 KO cells were isolated: 1.) monocytes 2.) monocytes that were fed with 1% CMG 14-12 conditioned media containing M-CSF for two days and 3.) monocytes that were fed with 1% CMG 14-12 and RANK-L (10ng/mL) for one day. cDNA was prepared from 1μg RNA using the iScript cDNA Synthesis Kit (Biorad) as per the manufacturer's protocol. Experimental genes were normalized to HPRT.

Statistical Analysis

Each experiment was performed in triplicate. We used Prism image analysis software to capture images, NIH image J (version 1.49) for quantitative analysis, one-way Anova test and Tukey test to determine statistical significance.
The results were considered statistically significant at *p <0.05, **p<0.01; ***p<0.001.

Results

HDAC7 KO cells trend toward increasing size and number to varying concentrations of M-CSF

To determine if HDAC7 KO osteoclasts were hypersensitive to levels of M-CSF, I cultured WT and HDAC7 KO osteoclasts in varying concentrations of M-CSF (2.5-10 ng/mL) and a constant amount of RANK-L (20 ng/mL). Cells were cultured for 5 days to allow for osteoclasts to differentiate into multinuclear cells. TRAP stained plates of WT and HDAC7 KO osteoclasts show a visual trend of increasing size and number of HDAC7 KO osteoclasts as concentrations of M-CSF increased from 2.5 to 10 ng/mL (Fig. 6A). Although not statistically significant, there was a trend in increased number of HDAC7 KO osteoclasts in each concentration of varying M-CSF (Fig. 6B). As the

number of osteoclasts increase, the opportunity for cell-cell fusion into multinucleated osteoclasts also increases. As shown in Fig. 6C, HDAC7 KO osteoclast size was significantly larger than WT osteoclasts in [10 ng/mL] and [2.5 ng/mL] of M-CSF.

