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MICRORNAS IN SKELETAL DEVELOPMENT (A DELANY, SECTION EDITOR)

MicroRNAs Are Critical Regulators of Osteoclast Differentiation

Henry C. Hrdlicka[1] · Sun-Kyeong Lee[2] · Anne M. Delany[1]

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Abstract

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.

Recent Findings Using unbiased approaches to identify miRNAs of interest and reporter-3′UTR assays to validate interactions, recent studies have elucidated the impact of specific miRNA-mRNA interactions during in vitro osteoclastogenesis. There has been a focus on signaling mediators downstream of the RANK and CSF1R signaling, and genes essential for 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 Validating miRNA expression patterns, targets, and impact in osteoclasts and other skeletal cells is critical for understanding basic bone biology and for fulfilling the therapeutic potential of miRNA-based strategies in the treatment bone diseases.

Keywords Osteoclast · Differentiation · Fusion · miRNA · Non-coding RNA · Microarray

Introduction

Osteoclast differentiation and function requires an orchestrated series of events including the following: 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 (NFκB), 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) (Fig. 1a) [1]. Moreover, pro-inflammatory cytokines (e.g., tumor necrosis factor α [TNF-α] and interleukin 1 β [IL-1β]) can enhance osteoclastogenesis while other anti-inflammatory cytokines dampen or inhibit differentiation completely (e.g., interferon β [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

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✉ Anne M. Delany
adelany@uchc.edu

