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The Role of microRNAs in Osteoclastogenesis

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The Role of microRNAs in Osteoclastogenesis

Tiziana Franceschetti, Ph.D.

University of Connecticut, 2014

ABSTRACT

microRNAs (miRNAs) are short sequences of RNA that function as negative post-transcriptional regulators of gene expression. Their effects are mediated through direct binding to target messenger RNAs. Studies describing the crucial role of miRNAs in osteoclasts are quite limited. The aim of this work was to investigate the basic mechanisms by which miRNAs control osteoclast differentiation and activity.

miR-29 Promotes Murine Osteoclastogenesis by Regulating Osteoclast Commitment and Migration. The expression of miR-29 family members increases during osteoclast differentiation, and its inhibition impairs commitment and migration of the progenitors. In an effort to understand how miR-29 exerts its function, we demonstrated that miR-29 negatively regulates RNAs critical for cytoskeletal organization and RNAs expressed in the macrophage lineage, as well as Calcitonin receptor, which controls osteoclast survival and resorption.

Pathway analysis of microRNA expression profile during murine osteoclastogenesis. Microarray analysis was used to profile the expression of miRNAs during the course of osteoclast differentiation, in an enriched population of osteoclast

progenitor cells from murine bone marrow. Computational analyses were used to predict functional pathways that may be regulated by clusters of miRNAs in osteoclasts. The most prominent pathways identified include those involved in cell motility, cell-matrix interactions, axon guidance, and cytoskeletal remodeling. These processes are critical for the migration of osteoclast precursors, their maturation, and bone resorbing activity.

These studies contribute to our understanding of miRNA function in the osteoclast lineage. This information could be used to develop therapies for skeletal diseases associated to alterations in the bone resorption compartment.

The Role of microRNAs in Osteoclastogenesis

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APPROVAL PAGE

Doctor of Philosophy Dissertation

The Role of microRNAs in Osteoclastogenesis

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CHAPTER 1:

INTRODUCTION

Osteoclast Biology and Regulation by microRNAs

Bone is a highly dynamic tissue, continuously remodeling itself to repair damaged bone matrix and to fulfill the metabolic needs of the organism. Bone remodeling is achieved through a delicate balance between bone formation, which is mediated by osteoblasts, and bone resorption, which is accomplished by osteoclasts (1). Specific factors are produced by osteoblasts and osteoclasts to ensure that bone resorption is coupled to bone formation during physiological bone remodeling, to preserve the microarchitecture of bone. Continuous cell-cell communication between osteoblasts and osteoclasts, through physical interaction and exchange of soluble factors, allows the bone cells to regulate each other's differentiation and activity.

Bone is remodeled to respond to hormonal cues or when nutritional modifications require mobilization of minerals from the skeleton. Furthermore, skeletal remodeling can regulate proliferation and differentiation of the cells of the bone marrow niche, a stem cell-rich compartment that is comprised of numerous hematopoietic and mesenchymal progenitors. Therefore, skeletal homeostasis is critical for the maintenance of bone health.

Alterations in osteoclast differentiation or resorption activity can lead to an imbalance in the bone remodeling compartment, which is associated with the development or progression of debilitating pathological conditions. These include osteoporosis, osteopetrosis, rheumatoid arthritis, and multiple myeloma (2-7). Decoding the complex regulatory pathways that underlie osteoclast differentiation and activity is a crucial step in the development of new therapeutic strategies to treat disorders of bone remodeling. Considerable research efforts have contributed to characterizing the processes that regulate osteoclastogenesis and bone resorption at the transcriptional and post-translational levels.

microRNAs (miRNAs) are a rather novel class of regulators of gene expression, and they control key cellular processes, including differentiation, proliferation, and survival. miRNAs are short (20-25 nucleotide-long), single-stranded sequences of RNA, which post-transcriptionally inhibit the expression of specific target genes (8). The importance of miRNAs in skeletal biology has been extensively investigated, with particular focus on osteoblasts and chondrocytes (9). However, the role of specific miRNAs in the osteoclast lineage is still widely uncharacterized.

The goal of this Introduction is to summarize our knowledge of the mechanisms regulating osteoclast differentiation and activity, and describe the most recent studies on the role of miRNAs in the osteoclast.

I. Osteoclasts.

I.1. Osteoclast differentiation.

Osteoclasts are specialized cells that resorb bone, and their differentiation is an intricate, multi-step process (Figure 1.1). Osteoclasts derive from multipotential hematopoietic precursors, which can also give rise to erythrocytes, platelets, and cells of the immune system. Osteoclastogenesis initiates with the differentiation of common myeloid progenitors, which

can form monocytes-macrophages, granulocytes, and megakaryocytes. In response to a number of osteoclastogenic stimuli, monocyte precursors proliferate, migrate, and commit to the osteoclast lineage, at the expense of the alternative macrophage fate. Osteoclast precursors then fuse into a multinucleated polykaryon, which can be activated to

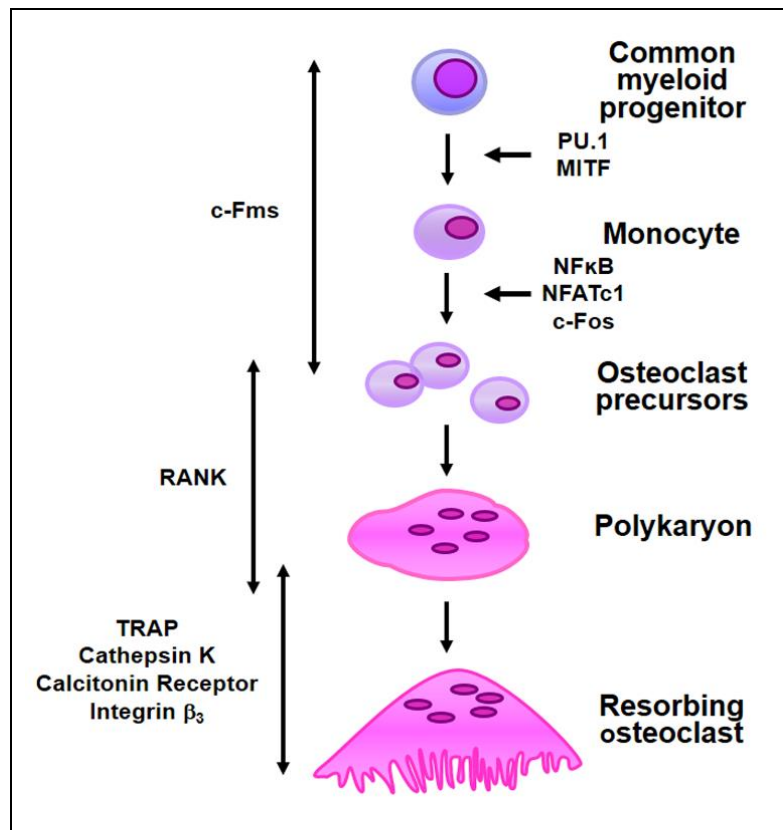


Figure 1.1. The process of osteoclastogenesis. Schematic representation of the various phases of osteoclast differentiation. Key transcriptional regulators and marker genes of each step are indicated.

resorb bone, triggering the bone remodeling process (2, 10). Although multinucleation is not necessary for bone degradation, it was shown that resorption efficiency is directly proportional to size of the mature osteoclast. The activated osteoclast has a limited life span, and will physiologically undergo apoptosis, to protect the skeleton from excessive bone resorption.

I.2. Osteoclast function.

Osteoclasts adhere to the bone surface through the interaction of integrin $\alpha_v\beta_3$ with Arg-Gly-Asp (RGD) motifs on extracellular matrix proteins, such as osteopontin and bone sialoprotein. Mature, activated osteoclasts then need to become polarized in order to begin the resorption process (Figure 1.1). To accomplish this, osteoclasts perform an extensive reorganization of the cytoskeleton (11, 12). Polarized osteoclasts, rich in mitochondria that produce energy necessary for resorption, position the nuclei in the apical part of the cell. In contrast, in the basal region, cytoskeletal remodeling allows the formation of specialized, actin-rich structures, called podosomes, used by the osteoclasts to attach to the bone matrix (Figure 1.1). Here, actin filaments interact with cytoskeletal proteins, including α -actinin and vinculin. The arrangement of several podosomes into a dynamic circular structure (podosome belt) leads to the formation of the sealing zone, which creates an isolated extracellular compartment, known as the bone resorption lacuna (11, 13, 14).

The basolateral membrane of the polarized osteoclast, called the ruffled border, is responsible for resorption. This is achieved by establishing an acidic environment in the resorption lacuna, with the combined action of carbonic anhydrase II, H^+ /ATPase proton pumps, ClC-7 chloride channels, and HCO_3^-/Cl^- exchangers. Carbonic anhydrase initiates the acidification process by producing protons and HCO_3^- , as a result of CO_2 hydration (15). By means of secretory vesicles and H^+ /ATPase pumps, H^+ ions are transported through the ruffled border into the resorption pit (16). Vacuolar H^+ /ATPase works in combination with ClC-7 channels to secrete Cl^- ions into the resorptive microenvironment (16). Energy-independent HCO_3^-/Cl^- exchangers, located in the apical membrane of the osteoclast, are responsible of maintaining the intracellular pH (17). The acidification step is necessary to dissolve the inorganic components of the extracellular mineralized matrix.

Different matrix proteases, such as matrix metalloproteinase 9 (MMP-9) and tartrate resistant acid phosphatase (TRAP), participate in the degradation of extracellular matrix proteins (Figure 1.1) (18, 19). However, a major role is played by Cathepsin K, a cysteine protease that is also secreted through the ruffled border, which degrades the organic constituents of bone, comprised primarily by type I collagen fibers (Figure 1.1) (4). The degradation products are endocytosed by the resorbing osteoclast, transported at the apical membrane, and secreted into the extracellular compartment.

Bone resorption is very dynamic, and consists of cycles of osteoclast adhesion to the bone surface, matrix degradation, and detachment. In a controlled manner, osteoclasts

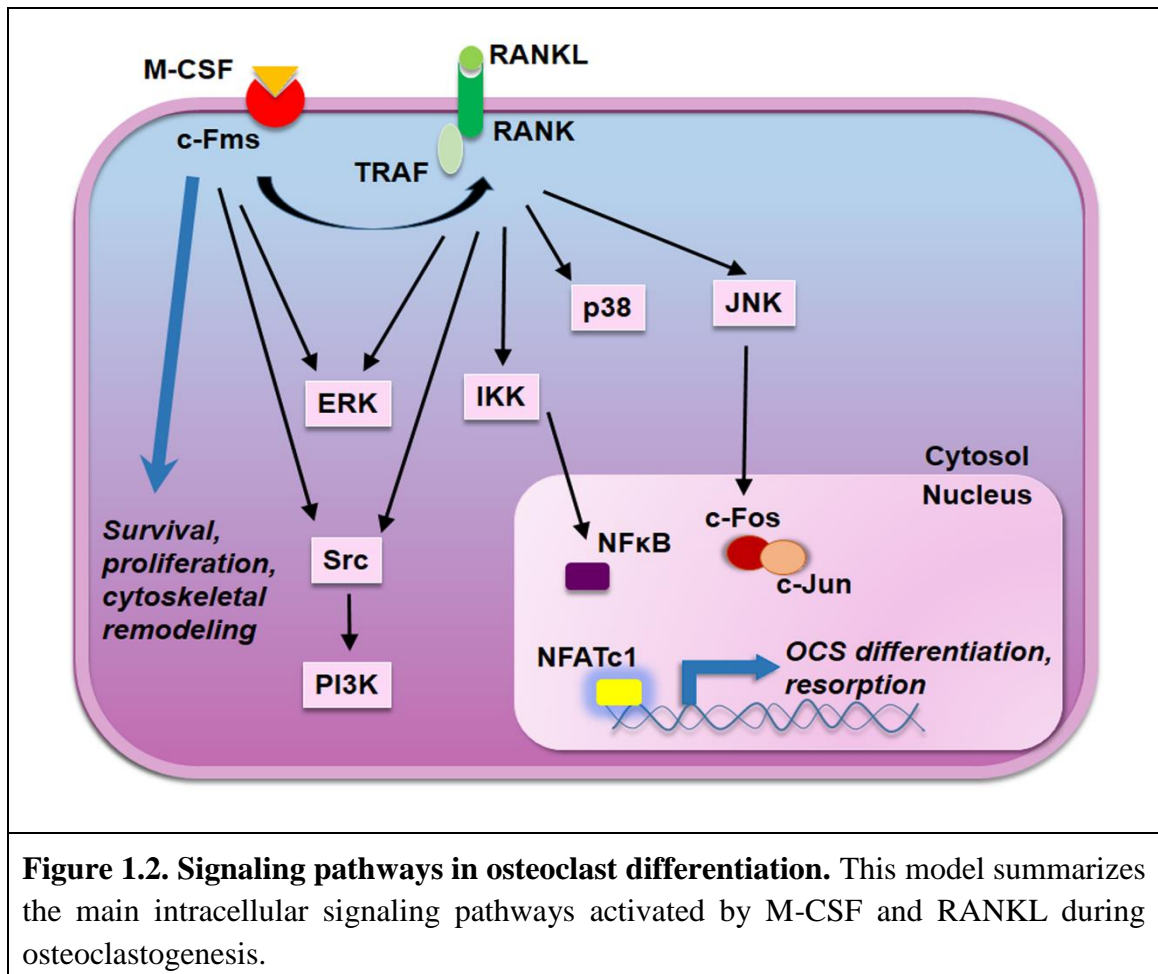
continuously reorganize the actin cytoskeleton into lamellipodia to migrate on the bone surface, and to form new sealing zones, until they undergo apoptosis (11).

I.3. Regulation of osteoclast differentiation and function.

Osteoclast differentiation, activation, and survival are tightly regulated by a combination of pro- and anti-osteoclastogenic hormones and cytokines. Among the pro-osteoclastogenic factors, Macrophage-colony stimulating factor (M-CSF, CSF1) and Receptor activator of nuclear factor kappa-B (NFκB) ligand (RANKL) are essential and sufficient for inducing osteoclast differentiation in vitro and in vivo (2, 20). In the skeleton, these and other cytokines are produced by cells of the mesenchymal lineage: stromal cells, osteoblasts, and osteocytes. However, osteoclast regulation is not limited to these non-hematopoietic cells, and in the past decade the importance of immune cells in the control of bone resorption has been described (21). Many of the molecules involved in regulating osteoclast differentiation and activity were identified through the analysis of animal models of naturally occurring genetic mutations, or transgenic gain-of-function or loss-of-function mouse models displaying an osteopetrotic phenotype.

M-CSF.

M-CSF is a cytokine important for both the macrophage and the osteoclast lineages. M-CSF signaling is activated upon binding to its receptor, c-Fms, which is a member of the receptor tyrosine kinase (RTK) superfamily (Figures 1.1 and 1.2). M-CSF



binding to its receptor triggers the activation of several intracellular pathways, thereby exerting its function on the osteoclast precursors. These complex signaling cascades are not completely understood, but include activation of c-Src (cellular Src kinase), Syk (spleen tyrosine kinase), PI3K (phosphoinositide 3-kinase), and ERK (extracellular signal-regulated kinase) pathways (11).

M-CSF supports the survival and proliferation of the osteoclast precursor cells. Further, M-CSF signaling promotes cytoskeletal reorganization and the migration of the

osteoclast precursors. Importantly, M-CSF also activates the expression of the RANK receptor on osteoclast progenitors, therefore priming them to respond to RANKL and initiate osteoclast differentiation (10).

RANKL.

RANKL signals through RANK, a member of the tumor necrosis factor (TNF) receptor superfamily, to promote osteoclast commitment, maturation, and survival (Figures 1.1 and 1.2). Further, RANKL supports cytoskeletal remodeling, motility, and bone resorption activity, thus acting as a key regulator of osteoclast differentiation and function. The RANKL/RANK interaction activates the expression of osteoclast marker genes, including TRAP, Cathepsin K, integrin β_3 , and calcitonin receptor (Figure 1.1) (2, 10, 22). Osteoprotegerin (OPG), also secreted by cells of the osteoblastic lineage and by immune cells, is a decoy receptor for RANKL, thus a negative regulator of osteoclastogenesis. Frequently, the relative abundance of OPG compared with RANKL can determine whether osteoclast formation may ensue (23).

Like M-CSF, RANKL signaling is transduced by numerous intracellular pathways, upon interaction of RANK with TNFR-associated cytoplasmic factors (TRAFs), including TRAF6. In osteoclasts, RANKL mediates the activation, for example, of the mitogen-activated protein kinase (MAPK) pathways involving c-Jun N-terminal kinase (JNK), p38 extracellular kinase (ERF), and ERK, as well as the inhibitor of NF κ B kinase (IKK) pathway, the PI3K pathway, and the Src pathway (11).

Downstream effectors of these signaling cascades include several transcription factors critical for the osteoclast lineage.

Transcriptional regulation of osteoclast differentiation.

Most of the transcription factors involved in the control of osteoclast differentiation act as heterodimers to control gene expression. Purine-rich nucleic acid binding protein 1 (PU.1) represents the earliest marker for osteoclastogenesis, and is essential for the commitment of the myeloid progenitors into macrophage and osteoclast precursors (24). Further, PU.1 regulates genes important for osteoclast function, including Cathepsin K and osteoclast-associated receptor (*Oscar*) (Figure 1.1) (25, 26). For the most part, PU.1 modulation of gene expression during the initial phases of osteoclast differentiation is mediated by its interaction with microphthalmia-associated transcription factor (MITF) (Figure 1.1) (26, 27). MITF has also been shown to participate in the regulation of the osteoclast genes *Clcn7*, and osteopetrosis-associated transmembrane protein 1 (*Ostm1*) (28).

RANKL signaling up-regulates the expression and the activation of NFκB, c-Jun, and c-Fos, which are required for osteoclast differentiation and to induce osteoclast marker genes (Figures 1.1 and 1.2). The NFκB family of transcription factors functions as heterodimers, and are translocated into the nucleus to regulate gene expression (29). c-Jun and c-Fos belong to the activator protein 1 (AP-1) family, which is composed of heterodimers of Fos and Jun proteins (30, 31). Importantly, NFκB, c-Jun, and c-Fos are

involved in inducing the initial up-regulation of Nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), the master regulator of osteoclastogenesis (30).

In addition to RANKL signaling, other co-stimulatory pathways can initiate NFATc1 expression during osteoclast differentiation. The RANKL-independent pathways include the Ca^{2+} signaling pathway and the immunoreceptor tyrosine-based activation motif (ITAM) proteins DNAX activation protein of 12 kDa (DAP12) and Fc-receptor common γ -subunit (FcR γ) (32). In the later stages of osteoclastogenesis, NFATc1 binds its own promoter to amplify its expression (32).

NFATc1 is involved in the regulation of several aspects of osteoclast maturation and activity (Figures 1.1 and 1.2). Either alone or in association with other transcription factors, NFATc1 stimulates the fusion of the precursors into multinucleated cells, through the up-regulation of the dendritic cell-specific transmembrane protein (DC-STAMP) and the d2 isoform of vacuolar ATPase V0 domain (Atp6v0d2) (32). In addition, NFATc1 regulates osteoclast migration and adhesion to the bone surface. This is achieved by directly promoting the expression of β_3 integrin and c-Src, which trigger intracellular pathways that converge on the reorganization of the cytoskeleton, orchestrated by small GTPases of the Rho family (32). NFATc1 also promotes the expression of proteins required for bone resorption: MMP-9, H^+ /ATPase, ClC-7, Cathepsin K, TRAP, calcitonin receptor, and OSCAR (32).

Osteoclast interaction with cells of the bone microenvironment.

As previously mentioned, bone resorption is coupled to bone formation, and osteoclast differentiation and activity are strongly regulated in vitro and in vivo by the action of cytokines, including M-CSF, RANKL, and OPG, produced by osteoblasts. Further, recent reports indicate that RANKL is also expressed at high levels by terminally differentiated osteocytes to support osteoclastogenesis (33, 34). Additionally, bi-directional signaling between osteoblasts and osteoclasts is achieved through the interaction of ephrins and ephrin receptors. EphB4 receptors, expressed on the osteoblasts, bind ephrin-B2, present on the surface of the osteoclasts. This interaction promotes osteoblast differentiation and, at the same time, inhibits osteoclastogenesis (35).

In the bone marrow niche, osteoclasts are in close relationship with cells of the immune system, which can secrete several regulatory cytokines (21). Macrophages produce inflammatory factors, including interleukin 1 (IL-1), IL-6, and Tumor necrosis factor α (TNF α). Although IL-1 alone cannot induce osteoclastogenesis, it stimulates TRAF6 and potentiates the activation of the RANKL signaling pathway (2, 21). Moreover, IL-1 promotes RANKL expression on cells of the osteoblast lineage, which confirms the tight interaction of the cells within the bone microenvironment (21). TNF α is a potent inducer of osteoclastogenesis, and it functions through a RANKL-independent mechanism. This was demonstrated by the observation that TNF α -mediated osteoclast differentiation is not blocked in the presence of OPG (36). Additionally, hematopoietic precursors from RANKL-, RANK-, and TRAF6-null mice can form mature osteoclasts when stimulated with TNF α (37).

Activated T lymphocytes express RANKL, a pro-osteoclastogenic factor, but also Interferon- γ (IFN- γ), IL-4, IL-10, IL-12, and IL-14, which are all inhibitory to osteoclast differentiation (21). Therefore, the role of T cells on osteoclast differentiation may depend on the ratios between these factors in specific biological conditions. Further, in rheumatoid arthritis, T cells of the synovium produce large amounts of IL-17, which induces RANKL secretion in synovial fibroblasts, and expression of TNF α , IL-1, and IL-6 in synovial macrophages. These cytokines stimulate osteoclast differentiation, promoting bone resorption and joint destruction (21, 38). Further, B lymphocytes are a major source of RANKL and OPG in the bone marrow (39, 40).

The effect of specific cytokines on osteoclastogenesis also depends on the cellular environment. For example, IL-7 has a direct inhibitory effect on osteoclastogenesis *in vitro* (41). However, under inflammatory condition, IL-7 production by stromal cells and osteoblasts stimulates the secretion of RANKL from T cells, which ultimately supports osteoclast differentiation (42).

I.4. Identification of osteoclast precursors.

In the perspective of analyzing the mechanisms that orchestrate osteoclast differentiation and activity, the use of a primary cell system is important. Peripheral blood and bone marrow represent the main reservoirs of primary osteoclast precursors. However, both of these sources are highly heterogeneous, which makes difficult the analysis of osteoclast-specific mechanisms. In an effort to decrease the heterogeneity of

primary cell cultures, several studies have adopted immunological techniques to deplete contaminations from other hematopoietic populations.

Cell surface markers are routinely used to distinguish osteoclast precursors from macrophages, dendritic cells, and B and T cells. A population of cells with high osteoclastogenic efficiency can be isolated from mouse bone marrow, peripheral blood, and spleen, which can be identified as negative for the lymphoid markers B220/CD45R and CD3, present of B and T cells, respectively (43). These cells also present negative or low expression of CD11b (also known as integrin alpha M, ITGAM), which is expressed on several myeloid lineages and natural killer lymphocytes. Osteoclast precursors express high levels of c-Fms (CD115) and c-Kit (CD117), a hematopoietic stem cell marker (43, 44). Additional studies have characterized the surface antigen profile of the osteoclast precursors. Recently, osteoclast precursors were identified as $CD3^{-}/B220^{-}/CD11b^{-/low}$ cells expressing high levels of Ly6C (lymphocyte antigen 6), a glycoprotein expressed on most hematopoietic cells, and CX₃CR1, a chemokine receptor expressed by monocytes and lymphocytes. These cells appear to be distinct from a population of progenitors that can give rise to not only osteoclasts, but also to macrophages and dendritic cells (45).

Species-specific differences in osteoclast cell surface antigens may also complicate their characterization. CD14, a co-receptor for lipopolysaccharide, is a commonly used monocyte marker used to isolate human osteoclast precursors (46, 47). More recently, the osteoclastogenic potential of human peripheral blood monocytes was differentiated based on the expression of DC-STAMP. This study showed that cells with high levels of DC-STAMP generate more osteoclasts than the negative population (48).

In conclusion, combinations of surface markers are necessary to isolate osteoclast precursor cells, in order to further investigate the mechanisms regulating osteoclastogenesis and bone resorption.

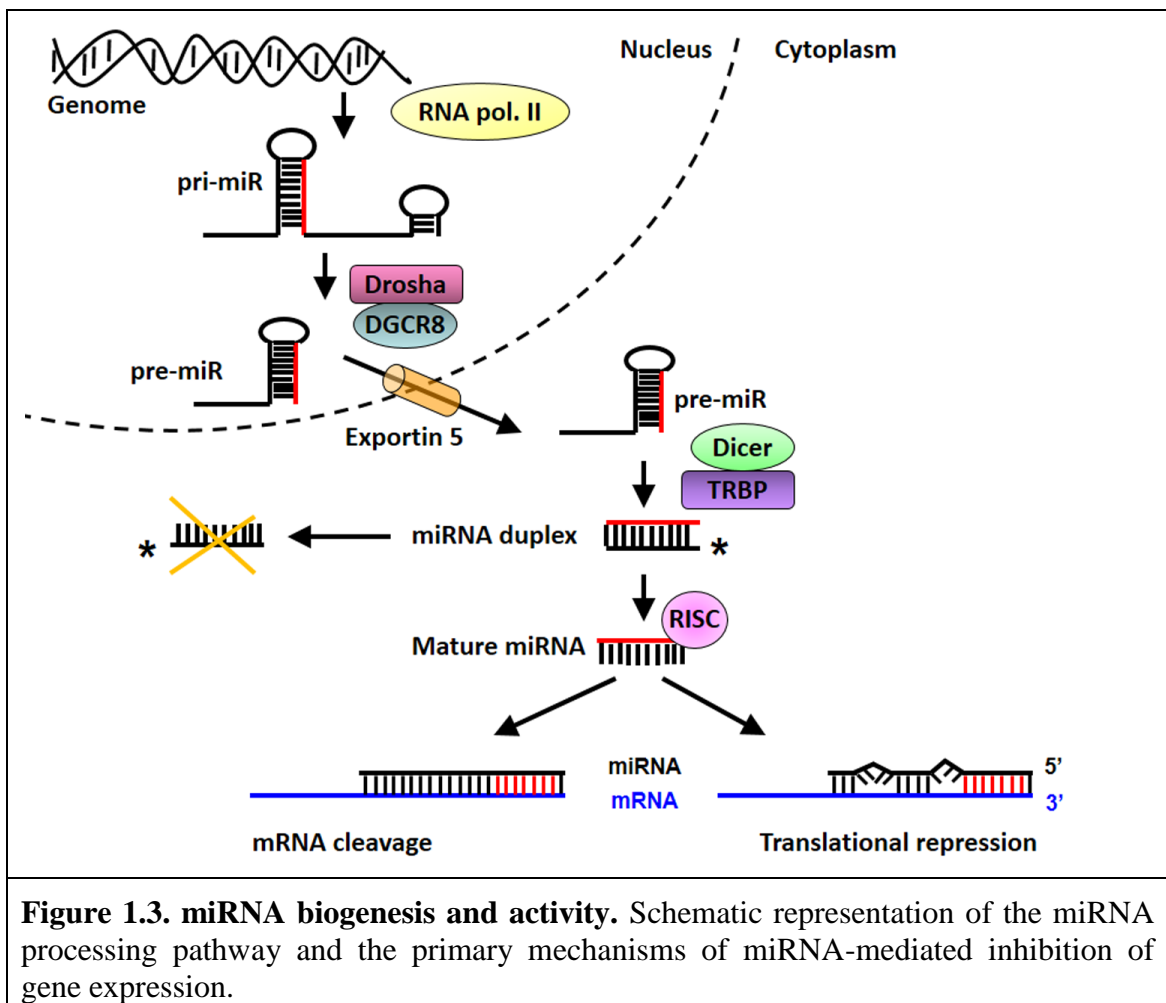
II. miRNAs.

II.1. miRNA biogenesis.

miRNAs are transcribed by RNA polymerase II as independent genes or included in introns or exons of protein coding genes (Figure 1.3). miRNA-coding genes are regulated similarly to protein-coding genes, and transcriptional control contributes to their expression in specific cells, or tissues, or stages of development (8). Long primary miRNAs (pri-miRNAs) contain typical hairpin structures, which, for the most part, are cleaved by the nuclear endonuclease Drosha and the RNA binding protein DiGeorge syndrome critical region gene 8 (DGCR8). The product, a ~60 nucleotide long precursor miRNA (pre-miRNA), is exported into the cytoplasm by Exportin 5. Here, pre-miRNAs are further cleaved by Dicer and the RNA binding protein transactivation-response RNA-binding protein (TRBP) into a miRNA duplex (8).

