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Osteoclastic microRNAs and their translational potential in skeletal diseases

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Abstract

Skeleton undergoes constant remodeling process to maintain healthy bone mass. However, in pathological conditions, bone remodeling is deregulated, resulting in unbalanced bone resorption and formation. Abnormal osteoclast formation and activation play a key role in osteolysis, such as in rheumatoid arthritis and osteoporosis. As potential therapeutic targets or biomarkers, miRNAs have gained rapidly growing research and clinical attention. miRNA-based therapeutics is recently entering a new era for disease treatment. Such progress is emerging in treatment of skeletal diseases. In this review, we discuss miRNA biogenesis, advances in the strategies for miRNA target identification, important miRNAs involved in osteoclastogenesis and disease models, their regulated mechanisms, and translational potential and challenges in bone homeostasis and related diseases.

Keywords

Osteoclast; microRNA; Rheumatoid arthritis

Introduction

Bone tissue is a highly dynamic organ that undergoes constant remodeling process to maintain healthy bone mass. Bone remodeling requires a delicate balance between bone resorption mediated by osteoclasts and new bone formation mediated by osteoblasts/osteocytes. Approximately 5–10% of adult skeleton is remodeled annually, which differs greatly from other tissues in the body and makes bone a unique organ. Normal bone remodeling provides a necessary mechanism for adapting the skeleton to changing

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biomechanical influences and repairing bone damage. However, in pathological conditions, bone remodeling is deregulated, which results in unbalanced bone resorption and formation. Many disease settings, including rheumatoid arthritis (RA), psoriatic arthritis, peri-odontitis, peri-prosthetic loosening, and osteoporosis, are associated with bone destruction [1, 2]. Bone loss (osteolysis) is a major cause of morbidity and disability in these patients and significantly reduces their quality of life (QOL) and increases risk of mortality.

Giant multinucleated osteoclasts, derived from monocyte/macrophage lineage, are exclusively responsible for bone resorption. In pathological conditions, abnormal osteoclast formation and activation play a pivotal role in osteolysis. Comprehensive understanding of the osteoclastic regulatory mechanisms in both homeostatic and disease settings will facilitate development of novel or complementary therapeutic strategies to benefit patients for suppressing pathologic bone resorption. A great amount of work has focused on the effects of protein-coding genes on osteoclastogenesis. Studies from the last decade demonstrate the importance of microRNAs (miRNAs) in various biological and pathological settings, such as cell differentiation, proliferation, apoptosis, cancers, inflammatory disorders, metabolic diseases, and neurological diseases [3–5]. As potential therapeutic targets or biomarkers, miRNAs have recently gained rapidly growing research and clinical attention [6–12]. Targeting miRNAs has shown promising therapeutic potential and efficacy in several clinical trials, such as in the treatment of cancer, diabetes, and hepatitis C [13–15]. This inspiring development highlights the clinical significance of a new era of miRNA-based therapeutics. In this review, we discuss miRNA biogenesis, advances in the strategies for miRNA target identification, important miRNAs involved in osteoclastogenesis and disease models, their regulated mechanisms, and translational potential and challenges in bone homeostasis and related diseases.

miRNA biogenesis

MicroRNAs (miRNAs) are small (~ 22 nucleotides), single-stranded non-coding RNAs that suppress the expression of their target mRNAs via post-transcriptional regulation [6, 16, 17]. miRNAs bind to partially complementary sequences in their target mRNAs but with perfect base pairing between the miRNA “seed region” (sequence of nucleotides 2–7 at the 5′ end of the miRNA) and the targeted sequences (microRNA response elements (MREs)) in 3′ untranslated region (3′-UTR) of mRNAs [18]. miRNAs repress gene expression by degradation or translational inhibition of specific target mRNAs, or a combination of these mechanisms [19]. Mature miRNAs are generated from long precursor RNAs, called primary miRNAs (pri-miRNAs), which are transcribed by RNA polymerase II [20]. In the nucleus, long pri-miRNAs are cropped by the microprocessor complex, which is consisted of the RNase III enzyme Drosha and RNA binding protein DiGeorge syndrome critical region 8 (DGCR8). This process produces hairpin-shaped pre-miRNAs (~ 60–70 nucleotides) [21, 22]. Pre-miRNAs are then exported to the cytoplasm by exportin 5 (XPO5) and further cleaved by the RNase III enzyme DICER [23]. DICER cleaves the double-stranded RNA stem and the terminal loop sequence of pre-miRNAs, and then a mature ~ 22 nucleotide miRNA/miRNA* duplex, containing a guide and a passenger strand (passenger strand designated with asterisk), is formed [24]. Subsequently, the guide strand of the mature miRNA is incorporated into miRNA-containing RNA-induced silencing complex (miRISC),

