REVIEW

MECHANISMS IN ENDOCRINOLOGY

Micro-RNAs: targets for enhancing osteoblast differentiation and bone formation

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Abstract

Osteoblast differentiation and bone formation (osteogenesis) are regulated by transcriptional and post-transcriptional mechanisms. Recently, a novel class of regulatory factors termed micro-RNAs (miRNAs) has been identified as playing an important role in the regulation of many aspects of osteoblast biology including proliferation, differentiation, metabolism and apoptosis. Also, preliminary data from animal disease models suggest that targeting miRNAs in bone can be a novel approach to increase bone mass. This review highlights the current knowledge of miRNA biology and their role in bone formation and discusses their potential use in future therapeutic applications for metabolic bone diseases.

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Introduction

Osteoblasts are bone-forming cells that are responsible for bone growth during development and bone formation during remodelling of the post-natal skeleton (1). Osteoblasts originate from stem cells within the bone marrow stroma lying on the abluminal surface of bone marrow sinusoids and are termed bone marrow stromal (skeletal or mesenchymal) stem cells (2). Understanding the mechanisms mediating osteoblast differentiation from mesenchymal stem cells (MSC) as well as regulation of osteoblastic cell functions is a prerequisite for developing new strategies to enhance bone formation and to treat systemic bone diseases such as osteoporosis (3, 4).

Throughout recent years, extensive molecular and genetic studies have unravelled several genetic and epigenetic mechanisms involved in osteoblast differentiation and functions. Differentiation of MSC into mature osteoblastic cells and regulation of osteoblast functions involve highly regulated processes mediated by a large number of hormones and locally produced growth factors. Regulatory factors for osteoblastic phenotype include the essential transcription factors, Runx2/Cbfa-1 and osterix/SP7 (5, 6, 7), and major signalling pathways, bone morphogenetic protein (BMP), Wnt and notch (8, 9, 10, 11, 12), as well as other growth factor-mediated kinase signalling pathways (13). Emerging evidence reveals an additional level of regulation that is mediated by small non-coding single-stranded RNAs termed micro-

RNAs (miRNAs) (14, 15). This review highlights the current knowledge about miRNAs and their involvement in bone development, osteoblast differentiation and functions. It also discusses the future potential of miRNA targeting to treat metabolic bone diseases.

miRNA biogenesis

miRNAs are an abundant class of evolutionarily conserved, short (~22 nt long), single-stranded RNA molecules that have emerged as important post-transcriptional regulators of gene expression (16). The founding members of the miRNA class, lin-4 and let-7, were discovered in *Caenorhabditis elegans* to regulate the developmental timing and progression of the nematode life cycle (17, 18, 19). Later studies have demonstrated that miRNAs are widely expressed in multicellular animals (metazoan eukaryotes) and plants (20). Most metazoan miRNAs bind to partially complementary sites in the 3'-UTRs of the target mRNAs, and thereby inhibit protein synthesis by translational repression and/or mRNA degradation. Perfect complementarity between the miRNA and its target site, as is the case for most plant miRNAs, leads to cleavage of the mRNA by miRNA-induced silencing complex (miRISC) (21).

Most miRNA genes are located in regions distant from annotated genes, suggesting that they derive from independent transcription units (22, 23). A minority of miRNA genes are in the introns of protein-coding

genes, preferentially in the same orientation as the mRNA, indicating that they are processed from the introns rather than transcribed from their own promoters (22, 24). In addition, a significant number of miRNA genes are clustered in the genome and transcribed as a multicistronic primary transcript. The miRNAs within the clusters are often functionally related (17, 25).

miRNAs are processed through a series of post-transcriptional biogenesis steps (26) (Fig. 1). They are transcribed in the nucleus by RNA polymerase II as a long primary miRNA (pri-miRNA) transcript consisting of the mature miRNA in an elongated RNA hairpin structure containing a loop structure directing its cleavage (27, 28). This loop is recognised by the RNase III family nuclease, Drosha, which is present in the ~500 kDa 'microprocessor complex' containing Drosha and double-stranded RNA binding protein (RBP), DGCR8, in humans or its homologue, Pasha, in *Drosophila melanogaster* and *C. elegans* (29, 30). Drosha cleaves the pri-miRNA into a ~70 nt-long stem-loop structure called precursor miRNA (pre-miRNA) (31). The pre-miRNA is actively transported from the nucleus to the cytoplasm by Ran-GTP-dependent nuclear export factor, exportin-5 (32, 33).

