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Regulation of Bone Metabolism by microRNAs

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Abstract

Purpose of Review The small non-coding microRNAs (miRNAs) have emerged as important post-transcriptional regulators of various physiological and pathological processes. The purpose of this article is to review the important recent advances on the role of miRNAs in bone remodeling and metabolic bone disorders.

Recent Findings In a physiological context, miRNAs regulate bone formation and bone resorption, thereby contributing to the maintenance of bone homeostasis. Under pathological conditions, an aberrant miRNA signaling contributes to the onset and progression of skeletal disorders, such as osteoporosis. Furthermore, miRNAs can be secreted to circulation and have clinical potential as non-invasive biomarkers. In a therapeutic setting, miRNA delivery or antagonism has been reported to affect several diseases under pre-clinical conditions thereby emerging as novel pharmacological tools.

Summary miRNAs are key regulators of bone remodeling in health and disease. The future perspectives in the field include the role of secreted miRNAs in cell-cell communication in the bone environment. Furthermore, the clinical potential of using miRNAs as diagnostic tools and therapeutic targets to treat metabolic bone diseases provides an attractive future direction.

Keywords Bone metabolism · miRNA · Osteoporosis · Biomarker · Bone formation · Bone resorption

Introduction

Under physiological conditions, bone mass is maintained by the coordinated and balanced activities of mesenchyme-derived matrix-producing osteoblasts and hematopoietic lineage-derived bone-resorbing osteoclasts, a continuous process called bone remodeling [1]. During aging, bone resorption increases while bone formation decreases, thereby reducing bone mass, which often leads to osteoporosis with subsequent fragility fractures. In addition, a wide range of pathological conditions including metastatic cancer, dietary malnutrition, or metabolic diseases alter bone metabolism leading to unbalanced bone remodeling. Overall, metabolic bone diseases are associated with bone weakening and represent a great medical and socioeconomic challenge worldwide [2].

Although bone mineral density (BMD) can be strikingly affected in mice and humans by mutations in single genes, several skeletal disorders are polygenic in nature. However, genetic variants can only partially explain the variance in BMD, suggesting that both genetic and environmental factors might contribute to the development of osteoporosis [3]. During the last decade, dynamic changes in the human epigenome have been identified that are crucial for the function of osteoblasts, osteoclasts, bone formation, and bone resorption and the pathogenesis of several metabolic bone disorders, including osteoporosis [4]. Epigenetic changes include DNA methylation and histone modifications that modulate gene expression, as well as non-coding RNAs. Non-coding RNAs present the vast majority of the human genome and act as post-transcriptional regulators of mRNA and protein abundance. Among the non-coding RNAs with epigenetic function are the long-non-coding RNAs and various types of small RNAs including small interfering RNAs and the microRNAs (miRNAs). Since their discovery in 1993, miRNAs have emerged as crucial regulators of various physiological processes including bone mass maintenance. In addition, dysregulation of miRNAs is implicated in numerous

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pathological conditions such as osteoporosis. This review outlines the recent understanding of the role of miRNAs in the regulation of bone metabolism and bone metabolic disorders with a specific focus on in vivo studies and studies involving patients.

Bone Metabolism and Osteoporosis

The skeleton is constantly dismantled and rebuilt through the coordinated activities of bone-resorbing osteoclasts and bone-forming osteoblasts [1]. In the growing skeleton, bone formation exceeds bone resorption. Once peak BMD has been reached in the young adult, bone mass is preserved for decades by balanced activities of osteoclasts and osteoblasts. During aging, the absolute volume of deposited bone decreases, resulting from a decline in osteoblast recruitment, differentiation, and a reduction of the amount of matrix synthesized by each osteoblast. Furthermore, osteoclast-mediated bone resorption increases during aging and in particular during osteoporosis in such a way that the ratio between bone formation and bone resorption becomes unbalanced since the amount of bone formed falls below the amount of bone resorbed. This is causative for the structural deterioration, loss of bone mass and BMD, and ultimately osteoporosis with its associated fragility fractures [5].

Secondary osteoporosis results from medical disorders or treatments that interfere with bone metabolism predisposing to accelerated bone loss. While long-term corticosteroids therapy is a well-defined risk factor of secondary osteoporosis and fragility fractures, an increasing list of dietary (e.g., anorexia nervosa), lifestyle (e.g., low physical activity), endocrine (e.g., diabetes mellitus, hyperthyroidism), metabolic, and other (e.g., rheumatoid arthritis) causes of bone mass deterioration has been identified [6]. Primary and secondary osteoporosis with the associated fragility fractures that substantially increase morbidity and mortality affect millions of individuals, thus representing a great and further increasing medical and socioeconomic challenge [7].