which comprises DICER, transactivating response RNA-binding protein (TRBP), and Argonaute proteins (AGO1–4) [25, 26]. miRISC is guided by the guide strand to complementary target mRNAs and induces mRNA degradation, translational repression, or a combination of the two mechanisms. Each miRNA can repress multiple target mRNAs. Those target mRNAs sharing the same MREs may compete for miRNA binding and thus affect the expression of each other.

Advances in the strategies for miRNA target identification

Individual miRNAs often target multiple mRNA targets to regulate various biological processes. Identification of miRNA targets can reveal molecular mechanisms and regulatory networks of each miRNA. Recently, several bioinformatics tools, such as TargetScan, miRanda, PicTar, and DIANA-microT, have been developed to predict miRNA targets [27–30]. These bioinformatics prediction algorithms are based on seed-pairing rules, the secondary structure of the 3′ UTR and evolutionary conservation. Traditional seed pairing rules predict miRNA-mRNA target recognition by a perfect complimentary sequence pairing between the miRNA “seed region” and the 3′-UTR of mRNAs [31]. The conventional ways identifying target mRNAs include (1) screening putative targets of each miRNA by bioinformatics tools, (2) validating the expression of target genes by qPCR and western blotting in combination with overexpression and/or inhibition of the miRNA of interest to examine whether there is an inverse correlation, and (3) a reporter assay testing the activity of the 3′ UTR of the predicted target genes (wild-type or mutated miRNA-pairing sequences) to corroborate a direct targeting by the miRNA. These bioinformatics tools, however, typically predict hundreds of targets for each miRNA, leading to a laborious and time-consuming way to identify authentic targets that have impacts on a biological process of interest.

Recent advances in high-throughput sequencing technology have developed several experimental approaches to identify endogenous miRNA targets on a genome-wide scale. Combination of the genome-wide profile of miRNA expression by miRNA-seq and genome-wide changes of mRNA expression levels by mRNA-seq following miRNA overexpression/inhibition significantly improve the efficiency and accuracy of miRNA target identification [32, 33]. In addition, tagged miRNA pull-down approach has successfully identified target mRNAs [34]. In this method, biotin-tagged microRNA mimics are introduced into cells and the biotinylated microRNA/mRNA complexes are purified by streptavidin-coated beads, then the captured mRNAs are analyzed by high-throughput sequencing. This approach is powerful yet not based on physiological conditions because of transfection of miRNA mimics, which needs attention when using this approach. On the other hand, several genome-wide approaches are developed based on immunoprecipitation of miRISC proteins, such as RIP-Seq (ribonucleoprotein immunoprecipitation followed by high-throughput sequencing) [35], HITS-CLIP (high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation) [36], PAR-CLIP (photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation) [37], and CLASH (crosslinking, ligation, and sequencing of hybrids) [38]. These techniques take advantage of crosslinking of endogenous miRNAs/mRNAs and proteins followed by immunoprecipitation with antibodies against miRISC proteins. Subsequently, fragmentation of the pull-downed RNAs

with RNase followed by high-throughput sequencing is performed to identify miRNA-binding sites. Furthermore, some groups have focused on studying translational inhibition by miRNA and developed a ribosome profiling approach using miRNA mimics to identify targets [39]. Because a high density of ribosomes usually binds to translationally active mRNAs, high-throughput sequencing of ribosome-bound mRNAs in the presence or absence of miRNA mimics is able to identify target mRNAs.

These advances in high-throughput sequencing technologies and computational approaches have enabled significant progress in the discovery of miRNA targets on a genome-wide scale. Biological function of these miRNA-target interactions needs further exploration in diverse settings. Nonetheless, these high-throughput sequencing strategies greatly benefit miRNA target identification.

