microRNA-29 Family: A Positive Regulator of Osteoclastogenesis

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Osteoclasts are large multinucleated bone cells of the myeloid lineage. Osteoclasts secrete proteases and create an acidic environment, causing bone resorption. Osteoclast differentiation, driven by MCSF and RANKL, is tightly regulated by numerous factors, including microRNAs (miRNAs, miRs). miRNAs are short non-coding RNAs that post-transcriptionally regulate gene expression by binding to complementary mRNA sequences, leading to mRNA degradation or destabilization. The aim of these studies was to investigate the function of the miRNA-29-3p family in osteoclasts both in vivo and in vitro.

We generated mice which globally express a miR-29-3p tough decoy which competitively inhibits miR-29-3p activity. The examination of both two-month-old and six-month-old animals allowed for determination of function in growing and skeletally mature animals. Histomorphometry failed to demonstrate an impact of the decoy on the osteoclast or bone marrow adipocyte lineages at either age. However, in two-month-old animals, we observed a significant decrease in bone formation rate and expression of osteoblastic genes in calvaria. By six-months, bone formation rate had normalized in the decoy mice, however, bone volume and trabecular number were decreased. Together, these studies indicate that global miR-29 inhibition more strongly affected osteoblast function.
We also studied the impact of miR-29-3p inhibition in these cell lineages in vitro. Despite our observations in vivo, miR-29 decoy did not affect the osteoblast lineage in vitro, suggesting the phenotype in vivo may be the result of extra-skeletal effects of global miR-29 inhibition. In the adipocyte lineage, marker gene expression was modestly increased by the miR-29 decoy, suggesting miR-29-3p inhibition may promote adipogenesis. Lastly, osteoclast differentiation was inhibited by the decoy in vitro, without changes in osteoclast function.

To better understand the mechanism by miR-29-3p regulates osteoclastogenesis, we performed both candidate gene studies and an RNA-seq study to identify potential miR-29-3p targets and affected pathways in the monocytic lineage. We identified novel targets in the E-cadherin/CDH1 signaling complex, as well as targets associated with macrophage polarization. These data suggest miR-29-3p may promote osteoclast differentiation, in part, by pushing osteoclast precursors away from the M2 lineage, towards the pro-inflammatory M1 macrophage lineage, which shares similar characteristics to bone resorbing osteoclasts.
microRNA-29 Family: A Positive Regulator of Osteoclastogenesis

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microRNA-29 Family: A Positive Regulator of Osteoclastogenesis

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CHAPTER 1
Introduction:

MicroRNAs are Critical Regulators of Osteoclast Differentiation

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ABSTRACT

\textbf{Purpose of review:} Our goal is to comprehensively review the most recent reports of microRNA (miRNA) regulation of osteoclastogenesis. We highlight validated miRNA-target interactions and their place in the signaling networks controlling osteoclast differentiation and function.

\textbf{Recent findings:} Using unbiased approaches to identify miRNAs of interest and luciferase assays to validate interactions, recent studies have been aimed at elucidating the impact of specific miRNA-mRNA interactions during \textit{in vitro} osteoclastogenesis. Specifically, there has been a focus on signaling mediators downstream of the RANK and CSF1R signaling, and genes essential for proper differentiation and function. For example, several miRNAs directly and indirectly target the master osteoclast transcription factor, Nfatc1 (e.g. miR-124 and miR-214) and Rho-GTPases, Cdc42 and Rac1 (e.g. miR-29 family).
Summary: Although the function of miRNAs in osteoclast biology is moving to the forefront, this field is still relatively underdeveloped compared to those of osteoblast and chondrocyte biology, requiring continued investigation.

1.1. Introduction

Osteoclast differentiation and function requires an orchestrated series of events including: osteoclast progenitor (OCP) commitment, pre-osteoclast motility, pre-osteoclast fusion, mature osteoclast attachment to the bone surface, and secretion of bone resorbing molecules. Differentiation, both in vivo and in vitro, is driven primarily by osteoblast-lineage derived macrophage-colony-stimulating-factor (M-CSF) and receptor-activator of NFκB-ligand (RANKL). These cytokines induce the activation and increased expression of nuclear-factor of activated T cells-1 (NFATc1), the master transcription factor of osteoclastogenesis. NFATc1 further promotes the activation and expression of other transcription factors, signaling pathways, and genes essential for osteoclast differentiation, such as nuclear factor κ B (NFkB), microphthalmia-associated transcription factor (MITF), phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), thrombospondin-1 (THBS1), tartrate-resistant acid phosphatase (TRAP; ACP5), and dendritic-cell specific transmembrane protein (DC-STAMP). Proteins essential for osteoclast function include cathepsin K (CTSK), and matrix metalloproteinase 9 (MMP9) (Figure 1.1A) (1). Moreover, pro-inflammatory cytokines (i.e. TNFα and IL-1β) can enhance osteoclastogenesis while other anti-inflammatory cytokines dampen or inhibit differentiation completely (i.e. IFNβ) (reviewed in detail in (2, 3)). In addition to this regulation by paracrine and inflammatory factors, osteoclast differentiation and function
is intrinsically regulated by microRNAs (miR; miRNA).

During the course of osteoclastogenesis many microRNAs are differentially expressed (4-7). These miRNAs are initially transcribed as long primary transcripts which undergo multiple processing steps in the nucleus and cytoplasm, resulting in the generation of mature 21-27 base miRNAs (reviewed in detail in (8, 9)). The mature miRNA strand is incorporated into the multiprotein RNA induced silencing complex (RISC), directing its activity to mRNA transcripts containing complementary sequences. The result of mRNA-RISC interaction includes inhibition of translation, mRNA cleavage and/or mRNA destabilization (reviewed in detail in (10, 11)). The importance of miRNA regulation in osteoclast differentiation was highlighted by Sugatani and Hruska in 2009. They observed that when OCPs were pretreated with siRNAs targeting key miRNA processing components (i.e. Dicer, DGCR8, or Ago2), osteoclastic differentiation was ablated almost entirely. In mice, conditional deletion of Dicer in OCPs resulted in mild osteopetrosis due to decreased osteoclast number and function (5).

In the osteoclast-miRNA literature, miRNAs are broadly divided into either positive or negative regulators of differentiation. These categories are further subdivided into miRNAs which are basally expressed in either precursors or mature osteoclasts and those which are induced or down-regulated in response to drug treatment or disease (e.g. glucocorticoid treatment, inflammation, osteoporosis, etc.).

The goal of this article is to comprehensively review recently reported positive and negative miRNA regulators of osteoclastogenesis, highlighting the cell source and context
Figure 1.1. miRNAs regulate osteoclastogenesis at multiple stages, targeting both positive and negative regulators of commitment, differentiation, and function. Osteoclast differentiation is induced by RANK and CSF1R signaling, resulting in signaling cascades that promote the expression of genes important for both differentiation and function. (A) Regulators of osteoclastogenesis targeted by miRNAs at each stage of differentiation, including: osteoclast precursor, precursor commitment and early differentiation, maturation, and osteoclast function and survival. Red text indicates negative regulators of differentiation and blue text indicates positive regulators. (B) RANK and CSF1R signaling cascades result in the activation and nuclear translocation of key transcription factors, including NFATc1 and NFkB, which in turn induce the expression of genes essential to osteoclast differentiation, maturation, motility, and function.
in which they are expressed. We believe it is important to consider the source of OCPs (bone marrow or peripheral blood mononuclear cells) and culture/osteoclast differentiation conditions because these can have a significant impact on the course of differentiation (12).

Overall, we restrict our discussion to miRNAs demonstrated to target specific proteins or pathways important for osteoclast commitment, differentiation, and survival. We further limit our review to validated miRNA-target interactions determined by 3' UTR-luciferase assays and those strongly suggested by miRNA manipulation followed by Western blot analysis; miRNA-target interactions suggested only by bioinformatics will not be discussed here.

1.2. miRNAs regulating osteoclast precursor commitment and early differentiation

To induce osteoclastogenesis, M-CSF and RANKL must first bind and activate their respective receptors, colony stimulating factor-1 receptor (CSF1R) and receptor activator of NFκB (RANK). Activation of CSF1R and RANK initiate several signaling cascades which simultaneously promote OCP commitment to the osteoclast lineage and early differentiation events (Figure 1.1A).

Upon RANK activation, the RANK adaptor molecule TNF receptor associated factor 6 (TRAF6), a ubiquitin ligase, complexes with TGF-β activated kinase-1 (TAK1) and TGF-β activated kinase-1 binding protein (TAB1). The formation of this complex subsequently recruits SMAD3 (13). The TRAF6-TAB1-TAK1-SMAD3 complex promotes the
downstream ubiquitination of the NFκB inhibitor, IκBα, allowing for NFκB translocation to
the nucleus. There, NFκB promotes transcription of NFATc1, the master transcription
factor of osteoclastogenesis (Figure 1.1B) (14). During differentiation, NFκB activation is
supported by the down-regulation of murine (mmu-) mmu-miR-145, which targets Smad3.
Additionally, the upregulation of human (hsa-) hsa-miR-99b supports NFκB activity by
targeting insulin like growth factor 1 receptor (Igf1r), whose signaling inhibits NFκB
activation (15-17).

hsa-miR-125a, however, has been reported to have conflicting roles in NFκB
signaling. In one study, miR-125a was reported to be upregulated over 21 days of
differentiation and to promote NFκB signaling by targeting TNF-α induced protein 3
(Tnfaip3), a deubiquitinating protease which induces TRAF6 degradation (16, 18). In
contrast, another study reported miR-125a was downregulated over 15 days of
differentiation and inhibited NFκB signaling by targeting Traf6 (19). Thus, miR-125a is
one miRNA reported to have conflicting roles in osteoclastogenesis, despite being
examined in similarly differentiated CD14+ human PBMCs.

As a master regulator of osteoclastogenesis, NFATc1 is responsible for driving early
differentiation as well as solidifying OCP commitment to the osteoclast lineage.
Downregulation of mmu-miR-124 in response to RANK signaling may support the
upregulation of NFATc1 in differentiating cells, as it has been suggested to target Nfatc1
(20). Successful NFATc1 signaling results in the activation of additional transcription
factors, including MITF. MITF amplifies NFATc1 signaling by upregulating many of the
same target genes as NFATc1. In the absence of MITF, few non-functional osteoclasts
form (21). MITF expression and activity during differentiation is supported by the downregulation of **mmu-miR-155** and **mmu-miR-340**, both of which have been reported to directly target **Mitf** (22, 23). Additionally, mmu-miR-155 downregulation results in the de-repression of suppressor of cytokine signaling-1 (**Socs1**). Increased SOCS1 activity subsequently reinforces osteoclast differentiation by antagonizing the inhibitory effects of interferon-β (IFNβ) and transforming growth factor-β1 (TGF-β1) on RANK signaling (22, 24-26).

In addition to RANK:RANKL signaling, CSFR:M-CSF signaling also induces PI3K/AKT signaling. PI3K/AKT signaling promotes the degradation of IκBα, again resulting in the translocation of NFκB and the increased **Nfatc1** transcription (27, 28). **mmu-miR-214** promotes these events by targeting phosphatase and tensin homolog (**Pten**), which converts active PIP₃ to inactive PIP₂ (29-31). **mmu-miR-34c** has also been reported to support PI3K signaling by targeting leucine rich repeat containing G protein-coupled receptor 4 (**Lgr4**), a receptor that can compete for RANKL. Moreover, LGR4 signaling prevents the inactivation of GSK3β; active GSK3β prevents the activation and nuclear translocation of NFATc1 (27, 32, 33).

As early differentiation occurs, additional immunomodulatory molecules which enhance differentiation are expressed downstream of RANK signaling. For example, IL-15, an autocrine cytokine, enhances osteoclastogenesis by promoting the upregulation of pro-inflammatory cytokines (e.g. TNFα, IL-1, and IL-6) (2). IL-15 expression and activity is supported by the downregulation of **hsa-miR-212**, which directly targets **Il15** (16). Monocytic cells transfected with a miR-212 inhibitor displayed decreased osteoclastic
differentiation in the presence of RANKL. Likewise, cyclooxygenase 2 (Cox2; PTGS2) is activated downstream of RANK signaling, and it is also targeted by hsa-miR-212, as well as hsa-miR-132 [16]. PTGS2 promotes the synthesis of prostaglandin E2 (PGE2). PGE2, like other prostaglandins, mediates inflammation through G-protein-coupled receptor (GPCR) subtypes EP-1, -2, -3, and -4. However, the outcome of this signaling is controversial, as PGE2 signaling has been shown to have both positive and negative effects on osteoclastogenesis [34-36].

During this time of early NFATc1 driven differentiation, osteoclast commitment is further supported by the miR-29 family, which consists of mmu-miR-29a, -29b, and -29c. These miRNAs are upregulated during RANKL induced differentiation in both murine BMMs and the RAW264.7 mouse monocyte cell line. The inhibition of miR-29 family members in murine cells results in the formation of smaller and fewer osteoclasts. The miR-29 family is thought to support commitment by targeting nuclear factor I/A (Nfia), which inhibits the differentiation of monocytes to both the macrophage and osteoclast lineages, and by targeting G-protein coupled receptor 85 (Gpr85) and Cd93, which are macrophage-specific gene transcripts [37].

While these data suggest miR-29 is a positive regulator of osteoclast differentiation, a contrasting study reported that during the differentiation of human PBMCs, hsa-miR-29b is significantly downregulated. In this system, miR-29b overexpression impaired osteoclast formation and inhibited induction of FOS and MMP2, although direct targeting of their mRNAs by miR-29b was not demonstrated [38-40]. Thus, miR-29 is an additional miRNA family reported to have differing roles in osteoclastogenesis, depending on the
Lastly, upregulation of hsa-miR-148a during osteoclastogenesis supports commitment by targeting MAF (avian musculoaponeurotic fibrosarcoma) BZIP Transcription Factor B (Mafb), a transcription factor which inhibits OCP differentiation by redirecting precursors to the macrophage lineage instead (41, 42).

1.3. miRNAs regulating pre-osteoclast maturation

As OCPs become committed to the osteoclast lineage and begin to differentiate they are considered pre-osteoclasts. Pre-osteoclasts are highly motile and form long pseudopodia in search of fusion partners. These events are facilitated, in part, by Rho-GTPase family members RHOA, CDC42, and RAC1. Rho-GTPases are small molecular switch proteins which mediate the actin remodeling events needed for migration and fusion, as well the formation of the actin ring and sealing zone needed for bone resorption. As with other GTPase molecules, Rho-GTPases are considered active when GTP bound and inactive when GDP bound (reviewed in detail in (43)). Several miRNAs have been reported to support osteoclast differentiation by fine-tuning the expression and activation of Rho-GTPase family members.

For example, in addition to causing the formation of smaller and fewer osteoclasts, inhibition of mmu-miR-29 in RAW264.7 cells inhibits their motility. The mmu-miR-29 family is thought to fine-tune actin remodeling required for motility and differentiation, in part, by targeting Rho-GTPase Cdc42 and SLIT-ROBO-GTPase activating protein-2 (Srgap2), a negative regulator of RAC1 Rho-GTPase (37).
As pre-osteoclasts migrate and extend membrane protrusions, they eventually find fusion partners and form large multinucleated cells. Osteoclast fusion is mediated by numerous proteins, including CD47-thrombospondin-1 (THBS1) interactions and DC-STAMP. CD47 and THBS1 are multifunctional proteins important for pre-osteoclast fusion (44). In human PBMCs, the contribution of THBS1 in fusion is fine-tuned by hsa-let-7e, which is upregulated with differentiation and targets Thbs1 transcripts (16).

DC-STAMP, one of the most prominent fusion markers, is induced by MITF part-way through differentiation, with its levels persisting throughout differentiation (45, 46). During this time, upregulation of DC-STAMP is supported by the downregulation of mmu-miR-7b, which has been suggested to target Dcstamp (47). In contrast, DC-STAMP expression is inhibited indirectly by the upregulation of mmu-miR-26a during differentiation. mmu-miR-26a targets connective tissue growth factor (Ctgf), which promotes osteoclastogenesis by inducing DC-STAMP expression (48, 49). Induction of both positive and negative regulators of osteoclast fusion during differentiation suggests that controlling osteoclast size is important, especially since osteoclast surface area is thought to correlate with osteoclast activity.

In addition to actin remodeling and fusion, as pre-osteoclasts migrate they interact with matrix proteins on the bone surface, including vitronectin, osteopontin, and fibronectin (50). Matrix proteins can influence osteoclast differentiation and function by interacting with integrins on the cell surface. In particular, integrin α4 (ITGA4) dimerizes with integrin β1 (ITGB1) to form VLA-4 (very late antigen-4; α4β1 integrin), which binds fibronectin (51, 52). These integrin interactions with fibronectin can have stage dependent
effects. For example, fibronectin is thought to hinder pre-osteoclast differentiation, but then upregulate mature osteoclast activity (50). These interactions are then fine-tuned by the upregulation of hsa-miR-let-7e during differentiation, which targets Itga4 (16).

1.4. miRNAs regulating osteoclast function and survival

Once mature-multinucleated osteoclasts are formed, integrin αvβ3 adheres the cell to the bone surface and forms a tight sealing zone around the area to be resorbed (53). This sealing zone is reinforced by the formation of dense actin rings. mmu-miR-31 inhibition significantly disrupts actin ring formation and bone resorption in mature osteoclasts and also results in increased RHOA protein, whose RNA is a putative miR-31 target. It is possible the de-repression of Rhoa by miR-31 inhibition increases the amount of active RHOA, which is known to inhibit the formation of the actin ring and sealing zone (43, 54).

Once the sealing zone is in place, protons and proteolytic enzymes are then secreted into the resorption area; as this occurs a distinct “ruffled border” of plasma membrane forms (53). The resorption area is acidified to pH 5 by the proton pump vacuolar-like H(+) -ATPase (V-ATPase) which is essential for bone resorption (55). In this acidic microenvironment, the inorganic components of the bone matrix begin to degrade and the secreted proteases become activated.

CathepsinK (Ctsk) and MMP9 are the primary proteases which degrade the organic component of the bone matrix. They are initially secreted through the ruffled border as pro-proteins. In the acidic resorption area, pro-cathepsinK autolytically cleaves into active cathepsinK. Active cathepsinK then cleaves pro-MMP9 into active MMP9; both proteases
are then free to digest the collagen fibers of the bone matrix (56). Several miRNAs have been reported to regulate the expression and secretion of these proteases. For example, mmu-miR-365 and mmu-miR-186 negatively regulate Mmp9 and Ctsk, respectively (57, 58). Further, mmu-miR-20a was found to target autophagy related 16 like 1 (Atg16l1) (59). While typically associated with the formation of the autophagosome during cellular stress, there is evidence that ATG16L1, and other members of the ATG family of proteins, participate in the packaging and secretion of proteases (i.e. cathepsinK and MMP9) at the ruffled border (60). Thus, these miRNAs appear to be negative regulators of osteoclast function.

Mature osteoclasts eventually reach a point where they have accumulated sufficient cellular damage and undergo programmed cell death, or apoptosis. In general, apoptosis is a complex process involving several pathways and mechanisms which result in the destruction of the cell. For an in depth review of osteoclast apoptosis please refer to (61).

One mechanism which promotes osteoclast apoptosis is the interaction of the cell-surface death-receptor FAS with its ligand, FAS-ligand (FASLG). FASLG binds to the FAS receptor on a “target” cell, which induces apoptosis of the target cell. OCPs, pre-osteoclasts, and mature osteoclasts express both FAS and FASLG. As expected, inhibition or deletion of either of these molecules increases osteoclast numbers (61, 62). Cell survival may be supported by mmu-miR-21, which is quickly upregulated with osteoclast differentiation and has been shown to directly target Faslg (63).

In parallel to FAS/FASLG signaling, programmed cell death 4 (PDCD4) also promotes apoptosis. Activation of PDCD4 inhibits protein translation initiation and cap-dependent
translation by binding to eukaryotic translation initiation factor 4A1 (EIF4A1). As with Faslg, miR-21 further promotes cell survival by targeting Pdcd4 (64, 65).