Differentiation and function of osteoblasts and osteoclasts are tightly regulated processes. Commitment of mesenchymal precursor cells to the osteoblast lineage and subsequent osteogenic differentiation occurs in a stage-dependent manner. Once committed, osteoblasts undergo early differentiation stages while actively synthesizing bone matrix, followed by matrix mineralization and terminal differentiation at which osteoblasts either die by apoptosis, become lining cells on the bone surface, or become matrix-embedded osteocytes [8]. Multiple signaling pathways including the canonical Wnt/β-catenin pathway control osteoblast differentiation and function in large part via activation of downstream transcription factors, including Runx2 and Osterix1 [1, 8]. Osteoclast differentiation is regulated by the receptor activator of nuclear factor kappa-B ligand (RANKL) and the macrophage colony-stimulating factor (MCS-F) signaling pathways and downstream signaling molecules, including the key transcription factor nuclear factor of activated T cells (NFATc1). In addition, post-transcriptional regulation of transcripts by microRNAs (miRNAs) has recently emerged as a crucial mechanism controlling osteoblast and osteoclast differentiation, function, and dysfunction [9, 10].

miRNA Biogenesis and Function

miRNAs are a class of evolutionary conserved short non-coding RNAs established as key regulators of various biological processes including bone remodeling in health and disease [11, 12•]. The human genome is estimated to encode more than 1800 miRNAs, and each miRNA is predicted to regulate several target genes [13–15]. Computational predictions indicate that more than 50% of all human protein-coding genes are potentially regulated by miRNAs [13, 16]. The abundance of mature miRNAs varies extensively from as few as 10 to more than 80,000 copies in a single cell which provides a high degree of regulation flexibility [17]. The regulation exerted by miRNA is reversible, as feedback/forward regulatory loops have been shown to exert modifying effects during translation [18, 19].

miRNAs are located in the genome as either independent miRNA genes or as miRNA clusters in intergenic regions, within introns of protein-coding genes or in the exons. The miRNAs within the clusters are transcribed as a multi-cistronic primary transcript and are often functionally related [20, 21]. Following transcription by RNA polymerase II, multiple post-transcriptional biogenesis steps are required to process the long polyadenylated primary miRNA (pri-miRNA) to a mature miRNA molecule (Fig. 1) [22]. Initially, pri-miRNA is processed in the nucleus into an approximately 70-nucleotides (nt) long pre-miRNA molecule by a ribonuclease Drosha [23, 24]. Pre-miRNA associates with a Ran-GTP-dependent nuclear export factor exportin-5, and is actively exported from the nucleus. In the cytoplasm, pre-miRNA is further cleaved by the ribonuclease II endonuclease Dicer to produce a short double-stranded structure composed of the sense *miRNA-5p* and the antisense *miRNA-3p* (previously referred as miRNA and miRNA*, respectively). Until recently, the *miRNA-5p* was thought to be the functional strand and the *miRNA-3p* to be degraded. However, both strands can be functional depending on the cellular context [25]. The functional ~22-nt-long mature miRNA strand is incorporated into the ribonucleoprotein complex known as RISC (RNA-induced silencing complex). The mature miRNA guides the RISC to the 3′ untranslated region (UTR) of