[1] Center for Molecular Oncology, UConn Health, Farmington, CT 06030, USA

[2] Center on Aging, UConn Health, Farmington, CT 06030, USA

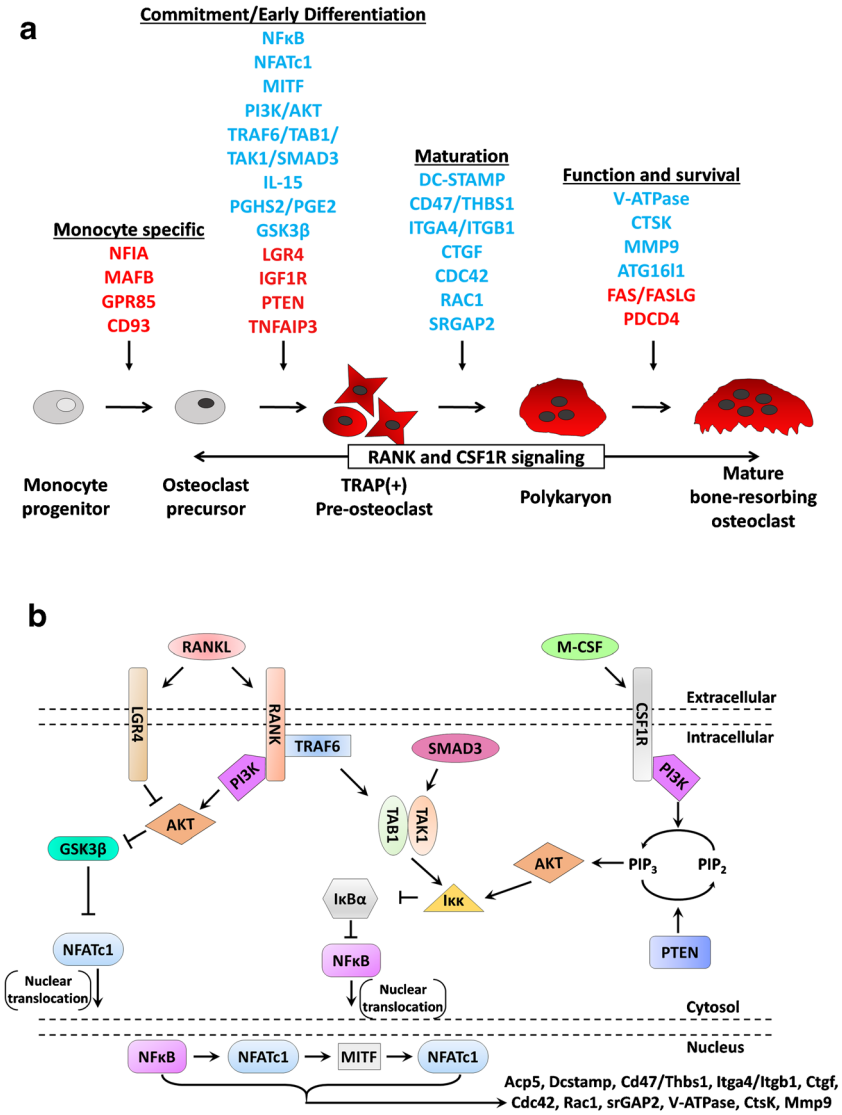

Fig. 1 miRNAs regulate osteoclastogenesis at multiple stages, targeting both positive and negative regulators of commitment, differentiation, and function. Osteoclast differentiation is induced by RANK and CSF1R signaling, resulting in signaling cascades that promote the expression of genes important for both differentiation and function. **a** Regulators of osteoclastogenesis targeted by miRNAs at each stage of differentiation, including the following: 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

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 downregulated in response to drug treatment or disease (e.g., glucocorticoid treatment, inflammation, and osteoporosis).

The goal of this article is to comprehensively review recently reported positive and negative miRNA regulators of osteoclastogenesis, highlighting the cell source and context in which they are expressed. We believe that 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.

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 initiates several signaling cascades which simultaneously promote OCP commitment to the osteoclast lineage and early differentiation events (Fig. 1b).

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 (Fig. 1b) [14]. During differentiation, NFκB activation is supported by the downregulation 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 that 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 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 transcription of Nfatc1 [27, 28]. mmu-miR-214 promotes these events by targeting phosphatase and tensin homolog (Pten), which converts active PIP_3 to inactive PIP_2 [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., TNF-α, 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••]. Cox2 promotes the synthesis of prostaglandin E2 (PGE_2). PGE_2, 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 PGE_2 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 that 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 cell system used.

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•].

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 as 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 decreases 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 DC-STAMP and the interaction of CD47 and thrombospondin-1 (THBS1) [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, the upregulation of DC-STAMP is supported by the downregulation of mmu-miR-7b, which has been suggested to target DC-STAMP [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 (Table 1).

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 [57]. 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; α4 β1 integrin), which binds fibronectin [58, 59]. 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 [57]. These interactions are then fine-tuned by the upregulation of hsa-miR-let-7e during differentiation, which targets Itga4 [16••].

Table 1 Summary of featured miRNA regulators of osteoclast differentiation

micoRNA	Target(s)	Reference
Positive regulators		
hsa-miR-let-7e	Thbs1, Itga4	[16••]
mmu-miR-21	Faslg, Pdcd4	[50–52]
mmu-miR-29 family	Nfia, Gpr85, Cd93, Srgap2, Cdc42	[37]
mmu-miR-31	Rhoa	[53]
mmu-miR-34c	Lgr4	[32]
hsa-miR-99b	Igf1r	[16••]
hsa-miR-125a	Tnfaip3	[16••]
hsa-miR-132	Pghs2	[16••]
hsa-miR-148a	Mafb	[42•]
hsa-miR-212	Il15, Pghs2	[16••]
mmu-miR-214	Pten	[29]
Negative regulators		
mmu-miR-7b	DC-STAMP	[47]
mmu-miR-20a	Atg16l1	[54]
mmu-miR-26a	Ctgf	[48]
hsa-miR-29b	–	[40]
mmu-miR-124	Nfatc1	[20]
hsa-miR-125a	Traf6	[19•]
mmu-miR-145	Smad3	[15]
mmu-miR-155	Mitf, Socs1	[22, 26]
mmu-miR-186	Ctsk	[55]
mmu-miR-340	Mitf	[23]
mmu-miR-365	Mmp9	[56]

miRNAs Regulating Osteoclast Function and Survival

Once mature-multinucleated osteoclasts are formed, the integrin αvβ3 adheres the cell to the bone surface and a tight sealing zone forms around the area to be resorbed [60]. 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 that 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, 53].