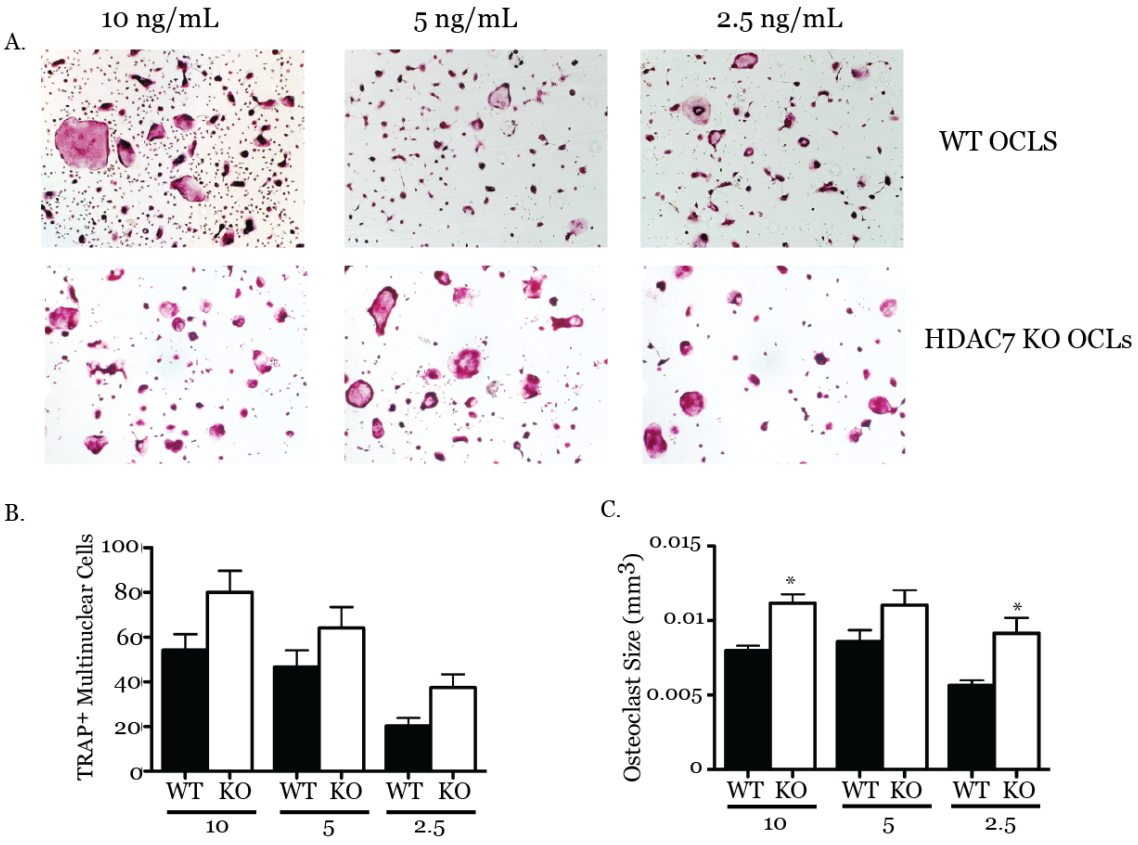

Fig. 6. HDAC7 KO and WT osteoclast differentiation with varying concentrations of M-CSF

To determine if the activity of the HDAC7 KO osteoclasts were hypersensitive to varying concentrations of M-CSF, osteoclasts were plated as described above for differentiation except that the cells were plated on calcium phosphate coated plates. Interestingly, our resorption data with varying concentrations of M-CSF, shows a trend that WT osteoclasts have increased resorption versus HDAC7 KO osteoclasts (Fig 7A). A trend of increased resorption in WT osteoclasts with varying M-CSF concentrations was shown in all three quantitative analyses: percent resorbed, number of resorption pits, and

size of resorption pits (Fig. 7 B, C, D). The [2.5 ng/mL] M-CSF was the only statistically significant concentration that showed a difference in resorption of HDAC7 KO and WT osteoclasts.