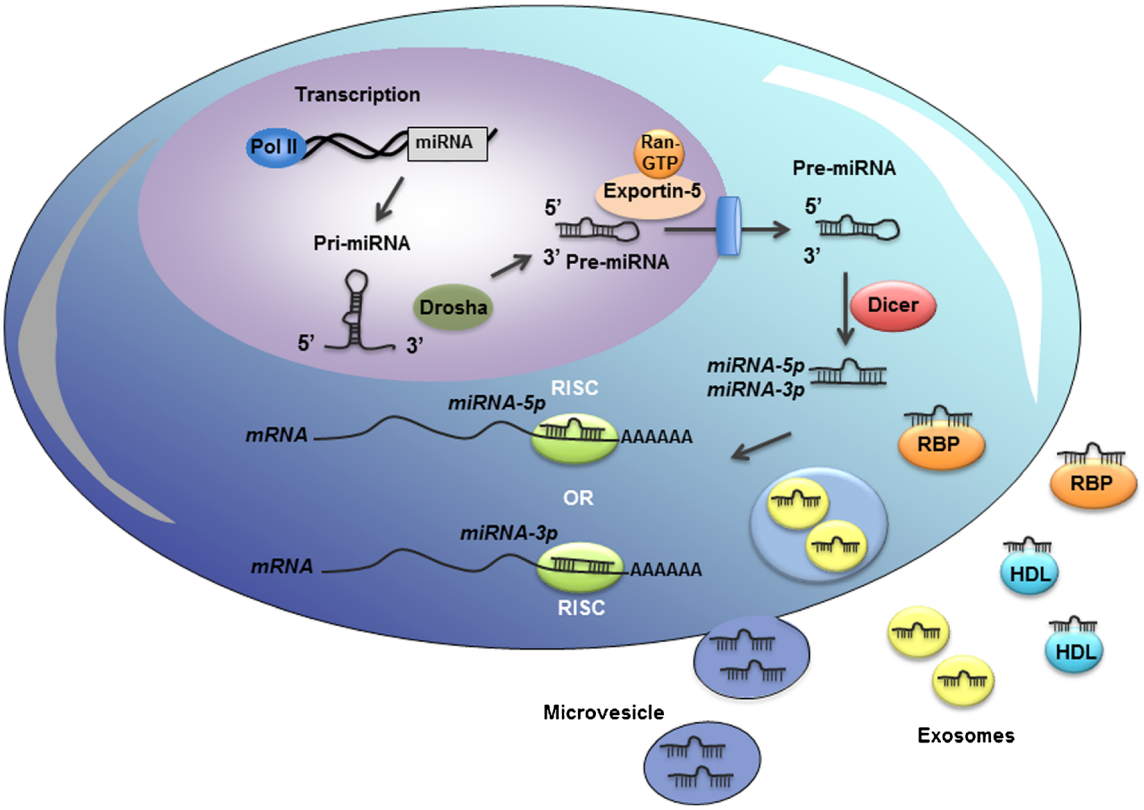

Fig. 1 miRNA biogenesis. miRNA genes are transcribed by an RNA polymerase II as long primary miRNAs (pri-miRNA). Pri-miRNA is processed by a ribonuclease Drosha into an approximately 70-nt-long pre-miRNA molecule. Pre-miRNA associates with a Ran-GTP-dependent nuclear export factor exportin-5, and is actively exported from the nucleus. In the cytoplasm, pre-miRNA is further cleaved by the endonuclease Dicer to produce *miRNA-5p* and *miRNA-3p* strands. The ~22-nt-long mature miRNA strand is incorporated into the RNA-induced silencing complex (RISC), which guides the miRNAs to the 3′ UTR of its target mRNA. Association of miRNA with its target results in either mRNA decay or repression of translation. In addition, miRNAs can be released from the cells encapsulated in microvehicles or exosomes or in vesicles formed by high-density lipoproteins (HDL) as well as in association with RNA-binding proteins (RBP). Pol II, RNA polymerase II; miRNA, microRNA; mRNA, messenger RNA; RISC, RNA-induced silencing complex; HDL, high-density lipoprotein; RBP, RNA-binding protein

its target mRNA [26]. Association of miRNA with its target results in mRNA de-adenylation and either mRNA decay or repression of translation [27].

An important feature of miRNAs is that they can be released from the cells encapsulated in extracellular vehicles, or in vesicles formed by high-density lipoproteins as well as in association with RNA-binding proteins [28–30]. Both secretion and uptake of miRNAs are highly regulated, selective, and energy-consuming processes [31]. To date, circulating miRNAs have been isolated from 12 different biofluids including serum, plasma, and urine [32]. Due to the vesicular encapsulation or protein binding, circulating miRNAs are considerably stable in biofluids even in harsh conditions such as acidic pH. Furthermore, circulating miRNAs are stable even after several freezing-thawing cycles and the expression level in serum is consistent among individuals. The presence and stability of circulating miRNAs in several biofluids make them attractive minimal or non-invasive source of biomarkers of various diseases, including bone disorders [33•].

miRNAs as Biomarkers in Osteoporosis

Despite the increased awareness and development of standardized tools in clinical routine, osteoporosis is still underdiagnosed and undertreated. Currently, fracture risk assessment is largely based on aBMD (areal BMD) measurement by dual energy X-ray absorptiometry (DXA) and assessment of clinical scores such as the WHO Fracture Risk Assessment Tool (FRAX) [34]. However, both methods have limitations [35]. Furthermore, serum markers for bone turnover such as procollagen type 1 *N* propeptide (P1NP) for osteoblast activity/bone formation and C-terminal telopeptide of type I collagen (CTX) for osteoclast activity/bone resorption have not been validated in diagnostics due to several limitations, including moderate association with fracture risk [36]. Therefore, novel biomarkers are needed to identify osteoporotic populations at increased fracture risk. Circulating miRNAs are emerging as promising candidates as biomarkers in bone diseases with several advantages as well as challenges as highlighted in a recent review [33•].

The first studies aiming to identify miRNA signatures associated with BMD focused on miRNAs in circulating peripheral blood monocytes (PBMCs). Analysis of 365 miRNAs in a small cohort of 20 postmenopausal Caucasian females revealed a significantly higher expression of miR-133a in women with low BMD assessed by DXA analysis [37]. Interestingly, miR-133a expression level in circulating B cells isolated from the same subjects was independent of BMD, suggesting that miR-133a might be a monocyte-specific marker to determine postmenopausal osteoporosis. These findings were confirmed in another study analyzing the expression of 721 miRNAs in PBMCs from patients with osteoporosis [38]. Besides monocytes, miR-133a was found to be upregulated in plasma of osteoporotic women in a Chinese cohort of 120 postmenopausal women [39]. Intriguingly, the miR-133a gene is located within two loci previously associated with osteoporosis in genome-wide association studies (GWAS) [40, 41] supporting the potential link between miR-133a and osteoporosis.

Additional miRNA that was identified in monocytes and subsequently found in serum is miR-21-5p [39, 42•]. However, while the expression of miR-21-5p was downregulated in PBMCs of osteoporotic women, it was significantly upregulated in the serum of osteoporotic patients [39, 42•]. Furthermore, miR-21-5p was highly expressed in bone tissue obtained from osteoporosis patients in a study comparing miRNA profile in serum and bone tissue in patients with recent osteoporotic hip fractures and control subjects with non-osteoporotic fractures [42•]. The initial screening of 83 miRNAs in two pooled samples from ten osteoporotic fracture patients and ten controls and the subsequent validation in a larger set of samples resulted in identification of nine miRNAs that were differentially expressed in serum biopsies of patients with osteoporotic compared to those with non-osteoporotic fractures. Out of these, five miRNAs (miR-21-5p, miR-23a-3p, miR-24-3p, miR-100-5p, and miR-125b-5p) were also upregulated in bone tissue biopsies of osteoporotic patients [42•]. Both miR-21-5p and miR-125b-5p were also upregulated in the serum of Spanish women with osteoporotic hip fractures compared to women with osteoarthritis undergoing hip replacement surgery, supporting their potential as valuable biomarkers for osteoporotic fractures [43].