The miRNA duplex is then loaded onto an Argonaute protein (AGO). Mature functional miRNAs can be derived from either the 5'-strand (indicated as -5p), or the 3'-strand (indicated as -3p), or both strands of the pre-miRNAs (49). For the most part, the functional miRNA strand is more abundant than the miRNA* or passenger strand, and can be identified as the least thermodynamically stable strand in the complex. The active

miRNA strand remains associated with AGO in an RNA-induced silencing complex (RISC), which increases its stability, whereas the miRNA* passenger strand is eventually degraded. The choice of the active miRNA strand is often tissue- or cell-specific (50). The mature miRNA drives the RISC complex to bind target messenger RNA (mRNA) sequences.



II.2. miRNA function.

Short, single-stranded mature miRNAs act, for the most part, as post-transcriptional, negative regulators of gene expression. Inhibition of gene expression is mediated by direct binding to complementary sequences located on target mRNAs (Figure 1.3). miRNA binding sites are frequently identified in the 3' untranslated region (UTR), although functional sites are recognized in the coding sequence and in the 5' UTR. Inhibition of gene expression is achieved through different mechanisms, depending on the degree of complementarity between the miRNA and the target mRNA. Near-perfect base-pairing frequently leads to the endonucleolytic cleavage of the mRNA sequence and its destabilization, followed by degradation. This mechanism is typically observed in plants, although it can also occur in mammalian cells (8).

More frequently, miRNA-target interactions are based primarily on the binding of the nucleotides in positions 2-8 of the miRNA sequence, which are known as the “seed region”. miRNA interaction with AGO determines the functional seed region. Here, miRNA binding leads to the blockage of translational initiation, and mRNA decay upon mRNA decapping or deadenylation. Additional features, other than base-pair complementarity, contribute to the final outcome of each miRNA-mediated repression of gene expression. These include the accessibility of the target site on the mRNA secondary structure, the distance of the binding site to the 3' end of the mRNA, as well as the cellular localization and stability of the miRNA (51, 52).

II.3. miRNAs in osteoclasts.

Since they were first discovered, miRNAs have been widely investigated in osteoblasts and in chondrocytes, where they are key regulators of skeletal development and bone cell differentiation and function (9). In contrast, there are relatively few reports on the role of miRNAs in osteoclasts, and a limited number of miRNA-target interactions have been examined in this cell type. One major reason for this shortcoming could be that the cell systems available for studying the mechanisms regulating osteoclastogenesis have several limitations. For example, most of the mechanistic studies aimed at analyzing miRNA function are performed using cell lines as tools. However, an immortalized cell line for mature osteoclasts is not available, and the existing osteoclast precursor cell lines have limited resorption activity. Further, as previously discussed, sources of primary osteoclast precursors, such as the bone marrow, are extremely heterogeneous, which makes necessary additional steps for cell isolation and sorting. These limitations likely contribute to the deficit in our understanding of miRNA function in osteoclasts.

miRNA biogenesis pathway in the osteoclast lineage.

Initially, two independent studies analyzed the global role of miRNAs in osteoclasts, by interfering with components of the miRNA biogenesis pathway. Sugatani and colleagues silenced the expression of DGCR8, Dicer1, and Ago2 in vitro in mouse primary bone marrow macrophages (BMMs) (53). These proteins are crucial for the processing of the miRNA precursors and generation of the active mature miRNAs. Knock-down of DGCR8, Dicer1, or Ago2 impaired the RANKL-mediated up-regulation

of transcription factors important for osteoclastogenesis, including PU.1, MITF, c-Fos, and NFATc1, as well as their recruitment to the NFATc1 and TRAP promoters. Further, the expression of the osteoclast markers TRAP, MMP-9, Cathepsin K, Calcitonin receptor, and integrin β_3 were strongly attenuated by the suppression of the DGCR8, Dicer1, or AGO2 levels. Consistently, osteoclast formation and bone resorbing activity were decreased upon silencing of DGCR8, Dicer1, or AGO2 (53). These results indicate the overall importance of the miRNA processing pathway in the osteoclast lineage.

In vivo, the role of miRNA biogenesis in osteoclasts was evaluated by generating mouse lines with cell type-specific deletion of Dicer. Dicer deletion in myeloid cells, using a CD11b promoter-driven cre, induced a mild osteopetrotic phenotype, with an increase in bone formation parameters and decreased osteoclast number (53). Similarly, Dicer ablation in mature osteoclasts using a Cathepsin K promoter-driven cre resulted in increased bone mass, due to reduced osteoclast number (54). Therefore, these in vivo phenotypes confirm that the miRNA biogenesis pathway is crucial for osteoclast formation, as observed in vitro.

There is limited understanding of the mechanisms by which miRNAs regulate the processes of osteoclast commitment, maturation, and function. Indeed, at the time of this writing, only 9 miRNAs have been investigated in the osteoclast lineage (Table 1.1). An overview of these miRNAs and their role in osteoclasts will be presented below.

Table 1.1. miRNAs investigated in the osteoclast lineage.				
miRNA	Known target genes in OCS	Role in OCS	Experimental system	References
miR-124	NFATc1	-	BMMs	(55)
miR-146a	TRAF6	-	PBMCs	(56)
miR-148a	MAFB	+	PBMCs; mouse	(57)
miR-155	MITF; SOCS	-	RAW264.7; BMMs; mouse	(58-60)
miR-21	PDCD4	+	BMMs	(61)
miR-223	NFIA	+	RAW264.7; BMMs	(53)
miR-29	CTR, Cdc42, srGAP2, NFIA, CD93, GPR85	+	RAW264.7; BMMs	Chapter 3; (62)
miR-31	RhoA	-	BMMs	(63)
miR-320a	ARF1	+	PBMCs	Chapter 5; (64)
miR-503	RANK	-	PBMCs; mouse	(65)

miR-223

miR-223 was the first miRNA with an identified role in osteoclasts. miR-223 expression is activated by PU.1, a transcription factor crucial in the early stages of myeloid differentiation (24, 66). The expression levels of miR-223 are sustained in primary bone marrow-derived osteoclast precursors and in the mouse monocytic cell line RAW264.7, but decrease in a time-dependent manner during RANKL-driven osteoclastogenesis (61, 67). In a study by Sugatani and colleagues, the authors reported

that inhibition of the mature miR-223 with an antisense oligonucleotide impairs osteoclastogenic differentiation in RAW264.7 cells, suggesting that miR-223 supports osteoclastogenesis (53).

The pro-osteoclastogenic role of miR-223 is mediated, in part, through the repression of the transcription factor NFI-A (53, 68). Down-regulation of NFI-A is required for the differentiation of several hematopoietic cell types, including osteoclasts, granulocytes, and monocytes. In contrast, NFIA is up-regulated during erythropoiesis (53, 69, 70). Further, NFI-A negatively regulates the expression of the M-CSF receptor, which is critical for osteoclast survival, maturation, and activity, as previously discussed (12, 69). Therefore, miR-223 promotes osteoclast differentiation, in part, by targeting NFIA.

miR-21

miR-21 expression is also induced by PU.1 and the AP-1 (activator protein 1) family member c-Fos, transcription factors important for osteoclast differentiation and function (71). miR-21 levels are up-regulated during the course of osteoclast differentiation in RAW264.7 cells and in mouse bone marrow macrophages (BMMs) (61, 67). BMMs lacking key components of the miRNA processing machinery, either DGCR8 or Dicer, display impaired osteoclastogenesis. Forced over-expression of miR-21 in these cells partially rescues their ability to differentiate into mature osteoclasts. Further, knock-down of miR-21 results in suppressed osteoclast formation and resorption activity of bone marrow-derived macrophage cultures (Figure 1.4) (61).

miR-21 represses the expression of PDCD4 (programmed cell death domain 4) in osteoclasts and other tissues (61, 72). PDCD4 inhibits the transcriptional activity of AP-1 family members, and its over-expression robustly suppresses osteoclast differentiation and function. Although other post-transcriptional targets of miR-21 may be involved, PDCD4 inhibition represents one mechanism by which miR-21 promotes osteoclastogenesis. In addition, miR-21 blocks the expression of FasL, an estrogen (E₂)-induced pro-apoptotic factor, thereby protecting osteoclast progenitors and mature osteoclasts from estrogen-mediated apoptosis. In a

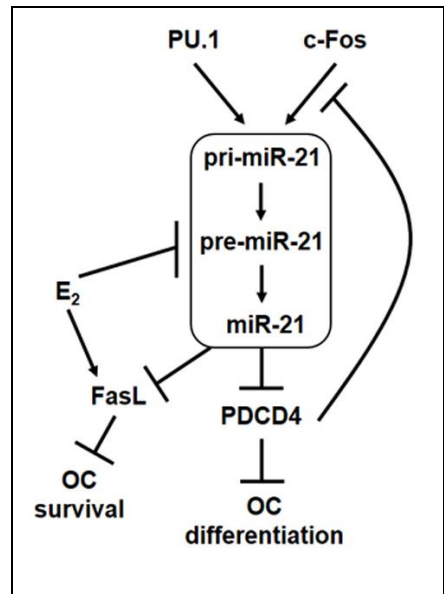


Figure 1.4. The function of miR-21 in osteoclasts. The diagram summarizes the regulatory loops that control the expression of the miR-21, a pro-osteoclastogenic miRNA.

regulatory loop, estrogen can inhibit miR-21 biogenesis, to limit its expression and allow FasL translation, in order to restrict osteoclast survival (Figure 1.4) (73).

miR-148a

miR-148a expression is strongly induced during osteoclastogenesis in CD14⁺ human PBMCs (57). Over-expression of miR-148a in human PBMCs, as well as in mouse BMMs, stimulates osteoclast differentiation and activity, whereas opposite effects are produced by a miR-148a inhibitor. One of the genes that miR-148a regulates in osteoclasts is the transcription factor MAFB (V-maf musculoaponeurotic fibrosarcoma

oncogene homolog B) (57). MAFB inhibits the RANKL-driven transcriptional program that promotes osteoclast differentiation (74). In vivo, inhibition of miR-148a, using a specific oligonucleotide, elevated bone mass by decreasing bone resorption parameters. This important in vivo experiment confirms the pro-osteoclastogenic role of this miRNA (57). In addition, miR-148a levels are increased in circulating mononuclear cells from systemic lupus erythematosus (SLE) patients, who display lower bone mineral density (BMD). PBMCs from SLE patients have augmented osteoclastogenic potential, which is restrained upon inhibition of miR-148a. Thus, elevated miR-148a likely contributes to the increased osteoclastogenesis in SLE patients (57).

miR-31

RANKL treatment of mouse BMMs induces a strong up-regulation of miR-31, a miRNA previously studied in cancer, but not in hematopoietic cells (63, 75). In osteoclasts, miR-31 supports actin ring formation and bone resorption. This is achieved because miR-31 tightly controls the expression of RhoA, a GTPase critical for cytoskeletal organization, formation of the sealing zone, and osteoclast activity (63, 76). Therefore, miR-31 promotes osteoclast formation and activity, and RhoA is a major mediator of its function.

miR-155

As regulators of skeletal homeostasis, miRNAs with a negative role in osteoclasts have also been identified. For example, pro-inflammatory signals, including TNF α and

Interferon β (IFN- β), activate the expression of miR-155 during monocytic differentiation and activation (59). miR-155 was shown to promote the commitment of monocyte precursors to the macrophage lineage, at the expense of osteoclastogenesis (58). In RAW264.7 cells, miR-155 over-expression impairs in vitro osteoclast formation and resorption activity after treatment with M-CSF and RANKL. In particular, over-expression of miR-155 blocks the early stages of osteoclastogenesis, prior to fusion of the precursors into multinucleated cells (58). In contrast, expression a miR-155 sponge construct, which inhibits the function of miR-155, enhances osteoclast formation in mouse BMM cultures (59).

The negative role of miR-155 on osteoclastogenesis is mediated by the repression of the transcription factor MITF. As discussed in the previous section, MITF induces the expression of key genes for osteoclast maturation and function: TRAP, OSCAR, and Cathepsin K (77). In osteoclasts, as well as in other myeloid cells, miR-155 also inhibits SOCS1 (suppressor of cytokine signaling 1), a protein that contributes to the signal transduction of cytokines, such as IFN- β (59). By repressing the negative effects of pro-inflammatory cytokines on the differentiation of the progenitor cells, SOCS1 supports osteoclastogenesis (78). Based on these findings, miR-155 down-regulates genes that are important for the differentiation of the progenitor cells to osteoclastogenesis, therefore favoring the macrophage fate.

Controversially, Blum and colleagues showed that, in an arthritis model, bone marrow precursors from miR-155-null mice have reduced osteoclast potential (60). Differences between this study and the in vitro findings by Zhang et al. suggest that miR-

155 could play distinct roles in different phases of the multi-step process of osteoclastogenesis. Therefore, a negative function of miR-155 on the commitment of the precursors to the osteoclast lineage does not exclude its ability to support osteoclast terminal differentiation. Alternatively, global miR-155-null mice may have decreased osteoclast differentiation potential due to the effects of the miR-155-null mutation on cells of other lineages, such as immune cells and osteoblasts.

miR-124

Similarly, miR-124 blocks osteoclast differentiation, and one of its targets is a major pro-osteoclastogenic factor, NFATc1 (55). Activation of the osteoclastogenic program by RANKL down-regulates miR-124 in mouse BMMs. Accordingly, over-expression of miR-124 inhibits osteoclast formation, whereas its knock-down promotes differentiation. Moreover, the proliferation and migration of the osteoclast precursors are diminished when miR-124 is over-expressed, suggesting that miR-124 regulates these important processes in osteoclasts (55).

miR-146a

Expression of miR-146a inhibits the RANKL- or TNF α -induced osteoclast differentiation of human PBMCs, as well as their bone resorption activity (56). miR-146a plays an important role in inflammation, and is up-regulated in activated macrophages (79). Additionally, in response to TNF α and IL-1 β stimuli, NF κ B drives miR-146 expression, and miR-146, in turn, targets TRAF6 (Tumor necrosis factor receptor-

associated factor 6) and IRAK1 (IL-1 receptor-associated kinase), in a negative feedback mechanism to limit NFκB signaling (80). TRAF6 is an ubiquitin ligase with a fundamental role in osteoclast maturation, and its targeting by miR-146a likely represents a key mechanism for repression of osteoclastogenesis. Further, in a mouse model of arthritis, systemic administration of a miR-146a mimic prevents joint destruction and bone erosion. This is achieved by reducing osteoclast number, providing an in vivo confirmation of the negative role of miR-146a on osteoclastogenesis (56).

miRNAs and bone mass: miR-503 and miR-133

To correlate the function of specific miRNAs with the development of pathological conditions, modifications in the miRNA expression profile of osteoclast precursors have also been evaluated in selected cohorts of osteoporosis patients. For example, dramatic down-regulation of miR-503 was detected in CD14⁺ peripheral blood mononucleated cells (PBMCs) of post-menopausal osteoporosis patients (65). In vitro, inhibition of miR-503 induces osteoclast differentiation of CD14⁺ PBMCs from healthy subjects. Moreover, over-expression of miR-503 decreases osteoclast formation in PBMCs from osteoporosis patients (65). miR-503 was found to regulate bone mass through direct targeting of RANK (65). In vivo, administration of a miR-503 inhibitor increases osteoclast formation, decreases bone mineral density (BMD), and reduces bone resorption parameters, whereas systemic injection of a miR-503 mimic caused the opposite bone phenotype (65). These results confirm that miR-503 expression negatively impacts osteoclastogenesis. Further, the expression of miR-503 in osteoclasts is induced

by estrogen, and decreased in ovariectomized (OVX) mice (65). Overall, these observations suggest that miR-503 may play an important role in the development of post-menopausal osteoporosis.

In contrast to miR-503, the expression of miR-133a is increased in circulating monocytes from post-menopausal women with low bone mineral density (BMD). This suggests that miR-133a may be a potential monocytic biomarker for post-menopausal bone loss. However, the authors of this study failed to identify direct targets of miR-133a which could mediate its function in bone resorbing cells, although this miRNA has been investigated in osteoblasts and chondrocytes, where it targets Runx2 (81).

Conclusions

Bone homeostasis depends on the coupling of bone formation and resorption, during the process of remodeling. Osteoclast differentiation, function, and survival must be finely regulated to assure proper bone resorption activity. Understanding the molecular mechanisms that participate in the regulation of osteoclastogenesis and bone resorption is vital for the development of therapeutics for bone diseases characterized by alterations in the osteoclast compartment.

Osteoblasts, osteocytes, and immune cells can regulate osteoclast differentiation and activity. This is achieved through the expression of cell surface molecules and the secretion of numerous soluble factors, including cytokines, hormones, growth factors, and the decoy receptor OPG. On the osteoclast, these factors can trigger complex intracellular signaling pathways, which converge in the activation of specific

transcription factors that promote the expression of genes important for osteoclast maturation and bone resorption.

However, post-transcriptional mechanisms also play an important role in osteoclast differentiation and function, and miRNAs are key components of this process. Only few miRNAs and target genes have been identified in the osteoclast lineage, where they can modulate multiple aspects of osteoclast biology, including commitment and migration of the precursors, maturation, resorption, and survival of the osteoclasts. Clearly, additional studies are required to gain a more comprehensive knowledge of the function of miRNAs in osteoclastogenesis.

CHAPTER 2:

SPECIFIC AIMS AND HYPOTHESES

The overall aim of the studies described in this dissertation is to improve our understanding of the basic mechanisms regulating the differentiation and function of osteoclasts. This work will describe the miRNA-mediated post-transcriptional regulation of key osteoclast genes, which could contribute to the development of novel therapeutic strategies for bone diseases characterized by alterations of bone resorption.

We hypothesize that specific miRNAs fine tune gene expression to regulate osteoclastogenesis, and, in particular, that: 1) miR-29 promotes osteoclast differentiation, and 2) miRNA profiling of differentiating osteoclast precursors will highlight novel miRNAs important for regulating osteoclast differentiation, and new genes and pathways that play a role in this process.

Chapter 3: Specific Aim 1. To test the hypothesis that miR-29 promotes osteoclastogenesis.

1a) To analyze the expression of the miR-29 family members during in vitro osteoclastogenesis, we will utilize quantitative RT-PCR and examine RNA from

differentiating cultures of mouse primary osteoclast precursors and of the monocytic cell line RAW264.7.

1b) To investigate the function of miR-29 in the osteoclast lineage, we will analyze osteoclastogenesis, proliferation, migration, and survival of RAW264.7 cells stably transduced with a doxycycline-inducible construct for miR-29 knock-down (miR-29 sponge).

1c) To identify novel miR-29 targets in osteoclasts, we will use miRNA target prediction algorithms (DIANA-microT 3.0, PicTar, miRanda, or TargetScan), and validate potential miR-29 targets in the monocytic RAW264.7 cell line using Luciferase Reporter-UTR assays.

Chapter 4: Specific Aim 2. To profile miRNA expression of differentiating osteoclast precursors, and identify novel potential regulators of osteoclast differentiation and function.

2a) To investigate the expression of mature miRNAs during osteoclastogenesis, we will perform a miRNA microarray analysis on a bone marrow population enriched for osteoclast precursor cells. We will determine miRNA expression in the early, middle, and late stages of RANKL-driven osteoclastogenesis. Further, we will profile miRNA expression in osteoclast cultures differentiated for 3 days with M-CSF and RANKL and in undifferentiated cells cultured with M-CSF alone. The expression of selected miRNAs will be confirmed by qPCR.

2b) To identify genes and functional pathways that are potentially regulated by miRNAs in the osteoclast lineage, we will perform a computational pathway prediction study for differentially expressed miRNAs that were identified by the microarray analysis.

CHAPTER 3:

miR-29 Promotes Murine Osteoclastogenesis by Regulating Osteoclast Commitment and Migration

This work has been published: Franceschetti, T., Kessler, C.B., Lee, S.K., Delany, A.M. J Biol Chem. 2013;288(46):33347-60.

CHAPTER ABSTRACT

Osteoclast differentiation is regulated by transcriptional, post-transcriptional, and post-translational mechanisms. microRNAs (miRNAs) are fundamental post-transcriptional regulators of gene expression. The function of the miR-29 (a/b/c) family in cells of the osteoclast lineage is not well understood. In primary cultures of mouse bone marrow-derived macrophages, inhibition of miR-29a, -29b, or -29c diminished formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts, and the osteoclasts were smaller. qRT-PCR showed that all miR-29 family members increased during osteoclast differentiation, in concert with mRNAs for the osteoclast markers *Trap* (*Acp5*) and Cathepsin K. Similar regulation was observed in the monocytic cell line RAW264.7. In stably transduced RAW264.7 cell lines expressing an inducible miR-29 competitive inhibitor (sponge construct), miR-29 knock-down impaired osteoclastic commitment and migration of pre-osteoclasts. However, miR-29 knock-down did not affect cell viability, actin ring formation, or apoptosis in mature osteoclasts. To better

understand how miR-29 regulates osteoclast function, we validated miR-29 target genes using Luciferase-3' UTR (untranslated region) reporter assays and specific miR-29 inhibitors. We demonstrated that miR-29 negatively regulates RNAs critical for cytoskeletal organization, including Cell Division Control protein 42 (*Cdc42*) and SLIT-ROBO Rho GTPase activating protein 2 (*Srgap2*). Moreover, miR-29 targets RNAs associated with the macrophage lineage: G protein-coupled receptor 85 (*Gpr85*), Nuclear Factor I/A (*Nfia*), and *Cd93*. In addition, Calcitonin receptor (*Calcr*), which regulates osteoclast survival and resorption, is a novel miR-29 target. Thus, miR-29 is a positive regulator of osteoclast formation, and targets RNAs important for cytoskeletal organization, commitment, and osteoclast function. We hypothesize that miR-29 controls the tempo and amplitude of osteoclast differentiation.

INTRODUCTION

Osteoclasts are the only cells able to resorb mineralized matrix. The activity of these cells is critical for bone growth, normal bone remodeling, and fracture repair. A fine balance between the number and activity of osteoblasts and osteoclasts is necessary for bone homeostasis (2). Pathologies associated with abnormal osteoclast number or function include osteopetrosis, osteoporosis, and inflammatory osteolysis. Osteopetrosis is caused by impaired resorption, due to insufficient osteoclast formation or activity, and results in augmented bone density. In this disorder, changes in bone morphology are

often accompanied by immunodeficiency and anemia, due to narrowing of the bone marrow cavity and reduced expansion of the hematopoietic cell populations (3, 4). Osteoporosis is caused by excessive bone resorption coupled with insufficient bone formation. Systemic loss of bone mass can be triggered and supported by hormonal imbalance, such as estrogen deficiency. In inflammatory osteolysis, signaling from immune cells in conditions such as rheumatoid arthritis and periodontal disease determine bone loss localized at the joints or in the oral cavity. Thus, abnormal osteoclastic activity can result in higher predisposition to fractures, impaired joint mechanics, and loss of teeth (5).

The differentiation of osteoclasts from hematopoietic precursors is a complex multistep process (2). It begins with the commitment of multipotent precursors to differentiation along the osteoclast lineage. These committed monocytic cells subsequently migrate and fuse together to form multinucleated mature osteoclasts (82). Bone resorption is initiated when the osteoclast polarizes and organizes the cytoskeletal structures that form the sealing zone and ruffled border. These dynamic structures, which in vitro appear as an actin-rich ring, mediate the degradation of the bone surface, creating an acidic environment and secreting proteolytic enzymes, to degrade the inorganic and organic components of bone matrix, respectively (11). Tight control of the complex osteoclast differentiation process is accomplished by the regulation of gene expression at multiple transcriptional, post-transcriptional, and post-translational levels (83).

Substantial progress has been made in describing the mechanisms of M-CSF and RANKL driven osteoclastogenesis and bone resorption, and key transcription factors

involved include c-Fos, NFATc1, and NFκB. In addition, several studies highlight the role of post-translational modifications, mainly phosphorylation, in regulating the activity of receptors and kinases important for transducing intracellular signals, such as the M-CSF receptor (c-Fms), Src, and c-Jun N-terminus kinase (JNK) (12, 83). However, in the last decade, the importance of an additional level of gene regulation has emerged: post-transcriptional control by microRNAs (miRNAs).

miRNAs are short sequences of non-coding, single-stranded RNA that can bind target messenger RNAs (mRNAs) based on sequence complementarity. This process involves the RNA-induced silencing complex (RISC), which, for the most part, mediates the inhibition of gene expression by decreasing translation and/or by decreasing mRNA stability (84). Often, miRNAs regulate biological functions by modulating the expression of multiple genes that participate in the same or correlated pathways (85). miRNA levels are rapidly altered during embryonic development, as well as in adulthood, resulting in prompt and efficient post-transcriptional control (8, 86).

The overall importance of the miRNA processing pathway in the osteoclast lineage was reported. In vitro silencing of key factors involved in miRNA processing, including DiGeorge syndrome critical region 8 gene (DGCR8), Argonaute2 (Ago2), and Dicer1, suppressed osteoclast differentiation and activity (53). In vivo, deletion of *Dicer* in the monocyte/macrophage lineage, using a CD11b promoter driven-cre recombinase, as well as in mature osteoclasts using a Cathepsin K promoter driven-cre, resulted in the development of a mild osteopetrotic phenotype (53, 54).

Recent studies identified specific miRNAs and miRNA targets involved in osteoclast commitment and differentiation. For example, miR-223 promotes osteoclast formation, at least in part, through the inhibition of NFIA (Nuclear factor 1/A) (53, 61). Decreased NFIA expression is necessary for the terminal differentiation of osteoclasts (53), as well as granulocytes and monocytes (68, 69). Further, miR-21 promotes osteoclast differentiation, and it was shown to target PDCD4 (programmed cell death domain 4). PDCD4 represses activator protein 1 (AP-1)-dependent transcription, and the AP-1 family member c-Fos is essential for osteoclastogenesis (83). Therefore, by suppressing AP-1 function, PDCD4 may exert a negative effect on osteoclast differentiation. Another report demonstrated a negative effect of miR-155 on osteoclastogenesis. miR-155 promotes the commitment of progenitor cells to the macrophage lineage, through repression of MITF (microphthalmia-associated transcription factor) (58). MITF is required in the later phases of osteoclast formation, where it promotes the expression of genes crucial for osteoclast maturation and function, like *Trap*, *Oscar* (osteoclast-associated immunoglobulin-like receptor), and Cathepsin K (77).

We and others have studied the role of the miR-29 family in cells of the osteoblast lineage. Although miR-29 family members target several critical extracellular matrix mRNAs and limit their expression, this miRNA family promotes osteoblastic differentiation, by targeting negative regulators of this process (87-89). We considered that miR-29 family members may also play a role in osteoclastogenesis, given that altered miR-29 levels were associated with hematopoietic malignancies. For example,

diminished miR-29 levels were found in patients with chronic lymphocytic leukemia (CLL), and correlate with advanced clinical features and poor prognosis in acute myeloid leukemia (AML) (90-93).

In this study, we characterized the expression of miR-29 family members during the differentiation of murine bone marrow-derived osteoclast cultures and an osteoclast precursor cell line. We show that miR-29 is important for cell migration, osteoclast commitment and differentiation, and we identified 6 novel miR-29 targets in osteoclastic cells.

MATERIALS AND METHODS

Cell Culture.

Primary osteoclast precursor cultures were established from bone marrow of 6-8 week old C57BL/6 male mice, which had been depleted of B220/CD45R-positive and CD3-positive cells (B and T lymphocytes, respectively). Briefly, bone marrow was isolated from femurs, tibias, and humeri (94). Cells were incubated with Phycoerythrin (PE)-conjugated primary antibodies for B220 and CD3 (eBioscience, San Diego, CA), and with magnetically labeled anti-PE microbeads (Miltenyi Biotec, Auburn, CA). Magnetic-Activated Cell Sorting (MACS®) Column Technology (Miltenyi Biotec, Auburn, CA) was used to capture CD45R and CD3 positive cells in the column, and the

flow-through contained a population of cells enriched for the monocytic and non-lymphoid lineage cells (43). Flow cytometric analysis confirmed that this procedure

depleted 93-95% of T and B cells, thereby decreasing the heterogeneity of the marrow cells that were subsequently plated for experiments (Figure 3.1). Cells were cultured in α -MEM (Gibco Life Technologies, Grand Island, NY) supplemented with 10% FBS (Fetal Bovine Serum, Atlas Biologicals, Fort

Collins, CO) and 30 ng/ml murine recombinant Macrophage Colony-Stimulating Factor (M-CSF) (eBioscience, San Diego, CA). Bone marrow-derived osteoclast precursor cells were plated in the presence of 30 ng/ml murine recombinant M-CSF and Receptor activator of nuclear factor kappa-B ligand (RANKL) (eBioscience) for up to 5 days.