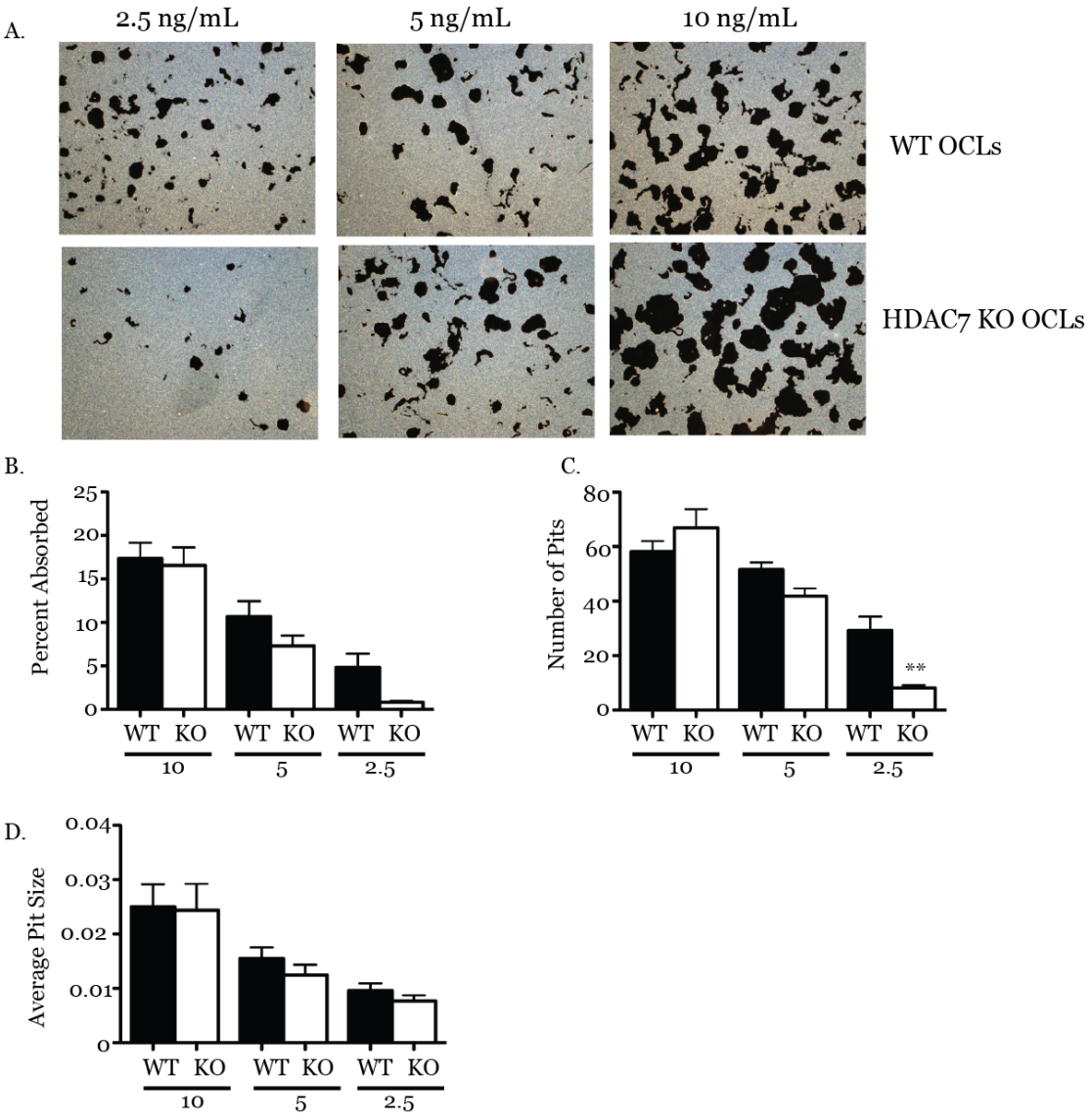

Fig. 7. HDAC7 KO and WT osteoclast resorption with varying concentrations of M-CSF

HDAC7 KO cells are hypersensitive to varying concentrations of RANK-L

To test if HDAC7 KO osteoclasts are hypersensitive to levels of RANK-L, I isolated and plated equal numbers of WT and HDAC7 KO osteoclasts in varying

concentrations of RANK-L (10-20 ng/mL) while keeping the amount of M-CSF constant. TRAP stained plates of WT and HDAC7 KO osteoclasts show a visual trend of increasing size and number of HDAC7 KO osteoclasts as concentrations of RANK-L increased from 10-20 ng/mL (Fig. 8A). There was a trend of increased number of HDAC7 KO osteoclasts as compared to WT and at a concentration of 20 ng/mL of RANK-L the increase in number was statistically significant (Fig. 8B). Again, an increase in number of osteoclasts allows for increased cell-cell fusion and thus larger osteoclasts. HDAC7 KO osteoclasts were significantly larger in size than WT in all three tested concentrations of RANK-L.