In the cytoplasm, the pre-miRNA is loaded into a complex of RNase III endonuclease Dicer and TRBP/Loquacious (34). This complex cleaves the loop from the pre-miRNA to produce a double-stranded structure composed of the miRNA and antisense miRNA* (35). The miRNA* strand is typically degraded, and the ~22 nt-long mature miRNA strand is incorporated into the argonaute protein (Ago 2)-containing ribonucleoprotein complex known as miRISC (36, 37). The mature

miRNA guides the RISC complex to the UTR of its target mRNA (21, 38). The seed sequence, comprising nucleotides 2–8 at 5'-end of the mature miRNA, is important for binding of the miRNA to its target site in the mRNA (39). Association of miRNA with its target results in mRNA cleavage or repression of translation (40, 41, 42). Recently, mRNA decay was suggested as a predominant reason for decreased protein levels of a target gene (43).

miRNA function

To date, 1424 miRNAs have been identified in human cells and each is predicted to regulate several target genes (44, 45). Computational predictions indicate that more than 50% of all human protein-coding genes are potentially regulated by miRNAs (39, 46). The abundance of mature miRNAs varies extensively from as few as ten to more than 80,000 copies in a single cell, which provides a high degree of regulation flexibility (47). The regulation exerted by miRNA is reversible, as feedback/forward regulatory loops have been shown to exert modifying effects during translation (48).

miRNAs play critical roles in diverse biological and cellular processes including metabolism, differentiation and apoptosis. Aberrant miRNA expression has been implicated in the pathogenesis of a large number of human diseases including cancer, diabetes, neurological disorders, heart failure, pulmonary hypertension and autoimmune diseases due to dysfunction of their target genes (49, 50, 51, 52). In addition, numerous miRNAs are associated with development and progression of cancer. Such cancer-associated miRNAs have been

Figure 1 miRNA biogenesis and function. miRNAs are transcribed as long precursors (pri-miRNAs) that are cleaved by Drosha to yield stem-loop pre-miRNAs. The pre-miRNAs are translocated from the nucleus to the cytoplasm by the exportin-5 complex. In the cytoplasm, pre-miRNAs are processed by Dicer into around 22-nucleotide long mature miRNAs. These are incorporated into the RNA-induced silencing complex (RISC), which guides the miRNAs to their target mRNAs, resulting in mRNA cleavage or repression of translation. miRNA, micro-RNA; RISC, RNA-induced silencing complex.

reported as both tumour suppressors and oncogenes (53). Due to aberrant miRNA expression in human disorders, miRNA expression profiling may be useful as diagnostic or prognostic tools and can help in treatment decisions in cancer and other diseases (54).

miRNAs are essential for vertebrate development, proven by studies demonstrating that universal disruption of *Dicer* in mice results in embryonic lethality and depletion of pluripotent stem cells, and deficiency of Argonaute results in severe defects in development (55, 56). Tissue-specific disruption of *Dicer* leads to developmental defects, and in most of the examined tissues, increased cell death, indicating that miRNAs play a crucial role in the development of various tissues including the heart, brain, muscle, lungs, limbs and T cells among others (57, 58).

Mechanisms of action of miRNAs

Mammalian miRNAs tend to have several isoforms (paralogues) encoded from one or more chromosome, suggesting that they are functionally redundant (59, 60). By differences in their expression pattern and 3'-end binding, they exert variable roles *in vivo* (61). Regulation is mainly exerted by binding to 3'-UTR of target mRNA, but miRNA binding to other positions on the target mRNA, e.g. in 5'-UTR or coding sequence and CDS region of transcription factors has also been reported (62, 63, 64). mRNA targeting by miRNA is highly specific, and even rare splice variants can be distinguished by spanning of exon-exon junctions (65). Interestingly, besides generally promoting mRNA cleavage or translational repression, miRNA binding to 3'-UTR can also induce translation of target mRNAs. Few miRNAs have been identified to repress translation during cell proliferation but to activate translation on cell cycle arrest by directing AGO-containing protein complexes to AU-rich elements in the 3'-UTR (66).