1.5. Hypothesis generating data-sets

Many of the individual miRNAs discussed herein were initially identified as differentially expressed in microarray analysis of M-CSF and RANKL-induced osteoclast differentiation (Table 1.1 and 1.2). While the authors of these reports further studied one or several of the identified miRNAs, these unbiased datasets often revealed ten to hundreds of differentially expressed miRNAs with putative roles in osteoclastogenesis. These datasets, and others, are publicly available for independent analysis. Here we will briefly discuss some studies that may be vital resources for generating novel hypotheses of mechanisms by which miRNAs regulate osteoclastogenesis (Table 1.3). As mentioned earlier, the source of OCPs and differentiation conditions affect the course of osteoclast differentiation. Therefore, we provide these details for consideration.

Osteoporosis is caused by an imbalance in the bone remodeling process, with bone resorption outpacing bone formation. Osteoporosis is often associated with a loss of estrogen, as frequently seen in post-menopausal women, or in mice which have undergone ovariectomy (66, 67). Several studies have compared differential miRNA expression in osteoporotic post-menopausal women versus their healthy counterparts, or ovariectomized (ovx) mice versus sham operated mice (15, 68, 69).

One unique study used miRNA microarray analysis to compare the miRNA expression profiles of osteoclasts, osteoblasts, and osteocytes, all collected from post-menopausal
women. To generate osteoclasts, CD14+ PBMCs were isolated from postmenopausal women and cultured in M-CSF and RANKL for 21-24 days, until multinucleated TRAP-positive cells had formed. At this time RNA was extracted from the mature osteoclasts. Primary osteoblasts were obtained from knee trabecular bone collected from postmenopausal women undergoing knee replacement. These bone tissues were cut into small pieces and placed into culture for approximately 3 weeks, until the osteoblast cultures were near confluence, and RNA was harvested. For osteocytes, the authors rationalized using RNA extracted from whole bone, as osteocytes account for approximately 90% of the cell mass in the bone matrix. For this, femoral bone was collected from postmenopausal women undergoing hip replacement; RNA was extracted fresh at the time of collection.

The subsequent analysis found dozen to hundreds of miRNAs uniquely expressed in each tissue, and similar numbers of miRNAs were common among all three cell types. For example, the human osteoclast cultures from these women differentially expressed 340 miRNA, 101 of which were also expressed in osteoblasts, and 196 of which were also found in whole bone (69). Osteoporosis is a complex disease. Whereas osteoclasts and cell autonomous effects contribute to the disease, there are likely contributions from the other skeletal cells and the potential cross talk between cell types is critical. Moreover, since exosomes transport miRNAs from one cell type to another, this dataset gives us a window into how miRNA expression profiles between cell types in the bone environment may contribute to the disease.

In addition to osteoporosis, another cause of pathological bone-loss is chronic
inflammation associated with autoimmune disorders, such as rheumatoid arthritis, Crohn’s disease, and systemic lupus erythematosus. (70-72). As already discussed, inflammation and the presence of inflammatory cytokines may enhance osteoclastogenesis (2). Two available datasets examine miRNA expression in murine BMMs and RAW264.7 cells treated with or without the inflammatory cytokine TNFα.

In the first study, miRNA microarray analyses were performed on RNA from RAW264.7 cells treated with RANKL, in the presence or absence TNFα for 0, 24, and 82 hours. 44 miRNAs were differentially expressed between untreated cells at 0 hours and RANKL+TNFα treated cells 82 hours later, whereas 52 miRNAs were differentially expressed between untreated cells and RANKL treated alone for 82 hours (73). Regarding mmu-miR-29b and mmu-miR-125a discussed earlier, both miRNAs were significantly upregulated during differentiation in response to both treatments. These observations were validated using qRT-PCR analysis of differentiating RAW264.7 cells and murine BMMs treated with RANKL+TNFα, and confirm previous reports that miR-125a and miR-29b are upregulated with differentiation (16, 37).

Recombination signal-binding protein for immunoglobulin-k-J (RBPJ) is a key negative regulator that restrains TNF-α-induced osteoclastogenesis and inflammatory bone resorption. In a second study, murine BMMs were isolated from Rbpj-floxed mice crossed with LysM-cre mice, deleting Rbpj specifically in the myeloid lineage. BMMs from these mice were treated with RANKL with or without TNFα for 24 hours, during which time 27 miRNAs were induced and 12 were suppressed by TNFα. Similar to the first study, both mmu-miR-29a and mmu-miR-125a were upregulated during differentiation in
this dataset (74). An interesting analysis would be a comparison of the two datasets to further identify similarly expressed miRNAs in response to TNFα treatment in the two cell systems.

In another study, a population of murine BMMs enriched for osteoclast precursors was treated with RANKL for 1, 3, and 5 days and the miRNA expression profiles were subsequently examined. The goal of this study was to gather a more complete miRNA signature during early, mid, and late differentiation. 93 miRNAs were differentially expressed and were divided into 7 clusters based on expression patterns. To validate the dataset, the expression and role of three differentially expressed miRNAs, mmu-miR-365, mmu-miR-99b, and mmu-miR-451, were further examined. First, using qRT-PCR analysis, the upregulation of miR-365 and miR-99b and the downregulation of miR-451 during differentiation was confirmed. Inhibition of mmu-miR-365 and mmu-miR-99b impaired osteoclast differentiation while the overexpression of mmu-miR-451 had no impact on differentiation (75).

In addition to miRNAs, other non-coding RNAs including long noncoding RNAs (IncRNA) and circular RNAs (circRNAs) are differentially regulated during osteoclastogenesis (76). Many IncRNAs can interact with DNAs, RNAs and proteins to alter chromatin accessibility and thereby transcription. In contrast, circRNAs regulate gene expression by acting as competitors for specific miRNAs or for transcriptional machinery (77). One recent study profiled mRNAs, IncRNAs and circRNAs in RAW264.7 cells treated for 0, 24, 72, and 96 hours with M-CSF and RANKL. Gene expression in undifferentiated cells was compared with that of pre-osteoclasts (24 hr RANKL), mature
osteoclasts (72 hr RANKL), or activated osteoclasts (96 hr RANKL). As expected, hundreds of members from each RNA subgroup were differentially expressed at each stage of differentiation. The authors generated co-expression networks of IncRNA-mRNA and circRNA-miRNA in an attempt to identify core regulation networks for osteoclastogenesis. This well executed study provides some candidate non-coding RNAs which may be further evaluated for an impact on osteoclast function using primary BMMs or human PBMCs (76).

Together, these studies provide datasets containing hundreds of miRNAs differentially expressed throughout osteoclastogenesis in RAW264.7 cells, murine BMMs, and human PBMCs and are all invaluable tools for the comparison of these cell systems. Importantly, the sequence of many mature miRNAs is conserved between mouse and human, broadening the potential utility of these unbiased data sets.

1.6. Conclusion

In recent years, nanoparticle technology has seen the development of the bone targeting peptide Asp₈, which can be conjugated to the surface of nanoparticles of various compositions (78). Asp₈ preferentially binds the crystallized hydroxyapatite common to bone resorption surfaces, and thus enhances the delivery of nanoparticles to bone resorbing osteoclasts while reducing off-target delivery and potential toxicity to the liver and kidneys, as compared to naked nanoparticles (30, 79). With the rise of the ability to more specifically target the specific bone surfaces, miRNA mimics and inhibitors have the potential to become powerful therapeutics for pathologies involving dysregulated osteoclast function. As such, validating and understanding miRNA expression patterns,
targets, and impact in multiple cells is important for fulfilling this therapeutic potential. While there has been increasing appreciation of the role of miRNAs in osteoclast biology over the last several years, the field is still relatively underdeveloped compared with the fields of osteoblast and chondrocyte biology and requires continued investigation.

Acknowledgements

This work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health [AR064867, AMD/SKL]; the National Institutes for Dental and Craniofacial Research [T90DE21989]; and the Center for Molecular Oncology at UConn Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors thank Dr. Julia Charles (Brigham and Women’s Hospital, Boston, MA) for her careful review of the manuscript.
Table 1.1. Summary of featured positive miRNA regulators of osteoclast differentiation.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Target(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-let-7e</td>
<td>Thbs1, Itga4</td>
<td>(16)</td>
</tr>
<tr>
<td>mmu-miR-21</td>
<td>Faslg, Pdc4</td>
<td>(63-65)</td>
</tr>
<tr>
<td>mmu-miR-29 family</td>
<td>Nfia, Gpr85, Cd93, Srgap2, Cdc42</td>
<td>(37)</td>
</tr>
<tr>
<td>mmu-miR-31</td>
<td>Rhoa</td>
<td>(54)</td>
</tr>
<tr>
<td>mmu-miR-34c</td>
<td>Lgr4</td>
<td>(32)</td>
</tr>
<tr>
<td>hsa-miR-99b</td>
<td>Igf1r</td>
<td>(16)</td>
</tr>
<tr>
<td>hsa-miR-125a</td>
<td>Tnfaip3</td>
<td>(16)</td>
</tr>
<tr>
<td>hsa-miR-132</td>
<td>Pghs2</td>
<td>(16)</td>
</tr>
<tr>
<td>hsa-miR-148a</td>
<td>Mafb</td>
<td>(42)</td>
</tr>
<tr>
<td>hsa-miR-212</td>
<td>Il15, Pghs2</td>
<td>(16)</td>
</tr>
<tr>
<td>mmu-miR-214</td>
<td>Pten</td>
<td>(29)</td>
</tr>
</tbody>
</table>

Table 1.2. Summary of featured negative miRNA regulators of osteoclast differentiation.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Target(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmu-miR-7b</td>
<td>Dcstamp</td>
<td>(47)</td>
</tr>
<tr>
<td>mmu-miR-20a</td>
<td>Atg16l1</td>
<td>(59)</td>
</tr>
<tr>
<td>mmu-miR-26a</td>
<td>Clgf</td>
<td>(48)</td>
</tr>
<tr>
<td>hsa-miR-29b</td>
<td>-</td>
<td>(40)</td>
</tr>
<tr>
<td>mmu-miR-124</td>
<td>Nfatc1</td>
<td>(20)</td>
</tr>
<tr>
<td>hsa-miR-125a</td>
<td>Traf6</td>
<td>(19)</td>
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<tr>
<td>mmu-miR-145</td>
<td>Smad3</td>
<td>(15)</td>
</tr>
<tr>
<td>mmu-miR-155</td>
<td>Mift, Socs1</td>
<td>(22, 26)</td>
</tr>
<tr>
<td>mmu-miR-186</td>
<td>Ctsk</td>
<td>(58)</td>
</tr>
<tr>
<td>mmu-miR-340</td>
<td>Mift</td>
<td>(23)</td>
</tr>
<tr>
<td>mmu-miR-365</td>
<td>Mmp9</td>
<td>(57)</td>
</tr>
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</table>
### Table 1.3. Hypothesis generating datasets.

<table>
<thead>
<tr>
<th>Year</th>
<th>Title</th>
<th>Study design</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010</td>
<td>Osteoclast-specific Dicer gene deficiency suppresses osteoclastic bone resorption.</td>
<td>Dicer\textsuperscript{fl/fl}:Cathepsin K-cre mice used to examine the contributions of miRNA processing on miRNA expression profiles in mature osteoclasts and the \textit{in vivo} phenotype.</td>
<td>(7)</td>
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<tr>
<td>2011</td>
<td>A microRNA expression signature of osteoclastogenesis.</td>
<td>miRNA expression profiles examined in murine BMMs treated with M-CSF with or without RANKL for 24 hours.</td>
<td>(64)</td>
</tr>
<tr>
<td>2013</td>
<td>Expression profiling of microRNAs in RAW264.7 cells treated with a combination of tumor necrosis factor alpha and RANKL during osteoclast differentiation.</td>
<td>miRNA expression profiles examined in RAW264.7 cells treated with RANKL, in the presence or absence of TNFα for 0, 24, and 82 hours.</td>
<td>(73)</td>
</tr>
<tr>
<td>2013</td>
<td>miR-148a regulates osteoclastogenesis by targeting V-maf musculoaponeurotic fibrosarcoma oncogene homolog B.</td>
<td>miRNA expression profiles examined in human PBMCs treated with or without MCSF+RANKL for 14 days.</td>
<td>(42)</td>
</tr>
<tr>
<td>2013</td>
<td>miR-31 controls osteoclast formation and bone resorption by targeting RhoA.</td>
<td>miRNA expression profiles examined in murine BMMs treated with M-CSF with or without RANKL for 24 hours.</td>
<td>(54)</td>
</tr>
<tr>
<td>2014</td>
<td>MiR-7b directly targets DC-STAMP causing suppression of NFATc1 and c-Fos signaling during osteoclast fusion and differentiation.</td>
<td>miRNA expression profiles examined in RAW264.7 cells treated with or without M-CSF and RANKL for 72 and 192 hours.</td>
<td>(47)</td>
</tr>
<tr>
<td>2014</td>
<td>Pathway Analysis of MicroRNA Expression Profile during Murine Osteoclastogenesis.</td>
<td>miRNA expression profiles examined in murine BMMs treated with M-CSF and RANKL for 1, 3, and 5 days.</td>
<td>(4)</td>
</tr>
<tr>
<td>2015</td>
<td>MicroRNA-26a regulates RANKL-induced osteoclast formation.</td>
<td>miRNA expression profiles examined in murine BMMs treated with M-CSF and RANKL for 0, 1, 2, and 3 days.</td>
<td>(48)</td>
</tr>
<tr>
<td>2015</td>
<td>miR-214 promotes osteoclastogenesis by targeting Pten/PI3k/Akt pathway.</td>
<td>miRNA expression profiles examined in RAW264.7 cells treated with or without RANKL for 48 hours.</td>
<td>(29)</td>
</tr>
<tr>
<td>Year</td>
<td>Study Title</td>
<td>Methodology</td>
<td>Reference</td>
</tr>
<tr>
<td>------</td>
<td>----------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>2015</td>
<td>NF-κB-direct activation of microRNAs with repressive effects on monocyte-specific genes is critical for osteoclast differentiation.</td>
<td>miRNA expression profiles examined in human PBMCs treated with M-CSF and RANKL for 0, 2, and 21 days.</td>
<td>(16)</td>
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<tr>
<td>2016</td>
<td>Changing expression profiles of lncRNAs, mRNAs, circRNAs and miRNAs during osteoclastogenesis.</td>
<td>Expression profiles of mRNAs, lncRNAs, circRNAs, and miRNAs examined in RAW264.7 cells treated for 0, 24, 72, and 96 hours with M-CSF and RANKL.</td>
<td>(76)</td>
</tr>
<tr>
<td>2016</td>
<td>RBP-J-Regulated miR-182 Promotes TNF-α-Induced Osteoclastogenesis.</td>
<td>miRNA expression profiles examined in BMMs treated with RANKL with or without TNFα for 24 hours.</td>
<td>(74)</td>
</tr>
<tr>
<td>2016</td>
<td>Validation of downregulated microRNAs during osteoclast formation and osteoporosis progression.</td>
<td>miRNA expression profiles examined in BMMs treated with M-CSF and RANKL for 0 and 5 days.</td>
<td>(68)</td>
</tr>
<tr>
<td>2018</td>
<td>Expression profiling of microRNAs in human bone tissue from postmenopausal women.</td>
<td>miRNA expression profiles of human osteoclasts, osteoblasts, and osteocytes isolated from postmenopausal women.</td>
<td>(69)</td>
</tr>
<tr>
<td>2018</td>
<td>Overexpressed miR-145 inhibits osteoclastogenesis in RANKL-induced bone marrow-derived macrophages and ovariectomized mice by regulation of Smad3.</td>
<td>miRNA expression profiles examined in BMMs treated with M-CSF with or without RANKL for 6 days.</td>
<td>(15)</td>
</tr>
</tbody>
</table>
CHAPTER 2

Research Aims and Hypothesis

Using *in vitro* models, our lab previously showed that the miR-29-3p family is a positive regulator of both osteoclast and osteoblast differentiation and function (37, 80, 81). While *in vitro* analyses are useful for determining cell autonomous effects and mechanisms, bone is a complex tissue in which cells representing both the mesenchymal and hematopoietic lineages interact and influence each other’s behavior. Thus, to examine the impact of the miR-29-3p family on the bone microenvironment, we generated a novel mouse line globally expressing a miR-29-3p competitive inhibitor known as a tough-decoy. We *hypothesize* that global inhibition of miR-29-3p activity will result in reduced osteoblast and osteoclast number and activity, and a low bone-turnover phenotype. Therefore, the overall aim of the studies described in this dissertation is to investigate the *in vivo* role of the miR-29-3p family in both osteoclast and osteoblast differentiation and function using the global miR-29 decoy mice.

Herein, we describe the impact of global miR-29-3p inhibition on the murine skeleton, and characterize the cell autonomous impact on osteoclasts, osteoblasts, and adipocytes *in vitro*. Furthermore, to investigate mechanisms responsible for miR-29-3p regulation of osteoclast differentiation, we validated a set of novel miR-29-3p targets selected using a candidate gene approach and as well as from a list of genes with increased expression in miR-29 decoy osteoclasts.
Research Aim 1: Investigate the skeletal phenotype of global miR-29 decoy mice in both growing and skeletally mature animals.

Aim 1a: To analyze the gross skeletal phenotype of the miR-29 decoy mice, we will use microCT analysis to evaluate the trabecular and cortical bone microarchitecture in two- and six-month old mice.

Aim 1b: To determine the impact of global miR-29-3p inhibition in the osteoclast and osteoblast lineages \textit{in vivo}, we will use static and dynamic histomorphometry to evaluate osteoclast and osteoblast number and activity, qRT-PCR analysis of whole calvaria will be used to examine expression of osteoclast and osteoblast marker genes.

Aim 1c: We will determine the impact of the miR-29 decoy on bone marrow adipose and total adiposity. To accomplish this, we will quantify bone marrow adipose in tibias from six-month-old wild-type and global decoy animals using osmium tetroxide staining followed by microCT analysis. Further, we will use TD-NMR to perform a longitudinal study measuring the total body composition of the decoy animals from two- to six-months of age.

Research Aim 2: Investigate the cell autonomous impact of the global decoy on the osteoblast, bone marrow adipocyte, and osteoclast lineages \textit{in vitro}.

Aim 2a: To evaluate the cell autonomous impact of the miR-29-3p decoy on the osteoblast and bone marrow adipocyte lineages, we will perform a series of differentiation assays for each lineage using bone marrow stromal cells. Differentiation will be evaluated using lineage specific stains and qRT-PCR analysis to examine the expression of lineage specific differentiation markers.
Aim 2b: To investigate the cell autonomous impact of the miR-29 decoy on the osteoclast lineage we will perform osteoclast differentiation assays, evaluate the expression of osteoclast differentiation markers by qRT-PCR and Western Blot analysis, assess formation of the osteoclast actin-ring, and use bone resorption assays to determine if osteoclast function is impacted.

Research Aim 3: Investigate the mechanisms by which miR-29-3p regulates osteoclast differentiation.

Aim 3a: miRNAs are known to regulate multiple molecules within a single pathway to modify cellular phenotype (82). With this rationale, we will use a candidate gene approach to identify miR-29-3p targets involved in Rho-GTPase signaling pathways, since Rho-GTPase Cdc42 and Rho-GTPase activating protein Srgap2 are known miR-29-3p targets. Potential miR-29-3p targets will be identified using the DIANA and RNA-Hybrid miRNA prediction algorithms. Predicted targets will then be validated in the RAW264.7 cell line using luciferase reporter assays. The mRNA and/or protein expression of validated targets will then be measured in osteoclast cultures derived from either wild-type or global decoy bone marrow monocytes.