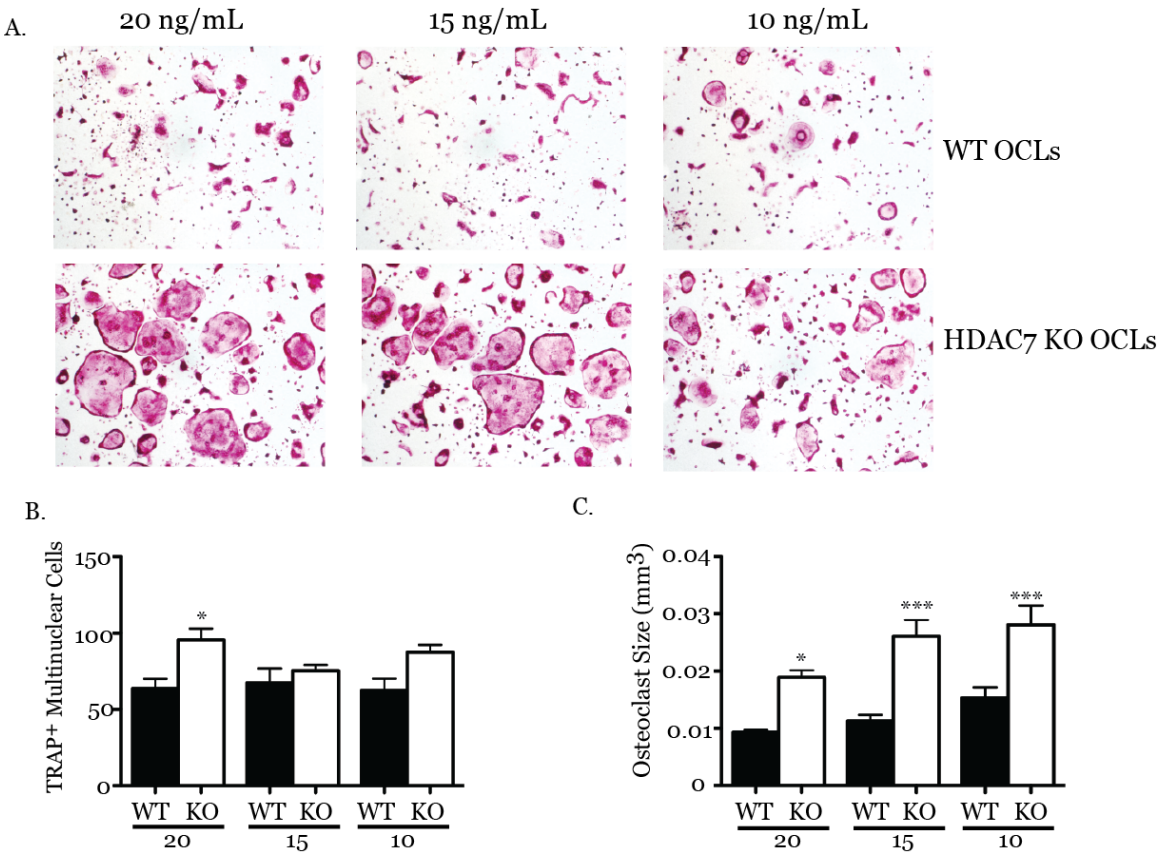

Fig. 8. HDAC7 KO and WT osteoclast differentiation with varying concentrations of RANK-L

Lastly, to determine if HDAC7 KO activity was hypersensitive to RANK-L

concentrations, I plated WT and HDAC7 KO osteoclasts on calcium phosphate coated plates in varying concentrations of RANK-L and a constant amount of M-CSF. Our resorption data shows a trend of increased resorption in HDAC7 KO osteoclasts in all three quantitative analyses: percent resorbed, number of resorption pits, and size of resorption pits. The 10 ng/mL concentration of RANK-L was statistically significant for increased number of pits and average size of pits in HDAC7 KO osteoclasts (Fig. 9).