While most of the studies so far have focused on identifying miRNA profiles in postmenopausal osteoporosis, two recent studies addressed whether miRNAs can discriminate fracture status in different bone fragility conditions [44, 45•]. Supporting the notion that miRNA signatures can be indicative of various types of skeletal fractures, Kicijan and coworkers identified a miRNA profile discriminative of patients with low-traumatic fractures, regardless of age and gender [44]. In this study, serum levels of 187 circulating miRNAs were analyzed in male and female patients with idiopathic osteoporosis and low-traumatic fractures as well as in patients with postmenopausal osteoporosis and low-traumatic fractures. All three subgroups displayed differences in the miRNA expression profile compared to healthy age- and sex-matched controls without fractures. Among 19 miRNAs that were commonly regulated in all three subgroups, 8 (miR-152-3p, miR-30e-5p, miR-140-5p, miR-324-3p, miR-19b-3p, miR-335-5p, miR-19a-3p, and miR-550-3p) were identified as significant discriminators of patients with low-traumatic fractures. Interestingly, several miRNAs correlated with bone turnover markers and BMD. For instance, miR-29b-3p, which promotes osteoblast differentiation and bone formation, positively correlated with P1NP [44]. Thus, miRNAs may be used not only as discriminators of patients with fractures but also as markers of bone turnover.

In a related study, serum miRNA signatures were investigated in a well-characterized cohort of 80 postmenopausal women with diabetic bone disease or postmenopausal osteoporosis [45•]. Both study arms comprised of two groups resulting in four groups: non-diabetic postmenopausal women without fractures, non-diabetic postmenopausal women with fragility fractures, type 2 diabetic postmenopausal women without fragility fractures, and type 2 diabetic postmenopausal women with fragility fractures. Expression analysis of 375 circulating miRNAs revealed that 48 miRNAs can differentiate fracture status in type 2 diabetic women and that various combinations of four miRNAs can discriminate diabetes-related fractures. In the non-diabetic cohort, 23 miRNAs were differentially expressed between subjects with and without fracture. Three miRNAs were upregulated and three miRNAs were downregulated in both type 2 diabetic and non-diabetic patients with fractures compared to controls. Further multivariate classification analysis and modeling resulted in identification of miR-550a-5p and miR-382-3p as the best discriminators of diabetic patients and miR-382-3p and miR-183p as the most promising circulating miRNAs for postmenopausal osteoporosis. Functionally, miR-382-3p, which was consistently downregulated in fracture patients, strongly enhanced osteoblast differentiation of human adipose tissue-derived mesenchymal stromal cells (MSCs). In contrast, the most upregulated miRNA, miR-550-5p, inhibited osteoblast differentiation [45•]. Although these findings need to be further validated, they suggest that circulating miRNAs not only serve as biomarkers but can also be functionally important and might, at least in part, explain the pathomechanisms of osteoporosis and other bone diseases.

miRNA Function in Osteoporosis

As outlined earlier, patients with skeletal disorders exhibit a distinct miRNA profile in serum and in bone tissue raising the question whether dysregulation of miRNAs is crucial and/or causative for bone diseases. Since the discovery of miRNAs in

osteoblasts and osteoclasts a decade ago, hundreds of studies have identified miRNAs that regulate bone cell differentiation in vitro [12, 46, 47]. Furthermore, genetic ablation of Dicer in the cells of the osteoblast and osteoclast lineages disturbs skeletal development and bone remodeling emphasizing the importance of mature miRNAs in the maintenance of bone homeostasis [11]. Given the important biological roles of miRNAs in the commitment, differentiation, and function of osteoblasts and osteoclasts, several investigators have recently explored the pathological role of miRNAs in bone degeneration in the context of both excessive bone resorption and reduced bone formation (Table 1).

miRNAs Regulating Bone Resorption

Several osteoclast-related miRNAs have been identified through expression profiles of human PBMCs, including miR-503 and miR-148a [38, 62]. miR-503 was reduced in

Table 1 miRNAs with a validated target and an in vivo function in primary or secondary osteoporosis