The mouse monocytic RAW264.7 (RAW) cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA) (TIB-71TM), and cultured in DMEM (Gibco Life Technologies, Grand Island, NY) supplemented with 10% FBS. Cells were cultured in α -MEM supplemented with 10% FBS and 30 ng/ml RANKL, to stimulate osteoclastic differentiation.

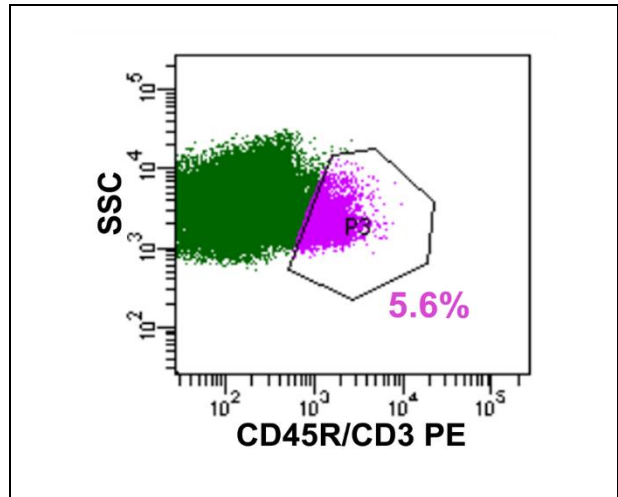


Figure 3.1. Flow cytometric analysis of the MACS sorted bone marrow cells. Expression of the lymphocyte-specific surface antigens CD45R and CD3 was evaluated by flow cytometry. SSC (side scattered light) is a measure of cell internal complexity. Percentage of CD45R/CD3⁺ cells in the total population is indicated.

The human embryonic kidney (HEK293T) PhoenixTM-Eco cell line was a gift from Nolan Lab, Stanford University, CA (95), and used for retrovirus production. These cells were cultured in DMEM supplemented with 10% FBS. The HEK293FT cell line was obtained from Invitrogen (Life Technologies, Grand Island, NY) and used for lentivirus production. 293FT cells were cultured in DMEM supplemented with 10% FBS.

In Vitro Osteoclast Formation Assay.

Cells were fixed in 2.5% glutaraldehyde in PBS, and TRAP activity was detected according to the manufacturer's instructions, using the Acid Phosphatase Leukocyte (TRAP) kit (Sigma-Aldrich). TRAP positive multinucleated cells containing more than three nuclei were counted as osteoclasts under microscopic examination. Osteoclast area was quantified using cellSens imaging software (Olympus, Center Valley, PA).

Quantitative Real time PCR.

Primary osteoclast precursors and RAW264.7 cells were plated at 53,000 cells/cm². Total RNA was isolated from differentiating cultures using the miRNeasy Mini kit (Qiagen, Valencia, CA). On-column DNase treatment was performed to reduce contamination with genomic DNA, and an additional treatment with RQ1 DNase (Promega, Madison, WI) was performed prior to gene expression analysis. miR-29 expression levels were analyzed with the TaqMan MicroRNA Assay (Life Technologies, Grand Island, NY). According to the manufacturer's instructions, 22.5 ng of RNA were

reverse transcribed with specific primers to generate cDNA. miR-29 expression was detected by qPCR in a MiQqPCR cycler (Bio-Rad) and normalized to U6 small nuclear RNA (RNUB6) levels using the absolute quantification method.

To quantify mRNA levels in total RNA, DNased RNA was reverse-transcribed using Moloney murine leukemia virus-reverse transcriptase (Invitrogen), and quantified by qPCR with iQ SYBR Green Supermix (Bio-Rad). The primer sets used are shown in

Table 3.1. RNA levels were

determined using absolute quantification and normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA. RNA experiments were performed at least twice, and each experiment contained biological triplicates. For qRT-PCR, each sample was analyzed in duplicate.

Table 3.1. Primer sets for gene expression analysis. The sequences of the primers used for quantitative RT-PCR analysis are reported [primers for HPRT, Cathepsin K, and TRAP as indicated in (96)]

PRIMER NAME	SEQUENCE (5' – 3')
HPRT FW	gttgatatgcccttgactataatga
HPRT RV	caacatcaacaggactcctcgatt
Cathepsin K FW	cgaaaagagcctagcgaaca
Cathepsin K RV	tgggtagcagcagaaacttg
TRAP FW	cgtctctgcacagattgcat
TRAP RV	aagcgcaaacggtagtaagg
GFP FW	gtgagcaagggcgaggagctgttc
GFP RV	gtaggtcagggtggtcacgaggg
F4/80 FW	tttctcgcctgcttcttc
F4/80 RV	ccccgtctctgtattcaacc
Mac-1 FW	tcctgtttaatgactctgcgttt
Mac-1 RV	ggctccactttggtctctgt

Retroviral Constructs.

To obtain miR-29 knock-down, double-stranded oligonucleotides targeting the miR-29a precursor were cloned into the retroviral vector pSilencer 5.1-H1 Retro (Ambion). The sequences of the oligonucleotides used are indicated in Table 3.2. The silencing construct was inserted into the pSilencer vector using BamHI and HindIII restriction enzymes. As negative control, pSilencer 5.1 Retro Scrambled was used (Ambion). Retrovirus was produced using the HEK293TPhoenixTM-Eco cell line (95).

miR-29 Knock-down and Osteoclast Formation.

Whole bone marrow was isolated from 6-8 week old C57BL/6 mice and plated overnight on a tissue culture plastic plate to limit the amount of stromal cells. The non-adherent population of cells was centrifuged on a Ficoll gradient, to enrich for macrophage precursors (97). These bone marrow-derived macrophages (BMMs) were seeded at 5,000 cells/well in 96-well plates, in α -MEM supplemented with 10% FBS in the presence of 30 ng/ml M-CSF. 48 hours after plating, cells were transfected with 50 nM anti-miRNA inhibitors (Dharmacon) using BioT reagent (Bioland Scientific, Paramount, CA). Alternatively, cells were transduced with retroviruses harboring a miR-29a knock-down construct or a scrambled control. Osteoclast differentiation was induced with RANKL treatment (10 ng/ml), and osteoclast formation was evaluated by TRAP staining.

Table 3.2. PCR cloning primers. GenBank reference numbers for the genes of interest are indicated. Restriction sites are underlined. MluI (FW) and HindIII (RV) restriction enzymes were used for all the genes, with the exception of Cdc42-UTR, for which SacI and MluI were used. In the oligonucleotides for miR-29 knock-down construct, BamHI and HindIII sites are indicated.		
RNA (MOUSE)	FW PRIMER (5' – 3')	RV PRIMER (5' – 3')
<i>Calcr</i> (NM_007588.2)	ggata <u>cgcg</u> tatgtgaagccacccaagca ttgtgatcc	cggca <u>agctt</u> catgtacacagcagaagcgttcaca c
<i>Trap</i> (NM_001102405.1)	tctac <u>gcg</u> tagatggattcatgggtggtg	tta <u>agctt</u> ctggaacctctgtcgctggc
<i>Cathepsin K</i> (NM_007802.3)	ttt <u>acgcg</u> tattccagccagccagcccatc	cgggcca <u>agctt</u> aaattgtaaagatactttattca aatacaag
<i>Srgap2</i> (NM_001081011.2)	ttt <u>acgcg</u> tctgcccacatcctctctcc	tta <u>agctt</u> aaggcgatggtggtgcacgttttaatttc
<i>Cdc42-CDS</i> (NM_009861)	tctac <u>gcg</u> tgacaagatctaattgaaatatta aaag	tcta <u>agctt</u> agttggtacatattccgatggg
<i>Cdc42-UTR</i> (NM_009861)	ttt <u>agctt</u> caaaggcctaagaatgtgaaac	tctac <u>gcg</u> tcatatacaaagagttgagacac
<i>Gpr85</i> (NM_145066.4)	ttt <u>acgcg</u> ttagggcgctgttcagcac	ccca <u>agctt</u> cacataatacagttcaatgctagc
<i>Cd93</i> (NM_010740.3)	ttt <u>acgcg</u> tgtgcccttaacttgcaaaag	tta <u>agctt</u> cctcccagaggcttactcg
<i>Nfia</i> (NM_010905.3)	ttt <u>acgcg</u> taccatcctccagacagacca	ccca <u>agctt</u> caggtgttgccatacatgtgt
miR-29a knock-down	<u>gatcc</u> gttcagagtcaatagaattctcaaga gaaattctattgactctgaactttttggaaa	<u>agctt</u> ttccaaaaagttcagagtcaatagaatttctct tgagaattctattgactctgaacg

pSLIK Lentiviral Constructs.

To knock-down the activity of all miR-29 family members, we generated a miR-29 “sponge”, which works as a competitive target for miR-29, relieving the repression of its endogenous target mRNAs (Figure 3.5) (98). The murine osteonectin 3' UTR contains

a pair of miR-29 binding sites, within cDNA bases 1083 to 1149. 3 copies of this tandem miR-29 binding site were cloned downstream of a GFP reporter gene, to generate the miR-29 sponge. The GFP alone control or GFP_Sponge cDNAs were subcloned in the pEN_Tmcs entry vector [plasmid 25751, Addgene, Cambridge, MA; (99)], which contains a tetracycline inducible promoter (tetracycline responsive element, TRE). These constructs were subjected to Gateway recombination with the lentiviral construct pSLIK, harboring a hygromycin resistance selectable marker gene [plasmid 25737, Addgene; (99)]. The Ubi-C (ubiquitin C) promoter in the pSLIK vector constitutively drives the expression of the tetracycline activator (rtTA3), which, in the presence of doxycycline (DOX), promotes the expression of the GFP or GFP_Sponge transgene from the TRE promoter.

Lentivirus Production and Transduction.

pSLIK constructs containing GFP alone or GFP_Sponge genes were co-transfected in the HEK293FT cell line, along with the expression vectors for the viral packaging proteins. These include the viral trans-activators Tat (pHDM-tat1b) and Rev (pRC/CMV-rev1b), the viral core polyprotein and reverse transcriptase, encoded by the GAG and POL genes respectively (pHDM-Hgpm2), and the VSV-G (vesicular stomatitis virus) envelope glycoprotein (pHDM-G) (gift from the Lee lab, Harvard Gene Therapy Initiative, MA). Culture medium containing the lentiviral particles was used to transduce RAW264.7 cells. Pools of stably transduced cells were established by culture in the presence of hygromycin (100 µg/ml).

miR-29 Sponge Expression and Osteoclast Differentiation.

GFP and GFP_Sponge RAW264.7 cell lines were seeded at a cell density of 1,000 cells/well in 96-well plates, in DMEM supplemented with 10% FBS. 24 hours later, osteoclast differentiation was stimulated by treatment with 30 ng/ml RANKL in α -MEM supplemented with 10% FBS. The expression of the transgene was induced by addition of 500 ng/ml doxycycline (DOX) to the culture medium (Sigma-Aldrich). Osteoclast formation was evaluated by TRAP staining.

Cell Viability Assay.

GFP and GFP_Sponge RAW264.7 cells were plated in 96-well plates at 5,000 cells/well, in DMEM supplemented with 10% FBS. 24 hours later, cells were treated with 30 ng/ml RANKL, to induce osteoclastogenesis, in the presence or absence of 500 ng/ml doxycycline, to activate transgene expression. Cell viability was assessed over 3 days by MTS assay using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit, as indicated by the manufacturer's instructions (Promega).

Phagocytosis Assay.

GFP and GFP_Sponge RAW264.7 cells were cultured in α -MEM supplemented with 10% FBS in the presence of 500 ng/ml of doxycycline and 30 ng/ml of RANKL for 24 hours prior to the assay. Cells were then plated at 100,000 cells/well in 96-well plates, and allowed to adhere to the plate for 3 hours. Culture medium was replaced with a

solution of pHrodo *S. aureus* bacterial particles (250 µg/ml), which will emit fluorescence when phagocytosed (Life Technologies). Cells were incubated for 1 hour at 37°C, and nuclei were stained with DAPI. Cultures were analyzed by fluorescence microscopy. Phagocytosis of pHrodo particles was quantified by measuring fluorescence emitted at 590 nm, and normalized to DAPI fluorescence at 460 nm.

Macrophage Commitment Assay.

GFP and GFP_Sponge RAW264.7 cells were plated at 26,000 cells/cm² in DMEM supplemented with 10% FBS. Subsequently, cells were cultured in α -MEM supplemented with 10% FBS in the presence of 500 ng/ml of doxycycline and 30 ng/ml of RANKL for 24 hours. Total RNA was isolated from the cultures using the miRNeasy Mini kit (Qiagen, Valencia, CA), and the expression of macrophage marker genes was analyzed by qPCR as previously described. The primer sets used are shown in Supplemental Table S1.

Cell Migration Assay.

GFP and GFP_Sponge RAW264.7 cells were cultured in DMEM, in the absence of FBS, and in the presence of 500 ng/ml of DOX for 24 hours prior to the assay. Cells were then plated at 200,000 cells/well on 8 µm pore polycarbonate membrane inserts in 6.5 mm Transwells (Corning, Tewksbury, MA). 30 ng/ml M-CSF was added to the bottom chamber, as a chemotactic agent. Cells were incubated overnight, in the presence

of DOX. Cells that did not migrate were removed from the top side of the transwell membrane using a cotton swab. Upon fixation with 3.7% formaldehyde, cells on the bottom side of the transwell membrane were stained with 0.05% Crystal violet solution. Crystal violet stain was solubilized using 100% methanol, and optical density was quantified at 540 nm.

Apoptosis Assay.

GFP and GFP_Sponge RAW264.7 were plated at 3,125 cells/cm² in DMEM supplemented with 10% FBS. 24 hours later, culture medium was switched to α -MEM supplemented with 10% FBS, with the addition of 30 ng/ml of RANKL. After 2 days of differentiation, expression of the transgene was induced with 500 ng/ml of DOX. 2 days later, osteoclast apoptosis was assessed by Caspase-3 Colorimetric Assay Kit, as indicated by the manufacturer's instructions (GenScript, Piscataway, NJ).

Actin Ring Formation Assay.

GFP and GFP_Sponge RAW264.7 cells were seeded on glass chamber slides at 3,125 cells/cm², in DMEM supplemented with 10% FBS. 24 hours later, culture medium was switched to α -MEM supplemented with 10% FBS, with the addition of 30 ng/ml of RANKL and 500 ng/ml of doxycycline. End point cultures were fixed with 3.7% formaldehyde and F-actin was labeled with Rhodamine Phalloidin conjugate (Life Technologies). Nuclei were visualized by using mounting medium containing DAPI

(4',6-Diamidino-2-Phenylindole) (Invitrogen, Life Technologies). Cultures were analyzed by fluorescent microscopy.

Luciferase Constructs.

Gene-specific PCR primers were used to amplify from mouse genomic DNA template the coding sequences (CDS) or untranslated regions (UTRs) for Calcitonin Receptor, Tartrate resistant acid phosphatase (*Trap*), Cathepsin K, *Cdc42*, *Srgap2*, *Gpr85*, *Cd93*, and *Nfia*, which contained potential miR-29 binding sites (primer sequences are reported in Table 3.2). Using the appropriate restriction enzymes, these fragments were cloned downstream from Luciferase, in the Cytomegalovirus promoter-driven Luciferase

reporter vector
pMIR-REPORT
(Ambion, Austin,
TX). Site-directed
mutagenesis, to
delete putative miR-
29 binding sites in
selected constructs,
was performed by
overlap extension

Table 3.3. Primer sets for site-directed mutagenesis of miR-29 targets. Forward primers were designed to delete miR-29 binding sites (see Table 3.4), and used in combination with complementary reverse primers.	
RNA (MOUSE)	FW MUTAGENESIS PRIMER (5' – 3')
<i>Calcr</i> (site 2565)	gtttataaagcagatctagcagtaagagaa
<i>Cdc42</i> -CDS	aattaagtgtgttggtgtaaaacatgtct
<i>Srgap2</i>	ccccagctctgggagctgacgcctgtgaga
<i>Gpr85</i>	gtgggtgaacactagagtatcagtgtctaaa
<i>Cd93</i> (site 4403)	agccaatggagccactattttcacatatat
<i>Nfia</i> (site 2125)	ttttaacttttagggaaatggttgggctg

(primer sequences are reported in Table 3.3). Luciferase plasmids containing the regions of interest were used as templates for mutagenesis. All constructs were verified by sequencing.

Luciferase Activity Assay.

RAW264.7 cells were plated at 58,000 cells/cm². After 24 hours, cells were co-transfected with Luciferase constructs and a constitutively expressing β -Galactosidase construct, as a control vector for transfection efficiency (GenBank accession number U02451) (Clontech, Mountain View, CA) using BioT (BioT:DNA ratio 1.5 μ l:1 μ g). In selected experiments, 50 nM anti-miR-29-a or -c or negative control (scrambled) miRNA inhibitors were also transfected. 6 hours post-transfection, cells were treated with or without RANKL (30 ng/ml) in α -MEM supplemented with 10% FBS. Following 48 hours, cell lysates were harvested using Reporter Lysis Buffer (Promega). Samples were analyzed for Luciferase activity using Luciferase Assay System (Promega), and normalized to β -Galactosidase activity, which was assessed using Galacton® reagent (Applied Biosystem, Foster City, CA). Each Luciferase experiment was performed at least three times, using n=6.

Table 3.4. Potential miR-29 binding sites in genes important for the macrophage/osteoclast lineage. Putative miR-29 sites were identified using miRanda, DIANA-mirExTra, PicTar, and RNAhybrid. miR-29 family members have identical seed binding (miRNA bases 2-8), and the sequence for miR-29a is shown in the table. The position of the miR-29 site in the target mRNA sequence is indicated. The miR-29 sites mutated using the primers reported in Table 3.3 are indicated (\pm).

RNA	miR-29 site	Sequence
<i>Calcr</i>	2095	3' UUGGCUAAAGUCUA----CCACGAU 5' miRNA : 5' GACCUAGUUCAGAUACAGGGUGCUCC 3' RNA
<i>Calcr</i>	2565(\pm)	3' UUGGCUAAAGUCUA--CCACGAU 5' miRNA 5' UACCUCUUGCCUUGGGUGCUAU 3' RNA
<i>Trap</i>	133	3' AUUGGCUAAAGU--CUACCACGAU 5' miRNA 5' AGAUGGAU-UCAUGGGUGGUGCUGC 3' RNA
<i>Ctsk</i>	9764	3' AUUGGCUAAAGUCUACCACGAU 5' miRNA 5' UUUCUCCUCUC-GUUGGUGCUU 3' RNA
<i>Cdc42</i>	166(\pm)	3' AUUGGCUAAAGUCUACCACGAU 5' miRNA : : : 5' UGUUGGU----GAUGGUGCUGU 3' RNA
<i>Cdc42</i>	683	3' AUUGGCUAAA-----GUCUAC-CACGAU 5' miRNA : : 5' GAACCGAAGAAGAGCCGAGGUGUGCUGC 3' RNA
<i>Cdc42</i>	1281	3' AUUGGCUAAAGUCUACCACGAU 5' miRNA 5' AACGUGUCCCCACCGUGGUCUC 3' RNA
<i>Cdc42</i>	1686	3' UUGG-CUA-----AAG-----UCUACCACGAU 5' miRNA : : : : 5' GACCUGGUACCUAGGGUCCUAACGGGAUGGUGUUA 3' RNA
<i>Srgap2</i>	6093(\pm)	3' UGGCUAAAGUCU-----ACCACGAU 5' miRNA 5' CCUAGCUGUCUCCUUAUCUCUGGUGCUU 3' RNA
<i>Gpr85</i>	2498(\pm)	3' AUUGGCUAAAGUCUACCACGAU 5' miRNA 5' AUAACGAAAGUAGCAGGUGCUA 3' RNA
<i>Cd93</i>	2421	3' AUUGGCUAAAGUCUACCACGAU 5' miRNA 5' CGAAGGAGUU--ACUGGUGCUA 3' RNA
<i>Cd93</i>	4403(\pm)	3' AUUGGCUAAAGUCUA--CCACGAU 5' miRNA : : 5' UCACUGUCCUAAAUGGGUGCUU 3' RNA
<i>Cd93</i>	5279	3' AUUGGCUAAAGUCUACCACGAU 5' miRNA 5' UCAUCACCAGCUCAGGGUGCUA 3' RNA
<i>Nfia</i>	2125(\pm)	3' UGGC-----UAAAGUCUACCACGAU 5' miR-29 : : 5' GACUGUCGUAUUUCUCAUGGUGCUG 3' RNA
<i>Nfia</i>	3056	3' UGGCUAAAGUCUACCACGAU 5' miRNA 5' AUCUAAUGGUGCUU 3' RNA
<i>Nfia</i>	7184	3' UGGC-----UAAAGUCUACCACGAU 5' miRNA : : 5' GACUGUCGUAUUUCUCAUGGUGCUG 3' RNA

Data Analysis.

Data are presented as mean \pm SEM. Data were analyzed by Student's *t* test or one-way ANOVA with Bonferroni post-hoc test as appropriate (KaleidaGraph, Synergy Software, Reading, PA).

RESULTS

miR-29 expression increases during in vitro osteoclast differentiation.

We analyzed the expression of miR-29 family members in mouse bone marrow depleted of B220 and CD3 positive cells, and cultured in the presence of M-CSF and RANKL for up to 5 days. In these cultures, osteoclasts were evident by day 3, and osteoclast number and size were highest at day 5 (Figure 3.2B, C, and D). qRT-PCR showed that all the miR-29 family members, miR-29a, -b, and -c, were expressed at a similar level, and that their expression was not increased until between days 3 and 5 of differentiation (Figure 3.2A). In contrast, mRNA levels for the osteoclast markers TRAP and Cathepsin K were increased from day 1 to day 3, and sustained at day 5 (Figure 3.2E). RANK expression was already elevated at day 1, and maintained during the course of osteoclast differentiation, whereas NFATc1 levels peaked at day 3, and then decreased (Figure 3.2F).

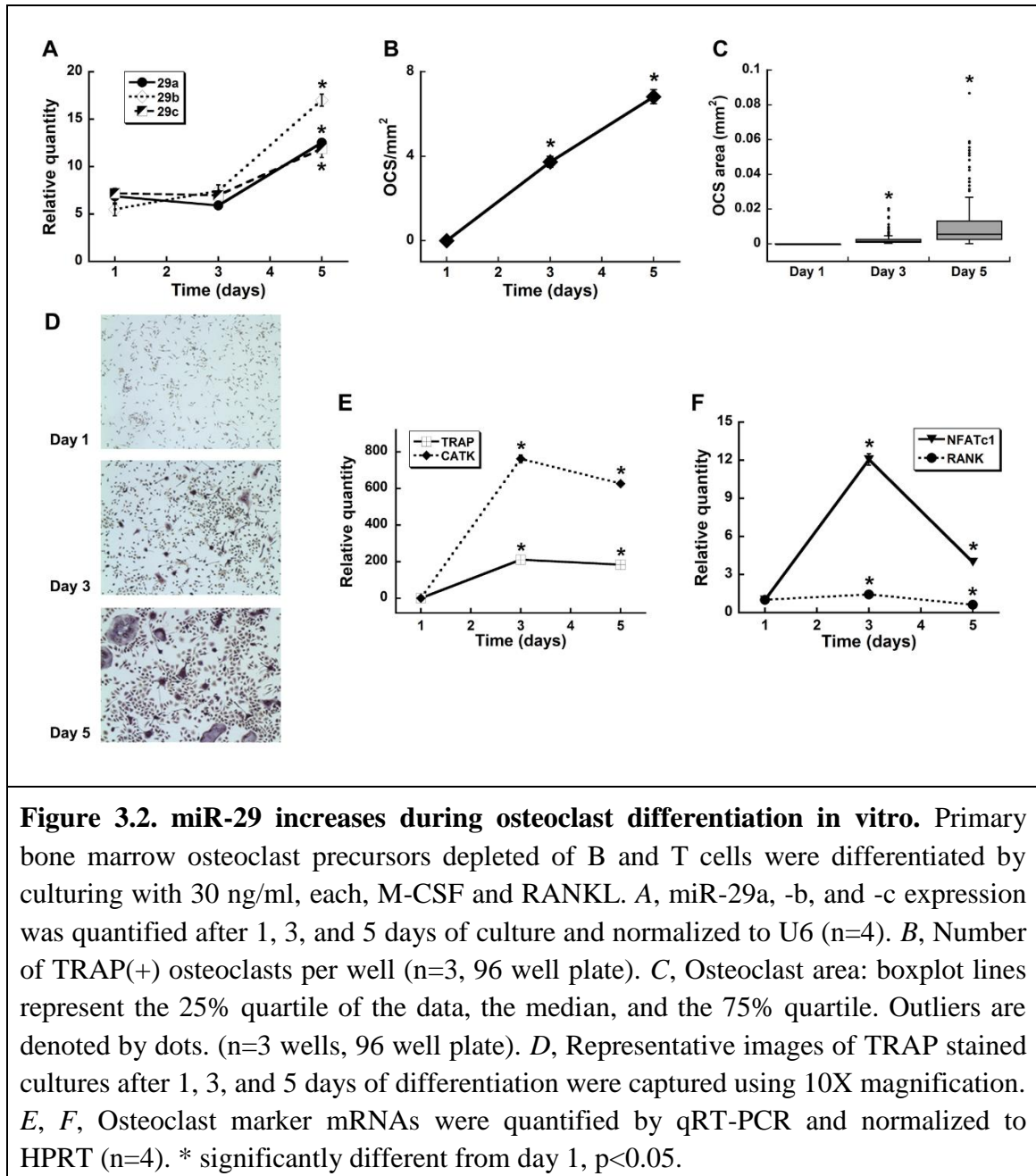


Table 3.5. miR-29 knock-down decreases osteoclast formation in vitro. Mature sequence of the non-targeting scrambled control (cel-miR-67) and the miR-29 family members is indicated. Divergent bases are underlined. Seed binding region (bases 2-8) is in *italic*. Primary BMMs were transfected with 50 nM anti-miR-29a, -b, or -c inhibitor or non-targeting scrambled control oligonucleotides. Cells were treated for 3 to 6 days with M-CSF (30 ng/ml) and RANKL (10 ng/ml). Osteoclast formation was evaluated by TRAP staining (n=4 wells, 96 well plate). * significantly different from 29a, -b, or -c inhibitor, p<0.01.

Inhibitor	Mature miRNA sequence	Day 3	Day 4	Day 5	Day 6
Scrambled	ucacaaccuccuagaaagaguaga	5.2 ± 1.6*	21.6 ± 6.3*	46.0 ± 4.3*	203.8 ± 17.9*
miR-29a	uagcacc <u>auc</u> gaaaucgguua	0	0	11.0 ± 1.7	17.3 ± 1.4
miR-29b	uagcacc <u>auu</u> gaaaucgguua	0.7 ± 0.5	2.8 ± 1.0	10.2 ± 1.9	17.8 ± 1.9
miR-29c	uagcacc <u>auu</u> gaaauc <u>ag</u> uguu	0	0	6.5 ± 2.3	17.8 ± 2.1

miR-29 is a positive regulator of osteoclastogenesis.

To investigate the role of miR-29 in osteoclast differentiation, we inhibited miR-29 activity in primary cultures of bone marrow-derived macrophage/osteoclast precursors (BMMs), using transiently transfected miR-29a, -b, or -c specific oligonucleotide inhibitors. Throughout the time course analyzed, miR-29 inhibition resulted in a significant reduction in the number of TRAP positive multinucleated osteoclasts compared to the scrambled, non-targeting control (Table 3.5; Figure 3.3A). These data suggest that miR-29 inhibition does not merely delay osteoclastogenesis, and that miR-29 activity is important for osteoclast formation. Consistent with these data, primary bone

marrow-derived precursors transduced with a retrovirus expressing a miR-29a inhibitor also showed decreased osteoclast formation compared with control (Figure 3.3B).

miR-29 family members have identical seed binding regions (miRNA bases 2-8). miR-29a and -29c differ by only one base, whereas miR-29b is more divergent. In the transient transfection studies shown in Table 1, the miR-29b inhibitor appeared to be somewhat less efficacious at the early time points, days 3 and 4. However, the isoform-specific inhibitors had similar effects at the later time points, days 5 and 6. Overall, the miR-29 isoform inhibitors had similar activity. Given the degree of conservation among the miR-29 isoforms, it may be difficult to tease out isoform-specific effects using an inhibitor strategy.