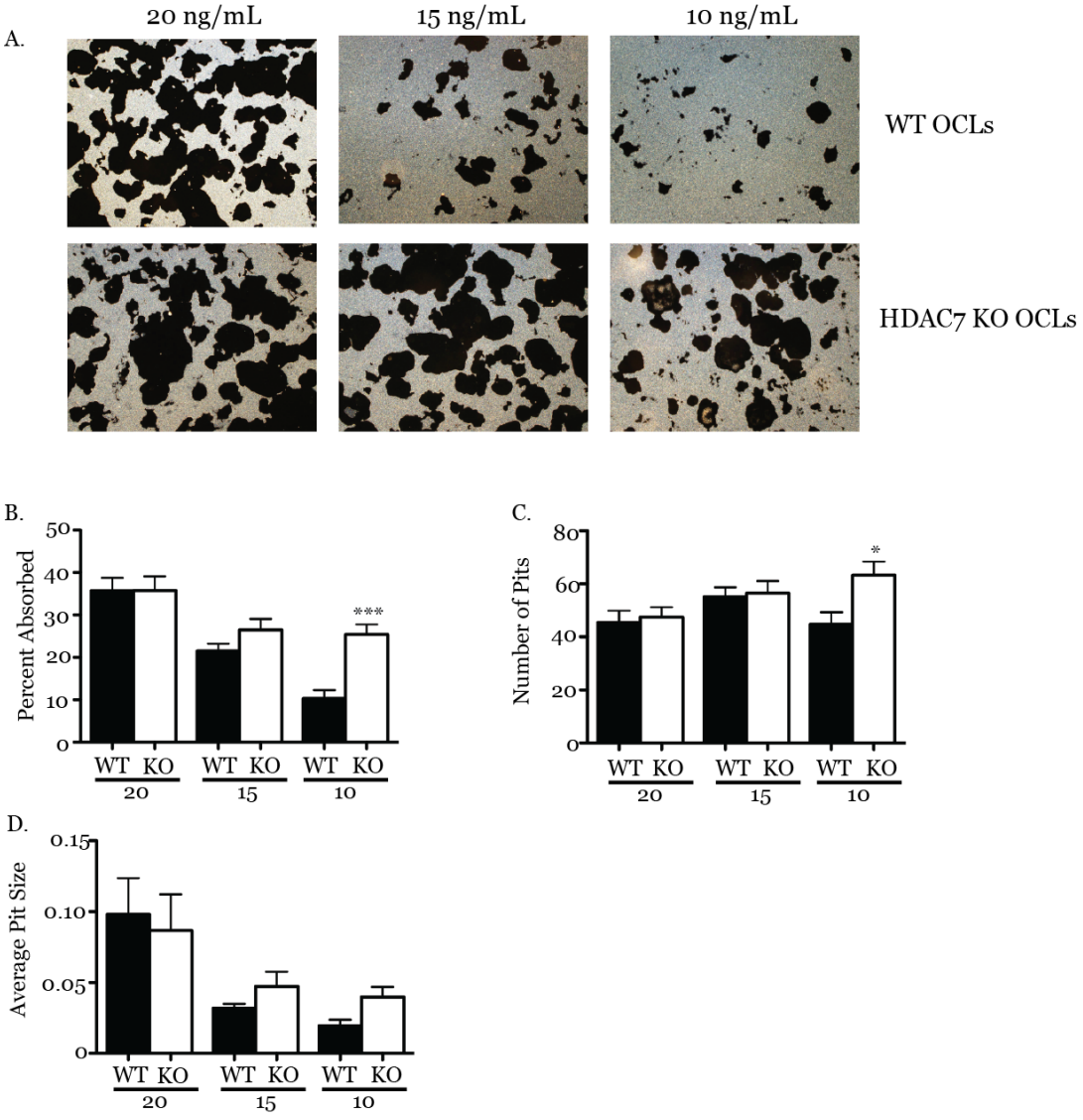

Fig. 9. HDAC7 KO and WT osteoclast resorption with varying concentrations of RANK-L

Increased expression of RANK and c-fms receptors in HDAC7 KO osteoclasts.

PU.1 and MITF have been shown to regulate expression *of c-fms* and *Rank* expression in osteoclasts [21, 23]. Data from the Mansky lab has demonstrated that HDAC7 can interact with and regulate PU.1 and MITF's ability to activate gene expression. To further investigate the mechanism by which HDAC7 KO osteoclasts are hypersensitive to M-CSF and RANK-L, we determined the differences in expression of *c-fms* and *Rank* in both WT and HDAC7 KO osteoclasts (Fig. 10). RNA was isolated from monocytes (mono), which were cells not treated with M-CSF or RANKL, Day 0 cells, which are monocytes that were treated with M-CSF for 2 days or Day 1 cells which are monocytes that were treated with M-CSF and RANK-L for 24 hours. Quantitative PCR was performed to measure expression of *c-fms* and *Rank* in these three populations of cells in either WT or HDAC7 KO cells. As shown in Figure 10A, there is no significance difference in *c-fms* expression in the three populations of cells tested; however, there was a trend for an increase in c-fms expression in the monocytes from the HDAC7 KO cells. In Figure 10B there was a significant increase in *Rank* expression in Day1 HDAC7 KO cells compared to Day1 WT cells.