Most of early studies used *in vitro* osteoclast cultures, while recent studies often take advantage of both *in vitro* and *in vivo* systems to investigate miRNA functions. Due to space limitation, we will discuss a few miRNAs that play important roles in osteoclast differentiation, fusion, resorbing activity, survival, osteoclast-to-osteoblast communication, and diseases associated with bone destruction.

miR-21

miR-21 is upregulated by receptor activator of nuclear factor-kappaB ligand (RANKL) and promotes osteoclastogenesis through targeting programmed cell death 4 (PDCD4) protein levels, which subsequently regulate the c-Fos-NFATc1 axis [40]. A later study showed that estrogen suppresses miR-21 biogenesis, which increases FasL protein levels because FasL is a target of miR-21. The enhanced autocrine FasL binds to Fas and induces apoptosis in osteoclasts. Thus, miR-21 also controls osteoclast survival in response to estrogen [41].

miR-31

miR-31, induced by RANKL, is a positive regulator for cyto-skeleton organization and bone resorptive activity of osteoclasts by targeting RhoA [42].

miR-155

miR155 is a well-studied miRNA that plays crucial roles in various immune cells in both innate and adaptive immunity [43–48]. In monocyte/macrophage lineage, miR155 is often induced by inflammatory stimuli, such as LPS and TNF, and regulates proliferation, differentiation, and function of macrophages and dendritic cells [43, 44, 49, 50]. In contrast to the expression pattern and activating function in macrophages, miR-155 is downregulated by RANKL signaling and impairs RANKL-induced osteoclast differentiation by targeting microphthamia-associated transcription factor (MITF) and PU.1. Interestingly, IFNβ induces miR-155, which represses osteoclast differentiation by targeting suppressor of cytokine signaling 1 (SOCS1) and MITF. Taken together, miR-155 acts as an inhibitory miRNA in osteoclastogenesis [51].

miR-223

miR-223 is first identified as a myeloid regulator, potentially controlled by the transcription factor PU.1 [52]. The expression of miR-223 is strongly upregulated in myeloid cells, including neutrophils and macrophages, and it is an important regulator of myeloid cell differentiation. Notably, miR-223 expression is elevated in RA patients [53, 54], and is overexpressed in CD68+ macrophages, CD14+ monocytes, and CD4+ T cells isolated from the synovium of RA patients [55]. miR-223 is expressed in osteoclast precursors and negatively regulates osteoclastogenesis by targeting nuclear factor I A (NFI-A) [56], which is required for upregulating M-CSF receptor levels that in turn induces the expression of PU. 1 and c-Fos [57].

miR-7b

The immune function of miR-7 in autoreactive B cells from systemic lupus erythematosus (SLE) patients was reported by Wu *et al.* [58]. B cell hyperresponsiveness in SLE is caused by enhanced B cell receptor (BCR) signaling, which is mediated by the Pten/ phosphatidylinositol 3-kinase (PI3K) pathway [59]. Wu et al. revealed that Pten expression is decreased in B cells from SLE patients and inversely correlated with disease activity. miR-7 expression is upregulated in the SLE B cells and targeting Pten by miR-7 contributes to B cell hyperresponsiveness in SLE.

Multinucleation formed by the cell-cell fusion of mononuclear osteoclast precursors is an important step for osteoclast maturation. Dendritic cell-specific transmembrane protein (DC-STAMP) is a key regulator of osteoclast precursor (OCP) fusion [60]. DC-STAMP expression is positively regulated by NFATc1, c-Fos, and strawberry notch homolog 2 (Sbno2) [61]. However, the post-transcriptional regulation of DC-STAMP expression is largely unclear. miR-7b is identified as a negative regulator of osteoclastogenesis and cell-cell fusion by directly targeting DC-STAMP [62]. Overexpression of miR-7b represses other fusogenic genes (CD47, ATP6v0d2 and OC-STAMP) as well as osteoclast-specific genes (Nfatc1 and OSCAR) via DC-STAMP inhibition [62].

miR-34a

miR-34a is known to modulate macrophage differentiation and functions. miR-34a is highly expressed in alveolar macrophages and mediates efferocytosis by targeting Axl, a receptor tyrosine kinase-recognizing apoptotic cells, and silent information regulator T1 (Sirt1) [63]. Another group reported the function of miR-34a in pre-B cell-to-macrophage transdifferentiation, in which miR-34a as a direct target of CCAAT/enhancer-binding protein-α (C/EBPα), together with miR223, inhibits Lef1 expression to achieve C/EBPα-mediated silencing of the B cell-specific gene program and transdifferentiation into functional macrophages [64, 65].