When BMMs were plated at a higher density, the formation of large osteoclasts (>8 nuclei) was significantly diminished in cells transiently transfected with miR-29c inhibitor [large osteoclast number (per well) in scrambled 108 ± 5 versus miR-29a inhibitor 74 ± 3 , $p < 0.01$]. This indicates that miR-29 knock-down negatively affects osteoclast size, suggesting that miR-29 activity plays a positive role in osteoclast maturation (Figure 3.3).

The primary cultures obtained from bone marrow are heterogeneous, even when depleted of lymphocytes (97). For this reason, we sought a more simplified and homogeneous model system for the purpose of evaluating the mechanisms by which miR-29 regulates osteoclast differentiation. Therefore, we chose to use the monocytic cell line RAW264.7. We first characterized the expression of miR-29 family members in these cells after treatment with RANKL for up to 4 days. We found that expression of all miR-29 family

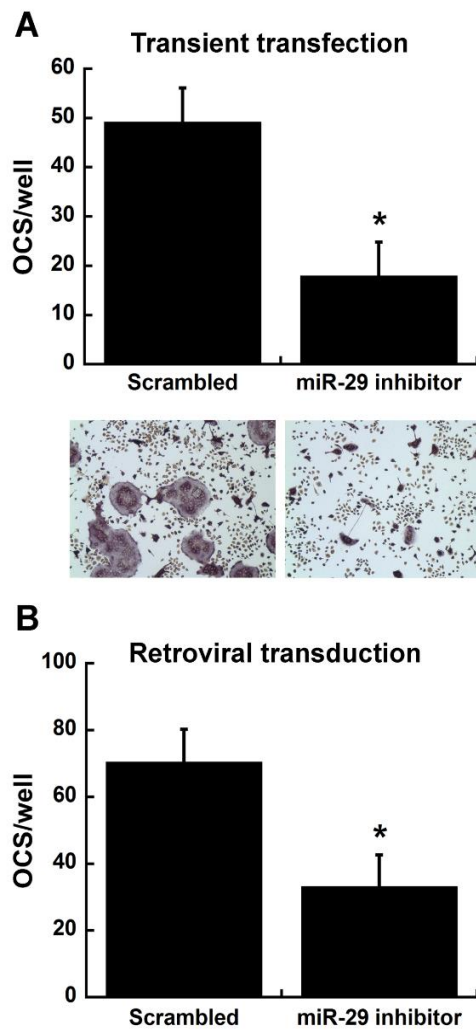


Figure 3.3. Inhibition of miR-29 represses osteoclast formation in vitro. *A*, BMMs were transiently transfected with a miR-29a inhibitor and differentiated for 3 days with M-CSF (30 ng/ml) and RANKL (10 ng/ml). Representative images of the cultures were captured using 10X magnification. *B*, BMMs were transduced with pSilencer retrovirus for miR-29a knock-down or a non-targeting scrambled control, and differentiated for 3 days. Osteoclast formation was evaluated by TRAP staining (n=6 wells, 96 well plate). * significantly different from scrambled, p<0.05.

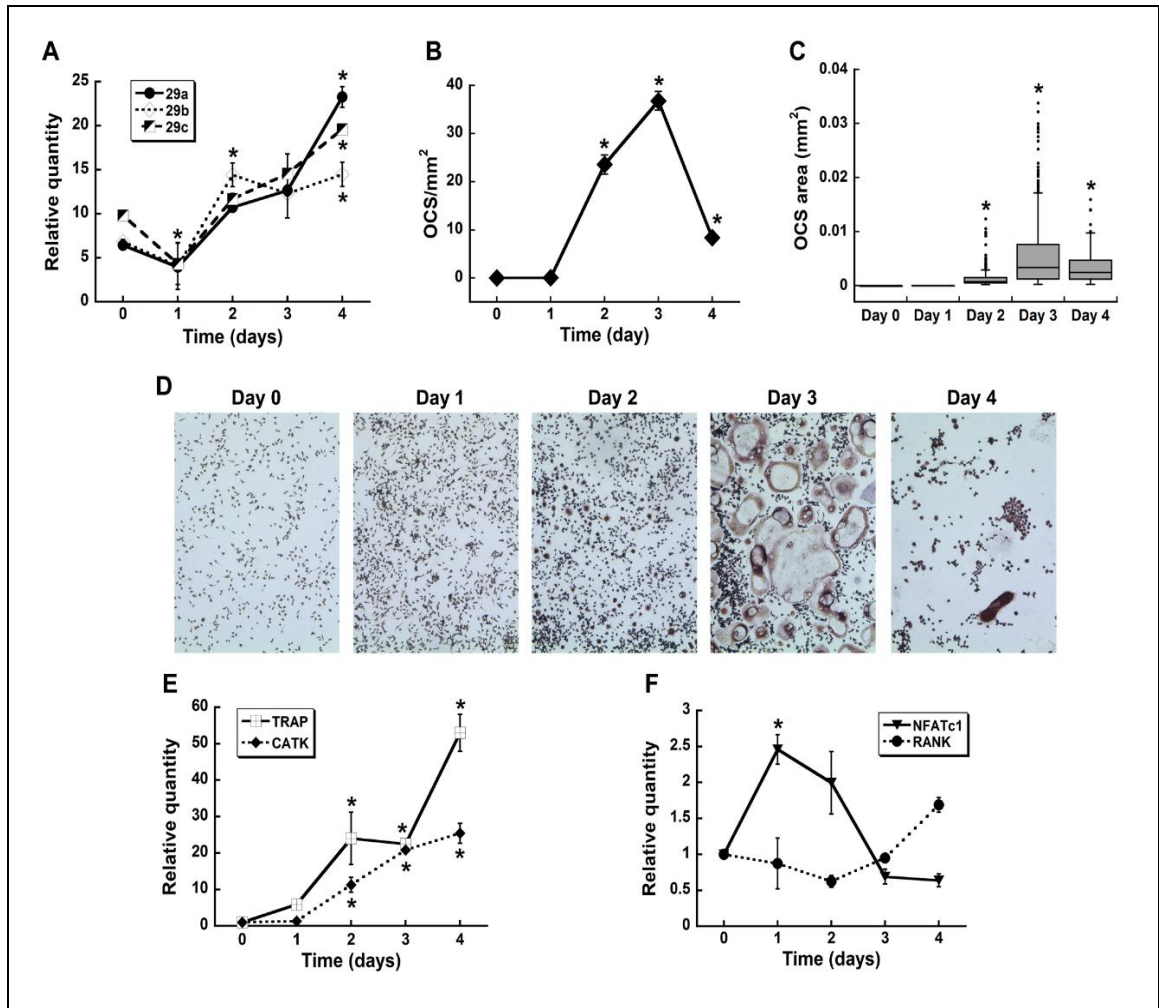
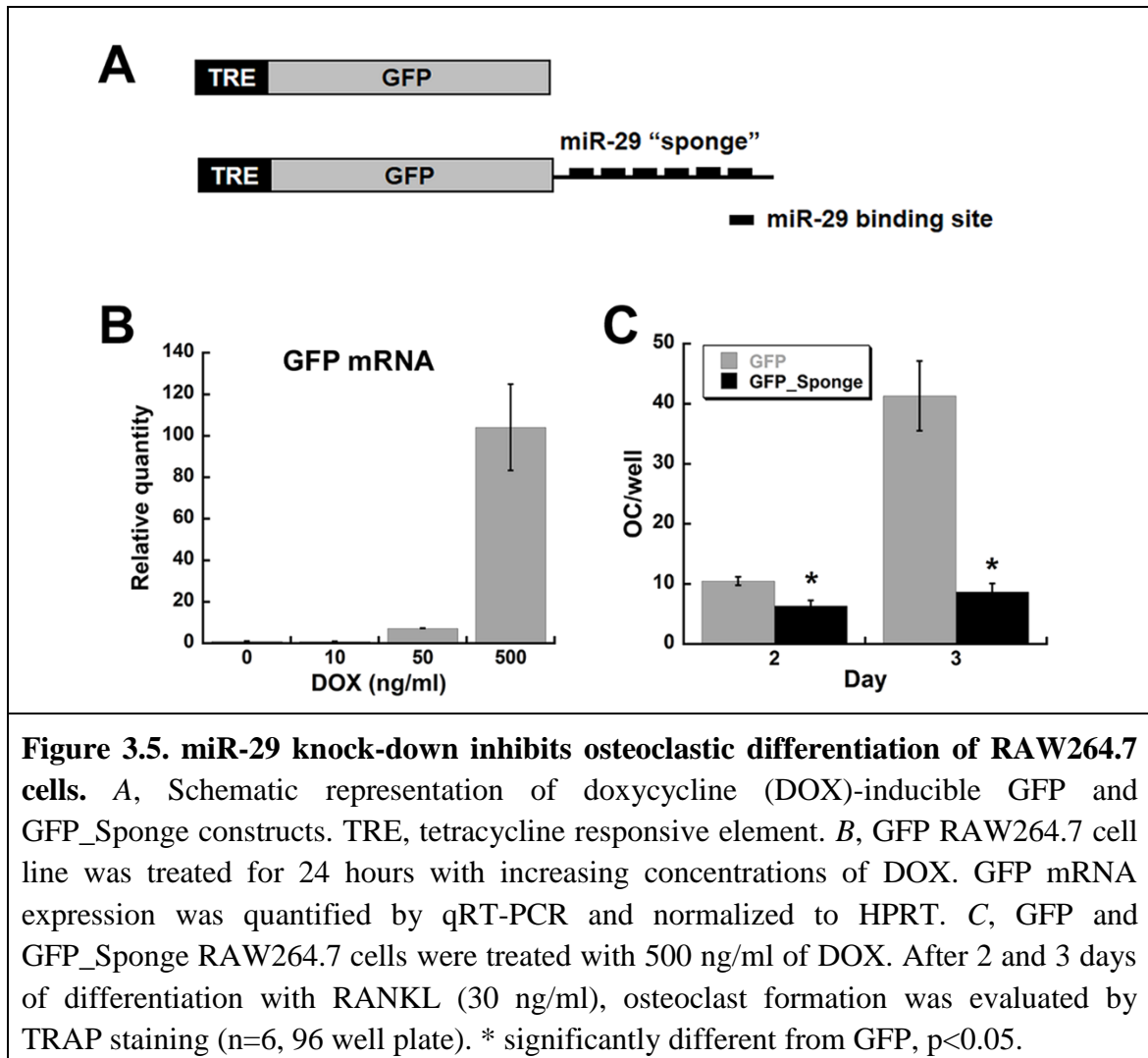


Figure 3.4. miR-29 expression in RAW264.7 cells recapitulates the pattern observed in primary osteoclast precursor cells. *A*, The expression of miR-29a, -b, and -c was analyzed over 4 days of differentiation with RANKL (30 ng/ml). *B*, Number of osteoclasts per well (n=3, 96 well plate). *C*, Osteoclast area: boxplot lines represent the 25% quartile of the data, the median, and the 75% quartile. Outliers are denoted by dots. (n=3 wells, 96 well plate). *D*, Representative images of TRAP stained cultures at days 0, 1, 2, 3, and 4 of differentiation, respectively, were captured using 10X magnification. *E*, *F*, Gene expression levels for osteoclast markers. * significantly different from day 0, $p < 0.05$.

members was modestly, but significantly decreased after 24 hours of RANKL treatment (Figure 3.4A). Levels of miR-29a, -b, and -c then increased as osteoclastogenesis progressed, in a trend similar to that observed in the primary cultures (compare with Figure 3.2A). In these cultures, osteoclast number and size peaked on day 3, and were decreased on day 4 due to apoptosis of mature osteoclasts (Figure 3.4B-D). As observed in the primary cells, the osteoclast marker genes, *Trap* and Cathepsin K, also increased with osteoclastic differentiation (Figure 3.4E). In contrast, RANK was highly expressed throughout the time course analyzed, and NFATc1 increased at day 1 of RANKL-treatment and was subsequently down-regulated (Figure 3.4F). These data suggested that RAW264.7 cells could be a valid surrogate for analyzing the mechanisms by which miR-29 regulates osteoclastogenesis.

Inhibition of miR-29 activity impairs osteoclastic differentiation of RAW264.7.

To further define the activity of miR-29 in osteoclastogenesis, we developed an inducible lentiviral knock-down construct, based on the miRNA “sponge” strategy (98). The miR-29 sponge consisted of 6 miR-29 binding sites cloned downstream of GFP, in a DOX-inducible lentiviral vector (Figure 3.5A). Expression of the GFP_Sponge RNA can work as a decoy or competitive inhibitor for all the members of the miR-29 family, which share sequence identity in the seed region (nucleotides 2-8 of the miRNA sequence). RAW264.7 cells were stably transduced with lentivirus harboring the miR-29 “sponge” (GFP_Sponge cells) or GFP alone (GFP cells).



To confirm DOX-inducible expression of the transgene, we quantified GFP mRNA levels in GFP cells treated for 24 hours with different doses of DOX. qRT-PCR verified that GFP RNA was induced in a dose-responsive fashion, in the presence of increasing concentrations of DOX (Figure 3.5B). The 500 ng/ml dose of doxycycline was chosen for the subsequent assays because it produced a level of transgene expression

similar to that of TRAP mRNA, while representing a DOX dose well below that recently reported to decrease osteoclast differentiation in bone marrow cells in vitro (100).

We used the GFP_Sponge RAW264.7 cells as a relatively homogeneous cell population to study the mechanisms by which miR-29 regulates osteoclastogenesis. In cells treated with DOX and RANKL, we observed reduced formation of TRAP-positive multinucleated cells in the GFP_Sponge cultures after 2 days, in comparison with the cells expressing the GFP transgene only (Figure 3.5C). After 3 days, this disparity was even more pronounced, as the formation of TRAP-positive multinucleated cells was increased only in the GFP RAW264.7 cultures. There was no difference in the ability of GFP and GFP_Sponge cells to form TRAP-positive multinucleated cells in the absence of DOX (data not shown). These results confirm the positive role of miR-29 in osteoclastogenesis, as observed in primary cells (Table 3.5; Figure 3.3).

To determine whether miR-29 knock-down in the GFP_Sponge cultures impaired

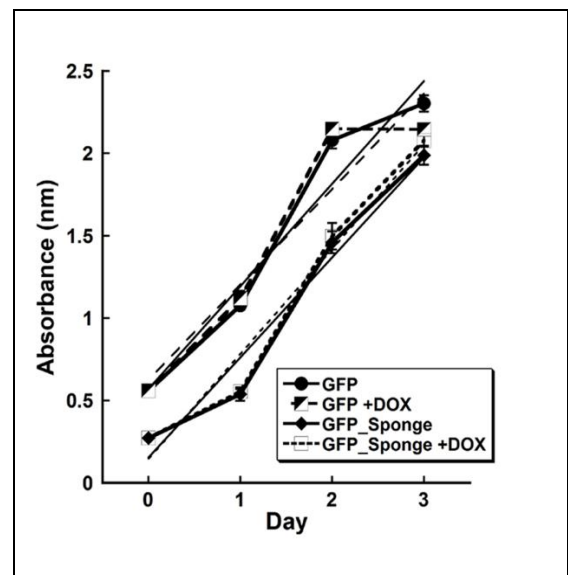


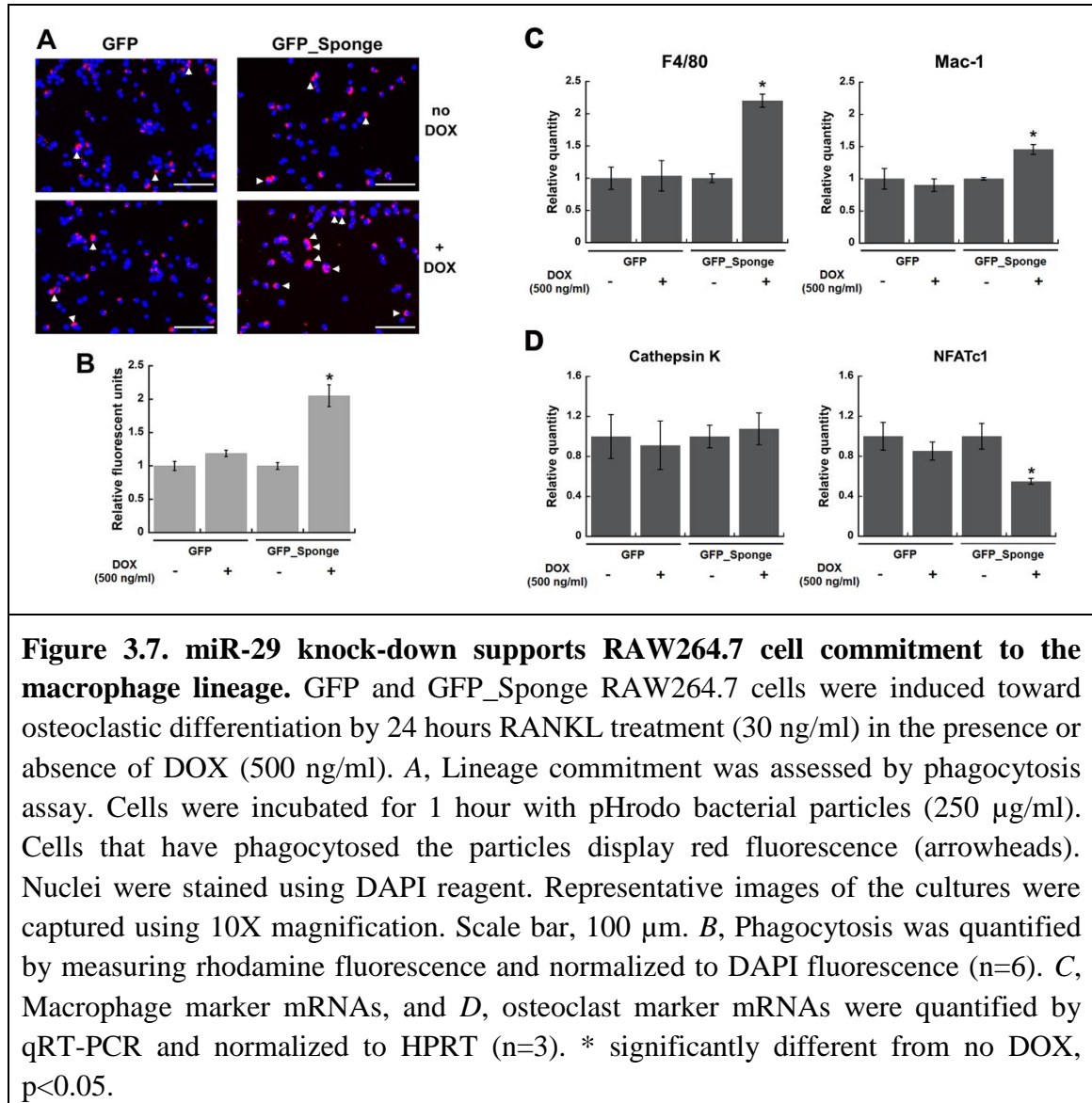
Figure 3.6. Inhibition of miR-29 does not affect cell viability. GFP and GFP_Sponge RAW264.7 cells were treated with RANKL (30 ng/ml) in the presence or absence of DOX (500 ng/ml). Cell viability was measured over 3 days by MTS assay (n=6). Growth curves in the presence or absence of DOX were superimposable. The lines without symbols illustrate linear growth curves with similar slopes.

osteoclast formation due to altered cell proliferation or viability, GFP or GFP_Sponge cells were cultured in the presence or absence of DOX for up to 3 days. Viable cells were quantified by MTS assay. All cultures displayed similar growth curves, suggesting that miR-29 did not promote osteoclast formation by regulating cell growth (Figure 3.6).

Inhibition of miR-29 activity impairs RAW264.7 cell commitment to the osteoclast lineage.

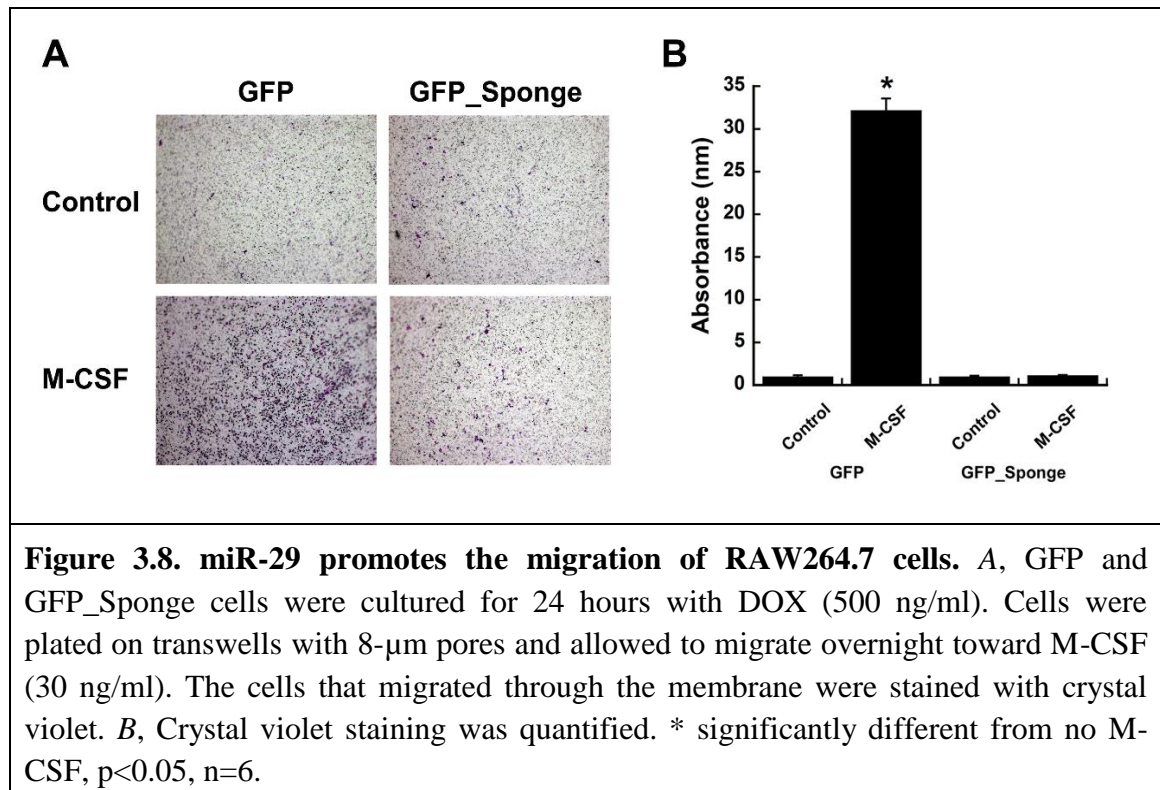
Some miRNAs regulate differentiation by promoting commitment to one cell fate at the expense of another. Since RAW264.7 cells have the potential to differentiate into osteoclasts or macrophages, we evaluated the effect of miR-29 knock-down on their lineage commitment. RAW264.7 cells have phagocytic properties, which are increased when committed to the macrophage fate. Therefore, we quantified phagocytosis in GFP and GFP_Sponge cells that had been induced toward osteoclastic differentiation by culture in the presence of RANKL for 24 hours. Phagocytosis was not altered in the GFP cells treated with DOX. However, when miR-29 activity was knocked-down in GFP_Sponge cultures treated with DOX, we observed increased phagocytic activity (Figure 3.7A, B). In addition, we analyzed the expression of genes associated with macrophage and osteoclast differentiation. DOX treatment of GFP_Sponge cells increased mRNA for macrophage markers F4/80 and Mac-1, and decreased mRNA for the early osteoclast marker NFATc1. In contrast, mRNA for the more mature osteoclast marker, Cathepsin K, was not affected (Figure 3.7C, D). These data suggest that miR-29

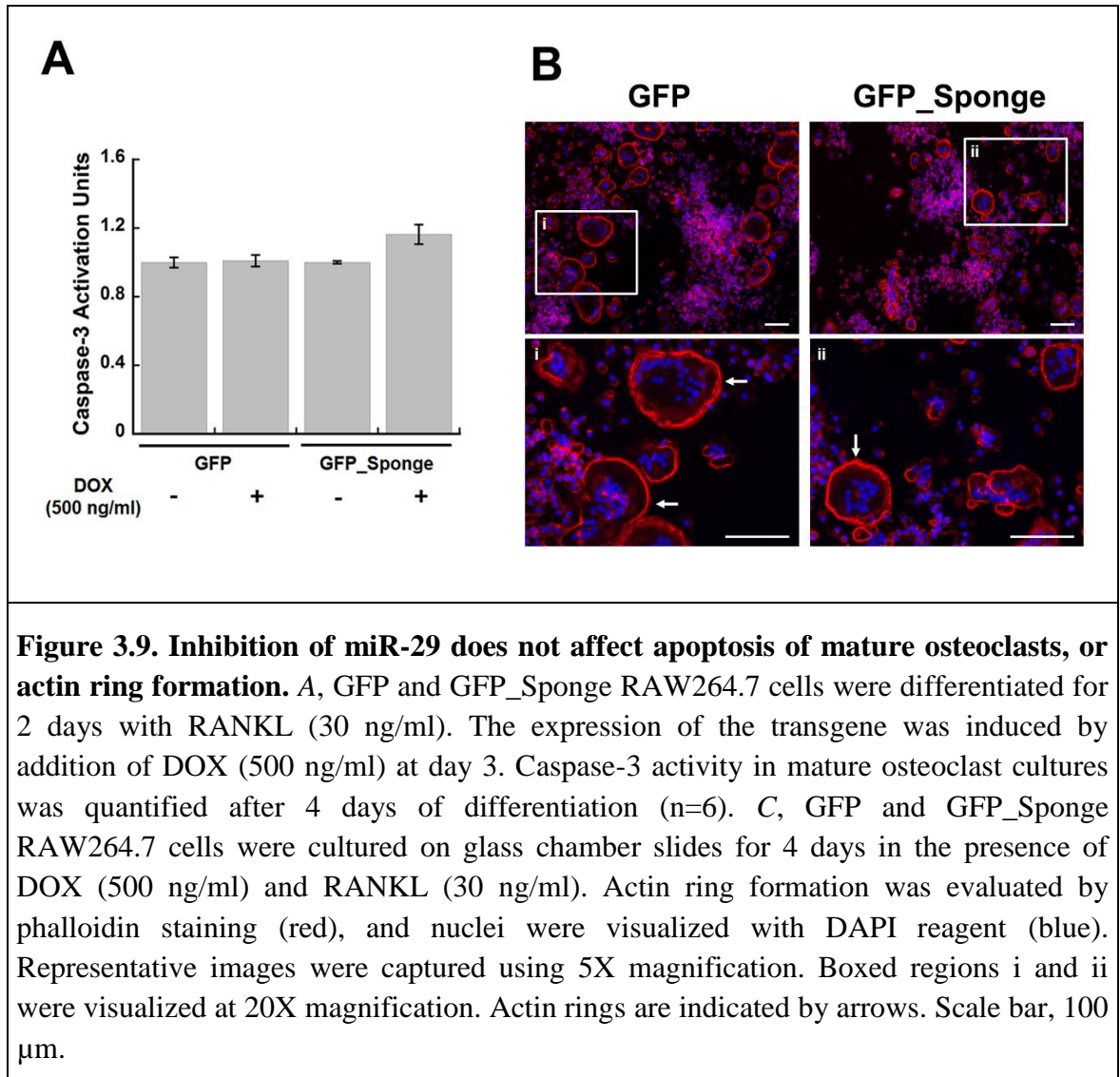
knock-down promotes the commitment of the RAW264.7 cell line to the macrophage lineage, at the expense of osteoclastogenesis.



Inhibition of miR-29 activity impairs RAW264.7 migration.

In order to form osteoclasts, the migration of precursor cells is critical. We analyzed the ability of GFP and GFP_Sponge cells to migrate in response to a chemotactic stimulus using a modified Boyden Chamber (Transwell) assay. We found that GFP-expressing RAW264.7 cells displayed robust migration toward M-CSF supplemented culture medium (Figure 3.8). However, expression of the miR-29 sponge completely abrogated the capability of these osteoclast precursor cells to respond to the chemotactic agent, strongly indicating that miR-29 expression supports cell migration (Figure 3.8).