miRNA	Expression profile	Target	In vivo function	Ref.
miRNAs regulating bone resorption				
miR-503	Reduced in PBMSc from osteoporotic patients.	RANK	Delivery of miR-503 impairs bone resorption and protects from ovariectomy-induced bone loss.	[37]
miR-148a	Increased during osteoclast differentiation.	MAFB	Antagonizing miR-148a suppresses bone resorption and enhances bone formation thereby increasing bone mass in osteoporosis.	[47]
miR-34a	Decreased in human and mouse osteoclasts upon RANKL stimulation.	Tgif2	Osteoclast-targeted overexpression of miR-34a suppresses bone resorption and protects from osteoporosis in mice. Delivery of miR-34a mimics attenuates ovariectomy-induced bone loss.	[48••]
miR-338-3p, miR-17/20a	Downregulated by glucocorticoids.	RANKL	Reduce glucocorticoid-induced osteoclast differentiation.	[49, 50]
miRNAs regulating bone formation				
miR-34b/c	Increased in mouse osteoblasts during differentiation and upon BMP-2 stimulation.	Runx2, Satb2, Notch1, Notch2	Osteoblast-targeted deletion of miR-34b/c promotes bone formation and increases bone mass. Overexpression causes an osteoporotic phenotype.	[51, 52]
miR-34a	Increased during osteoblast differentiation of human MSCs.	Jagged1	Overexpression of miR-34a in MSCs reduces while inhibition augments bone formation.	[53]
miR-138	Decreased during osteoblast differentiation of human MSCs.	PTK2 (FAK)	Delivery of miR-138 in MSCs reduces, and inhibition enhances osteoblast differentiation and bone formation.	[54]
miR-2861	Mutation causes an early onset osteoporosis.	HDAC5	Silencing of miR-2861 reduces bone mass and accelerates ovariectomy-induced osteoporosis.	[55]
miRNAs regulating bone resorption and bone formation				
miR-214	Increased in bone tissue and in serum of aged patients and osteoporotic mice. Secreted in exosomes by osteoclasts.	ATF4 (osteoblasts)	Overexpression in osteoblasts and osteoclasts impairs bone formation. Osteoblast- and osteoclast-targeted delivery of antagomiR-214 increases bone mass and restores osteoporosis-induced bone loss.	[56]
		PTEN (osteoclasts)	Overexpression of miR-214 in osteoclasts promotes bone resorption.	[57••, 58]
miR-29a	Decreased by glucocorticoids in mice and rats.	Dkk1, IGF-1, HDAC4	Overexpression and delivery of miR-29a promote osteoblast differentiation and bone formation, and attenuate glucocorticoid-induced bone loss in mice and rats.	[59–61]
		RANKL	Overexpression and delivery of miR-29a inhibit osteoclast differentiation and restore glucocorticoid-induced excessive bone resorption.	[59]

circulating PBMCs isolated from osteoporotic patients compared to healthy controls, suggesting a functional link between low miR-503 expression and high bone resorption [38]. Indeed, antagonizing miR-503 in PBMCs enhanced osteoclast differentiation whereas reconstituting PBMCs with miR-503 impaired RANKL-induced osteoclast differentiation. Further in vitro experiments revealed that miR-503 targets RANK rendering cells insensitive to RANKL stimulation. Consistently, reduced expression of miR-503 in ovariectomized mice was associated with an increased RANK abundance, which was further elevated by pharmacological inhibition of miR-503. As a consequence, antagonizing miR-503 enhanced bone resorption and reduced bone mass in vivo under physiological conditions and the effects were accelerated in ovariectomized mice. Consistently, replacement of miR-503 reduced RANK abundance and bone resorption and protected from ovariectomy-induced bone loss [38]. Although it remains to be elucidated whether low expression of miR-503 in osteoporotic patients results in increased RANK abundance and better response to RANKL, these studies demonstrate that miR-503 plays a role in the pathogenesis of postmenopausal osteoporosis. Opposite to miR-503, miR-148a expression was increased during osteoclast differentiation of PBMCs, suggesting a positive function in osteoclasts [62]. Indeed, miR-148a enhanced osteoclast differentiation and function in vitro through suppressing V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB), a negative regulator of NFATc1 and other osteoclast-promoting factors. In an in vivo preclinical model of osteoporosis, administration of antagomiRs against miR-148a concomitantly suppressed bone resorption and increased bone formation thereby augmenting bone mass [62].

An important group of miRNAs for bone metabolism consists of the miR-34 family members. The miR-34 family is highly conserved and among the best studied miRNA families thus far [63]. The miR-34 family comprises three highly related miRNAs: miR-34a, miR-34b, and miR-34c. miR-34a is encoded by a gene located on chromosome 4 in mouse and on chromosome 1 in human, whereas miR-34b and miR-34c are encoded by genes on mouse chromosome 9 and on human chromosome 11. Both chromosomal regions are positively regulated by the tumor suppressor p53 [63]. Consistently, the miR-34 family members slow the progression of several cancers, including the most prevalent primary tumor osteosarcoma [64]. In addition, miR-34 family members regulate both bone resorption and bone formation. However, partially conflicting results indicate that the regulation is complex and the family members exhibit distinct and even opposite functions.

Among the three family members, miR-34a has the highest expression in osteoclast precursors and the expression strongly decreases upon stimulation with RANKL [48••]. Functionally, synthetic miR-34a precursors reduced osteoclast differentiation in both human and mouse osteoclast precursors while an antisense miR-34a reversed the phenotype in both in vitro systems. Consistently, osteoclast-targeted gain-of-function of miR-34a suppressed osteoclast differentiation and bone resorption in vivo thereby increasing bone mass. Furthermore, germ-line deletion of one or both alleles of miR-34a as well as an osteoclast-targeted ablation of miR-34a augmented osteoclast differentiation and bone resorption indicating that miR-34a has a physiologically relevant function in osteoclasts. Mechanistically, miR-34a was shown to target the TGFB-induced factor homeobox 2 (Tgif2), a homeodomain protein with a novel positive function in osteoclasts. Genetic deletion of Tgif2 abrogated the increased bone resorption of miR-34a knock-out mice suggesting that Tgif2 is necessary for the miR-34a function in osteoclasts [48••].