Target prediction and validation

Several algorithms are provided online for the prediction of mRNA targets (39). PicTar and TargetScanS are the most commonly used and have been reported to produce comparable predictions of targets (67). Computational target prediction is primarily based on potential pairing of the miRNA seed sequence to a complementary site in the 3'-UTR of a target mRNA according to specific base-pairing rules. Another criteria for target prediction is cross species conservation of at least five species for the miRNA binding mRNA 3'-UTR target site.

Computational algorithms predict that over 50% of all human protein-coding genes may be regulated by miRNAs and that a single miRNA can have hundreds of target genes (46). A conventional low-throughput approach to validate miRNA targets is to clone the

target 3'-UTR into a luciferase reporter plasmid and detect luciferase activity in the presence of specific miRNA precursor and/or inhibitor. Novel immunoprecipitation (IP)-based methods were recently developed for target validation. Karginov *et al.* (68) combined RISC purification with microarray analysis of RISC-bound mRNAs. Ago HITS-CLIP method combines high-throughput sequencing to cross-linking IP to identify functional interaction sites between miRNA and target mRNA. Native Ago protein-RNA (miRNA or mRNA) complexes were covalently cross-linked by HITS-CLIP and the two data sets were combined with bioinformatics analysis. As a result, genome-wide interaction maps were generated for the 21 most abundant miRNAs in mouse brain (69). Cross-linking RBP and miRNA-containing ribonucleoprotein complexes (miRNPs) in a cell-type dependent fashion was used to transcriptome-wide identify miRNA-target interactions by a method called PAR-CLIP (70).

In RNP-IP approach, miRNA-RISC complexes were isolated and miRNA-bound mRNA transcripts were identified by amplification with seed sequence and 3'-UTR-derived primers (71). With RNP-IP method, Hassan *et al.* validated targets for two *Bmp2*-responsive miRNAs, miR-27a and miR-Let7/98, in mouse preosteoblastic cells. *Hlx* was identified as target for miR-27a and miR-Let7/98 family was shown to target several osteoblast-associated genes including *IGF2BP1*, *COL1A1* and *TGFBR1*. As a single miRNA may target several genes, it is evident that in many biological processes targeting only one gene is not sufficient for mediating miRNA-related biological functions. In support of this, Li *et al.* (72) demonstrated miR-29b as a key regulator of osteoblast differentiation by directly targeting several inhibitors of osteogenesis, histone deacetylase (HDAC4), TGF- β 3, ACVR2 and catenin- β -interacting protein 1 (CTNNBIP1) and thus promoting osteoblast phenotype.

Detection of miRNAs

miRNA profiling is a commonly used method for genome-wide miRNA expression analysis. Commercial oligonucleotide-based miRNA arrays provide an efficient platform for high-throughput profiling of miRNA expression (73). Most of the commercially available arrays are based on miRNAs available in miRBase (<http://www.mirbase.org/>) database that is updated regularly due to increasing number of identified miRNAs. miRNA expression profiling has been performed for various cell lines during the osteoblast differentiation process. In global microarray profiling in C2C12 mesenchymal cells, most of the significantly changed miRNAs were down-regulated in response to BMP2 (74, 75). In hMSCs, miRNA profiling with locked nucleic acid (LNA)-based microarray (76) revealed 33 miRNAs being significantly altered between undifferentiated and differentiated cells (77). Among them,

Table 1 miRNAs involved in osteoblastogenesis.