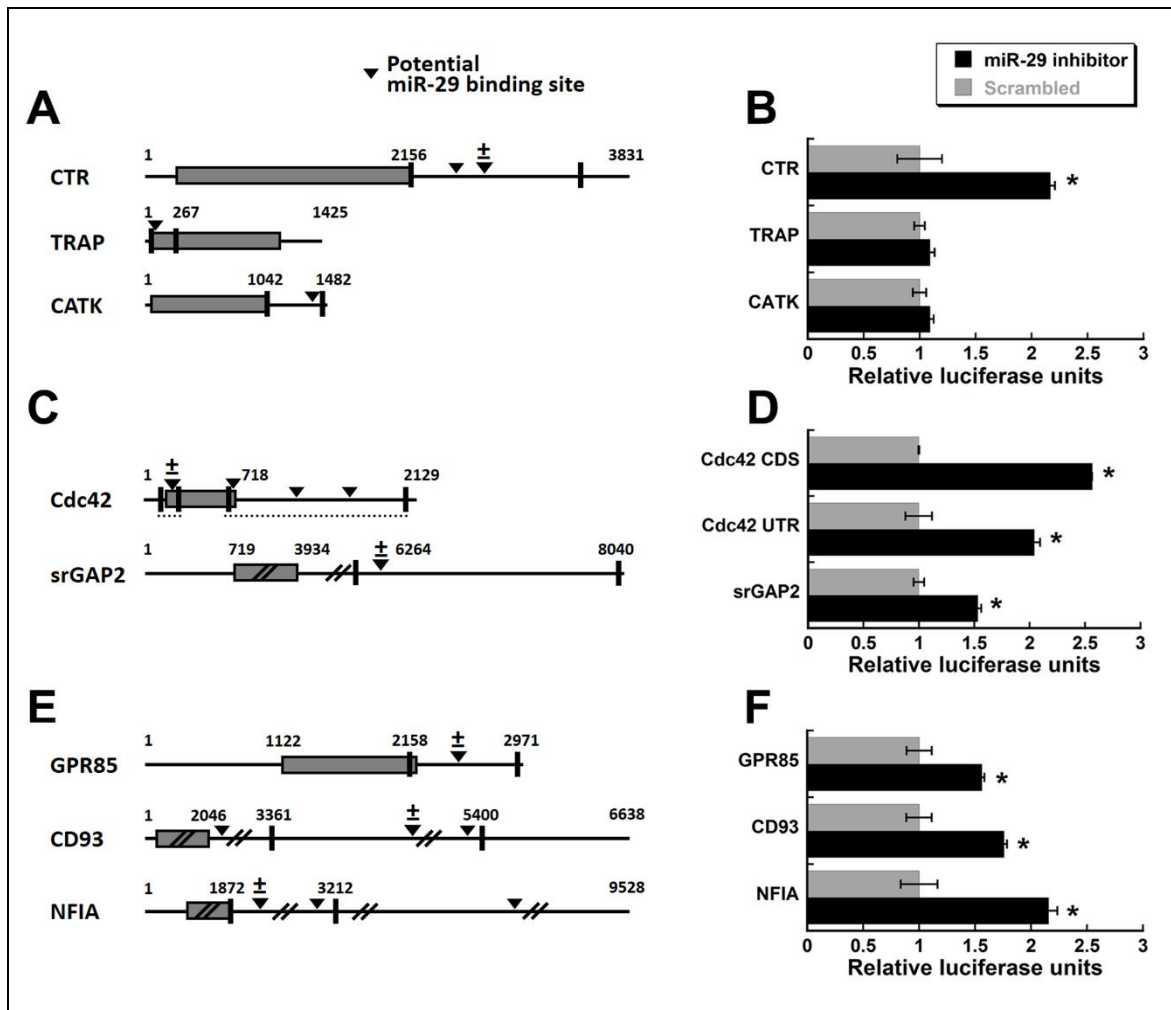


Figure 3.10. Luciferase analysis of miR-29 targets. Potential miR-29 targets were identified using a bioinformatic approach. Putative miR-29 binding sites (arrow heads) are present in the coding sequence and/or in the 3'UTR. The corresponding regions (between the vertical lines) were cloned into the pMIR-REPORT Luciferase vector, downstream of the Luciferase gene. The “±” symbol denotes the binding site deleted for the constructs shown in Figure 9. *A*, Putative miR-29 binding sites were identified in genes important for osteoclastogenesis. *B*, Luciferase activity was quantified in RAW264.7 cells co-transfected with a miR-29c inhibitor or a scrambled non-targeting control, and normalized to β-Galactosidase activity. Cells were treated with RANKL (30 ng/ml) for 48 hours after transfection. *C*, Putative miR-29 binding sites were identified in genes important for cytoskeletal remodeling and cell migration. *D*, Luciferase activity. *E*, Potential miR-29 target genes associated with the macrophage lineage. *F*, Luciferase activity.* significantly different from scrambled, $p < 0.05$, $n = 6$.

Inhibition of miR-29 activity does not affect the apoptosis of mature osteoclasts or actin ring formation.

Osteoclasts are terminally differentiated cells, and their ultimate fate is to undergo apoptosis (i.e. Figure 3.4B). It is possible that increased apoptosis could contribute to the decrease in number and size of osteoclastic cells observed in the presence of the miR-29 sponge. Therefore, we analyzed Caspase-3 activity in GFP and GFP_Sponge cells cultured for 4 days in the presence of RANKL. The miR-29 sponge was only expressed during days 3 and 4 of culture, to allow initiation of osteoclastic differentiation. We did not detect a significant difference in Caspase-3 activity in cells expressing the miR-29 sponge, compared with the other groups (Figure 3.9A). This suggests that miR-29 knock-down does not affect the survival of mature osteoclasts.

The formation of actin rings is a critical step for osteoclast-mediated bone resorption. To determine whether miR-29 knock-down affects actin ring formation, the GFP and GFP_Sponge were cultured for 4 days in the presence of RANKL and subjected to phalloidin staining. Although fewer and smaller multinucleated TRAP-positive cells were identified in miR-29 sponge cultures, their actin ring structures were intact (Figure 3.9B panels i, ii).

miR-29 targets RNAs important for the macrophage/osteoclast lineage.

Our functional assays indicated that miR-29 is important for osteoclastogenesis, and promotes cell migration and osteoclast commitment. To better understand the

underlying mechanisms, we focused on identifying the mRNAs that are targeted by miR-29 and whose functions are important in the macrophage/osteoclast lineage. We analyzed a list of genes expressed in osteoclastic cells (S-K Lee, unpublished data) for potential miR-29 targets, using several different algorithms for miRNA target prediction (miRanda, DIANA-mirExTra, PicTar, RNAhybrid). The list of potential targets was refined based on the ability of the miRNA to base pair with the target mRNA. We chose to clone and analyze 8 candidate genes with a well documented role in osteoclasts (Figure 3.10A), or with a role in cell migration (Figure 3.10C), or with a role in the macrophage lineage (Figure 3.10E) (Tables 3.2, 3.4).

We cloned the sequences containing the potential binding sites for miR-29 into a Luciferase reporter vector. Most constructs contained regions of interest ≥ 1 kb in length (Figure 3.10A, C, E). In these constructs, luciferase expression was driven by a strong, constitutive promoter, with the cloned regions serving as 3' UTR for the Luciferase gene. Therefore, luciferase activity represents the regulatory activity of the sequence of interest. RAW264.7 cells were transiently transfected with the luciferase reporter plasmids and miR-29c inhibitor oligonucleotides. Increased luciferase activity in the presence of the miR-29 inhibitor would suggest that miR-29 targets that RNA region.

Of the RNAs with a well known function in osteoclastic cells, Calcitonin receptor (*Calcr*, *Ctr*), *Trap*, and Cathepsin K (*Ctsk*, *Catk*) had potential miR-29 binding sites. The potential miR-29 binding site in the *Trap* RNA was in the coding region, while those for *Calcr* and *Ctsk* were in the 3' UTR. There were 2 potential miR-29 binding sites in the *Calcr* 3' UTR. miR-29c inhibitor only increased luciferase activity from the construct

containing the *Calcr* 3' UTR, suggesting that *Calcr* RNA is targeted by miR-29, whereas *Trap* and *Ctsk* are not (Figure 3.10B). The potential miR-29 binding site at base 2565 in the *Calcr* 3' UTR had the most complementarity to miR-29 family members. When we deleted this miR-29 binding site, the ability of the miR-29 inhibitor to increase luciferase activity was lost, indicating that this sequence is specific for miR-29-mediated regulation (Figure 3.11A).

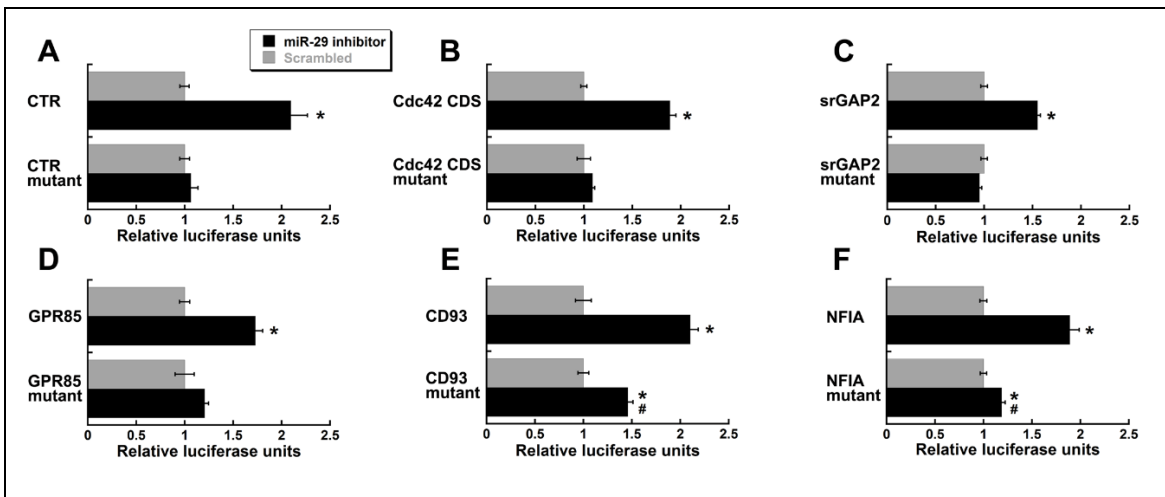


Figure 3.11. Deletion mutants of miR-29 binding sites. Putative miR-29 binding sites (marked with “±” in Figure 3.10) in the miR-29 target genes were deleted from pMIR-REPORT Luciferase vectors. Luciferase activity was quantified in the RAW264.7 cells co-transfected with a miR-29c inhibitor or a scrambled non-targeting control, and normalized to β -Galactosidase activity. Cells were treated with RANKL (30 ng/ml) for 48 hours after transfection. * significantly different from scrambled, # significantly different from 29c inhibitor in the wild type vector, $p < 0.05$, $n = 6$.

For RNAs important for cytoskeletal organization, we analyzed Cell Division Control protein 42 (*Cdc42*) and SLIT-ROBO Rho GTPase activating protein 2 (*Srgap2*) (Figure 3.10C). *Cdc42* is important for osteoclast function and migration and *srGAP2* participates in the same signaling pathway as *Cdc42* (35). Although expressed in

osteoclasts, srGAP2 has not been studied in the osteoclast lineage. There were potential miR-29 binding sites in the coding region and in the 3' UTR of *Cdc42*, and the miR-29 inhibitor increased luciferase activity from constructs containing either region, as well as the construct carrying the *Srgap2* 3' UTR (Figure 3.10D). Deletion of the potential miR-29 binding site in the coding region of *Cdc42* abolished the ability of the miR-29c inhibitor to relieve repression of luciferase activity, indicating that this is a miR-29 binding site (Figure 3.11B). Similarly, deletion of the potential miR-29 binding site in the *Srgap2* 3' UTR construct abrogated the response of the construct to miR-29c inhibitor, indicating that the *Srgap2* 3' UTR is targeted by miR-29 (Figure 3.11C). Deletion mutagenesis was not performed on the mouse *Cdc42* 3' UTR construct

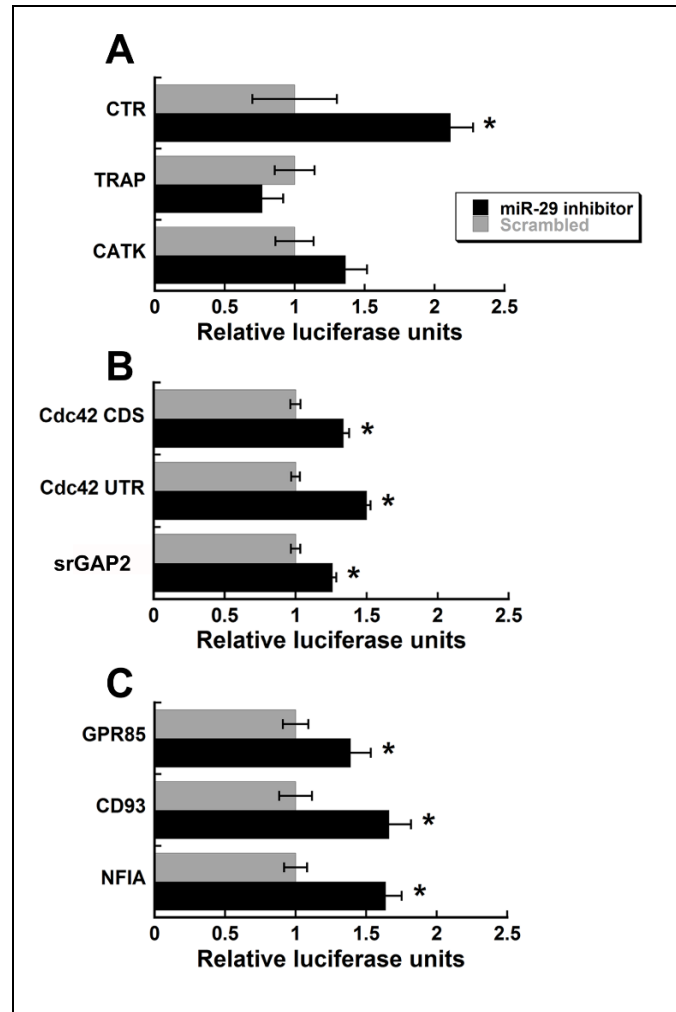


Figure 3.12. Luciferase analysis of miR-29 targets in the absence of RANKL. The luciferase constructs depicted in Figure 8 were co-transfected in RAW264.7 cells with a miR-29c inhibitor or a scrambled control. Luciferase activity was quantified and normalized to β -Galactosidase activity. * significantly different from scrambled, $p < 0.05$, $n = 6$.

because functional miR-29 binding sites in the human 3' UTR were previously reported (101).

With regard to genes associated with the macrophage lineage, we chose to examine G protein-coupled receptor 85 (*Gpr85*), Nuclear Factor I/A (*Nfia*), and *Cd93* (Figure 3.10E). NFIA expression inhibits both macrophage and osteoclast maturation, whereas GPR85 and CD93 are expressed during macrophage differentiation (53, 69, 102, 103). *Nfia* and *Cd93* had each 2 potential miR-29 binding sites in the 3' UTR segment analyzed, while *Gpr85* had one site. The miR-29c inhibitor increased luciferase activity for *Gpr85*, *Cd93*, and *Nfia* constructs, suggesting that these UTRs are miR-29 targets (Figure 3.10F). Deletion of the potential miR-29 binding site in the *Gpr85* 3' UTR eliminated the miR-29c inhibitor-mediated increase in luciferase activity, indicating that this site is directly regulated by miR-29 (Figure 3.11D). For *Cd93* (*ClqRp*), we deleted the potential miR-29 binding site at base 4403, and for *Nfia*, we deleted the miR-29 site at 2125. These sites were chosen because, of the 2 present in the UTR construct, they had the most complementarity to miR-29 family members. The ability of the miR-29c inhibitor to relieve repression of luciferase activity in the *Cd93* and *Nfia* mutant constructs was significantly decreased, but not totally abolished, likely due to the remaining functional miR-29 binding site in the construct (Figure 3.11E, F) (Table 3.4).

It should be noted that the transfection studies shown in Figure 10 were performed in cells treated with RANKL, and similar trends were also noted in the absence of RANKL (Figure 3.12). Moreover, similar results were obtained when miR-29a inhibitor was used instead of miR-29c inhibitor (data not shown). Overall, miR-29

may promote osteoclastogenesis by repressing RNAs important for differentiation to the alternative macrophage lineage. miR-29 targeting of RNAs important for osteoclast function and actin remodeling may allow subtle regulation of the rate of osteoclast differentiation (Table 3.6).

DISCUSSION

Osteoclast commitment and maturation is an intricate, multi-step process, modulated by the combined activity of numerous signaling pathways. Since miRNAs can control the expression of several genes working in one or multiple pathways, it is likely that miRNAs orchestrate many of the changes in gene expression or activity necessary for osteoclast differentiation. In this study, we demonstrate that miR-29 plays a positive role in osteoclastogenesis. Its expression increases during differentiation, and miR-29 knock-down impairs migration, commitment, and osteoclastogenesis. Our study is unique in that we validated a set of 6 novel miR-29 targets, which will contribute to our understanding of miR-29 function in osteoclasts and in other cell types (Table 3.6).

The miR-29 family consists of four genes that encode 3 mature miRNAs. These genes are organized in genomic clusters: miR-29a and miR-29b-1 are transcribed as a single polycistronic primary transcript from mouse chromosome 6, and miR-29b-2 and miR-29c are also transcribed as a polycistronic transcript from chromosome 1 (104, 105). The three mature miRNAs of this family, miR-29a, -b, and -c, present high sequence

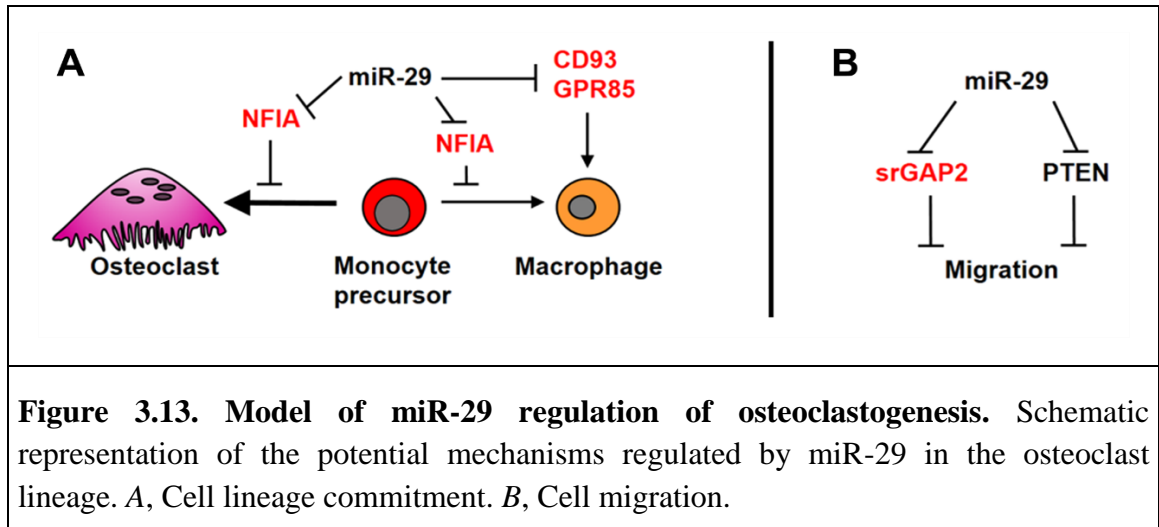
conservation and genomic organization in mouse, rat, and human, and nucleotides in positions 2-8, seed bases which are important for target recognition and binding, are identical (Table 3.5) (106). Although miR-29 family members may have overlapping targets, the mature miR-29 family members can be expressed at different levels, suggesting distinct transcriptional or post-transcriptional regulation of these genes (107, 108).

The expression of miR-29 family members increased during the osteoclast differentiation process, in both primary cultures and in RAW264.7 cells, and miR-29 knock-down decreased osteoclast formation, suggesting that this miRNA family plays a positive role in differentiation. Studies from our laboratory and others demonstrate a positive role of miR-29 in the differentiation of other lineages, including osteoblastic and myogenic (87, 89, 109, 110). More recently, miR-29 was identified as one of 7 miRNAs that, in concert, can restrict proliferation and promote differentiation (111). Thus, the increase in miR-29 expression seen in the later stages of osteoclast differentiation may be in response to RANKL-induced differentiation program and withdrawal from the cell cycle.

The *Calcr* 3' UTR is targeted by miR-29, and CTR plays an important role in osteoclast function and cell survival. CTR is a G protein-coupled receptor that mediates the anti-apoptotic effect of calcitonin on mature osteoclasts, while inhibiting their resorption activity (112). Thus, the targeting of *Calcr* by miR-29 in mature osteoclasts could promote resorption.

Table 3.6. Newly validated miR-29 targets and their biology.	
GENE	BIOLOGY
<i>Calcr, Ctr</i> <i>Calcitonin receptor</i>	Calcitonin decreases resorption and increases OC survival (112).
<i>Cdc42</i> <i>Cell division cycle 42</i>	Promotes OC survival and differentiation. Regulates rate of actin ring formation (113).
<i>Srgap2</i> <i>SLIT-ROBO Rho GTPase</i> <i>activator protein</i>	Induces membrane protrusions. Knock-down reduces cell adhesion and increases migration (114, 115).
<i>Gpr85</i> <i>G protein-coupled receptor</i> <i>85</i>	Transiently induced by LPS in macrophages (103, 116).
<i>Cd93</i> <i>complement component 1q</i> <i>receptor 1</i>	Promotes differentiation of monocytes into macrophages (102).
<i>Nfia</i> <i>nuclear factor I/A</i>	Negatively controls c-Fms. Over-expression inhibits osteoclast formation. Increased in <i>Dicer</i> ^{-/-} null osteoclast precursors (53, 69).

We identified 3 new miR-29 targets that may be involved in commitment of precursor cells to the osteoclast lineage: *Nfia*, *Cd93*, and *Gpr85* (Figures 3.10 and 3.11, Table 3.6). Transcript levels for these genes are decreased in primary cultures of osteoclast progenitors treated for 4 days with RANKL (S.K. Lee, unpublished data). NFIA is known to repress the differentiation of hematopoietic cells, including granulocytes and monocytes, and NFIA is a negative regulator of the M-CSF receptor in osteoclasts (68, 69). Since the M-CSF receptor is a positive regulator of osteoclastogenesis, the targeting of *Nfia* by miR-29, like miR-223, could contribute to differentiation (Figure 3.13A) (53).



In hematopoietic cells, the role of the other two miR-29 targets, CD93 and GPR85, is less well characterized. However, it is known that CD93 (C1qRp) is a transmembrane receptor regulating phagocytosis and cell adhesion, and is present on cells of the myeloid lineage (117). CD93 expression is increased with monocyte differentiation and macrophage activation (102, 118). Targeting of *Cd93* by miR-29 could promote commitment to osteoclastogenesis, preventing monocytic differentiation. GPR85, also called SREB2 (superconserved receptor expressed in brain 2) is a G protein-coupled receptor abundant in neurons, and involved in determining brain size and functionality (116). In macrophages, GPR85 expression increases upon inflammatory stimulation with LPS (lipopolysaccharide) (103). Here, we showed that inhibition of miR-29 activity promotes the commitment of the RAW264.7 cells to the macrophage fate (Figure 3.7). It is possible that down-regulation of NFIA, CD93, and GPR85 by miR-29 could play a role in decreasing the potential of the cell to differentiate into the macrophage lineage, thus promoting osteoclastogenesis (Figure 3.13A).

Osteoclast migration and fusion require extensive cytoskeletal reorganization, as does polarization and actin ring formation, initial steps for bone resorption. In this study, we demonstrated that knock-down of miR-29 family members, through the expression of an inducible miRNA sponge construct, suppressed the chemotactic migration of RAW264.7 cells (Figure 3.8). Decreased cell motility in the presence of the miR-29 sponge likely contributed to the observed decrease in osteoclast size. However, not all functions involved in cytoskeletal remodeling, such as actin ring formation, were affected by knock-down of miR-29 activity (Figure 3.9B). It is possible that the factors or pathways specific for regulating actin ring formation may be less affected by miR-29 in osteoclasts.

Cdc42 was identified as a miR-29 target in humans, and we confirmed miR-29 targeting of the mouse homolog (Figures 3.10 and 3.11) (101). *Cdc42* is a small GTPase that regulates actin remodeling, as well as cell cycle control and survival (119). Although not required for actin ring formation, *Cdc42* regulates the rate of formation, as well as cell polarization (113). *Cdc42* also regulates migration by controlling podosome turnover, and it is important for the movement of hematopoietic progenitors and macrophages toward chemotactic signals (120, 121). Although *Cdc42* is critical for osteoclast formation and survival (113), *Cdc42* transcript levels in osteoclasts are less than those found in macrophages or monocytes (122). Further, the levels of *Cdc42* mRNA do not change dramatically during osteoclastic differentiation (123). It is possible that translational regulation by miR-29 family members could play a role in fine-tuning the *Cdc42* levels during osteoclastogenesis. However, it is also important to consider that

Cdc42 transcripts are subject to alternative splicing, which can give rise to alternative 3' UTRs. There is little known about *Cdc42* splice variants in cells of the osteoclast lineage. It is possible that alternative 3' UTR usage may be one means to vary the ability of *Cdc42* to be targeted by miRNAs.

srGAP2 is one of the novel miR-29 targets identified in this study. srGAP2 is a Rho-GTPase activating protein that participates in the same signaling pathway as Cdc42 (114). Although srGAP2 function in osteoclasts has not been investigated, it has been shown to repress cell migration during neuronal development (114, 124) (Figure 3.13, Table 3.6). Knock-down of miR-29 activity could decrease cell motility, in part, by causing an increase in srGAP2. Intriguingly, in other cell systems, miR-29 was shown to target PTEN (tumor-suppressor phosphatase and tensin homolog), a lipid phosphatase involved in the phosphatidylinositol metabolism. Recent studies identified the suppression of PTEN as one mechanism by which miR-29 promotes migration in endothelial cells, breast cancer cells, and hepatocellular carcinoma cells (125). In RAW264.7 cells, activation of PTEN inhibits RANKL-mediated osteoclastogenesis and osteopontin-induced migration (126). It is possible that miR-29 knock-down could increase PTEN levels, and contribute to repressed migration of RAW264.7 cells (Figure 3.13).

It should be noted that our results appear to conflict with a recent report that lentiviral mediated over-expression of miR-29b in human CD14⁺ peripheral blood mononuclear cells reduced osteoclast formation (127). In that paper, Rossi et al. reported that miR-29b expression decreased during osteoclast formation, and that constitutive

over-expression of miR-29b decreased the expression of osteoclast marker genes and impaired collagen degradation. However, the miR-29 over-expression study by Rossi et al. differs from our work in several key areas. First, Rossi et al. used semi-sorted circulating human osteoclast precursors from the periphery, whereas we used murine bone marrow resident osteoclast precursors, which may not circulate. Second, the study by Rossi et al. did not report on the expression of other miR-29 family members, which should be present, since miR-29b is transcribed on the same pri-miRNA as miR-29c and miR-29a. Third, Rossi et al. over-expressed miR-29b. It has been shown that super physiological expression of a particular miRNA can alter global recruitment of miRNAs to the RISC (RNA-induced silencing complex), which can confound interpretation of a resulting cell phenotype (128, 129). Further, knock-down of components important for miRNA processing, such as DGCR8, Ago2, and Dicer1, inhibits osteoclastogenesis (53). It is possible that the miR-29b precursor over-expressed by Rossi et al. could compete for miRNA processing machinery, providing an alternative explanation for the inhibition of osteoclastogenesis observed by those investigators.

In our study, we observed similar inhibitory effects on osteoclastogenesis when miR-29a, -29b or -29c were individually targeted by transiently transfected inhibitors (Table 3.5, Figure 3.3) and when their activity was inhibited by the miR-29 sponge competitive inhibitor (Figure 3.5C). These data, and the rest of the data herein, strongly support the conclusion that miR-29 family members promote osteoclastogenesis by several mechanisms. Whereas some miRNAs may act as “switches” for the commitment to one cell fate or another, many miRNAs are more subtle regulators of gene expression,

modulating the amplitude and tempo of a differentiation program (53, 58). miR-29 family members are likely subtle regulators of multiple osteoclast mRNA targets.

In conclusion, we demonstrated that miR-29 family members sustain migration and commitment of the precursor to osteoclastogenesis, and we validated 6 novel targets for this miRNA family. These data contribute to our understanding of the basic mechanisms regulating osteoclast differentiation, and provide insight into the function of miR-29 family members in cells of the hematopoietic lineage and in other tissue types. Dysregulation of miR-29 family members is implicated in the pathology of multiple malignancies, and in conditions such as diabetes and fibrosis, and aging (130). Additional studies, in vivo, will better define the role of miR-29 in osteoclastogenesis. It is possible that increased miR-29 levels could contribute to increased osteoclast formation with aging (131).