Aim 3b: To more broadly investigate the regulation of miR-29-3p in the osteoclast lineage, we will use results from a previously performed RNA-sequencing experiment to generate a list of genes up regulated in miR-29 decoy osteoclasts. These genes will be evaluated for miR-29-3p binding sites using the DIANA and RNA-Hybrid miRNA prediction algorithms. Predicted targets will then be validated in the RAW264.7 cell line using luciferase reporter assays. The mRNA and/or protein expression of selected
validated targets will then be measured in osteoclast cultures derived from either wild-type or miR-29-3p decoy bone marrow monocytes.
CHAPTER 3
Inhibition of MiR-29-3p Isoforms via Tough Decoy Suppresses Osteoblast Function in Murine Bone

Henry C. Hrdlicka, Bongjin Shin, Siu-Pok Yee, Alix C. Deymier, Sun-Kyeong Lee, Anne M. Delany

ABSTRACT

The microRNA-29-3p family has been shown to promote osteoblast differentiation \textit{in vitro}, while the role of miR-29 in osteoclast differentiation is less well-defined. Previous investigations of miR-29 function in the skeleton have primarily involved miR-29 over expression models. Here we describe the skeletal phenotype of a novel mouse line globally expressing a miR-29-3p tough decoy, which decreases activity of all three miR-29-3p isoforms: miR-29a, miR-29b, and miR-29c. Growing two-month-old global miR-29 decoy mice had normal bone microarchitecture, no changes to osteoclastic parameters, but had significantly decreased bone formation rate. These mice also displayed decreased \textit{Runx2} mRNA and an increase in the \textit{Rankl/Opg} mRNA ratio. In six-month-old skeletally mature mice, the global decoy caused a significant reduction in trabecular bone volume, whereas cortical bone was unaltered. These data verify the positive role of miR-29-3p in osteoblast function \textit{in vivo}, and indicate that the osteoblastic lineage is sensitive
to perturbations which decrease miR-29-3p levels. Others have reported that in mice subjected to estrogen deficiency or glucocorticoid excess, both of which cause bone loss, miR-29-3p isoforms are decreased. Our data suggest that decreased miR-29-3p levels could contribute to osteopenia in these conditions.

3.1. Introduction

The skeleton is a dynamic organ which continuously remolds through the coupled processes of bone resorption and bone formation (83). This homeostasis is dependent on the coordinated efforts of both hematopoietic and mesenchymal lineage cells. The hematopoietic lineage gives rise to bone marrow monocytes (BMMs), which can further differentiate into multinucleated bone-resorbing osteoclasts. The mesenchymal lineage gives rise to bone marrow stromal cells (BMSCs), which can further differentiate into bone-forming osteoblasts. Maintaining a balance between osteoclast and osteoblast number and activity is needed for normal bone remodeling (83, 84).

microRNAs (miRNAs; miRs) are small, single-stranded, non-coding RNAs which post-transcriptionally fine-tune gene expression. This class of small RNA is now well recognized as an important regulator of both osteoclast and osteoblast lineages (37, 40, 85-87). miRNA-mediated regulation is achieved by incorporation of the mature miRNA strand into the multiprotein RNA-induced silencing complex (RISC), which targets mRNA transcripts with sequences complementary to the miRNA. RISC-miRNA:mRNA interactions can result in translational repression, transcript de-stabilization, and/or transcript degradation (83). Further, individual miRNAs frequently target multiple components of signaling pathways, resulting in wide-ranging regulation of cellular
differentiation and function (87). One miRNA family shown to play a substantial role in the
differentiation and function of osteoblasts and osteoclasts is miRNA-29-3p, an
evolutionarily conserved family consisting of the isoforms: miR-29a, -29b, and -29c (37, 40, 85, 86, 88).

In the osteoclast lineage, we and others have previously reported that the miR-29-3p family is upregulated in primary BMMs during macrophage colony stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL) induced osteoclastogenesis (37, 89). We showed that inhibition of miR-29-3p activity decreases osteoclast formation in vitro, indicating the miR-29-3p family is a positive regulator of osteoclast differentiation (37). The miR-29 family promotes osteoclast differentiation and function, in part, by targeting Gpr85, Cd93, and Nfia, which negatively impact the commitment of BMMs to the osteoclast lineage, and by targeting Calcr, which mediates the anti-resorptive effects of calcitonin. Cdc42 and srGAP2, which regulate actin-remodeling and podosome formation necessary for motility and actin ring formation, are also miR-29 targets; regulation of these targets may be necessary to regulate osteoclast size (37).

In the osteoblast lineage, we and others previously reported that the miR-29-3p family is upregulated with osteoblastogenesis and that it positively regulates osteoblast formation and function in both mouse and human cells (80, 86, 90-92). Upregulation of the miR-29 family promotes osteoblast differentiation, in part, by targeting negative regulators of this process, including Hdad4, Tgfb3, and several negative regulators of Wnt-signaling (80, 86, 90-92).

To date, the majority of studies investigating the function of the miR-29 family in bone
were performed \textit{in vitro}. To better understand the function of the miR-29 family \textit{in vivo}, we developed a mouse model globally expressing a miR-29-3p tough decoy (miR-29 decoy; global decoy), which serves as a competitive inhibitor of miR-29-3p activity (93-95). In the present study, we report on the skeletal phenotype of these global miR-29 decoy mice. Global expression of the miR-29 decoy results in decreased bone formation at two-months of age and a significant decrease in trabecular bone volume at six-months, reinforcing the concept that the miR-29 family plays an important role in the skeleton.

3.2. Materials and Methods

3.2.1. Animal care

All mice were in the C57BL/6 background and were housed in the Center for Comparative Medicine at UConn Health under a 12-h light/dark cycle and were provided with standard mouse chow and water \textit{ad libidum}. Except when stated otherwise, female littermates were used for all experiments. To evaluate bone formation via dynamic histomorphometry, mice were injected with 10 mg/kg calcein (Sigma) 7 and 2 days prior to the euthanasia of two-month-old animals, and 10 and 3 days prior to the euthanasia of six-month-old animals. All animal protocols were approved by the Institutional Animal Care Committee at UConn Health.

3.2.2. Generation of conditional miR-29 decoy allele

A miRNA tough decoy consists of a hairpin with an internal bulge that contains two imperfect miRNA binding sites (94-97). These decoys decrease the abundance of target miRNAs, as well as sequester the target miRNAs by acting as competitive inhibitors.
A Rosa26-specific donor vector containing the tdTomato coding sequence followed by three-copies of a miR-29-3p tough decoy was prepared and used to generate the miR-29 decoy mouse line using CRISPR mediated gene editing at the UConn Health Center for Mouse Genome Modification. Figure 3.1 and Appendix A provide additional details about generation of the construct and mice. The decoy was designed to interact with all miR-29-3p isoforms, with additional modifications to improve miRNA binding site accessibility.

A line of mice displaying germline transmission of the knock-in allele were bred with Hprt-Cre mice (Jax stock no: 004302; C57BL/6 background) to remove the STOP sequence (three copies of the SV40 polyadenylation signal sequence), placing the
tdTomato-miR-29-3p decoy under the transcriptional control of the CAG promoter, thus accomplishing global overexpression (Figure 3.2B-D and 3.3). Mice heterozygous for the globally expressed miR-29 decoy were crossed with each other and the genotype of the resulting pups was determined by PCR. Genotyping primers can be found in Appendix B, Table B.2. Wild-type mice and littermates homozygous for the decoy allele were retained for analysis.

3.2.3. Static and dynamic histomorphometry

For static histomorphometry, right femurs from global miR-29 decoy mice and littermate controls were fixed for 7 days in 4% paraformaldehyde at 4°C. After fixation, femurs were demineralized for 14 days in a 14% EDTA and 2.7% NH₄OH solution. Femurs were subsequently dehydrated, paraffin embedded, and sectioned using a Leica microtome; 7 µm thick sections were TRAP stained with a hematoxylin counterstain.

Left femurs were fixed 7 days in 4% paraformaldehyde at 4°C, incubated 24-36 hours in 30% sucrose, embedded in O.C.T. (Thermofisher) and flash frozen in super-cooled 2-methylbutane (Fisher); 7 µm sections were collected using a Leica cryostat and Cryofilm II tape transfer system (Section Lab Co. Ltd., Hiroshima, Japan). Sections were cross-linked to glass slides using Norland Optical Adhesive and mounted using 50% glycerol solution.

Static and dynamic histomorphometric parameters were measured using Osteomeasure software (OsteoMetrics) and were quantified 200 µm from the growth plate and cortices, as recommended by the Nomenclature Committee of the American Society
for Bone and Mineral Research (98). Fluorescence and light micrographs were obtained using an Axiocam MRc digital camera and AxioVision software (Zeiss).

3.2.4. **Micro-computed tomography (microCT)**

Right femurs from global miR-29 decoy mice and littermate controls were fixed in 70% ethanol at 4°C until analysis. The metaphyseal region of the distal femur was analyzed at 8 µm voxel resolution using micro-CT (µCT40, Scanco Medical AG, Bassersdorf, Switzerland) to quantify trabecular and cortical microarchitecture (99).

3.2.5. **Quantitative Real-Time PCR (qRT-PCR)**

For whole bone gene expression, calvaria were harvested, cleaned of soft tissue, flash frozen, homogenized in TRIzol reagent (Life Technologies), and RNA was extracted as directed by the manufacturer. As previously described, total RNA was quantified, DNase treated (Promega), and subjected to random hexamer primed reverse transcription for Sybr green based quantitative Real-Time PCR (qRT-PCR) (85). For Sybr green based qRT-PCR, gene expression was normalized to 18S rRNA. Primer sets are shown in **Appendix B, Table B.2**. Expression of miRNA-29a-3p, miRNA-29b-3p, miRNA-29c-3p, miRNA-365-3p and miRNA-30a-5p were analyzed using miRNA-specific TaqMan microRNA assays (Life Technologies). Briefly, miRNAs were reverse transcribed from total RNA using miRNA specific primers to create cDNA, which was quantified by qRT-PCR. miRNA expression was normalized to RNU6B. Relative quantities were determined using standard curves and samples were assayed in duplicate.
3.2.6. **Raman spectroscopy**

To examine bone composition, Raman spectroscopy was performed on the cortical bone of femurs from five-month-old female mice (100). Briefly, 20 µm frozen sagittal sections of were analyzed using a Witec alpha 300 Raman system with a 785 nm⁻¹ laser at an output power of 60-70 mW. Using a 100x objective, 5 measurements were collected along the long axis of the bone at the mid-diaphyseal region of the femoral cortex, approximately 20 µm apart.

Cosmic ray corrections and background subtractions were performed on all Raman spectra before analysis. Peaks of interest, including 960, 1000, 1070, 1451, and 1670 Δcm⁻¹, were fit as Lorentzian peaks using the Witec Control 5.1 fitting program. Starting parameters such as peak height and width were selected to obtain a visually determined best fit, after which the program was allowed to perform up to 200 fitting iterations to obtain a best fit. Peak center, width, and height for the five peaks of interest were recorded (100). Since Raman peak height can be affected by numerous factors, peak height ratios were reported in order to account for any scattering variations. The mineral to matrix ratio is represented as the ratio of the 960/1000 and 960/1670 peak height ratios and the mineral carbonate to phosphate ratio is represented by the 1070/960 peak height ratio.

3.2.7. **Whole body and bone marrow adiposity**

Bone marrow adipose tissue was quantified in whole tibia of six-month old animals using osmium tetroxide staining, essentially as described by Scheller et al (101). Safety
Figure 3.2. Global miR-29 decoy mice. (A) Schematic of the miR-29-3p decoy cassette that was knocked into the Rosa26 locus of C57BL/6 mice. It consists of the CAG promoter, three copies of a transcription stop sequence flanked by pLox sites (F), and the coding sequence for tdTomato (red fluorescent reporter), with three copies of a miR-29 tough decoy in its 3’-UTR. Each tough decoy contains two miR-29 binding sites (in green). WPRE was added to enhance expression of the decoy. (B-D) Representative images of tdTomato fluorescence in bone from two-month-old mice: femur (B), knee joint (C), and calvarium (D). (E-F) Quantification of tdTomato mRNA (E) and miRNA (F) expression in calvaria from two-month-old wild-type and global miR-29 decoy mice. Mean ± standard deviation. WT n=9; Decoy n=9.
protocols for proper use and disposal of osmium tetroxide were developed in coordination with the UConn Health Environmental Health and Safety. Briefly, right tibias from global miR-29 decoy mice and controls were fixed 24 hours in 4% paraformaldehyde at 4°C. After fixation, tibias were demineralized 21 days in a 4.14% EDTA and 0.44% NaOH solution; the solution was refreshed every three days. After demineralization, the bones were stained with 1% osmium tetroxide (4% aqueous osmium tetroxide diluted in 5% potassium dichromate) for 48 hours at room temperature. Bones were rinsed copiously with water prior to analysis. Stained tibias were scanned at 8 µm voxel resolution using micro-CT (µCT40). For analysis, the volume of interest consisted of 600 total slices (300 slices above and 300 slices below the tibia-fibula junction). The fractional volume of osmium-stained adipose tissue was determined using the Scanco 3D bone morphometry evaluation program with a threshold of 550 and Gaussian filtering (σ = 0.8, support = 1).

Whole body composition of lean and fat mass was measured using a mq7.5 TD-NMR Analyzer (Bruker BioSpin Corporation, Billerica, MA, USA), according to manufacturer's instructions. A cohort of mice was analyzed longitudinally from two-months through six-months of age.

3.2.8. Statistics

Data shown in bar graphs are mean ± standard deviation. For data shown in box-and-whisker plots, the mid-line represents the median, the top and bottom of each box represents the 75% and 25% quartiles respectively, the whiskers mark the minimum and maximum values within the data set, and open circles represent outliers. Statistical significance was determined using two-tailed Student's t-test (KaleidaGraph, Synergy...
3.3. Results

3.3.1. Generation of the global miR-29 decoy mice.

miR-29 family members are expressed from two loci in mouse: miR-29b-1 and miR-29a from chromosome 6, and miR-29c and miR-29b-2 from chromosome 1. The miR-29b loci give rise to identical miR-29b-3p isoforms. The miR-29-3p family members display remarkable sequence identity, such that miR-29a-3p and -29c-3p differ by a single nucleotide, whereas the 3’-end of miR-29b-3p shows the most variation (Figure 3.1 top). For all three isoforms, the seed binding region (bases 2-8), which is thought to nucleate interactions with mRNA targets, is identical (102-104). This suggests that miR-29 family members have the ability to target many of the same transcripts.

In an effort to inhibit the activity of the miR-29-3p family members, we designed a miR-29 tough decoy to serve as a competitive inhibitor for all three isoforms (Figure 3.1 middle). We chose this strategy because it has been shown to be effective in knocking down the activity of miRNAs in vivo and in vitro (93-95). The decoy cassette consisted of the coding sequence for the fluorescent tdTomato reporter gene, with three miR-29-3p tough decoys in place of its 3'-UTR. This decoy cassette was knocked into a known PAM sequence in the Rosa26 locus (Figure 3.1 bottom) using CRISPR-Cas9, in a manner such that its expression can be activated by Cre-mediated recombination of a floxed transcription stop consisting of three copies of the SV40 polyA signal sequences (Appendix A, Figure 3.2A). A line of mice displaying germline transmission of the knock
Figure 3.3. Global miR-29 tough decoy is ubiquitously expressed. (A-E) To verify the miR-29 tough decoy is globally expressed under Hprt-cre activation and not present in littermate control mice, a series of organs (including heart (A), kidney (B), liver (C), spleen (D), and skeletal muscle (E)) were harvested and screened for tdTomato fluorescence.
-in allele was crossed with HPRT-cre mice, resulting in global expression of the decoy cassette, demonstrated by ubiquitous tdTomato fluorescence in all tissues (Figure 3.3). Global miR-29 decoy mice were born at normal Mendelian ratios and were viable and fertile (data not shown).

Images from long bones and calvaria confirm tdTomato fluorescence in all cells of the skeletal elements (Figure 3.2 B-D). tdTomato mRNA expression in the calvaria of wild-type and decoy animals was also quantified by qRT-PCR (Figure 3.2E). Examining the expression of mature miR-29-3p, we found all three isoforms were significantly down-regulated in the calvaria of decoy animals compared to wild-type littermates (Figure 3.2F). In comparison, levels of miR-30a-5p and miR-365-3p, two miRNAs normally expressed in both osteoclasts and osteoblasts (4, 85, 105), were unchanged (Figure 3.3F). This suggests that expression of the decoy results in specific knockdown of miR-29-3p isoforms.

3.3.2. Impact of the global decoy at two-months of age.

We first assessed the microarchitecture of femoral trabecular and cortical bone at two-months of age, the time of peak trabecular bone volume in C57BL/6 mice (106). MicroCT analysis did not reveal a significant impact of the global miR-29 decoy on trabecular or cortical bone compartments in neither female nor male mice at this age (Figure 3.4 and Figure 3.5).

Since the miR-29 family positively regulates both osteoclast and osteoblast differentiation and function in vitro, histomorphometry and serum markers were used to
examine cellular parameters of bone turnover in vivo (Figure 3.6). Examining osteoclast parameters, static histomorphometry (Figure 3.6A, B) did not reveal a difference in osteoclast surface or eroded surface between wild-type and global decoy mice (Figure 3.6K, L). Although statistical significance was not achieved, serum CTX (marker of systemic bone resorption) trended higher in the decoy mice compared to wild-type (Figure 3.6I). Dynamic histomorphometry (Figure 3.6G, H) showed significantly
decreased mineralizing surface and bone formation rate in global decoy mice compared to wild-type controls (Figure 3.6D, E). While statistical significance was not achieved, mineral apposition rate (Figure 3.6F) also trended lower in the decoy mice, as did osteoblast surface (Figure 3.6C). Serum P1NP (marker of systemic bone formation) was not altered in the decoy mice (Figure 3.6J). Together, these data suggest the major impact of the global miR-29 decoy in vivo may be occurring in the mesenchymal lineage.
Figure 3.6. Histomorphometric analysis of femurs from two-month-old wild-type and global miR-29 decoy mice. (A, B) Representative images of TRAP stained sections from wild-type (A) and global miR-29 decoy (B) mice used for static histomorphometry. (G, H) Representative images of calcein-double labeling in wild-type (G) and global miR-29 decoy (H) mice used for dynamic histomorphometry. (C, K, L) Static histomorphometry measurements include: osteoclast surface (K), eroded surface (L), and osteoblast surface (C); WT n=7; Decoy n=7. (D-F) Dynamic histomorphometry measurements include: mineralizing surface (D), bone formation rate (E), and mineral apposition rate (F); WT n=4; Decoy n=5. (I, J) Serum markers of bone remodeling, serum CTX (I) and serum P1NP (J) were measured by ELISA; mean ± standard deviation; WT n=6-7; Decoy n=7. Data on serum markers of bone turnover were provided by Dr. Bongjin Shin.
To examine this more closely, we evaluated expression of osteoblast marker genes *Runx2* (master transcription regulator of osteoblastogenesis) and *Bglap* (osteocalcin; an osteoblast differentiation marker) in the calvaria of two-month old wild-type and global decoy mice. We observed a significant decrease in *Runx2* expression in global decoy mice, while *Bglap* only trended lower (Figure 3.7A, B). Together, the dynamic histomorphometry and gene expression data suggest that osteoblasts in global decoy mice are less functional.

To further examine osteoblast function, we evaluated the mRNA expression of *Rankl* (driver of osteoclastogenesis) and *Opg* (soluble RANKL decoy receptor). While *Rankl* was not changed, *Opg* was significantly decreased in the global decoy mice, resulting in

Figure 3.7. Expression of osteoblastic genes in calvaria from two-month-old global miR-29 decoy mice. (A, B) mRNA for osteoblast marker genes *Runx2* (A) and *Bglap* (osteocalcin) (B) (C-E) mRNA for *Rankl* (C), *Opg* (D), and the *Rankl/Opg* ratio (E). Mean ± standard deviation; WT n=8; Decoy n=8.
an increased Rankl/Opg ratio (Figure 3.7C-E). While this increase in the Rankl/Opg ratio would suggest a pro-osteoclastic environment, significant alterations in osteoclast parameters were not observed at two months of age (Figure 3.6K, L).