Besides regulating physiological bone resorption, miR-34a is implicated in pathological bone remodeling. Overexpression of miR-34a in the osteoclast lineage protected from the ovariectomy-induced increase in bone resorption and subsequent bone loss [48••]. Furthermore, in a therapeutically relevant treatment setting, systemic administration of miR-34a-conjugated chitosan nanoparticles attenuated bone loss caused by estrogen depletion. These significant findings suggest that miR-34a could be a potential target that protects bone integrity in osteoporosis and other bone loss conditions involving excessive bone resorption.

Opposite to the expression of miR-34a, a recent study demonstrated that miR-34c is upregulated during osteoclast differentiation in vitro [65]. Functional assays revealed an enhanced osteoclast differentiation upon delivery of miR-34c mimics while suppression of miR-34c inhibited the differentiation process. However, the miR-34a/b/c triple knock-out mice exhibited accelerated bone resorption thus recapitulating the loss-of-function phenotype of miR-34a suggesting that miR-34a is predominant in bone resorption in vivo, which might be explained by a higher baseline expression of miR-34a than miR-34b/c in osteoclasts [48••]. Intriguingly, in context of bone formation, miR-34b/c appears to play a more prevalent role, which will be discussed in the next chapter.

miRNAs Regulating Bone Formation

Similar to osteoclasts, a number of studies have established the importance of specific miRNAs in controlling differentiation and function of bone-forming osteoblasts [10]. Notably, several independent studies identified a consistent regulation of the miR-34 family in osteoblasts [51–53]. In mouse cells, miR-34b and miR-34c were strongly upregulated during osteoblast differentiation and induced by BMP-2 [51, 52]. While miR-34a displayed only a modest regulation in these systems, it was increased during osteoblast differentiation of human MSCs, suggesting that the regulation might have species-specific features [53]. Functionally, miR-34b and miR-34c

inhibit cell-autonomous osteoblast differentiation in various mouse cell lines and primary cells through direct targeting of several important osteoblast-related factors including Runx2, Satb2, Notch1, and Notch2. Subsequent in vivo gain-of-function and loss-of-function studies established miR-34b and miR-34c as important regulators of skeletal development as well as bone remodeling in an adult skeleton [51, 52]. In a series of experiments, osteoblast-targeted ablation of miR-34b/c was shown to enhance bone formation and increase bone mass while overexpression of miR-34b/c in the osteoblast lineage reduced osteoblast function leading to an osteoporotic phenotype. In these models, miR-34a was shown to be dispensable for osteoblast function, which was recently confirmed in several additional genetic models by Kresinsky and co-workers [48••]. However, in human MSCs, miR-34a inhibited osteoblast differentiation by targeting a Notch ligand Jagged1 leading to a reduced bone formation in an in vivo model of heterotopic bone formation [53].

In a similar in vivo system, we identified miR-138 as a negative regulator of human MSC differentiation and bone formation [54]. The inhibitory effect was exerted through targeting of focal adhesion kinase (FAK) and subsequent suppression of the ERK pathway and Osterix transcriptional activity. Delivery of synthetic locked nucleic acid (LNA)-based miR-138 inhibitors in human MSCs strongly enhanced bone formation in vivo, suggesting that antagonizing miR-138 might be an attractive bone anabolic approach. Whether miR-138 restores bone mass in osteoporosis remains to be elucidated, however, the LNA technology represents an approach to target miRNAs in a clinically valid setting [66].

Another bone anabolic strategy was introduced in a context of miR-214. miR-214 expression increased in bone tissue and in serum of aged human and osteoporotic mice [56, 57••]. Furthermore, miR-214 levels negatively correlated with bone formation in both human and bone specimen, indicating an inhibitory effect on bone formation. Indeed, osteoblast-targeted overexpression of miR-214 decreased bone mass in mice due to reduced osteoblast parameters and impaired bone formation [56]. The inhibitory effect was mediated by the activating transcription factor 4 (ATF4), an important transcription factor driving osteoblast differentiation and a bona fide target of miR-214. The bone phenotype was restored by targeted delivery of antagomiR-214 inhibitors in osteoblasts using an elegant delivery system, in which antagomiR-214 was encapsulated in aptamer-functionalized lipid nanoparticles that have increased affinity to osteoblasts [67]. Furthermore, targeted delivery of antagomiR-214 increased bone mass in healthy animals and restored osteoporosis-induced bone loss in an ovariectomy model [68]. While these results clearly suggest that miR-214 regulates physiological and pathophysiological bone formation in an osteoblast cell-autonomous manner, the follow-up studies revealed a more complex mechanism.

Apart from osteoblasts, miR-214 is expressed in osteoclasts, in which it promotes differentiation through inhibition of the phosphatase and tensin homolog (PTEN) and subsequent activation of the PI3K/AKT signaling pathway [58]. Consistent with the in vitro findings, osteoclast-targeted overexpression of miR-214 promoted bone resorption in vivo [57••]. Interestingly, these animals also exhibited decreased bone formation due to a reduced osteoblast number and function raising a question whether miR-214 is secreted from osteoclasts and acts on local or distant osteoblasts. This hypothesis was addressed by a series of in vitro and in vivo experiments demonstrating that miR-214 is secreted in exosomes into circulation and is indeed taken up by the osteoblasts [57••]. Not only was miR-214 transferred into osteoblasts but it was also fully functional in the target cells as evidenced by an impaired bone formation in mice treated with exosomes isolated from mice overexpressing miR-214 in osteoclasts. Further support was provided by an increased bone formation after systemic administration of antagomiR-214 encapsulated in an osteoclast-targeted (D-Asp)8-liposome [57••]. Given that bone formation was also strongly increased when antagomiR-214 inhibitors were targeted to osteoblasts, targeting antagomiR-214 into active bone surfaces, i.e., osteoblasts and osteoclasts might have potential as an anabolic or anti-catabolic strategy to reverse established osteoporosis.