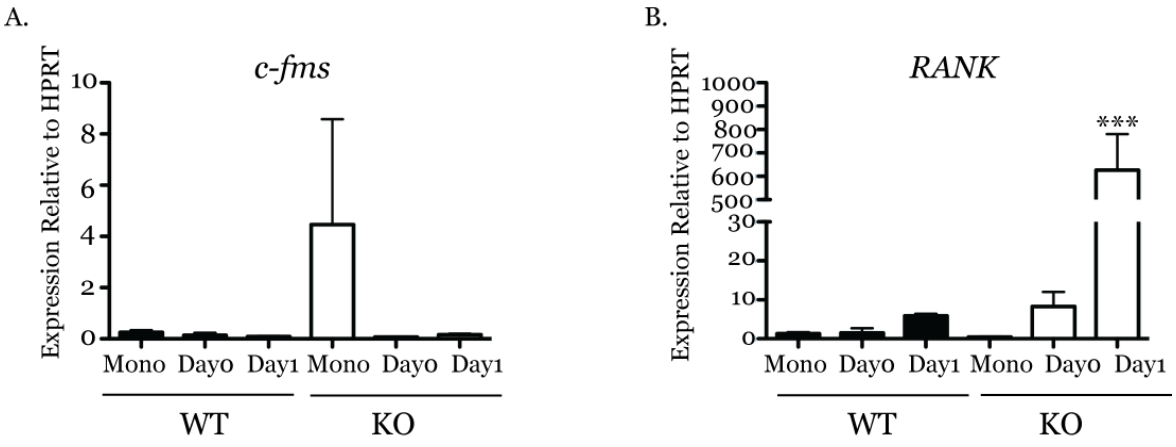

Fig. 10. Expression of *c-fms* and *RANK* receptors on HDAC7 KO and WT osteoclasts.

Discussion

Understanding osteoclastogenesis and its regulation has piqued the interest of researchers for years given the prevalence of bone homeostasis dysregulation seen in osteoporosis, Paget's disease, cancer metastasis, rheumatoid arthritis, etc. One of the groups of genes recently identified to be involved in regulation of osteoclastogenesis, HDACs, have been intensely studied in other cell types in order to provide therapies for various diseases. Although much more research is needed to provide specific and efficacious treatment involving HDAC inhibitors, we aimed in this study to understand better the role of HDAC7 in osteoclastogenesis when subjected to varying [M-CSF] and [RANK-L] and if the hypersensitivity seen was due to increased expression of *Rank* and *c-fms*.

As previously mentioned, Jensen et al. demonstrated HDAC7 regulates osteoclast differentiation by inhibiting the activity of transcription factor MITF. In other words, in HDAC7 KO osteoclasts, MITF activity is upregulated. One of MITF's role in regulating osteoclastogenesis is regulating cell fusion. DC-STAMP (dendritic cell-specific transmembrane protein) is essential for the cell-cell fusion of osteoclasts and transcription of DC-STAMP is positively regulated by MITF [9, 19]. This relative upregulation of MITF in HDAC7 KO osteoclasts and thus increase in cell-cell fusion may partially explain the larger size of the multinucleated osteoclasts in HDAC7 KO in both varying [M-CSF] and varying [RANK-L] experiments.

One study by Mochizuki A, et.al. may explain in part the increased differentiation seen in varying [RANK-L] HDAC7 KO osteoclasts. This particular study looked at cell adhesion and the regulation of RANK receptors. Subjected to adherent and non-adherent

conditions, bone-marrow derived macrophages were used as osteoclast precursors and were found to show increased *Rank* receptor expression and osteoclast differentiation on the adherent condition and markedly reduced differentiation in the non-adherent condition. In fact, bone-marrow macrophages (BMMs) transferred from adherent to non-adherent conditions showed downregulation in RANK receptor expression and vice versa. BMMs transferred from non-adherent to adherent conditions showed significant increase in the level of RANK expression. This study suggests cell adhesion signaling may in part regulate *Rank* expression and that cell adhesion is required for *Rank* expression in osteoclast precursors, which is essential for osteoclast differentiation. In our study, increased RANK receptors expression in HDAC7 KO may in turn increase adhesion and thus contribute to larger cells and increased resorption seen in HDAC7 KO osteoclasts with varying [RANK-L]. In fact, in unpublished data, the Mansky lab has demonstrated that HDAC7 KO osteoclasts are more adherent than WT osteoclasts.