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 [60]. The resorption area is acidified to pH 5 by the proton pump vacuolar-like H(+)-ATPase (V-ATPase) which is essential for bone resorption [61]. In this acidic microenvironment, the inorganic components of the bone matrix begin to degrade and the secreted proteases become activated.

Cathepsin K and MMP9 are the primary proteases which degrade the organic component of bone matrix. They are initially secreted through the ruffled border as pro-proteins. In the acidic resorption area, pro-cathepsin K autolytically cleaves into active cathepsin K. Active cathepsin K then cleaves pro-MMP9 into active MMP9; both proteases are then free to digest the collagen fibers of the bone matrix [62]. 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 [55, 56]. Further, mmu-miR-20a was found to target autophagy related 16 like 1 (Atg16l1) [54]. 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., cathepsin K and MMP9) at the ruffled border [63]. 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 [64].

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 [64, 65]. Cell survival may be supported by mmu-miR-21, which is quickly upregulated with osteoclast differentiation and has been shown to directly target Faslg [50].

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 [51, 52].

Hypothesis Generating Datasets

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 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 2). 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 postmenopausal women, or in mice which have undergone ovariectomy [71, 72]. Several studies have compared differential miRNA expression in osteoporotic postmenopausal women versus their healthy counterparts, or ovariectomized (ovx) mice versus sham-operated mice [15, 69, 70].

One unique study used miRNA microarray analysis to compare the miRNA expression profiles of osteoclasts, osteoblasts, and osteocytes, all collected from postmenopausal 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

Table 2 Hypothesis generating datasets

Year. Title	Study design	Reference
2010. Osteoclast-specific Dicer gene deficiency suppresses osteoclastic bone resorption	$Dicer^{fl/fl}$:Cathepsin K-cre mice used to examine the contributions of miRNA processing on miRNA expression profiles in mature osteoclasts and the in vivo phenotype	[7]
2011. A microRNA expression signature of osteoclastogenesis	miRNA expression profiles examined in murine BMMs treated with M-CSF with or without RANKL for 24 h	[51]
2013. Expression profiling of microRNAs in RAW264.7 cells treated with a combination of tumor necrosis factor alpha and RANKL during osteoclast differentiation	miRNA expression profiles examined in RAW264.7 cells treated with RANKL, in the presence or absence of TNF-α for 0, 24, and 82 h.	[66]
2013. miR-148a regulates osteoclastogenesis by targeting V-maf musculoaponeurotic fibrosarcoma oncogene homolog B	miRNA expression profiles examined in human PBMCs treated with or without M-CSF+RANKL for 14 days	[42•]
2013. miR-31 controls osteoclast formation and bone resorption by targeting RhoA	miRNA expression profiles examined in murine BMMs treated with M-CSF with or without RANKL for 24 h	[53]
2014. MiR-7b directly targets DC-STAMP causing suppression of NFATc1 and c-Fos signaling during osteoclast fusion and differentiation	miRNA expression profiles examined in RAW264.7 cells treated with or without M-CSF and RANKL for 72 and 192 h	[47]
2014. Pathway analysis of microRNA expression profile during murine osteoclastogenesis	miRNA expression profiles examined in murine BMMs treated with M-CSF and RANKL for 1, 3, and 5 days	[4]
2015. MicroRNA-26a regulates RANKL-induced osteoclast formation	miRNA expression profiles examined in murine BMMs treated with M-CSF and RANKL for 0, 1, 2, and 3 days	[48]
2015. miR-214 promotes osteoclastogenesis by targeting Pten/PI3k/Akt pathway	miRNA expression profiles examined in RAW264.7 cells treated with or without RANKL for 48 h	[29]
2015. NFκB-direct activation of microRNAs with repressive effects on monocyte-specific genes is critical for osteoclast differentiation	miRNA expression profiles examined in human PBMCs treated with M-CSF and RANKL for 0, 2, and 21 days	[16••]
2016. Changing expression profiles of lncRNAs, mRNAs, circRNAs, and miRNAs during osteoclastogenesis	Expression profiles of mRNAs, lncRNAs, circRNAs, and miRNAs examined in RAW264.7 cells treated for 0, 24, 72, and 96 h with M-CSF and RANKL	[67••]
2016. RBP-J-regulated miR-182 promotes TNF-α-induced osteoclastogenesis	miRNA expression profiles examined in BMMs treated with RANKL, with or without TNF-α, for 24 h	[68]
2016. Validation of downregulated microRNAs during osteoclast formation and osteoporosis progression	miRNA expression profiles examined in BMMs treated with M-CSF and RANKL for 0 and 5 days	[69]
2018. Expression profiling of microRNAs in human bone tissue from postmenopausal women	miRNA expression profiles of human osteoclasts, osteoblasts, and osteocytes isolated from postmenopausal women	[70]
2018. Overexpressed miR-145 inhibits osteoclastogenesis in RANKL-induced bone marrow-derived macrophages and ovariectomized mice by regulation of Smad3	miRNA expression profiles examined in BMMs treated with M-CSF with or without RANKL for 6 days	[15]