miRNAs in Secondary Osteoporosis

Glucocorticoid-induced osteoporosis (GIOP) is the most common form of secondary osteoporosis [69]. Patients with GIOP develop bone loss with an increased risk of spine and hip fractures [6]. Mechanistically, glucocorticoids promote osteoclast differentiation and bone resorption by increasing the expression of RANKL and decreasing the expression of its decoy receptor, osteoprotegerin (OPG) [69]. Interestingly, this pathological increase of RANKL can be reversed by miRNAs. Both miR-338-3p and miR-17/20a target RANKL in osteoblasts and thereby reduced the glucocorticoid-induced osteoclast differentiation and function [49, 50]. Besides inducing bone resorption, glucocorticoids inhibit bone formation by reducing the number and function of osteoblasts. Mechanistically, glucocorticoids impair Wnt signaling activity by increasing the expression of several Wnt inhibitors such as Dkk1 and reduce the expression growth factors that stimulate osteoblasts, including the insulin-like growth factors (IGFs) [69]. Furthermore, the glucocorticoid-induced suppression of osteoblast function is controlled by miRNAs. Several miRNAs are regulated upon treatment with glucocorticoids, including miR-29a, which is downregulated in vitro and in vivo in rats and mice [70–72]. Under physiological conditions, miR-29a promotes osteoblast differentiation through a positive feedback loop involving the Wnt inhibitor Dkk1 and the subsequent activation of Wnt signaling [73]. In a

pathological setting, lentivirus-mediated delivery of miR-29a attenuated glucocorticoid-induced loss of BMD and trabecular bone mass and reduced cortical bone porosity [59]. As a consequence, miR-29a-treated rats were protected from the deleterious effects of glucocorticoids on peak load of bone tissue. These findings were corroborated in mice overexpressing miR-29a [60]. On a molecular level, three mechanisms have been proposed to explain the effect on osteoblast function and bone formation. First, miR-29 treatment decreased the level of serum Dkk1 and activated Wnt signaling. In addition, miR-29a increased the expression of IGF-1, one of the important regulators of glucocorticoid-induced impairment of osteoblast differentiation. A third mechanism by which miR-29a protects from the glucocorticoid-induced reduction of osteoblast differentiation is through the inhibition of histone deacetylase 4 (HDAC4) and subsequent restoration of acetylated Runx2 and β-catenin abundance [61]. The beneficial effects of miR-29a in bone were not limited to osteoblasts, but miR-29a also reduced osteoclast function cell autonomously and non-cell autonomously by attenuating the glucocorticoid-induced increase of RANKL expression in osteoblasts [59].

Despite the evidence that several miRNAs regulate glucocorticoid-induced bone loss, inhibition of microRNA biogenesis and subsequent maturation of miRNAs by deletion of Dicer in the osteoblast lineage had only a minor relevance in the pathogenesis of glucocorticoid-induced inhibition of bone formation in vivo [72]. Deletion of Dicer in Runx2-expressing osteoblast precursors resulted in growth retardation and reduced bone formation; however, glucocorticoids exerted similar effects in Dicer-deficient and wild-type mice [72]. While these findings suggest that Dicer in osteoblasts is dispensable for glucocorticoid-induced bone loss, they do not hamper the importance of mature miRNAs in the process. In fact, several functionally important miRNAs, including miR-29a and miR-17/20a, are already pathologically downregulated by glucocorticoids and thus the complete ablation might have only minor additional effects.

Beyond the effects on bone cells, glucocorticoids increase the number and size of adipocytes in the bone marrow compartment. Although the contribution of miRNAs in bone marrow lipid metabolism is not well understood, recent evidence suggests that miR-29a attenuates the glucocorticoid-induced excessive formation of bone marrow lipids [60]. In contrast, miR-27b suppresses the browning of white adipose tissue and promotes body fat accumulation. Antagonizing miR-27b reversed the effects of glucocorticoids on fat accumulation and might thus prevent from glucocorticoid-induced metabolic dysfunction [74]. Although beyond this review, the role of miRNAs in energy metabolism is an understudied and exciting research area.

miRNA Mutations and miRNA Binding Site Polymorphisms Associated with Osteoporosis

Skeletal diseases are often highly polygenic in nature. In addition to mutations and single-nucleotide polymorphisms (SNPs) in the coding regions, genetic variants can affect or interfere with miRNA function in various conditions. An increasing number of studies have identified SNPs that affect miRNA function [75•]. Such polymorphism can occur in the miRNA promoter (miR-P-SNP) altering their biogenesis and function, in the miRNA-targeting site of the mRNA 3′ UTRs (miR-TS-SNP) interfering with the miRNA binding, or in the miRNA gene (miR-SNPs).