microRNA	Target	Supporting observations	Cell source	References
Let-7	–	More regulated in osteogenic than adipogenic and chondrogenic cells	hMSC	(128)
miR-9, miR-98		Clinically identified as varying between OA and normal tissue	Human tissues	(117)
miR-20b ^s	PPAR γ Bambi Crim1	Upregulates BMP2 and Runx2	hMSC	(129)
miR-23a ~ 27a ~ 24-2	Runx2	Down-regulate SATB2 that synergizes with Runx2 to facilitate bone formation	rOB, MC3T3-E1	(105)
miR-26a	SMAD1	Decreases SMAD1 protein levels by diminishing availability to active SMAD1	hASC	(97)
miR-27 ^s	APC	Activates Wnt signalling through accumulation of β -catenin	hFOB	(130)
miR-29a	Osteonectin, sFRP2, Dkk1	Decreases ON levels by 3'-UTR binding, down-regulates Wnt antagonists	hFOB hOB mOB	(102, 103)
miR-29b ^s	HDAC4, TGF- β 3, ACVR2A, CTNNBIP1, DUSP2	Up-regulated at the matrix maturation stage, down-regulated during mineralisation Down-regulates several inhibitors of OB differentiation	mBMS, MC3T3-E1	(72, 110)
miR-30c, miR-34	Col1A1 Col5A3 Col4A2 Runx2	Group of miRNAs target Runx2 and inhibit osteoblastogenesis	MC3T3-E1	(94)
miR-125b	ErbB2	Inhibits OB differentiation	ST2	(131)
miR-133	Runx2	Belongs to group of Runx2 targeting miRNAs	C2C12	(75, 94)
miR-135	SMAD5, Runx2	Decreases SMAD5 phosphorylation and Runx2	C2C12, MC3T3	(75, 94)
miR-135b		Decreases mineralisation	hUSSC	(132)
miR-138	PTK2	Inhibits FAK/ERK pathway and Runx2 activation	hMSC	(77)
miR-140	HDAC4 ADAMTS5	Accumulates in cartilage, down-regulated in OA	Mouse embryos	(109, 120, 121)
miR-141, miR-200a	Dlx5	Down-regulate Dlx5 and osterix	MC3T3-E1	(101)
miR-146	–	Inhibits NF- κ B activation and inhibits mineralisation	hASC	(133)
miR-148b ^s , miR-27a, miR-489		miR-27a and miR-489 inhibit osteogenesis through repressing grancalcin	hMSC	(108)
miR-155	SMAD1, SMAD5	Inhibits endogenous SMADs and decreases ID3	MUTU I, A5499	(134)
miR-196a ^s	HoxC8	Reduces osteoclast number and causes trabeculae thickening Down-regulates HoxC8, a transcriptional repressor of osteogenesis	Dicer deficient OB and OC hASC	(135) (106)
miR-199a	SMAD1	Regulates chondrogenesis and may play an important role in osteogenesis	C3H10T	(136)
miR-199a, miR-346	LIF	Decrease LIF and induce differentiation by unidentified pathway	hBMS	(137)
miR-204, miR-205 miR-206	Runx2 Cx43	Belong to group of Runx2 targeting miRNAs Down-regulates osteocalcin BMP2 prevents Pri-miR-206 maturation process	MC3T3 C2C12 MC3T3-E1, ST2	(94) (74, 138)
miR-208	Ets1	Down-regulates OPN, Runx2, PTHrP, tenascin-C and type I procollagen	MC3T3-E1 mOB	(139)
miR-210 ^s	ActR1B	Inhibits the TGF- β /actin signalling pathway	ST2	(107)
miR-217, miR-218, miR-338	Runx2	Belong to group of Runx2 targeting miRNAs	MC3T3-E1	(94)
miR-335-5p ^s	Dkk1	Promotes osteogenesis by activating Wnt signalling	MC3T3-E1, MLO-Y4	(78)
miR-378	GalNT-7	Increases nephronectin	MC3T3-E1	(140)

Table 1 Continued

microRNA	Target	Supporting observations	Cell source	References
miR-2861 [§]	HDAC5	Causes Runx2 accumulation Mutations cause osteoporosis in humans	ST2	(63)
miR-3960 [§]	Hoxa2	Induced by Runx2 and promotes osteoblastogenesis	ST2	(104)

C2C12, murine myoblast cell line; MC3T3-E1, murine pre-osteoblastic cell line; 3T3-L1, sub-line of murine embryonic fibroblast cell line; ST2, murine fetal liver-derived stromal cell line; hASC, human adipose tissue-derived stem cells; hFOB, human fetal osteoblasts; hPLSC, human periodontal ligament stem cells; hUSSC, human unrestricted somatic stem cells; m- and hBMSC, murine and human bone marrow mesenchymal stem cells; OB, osteoblast; OC, osteoclast; OA, osteoarthritis. [§], microRNAs that positively regulate osteogenesis.

an almost equal number of miRNAs was up- and down-regulated during differentiation (15 up- and 18 down-regulated). In contrast to MSC, a great number of miRNAs are up-regulated during osteoblast maturation and mineralisation. In preosteoblastic calvarial cells, the majority of miRNAs (58 of 68 significantly altered miRNAs) were up-regulated during mineralisation stage (72), and in mouse pre-osteocyte-like MLO-A5 cells, 14 out of 20 miRNAs were up-regulated in response to ascorbic acid treatment (78). One of the most sensitive methods for miRNA profiling and identification of novel miRNAs is ultra high-throughput sequencing or 'deep sequencing' (79). Deep sequencing produces millions of sequencing reads encompassing the transcriptome of choice for mapping and quantitative and qualitative analysis of small RNA fragments (80). Several programs are available to distinguish miRNA sequences from other non-coding RNAs, mRNAs and degraded RNA, including publicly available programs miRDeep and miRanalyser (81, 82).