In our study, we examined osteoclast differentiation and resorption; because the cells were plated on different surfaces: differentiation on plastic and resorption on calcium phosphate, the results cannot be directly compared. For this reason, and according to our varying M-CSF results, we cannot assume a trend in increased differentiation will lead to increased resorption. In our results, the resorption data varying M-CSF interestingly shows little difference and actually a trend in less resorption in HDAC7 KO osteoclasts compared to WT. This trend could indicate the osteoclasts are undergoing apoptosis or there is actually a change in the resorptive activity of the HDAC7 KO osteoclasts. Further studies are needed to elucidate the mechanism behind this trend. In our study, RANK/RANK-L signaling pathway appears to be more affected by loss of HDAC7 expression in osteoclasts.

In HDAC7 KO osteoclasts, we see enhanced differentiation with varying concentrations of M-CSF while RANK-L is being held constant and with varying concentrations of RANK-L while M-CSF is being held constant. We postulated that the hypersensitivity was possibly due to the HDAC7 KO osteoclasts having more C-FMS and RANK expression. The second part of this research project was designed to look at the receptor amounts to see if our hypothesis was true. We wanted to see if and how early in osteoclast differentiation do we see an increase in receptor amounts and if/when do the receptor amounts go back down to WT levels after the monocytes become macrophages and osteoclasts. The increase in C-FMS receptors in HDAC7 KO monocyte cells may indicate their enhanced drive toward osteoclast cell fate. Levels of *c-fms* expression in HDAC7 KO return to the level of WT in the Day 0 and Day 1 cells, further indicating a more prominent role of C-FMS/M-CSF early on in osteoclast differentiation. This data echoes what is already known of M-CSF: it's main role is provide proliferation and survival signals during osteoclastogenesis and its receptor (C-FMS*)* reflects the stage at which M-CSF may exert its primary role [13]. The increase in *Rank* expression in HDAC7 KO Day 1 cells (osteoclasts) may explain why our differentiation and resorption data showed hypersensitivity to RANK-L because there are more RANK receptors to bind RANK-L. This increase in *Rank* receptors later on Day 1, also further corroborates the primary role of RANK-L; to provide differentiation signals and activate multiple pathways during osteoclastogenesis [13].

There is a trend toward increased *C-fms* expression in HDAC7 KO monocytes and and a significant increase in *Rank* expression in HDAC7 KO Day 1 osteoclasts. Because PU.1 regulates *c-fms* expressions and PU.1 and MITF may cooperatively regulate *Rank*

expression [33, 35]; it appears HDAC7 targets the PU.1 and MITF complex.

Mechanistically the hypersensitivity may be explained because HDAC7 KO osteoclasts had increased *c-fms* and *Rank* expression compared to wild type osteoclasts. Further research is necessary to determine if the hypersensitivity is due to increased gene expression by transcription factors MITF and PU.1. Such studies may seek to decrease/modify the pathway or one part of the pathway that contributes to expression of *Rank*/c-fms for example down regulate MITF specifically and evaluate if there are changes to the expression levels of *Rank* and *c-fms*.

The more we understand HDACs role in osteoclastogenesis, the more we can understand their possible therapeutic targets and clinic usefulness. In orthodontics, tooth movement occurs by bone resorption in front of the tooth, in the direction of tooth movement, and bone apposition occurs behind the tooth, when the desired tooth movement is achieved. Application of HDACi may allow us in the future to control the balance of bone remodeling at a cellular and local level, ultimately affecting tooth movement, post-surgical healing, the need for bone grafting, and suture remodeling.

Conclusions

1.) HDAC7 KO cells trend toward increasing size and number to varying concentrations of M-CSF

2.) HDAC7 KO osteoclast differentiation and resorption are both hypersensitive to varying [RANK-L] compared to wild type osteoclasts

3.) RANK receptor expression is significantly upregulated in HDAC7 KO osteoclasts as compared to wild type osteoclasts, likely contributing to HDAC7 hypersensitivity to varying [RANK-L].

4.) Data from others suggests PU.1 may regulate Rank expression in cooperation with MITF. Upregulation of Rank expression in HDAC7 KO osteoclasts, seen in this study suggests the transcription factors PU.1 and MITF are the target of HDAC7 inhibition.

5.) The RANK/RANK-L signaling pathway appears to be the main target of HDAC7 due to the statistically significant hypersensitivity of HDAC7 KO to varying RANK-L and the statistically significant increase in Rank receptors in day 1 HDAC7 KO osteoclasts.

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