Pathological osteoclast differentiation contributes to both osteoporosis and osteolytic bone metastases of cancer. Krzeszinski *et al.* identified miR-34a as a novel negative regulator of osteoclastogenesis, bone resorption, and the bone metastatic niche [66]. miR-34a expression level is decreased during osteoclast differentiation. Osteoclastogenesis from both mouse

bone marrow-derived macrophages (BMMs), and human peripheral blood mononuclear cells is suppressed by miR-34a overexpression but promoted by miR-34a inhibition. Osteoclastic miR-34a-overexpressing transgenic mice exhibit reduced bone resorption and elevated bone mass. On the other hand, miR-34a knockout mice show a complementary bone phenotype. Under pathological conditions, such as ovariectomy (OVX)-induced osteoporosis and bone metastasis of breast or skin cancers, osteoclastic miR-34a overexpression impedes pathological bone resorption and bone metastasis. Moreover, treatment with miR-34a encapsulated in chitosan-nanoparticles effectively attenuates both osteoporosis and cancer bone metastases. Mechanistically, transforming growth factor-β-induced factor 2 (Tgif2) is identified as a direct target of miR-34a in these settings.Tgif2 knockout mice exhibit reduced bone resorption and higher bone mass. Tgif2 deletion abolishes the anti-osteoclastogenic effects of miR-34a. Taken together, miR-34a is a key suppressor of osteoclastogenesis and augmentation of miR-34a activity has a strong therapeutic potential for pathological bone resorption associated with osteoporosis and cancer bone metastases.

miR-124

miR-124 was originally identified as a key regulator that controls the immune function of microglia, tissue-resident macrophages in the central nervous system (CNS), by directly suppressing the transcription factor CCAAT/enhancer-binding protein-α (C/EBP-α) and its downstream target PU.1 [67]. miR-124 expression is decreased during osteoclastogenesis. miR-124 suppresses osteoclastogenesis of mouse BMMs by inhibiting the protein expression of Nfatc1, the master transcription factor for osteoclast differentiation [68]. Furthermore, Nakamachi *et al.* [69] reported that miR-124 is downregulated in the ankles of adjuvant-induced arthritis (AIA) rats. Injection of pre-miR-124 into the ankles of AIA rats strongly ameliorates bone destruction with attenuation of synoviocyte proliferation, leukocyte infiltration into synovial tissue and the destruction of cartilage. Osteoclastogenesis in the joints of AIA rats is also suppressed with pre-miR-124 treatment via directly targeting Nfatc1.

miR-141

miR-141 is as an important regulator involved in osteolytic bone metastasis, which occurs frequently in late stage of breast and bladder cancers, often leading to pathological bone fractures. By genome-wide screening of miRNAs in osteoclasts in response to conditioned medium (CM) obtained from the culture of bone metastatic tumors, such as 4T1.2 and TSU-Pr-B2 cells, Ell et al. found that miR-141 was downregulated by the CM treatment [70]. Ectopic expression of miR-141 inhibits osteoclast differentiation through inhibiting the expression of Mitf and Calcr (calcitonin receptor). Systemic treatment of miR-141 inhibits *in vivo* osteoclast differentiation and increases trabecular bone. miR-141 treatment in a mouse model of breast cancer bone metastasis also downregulates *in vivo* osteoclastogenesis and suppresses bone metastasis [70]. Recently, Yang and colleagues showed the importance of miR-141 in osteoclast differentiation and bone resorption in aged rhesus monkeys [71]. They found that the expression levels of miR-141 were downregulated in aged osteoporotic patients and aged rhesus monkeys. Selective delivery of miR-141 into the aged rhesus monkeys using Asp (Aspartic acid) 8-PU (polyurethane) nanoparticles, which specifically

target bone-resorption surfaces, inhibited in vivo osteoclast differentiation and increased trabecular bone mass. Mechanistically, miR-141 inhibits osteoclast differentiation by targeting Calcr and EphA2 (ephrin type-A receptor 2 precursor). Collectively, these studies indicate an important role for miR-141 in suppressing bone resorption in primates and provide experimental evidence for future clinical application of miRNAs in treatment for osteolytic bone metastasis and osteoporosis.