Validation of miRNA expression data is commonly performed with small RNA northern blots, RNase protection assays or qRT-PCR analysis. *In situ* hybridisation provides an approach to detect expression and *in vivo* distribution of miRNA. Wienholds *et al.* (83) compared *in situ* expression patterns with expression profiles obtained from miRNA array in zebrafish and were able to confirm more than 77% of the *in situ* expression patterns by at least one microarray data set. By *in situ* hybridisation, Inose *et al.* (74) confirmed miRNA array observations and detected miR-206 expression in perichondrium in E14.4 old mice with decreased expression during *in vivo* skeletogenesis. miR-335-5p was observed in perichondrial cells in E13.5 embryos and in E16.5 in mandible and in osteoblasts aligning on the surface of cranial base cartilage supporting its role as a positive regulator of osteogenesis (78).

Regulation of osteoblast differentiation and bone development by miRNA

The osteogenic differentiation of MSCs is a coordinated process defined by four stages: cellular commitment, proliferation, matrix maturation and mineralisation. Osteoblast differentiation is tightly regulated by hormones such as parathyroid hormone and by local

growth factors including BMPs, IGF and FGFs (84, 85, 86). These factors activate specific intracellular pathways that trigger the expression of several osteoblast-specific transcription factors. Runx2 is an essential transcription factor for the differentiation of MSCs into the osteogenic lineage and for bone formation (87, 88). It regulates the expression of several osteoblastic genes, such as *COL1A1*, alkaline phosphatase (*ALPL*), bone sialoprotein (*IBSP*), *SPP1* (osteopontin) and *BGLAP* (osteocalcin) (89, 90). Runx2 transcriptional activity is negatively regulated by HDACs. Osterix/SP1 is another crucial transcription factor required for the differentiation of preosteoblasts into fully functioning osteoblasts (91). Other important transcription factors involved in osteoblast differentiation include activating transcription factor 4, transcriptional modulator (TAZ), TWIST and homeodomain proteins Msx1, Msx2, Dlx5 and Dlx6 (6, 7, 92).

Role of miRNAs in mesenchymal precursor cell differentiation

An increasing number of miRNAs have been identified to regulate osteoblast differentiation and bone formation positively by targeting negative regulators of osteogenesis or negatively by targeting important osteogenic factors (Table 1 and Fig. 2). Several studies have demonstrated that miRNAs target the principal transcription factors and signalling molecules involved in osteoblast differentiation of MSCs and osteoblast functions (Figs 2 and 3). miRNAs 133 and 204/211 attenuated osteoblast differentiation by directly targeting *Runx2* in C2C12 mesenchymal progenitor cells and MSCs respectively (75, 93). Recently, a group of 11 miRNAs was discovered to control Runx2 levels during osteoblast and chondrocyte differentiation suggesting complex regulation of lineage commitment by multiple miRNAs targeting key lineage-specific transcription factors (94). In addition, several miRNAs indirectly affect *Runx2* expression or activation to modulate osteoblast differentiation. Focal adhesion kinase is a key activator of extracellular signal-related kinase (ERK) pathway in extracellular matrix (ECM)-induced osteoblast differentiation (95, 96). By directly targeting *PTK2*, a gene encoding FAK, miR-138 was shown to attenuate the ERK-dependent pathway, phosphorylation of

Figure 2 Diagrammatic representation of miRNAs regulating osteoblast differentiation. miRNAs are involved in various steps during MSC differentiation to osteoblasts. miRNAs regulate each differentiation step by targeting important transcription factors, growth factors and other molecules. Depending on the target, miRNAs have either a positive or negative effect on osteoblast differentiation. miR, micro-RNA; Osx, osterix; HDAC, histone deacetylase; BMP, bone morphogenetic protein; ECM, extracellular matrix; BSP, bone sialoprotein. §, miRNAs that positively regulate osteogenesis. Red font indicates direct target and black font indicates indirect effect.