CHAPTER 4:

Pathway analysis of microRNA expression profile during murine osteoclastogenesis

CHAPTER ABSTRACT

Osteoclasts are multinucleated cells specialized in degrading the mineralized bone matrix. Osteoclast differentiation and function are tightly regulated, to prevent excessive or insufficient bone resorption. Several control mechanisms participate in modulating osteoclastogenesis, and an increasing number of reports describe the role of microRNAs (miRNAs) in this process. Disrupting the expression of specific miRNAs can result in alterations of osteoclast formation and bone homeostasis. We and others have previously characterized 9 miRNAs whose levels change during osteoclast differentiation, and identified some of the target genes that mediate their function. However, little is known about changes in the miRNA expression profile during osteoclastogenesis. In this study, we isolated a murine primary bone marrow population enriched for osteoclast precursors, and used the Agilent microarray platform to analyze the expression of mature miRNAs after 1, 3, and 5 days of RANKL-driven differentiation. 93 miRNAs showed greater than 2 fold-change during these early, middle, and late stages of osteoclastogenesis. Further, we compared the miRNA expression profile in cultures differentiated for 3 days with M-

CSF and RANKL and undifferentiated cells cultured with M-CSF alone. 17 miRNAs showed more than 2 fold-change with RANKL treatment. Many of the miRNAs differentially regulated in the array were detected for the first time in osteoclasts, and we validated the expression of selected miRNAs by quantitative RT-PCR. We identified clusters of differentially expressed miRNAs during the course of osteoclastogenesis, and performed computational analyses to predict functional pathways that may be regulated by these miRNAs. Several miRNAs were predicted to regulate genes involved in cytoskeletal remodeling, a crucial mechanism for the migration of osteoclast precursors, their maturation, and bone resorbing activity. Our results suggest that clusters of miRNAs differentially expressed during the course of osteoclastogenesis converge on the regulation of several key functional pathways. Overall, this study identified miRNAs expressed during early, middle, and late osteoclastogenesis contributing to our understanding the molecular mechanisms regulating this complex differentiation process.

INTRODUCTION

The maintenance of bone homeostasis requires a tight control of the number and activity of osteoblasts, the bone-forming cells, and osteoclasts, the only cells able to resorb mineralized bone matrix. Osteoclast differentiation is an intricate process, regulated at multiple levels by transcription factors and post-translational modifications. In this process, myeloid progenitor cells differentiate into monocytes, commit to the

osteoclast lineage, migrate, and then fuse into multinucleated polykaryons, at the expense of the alternative macrophage fate. Several cytokines, including macrophage-colony stimulating factor (M-CSF, CSF1) and receptor activator of nuclear factor kappa-B ligand (RANKL), are responsible for driving osteoclastogenesis from multipotential hematopoietic progenitors. Several intracellular signaling pathways promote commitment to the osteoclast lineage, and converge on the activation of NFATc1, the master transcriptional regulator of osteoclastogenesis. NFATc1, in combination with other transcription factors, including PU.1, MITF, NFκB, and c-Fos, coordinates the expression of genes necessary for bone resorption, such as Cathepsin K, Tartrate-resistant acid phosphatase (*Acp5*, *Trap*), and Calcitonin receptor (2, 12, 83).

More recently, a growing number of reports have demonstrated the important role of microRNAs (miRNAs) in osteoclast biology. miRNAs are short sequences of single-stranded, non-coding RNA that act, for the most part, as post-transcriptional regulators of gene expression. This is achieved primarily by binding target mRNAs at sites frequently located in the 3' untranslated region (UTR). However, miRNA binding sequences have been identified also in the coding region and in the 5' UTR (84, 132). miRNA activity requires its incorporation in a RNA-induced silencing complex (RISC). Target recognition by the miRNA relies mainly on near-perfect base pair complementarity of the mRNA with the miRNA "seed region", a 6-8 nucleotide-long sequence in the 5' end of the miRNA. Upon target binding, repression of gene expression is accomplished by suppressing translation, and/or decreasing the stability of the mRNA.

The critical role of the miRNA processing pathway in the osteoclast lineage was described. In vitro, silencing of DiGeorge syndrome critical region 8 gene (DGCR8), Argonaute2 (Ago2), and Dicer1, key miRNA processing factors, decreased osteoclastogenesis and bone resorption (53). In vivo, deletion of Dicer in the myeloid lineage, using a CD11b promoter driven-Cre recombinase, and in mature osteoclasts, using a Cathepsin K promoter driven-Cre, led to the development of mild osteopetrosis, due to impaired osteoclast differentiation and activity (53, 54). These studies highlight the overall importance of miRNAs in regulating osteoclast biology, and allude to their potential as therapeutic targets for pathologies caused by excessive or insufficient osteoclast activity. However, little is known about the function of individual miRNAs in osteoclastogenesis.

At the time of this writing, 9 miRNAs and only a few target genes have been analyzed in the osteoclast lineage (53, 55-58, 61-63, 65). Hundreds of miRNAs have been identified, and each miRNA can potentially regulate hundreds of mRNAs. Further, limited information is available about the miRNA expression profile during osteoclastogenesis, and how it changes during the course of differentiation.

In the present study, we profiled miRNA expression during the early, middle, and late stages of osteoclastogenesis, in a population of primary murine bone marrow cells enriched for osteoclast progenitors. We also analyzed changes in miRNA expression in osteoclast precursors differentiated for 3 days with M-CSF and RANKL, when compared with undifferentiated cultures. Clusters of differentially expressed miRNAs were identified, and computational target prediction tools suggest that a set of miRNAs

expressed in the osteoclast lineage likely regulate pathways critical for cell motility, cell-matrix interactions, axon guidance, and regulation of the actin cytoskeleton.

MATERIALS AND METHODS

Cell Culture.

Primary osteoclast precursor cultures were established using bone marrow from 6-8 week old C57BL/6 male mice, which had been enriched for osteoclast precursors by depletion of B220/CD45R-positive and CD3-positive cells (B and T lymphocytes, respectively). Briefly, bone marrow was isolated from femurs, tibias, and humeri, and depleted of erythrocytes by treatment with ammonium-chloride-potassium (ACK) buffer (Gibco Life Technologies, Grand Island, NY) (94). Cells were incubated with Phycoerythrin (PE)-conjugated primary antibodies for CD45R and CD3 (eBioscience, San Diego, CA), and with magnetically labeled anti-PE microbeads (MiltenyiBiotec, Auburn, CA). Magnetic-Activated Cell Sorting (MACS®) Column Technology (MiltenyiBiotec, Auburn, CA) was used to capture CD45R and CD3 positive cells in the column, and the flow-through contained a population of cells enriched for monocytic and non-lymphoid lineage cells. Flow cytometric analysis was performed to analyze the presence of CD45R and CD3 positive cells. Standard staining procedures were used to label the cells for flow cytometry. Non viable cells were identified by their ability to incorporate propidium iodide (PI). Flow cytometry was performed using a BD-FACS

Aria (BD Biosciences, San Jose, CA, USA), and data were analyzed using FlowJo software from Tree Star Inc (Ashland, OR, USA). Cells were cultured in α -MEM (Gibco Life Technologies, Grand Island, NY) supplemented with 10% FBS (Fetal Bovine Serum, Atlas Biologicals, Fort Collins, CO). Bone marrow-derived osteoclast precursor cells were cultured in the presence of 30 ng/ml recombinant Macrophage Colony-Stimulating Factor (M-CSF) and 30 ng/ml murine recombinant RANKL (eBioscience, San Diego, CA) for up to 5 days. Bone marrow precursors were also cultured in the presence of 30 ng/ml M-CSF alone for 3 days.

In Vitro Osteoclast Formation Assay.

Cells were fixed in 2.5% glutaraldehyde in PBS, and TRAP activity was detected according to the manufacturer's instructions using the Acid Phosphatase Leukocyte (TRAP) kit (Sigma-Aldrich). Osteoclast cultures were imaged using light microscopy.

miRNA Microarray.

Primary osteoclast precursors were plated at 53,000 cells/cm². Total RNA was isolated from differentiating cultures using the miRNeasy Mini kit (Qiagen, Valencia, CA). On-column DNase treatment was performed to reduce contamination with genomic DNA, and an additional treatment with RQ1 DNase (Promega, Madison, WI) was performed prior to gene expression analysis. RNA concentration and purity were assessed by spectrophotometric analysis. The quality of small RNAs in each sample was

determined using the 2100 Bioanalyzer assay (Agilent Technologies, Santa Clara, CA). 200 ng of total RNA were labeled using miRNA Microarray System with miRNA Complete Labeling and Hyb Kit (Agilent Technologies). According to the manufacturer's instructions, the samples were hybridized for 20 hours onto a mouse miRNA Microarray, Release 15.0, 8x15K (based on Sanger miRBase release 15.0), containing 627 mouse mature miRNA probes (Agilent Technologies). Hybridized and washed array slides were scanned at 5 μ m resolution using an Agilent SureScan Microarray Scanner (Agilent Technologies). Image processing was completed using Feature Extraction Software (Agilent Technologies). We acknowledge Dr. David Willmot for technical assistance with the microarray analysis.

Microarray Data Analysis.

Microarray data were normalized and analyzed using the GeneSpring GX software (Technology 29152_v.17_0, Agilent Technologies). 4 biological replicates were used for each sample set. miRNAs detected in at least one sample were subjected to quantile normalization to allow comparison between the microarray chips, and relative expression is presented as log(base 2). One-way Analysis of Variance (ANOVA) was performed on miRNAs expressed during the time course of osteoclast differentiation analyzed (days 1, 3, and 5). Student's t test was performed on miRNAs expressed in undifferentiated (cultured in the presence of M-CSF alone) and differentiated (cultured in the presence of M-CSF and RANKL). For both data sets, miRNAs showing >2 or <-2 fold-change, with $p < 0.01$, were considered statistically significant. A hierarchical

clustering analysis was used to organize the genes based on similarities in their expression profiles. A list of putative miRNA targets was identified using the prediction algorithm DNA Intelligent Analysis (DIANA) DIANA-microT-CDS (v5.0). The predicted miRNA targets were annotated into functional pathways using DIANA-miRPath (v2.0) (<http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/index>). The complete data set has been submitted to NCBI-GEO (National Center for Biotechnology Information – Gene Expression Omnibus), and is available at the accession number GSE53017.

Quantitative Real time PCR.

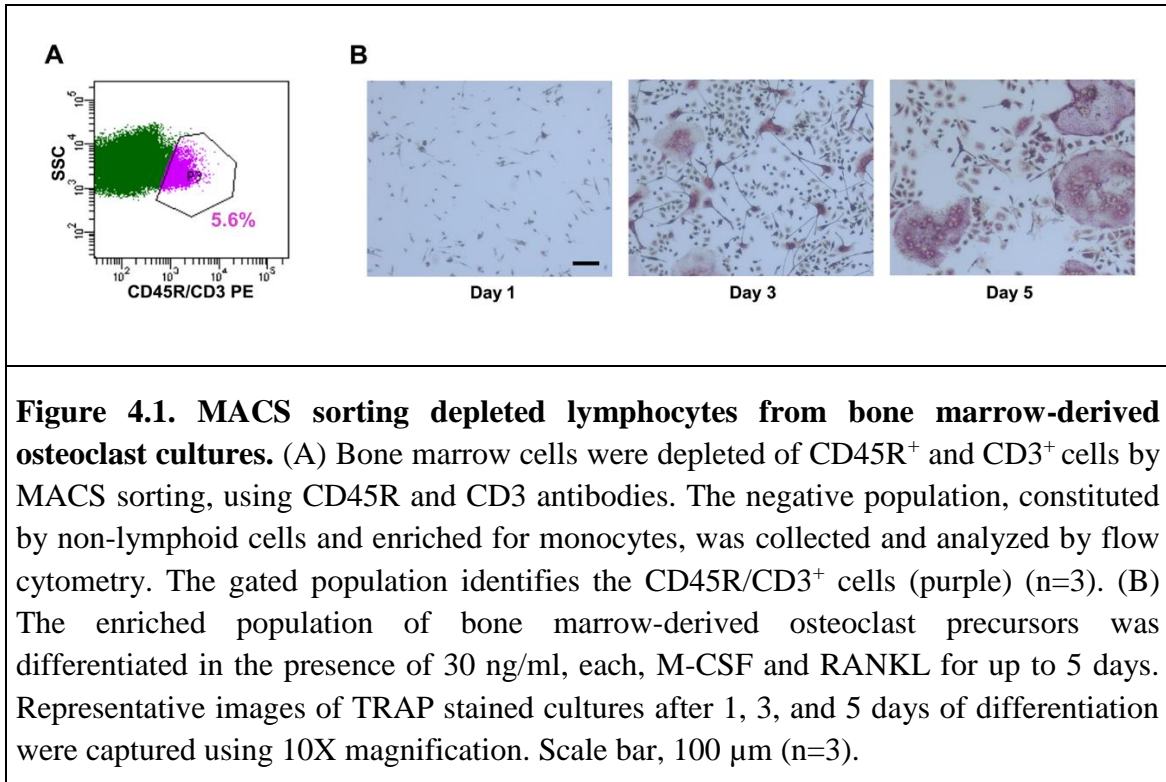
miRNA expression levels were analyzed using the TaqMan MicroRNA Assay (Life Technologies, Grand Island, NY). According to the manufacturer's instructions, 22.5 ng of RNA were reverse transcribed with specific primers to generate cDNA. The expression of miR-365, miR-99b, and miR-451 was detected by qPCR in a MiQ qPCR cyclers (Bio-Rad) and normalized to U6 small nuclear RNA (RNUB6) levels, using the absolute quantification method. Data are presented as mean \pm SEM. Data were analyzed by one-way ANOVA with Bonferroni post-hoc test as appropriate (KaleidaGraph, Synergy Software, Reading, PA).

RESULTS AND DISCUSSION

Differential miRNA expression during in vitro osteoclastogenesis.

Mouse bone marrow is a widely used source of primary osteoclast progenitors for in vitro analyses. However, bone marrow represents a highly heterogeneous population, containing not only monocytes, but also megakaryocyte precursors, terminally differentiated macrophages and neutrophils, and, in higher percentage, lymphocytes. Our unpublished results indicate that 25-30% of total bone marrow is B220⁺ and 5-7% is CD3⁺ after erythrocyte depletion (Dr. S.K. Lee, personal communication, unpublished data). In addition, the majority of the efficient osteoclast precursors is contained within the B220⁻/CD3⁻/CD11b^{-/lo} population in the bone marrow (43, 133). Therefore, we sought to decrease the heterogeneity of this precursor population, prior to the initiation of osteoclastogenesis, by depleting the lymphocytic cells. Mouse bone marrow cells were subjected to MACS sorting using CD45R and CD3 antibodies. Flow cytometric analysis confirmed that this procedure depleted 93-95% of T and B cells, thereby decreasing the heterogeneity of the marrow cells that were subsequently plated for experiments (Figure 4.1A). We cultured mouse bone marrow-derived osteoclast precursors in the presence of M-CSF and RANKL for up to 5 days. At days 1, 3, and 5 of culture, osteoclast formation was monitored by TRAP staining, and total RNA was harvested (Figure 4.1B). Under these conditions, osteoclast number increased progressively, and mRNA levels for the osteoclast markers TRAP and

Cathepsin K were increased from day 1 to day 3, and maintained at day 5, as we previously reported (62) (Figure 3.2E,F).



The Agilent mouse miRNA microarray that we used contained 627 probes for mature miRNAs. In our sample set, 258 miRNAs were significantly detected at least at one time point. 142 of these miRNAs were significantly changed during the time course investigated, and several were identified for the first time in the osteoclast lineage. Among the significantly changed miRNAs, 93 miRNAs showed $> \pm 2$ fold-change. 49 miRNAs were up-regulated over time, whereas 44 were down-regulated (Figure 4.2).

In regards to miRNA expression after 3 days in culture, 43 miRNAs were significantly changed with M-CSF and RANKL treatment in comparison with M-CSF

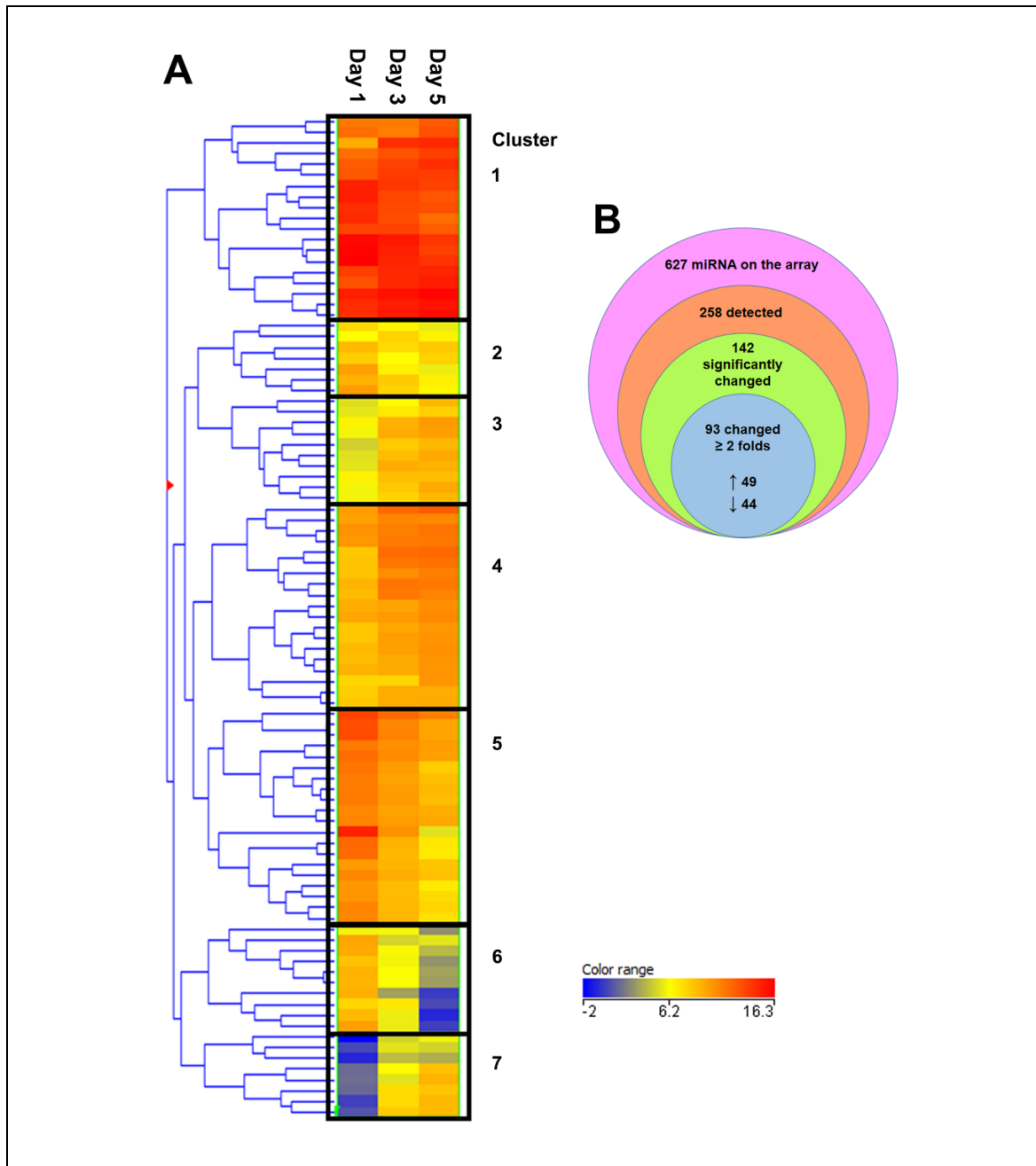


Figure 4.2. miRNA expression profiles during osteoclastogenesis. (A) Heat map of the 93 miRNAs showing $> \pm 2$ fold-change over 5 days of osteoclast differentiation. Fold-change was calculated between day 1 and day 3, day 1 and day 5, and day 3 and day 5. Hierarchical cluster analysis on gene expression divided the miRNAs in 7 groups. Blue represents low expression, red high expression, and yellow intermediate expression. (B) Schematic overview of the microarray results.

treatment alone, and 17 miRNAs showed $> \pm 2$ fold-change. 9 miRNAs were up-regulated in the presence of RANKL, whereas 8 were decreased (Figure 4.3).

Verification of the microarray results.

To verify the results of the microarray experiment, we used quantitative RT-PCR (qRT-PCR) to confirm changes in the levels of 3 miRNAs, the expression of which had not been previously reported in the osteoclast lineage: miR-365, miR-99b, and miR-451 (Figure 4.4, Table 4.1). We observed that miR-365-3p and miR-99b-5p were 2 miRNAs strongly up-regulated in day 5 cultures (Figures 4.8A, 4.9A). qRT-PCR confirmed that their levels increased 12 fold in the differentiating cultures (Figure 4.4A). Further, the expression of miR-365 and miR-99b was significantly induced by 3 days of M-CSF and RANKL treatment, when compared with cells cultured in the presence of M-CSF alone (Figure 4.4C,D). This suggests that the up-regulation of miR-365 and miR-99b levels during the course of osteoclastogenesis is likely associated with the progression of the differentiation program, rather than time in culture.

The function of miR-365 in osteoclastogenesis or in hematopoietic cells has not been investigated. However, we found this miRNA was of interest because, although mature miR-365 is transcribed from two independent genetic loci (on mouse chromosomes 11 and 16), the expression of miR-365 from the chromosome 16 locus is activated by Sp1 and NFκB, two transcription factors that promote osteoclastogenesis (29, 134). miR-99b was of interest because it is up-regulated in dendritic cells and monocytes during inflammation (135, 136). Further, TNFα (tumor necrosis factor α) signaling is a key pathway in promoting osteoclastogenesis, and *Tnfa*, *Tnfrsf4* (Tumor Necrosis Factor Receptor Superfamily, Member 4), and *Traf2*

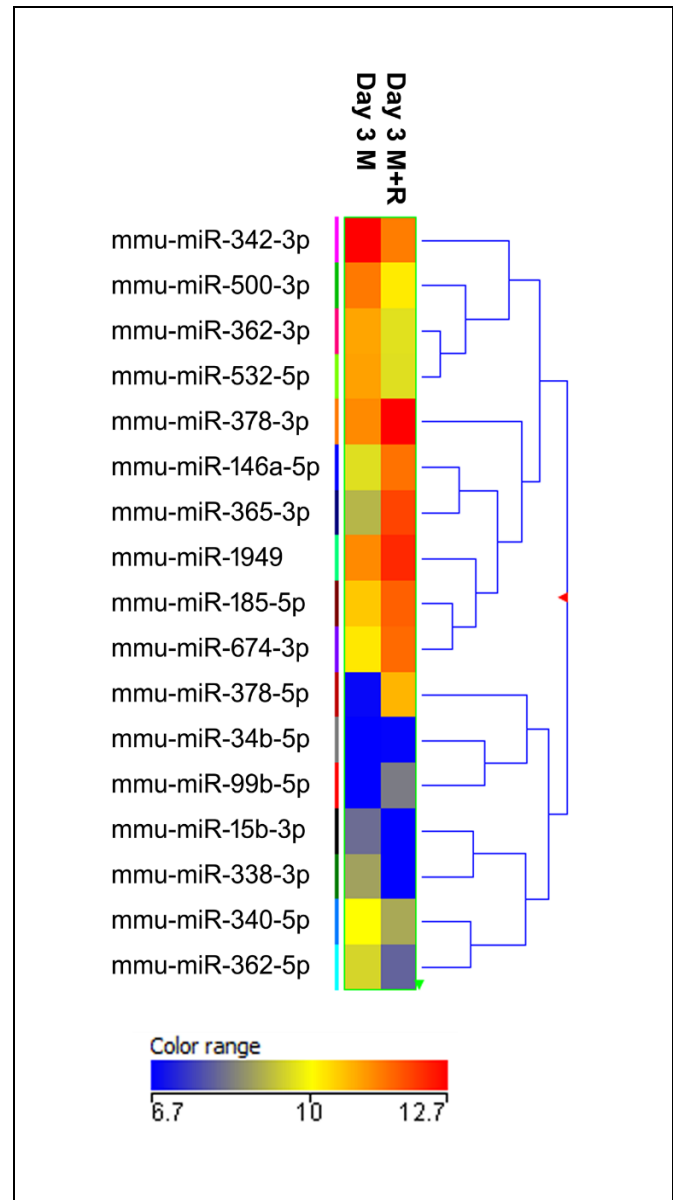


Figure 4.3. miRNA expression profile after 3 days of culture with M-CSF and RANKL. Heat map of the 17 miRNAs showing $> \pm 2$ fold-change in the presence or absence of RANKL. Hierarchical cluster analysis grouped the miRNAs based on their expression levels. Blue represents low expression, red high expression, and yellow intermediate expression.

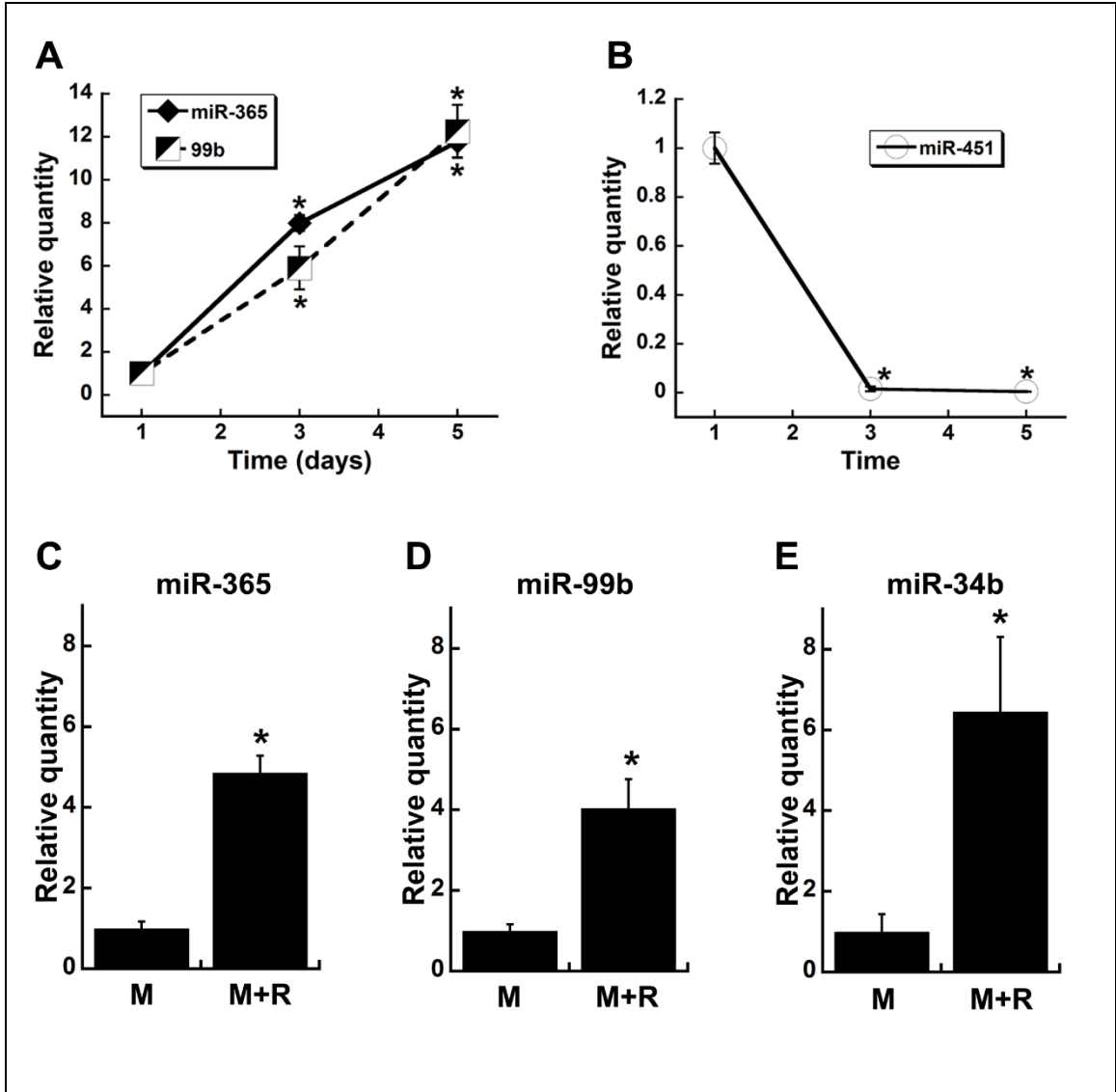


Figure 4.4. Validation of miRNA microarray results. (A,B) The expression of selected miRNAs that changed $> \pm 2$ folds during the course of osteoclastogenesis was analyzed by qPCR. (A) miR-365 and miR-99b expression; (B) miR-451 expression. (C-E) Selected miRNAs that showed greater than ± 2 fold-change in cells treated with M-CSF and RANKL (M+R) versus cells treated with M-CSF (M) alone for 3 days were quantified. (C) miR-365 expression; (D) miR-99b; (E) miR-34b. Gene expression was normalized to U6. $n=4$; * significantly different from day 1 ($p<0.01$).