miR-503

miR-503 was first identified as one of the tumor-associated miRNAs inducing dendritic cell apoptosis by targeting Bcl2 [72]. Later, Chen et al. [73] performed miRNA microarray to compare the miRNA expression profiles in CD14+ peripheral blood mononuclear cells (PBMCs) from postmenopausal osteoporosis patients and postmenopausal healthy women. They found that miR-503 shows the most dramatic downregulation in CD14+ cells isolated from osteoporosis patients. RANKL-induced osteoclastogenesis is inhibited by overexpression of miR-503 in CD14+ PBMCs. Conversely, miR-503 inhibition in CD14+ PBMCs enhances osteoclastogenesis. miR-503 directly targets receptor activator of nuclear factor κ B (RANK), the receptor for RANKL. Specific agomir for miR-503 prevents OVX mice from significant bone loss. Thus, miR-503 may contribute to the pathogenesis of postmenopausal osteoporosis and could be a potential biomarker and therapeutic target for postmenopausal osteoporosis.

miR-214

Osteoclasts and osteoblasts control bone homeostasis by communicating with each other through coupling factors, such as RANKL, OPG, TGF-β1, IGF-1, Sema4D, Sema3A, EphrinB2, Cthrc1, and Wnt16 [74–79]. Exosomes are emerging as essential messengers delivering bioactive molecules, such as mRNAs, microRNAs, and proteins, to mediate inter-cellular communication [80]. Recently, miR-214–3p was identified as an osteoclast-derived exosomal miRNA communicating with osteoblasts [81]. Osteoclastic miR-214–3p is increased with elevation of serum exosomal miR-214–3p in elderly women with fractures and in OVX mice [81]. Osteoclast-specific miR-214–3p overexpression mice (OC-miR-214–3p) exhibit increased serum exosomal miR-214–3p and reduced bone formation. Injection of antagomiR-214–3p enveloped by (D-Asp8)-liposome [82], which can target osteoclasts, rescues low-bone-mass phenotypes in OC-miR-214–3p mice. Moreover, in co-culture system, osteoclast-derived exosomal miR-214–3p is able to transfer to osteoblasts and inhibit osteoblast activity. Treatment with supernatant exosomes derived from the cultures of primary OC-miR-214–3p osteoclasts reduces bone formation *in vivo*, whereas osteoclast-targeted antagomiR-214–3p treatment enhances bone formation in aged OVX mice. The exosomal miR-214–3p derived from osteoclasts inhibits osteoblast activity via inhibiting the protein expression of an important osteogenic transcription factor ATF4, a target of miR-214–3p in osteoblasts [81, 83, 84]. In addition, miR-214–3p expression is increased during osteoclastogenesis and plays a positive role in osteoclast differentiation and activity by directly targeting phosphatase and tensin homolog (Pten) and regulating PI3K/Akt pathway [85]. Previous studies show that miR-214–3p also targets Pten to enhance T cell activation and proliferation [86, 87]. Osteoclast-specific miR-214 transgenic mice exhibit

increased osteoclastogenesis and resorption activity with reduced Pten protein levels and reduced bone mass. Upregulated miR-214–3p expression is also observed in bone specimens from breast cancer patients with osteolytic bone metastasis [88]. The increase of miR-214–3p is correlated with the elevation of bone resorption during the development of osteolytic bone metastasis in nude mice xenografted with human breast cancer MDA-MB-231 cells. Osteoclast-specific miR-214–3p-deficient nude mice show resistance to osteolytic bone metastasis. In this study, TNF receptor-associated factor 3 (Traf3) is identified as a direct target of miR-214–3p, and the increased bone resorption observed in OC-miR-214–3p mice is attenuated with administration of Traf3 3′UTR-containing plasmid encapsulated with (D-Asp8)-liposome [88]. Delivery of osteoclast-targeted antagomiR-214–3p recovers TRAF3 protein levels and attenuates bone resorption and osteolytic bone metastasis. Taken together, these studies strongly indicate the therapeutic benefits of targeting miR-214–3p through regulating both osteoclasts and osteoblasts for patients with abnormal bone remodeling especially in cancer bone metastasis.