Runx2, OSX expression and, subsequently, inhibit osteoblast differentiation and bone formation of MSCs *in vitro* and *in vivo* (77). miR-26a and miR-135 functionally repress osteoblast differentiation by targeting Smad1 and Smad5 respectively (75, 97).

Since osteoblasts and adipocytes share common stem cells (MSCs), miRNA regulation of OB differentiation may regulate adipocytic differentiation indirectly. miR-138 was highly expressed in undifferentiated MSCs and down-regulated upon differentiation to osteoblastic, adipogenic and chondrogenic lineages. Based on its expression profile and functional inhibition of osteogenesis and adipogenesis (77, 98), it is thus possible that miR-138 maintains MSCs in undifferentiated stage. Similarly, miR-335 down-regulation was shown to be critical for MSC differentiation, and over-expression of miR-335 inhibited both osteoblastic and adipogenic differentiation of MSCs (99). A hypothetical model was proposed by Tome *et al.* in which miR-335 is down-regulated in response to tissue damage signals leading to de-repression of its target genes and MSC migration and differentiation. While the above-mentioned miRNAs inhibited both osteoblastic and adipogenic differentiation, miR-204/211 and miR-637 were shown to play a role in the inverse relationship

between osteoblastogenesis and adipogenesis by targeting Runx2 and Osx respectively (93, 100).

miRNAs regulating osteoblast maturation and function

In addition to regulating MSC differentiation to osteoblastic lineage, miRNAs contribute to osteoblast maturation (14). miRNAs miR-141 and miR-200a were down-regulated during preosteoblast differentiation and inhibited osteoblastogenesis by targeting Dlx5 (101). Inose *et al.* (74) identified miR-206 in perichondral cells and not in mature osteoblast and accordingly demonstrated inhibition of osteoblast maturation and *in vivo* bone formation with miR-206.

Activation of canonical Wnt signalling is crucial for osteoblast function. miR-335-5p was shown to directly target and down-regulate Wnt inhibitor DKK1, enhance Wnt signalling and promote osteogenesis in a cell- and development-dependent manner (78). miR-29a potentiates osteoblastogenesis by modulating Wnt signalling through a positive feedback loop (102). While canonical Wnt signalling induced miR-29 expression, miR-29 targets negative regulators of Wnt signalling: Dkk1, Kremen and sFRP2, thus further

Figure 3 Regulation of Runx2, BMP and Wnt signalling pathways by miRNAs. (A) miRNAs regulate positively and negatively Runx2, the key transcription factor of osteogenesis. Positive regulation of Runx2 is obtained by miRNAs targeting Runx2 inhibitors HDACs. Direct binding of miRNAs to Runx2 3'-UTR results in negative regulation. Both negative and positive regulatory loops are shown between Runx2 and miRNAs. (B) miRNA regulation of BMP/Smad signalling results in inhibition of osteoblastogenesis. (C) miRNAs can activate Wnt signalling by targeting Wnt inhibitors. miR-29a expression is activated by Wnt signalling leading to a positive regulatory loop. miR, micro-RNA; HDAC, histone deacetylase; BMP, bone morphogenetic protein; APC, adenomatous polyposis coli.

promoting Wnt activity and osteoblast differentiation (103). Additional regulatory loops were recently identified involving miRNAs and Runx2, implying sophisticated regulatory mechanisms between miRNAs and their targets. miR-3960/miR-2861/Runx2 form a positive regulatory loop promoting osteogenesis (104) while the miR-23a-27a-24-2/Runx2/SATB2 loop functions as a negative regulator of the osteoblast differentiation program. However, Runx2 down-regulates this miR cluster to promote differentiation (105).