(TNF receptor-associated factor 2), were recently identified as miR-99b targets in other cell types (136-139). Interestingly, miR-99b is transcribed in an evolutionary conserved cluster that includes let-7e and miR-125a, all of which were significantly up-regulated during osteoclastogenesis, as assessed by our microarray (Figure 4.7A) (140).

In contrast, miR-451 expression was dramatically decreased over 5 days of osteoclast differentiation (Figure 4.4B). Several reports have revealed that miR-451 expression is required for erythroid differentiation and homeostasis (141, 142). Therefore, it is possible that the high levels of miR-451 detected at day 1 are due to the presence of erythrocyte precursors in the cultures, which will not survive the differentiation with M-CSF and RANKL. Indeed, we could not detect expression of miR-451 by qRT-PCR in the bipotential mouse monocytic cell line RAW264.7 (data not shown).

In regard to miRNAs significantly changed at day 3 of differentiation with M-CSF and RANKL, in comparison with undifferentiated cultures, we confirmed that miR-34b-5p is significantly increased in the presence of RANKL (Figure 4.3, Figure 4.4E). miR-34b belongs to the miR-34 family of miRNAs, which were previously identified as principal players in the p53-mediated regulation of apoptosis (143). The role of miR-34b in the osteoclast lineage is unknown. However, the function of miR-34b in the regulation of hematopoiesis has been investigated. The genomic region encoding for the miR-34b gene is frequently deleted in chronic lymphocytic leukemia, and miR-34b targets the anti-apoptotic gene *Tcl-1* (T-cell leukemia/lymphoma 1) (144). In addition, miR-34b inhibits the expression of CREB (cAMP response element-binding protein), a key transcriptional

Table 4.1. Changes in miRNA profile during the progression of murine osteoclastogenesis, confirmed by other microarray studies. Published studies in RAW264.7 cell line and mouse bone marrow macrophages (BMMs) confirm the expression pattern for 17 of the 97 miRNAs regulated during the course of osteoclastogenesis (61, 67).

miRNA	Expression during osteoclastogenesis	Experimental system	References
let-7a-5p	↑	BMMs, RAW264.7	(61, 67)
let-7e-5p	↑	BMMs, RAW264.7	(61, 67)
let-7f-5p	↑	BMMs, RAW264.7	(61, 67)
miR-100-5p	↑	RAW264.7	(67)
miR-125a-5p	↑	RAW264.7	(67)
miR-125b-5p	↑	RAW264.7	(67)
miR-146a-5p	↑	RAW264.7	(67)
miR-146b-5p	↓	RAW264.7	(67)
miR-185-5p	↑	RAW264.7	(67)
miR-29b-3p	↑	RAW264.7, bone marrow-derived osteoclast precursors	(62, 67)
miR-338-3p	↓	RAW264.7	(67)
miR-365-3p	↑	RAW264.7	(67)
miR-378-3p	↑	RAW264.7	(67)
miR-674-3p	↑	RAW264.7	(67)
miR-689	↓	RAW264.7	(67)
miR-98-5p	↑	BMMs, RAW264.7	(61, 67)
miR-99a-5p	↑	RAW264.7	(67)

regulator of hematopoiesis (145). These observations indicate that proper expression of miR-34b is necessary for the progression of hematopoietic differentiation. Therefore, changes in the expression of miR-34b may be involved in the control of osteoclastogenesis.

We compared our microarray results with other published murine miRNA profile analyses in osteoclastic cultures. The expression pattern that we observed for 17 of the significantly regulated miRNAs was similar to what observed in a 24 or 82 hour RANKL-driven RAW264.7 cell differentiation, and in a 24-hour RANKL-treatment of bone marrow macrophages (Table 4.1) (61, 67). However, a few discrepancies were noted in miRNA expression trends between our microarray data and those published by other investigators. These are likely attributable to differences in the experimental designs, most notably differences in the percentage of osteoclast precursor cells used and the time in culture. Our study is unique in that we analyzed an enriched population of primary osteoclast precursors from the bone marrow, and evaluated miRNA expression during early, middle, and late phases of differentiation.

Target prediction and correlated pathways for miRNA expression clusters.

The 93 miRNAs that showed greater than ± 2 fold-change with osteoclast differentiation were analyzed by hierarchical clustering. Based on their level of expression and change during differentiation, the miRNAs were divided into 7 clusters (Table 4.2, Figures 4.5-4.11, Figure 4.2). In an attempt to understand how these changes

in miRNA expression might influence osteoclastogenesis, we performed a computational target prediction analysis. Potential target RNAs for up- or down-regulated miRNAs from each cluster were identified using the prediction algorithm DIANA-microT-CDS (v5.0). This algorithm recognizes potential miRNA

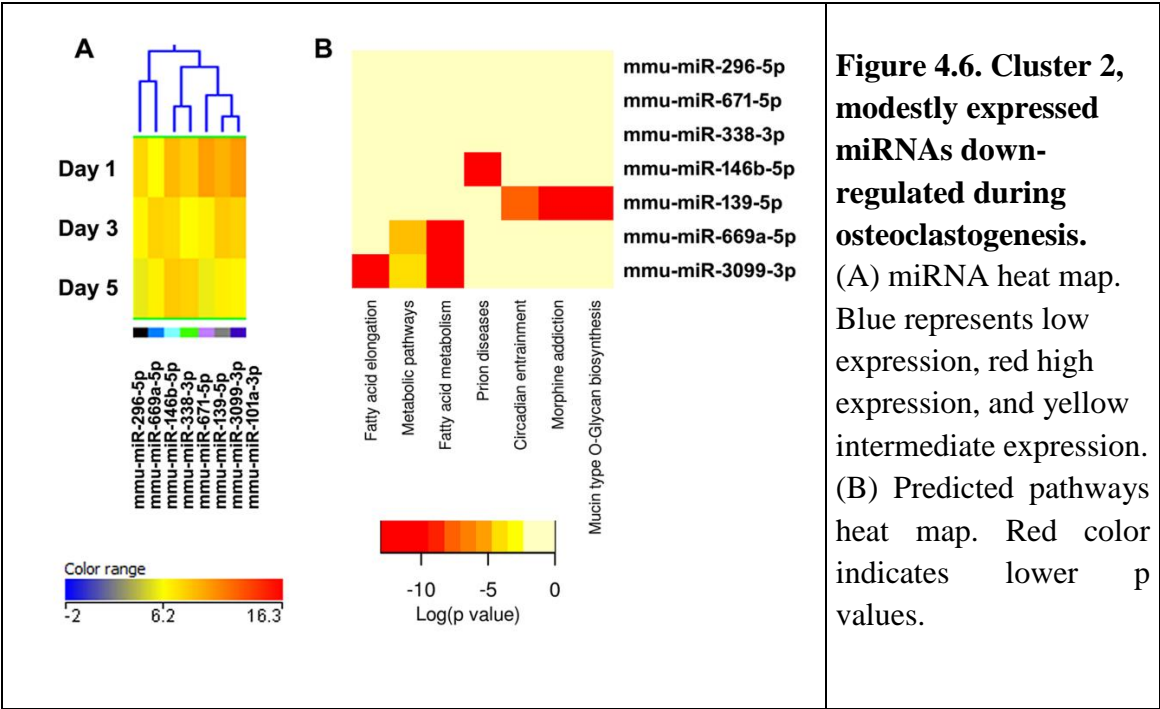
Table 4.2. miRNA clusters. Hierarchical clustering of the miRNAs significantly changed during osteoclast differentiation generated 7 subgroups.

Cluster number	Name/Description
1	Highly expressed
2	Modestly expressed down-regulated
3	Modestly expressed up-regulated
4	Well expressed up-regulated
5	Well expressed down-regulated
6	Most down-regulated over time
7	Most up-regulated over time

binding sites located in the coding sequence and in the 3' UTR of an mRNA, based on complementary pairing with nucleotides in position 1-9 at the 5' end of the miRNA (i.e. the seed binding region). Additional features taken into consideration include conservation of the sequence element across species and accessibility of the target site (146, 147). The potential miRNA targets for each miRNA cluster were then subjected to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis, using the DIANA-miRPath (v2.0) software. This algorithm calculates the significance for all the miRNA-mRNA pairs in a pathway, and then combines them into a merged P-value for each pathway (148). The results are reported as heat maps, and the pathways are clustered based on significance levels. The more intense red color indicates higher probability that a specific pathway is significantly enriched with target genes for a certain miRNA.

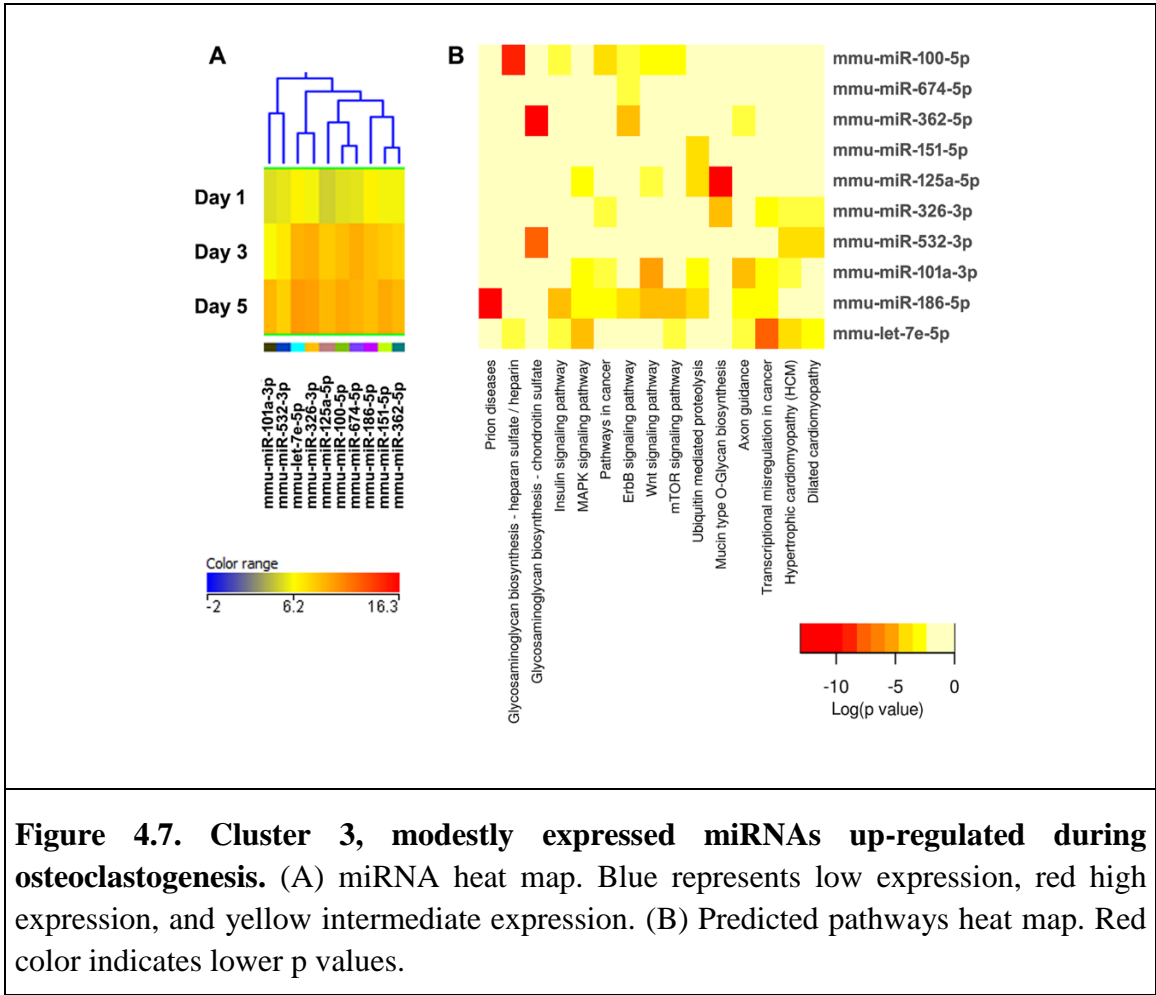
Cluster 1 is composed of highly expressed miRNAs, and contains miRNAs that are both up- and down- regulated during osteoclastogenesis. Therefore, separate pathway analysis was performed for the up- and down- regulated miRNAs. Among the highly expressed and up-regulated miRNAs is miR-29b, a member of the miR-29 family (Figure 4.5A). We and others previously showed that the expression of all miR-29 family members increases during osteoclastogenesis (62, 67) (Figure 3.2A). In addition, we demonstrated that miR-29 promotes osteoclast commitment and migration, and is critical for osteoclast formation. We validated several novel miR-29 targets in the osteoclast lineage, including calcitonin receptor. Further, miR-29 targets genes important for the macrophage lineage, *Nfia* (Nuclear factor 1/A), *Cd93*, and *Gpr85* (G protein coupled receptor 85); and genes modulating cell migration, including *Cdc42* (Cell division control protein 42) and *Srgap2* (SLIT-ROBO Rho GTPase activating protein 2) (62). With regard to miR-29b, the pathway analysis suggests that functions such as cell-matrix interactions, focal adhesion, and PI3K/Akt, to be most significantly associated with miR-29 (Figure 4.5B). Several predicted miR-29 targets within these pathways have been experimentally confirmed. These include mRNAs for a large number of extracellular matrix proteins (collagens, laminins), the tumor suppressor *Pten*, *Igf1* (insulin growth factor 1), and *Mcl1* (myeloid cell leukemia 1) (149-152). Overall, these observations suggest that the pathway prediction analysis for the individual miRNAs is, at least in part, validated by experimental data.

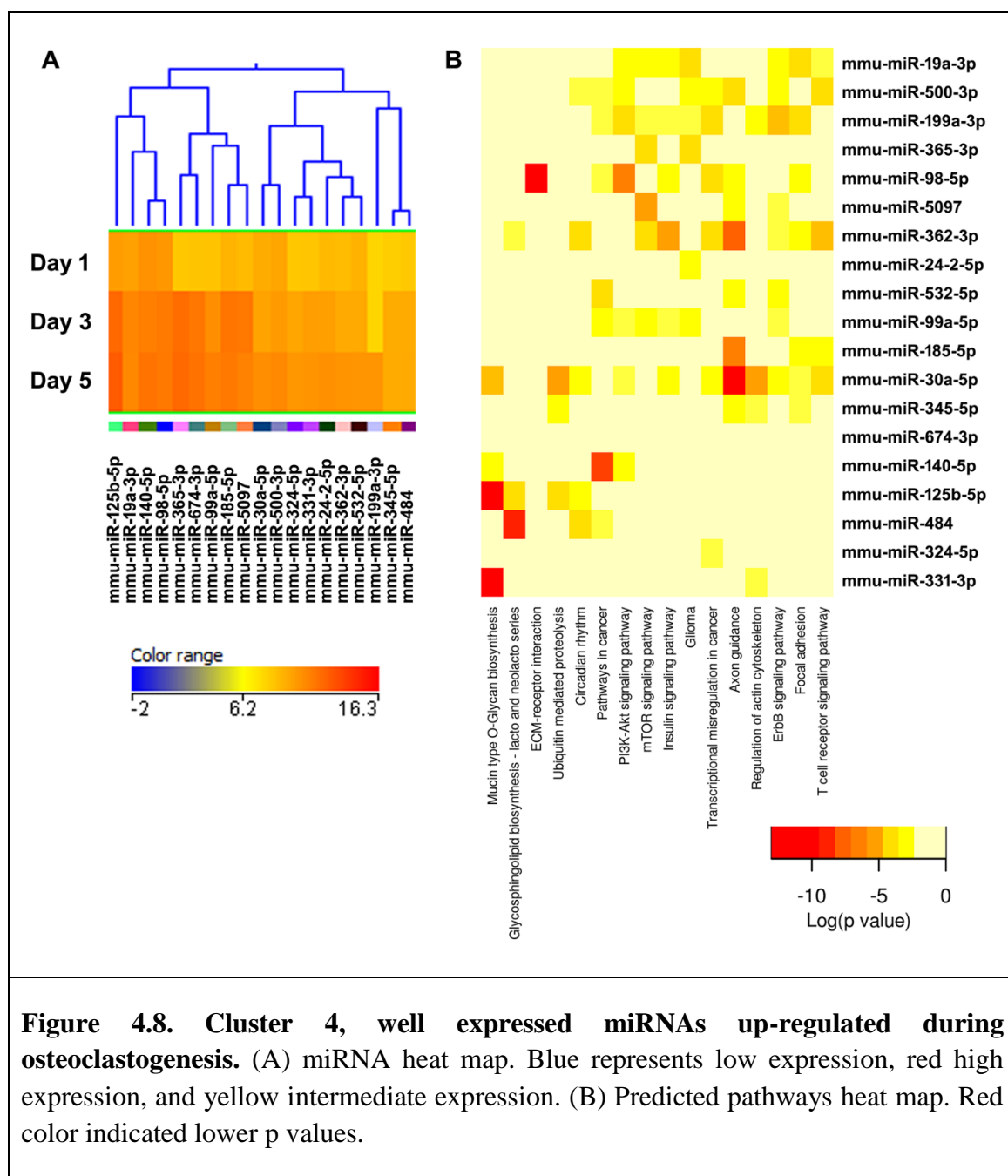
The validity of this pathway clustering approach is further supported by analysis of the miRNA family formed of miR-99a, miR-99b, and miR-100. Each these miRNAs is

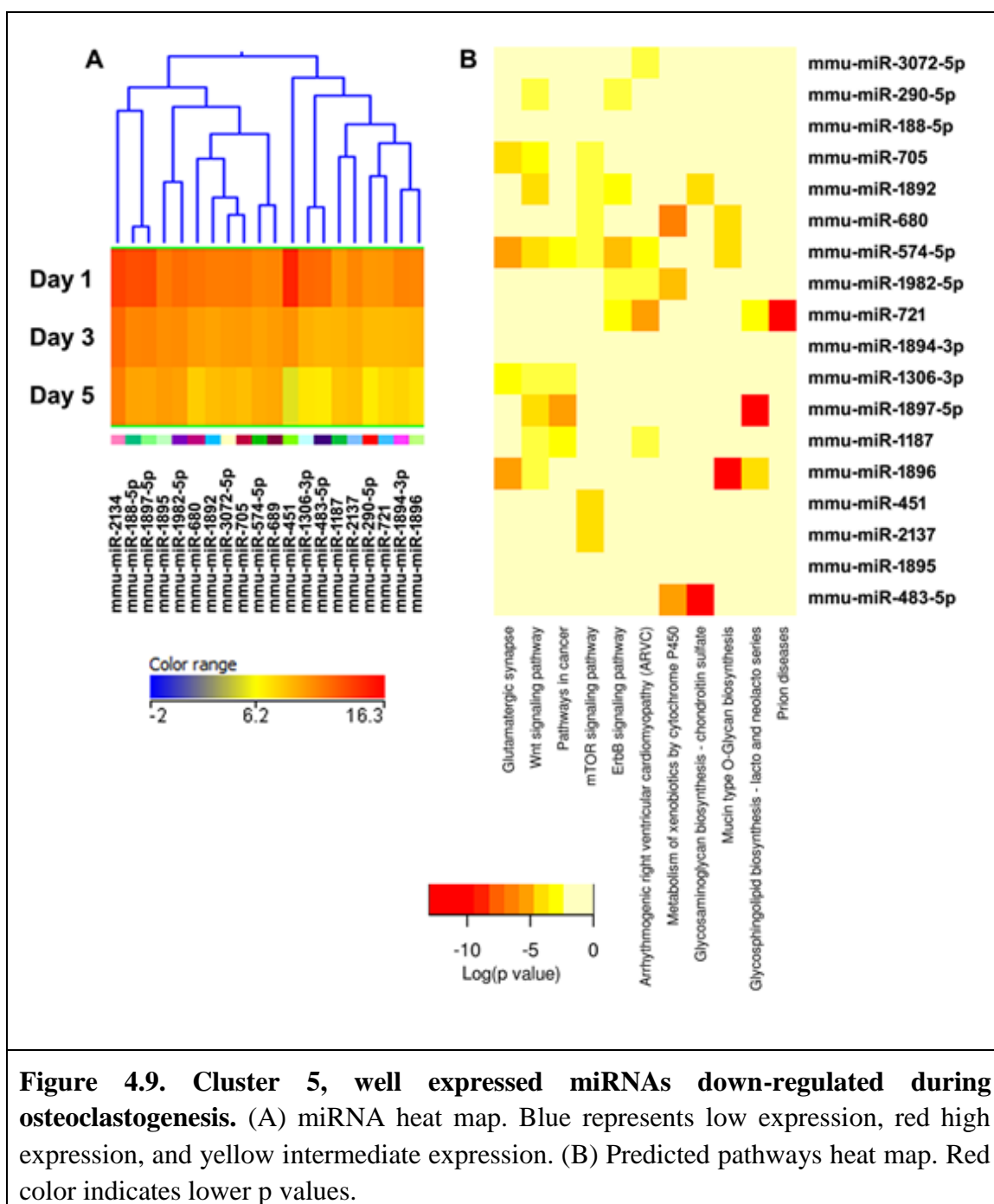


up-regulated during the course of osteoclastogenesis, although with different amplitude. miR-99a-5p belongs to expression cluster 4, miR-99b-5p to cluster 7, and miR-100-5p to cluster 3 (Figures 4.7A, 4.8A, and 4.11A). KEGG pathway analysis predicted, with a high degree of confidence ($p < 0.001$), miR-99b regulation of the mTOR (mammalian target of rapamycin) pathway, whereas association of miR-99a and miR-100 with the mTOR pathway was predicted with lower confidence ($p < 0.005$) (Figures 4.7B, 4.8B, and 4.11B). Remarkably, several studies demonstrated the role of the miR-99 family in repressing mTOR signaling in different cell systems, including wound healing keratinocytes, as well as prostate, endometrial, and pancreatic cancer cells (153-156). In our study, numerous clusters of miRNAs, both up- and down-regulated during the course of osteoclast differentiation, were predicted to target components of the mTOR pathway.

Cluster 4 appears to be particularly enriched in miRNAs with potential targets in the mTOR pathway (Figure 4.8B). Clusters of differentially regulated miRNAs are predicted to target both positive and negative regulators of the mTOR pathway, as represented in the diagram in Figure 4.12.







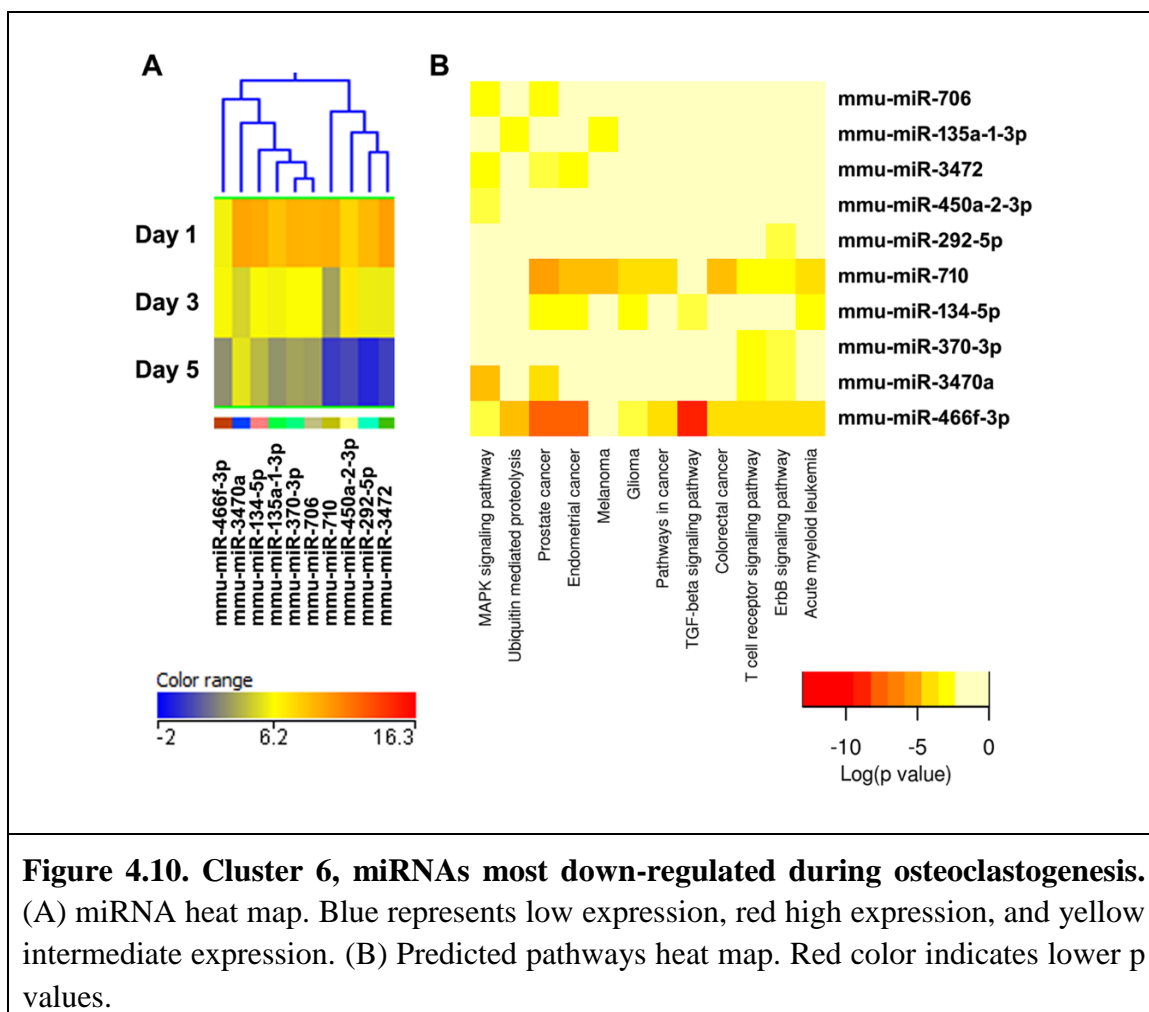


Figure 4.10. Cluster 6, miRNAs most down-regulated during osteoclastogenesis. (A) miRNA heat map. Blue represents low expression, red high expression, and yellow intermediate expression. (B) Predicted pathways heat map. Red color indicates lower p values.

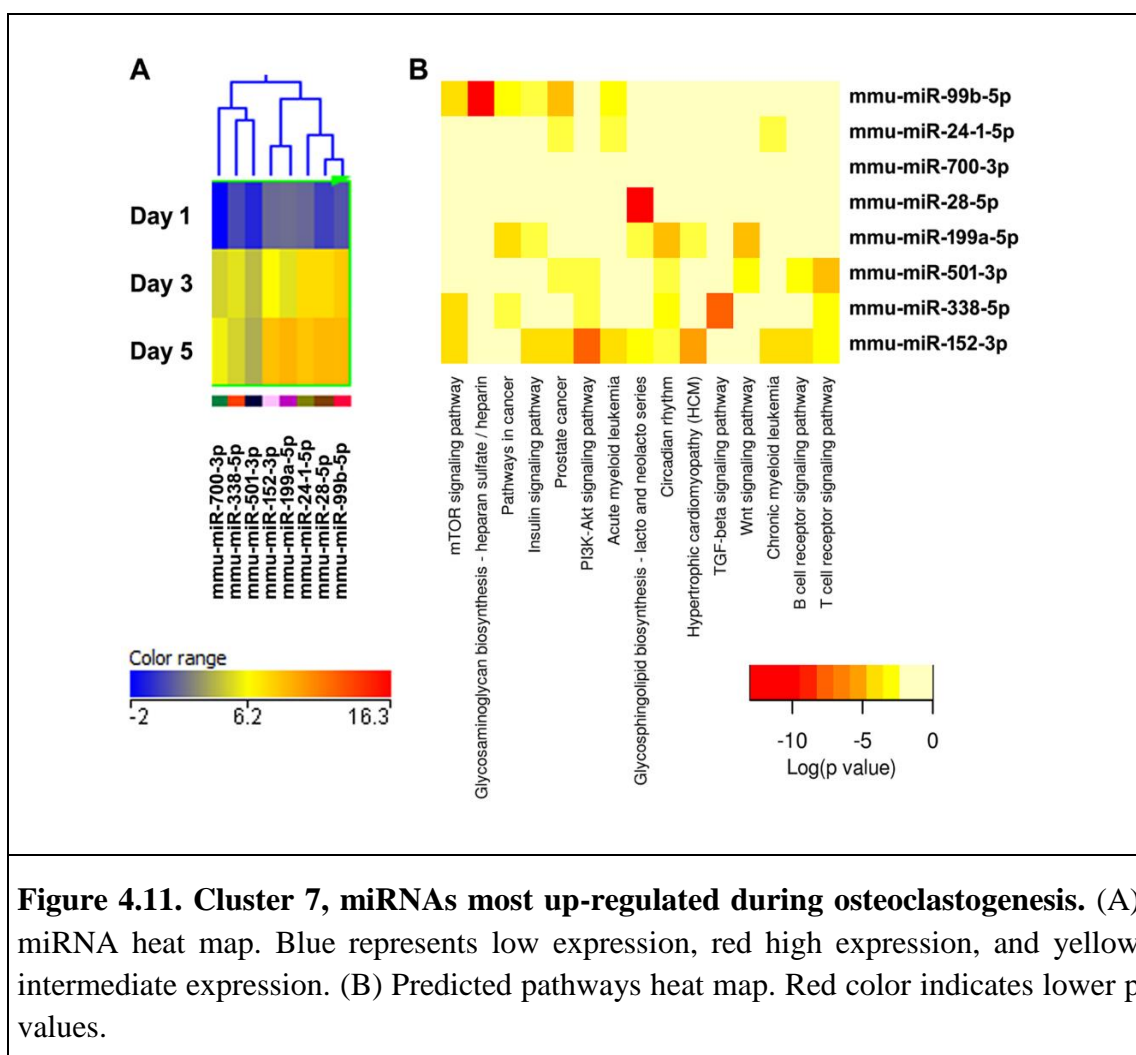


Figure 4.11. Cluster 7, miRNAs most up-regulated during osteoclastogenesis. (A) miRNA heat map. Blue represents low expression, red high expression, and yellow intermediate expression. (B) Predicted pathways heat map. Red color indicates lower p values.