### 3.3.3. Impact of the global decoy in skeletally mature animals.

While femoral trabecular and cortical bone microarchitecture was unaltered in two-month-old decoy mice, these mice displayed a robust decrease in bone formation rate. To determine whether this might contribute to differences in the skeleton of mature mice, we analyzed six-month-old animals (Figure 3.8A). Although microCT analysis did not demonstrate changes in cortical bone parameters (Figure 3.8G-I), we observed significant decreases in both bone-volume fraction (Figure 3.8C) and trabecular number (Figure 3.8E), whereas trabecular spacing (Figure 3.8D) was increased. Trabecular thickness (Figure 3.8F) and total bone volume (Figure 3.8B) were unaltered.

We then used histomorphometry and serum markers to determine if cell parameters were altered in six-month-old mice (Figure 3.9). Unlike the two-month-old animals, dynamic histomorphometric analysis of six-month-old mice (Figure 3.9E, F) did not reveal significant differences in mineralizing surface, bone formation rate, or mineral apposition rate between wild-type and global decoy animals (Figure 3.9H, K, L); osteoblast surface and serum P1NP were also unchanged (Figure 3.9G, J). Similarly, osteoclast surface and eroded surface were not different between six-month-old wild-type and global decoy animals (Figure 3.9C, D). While serum CTX trended higher in the six-month decoy mice compared to wild-type, it did not achieve significance (Figure 3.9I).
Although cortical bone parameters were not different at the level of microarchitecture (Figure 3.8G-I), alterations in bone matrix properties can have a significant impact on biomechanical properties. Therefore, Raman spectroscopy was used to determine the mineral-to-matrix ratio, a primary determinant of bone quality, as well as the carbonate-to-phosphate ratio, in the cortical bone of femur from five-month-old mice. However, significant differences between wild-type and miR-29 decoy mice in the mineral-to-matrix ratio or the carbonate-to-phosphate ratio were not detected (Figure 3.9M, N). These data
suggest remodeling may not be substantially altered in the cortical bone of decoy mice.

3.3.4. Impact of the global decoy on adiposity.

The miR-29 family was previously reported to be downregulated during adipocyte differentiation and that it negatively regulates adipocyte differentiation. *In vitro* models
have shown downregulation of the miR-29 family is necessary for adipocyte
differentiation, and that miR-29 targets several transcription factors needed for
adipogenesis (107-111). To determine if global expression of the miR-29 decoy impacted
adiposity, we performed a longitudinal study of whole-body composition using TD-NMR
(Figure 3.10). Both wild-type and decoy mice showed significantly increased total mass
from two- to six-months of age (p<0.01), although there was no difference between the
genotypes (Figure 3.10A). Percent lean mass (Figure 3.10B), and percent fat mass
(Figure 3.10C) remained similar from two to six months of age, and were also unaffected
by genotype, suggesting that, up to six-months of age, global expression of the miR-29
decoy does not impact whole body composition.

Osteoblasts and marrow adipocytes originate from the same precursors (112, 113).
Although osteoblast surface was not different between wild-type and miR-29 decoy mice
at any age analyzed, we determined whether expression of the decoy had an impact on
bone marrow adiposity using osmium tetroxide staining in tibias. This heavy metal
compound interacts with lipids and is opaque when visualized by microCT, allowing
reliable quantification of marrow adipose (101). Although marrow adipose was readily
detected, there was no difference between global miR-29 decoy mice and wild-type
littermates at either 2 months (not shown) or 6 months of age (Figure 3.10D, E).

### 3.4. Discussion

Skeletal homeostasis is an intricate process involving the interplay of osteoblasts and
osteoclasts. The differentiation and function of these cell types is regulated at multiple
levels, and miRNAs play a vital role in post-transcriptional regulation of gene expression in bone cells (83). The miR-29 family is expressed in both the osteoblast and osteoclast lineages. The goal of the current study was to better understand the role of the miR-29-3p family in osteoclast and osteoblast biology in vivo, in healthy mice. To accomplish this, we generated a novel mouse line in which a miR-29-3p tough decoy, capable of decreasing the abundance and activity of all three miR-29-3p isoforms, was knocked into the Rosa26 locus of C57BL/6 mice and globally expressed. In growing mice, expression of the miR-29-3p decoy caused decreased bone formation in the absence of significant changes in osteoblast surface, which led to decreased trabecular bone volume in mature mice. An impact of the miR-29-3p decoy on the osteoclast lineage in vivo was not detected in growing or mature animals. These data verify the positive role of miR-29-3p in osteoblast function in vivo, and suggest that miR-29-3p isoforms may play a more prominent role in the regulation of osteoblasts.

Expression of miR-29-3p isoforms increases with osteogenic differentiation in human, mouse and rat cells (80, 86, 90). In the context of osteogenic differentiation, overexpression of miR-29b in a mouse pre-osteoblastic cell line enhanced osteoblastic differentiation by targeting several negative regulators of this process, including HDAC4, TGFβ3, and beta catenin-interacting protein 1 (86). Studies in a human osteoblastic cell line demonstrated that the promoter of the miR-29a-b1 cluster contains several Tcf/Lef binding elements that are instrumental in the up regulation of miR-29a in response to canonical Wnt signaling. Moreover, miR-29 directly targets additional negative regulators of Wnt signaling in these human osteoblastic cells, establishing a positive feed-back loop that amplifies Wnt signaling (90).
Somewhat paradoxically, the miR-29 family is known for its ability to target extracellular matrix mRNAs, including type I collagen mRNAs, as well as RNAs for non-collagenous extracellular matrix components that regulate fibril formation, mineralization, and the activity of specific growth factors \( (86, 90, 114, 115) \). *In vitro*, miR-29-3p levels are low in early osteoblasts, which are responsible for depositing large amounts of extracellular matrix. The expression of the miR-29-3p family members then increases as the matrix matures and becomes mineralized \( (90) \). It is well established that miRNAs regulate the tempo and amplitude of gene expression programs \( (116, 117) \). Certainly, miRNA-mediated regulation provides an efficient means for rapidly decreasing the production of proteins, a concept especially relevant to the regulation of very stable mRNAs, such as those coding for extracellular matrix components \( (90) \).

Compared to osteoblasts, miRNA function and regulation in the osteoclast lineage is less well understood \( (87) \). Indeed, the function of the miR-29 family in osteoclasts remains somewhat controversial. For example, Rossi and coworkers reported that miR-29b was downregulated in human CD14\(^+\) peripheral blood mononuclear cells after 18 days of RANKL-induced osteoclastogenesis *in vitro*. When miR-29b was overexpressed via lentivirus, osteoclast differentiation was impaired and expression of RANK was decreased \( (40) \). On the contrary, we observed that all miR-29-3p family members are upregulated during RANKL-induced osteoclast differentiation of primary BMMs, as well as in a murine monocytic cell line. Inhibition of miR-29a, -29b, or -29c in these cells *in vitro* impaired differentiation, resulting in fewer and smaller osteoclasts. Inhibition of miR-29-3p was found to decrease both precursor commitment to the osteoclast lineage and osteoclast precursor motility, which is required for fusion \( (37) \). Differences in methodology between
these studies could account for some of the divergent findings; these methodological differences include the source of osteoclast precursors (species and compartment), overexpression versus knock-down models, and the length of time the cells were in culture. Further, whereas miR-29-3p isoforms were studied in the mouse culture system, it is not clear whether the studies in human cells involved miR-29b-3p or -5p. Nonetheless, additional in vitro and in vivo studies will be key to sorting out the function of miR-29 family members in osteoclasts.

Our study of miR-29-3p in bone was performed in healthy mice, both growing and mature. In captivity, healthy mice have few stresses on their skeleton. It will be of interest to use the miR-29-3p decoy mice to probe the function of miR-29-3p in models of skeletal disease. For example, lipopolysaccharide (LPS), often used to generate an inflammatory response in vitro and in vivo, was recently shown to induce miR-29b-3p expression in osteoclasts. In mouse osteoclasts, overexpression of miR-29b promoted cell survival, whereas inhibition of miR-29b activity was shown to be pro-apoptotic; BCL2 modifying factor, an apoptosis activator, was validated as a novel miR-29 target (89). This study suggests that the miR-29 decoy mice may be more susceptible to bone loss in models of localized or systemic inflammation (89).

Glucocorticoid excess is a common cause of secondary osteopenia. In vitro, supraphysiologial doses of glucocorticoids can decrease the expression of miR-29a in the osteoblast lineage (91). Similarly, miR-29a levels in bone are decreased by glucocorticoid excess in both mice and rats (113, 118). In mice globally overexpressing miR-29a-3p under the control of the phosphoglycerate kinase (PGK) promoter, bone loss caused by pharmacologic doses of glucocorticoid was attenuated. This effect appeared
to be due to decreased osteoclast number, with a more modest impact on bone formation rate (113, 119). Further, overexpression of miR-29a in rats using a lentiviral vector also protected against glucocorticoid mediated bone loss, demonstrating enhanced bone formation, as well as attenuated osteoclast differentiation in response to glucocorticoid treatment (118).

Estrogen deficiency is also a prominent cause of bone loss, and miR-29a was recently shown to be down regulated in bone from mice subjected of ovariectomy (120). In transgenic mice overexpressing miR-29a under the control of the osteocalcin promoter, in mature osteoblasts, ovariectomy-induced bone loss was partially rescued. Interestingly, miR-29a overexpression in osteoblasts increased bone formation rate and decreased osteoclast number. Rankl was subsequently identified as a novel miR-29 target, contributing to an indirect mechanism of miR-29 regulation of osteoclast number in vivo (120). Although we did not observe a significant increase in Rankl mRNA in bone from the miR-29-3p decoy mice, Opg mRNA was decreased. This resulted in an increased Rankl/Opg ratio, which could favor osteoclast formation. This may be interpreted as a potential compensatory response by the osteoblast lineage to maintain skeletal homeostasis by promoting osteoclast number and function in the face of decreased miR-29-3p activity in osteoclasts, themselves. These in vivo studies indicate an impact of miR-29 on both osteoblast and osteoclast lineages, and highlight the ability of osteoblasts to regulate osteoclast formation and function through the expression of paracrine factors.

Expression of miR-29 family members is altered in models of skeletal disorders, including inflammatory disease, estrogen deficiency and glucocorticoid excess (89, 113,
In the previously discussed models, the impact of miR-29 on disease phenotype was evaluated when miR-29 family members were overexpressed. Overexpression of a miRNA may affect the ability of other miRNAs to access the RISC and/or could lead to the engagement of lower affinity miRNA targets (121). Our approach involved inhibiting miR-29-3p family members using a tough decoy. Expression of the decoy cassette decreased the abundance of detectable miR-29-3p isoforms from 30 to 60%, a level similar to that seen with other miRNA decoy constructs (94). Sequestration of miRNA-mRNA complexes in P-bodies may also provide additional relief from miRNA targeting (94, 122). Thus, our miR-29-3p decoy model represents a modest loss-of-function that had a more pronounced effect on the osteoblast lineage compared to the osteoclast lineage. It is possible that the osteoblastic differentiation may be more sensitive to perturbations in miR-29-3p family abundance. Whereas other groups have created mice with targeted deletion of one or both miR-29 loci, studies of the impact on the skeleton have yet to be reported (123-126).

We examined the skeletal phenotype of mice globally expressing a miR-29-3p inhibitor. miR-29-3p family members are broadly, but not ubiquitously, expressed (127-130). Bone homeostasis is regulated by multiple organs, including brain, gut, kidney, liver and adrenal gland (84). Expression of the miR-29-3p decoy in extra-skeletal tissues may make it more difficult to isolate the role of miR-29 in bone cells. Our data provide a strong rationale for future studies using lineage restricted cre-drivers (i.e. LysM-cre or osteocalcin-cre) to activate expression of the miR-29 decoy. This would allow us to better understand the role of miR-29 in the osteoblast and osteoclast lineages independently.
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Author Contributions

HCH: Investigation, Visualization, Writing-Original Draft. BS: Investigation, Validation. 
SPY: Methodology. ACD: Investigation. SKL: Conceptualization, Project Administration, Funding acquisition, Writing-Review & Editing. AMD: Conceptualization, Methodology, Project Administration, Funding acquisition, Writing-Review & Editing.

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CHAPTER 4

Global miR-29-3p inhibition decreases osteoclastic potential while increasing the adipogenic potential of bone marrow cultures in vitro

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ABSTRACT

The miRNA-29-3p family is a key regulator in the differentiation of osteoblasts, adipocytes, and osteoclasts in vitro. In our previous study of mice globally expressing a miR-29-3p tough decoy, decreased trabecular bone volume and bone-formation rate were observed, with no changes to osteoclast or osteoblast surface, or bone marrow adiposity. Given the global nature of the miR-29 decoy, it is likely that these findings not only reflect the impact of the miR-29 decoy in the osteoblasts, osteoclasts, and adipocytes, but also the indirect effects of the decoy in miR-29-3p-expressing extraskeletal tissues. In the current study, we sought to characterize the cell autonomous effect of the global miR-29 decoy in skeletal cells separated from the influence of systemic factors. In whole bone marrow cultures undergoing osteogenic differentiation in vitro, expression of the global miR-29 decoy did not impact osteoblast differentiation or function. In cells undergoing adipogenic differentiation, expression of the decoy modestly increased adipocyte marker gene expression. In contrast, in bone marrow monocytes undergoing osteoclastogenesis, expression of the miR-29 decoy decreased osteoclast formation without affecting osteoclast function. Together, these data reinforce the
rationale for utilizing lineage restricted cre-drivers in our future studies as we continue to study the specific role of miR-29-3p in these lineages.

4.1. Introduction

In the previous chapter, we presented data on the skeletal phenotype of our novel miR-29-3p tough decoy (miR-29 decoy; global decoy; decoy) mice. These mice globally express a miR-29-3p tough decoy, which competitively inhibits endogenous miR-29-3p activity. At two-months of age, we observed decreased bone formation rate in the global decoy mice, which was accompanied by a significant decrease in Runx2 mRNA expression and a marked increase in the Rankl/Opg mRNA ratio in calvaria. By six-months of age, the trabecular bone volume was significantly decreased in the decoy mice. While our analysis indicated that the miR-29 decoy has a negative impact on the osteoblast lineage in vivo, none of the measured in vivo osteoclast or adipocyte parameters were significantly altered by the miR-29 decoy at either two or six months of age. These data reinforce the concept that the miR-29 family plays an important role in the skeleton, but drawing direct conclusions about the impact of the decoy on these specific skeletal cell lineages may be confounded by miR-29-3p inhibition in extra-skeletal tissues which influence bone homeostasis (84).

To address this, we characterized the cell-autonomous impact of the global miR-29-3p tough decoy on the osteoblast, adipocyte, and osteoclast lineages in vitro. As outlined in the previous chapter, the miR-29-3p family is upregulated during both osteoblastogenesis and macrophage colony stimulating factor (MCSF) and receptor activator of NFκB ligand (RANKL) induced osteoclastogenesis (37, 80, 86, 89-92). In vitro,
miR-29-3p positively regulates osteoblastogenesis and function in both mouse and human cells, in part, by targeting negative regulators of differentiation, including Hdac4, Tgfb3, and several negative regulators of Wnt-signaling (80, 86, 90-92). miR-29-3p positively regulates osteoclastogenesis in vitro; its ability to target negative regulators of osteoclast differentiation, including Nfia and Calcr, likely contribute to this effect. Further, miR-29-3p fine-tunes osteoclast formation by targeting Cdc42 and Srgap2, which regulate actin-remodeling and podosome formation necessary for motility, cell-fusion, and actin ring formation. Regulation of these targets may be necessary to control osteoclast size and activity (37, 43, 131).

In the adipocyte lineage, in vitro models have shown that the miR-29-3p family is down-regulated during adipocyte differentiation and that sustained miR-29-3p expression, using miRNA mimics, impairs differentiation. This downregulation is thought to be necessary, in part, as miR-29-3p targets several transcription factors needed for adipogenesis, as well as the glucocorticoid receptor and glucocorticoid-receptor signaling-mediators (107-111).

The present in vitro studies indicate that expression of the global miR-29-3p decoy decreases osteoclast formation and modestly increases expression of adipocyte marker genes. In contrast, the osteoblast lineage was unaffected by miR-29-3p decoy expression. This suggests that the skeletal phenotype observed in vivo results from the inhibition of miR-29-3p activity in both skeletal and extra-skeletal tissues.

4.2. Materials and Methods

4.2.1. Animal care
All mice were in the C57BL/6 background and were housed in the Center for Comparative Medicine at UConn Health under a 12-h light/dark cycle and were provided with standard mouse chow and water ad libitum. Mice globally expressing a miR-29-3p tough decoy were generated as previously described in Chapter 3 of this dissertation and in Appendix A. For all experiments, whole bone marrow was isolated from six-to-ten-week-old wild-type and global-decoy female littermates. All animal protocols were approved by the Institutional Animal Care Committee at UConn Health.

4.2.2. In vitro osteoclast differentiation

Primary murine osteoclast cultures were prepared from bone marrow monocytes (BMMs) isolated from non-adherent bone marrow cells as previously described (132). Briefly, whole bone marrow was flushed from femurs and tibias, passed through a cell strainer, and cultured overnight on tissue culture plates in complete α-MEM (Gibco) [10% heat-inactivated FBS (Hyclone) and 1% antibiotic-antimycotic cocktail (Gibco #15240062)]. The following day, non-adherent cells were collected and bone marrow monocytes (BMMs) were prepared using Ficoll-Paque (GE Healthcare; Sigma-Aldrich) density gradient centrifugation. BMMs were collected from the Ficoll-Paque:media interface and then rinsed with serum free α-MEM before final resuspension and plating.

For osteoclast differentiation assays, BMMs (2x10^4 cells/well in a 96 multi-well plate or 1.5x10^6 cells/well in 6 multi-well plate) were cultured in complete α-MEM supplemented with recombinant human M-CSF (30 ng/mL) and human RANKL (30 ng/mL). Both were expressed in our laboratory using the constructs kindly provided by Dr. D. Fremont from Washington University and Dr. Y. Choi from University of Pennsylvania, respectively.
Cells were cultured up to 5 days, with a media change at day 3. At indicated days, cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton-X, and stained with TRAP staining solution (0.1 mg/ml Napthol AS phosphate, 120 mM Na-Acetate, 12 mg/mL Na-Tartrate, 0.5 mg/mL Fast Red Violet dye) for 3-6 minutes at 37°C. TRAP-positive cells, containing 3 or more nuclei, were counted as osteoclasts.

To assess osteoclast function in vitro, we performed bone resorption assays. BMMs were plated in equal numbers (2x10^4 cells/bone chip) on devitalized bone chips prepared from bovine cortical bone. BMMs on the bone chips were cultured in complete α-MEM supplemented with recombinant human M-CSF and RANKL (30 ng/mL each) for 5 days. After 5 days, cells were removed using 10% bleach and bone resorption pits visualized using toluidine blue stain (1 g/mL Toluidine blue, 1 g/mL sodium-borate-decahydrate). Resorption pits were then viewed using an Olympus BX53 light microscope and measured using Olympus CellSens imaging software.

To assess actin ring formation in vitro, BMMs were cultured in 96 well plates or bone chips in α-MEM supplemented with recombinant human MCSF and RANKL (30 ng/mL each) for 6 days. After 6 days, cells were fixed, permeabilized, and stained with rhodamine-phalloidin (1:40 dilution). Fluorescent photomicrographs were obtained using an Axiocam MRc digital camera and AxioVision software (Zeiss).

4.2.3. In vitro osteoblast and adipocyte differentiation assays

Primary osteoblast and adipocyte cultures were prepared from total bone marrow flushed from murine femurs and tibias. Whole marrow was passed through a cell strainer,
rinsed, and re-suspended in complete α-MEM. For all osteoblast and adipocyte differentiation experiments, freshly harvested whole bone marrow was plated at 4×10⁶ cells/well in 6 multi-well plates. Cells were cultured for 7 days to allow for cell expansion, after which, the media was changed to either osteoblast or adipocyte differentiation media (differentiation day 0). Media changes were performed every 3-4 days.