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 expressed 340 miRNAs, 101 of which were also expressed in osteoblasts, and 196 of which were also found in whole bone [70]. 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 can carry 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 [73–75]. 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 TNF-α.

In the first study, miRNA microarray analyses were performed on RNA from RAW264.7 cells treated with RANKL, in the presence or absence TNF-α for 0, 24, and 82 h. Forty-four miRNAs were differentially expressed between untreated cells at 0 h and RANKL+TNF-α-treated cells 82 h later, whereas 52 miRNAs were differentially expressed between untreated cells and RANKL treated alone for 82 h [66]. 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 qPCR analysis of differentiating RAW264.7 cells and murine BMMs treated with RANKL+ TNF-α, confirming previous reports that miR-125a and miR-29b are upregulated with differentiation [16••, 37].

Recombination signal binding protein for immunoglobulin-κ-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 TNF-α for 24 h, during which time 27 miRNAs were induced and 12 were suppressed by TNF-α. Similar to the first study, both mmu-miR-29a and mmu-miR-125a were upregulated during differentiation in this dataset [68]. An interesting analysis would be a comparison of the two datasets to further identify similarly expressed miRNAs in response to TNF-α 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. Ninety-three miRNAs were differentially expressed and were divided into seven 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 qPCR analysis, the upregulation of miR-365 and miR-99b and the downregulation of miR-451 during differentiation were 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 [76].

In addition to miRNAs, other non-coding RNAs including long non-coding RNAs (lncRNA) and circular RNAs (circRNAs) are differentially regulated during osteoclastogenesis [67••]. Many lncRNAs 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, lncRNAs, and circRNAs in RAW264.7 cells treated for 0, 24, 72, and 96 h with M-CSF and RANKL. Gene expression in undifferentiated cells was compared with that of pre-osteoclasts (24 h RANKL), mature osteoclasts (72 h RANKL), or activated osteoclasts (96 h 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 lncRNA-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 [67••].

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 resources for the comparison of these cell systems. Importantly, the sequence of many mature miRNAs is conserved between the mouse and human, broadening the potential utility of these unbiased datasets.

Conclusion

In recent years, nanoparticle technology has seen the development of the bone targeting peptide Asp_8, which can be conjugated to the surface of nanoparticles of various compositions [78•]. Asp_8 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 increasing ability to more specifically target 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 are important for fulfilling this therapeutic potential. While there has been growing 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.

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Compliance with Ethical Standards

Conflict of Interest Henry C. Hrdlicka, Sun-Kyeong Lee, and Anne M. Delany each declare no potential conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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