In osteoclasts, mTOR has been implicated in the regulation of apoptosis. Crucial signaling pathways triggered by M-CSF, RANKL, and TNF α converge on the activation of S6K (p70 ribosomal protein S6 kinase), a main effector of the mTOR signaling cascade. By regulating the process of translation, mTOR promotes osteoclast differentiation, survival, and bone-resorbing activity (157, 158). Although miRNA-mediated modulation of mTOR factors has been widely investigated in other biological systems, this represents a novel area of research in the bone field.

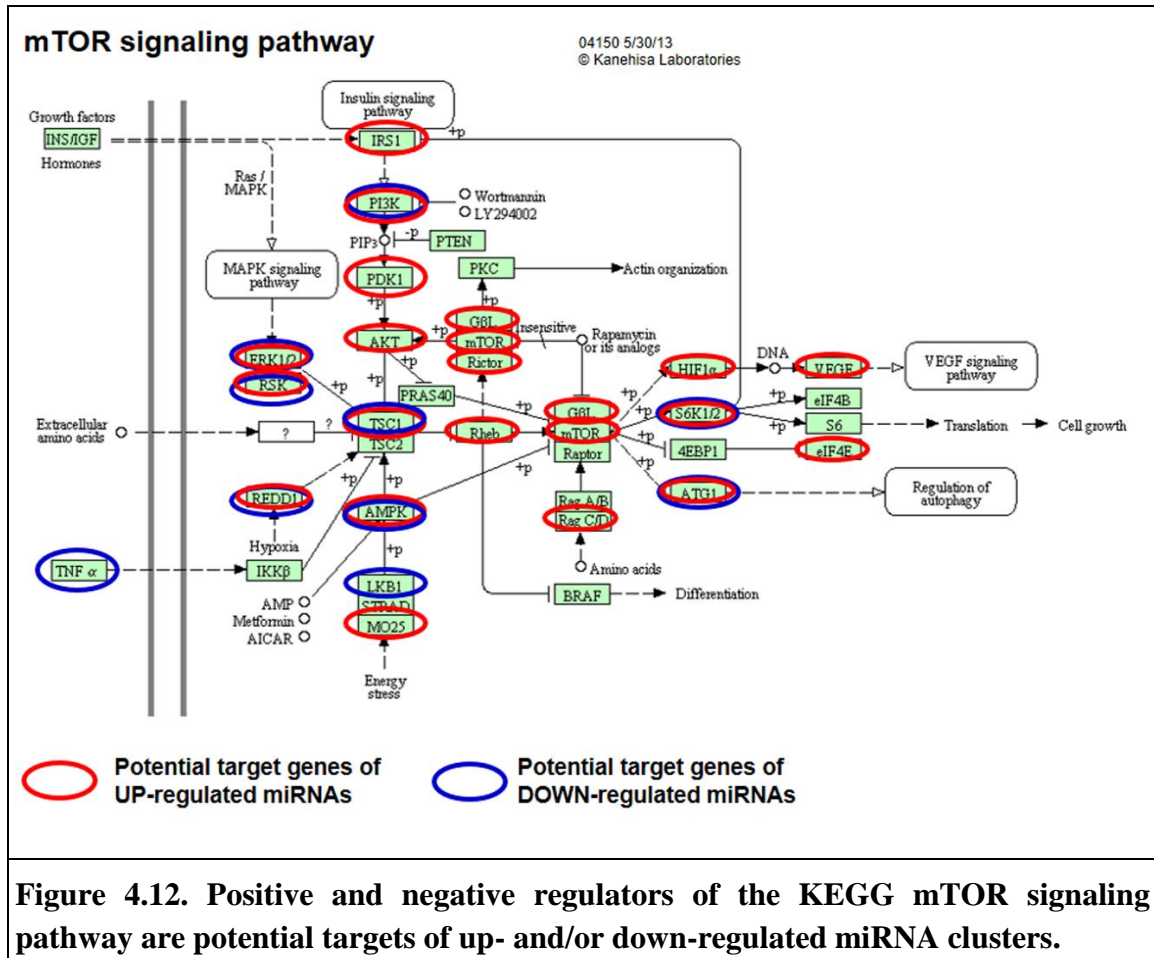
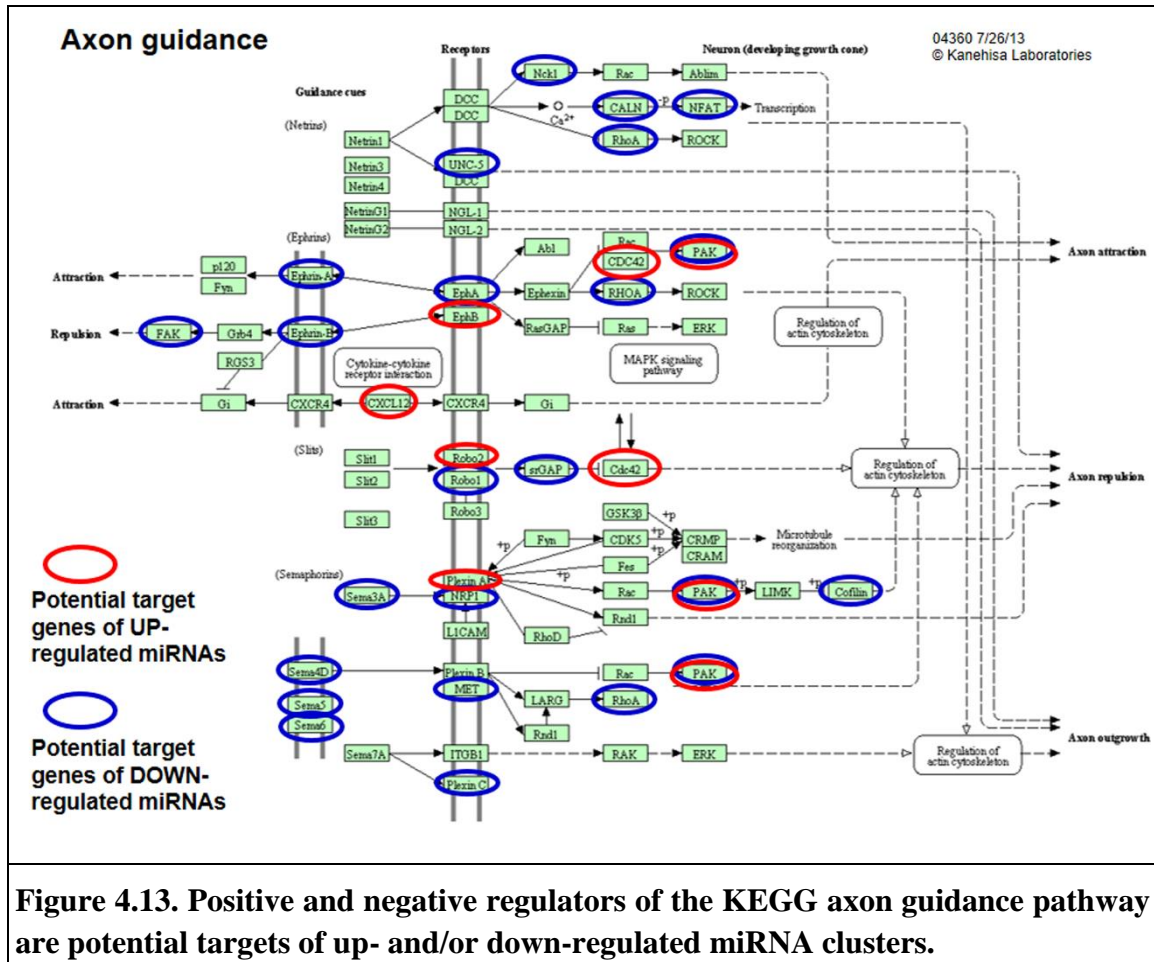


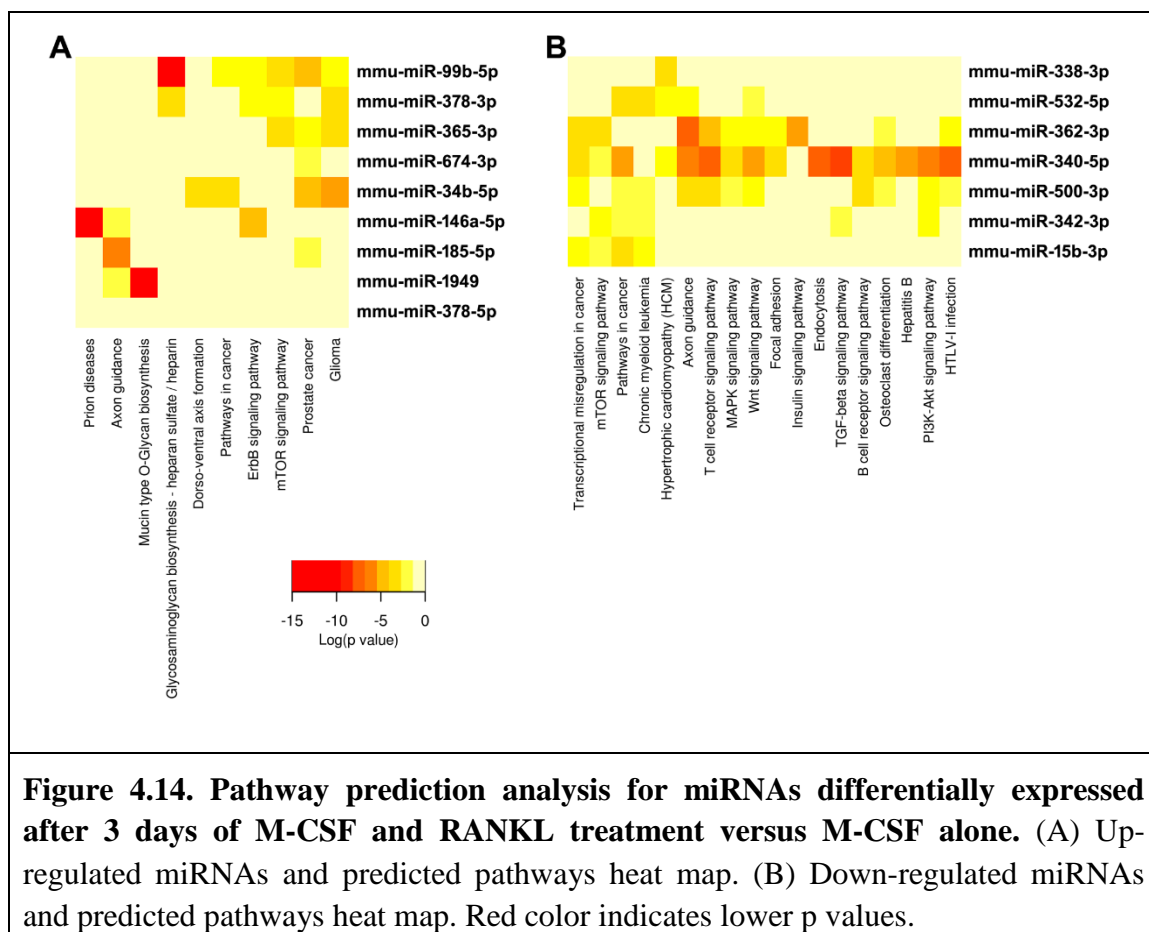
Figure 4.12. Positive and negative regulators of the KEGG mTOR signaling pathway are potential targets of up- and/or down-regulated miRNA clusters.

KEGG pathway analysis showed that several miRNAs from clusters 1, 3, and 4 were predicted to target extracellular matrix-receptor interactions, regulators of the actin cytoskeleton, focal adhesion, and axon guidance (Figures 4.5B,C, 4.7B, 4.8B). As a representative example, the predicted target genes in the axon guidance pathway are depicted in Figure 4.13. Pathway prediction analysis indicated that both genes with a positive and negative role in axon guidance were predicted to be regulated by differentially expressed miRNAs. This suggests that further analyses are necessary to



validate the individual miRNA-target interactions and the function of clusters of miRNAs in regulating this pathway.

Likewise, pathway analysis for miRNAs up- and down-regulated after 3 days of osteoclastogenic differentiation with M-CSF and RANKL indicated that focal adhesion and axon guidance were predicted targets for various miRNAs (Figure 4.14). Osteoclasts do not use focal adhesions to adhere to the bone surface. However, several proteins that belong to the KEGG functional pathway for focal adhesion participate in the formation of



podosomes and actin rings, which are critical for osteoclast adhesion. These include integrins and proteins of the Rho GTPase signaling pathway. Similarly, although axon guidance is usually studied in regard to neuronal development, numerous factors within this KEGG pathway play an essential role in the osteoclast lineage, such as ephrins, semaphorins, and Rho GTPases (76, 159, 160). Moreover, many signaling cascades that regulate focal adhesion and axon guidance, as well as extracellular matrix-receptor interaction, converge on reorganization of the actin cytoskeleton. This is a key process that regulates a variety of biological functions, including cell motility, morphology, and attachment, as well as gene expression, differentiation, and apoptosis. Cytoskeletal

remodeling and cell migration are fundamental for osteoclast formation and bone resorption activity, and they are tightly controlled at multiple levels (161). We and others have previously shown that miRNAs can modulate osteoclast motility and activity (55, 62, 63). However, a complete understanding of the miRNAs involved in fine tuning the regulation of cytoskeletal reorganization is lacking. Our study contributes to the identification of miRNAs that may play a role in this function.

We performed pathway analysis on miRNAs down-regulated upon 3 days of RANKL treatment. This revealed that the KEGG pathway for osteoclast differentiation was predicted to be regulated by a subset of miRNAs, miR-340-5p, miR-362-3p, miR-500-3p, which had previously not been investigated in osteoclasts or in hematopoiesis (Figure 4.14B). Predicted targets for these miRNAs include proteins involved in signal transduction for the PI3K/Akt signaling and the MAPK signaling, transcription factors such as *Nfatc1*, *c-Fos*, and *Nfkb*, and osteoclast markers like calcitonin receptor and integrin β_3 . Although, further experimental validation is needed, these data suggest that this subset of miRNAs may be critical in the regulation of osteoclastogenesis.

CONCLUSIONS

Osteoclastogenesis is an intricate multi-step process, initiating with the proliferation and commitment of mononucleated precursors, and culminating in the formation of large bone-resorbing polykaryons. In this study, we identified a profile of

miRNAs differentially expressed in the various stages of osteoclastogenesis in primary cultures. Pathway analysis showed that a large number of miRNAs detected in the microarray were predicted to target genes involved in the interaction between cells and extracellular matrix, axon guidance, focal adhesion, and remodeling of the actin cytoskeleton. An increasing number of studies have revealed the relevance of miRNAs in osteoclast biology, making them appealing targets for the development of therapeutic strategies for bone disease. However, our knowledge of the function of specific miRNAs in this lineage is still limited. This study provides important information on miRNAs with the potential to regulate osteoclast differentiation; such information will contribute to the development of therapies for skeletal pathologies caused by alterations in bone resorption activity.

CHAPTER 5:

SUMMARY, SIGNIFICANCE, AND CONCLUSIONS

Summary

The purpose of the studies described in this dissertation was to improve our knowledge of the molecular mechanisms by which miRNAs regulate genes important for osteoclast differentiation and function.

First, we determined that the miR-29 family members, miR-29a, -29b, and -29c, increase during murine osteoclastogenesis. We also showed that inhibition of miR-29 family members impairs osteoclast formation in vitro. In addition, we developed a stably transduced RAW264.7 cell line expressing a doxycycline-inducible miR-29 competitive inhibitor (sponge construct). Using this cell line as a tool, we showed that miR-29 knock-down inhibits commitment and migration of osteoclasts precursors. However, miR-29 knock-down does not affect cell viability, actin ring formation, or apoptosis in mature osteoclasts. We also identified six novel miR-29 targets, which play a role in mediating its function in the osteoclast lineage. These include RNAs critical for cytoskeletal organization, such as Cell Division Control protein 42 (*Cdc42*) and SLIT-ROBO Rho GTPase activating protein 2 (*Srgap2*). Moreover, miR-29 targets RNAs associated with

the macrophage lineage: G protein-coupled receptor 85 (*Gpr85*), Nuclear Factor I/A (*Nfia*), and *Cd93*. In addition, Calcitonin receptor (*Calcr*), which regulates osteoclast survival and resorption, is a novel miR-29 target. Thus, we identified miR-29 as a positive regulator of osteoclast formation, which inhibits RNAs important for cytoskeletal organization, commitment, and osteoclast function (Chapter 3) (62).

Second, we isolated a murine primary bone marrow population enriched for osteoclast precursors, and analyzed the expression of mature miRNAs during the early, middle, and late stages of osteoclastogenesis. Further, we compared the miRNA expression profile in day 3 undifferentiated and differentiated cultures of osteoclast precursors. We identified clusters of differentially expressed miRNAs during the course of osteoclastogenesis, and performed computational analyses to predict functional pathways that may be regulated by these miRNAs. Several miRNAs were predicted to regulate genes involved in cytoskeletal remodeling, a crucial process for the migration of osteoclast precursors, their maturation, and bone resorbing activity. Therefore, miRNA profiling of differentiating osteoclast precursors highlighted novel miRNAs that may regulate osteoclast differentiation and function (Chapter 4).

The main challenge in understanding the functional significance of alterations in the miRNA expression profile is the identification of target mRNAs. A single miRNA can regulate the expression of several hundred genes, and validation of true miRNA-mRNA target interactions requires experimental approaches similar to the ones used in Chapter 3. To determine potential miRNA-mRNA interactions for analysis, computational target prediction is usually the first step, since it helps develop the

hypotheses. This approach is biased by the design of the algorithm used, and frequently by the investigator's interest in specific genes or biological mechanisms. Alternatively, pull-down assays for the proteins involved in miRNA biogenesis, such as Argonaute2, can be used to identify physical interactions between miRNAs and mRNA sequences (162). Both of these methods present advantages and limitations.

For example, computational analysis evaluates hundreds of genes at the same time; although the results of such analyses include a large number of false positive and false negative interactions. Thus, it is necessary to directly validate the predicted miRNA-mRNA interactions (162, 163). Pull-down assays, on the other hand, can lead to the identification of direct miRNA binding sequences in target mRNAs in the cell. However, this type of analysis is technically challenging, demands a high level of expertise, and additional tests are required to confirm that the identified miRNA-mRNA interactions actually regulate the protein product (163).

A more comprehensive approach would combine proteomic analyses with computational target prediction and miRNA expression data, generated by microarray or deep-sequencing. This approach could reveal an inverse relationship between the expression of a certain miRNA and potential mRNA and protein targets in the data set. For example, a recent study by Ou et al. integrated miRNA deep-sequencing results on PBMCs from osteopetrotic patients with quantitative proteomics and bioinformatics for target prediction. Here, a reciprocally expressed miRNA-target pair was identified. miR-320a was decreased in osteopetrosis, whereas the Ras-like GTPase ARF1 (ADP ribosylation factor 1) was up-regulated. *Arf1* was identified as a novel miR-320a target,

and it belongs to a family of GTPases important in the regulation of osteoclast polarization and formation of the sealing zone (64, 164). Such studies in osteoclasts could help identify new miRNAs and target genes important in the development of pathological conditions, and potential candidates for therapy.

Significance

We and others demonstrated that miRNAs are essential regulators of cell differentiation and activity. In addition, alterations in miRNA expression are often detected in pathological conditions, including degenerative diseases, cancer, and autoimmune conditions (8). Due to their ability to regulate cell physiology and their documented dysregulation in disease, miRNAs appear as attractive candidates for the development of diagnostic and prognostic tools, as well as for the development of miRNA-based therapeutics. With regard to miRNA-based therapeutics, the potentially pathological effect of a miRNA over-expressed during disease could be blocked by specific inhibitors, whereas the levels of a miRNA down-regulated during pathogenesis could be restored using mimics. In this way, it may be possible to modify the miRNA levels and, as a consequence, change the phenotype of a particular cell or tissue.

We showed that the miR-29 family of miRNAs promotes osteoclast differentiation (62). Therefore, understanding the mechanisms by which miR-29 exerts its positive effect on osteoclastogenesis may aid in the design of therapeutic strategies for bone diseases associated with alterations of the osteoclast compartment.

miRNA-based therapeutics in clinical trials.

At the time of this writing, two miRNA-based therapeutics have been used in clinical trials. Miravirsen, a locked nucleic acid (LNA) inhibitor oligonucleotide for miR-122, is the most clinically advanced miRNA-based drug. Currently in clinical phase 2a trial, Miravirsen is designed to repress miR-122, which is highly expressed in the liver. Here, miR-122 targets the hepatitis C virus (HCV) in infected patients and protects it from degradation. By sequestering miR-122, Miravirsen blocks HCV replication, and repress the viral infection (Santaris Pharma A/S) (165).

Recently, the first miRNA mimic entered clinical phase 1 trial. MRX34 is a double-stranded RNA mimicking the sequence of miR-34. miR-34 is down-regulated in a variety of tumors, and more than 20 oncogenes were identified as direct targets. MRX34 is delivered using an amphoteric liposomal carrier formulation, which is positively charged in an acidic environment. Since tumors often have low pH, this design facilitates MRX34 uptake in cancer cells, but not in normal cells (miRNA Therapeutics, Inc.) (166).

The development of therapeutics based on miRNAs faces many challenges. In pre-clinical studies, most approaches have used oligonucleotides designed to inhibit a specific miRNA or to mimic its sequence. For safety reasons, this strategy is usually preferred to the use of viral expression constructs. However, chemical stabilization of the oligonucleotide RNA molecules is necessary to avoid their degradation and decrease immunogenicity (167). Another difficulty centers on the delivery of the miRNA therapeutic.

Delivery of miRNA-based therapeutics.

The most recent delivery methods for nucleic acid-based drugs, investigated primarily for small interference RNAs (siRNAs), consist of the conjugation of the RNA molecules with cationic lipids or natural and synthetic polymers, to form nanoparticles. Lipids and polymers may be used both as delivery carriers and as modification agents. Common polymers used include polyethylenimine (PEI), composed of repeated amine groups, poly(lactide-co-glycolide) (PLGA), chitosan, atelocollagen, and protamine. Alternatively, the RNA sequences can be fused to proteins or aptamers, which are structured nucleic acid or peptide molecules, which can be recognized by specific surface receptors. These delivery methods have been explored in several cell types, including hepatocytes, breast cancer cells, T lymphocytes, and osteoblasts (167).

In selecting an appropriate delivery system, it is important to choose an approach that is efficient, but that also allows the targeting of the nucleic acid to the tissue of interest. Localized delivery and release of the miRNA mimics or inhibitors is necessary to reduce systemic side effects. For the same reason, in the perspective of using miRNAs for the development of therapeutics, the identification of miRNA target genes is critical. Although the function of a specific miRNA is known in a particular cell type or tissue, unpredictable effects could occur due to the regulation of unknown targets.

In the skeleton, cell-specific delivery is especially difficult because of the complexity of the microenvironment. Recently, RNA-based molecules have been used to improve bone formation in an animal model. siRNA for pleckstrin homology domain-containing family O member 1 (*Plekho1*) was selectively delivered to osteoblasts, but not

to osteoclasts, using cationic liposomes conjugated with six repetitive sequences of aspartate, serine, serine, (Asp,Ser,Ser)₆ (168). This oligopeptide was shown to preferentially bind lowly crystallized hydroxyapatite and amorphous calcium phosphonate, which are characteristics of bone-forming surfaces (169). In contrast, a sequence of eight repeats of aspartate (Asp₈) has strong affinity for highly crystallized hydroxyapatite, such as that found on osteoclast-rich bone-resorbing surfaces (170). These differences allow the specific targeting of surfaces covered mainly by osteoblasts or osteoclasts.

In another study, siRNA against RANK was delivered to osteoclast precursors and mature osteoclasts in vivo, using PLGA microparticles and calcium-based injectable cement. Here, the siRNA retained its biological activity, and repressed the expression of RANK (171). In vitro, siRNA for RANK was shown to inhibit osteoclast formation and bone resorption activity (172). However, the efficacy of these molecules on osteoclast differentiation and function has not been tested in an in vivo model.

Targeting common precursors, rather than differentiated osteoclasts, could represent another strategy for affecting osteoclast number. A few studies have selectively delivered siRNA molecules to leukocytes, taking advantage of antibodies directed against cell-surface antigens, such as integrin lymphocyte function-associated antigen-1 (LFA-1), fused to protamine molecules (173). In another report, Peer and colleagues delivered siRNA by means of liposomes covalently bound to anti- β_7 integrin antibodies (174). Although these and other strategies can be used to deliver RNA-based drugs to

hematopoietic cells, selective targeting of cells of the monocytic lineage has not been reported.

We showed a positive role for miR-29 in the osteoclast lineage, and demonstrated that its knock-down strongly affects osteoclast differentiation in vitro. Our data suggest that miR-29 could be targeted in the development of therapies for pathological conditions characterized by extensive, aberrant bone resorption. Systemic bone loss can be caused by excessive osteoclast number or activity, as in the case of post-menopausal osteoporosis. In contrast, localized bone loss can occur in rheumatoid arthritis and periodontal disease, and cause joint damage and tooth loss. miRNA-based therapies appear especially appealing for structures like the articular joints or the mandible, since the delivery of the miRNA mimics or inhibitors could be confined to these localized areas.

However, our laboratory and others previously demonstrated that miR-29 also mediates the differentiation of osteoblasts. Therefore, in the use of miR-29 inhibitors to repress osteoclastogenesis, it would be particularly important to deliver the RNA molecules only to the bone resorbing cells. Potentially, the delivery of a miR-29 inhibitor to the bone remodeling units would result in reduced bone turnover, leading to increased bone fragility.

Conclusions

The studies presented in this dissertation are unique in that we described the molecular mechanisms by which a family of miRNAs, miR-29a, -29b, and -29c, supports osteoclastogenesis, and we identified six novel target genes that may mediate this function. This work not only expands our knowledge of the role of miRNAs in the osteoclast lineage, but it also helps us better understand the basic molecular workings regulating cell differentiation, which could be applicable in other cell types.

In addition, we profiled miRNA expression in a primary bone marrow population enriched for osteoclast precursors, and characterized how this profile changes during the early, middle, and late phases of osteoclastogenesis. We showed that clusters of differentially expressed miRNAs could potentially regulate numerous genes involved in actin reorganization and cell motility. Thus, we highlighted novel miRNAs that may play a critical role in osteoclasts.

Maintaining the delicate balance between bone formation and resorption is critical for bone health. In pathological conditions this becomes unbalanced, and the development of therapeutic strategies able to restore this homeostasis relies on a thorough understanding of the molecular mechanisms of osteoblast and osteoclast differentiation. Our research contributes to this goal by providing new data on the function of miRNAs in the osteoclast lineage.

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