For osteoblastic differentiation, complete α-MEM was supplemented with 50 µg/mL Ascorbic acid (100 µg/mL total) (Sigma-Aldrich) and 7 mM β-Glycerophosphate (Sigma-Aldrich). To assess early-osteoblast differentiation, cultures were stained for alkaline phosphatase activity at day 0 using a Leukocyte-Alkaline-Phosphatase-Kit (Sigma-Aldrich). To measure osteoblast function in vitro, at days 7, 14, and 21 of culture, cells were fixed using 4% paraformaldehyde and calcium deposits were detected using 1% Alizarin-red stain pH 4.2 (Sigma-Aldrich). To quantify differences between time-points and genotypes, Alizarin-red stain was eluted from tissue culture wells using 10% acetic acid, neutralized using 10% NH₄OH, and measured on a plate reader at 405 nm⁻¹. After Alizarin-red elution, cells were stained with 0.5% crystal violet to visualize all cells. Crystal violet stain was eluted in 100% methanol and measured at 570 nm⁻¹. Alizarin red values were normalized to their corresponding crystal violet values to account for potential differences in cell number.

For adipocyte differentiation, complete α-MEM was supplemented with 1 µM Insulin (Sigma-Aldrich) and 500 nM Rosiglitazone (Sigma-Aldrich). To measure adipocyte differentiation, adipocyte cultures were fixed with 4% paraformaldehyde at days 0, 3, 5, and 7. After fixation, cells were rinsed with water, air dried, and then stained with Oil-red
O (0.18% Oil-red O in 60% isopropanol) (Sigma-Aldrich). Oil-red O was then eluted from tissue culture wells using 100% isopropanol and measured at 492 nm⁻¹.

### 4.2.4. Quantitative Real-Time PCR (qRT-PCR)

Cells were lysed directly in TRIzol (Invitrogen). For osteoblast cultures, the TRIzol lysate was immediately homogenized (Polytron) to disrupt multilayered colonies and mineral deposits. RNA was then extracted as directed by the manufacturer. As previously described, total RNA was quantified, DNase treated (Promega), and subjected to random-hexamer-primed reverse-transcription for Sybr green based quantitative Real-Time PCR (qRT-PCR) (85). For Sybr green based qRT-PCR, gene expression was normalized to 18S rRNA. Primer sets are shown in Appendix B, Table B.2. Expression of miRNA-29a-3p, miRNA-29b-3p, and miRNA-29c-3p were analyzed using miRNA-specific TaqMan microRNA assays (Applied Biosystems). Briefly, miRNAs were reverse transcribed from DNase treated total RNA using miRNA-specific primers to create cDNA, which was quantified by qRT-PCR. miRNA expression was normalized to RNU6B. Relative quantities were determined using standard curves and samples were assayed in duplicate.

### 4.2.5. Western Blotting

Primary BMMs were differentiated into osteoclasts in 6 multi-well plates and were harvested in protein lysis buffer (10% glycerol, 1% Triton-X 100, 150 mM NaCl, 20 mM HEPES, and 1X Halt protease and phosphatase inhibitor cocktail (ThermoFisher)). After a freeze-thaw cycle, protein lysates were quantified using a BCA kit (ThermoFisher). 15
µg of protein lysate was denatured and loaded on a 10% SDS-PAGE gel, subjected to electrophoresis, and transferred to PVDF membrane. Membranes were then blocked in 5% BSA in 0.1% TBST for 1 hour prior to incubation with primary antibody. The following antibodies were used for Western blotting: mouse anti-NFATc1 (BD Biosciences); rabbit anti-c-SRC, rabbit anti-GAPDH, rabbit anti-CDC42, and HRP-conjugated goat anti-Rabbit (Cell Signaling Technology); Rabbit polyclonal anti-SRGAP2 antibody generated in-house by Dr. Franck Polleux was previously described (133). Complete antibody data product information found in Appendix B, Table B.1.

4.2.6. Statistics

Data are shown as mean ± SEM. Statistical significance was determined using one-way analysis of variance (ANOVA) with Student-Newman-Keuls Multiple Comparison post hoc test or Student’s t test as appropriate (KaleidaGraph, Synergy Software, Reading, PA). For data analyzed by ANOVA, only the relevant comparisons were indicated.

4.3. Results

4.3.1. Characterizing the cell autonomous effects of the global miR-29 decoy in the osteoblast and adipocyte lineages

Based on the impaired bone formation demonstrated in the global miR-29 decoy mice in vivo, and our prior work using transiently transfected miR-29-3p inhibitors in the human osteoblastic hFOB1.19 cell line (80), we expected to see impaired osteoblastic differentiation in vitro in mesenchymal cells from global decoy mice. To assess this, we
performed osteoblast differentiation assays using wild-type and global decoy whole bone marrow cell cultures differentiated towards the osteoblast lineage for up to 21 days. At the start of differentiation (D0), we assessed early osteoblast differentiation by staining cultures for alkaline phosphatase activity (Figure 4.1A, far left column). At this early time point, there appeared to be increased alkaline phosphatase staining in the decoy cultures,
however crystal violet staining of the same wells (data not shown) suggested that this
different may have been due to a difference in cell number. For the subsequent time-
points (D7-21), we used alizarin red staining (Figure 4.1A) to assess the formation of
mineralized nodules as the cells differentiated. Once eluted and quantified, the alizarin
red stain revealed that both wild-type and global decoy cultures deposited progressively
more mineral throughout differentiation, however, there was no difference in the
osteogenic potential between wild-type and global decoy cells (Figure 4.1B).

We then examined the gene expression of Runx2 (master transcription regulator of
osteoblastogenesis) and Bglap (osteocalcin; osteoblastic differentiation marker) in
similarly treated whole bone marrow cultures differentiated towards the osteoblast linage
for up to 21 days. For both wild-type and global decoy cultures, expression of both Runx2
and Bglap peaked at day 14 of differentiation, followed by a robust decrease at day 21
(Figure 4.1C and D). Similar to the alizarin red staining, the expression of these
osteogenic markers genes was not significantly different between the wild-type and global
decoy cells. This suggests that the observed decrease in Runx2 and Bglap mRNA
expression in calvaria collected from two-month old miR-29 decoy mice (Chapter 3
Figure 3.7) may reflect the contributions of extra-skeletal factors. It may also reflect a
possible difference in bone compartment, calvaria versus long-bone.

While in the previous chapter we did not demonstrate a significant impact of the miR-
29 decoy on bone marrow adiposity in vivo (Chapter 3 Figure 3.10), we wanted to
determine if we could detect a cell autonomous effect on the adipocyte lineage in vitro.
To assess this, we cultured wild-type and global decoy whole bone marrow cells with
Insulin and Rosiglitazone for up to 7 days, in order to drive them towards the adipogenic lineage. Using oil-red-O staining (Figure 4.2A) we found that both wild-type and global decoy cells accumulated lipid droplets when differentiated towards the adipocyte lineage. Once eluted and quantified (Figure 4.2B), the oil-red-O staining revealed that at day 5 of differentiation, the global decoy cultures had a significant increase in oil-red-O stain compared to the day 0 cultures, whereas the wild-type cultures only trended towards an increase. By the end of differentiation at day 7, both wild-type and global decoy adipocyte cultures accumulated significantly more oil-red-O stain compared to day 0. Furthermore, at day 7, the global decoy cultures showed a trend towards increased oil-red-O accumulation compared to the wild-type cells. These data suggest that while in vitro lipid accumulation is not robustly different between wild-type and global decoy adipocytes cultures, there may be slightly increased adipogenic potential in the miR-29 decoy cells (Figure 4.2A and B).

We then examined the gene expression of Pparγ (peroxisome-proliferation-activated-receptor-gamma; master transcription regulator of adipogenesis) and Cebpα (CCAAT/enhancer-binding-protein-alpha; adipocyte differentiation marker) in similarly treated whole bone marrow cultures differentiated towards the adipocyte lineage for up to 7 days. Similar to the oil-red-O staining, both genes peaked in expression at day 7 (Figure 4.2C and D). Interestingly, at days 3 and 7 of differentiation, we observed a significant increase in Pparγ expression in global decoy cultures compared to wild-type; on day 5 it only trended higher (Figure 4.2C). Furthermore, only in the global decoy cultures, and not wild-type, was Cebpα expression significantly increased at day 7 compared to day 0 (Figure 4.2C and D). These data, along with the oil-red-O staining,
suggest that inhibition of miR-29-3p activity by the global decoy results in a modest cell autonomous increase in adipogenic potential.

4.3.2. Characterizing the cell autonomous effects of the global miR-29 decoy in the osteoclast lineage.

Based on our prior work using transiently transfected miR-29-3p inhibitors in primary BMMs (37), we anticipated that the global decoy would negatively impact osteoclastogenesis in a cell autonomous manner in vitro, despite not seeing significant
changes in osteoclast number or activity in vivo (Chapter 3, Figure 3.6 and 3.9). Indeed, osteoclast formation assays demonstrated a significant decrease in osteoclast formation in global decoy BMMs compared to wild-type (Figure 4.3A-C), corroborating our previous findings.

We further characterized our in vitro cultures by examining the expression of mature miR-29-3p isoforms at days 1, 3, and 5 of RANKL induced osteoclastogenesis. At each timepoint, miR-29a-3p, miR-29b-3p, and miR-29c-3p were significantly down-regulated in the decoy cells compared to wild-type (Figure 4.3D-F). Furthermore, tdTomato mRNA (Figure 4.3G), which is used as an indirect measurement of the miR-29 decoy located in its 3’-UTR, was easily detectable in the decoy cells and did not change with differentiation. This decrease in mature miR-29-3p in the decoy cells may result from direct interactions of the miR-29 decoy and RISC loaded miR-29-3p family members, leading to the degradation of the miRNAs. Moreover, if expression of the decoy caused de-repression of miR-29 targets, leading to impaired osteoclastogenesis, it is possible that this impaired differentiation status could contribute to decreased expression of the miR-29-3p family members. In either case, these data demonstrate the function of the decoy.
Examining osteoclast marker gene expression, levels of a key osteoclast differentiation marker gene *CathespinK* (cysteine protease needed for bone-resorption) peaked at day 3 in both genotypes, and was decreased at day 5, when mature osteoclasts start undergoing apoptosis. Despite the observed decreases in osteoclast number and miR-29-3p levels, *CathespinK* mRNA was modestly increased in global decoy osteoclast cultures at day 5; this may reflect a delay in the peak of osteoclast formation and subsequent apoptosis. Like *CathespinK*, *Ocstamp* (molecule needed for pre-osteoclast fusion) mRNA expression peaked in cultures at day 3, but there was no difference between wild-type and decoy cells at any time point (Figure 4.3H, I). Further, NFATc1 (master transcription regulator of osteoclast differentiation) and c-SRC (tyrosine kinase responsible for actin remodeling needed for actin ring formation) protein levels at day 4 of osteoclast differentiation did not show significant differences between genotypes (Figure 4.3M-O).

In our previous work, we identified several novel miR-29 targets, including *Nfia* (a negative regulator of c-FMS expression), *Cdc42* (Rho-GTPase family member; regulates actin remodeling), and *Srgap2* (negative regulator of the Rho-GTPase Rac1) (37). This prompted us to examine the mRNA expression of these targets in the wild-type and decoy cells at days 1, 3, and 5 of osteoclast differentiation. We observed a significant increase in *Nfia* (nuclear factor I/A) mRNA expression in decoy cells compared to wild-type cells at day 5 of differentiation (Figure 4.3J), however, we found no difference in *Cdc42* or *Srgap2* mRNA expression between genotypes (Figure 4.3K, L). It is widely known that changes in miRNA expression or activity may not necessarily alter the abundance of mRNA, but rather it may more prominently change protein levels (134). However,
Figure 4.4. Characterization of cell-autonomous effects of the global miR-29 decoy on the osteoclast function. (A) Representative fluorescent micrographs of rhodamine-phalloidin stained actin-rings in day 6 wild-type or global decoy osteoclasts grown on either cell-culture plastic or on devitalized bovine bone-chips. (B) Representative photomicrographs of Toluidine-blue stained resorption pits formed by wild-type (left) or global decoy (right) osteoclasts by day 5 of differentiation. (C) The area of non-overlapping resorption pits was measured using Olympus CellSens imaging software and the mean resorption pit area per osteoclast is displayed; mean ± SEM; WT n=8; Decoy n=6. Data on in vitro bone resorption were provided by Dr. Bongjin Shin Ph.D.
Western blot analysis of day 4 osteoclast cultures did not reveal significant differences in either SRGAP2 or CDC42 between genotypes (Figure 4.3P and Q).

Since the miR-29 decoy decreased osteoclast formation in vitro, we determined if the global decoy had an impact on osteoclast function in vitro. First, we examined if osteoclasts formed from global decoy BMMs were able to appropriately form actin rings, which are needed for bone resorption. We cultured BMMs on both cell-culture plastic and on bone-chips, and after 6 days of differentiation, cells were stained for actin-ring formation using rhodamine-phalloidin. These assays demonstrated that actin ring formation is grossly similar between genotypes on either surface (Figure 4.4A).

With actin ring formation unaffected, we then determined if the global decoy had any impact on bone resorption activity. To examine this, equal numbers of wild-type and global decoy BMMs were differentiated on devitalized bovine bone-chips for 5 days, after which the cells were removed and the bone chips stained with toluidine blue. Toluidine blue stains newly exposed collagen fibers more vibrantly than the intact bone surface, allowing visualization of resorption pits. Given the known decrease in osteoclast formation from decoy BMMs, the area of only non-overlapping resorption tracks, made by single osteoclasts, was quantified. Similar to our in vivo observations (Chapter 3 Figure 3.6 and 3.9), this assay did not demonstrate differences in bone resorption area between genotypes (Figure 4.4B-C).

4.4. Discussion

The goal of the current study was to determine the cell-autonomous effect of the global
miR-29-3p tough decoy on the osteoblast, adipocyte, and osteoclast lineages \textit{in vitro}. To address this, we examined the osteoblastic, adipogenic, and osteoclastic potential of precursor cells harvested from the bone marrow of global decoy mice. In contrast to our observations \textit{in vivo}, expression of the global decoy had no impact on either osteoblast differentiation or function \textit{in vitro}. In the adipocyte lineage, expression of the decoy \textit{in vitro} modestly increased \textit{Pparγ} mRNA levels, suggesting an increase in adipogenic potential. Expression of the global decoy in the osteoclast lineage \textit{in vitro} decreased osteoclast formation, while osteoclast function was not impaired.

In whole bone marrow cell cultures differentiated towards the osteoblast lineage, we did not observe differences between genotypes, indicating that global miR-29 decoy expression does not incur a cell autonomous osteoblast phenotype \textit{in vitro}. This is in contrast to our \textit{in vivo} data, in which we observed a decrease in bone formation rate and as well as a decrease in \textit{Runx2} and \textit{Bglap} mRNA expression in calvarial bone. These apparently contradictory findings likely stem from difference in experimental design. Whereas the RNA for the \textit{in vitro} experiments was isolated from bone marrow cell cultures differentiated towards the osteoblast lineage over the course of weeks, the RNA from calvaria are derived from cells influenced by systemic signals and potentially reflect a more heterogeneous cell population.

While the mesenchymal lineage cells present in both the calvaria and bone marrow are similar in nature, they form bone through two very different processes. The mesenchymal lineage cells of the bone marrow are a multipotent progenitor population capable of forming bone through endochondral ossification, where an initial hyaline
cartilage scaffold consisting mainly of type-II and type-X collagen is formed. This scaffold is then replaced over time by bone consisting of mineralized matrix proteins, primarily type-I collagen. The mesenchymal lineage cells of the calvaria are a more committed, less multipotent, osteoprogenitor population that undergo intramembranous ossification, which forms bone directly (135). These two populations of cells are regulated by very different transcriptional profiles. For example, endochondral ossification is tightly regulated by the spatiotemporal expression of several growth factors and matrix-proteins, such as collagens (including Type-I, -II, and -X), fibroblast-growth-factors (Fgfs; including Fgf-2, -7, -9, -10, and -18), Fgf-receptors (Fgfrs; including Fgfr-1, -2b, -2c, and -3), bone-morphogenic proteins (BMP; including BMP-2 and -4), Indian-hedgehog (Ihh), parathyroid-hormone-related-protein (Pthrp), and Runx2. On the other-hand, intramembranous ossification forms bone directly and, as such, requires the coordinate expression of fewer factors, such as type-I collagen, Fgfs (including Fgf-2, -9, and -18), Fgfrs (including Fgfr-1 and -2), Msh Homeobox (Msx)1/2, and Runx2 (136, 137). These differences in the experimental systems could contribute to the differing observations of in vitro gene expression and function compared to what was observed in vivo. It is possible then, that examination of isolated calvarial osteoblastic cells may demonstrate a genotype-mediated difference in differentiation or function.

In contrast to our in vitro osteoblast experiments, examination of the adipocyte lineage demonstrated that expression of the global miR-29 decoy elicits a modest cell autonomous increase in the adipogenic potential of bone marrow cultures. This observation is in agreement to previously reported in vitro studies using miR-29-3p inhibitors in primary adipocytes and cell lines (107-111). Our data is highlighted by an
increase in *Pparγ* mRNA expression in the decoy cultures compared to wild-type. While our healthy global decoy mice did not show any difference in bone marrow adiposity or body composition *in vivo*, these *in vitro* data suggest that if the mice were challenged with a high-fat-diet, there may be increased adiposity in the decoy mice (138, 139).

Using BMM cultures treated with RANKL and MCSF, we demonstrate that global miR-29 decoy expression results in fewer multinucleated TRAP positive osteoclasts, indicating these cells have a reduced osteoclastogenic potential. While *Ocstamp* was unchanged between genotypes, we observed a significant increase in *CathepsinK* expression in the global decoy cultures at day 5 of differentiation, which may seem somewhat paradoxical. However, due to pharmacological doses of MCSF and RANKL in cultures, osteoclasts *in vitro* undergo dynamic changes that result in oscillations of peak osteoclast formation followed by apoptosis (140). The significant increase in *CathepsinK* expression in global decoy cultures at day 5 may suggest that the osteoclast differentiation kinetics are delayed, such that if we extended the culture time past 5 days, we may observe more osteoclasts form in the decoy cultures as the wild-type cells undergo apoptosis. In future experiments, measuring apoptosis markers and extending the culture time may reveal if this is the case (140).

miR-29 inhibition results in decreased osteoclast differentiation *in vitro* (37). However, *in vivo* osteoclast surface and eroded surface were unchanged in miR-29 global decoy mice (Chapter 3, Figure 3.6). Further, we showed an increase in the *Rankl/Opg* mRNA ratio in the calvaria of global decoy mice, an effect driven by reduced *Opg* expression (Chapter 3, Figure 3.7). This suggests a skeletal environment primed for enhanced
osteoclastogenesis in the decoy mice. It is possible that if osteoclastogenesis is impaired in vivo as a result global decoy expression, an increased Rankl/Opg mRNA ratio in osteoblastic cells may be a response to normalize osteoclast balance. To confirm this, the Rankl/Opg mRNA ratio could be measured in RNA harvested from femurs, the same skeletal compartment where the dynamic histomorphometry was measured. Furthermore, a recent study showed that miR-29-3p may target the Rankl 3’-UTR (120).

It would be pertinent to examine the abundance of RANKL protein in osteoblasts derived from the global decoy mice, as Rankl mRNA has already been shown to be unchanged in calvaria (Chapter 3, Figure 3.7).