miR-182/miR-183

miR-183 cluster is comprised of miR-182, miR-183, and miR-96 [89]. miR-96 is undetectable in osteoclastogenesis [32]. miR-183 expression is elevated by RANKL and positively regulates RANKL-induced osteoclastogenesis via heme oxygenase-1 (HO-1) suppression in *in vitro* osteoclastogenesis [90]. The important function of miR-182 in cell growth, cell fate decision, cancer, T lymphocyte expansion, and Th17 function was just recently appreciated [91, 92]. We initially performed high-throughput miRNA-sequencing and obtained a genome-wide profile of miRNA expression induced by TNFα in mouse BMMs [32]. Based on this database, we identified that miR-182 is a new regulator in TNF-induced inflammatory osteoclast differentiation *in vitro* [32]. We then elucidated the role of miR-182 *in vivo* in physiological bone metabolism and pathological conditions, such as those that occur in osteoporosis and inflammatory arthritis [93]. Pathologic bone destruction is a severe consequence and characteristic of diseases such as rheumatoid arthritis (RA) and postmenopausal osteoporosis, in which osteoclasts are directly responsible for osteolysis. miRNA-based therapeutics is recently entering a new era for disease treatment; however, such progress is quite underdeveloped in treatment of skeletal diseases. We identified miR-182 as a key osteoclastogenic regulator, provided strong translational implications of targeting miR-182 in pathologic bone destruction, uncovered a novel miRNA-orchestrated regulatory network that controls interferon pathway in skeleton, and revealed significant correlation between miR-182 and human RA disease.

The principal findings of our study on miR-182 and their significance include (1) strong translational implications: using complementary gain and loss-of-function approaches (myeloid-specific miR-182 KO and Tg mice), we identify miR-182 as a key positive regulator of osteoclastogenesis, bone homeostasis, and pathologic bone destruction. To investigate translational significance of targeting miR-182, we applied two disease models in our study: ovariectomy (OVX)-induced osteoporosis that mimics postmenopausal osteoporosis and inflammatory arthritis that mimics RA. Inhibition of miR-182 by genetic ablation or pharmacological inhibitors completely counteracts bone loss in both disease models, indicating a robust bone protection effect by miR-182 inhibition. Of note, this

protection is not attributed to the basal bone mass level in the miR-182-deficient mice. To increase the stability, efficacy, and specificity of cellular targeting of the miR-182 inhibitors, we utilized chitosan nanoparticles as delivery vehicles. The chitosan nanoparticles used were packaged with a specific formula that enables them to have the highest bio-distribution in bone marrow and target myeloid osteoclast cell linage. Importantly, the nanoparticles per se are safe and do not affect bone mass. Inhibition of miR-182 does not show undesired side effects, such as immune suppression in these disease models. Thus, our findings highlight promising therapeutic implications of miR-182 inhibition in these diseases and provide proof-of-principle that targeting miR-182 may have clinical utility to treat bone loss. (2) Novel molecular mechanisms: we identify PKR (protein kinase double-stranded RNA-dependent) as a new and essential miR-182 target that is a novel inhibitor of osteoclastogenesis. PKR attenuates osteoclast differentiation via regulation of endogenous IFN-β-mediated autocrine feedback loop. Osteoclastogenesis is determined by the balance between osteoclastogenic and anti-osteoclastogenic factors. Although previously unclear how IFN-β mediated inhibitory loop was downregulated, our studies, for the first time, uncovered an important mechanism that miR-182-PKR axis is responsible for suppressing autocrine IFN-β signaling. These findings indicate a conceptually new model, in which a previously unrecognized regulatory circuit, orchestrated by miR-182-PKRIFN-β axis, fine tunes the osteoclastogenic network. (3) Significant disease correlation: for the first time, our results reveal significant changes of the miR-182-PKR-IFN-β axis with higher miR-182 levels, and lower PKR and IFN-β levels in the PBMCs isolated from RA patients than from healthy donors. Serum TNF levels affect the expression of this axis, and TNFi therapy (Enbrel) reverses the expression of miR-182, PKR, and IFN-β towards healthy donors' levels. Importantly, the osteoclastogenic capacity of RA PBMCs is strongly correlated with the expression levels of miR-182 (positively), PKR (negatively), and IFN-β (negatively). Functional analysis of miR-182 in human PBMCs by gene silencing reveals its key osteoclastogenic role. Thus, as evidenced from our murine and human data, both the regulatory pattern of the miR-182-PKR-IFN-β axis and the miR-182 function are well conserved. These human RA data therefore further support a translational promise of targeting miR-182 in diseases associated with bone loss, such as RA.