Most commonly, SNPs are identified in miRNA target sites within the 3′ UTR of the target mRNA. In this case, polymorphism can abrogate an existing miRNA-binding site or create a new site altering the expression of the miRNA target protein. For instance, natural genetic variation in three miR-TS-SNPs was associated with variation in femoral neck BMD in a large group of Caucasian subjects [76]. These SNPs contain seed sequences for nine miRNAs, including miR-146a and miR-146b in the 3′ UTR of the fibroblast growth factor 2 (FGF2) mRNA. Consistently, polymorphism-related changes in the expression of FGF2 correlated with differences in bone mineral density [76].

In a set of two studies, a miR-TS-SNP rs1054204 located in the 3′ UTR of osteonectin gene was associated with low bone mass in a cohort of Caucasian men with idiopathic osteoporosis [77, 78]. The rs1054204 C/G polymorphism creates a new functional target site for miR-433 as evidenced by a series of in vitro and in vivo experiments. Mice carrying the minor allelic variant rs1054204G had reduced level of osteonectin accompanied with decreased trabecular bone volume and reduced bone formation rate compared with mice carrying the protective rs1054204C allele [78]. Thus, microRNA-related gene polymorphism in bone-related genes may predispose to osteoporosis susceptibility.

Currently, there are no reports showing a validated association of miR-SNPs with bone mass. However, an important study revealed that a mutation in the genomic locus coding for miR-2861 causes a rare form of familial early-onset osteoporosis in two adolescent patients [55]. Both individuals carried a homozygous C-G mutation in the leading strand of pre-miR-2861, which hampered miRNA processing and reduced mature miR-2861 levels. Loss of miR-2816 in bone biopsies of the two patients correlates with elevated expression of the miR-2816 target HDAC5 and decreased Runx2 levels. Furthermore, silencing of miR-2861 in vivo reduced bone mass and aggravated ovariectomy-induced bone loss recapitulating the effects observed in patients [55]. Hence, dysfunction of miRNAs can disrupt skeletal remodeling during postnatal growth and contribute to osteoporosis.

Clinical Potential of miRNAs in Metabolic Bone Disorders

As highlighted in this review, aberrant miRNA expression has been implicated in the pathology of osteoporosis. Although the role of miRNAs is other bone metabolic disorders than osteoporosis has been poorly investigated, dysregulation of miRNAs is implicated in certain genetic diseases such as osteogenesis imperfecta and osteopetrosis [79, 80]. Furthermore, miRNAs have been revealed as important pathological factors in primary and secondary bone tumors [64, 81, 82]. In vivo delivery of miRNA mimics or miRNA antagonists provides an attractive therapeutic tool to reverse bone tissue degeneration. Thus, miRNAs have great potential for diagnosis and treatment of bone metabolic disorders.

Currently, 164 clinical studies related to miRNAs are recruiting patients, active or completed (www.clinicaltrials.gov). Only three of them are investigating miRNAs in osteoporosis, indicating that the field is still its infancy. The majority of active studies, including the three osteoporosis-related, are exploring the role of miRNAs as biomarkers in various diseases. Once certain challenges such as reliable clinical validation, implementation of reference values and methods, and cost effectiveness have been overcome, circulating miRNAs have potential as non-invasive biomarkers for osteoporosis and other skeletal disorders [33•].

Despite the encouraging results in animal models, only few miRNAs have entered clinical trials as therapeutic targets. The most advanced phase II clinical study has been completed with a positive outcome [66]. In this study, the safety and efficacy of miravirsen, an LNA-modified antisense nucleotide that inhibits miR-122 function, was studied in 36 patients with chronic hepatitis C virus (HCV). Miravirsen treatment resulted in significant virologic response, and no adverse effects were observed [66]. These results clearly show a therapeutic effect by targeting an endogenous miRNA. Importantly, due to the broad applicability of the LNA technology, it is likely that the strategy may be used for diseases other than HCV infection. Although there are still several hurdles to be solved, in vivo delivery of synthetic miRNA mimics or miRNA antagonists is an attractive therapeutic tool potential as novel therapeutic targets in various diseases, including skeletal disorders.

Conclusions

Bone is a dynamic tissue that is constantly dismantled and rebuilt throughout life by the coordinated and balanced activities of matrix-resorbing osteoclasts and bone-forming osteoblasts. Unbalanced bone metabolism leads to decreased bone mass and BMD, and often further develops to low bone mass diseases such as osteoporosis. Mechanisms underlying bone physiology and pathology include interconnected signaling pathways, transcription factors, secreted regulators, genetic determinants, and epigenetic cues including non-coding RNAs. Especially, miRNAs have emerged as crucial modulators of osteoblast-mediated bone formation and osteoclast-related bone resorption, thereby contributing to the maintenance of bone mass maintenance. Under pathological conditions, an aberrant microRNA signaling network can contribute to the onset and progression of diseases such as primary and secondary osteoporosis. Furthermore, miRNAs can be secreted from the cells to regulate cell-cell communication in the bone environment. As a consequence, miRNAs have clinical potential as disease-specific biomarkers. In addition, manipulation of dysregulated miRNAs offers an attractive therapeutic potential to correct the detrimental reduction of bone mass in several bone metabolic disorders.

Compliance with Ethical Standards

Conflict of Interest Hannah Taipaleenmäki declares no conflict of interest.

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

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- • Of importance
- •• Of major importance

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