Moreover, bone marrow adipocytes can also express Rankl and Opg, while other peripheral adipose depots do not (112, 141-143). Bone marrow adipocytes may be an additional source of RANKL that could rescue, or normalize, any inherent deficits of global decoy BMMs to form osteoclasts in vivo. To determine if this may be the case, we could measure the abundance of Rankl mRNA and protein in bone marrow adipocyte cultures in vitro. This could be followed by the use of flow cytometry to determine if global decoy expression alters the abundance of RANKL expressing bone marrow pre-adipocytes in vivo, by gating for Rankl and Preadipocyte-factor-1 (Pref-1) double-positive cells (141).

Using osteoclast cultures, we also measured the abundance of mature miR-29-3p family members, confirming the miR-29-3p family members are upregulated during osteoclast differentiation (Figure 4.3D-F) (37). Expression of the decoy resulted in a decreased abundance of all mature miR-29-3p isoforms. As we discussed in the previous chapter, this may be a result of miR-29-3p degradation caused by interactions of the miR-
29 decoy and RISC loaded miR-29-3p family members. Further, disruption of regulatory loops elicited by the de-repression of critical miR-29 targets may impair expression of transcription factors necessary to drive osteoclast differentiation and/or miR-29-3p expression (144). In either case, these data confirm the inhibitory activity of the miR-29 tough-decoy.

To determine the functional outcome of the decreased miR-29 expression we examined the RNA and protein expression of several miR-29 targets in the osteoclast lineage. Of the targets examined, only Nfia mRNA was significantly altered between genotypes; it was increased in global decoy cells at day 5 of differentiation (Figure 4.3J). NFIA negatively regulates both osteoclast and macrophage differentiation by inhibiting the expression of c-Fms, the MCSF receptor, which is needed for osteoclast precursor survival and proliferation (5, 145, 146). By limiting c-Fms expression, increased abundance of NFIA protein may contribute to the impaired osteoclastogenesis of global decoy BMMs by promoting increased apoptosis of mature osteoclasts or osteoclast precursors. It could also limit the amount of cell-proliferation and/or commitment of precursors to the osteoclast lineage, which could then limit the number of fusion partners available to form mature osteoclasts (147-149).

Using our novel global miR-29-3p global decoy mice, we studied the cell autonomous phenotype of three cell lineages important for skeletal homeostasis: osteoblasts, adipocytes, and osteoclasts. While we did not observe significant changes to osteoblasts in vitro, we did observe modest changes to the adipocyte and osteoclast lineages. To better understand the mechanisms by which miR-29-3p regulates these lineages, these
data strengthen the rationale for using lineage restricted cre-drivers in our future studies. For example, we could use osteocalcin-cre to more specifically study the impact of the miR-29 decoy in the osteoblast lineage (150). To target the adipocyte lineage, we could use adiponectin-cre or Pref-1-cre, which would target all fat depots including bone marrow adipose (142, 151). Prx-1-cre, which primarily targets the mesenchymal progenitors of the forming limb-bud, would result in more targeted expression of the decoy in both osteoblasts and bone marrow adipocytes (152).

For the osteoclast lineage, use of LysM-cre would be appropriate, as it targets the common-myeloid progenitor that gives rise to both macrophages and osteoclasts (153, 154). In fact, others in our group have already begun these studies and preliminary data suggest that the LysM-conditional miR-29 decoy mice display a similar decrease in osteoclast differentiation in vitro, with no change in osteoclast surface in vivo. However, there is a significant decrease in osteoclastic activity in vitro, as well as in vivo, as shown by decreased eroded surface and serum CTX (Dr. Bongjin Shin, unpublished data). Since the global miR-29 decoy animals did not present altered osteoclastic parameters in vivo and showed only a modest impact in vitro, the differing cell-autonomous effect in these two-models is particularly intriguing. It is possible that the effect of the global decoy is somehow being dampened, maybe through epigenetic regulation of the decoy locus, or that the global miR-29 decoy may be impacting a population upstream of the monocyte/macrophage progenitor.
CHAPTER 5
Validation of miR-29-3p Targets in the E-Cadherin Signaling Complex and Within the Macrophage Polarization Pathway

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ABSTRACT
The miR-29-3p family has been shown to positively regulate osteoclastogenesis, although the underlying mechanisms are not well understood. Identification and validation of miR-29-3p targets in the monocytic lineage is key to understanding its mechanisms of action. Since miRNAs often target multiple components of a single pathway, and miR-29 targets Cdc42 and Srgap2 in the Rho GTPase pathway, a candidate gene approach was implemented to explore targets related to Rho GTPase signaling. Through this, we identified novel miR-29-3p targets in the CDH1 signaling complex, including Cdh1, Ctnnb1, and Fmnl2. Furthermore, using the rationale that miR-29-3p targets may be upregulated when miR-29-3p activity is inhibited, we utilized an RNA-seq study of wild-type and global miR-29-3p tough decoy osteoclasts to identify putative miR-29-3p targets amongst the differentially upregulated genes. Of the upregulated genes, we examined eight which had previously been reported to have roles in macrophage polarization, especially M2 polarization, of which, we identified putative miR-29-3p in six genes. Together, our studies expand our understanding of miR-29-3p regulation of
osteoclastogenesis, possibly through modulating lineage commitment and macrophage polarization.

5.1. Introduction

Monocytes are multipotent cells of the hemopoietic lineage that are able to form both bone resorbing osteoclast and immune-supporting macrophages. In response to macrophage colony stimulating factor (MCSF) and receptor activator of NFκB ligand (RANKL), monocytes differentiate into osteoclasts through a series of coordinated steps, including progenitor commitment, pre-osteoclast motility and fusion, mature osteoclast attachment to the bone surface, and secretion of bone resorbing molecules (12, 37, 131, 155). Alternatively, when treated with MCSF and pro-inflammatory cytokines (such as IFNγ, LPS, and TNFα), monocytes will differentiate into pro-inflammatory M1 macrophages (156). However, when stimulated with MCSF and anti-inflammatory cytokines (such as TGFβ, IL-4, IL-10, and IL-13), monocytes will differentiate into anti-inflammatory M2 macrophages (156). Macrophage differentiation and lineage commitment to either M1 or M2 is known as polarization. In addition to cytokines, these processes are further regulated by intrinsic factors, including microRNAs (5, 37, 43, 64, 75, 87).

MicroRNAs (miRNAs; miRs) are small non-coding RNAs, approximately 21-27 bases in length, which act as post-transcriptional regulators of gene expression (83). miRNAs are first transcribed from the genome and, through a series of processing steps, result in a mature miRNA duplex consisting of both -5p and -3p strands. The duplex undergoes strand selection and either the -5p or -3p miRNA strand is incorporated into the
multiprotein RNA-induced-silencing-complex (RISC) (83). As a part of RISC, the mature miRNA guides RISC activity to transcripts containing complementary sequences. Depending on the extent of complementarity between the miRNA and mRNA, the RISC:miRNA:mRNA interaction will result in mRNA de-adenylation and destabilization, cleavage of the interacting miRNA and mRNA, and/or translational repression caused by steric hindrances between RISC and the protein machinery (10, 83).

The outcome and relative efficacy of these interactions is dictated by multiple factors. Most prominent is the degree of complementarity between the miRNA seed sequence, miRNA bases 2-8, and the mRNA target. At a minimum, all 6 nucleotides of the seed sequence should have Watson-Crick base pairs in the mRNA sequence (6mer) (10, 157). However, the presence of either an adenosine in the target mRNA adjacent to miRNA nucleotide 1 (7mer-A1), Watson-Crick pairing at nucleotide 8 (7mer-m8), or a combination of the two (8mer) increase the stability and efficacy of the miRNA:mRNA interactions. Further, complementary binding in the 3’ end of the miRNA can further increase the stability of these interactions, but is not essential for the initial interactions to occur (10, 157). While these basic “rules” describe many functional miRNA-target interactions, there are exceptions to these rules.

Location of the binding site is also thought to play a role in miRNA efficacy. For example, binding sites in the proximal or distal end of the transcript 3’-UTR are more likely to have secondary and tertiary conformations more favorable for RISC binding, whereas binding sites in the middle of the 3’-UTR may be less favorable as they are likely to have complex secondary structures that hinder RISC binding (10). Further, binding sites in the
transcript 5’-UTR, or coding sequence, are likely to conflict with capping proteins and/or ribosomal machinery that would displace RISC. Additionally, a high abundance of local AU nucleotides surrounding the binding site further favors miRNA binding (10, 157).

Accounting for these factors, as well the extent that the miRNA binding site is evolutionarily conserved, prediction algorithms suggest putative miRNA:mRNA target interactions (10). Based on these predictions, it is recognized that a single mRNA transcript may contain multiple miRNA target sequences and, conversely, a single miRNA may be predicted to target hundreds of transcripts (10). However, many of these predicted binding sites do not withstand experimental validation (10, 134, 157). Therefore, experimental validation of predicted miRNA:mRNA interactions is an important step in understanding the biological relevance of these interactions and remains a critical gap in knowledge.

A miRNA of particular interest in the monocyte/macrophage/osteoclast lineage is the miR-29-3p family, consisting of miR-29a-3p, -29b-3p, and -29c-3p (38, 39, 80, 88, 90, 92, 110, 115, 119, 120, 126, 158-161). Our previous work shows that the miR-29-3p family is upregulated during osteoclast differentiation in vitro (37). We demonstrated that the miR-29 family is a positive regulator of osteoclast differentiation and that miR-29 inhibition resulted in less motile osteoclast precursors and fewer mature osteoclasts. Further, we previously identified several novel miR-29-3p targets, including the Rho-GTPase Cdc42 and Srgap2, a negative regulator of Rho-GTPase Rac1, as miR-29 targets. Rho-GTPases are potent regulators of the actin-remodeling necessary for cell motility and fusion (37).

The goal of the present study is to better understand the function of miR-29-3p in
osteoclastogenesis, by validating additional miR-29-3p targets that play a role in osteoclast commitment, differentiation, and/or function. With the understanding that miRNAs tend to regulate multiple targets in the same pathway, we employed a candidate gene approach, using literature searches and bioinformatics, to identify additional miR-29 targets within Cdc42 and Rac1/Srgap2 related pathways. This led us to investigate the components of the CDH1, or E-cadherin, signaling complex, important for the formation of adherens-junctions required for cell adhesion and cell-cell interactions (162-164). CDC42 and RAC1 contribute to this signaling complex by mediating actin-remodeling at these sites (165-169). From this pathway, we validated several novel miR-29 targets, including Cdh1. Additionally, using a list of differentially expressed genes from a previously performed RNA-seq analysis of osteoclasts expressing a miR-29-3p tough decoy or wild-type controls, we validated additional novel miR-29-3p targets that may function in lineage commitment and osteoclast differentiation.

5.2. Methods and Materials

5.2.1. Animals and Cell Lines

All mice were in the C57BL/6 background and were housed in the Center for Comparative Medicine at UConn Health under a 12-h light/dark cycle and were provided with standard mouse chow and water ad libidum. Mice globally expressing a miR-29-3p tough decoy (global miR-29 decoy; global decoy; gDecoy) were generated as previously described in Appendix A and Chapter 3 of this dissertation. Briefly, the miR-29 decoy/fl/fl line was crossed with HPRT-cre expressing mice, resulting in a line of mice with germ-line transmission of the recombined and activated decoy cassette. Mice conditionally
expressing the miR-29 decoy (conditional miR-29 decoy; conditional decoy, cDecoy) were generated by breeding the miR-29 decoy^{fl/fl} line with Lysozyme-M-cre (LysM-cre) expressing mice. LysM-cre is expressed in common-myeloid-progenitor cells, which give rise to all myeloid cells including macrophages and osteoclasts. For all experiments, whole bone marrow was isolated from six-to-ten-week-old wild-type and decoy female littermates. All animal protocols were approved by the Institutional Animal Care Committee at UConn Health.

5.2.2. Osteoclast differentiation, qRT-PCR Analysis, and RNA-seq analysis

Primary murine osteoclast cultures were prepared from bone marrow monocytes (BMMs) isolated from non-adherent bone marrow cells as previously described (132). Briefly, whole bone marrow was flushed from femurs and tibias, passed through a cell strainer, and cultured overnight on tissue culture plates in complete α-MEM (Gibco) [10% heat-inactivated FBS (HyClone) and 1% antibiotic-antimycotic cocktail (Gibco)]. The following day, non-adherent cells were collected and bone marrow monocytes (BMMs) were prepared using Ficoll-Paque (GE Healthcare; Sigma-Aldrich) density gradient centrifugation. BMMs were collected from the Ficoll-Paque:media interface and then rinsed with serum free α-MEM before final resuspension and plating.

For osteoclast differentiation assays, BMMs (1.5x10^6 cells/well in 6 multi-well plate) were cultured in complete α-MEM supplemented with recombinant human MCSF (30 ng/mL) and human RANKL (30 ng/mL). Both were expressed in our laboratory using the
constructs kindly provided by Dr. D. Fremont from Washington University and Dr. Y. Choi from University of Pennsylvania, respectively. Cells were cultured up to 5 days, with a media change at day 3.

For qRT-PCR analysis, cells were lysed directly in TRIzol (Invitrogen). RNA was then extracted as directed by the manufacturer. As previously described, total RNA was quantified, DNase treated (Promega), and subjected to random-hexamer-primed reverse-transcription for Sybr green based quantitative Real Time PCR (qRT-PCR) (85). For Sybr green based qRT-PCR, gene expression was normalized to 18S rRNA. Primer sets are shown in Appendix B, Table B.2. Relative quantities were determined using standard curves and samples were assayed in duplicate.

An RNA-seq study was previously performed in our lab. It utilized osteoclasts cultured from a population of bone marrow cells isolated from wild-type and global miR-29 decoy mice, enriched for osteoclast precursors (B220-CD3-CD11blowCD115+ via FACS), isolated from wild-type and global miR-29 decoy mice (12). These cells were cultured in the presence of MCSF and RANKL for 3 days prior to RNA isolation and analysis by RNA-seq. The library preparation, sequencing, and preliminary data analysis were performed by the UConn Center for Genome Innovation (Storrs, CT) on a fee-for-service basis. Four biological replicates (one mouse for each replicate) were analyzed. These unpublished data were used to select candidate genes for analysis of miR-29-3p regulation.
5.2.3. Luciferase Constructs and Reporter Assay

Gene-specific PCR primers (Appendix B, Table B.3) were designed to amplify genomic DNA fragments containing putative miR-29-3p binding site(s) in the 3’-UTR and/or coding sequence (CDS) of Cdh1, Fmnl2, Ctnnb1, Rab44, Serp1, and Tnfr1a (Table 5.3 and 5.5). Coding region fragments for Ltf (bases 1261-2155), Stab1 (bases 5041-5510), and Msr1 (661-1495) were synthesized as gBlocks by IDT. Using the appropriate restriction enzymes, all fragments were cloned into the pMIR-REPORT™ miRNA Expression Reporter Vector (Thermofisher #AM5795). Deletion mutants of selected constructs were made using standard PCR techniques. The final constructs were verified by Sanger sequencing.

Using BioT transfection reagent (1.5 µL/µg DNA) (Bioland Scientific), RAW264.7 cells (ATCC) were co-transfected with the luciferase construct, a constitutive β-galactosidase construct used for transfection control (Clontech #631719), and 50 nM miR-29b-3p inhibitor or non-targeting control #1 (Dharmacon). Six hours post-transfection, cells were treated with 30 ng/mL RANKL in complete DMEM (Gibco) [10% heat-inactivated FBS (Hyclone) and 1% antibiotic-antimycotic cocktail (Gibco)]. After 48 hours, following manufacturers’ instructions, luciferase activity was assessed using the Luciferase Assay System (Promega) and was normalized to β-galactosidase activity using Galacton-Plus™ Substrate (Invitrogen). Each luciferase experiment contained 6 biological replicates and was performed at least 3 times.
5.2.4. Western Blotting

Primary BMMs were differentiated into osteoclasts in 6 multi-well plates and were harvested in protein lysis buffer (10% glycerol, 1% Triton-X 100, 150 mM NaCl, 20 mM HEPES, and 1X Halt protease and phosphatase inhibitor cocktail (ThermoFisher)). After a freeze-thaw cycle, protein content was quantified using a BCA kit (ThermoFisher). 15 µg of protein lysate was denatured and loaded on a 10% SDS-PAGE gel, subjected to electrophoresis, and transferred to PVDF membrane. Membranes were then blocked in 5% BSA in 0.1% TBST for 1 hour prior to incubation with primary antibody. The following antibodies were used for Western blotting: mouse anti-CTNNB1 (Millipore); mouse anti-CDH1 and mouse anti-NFATc1 (BD Biosciences); rabbit anti-c-SRC, rabbit anti-GAPDH, rabbit anti-CDC42, and HRP-conjugated goat anti-Rabbit (Cell Signaling Technology); Rabbit polyclonal anti-SRGAP2 antibody generated in-house by Dr. Franck Polleux was previously described (133). Complete antibody product information is available in Appendix B, Table B.1.

5.2.5. Statistics

Data for qRT-PCR experiments and luciferase assays are shown as mean ± SEM. Statistical significance was determined using one-way analysis of variance (ANOVA) with Student-Newman-Keuls Multiple Comparison post hoc test or Student's t test as appropriate (KaleidaGraph, Synergy Software, Reading, PA). For data analyzed by ANOVA, only the relevant comparisons were indicated.
5.3. Results

5.3.1. Identification of miR-29-3p targets in the CDH1 signaling complex

Previously, we identified the Rho-GTPase CDC42 and SRGAP2, a negative regulator of Rho-GTPase RAC1, as miR-29-3p targets (37). By examining known interactomes involving these Rho-GTPases for potential miR-29-3p targets, the CDH1 signaling complex was identified (Figure 5.1A) (165-169). Cadherins are transmembrane proteins that mediate strong cell-cell interactions through homophilic and heterophilic binding of cadherins on adjacent cells (162-164). CDH1 is the most abundant cadherin in pre-osteoclasts and, by mediating the cell-cell interactions which precede pre-osteoclast fusion, it is essential for osteoclast differentiation (170, 171).

At the cell membrane, the CDH1 signaling complex consists of several key components including CTNNA1, CTNNB1, and CTNND1 (α-, β-, and δ-catenin), Rho-GTPases (including CDC42 and RAC1), Rho-GTPase regulatory proteins (such as SRGAP2), and actin remodeling proteins including FMNL2 (formin-like 2). Using bioinformatics tools (DIANA, Tarbase, miRanda, and RNAhybrid (103, 104, 172-178)) we analyzed the mRNA sequences of these complex members for miR-29-3p binding sites, finding several possible candidates in Cdh1, Ctnnb1, and Fmnl2 (Table 5.1). Ctnnd1 (p120-catenin; delta-catenin), was also observed to contain putative miR-29-3p binding sites (data not shown), but we chose not to pursue it as this time.
Figure 5.1. Cartoon representation of the CDH1 signaling complex and murine Cdh1 3' UTR isoforms and miRNA binding sites. (A) The key components of the complex include CDH1, CTNNA1, CTNNB1, and CTNND1 (α-, β-, and δ-catenin), Rho-GTPases (including CDC42 and RAC1), Rho-GTPase regulatory proteins (such as SRGAP2), and actin remodeling proteins like FMNL2. Upon binding a cadherin family member on an adjacent cell, CDH1 undergoes a conformational change that recruits the catenin family members, which in turn recruit Rho-GTPase regulatory proteins. The activation of Rho-GTPases allows for the recruitment of actin remodeling proteins, such as FMNL2. The actin remodeling proteins localize actin filament assembly, stiffening the cell membrane, thus strengthening and prolonging the cell-cell interactions. (B) Alternative Cdh1 3' UTRs and known miRNA binding sites. The miRNAs shown are known to both target Cdh1 and be important for osteoclast differentiation. The first base of each miRNA binding site is noted.
Table 5.1. RNA Hybrid alignments of predicted miR-29-3p binding sites in Cdh1 signaling members. The mRNA reference sequences for Cdh1 complex member transcripts were first analyzed for predicted miR-29-3p binding sites using the miRanda prediction algorithm available at microRNA.org. If a conserved putative binding site was found, the site was cross-referenced with predicted miR-29-3p binding sites generated by RNA Hybrid. If present in both, the site was targeted for future luciferase experiments. Below are the RNA Hybrid alignments for transcripts that met these criteria. Also shown, are the mRNA start position based on the RefSeq and the predicted MFE.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Start Position</th>
<th>miR-29-3p Seed Binding Alignments</th>
<th>MFEa (kcal/mol)</th>
</tr>
</thead>
</table>
| Cdh1 (NM_009864.3) | 3804 (MBS1b) | target 5' U C UU A C 3'
|              |                | miRNA 3' U U UAA U 5' | -19.5 |
| Cdh1 (NM_009864.3) | 4308 (MBS2b) | target 5' U GUG UGUGUGUG A 3'
|              |                | miRNA 3' UUGU AAA UUA 5' | -17.6 |
| Ctnnb1 (NM_007614.3) | 3167 | target 5' U U U A 3'
|              |                | miRNA 3' UAAAGU 5' | -22.1 |
| Fmnl2 (NM_172409.2) | 4859 | target 5' A UU CUAUA U A 3'
|              |                | miRNA 3' UUAAG U 5' | -17.0 |

a Minimum free energy, measurement of the energetic stability of the predicted miRNA:mRNA interaction.
b MBS: miR-29-3p binding site present in luciferase assays shown in Figure 5.2.