Perspectives and challenges of miRNA-based therapeutics

miRNAs exert functions through their specific targets and the downstream pathways mediated by the targets. By binding to complementary "seed" region in target mRNAs, different miRNAs target different genes. However, the target genes regulated by the same miRNA are often variable depending on cell and tissue types. Even in the same cell type, the same miRNA targets can be different in response to distinct stresses or disease settings, presumably due to diverse gene expression and regulation profiles in different conditions. Thus, miRNA-targeted gene regulation is highly specific and quite sensitive to environmental changes. In addition, the same biological process, for example osteoclast differentiation, can be regulated by multiple miRNAs, whose functions may or may not compensate each other in this process. It is therefore important to take into consideration of potential side effects from variable targets by a specific miRNA targeting in preclinical development and clinical trials. Recent studies have shown that mRNA, transcribed

pseudogenes, long non-coding RNAs (lncRNA), and circular RNAs (circRNA) sequester miRNAs and block them from binding to their mRNA targets. Those RNAs are referred to as competing endogenous RNAs (ceRNAs) [94, 95], functioning as molecular sponges for miRNAs and de-repress all target genes of a specific miRNA. These findings add another layer of complexity of miRNA-mediated gene regulation. Therefore, understanding of not only miRNA-target mRNA interactions but also miRNA-ceRNA networks will help fully delineate the mechanisms of miRNA-mediated gene regulation.

Despite a significant progress in miRNA therapeutics, only a small number of miRNA mimics or inhibitors have entered clinical development. Another challenge is the design of miRNA delivery approaches that can ideally make the miRNA-based drugs stable and enable tissue-specific targeting, meanwhile minimizing potential toxicities and off-target effects. Naked small RNA molecules are easily degradable. Chemical modification of the nucleotide back-bone of miRNA mimics or inhibitors, such as modification with locked nucleic acid (LNA), have improved their binding affinity and stability. For example, in our studies, the miR-182 inhibitor has LNA modification. The initial preliminary results however showed that a large amount of the miR-182 inhibitors (~ 1 mg daily) was required to suppress osteoclastogenesis *in vivo*, indicating a delivery vehicle is necessary to reduce amount. Indeed, recent *in vivo* delivery technologies, including nanoparticle systems, have enabled the first generation of miRNA-based agents to move into the pre-clinical development and clinic trials. Chitosan is a cationic polymer derived from chitin and has been extensively used for small RNA delivery in preclinical studies due to its biodegradability and low cellular toxicity. We applied chitosan nanoparticles to incorporate miR-182 inhibitors and reduced approximately 600 times of the amount of miR-182 inhibitors down to 5 μg every 3 days to suppress osteoclastogenesis. The low amount of miR-182 inhibitors using chitosan delivery system not only functions efficiently but also could lower off-targeting effects and cellular toxicity. Chitosan is FDA-approved safe for wound dressing and dietary use. Several animal toxicity studies, including our results, reported good safety *in vivo* [93, 96]. The nanoparticle formula decides the particle size and weight that usually delicately determine the specificity of targeting certain cells. For example, the chitosan formula can be optimized to facilitate targeting monocytes/macrophages and bone marrow [66, 93]. Other groups were successful by using aptamer-functionalized lipid nanoparticles, such as (D-Asp8)-liposome, or Asp (Aspartic acid) 8-PU (polyurethane) nanoparticles that specifically target bone-resorption surfaces, as delivery vehicles in animal models to target osteoclasts [71, 82, 88]. Nonetheless, in order to eventually achieve a successful clinical application, precise identification of miRNA targets in different diseases and development of more osteoclast-specific targeting delivery approaches should be conducted when developing therapeutic applications to treat osteolysis.

The challenges, as described above, give rise to new opportunities for miRNA-based therapeutics. As reviewed in this article, recent miRNA studies provide a proof of concept for the efficacy of therapeutic targeting of miRNAs to prevent or treat bone loss based on the genetic evidence from both *in vitro* and *in vivo* systems, correlation between miRNA expression levels and osteoclastogenic capacity in skeletal diseases, and the *in vivo* pharmacological results obtained from various animal disease models. These promising

studies highlight the translational implications of miRNA-based therapeutics in treating osteolytic diseases, especially the refractory bone resorption such as that occurs in RA, or life-threatening bone destruction associated with cancer bone metastasis.

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