5.3.2. Validating newly identified miR-29-3p targets in CDH1 complex members

To validate the predicted miR-29-3p-target interactions (Figure 5.2A, Table 5.1), we generated 3'-UTR luciferase reporter constructs for Cdh1, Ctnnb1, and Fmnl2 (Figure 5.2B-D). For Cdh1, there are 3 reported isoforms (2 coding; 1 non-coding; ENSMUSG00000000303) (179). While the coding transcripts generate identical proteins,
alternative splicing results in one isoform having a substantially smaller 3'-UTR (432 vs 1631 bases), containing fewer potential regulatory elements, including miRNA binding sites (Figure 5.1B). For our studies, we cloned the longer of the two splice variants.

For the luciferase assay, RAW264.7 cells were transiently transfected with the luciferase plasmids, a constitutively expressed beta-galactosidase control plasmid, and either a miR-29b-3p inhibitor or non-targeting control. The miR-29 inhibitor significantly increased the luciferase activity for the Cdh1 (Figure 5.2B), Fmnl2 (Figure 5.2C), and Ctnnb1 3'-UTR (Figure 5.2D) luciferase constructs compared to the non-targeting control. This derepression of the 3'-UTR constructs by miR-29b-3p inhibitors indicates they contain one or more miR-29-3p binding sites.

To verify the activity of the individual predicted miR-29-3p binding site(s) (Figure 5.2A, Table 5.1), we generated deletion mutants for each site (Figure 5.2E-G). Since the Cdh1 3'-UTR construct had two predicted targets, we generated single deletion mutants for each site, as well as a compound mutant in which both sites were deleted. When the first miR-29-3p binding site (Site 1) was deleted from the Cdh1 3'-UTR construct, derepression mediated by miR-29b-3p inhibitor was modestly, yet significantly reduced compared to the parent construct (Figure 5.2E). This effect was more pronounced by independent deletion of miR-29-3p binding Site 2. When the compound deletion mutant (deletion of both Sites 1 and 2) was tested, the ability of the miR-29b-3p inhibitor to relieve repression of luciferase activity was similar to that of the Site 2 construct. This suggests that Site 2 is strongly targeted by miR-29-3p, while Site 1 may be a weaker target. Further, since effect of the miR-29 inhibitor wasn’t completely ablated, these data suggest there
Deletion of the predicted miR-29-3p binding site in the Ctnnb1 3'-UTR luciferase construct significantly decreased the ability of the miR-29-3p inhibitor to derepress luciferase activity, demonstrating the activity of the miR-29-3p binding site (Figure 5.2F).
In contrast, deletion of the predicted miR-29 binding site from the *Fmnl2* 3'-UTR luciferase construct did not alter the responsiveness of the construct to the miR-29 inhibitor (Figure 5.2G). This suggests that, while *Fmnl2* is a miR-29 target, the predicted miR-29 binding site we deleted is not legitimate and other potential binding site(s) remain to be identified.

**Figure 5.3.** mRNA and protein expression of novel miR-29-3p targets during osteoclastogenesis in osteoclast cultures derived from global miR-29 decoy animals. (A-B) mRNA expression of novel miR-29-3p target *Fmnl2* (A) and *Cdh1* (B) in wild-type and heterozygous global miR-29 decoy osteoclast cultures at days 1, 3, and 5 of osteoclastogenesis; mean ± SEM; n=4 for both genotypes. (C) *Cdh1* mRNA in wild-type and homozygous global miR-29 decoy osteoclast cultures at days 1, 3, and 5 of osteoclastogenesis; mean ± SEM; n=6 for both genotypes. (D) Representative Western blot of CDH1 (~120 kDa), CTNNB1 (~75-100 kDa), and GAPDH (37 kDa) in wild-type and homozygous global miR-29 decoy osteoclast cultures at days 2-5 of osteoclast differentiation. The ratio of CDH1:GAPDH and CTNNB1:GAPDH densitometry is shown.

5.3.3. Expression of *Cdh1* complex members in the global-decoy osteoclasts

Luciferase assays allow us to confirm if a particular transcript is targeted by miR-29-
3p. However, to determine the impact of miR-29-3p regulation in a biological context, we performed preliminary studies examining the RNA and/or protein expression of these newly validated miR-29-3p targets in BMMs derived from wild-type and global miR-29-3p decoy mice and cultured in the presence of MCSF and RANKL up to 5 days (Figure 5.3). In wild-type cultures, Fmnl2 mRNA (Figure 5.3A) expression was stable between days 1 and 3 of osteoclast culture, but was significantly decreased by day 5. In contrast, Fmnl2 expression was significantly higher in the global decoy heterozygous cultures compared to the wild-type at day 1 of culture, but was then significantly lower in the later time points. Since Fmnl2 is miR-29-3p target, its increased mRNA levels at day 1 may suggest that expression of the decoy relieved miR-29-3p repression of Fmnl2 mRNA in pre-osteoclastic cells. In later time points then, when miR-29-3p inhibition is known to impair osteoclast formation, de-repression of other miR-29-3p targets that impair osteoclast formation may also have impaired the sustained expression of Fmnl2. Given this experiment was completed using osteoclasts derived from heterozygous mice, it is also possible that, as miR-29-3p levels increased with osteoclast differentiation, the level of decoy expression may not have been sufficient to maintain suppression of miR-29-3p activity. We also attempted to examine FMNL2 protein in these cells, but due to antibody quality, we were unsuccessful.

We then examined Cdh1 mRNA expression in wild-type cells and compared it to both global decoy heterozygous and homozygous cultures (Figure 5.3B, C). In wild-type cells, Cdh1 mRNA was at its highest at the start of differentiation and steadily decreased as differentiation progressed. In the decoy cells, we observed that Cdh1 mRNA expression was significantly lower in homozygous decoy cells (Figure 5.3C) at day 1 of culture.
compared to wild-type, whereas it only trended towards a decrease in heterozygous decoy cells (Figure 5.3B). Beyond that, expression of the global decoy did not dramatically alter Cdh1 mRNA expression during osteoclast differentiation.

Unlike Cdh1 mRNA which quickly decreases with osteoclast differentiation (Figure 5.3B, C), Western blot analysis demonstrates that CDH1 protein levels persist in the cell,
even as Cdh1 mRNA levels are substantially decreased (Figure 5.3D). It is known in the literature that CDH1 is a relatively stable protein which is recycled through endocytosis, allowing one protein molecule to go through multiple signaling events (178, 180, 181). In wild-type cultures, we observed that CDH1 protein expression was relatively stable days 2 through 4 of differentiation, a time when pre-osteoclasts and maturing osteoclasts are undergoing rapid fusion. Around day 5 of differentiation, CDH1 protein levels decreased somewhat. This decrease coincides with the decreased need for fusion in mature osteoclasts, as well as increasing cell death, as the mature osteoclasts undergo apoptosis in culture. However, in homozygous decoy cells, CDH1 protein decreased soon after differentiation day 2 (Figure 5.3D, middle panel). The Cdh1 mRNA and CDH1 protein data demonstrate that homozygous expression of the miR-29-3p global decoy appeared to have a negative effect on expression, especially at the protein level. This is contrary to what was anticipated and could represent miR-29-3p regulation of other RNAs impacting CDH1 levels.

Using homozygous global decoy osteoclast cultures, we also examined the protein expression of CTNNB1, another novel miR-29-3p target (Figure 5.3D, bottom panel). CTNNB1 protein levels were relatively constant in wild-type cells through day 4 of differentiation, then appeared to decrease at day 5. In homozygous global decoy cells, CTNNB1 was highest at day 2 of differentiation, then gradually tapered off during differentiation. Similar to what was observed with Fmnl2 mRNA, these CTNNB1 protein data suggest that expression of the decoy may relieve miR-29-3p repression of Ctnnb1 mRNA in pre-osteoclasts, resulting in the observed increase in CTNNB1 protein. More studies are needed to confirm these observations.
5.3.4. Identification of miR-29-3p targets from mRNAs significantly increased in miR-29-3p decoy osteoclasts

In an effort to better understand the function of miR-29-3p in osteoclastogenesis, we had previously performed an RNA-seq study of an enriched population of osteoclast precursors derived from either wild-type or homozygous global decoy mice, cultured with MCSF and RANKL for 3 days. Comparing the gene expression between the two

![Figure 5.5. RNA-seq dataset is enriched for several genes involved with macrophage differentiation and polarization. From the RNA-seq dataset, we selected 8 genes for further analysis. These included Msr1 (A), Stab1 (B), Chil3 (C), Ltf (D), Serpine1 (E), Rab44 (F), Thbs1 (G), and Tnfrsf1a (H). n=4 for both genotypes; midline represents the mean; RPKM: Reads Per Kilobase of transcript, per Million mapped reads. Data provided by Dr. SK Lee.](image)
genotypes, using a false discovery adjusted p-value of 0.05 as a cutoff, 60 genes were found to be differentially expressed. Of the previously identified miR-29-3p targets (37), only \texttt{Cd93} (Figure 5.4A) and calcitonin receptor (\texttt{Calcr}; Figure 5.4B) were represented within this differentially expressed gene set; \texttt{Cd93} was significantly increased in the global decoy cells, while \texttt{Calcr} was significantly decreased. \texttt{Cd93} is macrophage receptor that aides in the adhesion and removal of apoptotic cells (182), a feature similar to that of M2 anti-inflammatory macrophages. Increased \texttt{Cd93} could suggest a possible increase in monocyte commitment to the macrophage lineage, as we previously hypothesized (37). Further, up-regulation of \texttt{Cd93} in miR-29-3p decoy cells suggests that it is a biologically relevant miR-29 target.

Using the RNA-seq dataset, we also analyzed the expression of other previously and newly identified miR-29-3p targets (Figure 5.4C-I). We first examined the other previously reported miR-29-3p targets, including \texttt{Nfia} (Figure 5.4C), \texttt{Gpr85} (Figure 5.4D), \texttt{Cdc42} (Figure 5.4E), and \texttt{Srgap2} (Figure 5.4F), the expression of which was not significantly different between genotypes. We also examined the newly identified targets in the CDH1 signaling complex, \texttt{Cdhh} (Figure 5.4G), \texttt{Ctnnb1} (Figure 5.4H), and \texttt{Fmnl2} (Figure 5.4I). Similar to our other analyses using qRT-PCR and Western blot (Figure 5.3), the expression of these three targets was unchanged between genotypes in the RNA-seq dataset.

With the rationale that the pool of significantly increased RNAs in miR-29 decoy osteoclasts may be enriched with miR-29-3p targets, we assembled a list of genes that were upregulated in the miR-29 decoy osteoclasts and are known to promote
macrophage differentiation and/or inhibit osteoclast differentiation and/or function. In a subset of genes of interest, RNA Hybrid was used to screen the UTRs and coding sequences (CDS) for potential miR-29-3p binding sites. Genes with the potential to interact with miR-29-3p isoforms included: macrophage scavenger receptor-1 (Msr1, Figure 5.5A), stabilin-1 (Stab1, Figure 5.5B), chitinase-like-3 (Chil3, Figure 5.5C), lactotransferrin (Ltf, Figure 5.5D), serpin-peptidase-inhibitor, clade E, member 1 (Serpine1).

Table 5.2. Cellular function of the 8 selected target genes.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Aliases</th>
<th>Gene Function in Macrophages and Osteoclasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage scavenger receptor 1, class A scavenger Receptor</td>
<td>Msr1; SR-A1; SCARA1</td>
<td>Promotes M2 macrophage polarization. Positive regulator of early osteoclastogenesis; KO mice display increased bone volume.</td>
</tr>
<tr>
<td>Fasciclin Egf-Like, Laminin-Type Egf-Like, And Link Domain-Containing Scavenger Receptor-1</td>
<td>Stabilin 1; Stab1; FEEL-1; MS-1 Antigen</td>
<td>Promotes M2 macrophage polarization. Osteoclasts from knock-out animals display increased activity in vitro, but there is no in vivo phenotype when knocked-out.</td>
</tr>
<tr>
<td>Chitinase-like-3</td>
<td>Chil3; Chi3l3; Ym1</td>
<td>Marker of M2 polarized macrophage. No studies have directly examined the role of Chil3 in osteoclasts.</td>
</tr>
<tr>
<td>Lactotransferrin</td>
<td>Ltf; Lactoferrin</td>
<td>Promotes the transition from M1 to M2 macrophages. Inhibits osteoclastogenesis, but has no impact on mature osteoclast function.</td>
</tr>
<tr>
<td>Serpin peptidase inhibitor, clade E, member 1</td>
<td>Serpine1; Serpin Family E Member 1; Plasminogen activator inhibitor-1; PAI1</td>
<td>Promotes M2 macrophage migration and polarization. Negatively regulates osteoclast differentiation</td>
</tr>
<tr>
<td>RAB44, Member RAS Oncogene Family</td>
<td>Rab44; Ras-Related Protein Rab-44; RAS-Like, Family 13</td>
<td>No studies have examined its role in the macrophage lineage. Negatively regulates osteoclast differentiation.</td>
</tr>
<tr>
<td>Thrombospondin 1</td>
<td>Thbs1; Tsp1; Glycoprotein G</td>
<td>Promotes M1 macrophage polarization when it binds to CD36, which results in TNFα production. Positive regulator of osteoclastogenesis</td>
</tr>
<tr>
<td>TNF Receptor Superfamily Member 1A</td>
<td>Tnfr1a; Tnfrsf1a; Tnfr1; TNF-R1</td>
<td>Marker and promoter of M1 macrophage polarization. Enhances osteoclast differentiation</td>
</tr>
</tbody>
</table>
Serpine-1, Serp1, Ras-Related-Protein-Rab-44 (Rab44, Figure 5.5F), and thrombospondin-1 (Thbs1, Figure 5.5G). Detailed descriptions of these genes can be found in Table 5.2, and potential miR-29 binding sites are shown in Table 5.3. Despite not being significantly different between genotypes, we included TNF-receptor-superfamily-1-alpha (Tnfrsf1a; Tnfr1, Figure 5.5H) for further analysis, as it was shown to be a miR-29-3p target in human cells. Further, we observed significant increases in

Figure 5.6. Luciferase reporter assays of putative miR-29-3p targets involved with macrophage differentiation and polarization identified by RNA-seq. Genes that were differentially expressed in the RNA-seq experiment and observed to have putative miR-29 binding sites (MBS) were selected for luciferase assays. (A) These schematics show the transcript structure, including CDS, 5'- and 3'-UTRs, the cloned regions of interest as denoted by the paired dotted lines, and the relative location of the putative miR-29-3p binding site(s) (MBS) in those regions. Of the identified regions, we have been able to test the Ltf gene-block (B), Stab1 gene-block (C), Rab44 3'-UTR (D) and Tnfr1a 3'-UTR (E); mean ± SEM; n=5-6. The other regions of interest shown in Table 5.6 will be studied in future experiments.
TNFR1a protein in osteoclasts derived from LysM-cre conditional miR-29-3p decoy mice (not shown).

5.3.5. Luciferase validation of newly identified miR-29 targets

Of the candidate genes selected for further investigation (Figure 5.6A, B), we chose to initially clone and analyze a subset of the regions of interest including, the Ltf CDS (Figure 5.6C), Stab1 CDS (Figure 5.6D), Rab44 3’-UTR (Figure 5.6E), and Tnfr1a 3’-UTR (Figure 5.6F). Luciferase-3’ UTR assays showed that introduction of the miR-29-3p inhibitor significantly increased the luciferase activity compared to the non-targeting controls for each construct. This demonstrates that these genes, which the RNA-seq showed to be significantly upregulated in global decoy osteoclasts compared to wild-type cells, are legitimate miR-29-3p targets. In addition to these genes and regions, we plan to analyze the other identified regions in future experiments (Table 5.3, Figure 5.6A).

Table 5.3. RNA Hybrid alignments of predicted miR-29-3p binding sites in genes were identified through RNA seq analysis of wild-type and global decoy osteoclasts.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Start Position</th>
<th>miR-29-3p Seed Binding Alignments</th>
<th>MFE a (kcal/mol)</th>
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5.3.6. qRT-PCR validation of newly validated miR-29-3p targets and other macrophage related genes.

To validate the changes in gene expression observed in the RNA-seq dataset, wild-type and LysM-cre conditional decoy BMMs were treated with MCSF and RANKL for 3 and 4 days to induce osteoclast differentiation. Using qRT-PCR, we first analyzed the expression of the two newly identified miR-29-3p targets, Ltf and Stab1 (Figure 5.7A and B). Expression did not differ between genotypes at both day 3 and 4 of osteoclastogenesis, and largely did not change between time points. Thbs1 and Chil3, macrophage specific genes differentially expressed in the RNA-seq dataset, were similarly unchanged between genotypes at both time points (Figure 5.7C and D), although, Chil3 expression did trend upwards between timepoints. Msr1 expression was also unaffected by decoy expression, however Msr1 expression was more dynamic than
the other genes analyzed, in that, Msr1 expression significantly increased between timepoints in both genotypes (Figure 5.7E).

5.4. Discussion

The goal of the present study was to identify novel miR-29-3p targets, in order to better understand the mechanisms by which this miRNA family regulates osteoclastogenesis. To accomplish this, we first used known miR-29-3p targets, Cdc42 and Srgap2, to employ a candidate gene approach to identify their associated signaling pathways and potential miR-29-3p targets among the signaling components. Through
this, we validated \textit{Cdh1}, \textit{Ctnnb1}, and \textit{Fmnl2} to have miR-29-3p targets within their respective 3'-UTRs.

Moreover, using RNA-hybrid analysis, we identified putative miR-29-3p binding sites in \textit{Msr1}, \textit{Ltf}, \textit{Stab1}, \textit{Rabb44}, \textit{Serpine-1}, and \textit{Tnfr1a}, of which, we validated \textit{Ltf}, \textit{Stab1}, \textit{Rab44}, and \textit{Tnfr1a} to be miR-29-3p targets. Using qRT-PCR analysis, we examined the RNA expression of a subset of the genes upregulated in the RNA-seq data in osteoclasts derived from LysM-conditional miR-29 decoy mice. However, none of the examined genes were significantly different between genotypes.

As previously discussed, a single miRNA can target hundreds of transcripts (10). Frequently, those targets are found within shared signaling pathways (87). Multiple strategies exist for identifying novel miRNA targets and their associated pathways, including a candidate gene approach and RNA-seq analyses using specific miRNA knock-down or deletion, both of which we used in this study. Each come advantages and disadvantages. The benefit of the candidate gene approach is that it allows one to expand upon prior findings and directly investigate pathways of interest associated with a known miRNA target. One drawback is that this approach is more susceptible to selection bias when identifying potential targets, such that pertinent pathways and targets may be missed. On the other-hand, RNA-seq analysis is less susceptible to selection bias as it broadly evaluates changes in gene expression. Using these data, one may further identify common themes and pathways among the genes differentially expressed in response to miRNA over-expression or knockdown, allowing for less-biased decisions to be made about which targets to pursue. The drawback, however, is that it may be difficult to clearly
interpret the data and develop a cohesive picture of what pathways are being regulated and the known and/or novel miRNA targets in those pathways. By employing both methods, we expanded upon our previous findings and identified new targets and pathways to explore in the future (Figure 5.8).

The genes chosen for further examination from the RNA-seq data set have varied roles in both osteoclast differentiation and macrophage polarization. For example, Msr1 has been associated with the positive regulation of macrophage-derived foam cell

![Figure 5.8. Working Model of miR-29-3p Regulation of Macrophage Polarization and Osteoclastogenesis. Monocytes are multipotent cells of the hemopoietic lineage. MCSF treatment promotes monocyte survival and proliferation, priming them for further differentiation. When treated with RANKL and/or TNFα, expanded monocytes will form large, multinucleated, bone-resorbing osteoclasts (red). Alternatively, when treated with pro-inflammatory cytokines (such as IFNγ, LPS, and TNFα), monocytes will differentiate into pro-inflammatory M1 macrophages (orange). However, when stimulated with anti-inflammatory cytokines (such as TGFB, IL-4, IL-10, and IL-13), monocytes will differentiate into anti-inflammatory M2 macrophages (yellow). miR-29-3p fine-tunes these processes by regulating the underlined validated miR-29-3p targets. Regulation of the other putative miR-29-3p targets shown may further contribute to this process, but these need to be empirically tested before a determination can be made.](image)
differentiation. Foam cells are specialized macrophages typically localized to fatty deposits on blood vessel walls, where they ingest modified low-density lipoproteins. This results in the formation of lipid droplets similar to adipocytes, giving them a “foamy” appearance. MSR1 is one of several scavenger receptors that binds and promote the phagocytosis of modified (i.e. hydrolyzed, oxidized, and/or acetylated) lipoproteins. Foam cells also produce molecules which promote plaque growth in the arterial wall; their death increases local inflammation, further contributing to cardiovascular disease (183, 184).

In addition to binding modified low-density lipoproteins, Msr1 expressing macrophages are also linked more generally to immunological tolerance and maintenance. While macrophages phagocytose apoptotic cellular debris, Msr1 is thought to target and sequester hydrolyzed and oxidized auto-antigens. In turn, this contributes to immune tolerance by limiting the amount of available autoantigen to be presented to B cells by antigen presenting cells, such as dendritic cells (185). This immune tolerance effect is also supported by evidence that Msr1 expression is important for maintaining M2 macrophage polarization through the PI3K/AKT signaling pathway. M2 macrophages express anti-inflammatory molecules and promote tissue repair, while M1 macrophages express pro-inflammatory molecules and promote tissue damage (186).

In the osteoclast lineage, others have shown that Msr1 is expressed by osteoclast precursors and pre-osteoclasts during early osteoclastogenesis, but is not detectable in mature osteoclasts (187). Furthermore, Msr1-null mice show increased bone volume and bone density, as a result of decreased osteoclast number and erosion in vivo (187, 188), indicating Msr1 is a negative regulator of osteoclastogenesis.
Stab1 is another scavenger receptor that plays a diverse role in many cell processes, including clearance of apoptotic cells and cell fusion (189). In polarized anti-inflammatory M2 macrophages, Stab1 has been shown to mediate macrophage phagocytosis and clearance of apoptotic cells through the recognition and binding of plasma membrane bound phosphatidylserine on apoptotic cells, an indicator of approaching cell death (189, 190). Stab1 inhibition in M2-macrophages markedly decreases their ability to remove apoptotic cells. In addition to mediating phagocytosis, Stab1 mediates the endocytosis of modified lipoproteins, similar to Msr1, as well as the common regulator of tissue remodeling, osteonectin/SPARC (190). Further, Stab1 inhibition induces the expression of pro-inflammatory cytokines in the typically anti-inflammatory M2 macrophages (189).

Like the other scavenger receptor Msr1, Stab1 is detectable in osteoclast precursors and pre-osteoclast, but decreases as differentiation progresses (191). When Stab1 is globally knocked-out, there is no impact on osteoclast differentiation in vitro, but there is a significant increase in osteoclast activity. Despite this, no skeletal changes have been observed in global Stab1-null mice (191).

Chil3, also known as Ym1, was first recognized in mice for its ability to bind chitin; it has also been shown to bind heparin. In mice, Chil3 is used as a marker for anti-inflammatory M2 macrophages (192). It is considered an M2 marker as the anti-inflammatory cytokines, IL-4 and IL-13, upregulate its expression through the IL-4-receptor and Stat6, two additional markers of M2 macrophages (193, 194). However, Chil3 is not expressed in humans and the closest homolog, ECF-L, is not regulated by IL-4 in human M2 macrophages (194). Further, only a limited number of studies have
examined the indirect or paracrine effect of Chil3 on osteoclastogenesis (195, 196); to our knowledge direct examination of the expression and role of Chil3 in osteoclast differentiation and function has not been reported.

Lactotransferrin (Ltf), also commonly referred to as lactoferrin, is a multifunctional iron binding transport protein with strong antimicrobial and immunomodulatory activity (197). Although the mechanism is still not understood, Ltf treatment has an anti-inflammatory effect in various in vitro models by reducing expression of pro-inflammatory cytokines, such as IL-6 and IL-1β, in macrophages (197). Additionally, in vitro studies using the Thp1 macrophage cell line, have shown that Ltf treatment is capable of promoting macrophage transition from pro-inflammatory M1 macrophages towards the anti-inflammatory M2 macrophages (198).

Interestingly, in the skeleton Ltf has contrasting roles in the differentiation and function of osteoclasts and osteoblasts (199-201). By binding the Ltf receptor, lipoprotein-receptor-protein-1 (LRP1), LTF promotes osteoblast survival, proliferation, and differentiation in both primary osteoblast cultures and in vivo. In the osteoclast lineage, however, LTF inhibits osteoclast differentiation without impacting osteoclast activity (199-201). The mechanism of this inhibition in the osteoclast lineage is unclear, as the addition of an LRP1 inhibitor to osteoclast cultures did not prevent LTF mediated inhibition of osteoclast differentiation (199-201), suggesting that LTF may have additional receptors.

Serpine-1, also widely known Plasminogen activator inhibitor-1, is a serine protease inhibitor. It has diverse roles, including blood clot degradation (202), regulation of cell adhesion, spreading, migration (203, 204), and regulation of odontoblast differentiation.
While not a marker of macrophage differentiation, it has been shown to have a pro-tumorigenic effect through the recruitment and polarization of M2 anti-inflammatory macrophages. By limiting, the inflammatory response, it is thought that M2 macrophages allow tumors cells to persist (206).

In the osteoclast lineage, Serpine-1 is reported to be a negative regulator of osteoclastogenesis. Cells from Serpine-1 global knock-out mice display significantly upregulated osteoclastogenesis in vitro (207). Further, Serpine-1 knockout increased RANKL expression in primary osteoblast cultures, resulting in an increased RANKL:OPG ratio, which is indicative of a pro-osteoclastic environment (207).

Rab44 is a member of the Rab GTPase family, and like other Rab GTPases, it helps regulate intracellular membrane trafficking (208). Rab44 is considered to be an atypical Rab family member, as it is one of few “large” Rab-GTPases, containing additional domains and motifs not found in Rab GTPases 1-43 (209). While its existence has been known for some time, it is a relatively new gene of interest in osteoclast biology (210) and its role has yet to be explored in the macrophage lineage. However, it has been reported that Rab44 knock-down promotes osteoclast differentiation in both primary osteoclast cultures and the RAW264.7 mouse monocytic cell line. Additionally, Rab44 knock-down enhanced primary osteoclast function in vitro. In contrast, Rab44 overexpression nearly ablates the osteoclastic differentiation of RAW264.7 cells (210).

Thbs1 is a heparin binding glycoprotein which mediates cell-cell interactions through the binding of one of several receptors, including CD36 and CD47. Both Thbs1 and its receptors are co-expressed in many cells, including macrophages. In contrast to the
previous genes which promote M2 macrophage polarization, THBS1 binding to the CD36 receptor promotes M1 macrophage polarization, results in increased expression and secretion of TNF-α, and further promotes a pro-inflammatory environment \( (211, 212) \).

As in macrophages, \( Thbs1 \) is expressed by osteoclasts \( (16, 213) \). \( Thbs1 \)-null mice show increased bone mass and cortical thickness, which results from impaired osteoclast differentiation and function \( (213) \). Our understanding of the exact mechanism of \( Thbs1 \) regulation of osteoclasts is incomplete, but it has been suggested that \( Thbs1 \) is a positive regulator of osteoclastogenesis as the THBS1:CD47 interaction may promote osteoclast fusion, while THBS1:CD36 interactions promote osteoclastic bone resorption. Further, there is evidence that THBS1 acts as a paracrine factor, as bone forming osteoblasts deposit THBS1 into the forming bone matrix, where is later released by osteoclasts during bone resorption \( (213) \).

TNFα is a pro-inflammatory antiviral cytokine that induces apoptosis upon binding the TNF receptor. \( Tnfr1a \), which is ubiquitously expressed, must be bound in order for apoptosis to be efficiently induced through its cytosolic death domain \( (214, 215) \). \( Tnfr1b \) expression is more restricted to immune cells and some endothelial cells, such as lung endothelium \( (216) \). TNFR1b does not harbor a death domain, so TNFR1b signaling does not induce apoptosis, but rather induces a TNFα specific signaling cascade, including NFκB activation \( (214) \). As would be expected, TNFα signaling promotes pro-inflammatory M1 macrophage polarization, resulting in TNFα expression and further autocrine and paracrine signaling \( (217, 218) \).

In the case of osteoclast differentiation, there is a consensus that TNFα signaling
enhances osteoclastogenesis both in vivo and in vitro, however, when TNFα needs to be introduced in order to consistently promote this effect is uncertain (219). Nevertheless, this effect is mediated more prominently through TNFR1α rather than TNFR1β (219). This stimulatory role of TNFα in osteoclastogenesis is consistent with the skeletal manifestations of several diseases, including the inflammatory osteolysis seen in rheumatoid arthritis (218, 220). Indeed, a common treatment for these disorders are monoclonal anti-TNFα antibodies, such as adalimumab (Humira), infliximab (Remicade), and golimumab (Simponi) (218, 220).

When we tried to independently validate the expression of a subset of the differentially expressed genes identified in the RNA-seq study by qRT-PCR, we observed no differences between genotypes. However, there are experimental differences that could contribute to these discrepancies. Specifically, the cell source and preparation of osteoclast precursors for each experimental model differed. In the RNA-sequencing experiment, FAC sorting was used to isolate a more homogenous population of osteoclast precursor cells from the whole bone marrow of wild-type and global decoy mice. This population of cells is known to efficiently differentiate into osteoclasts (12). In the qRT-PCR experiments, we used BMMs isolated from the bone marrow of conditional decoy mice. BMMs are a heterogeneous preparation consisting of an amalgam of cells coming from multiple lineages (B-cell, T-cell, macrophage, dendritic cell, granulocytes, etc), at different levels of differentiation and activation. Some, but not all, of these lineages are able to differentiate into osteoclasts, but at a much lower efficiency due to the heterogeneity (12). Additionally, the difference in animal models may also impact the direct comparison of the RNA-seq data with qRT-PCR data. The RNA-seq data were
acquired using cells from global miR-29-3p decoy mice, whereas some of the validation work was performed using cells from the LysM-Cre conditional decoy mice.

We also examined the RNA and protein expression of the newly identified miR-29-3p targets in the Cdh1 signaling complex, Cdh1, Ctnnb1, and Fmnl2. For this we used osteoclasts derived from global decoy BMMs to determine if inhibition of miR-29-3p activity would result in derepression and increased expression of these targets. In general, expression of these targets was largely unchanged between genotypes or was significantly lower in the global decoy cells. This was particularly evident when looking at CDH1 protein and mRNA. This seemingly contradictory response to miR-29-3p inhibition may suggest the existence of a compensatory mechanism to keep CDH1 levels at a biological level, as CDH1 overexpression in osteoclasts would be expected to enhance cell-cell interactions, leading to more and larger osteoclasts. As demonstrated by Pagetic osteoclasts, which may contain hundreds of nuclei (221), increasing the number and size of osteoclasts would have detrimental effects on bone homeostasis.

Furthermore, as we mentioned previously, there are three Cdh1 isoforms (2 coding; 1 non-coding; ENSMUSG00000000303) (179). While the coding sequence for each isoform generates identical proteins, alternative splicing results in the inclusion, or exclusion, of 2 additional terminal exons. These terminal exons code for alternative 3’-UTR isoforms (432 vs 1631 bases). In cases of alternative 3’-UTR isoforms, longer 3’-UTR sequences usually contain more regulatory elements, tend to be expressed in more differentiated cells, and are more unstable and prone to degradation (105, 222). Indeed, the longer Cdh1 3’-UTR (1631 bases) has miRNA binding sites for multiple miRNAs.
known to be important for osteoclastogenesis. In addition to miR-29-3p, these miRNAs include miR-214 (29, 223), miR-186 (4, 224), miR-9 (225, 226), and miR-199a-5p (223, 227, 228).

However, the expression pattern of the Cdh1 3′-UTR isoforms during osteoclastogenesis is not known. Differential expression of these isoforms could result in regulation by miR-29-3p only when the longer isoform is expressed. This kind of varied regulation due to alternative splicing has been previously observed, for example, between miR-29-3p and the regulation of alternatively expressed Igf1 3′-UTR isoforms in osteoblasts (105).

Investigating the expression patterns of Cdh1 isoforms is also of translational interest as the human CDH1 gene has 4 isoforms which display similar alternative 3′-UTR splicing to murine Cdh1 (ENSG00000039068) (179, 229). Furthermore, our findings that the Cdh1 signaling complex is regulated by miR-29-3p is not only important for the osteoclast lineage, but is also relevant in all cell lineages that concomitantly express miR-29-3p and Cdh1, such as kidneys, liver, lung, and gastrointestinal tract (https://www.proteinatlas.org/ENSG00000039068-CDH1/tissue) (88, 230).

Together, these data suggest that miR-29-3p may promote osteoclast differentiation, in part, by 1.) limiting how large osteoclasts become by regulating cell-cell interactions as the osteoclast matures and 2.) by pushing osteoclast precursors away from the M2 lineage, towards the pro-inflammatory M1 macrophage lineage, which shares similar characteristics to bone resorbing osteoclasts.
Appendix A: Generation of the miR-29-3p Decoy Conditional Overexpressing Mice

The miR-29 decoy donor vector contains the conditional overexpression (cOE) cassette flanked by 5' and 3' arms (1.2 and 4.2 Kb respectively) homologous to intron 1.
of Rosa26. The cOE cassette included: a CAG promoter followed by a LoxP-STOP-LoxP sequence, tdTomato-miR-29 tough decoy (tdTomato-TuD) sequence, woodchuck hepatitis virus posttranscriptional regulatory element, and a bovine growth hormone polyadenylation signal sequence.

To generate the miR-29 decoy cOE mouse strain, the miR-29 decoy donor vector and Rosa26 specific CRISPR RNP (sgRNA/Cas9 protein) were microinjected into the pronucleus of C57BL/6 one-cell embryos, then transferred to foster females for subsequent development. Live born pups were initially genotyped using primer pairs specific to the tdTomato-miR-29 decoy sequence. Their genotypes were further confirmed by long-range PCR of both arms using primers specific to the donor and outside both homologous 5’ and 3’ arms.

The donor vector is based on Ai9 (Addgene plasmid: 22799) as described by Madisen et al. (PubMed PMID: 22446880). This vector has been used successfully by many laboratories, including ours, to generate conditional overexpression mouse strains using either ES cells or CRISPR/Cas9. This plasmid was used as template for PCR amplification and conventional cloning techniques were used to prepare a Rosa26 cOE vector with a unique AsiSI restriction site between Lox-STOP-Lox and WPRE sequences. A DNA fragment containing three copies of a miR-29-3p tough decoy with AsiSI restriction sites in both ends was synthesized by GenScript (Piscataway, NJ). This sequence was then inserted into Rosa26 cOE to generate the final miR-29 decoy cOE donor vector for CRISPR gene editing.

A line of mice displaying germline transmission of the miR-29 decoy cOE cassette
was bred with Hprt-Cre mice (Jax stock no: 004302) to remove the STOP sequence, which consists of three copies of a SV40 polyadenylation signal sequence, to place the tdTomato-miR-29 decoy sequence under the transcriptional control of the CAG promoter, to accomplish global overexpression. The Hprt-Cre mice were backcrossed with C57BL6 mice for over 30 generations, so all mice are in the C57BL/6 background.
### Appendix B: Antibodies and Primers

Table B.1. Information for antibodies referenced in Chapters 4 and 5.

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Table B.2. Sequences and annealing temperatures of qRT-PCR primers and genotyping primers referenced in Chapters 3, 4, and 5.

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<td>TGTTGGATGATGATTTTCA</td>
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<tr>
<td>Genotyping 1(^i)</td>
<td>GGGTTCCGGTCTCCTGGCTGTG</td>
<td>CATGGAGCCCTCCATGCGCACC</td>
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<tr>
<td>Genotyping 2(^ii)</td>
<td>CTCTGCTGCTCGCTGCTCTGAG</td>
<td>CTCCGAGGCGATCACAAGC</td>
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\(^*\) For genotyping, Touchdown PCR was used with initial annealing beginning at 65°C. The annealing temperature was decreased in 5-degree increments, every 5 cycles, for a total of 30 PCR cycles.

\(^i\) Genotyping primer pair 1 was used to confirm cre recombination of the floxed transcription stop sequence.

\(^ii\) Genotyping primer pair 2 was used to confirm homozygosity. Only WT alleles will amplify this product, so lack of band confirms homozygosity.
Table B.3. Sequence and annealing temperatures cloning primers and deletion primers referenced in Chapter 5.

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Strand</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
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<tr>
<td>Cdh1 3' UTR (parent)</td>
<td>Forward</td>
<td>5'/AATAATACTAGTGGGACTAGCAAGTCTCCCCCGTG/3'</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'/TATATAAAGCCTGCATCTTTAGAGAACGGTTTCAATGG/3'</td>
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<tr>
<td>Ctnnb1 3' UTR (parent)</td>
<td>Forward</td>
<td>5'/TCTCTCACTAGTGCTGGCTGGTTTGA/TACTG/3'</td>
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<tr>
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<td>Reverse</td>
<td>5'/TCTCTACGCGTACGCATCTGGTGAAGCATGG/3'</td>
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</tr>
<tr>
<td>Fmnl2 3' UTR (parent)</td>
<td>Forward</td>
<td>5'/TACTATACTAGTGGACAGTGGAGACGGGC/3'</td>
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<td></td>
<td>Reverse</td>
<td>5'/GGAGGGAAGCTTGACTCAACAGACGAATCTTGG/3'</td>
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<tr>
<td>Rab44 3' UTR (parent)</td>
<td>Forward</td>
<td>5'/GTTTGTAATGCTGGCTGGCTGCC/3'</td>
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<td>Tnfrsf1a 3' UTR (parent)</td>
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<td>5'/GCGCGGACTAGTGAATTATCCTTGAG/3'</td>
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<td>Reverse</td>
<td>5'/GCGGCACCGTGACTCCATAAGGTC/3'</td>
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<td>Forward</td>
<td>5'/GAAATGCAACGCCCTCCACAGAAATGC/3'</td>
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<td>Cdh1 site 1 deletion</td>
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<td>Reverse</td>
<td>Forward</td>
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<td>Ctnnb1 deletion</td>
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<td>5’/GATCTTTAAAATGTACAGATTTTTTTATGGTTTTTACAC/3’</td>
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REFERENCES


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https://www.nature.com/articles/srep43191#supplementary-information.


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