Although most of the miRNAs identified thus far negatively regulate osteoblast differentiation, several miRNAs are capable of enhancing osteoblast differentiation and osteogenesis (Table 1). miR-29b, miR-148b, miR-196a, miR-210, miR-335-5p, miR-2861 and miR-3960 have been reported to target and cause down-regulation of various inhibitors of osteoblast differentiation and hence exert stimulatory effects (72, 102, 104, 106, 107, 108). The effects are applied through several mediators, such as HDAC4, HDAC5, TGF- β 3, HoxC8, ActR1b, PEX7 and grancalcin, a Ca^{2+} -binding protein abundantly expressed by neutrophils and macrophages. Interestingly, miR-29b exhibits differentiation stage-dependent expression where it is up-regulated during matrix maturation and down-regulated during matrix mineralisation (72). Several inhibitors of osteoblast differentiation were identified as direct targets for miR-29b: HDAC4, TGF- β 3, activin A receptor type IIA (ACVRA2A) and CTNNBIP1, suggesting that not only multiple proteins but also various signalling pathways can be affected by specific miRNAs.

miRNAs in skeletal development

The importance of miRNAs in skeletal development has been addressed by generating conditional limb

mesenchyme- and osteoblast-specific *Dicer* knockout mice. The disruption of *Dicer* in chondrocytes resulted in an abnormal cartilage phenotype with impaired chondrocyte proliferation and accelerated maturation, indicating that miRNAs play a crucial role in chondrogenesis by maintaining chondrocyte proliferation and inhibiting their premature hypertrophy (58). Tuddenham *et al.* (109) provided evidence for miR-140 accumulation in the cartilage of developing long and flat bones of mice embryos. During bone formation, miRNAs are important in two periods of the process: in promoting the osteoblast differentiation and in controlling bone accrual in a post-natal organism (110). Gaur *et al.* (110) additionally showed that miR-29b and let-7a are up-regulated at matrix maturation stage (d19) and down-regulated during mineralisation (d27) in developing mice. However, our current knowledge on expression and function of specific miRNAs during bone development *in vivo* is still limited. miR-206 expression was detected in developing mouse embryos with decreased expression during osteoblast differentiation and bone formation. Correspondingly, over-expression of miR-206 resulted in a low bone mass due to defected bone formation in mice (74).

Utility of miRNA targeting for treatment of skeletal diseases

miRNAs as therapeutic targets

miRNAs may represent novel therapeutic targets for pharmacological control of bone cell functions and enhancement of bone formation. Several approaches are currently being investigated to be utilised in therapeutic applications (111). By expression of a short hairpin RNA containing either the miRNA from

a plasmid or viral vector with either a polymerase II or III promoter upstream, Dicer could modify the hairpin structure to produce the mature miRNA (112). The use of miRNA sponges to inhibit miRNA function was first described by Ebert *et al.* (113). By transfection with a sponge plasmid containing multiple tandem binding sites for specific miRNA(s), cells expressing sponge RNAs sequester the miRNA, thereby preventing the interaction between targets and endogenous miRNAs. Chemically modified antisense oligonucleotides complementary to the mature miRNA (designated as anti-miRs) provide another approach to silence specific miRNA in cultured cells and *in vivo* (114). Most advanced studies using LNA-modified anti-miRs were performed with miR-122, abundant liver-expressed miRNA. miR-122 binds in the 5'-non-coding region of the hepatitis C virus (HCV) genome, resulting in the up-regulation of viral RNA levels (115). Targeting miR-122 with anti-miRs resulted in decreased cholesterol levels in mice and African monkeys and was recently shown to lead to long-lasting suppression of HCV (116).

miRNAs as targets in skeletal disease

Few specific miRNAs have been identified as playing a role in skeletal diseases, e.g. osteoporosis and osteoarthritis (OA) (117, 118). A novel miRNA, miR-2861, was identified by Li *et al.* (63) to contribute to osteoporosis in mice and humans. miR-2861 promoted osteoblast differentiation by targeting HDAC5 and thereby increasing levels of Runx2. *In vivo* silencing of miR-2861 inhibited bone formation and resulted in decreased bone mass in mice. Consistently, mutations in pre-miR-2861 were associated with early osteoporosis in humans. Not only mutations in the miRNA gene but also in the 3'-UTR of the target gene can predispose to diseases such as osteoporosis (119). Polymorphisms in the 3'-UTR may alter miRNA binding leading to dysregulation of the target gene and aberrant protein level. By comparing known polymorphisms in miRNA target sites (poly-miRTSs) and osteoporosis, Lei *et al.* recently discovered three polymorphisms in the *FGF2* gene that were significantly associated with femoral neck bone mineral density (BMD). These poly-miRTSs harboured binding sites for nine miRs whose binding is potentially altered due to polymorphisms, which can contribute to susceptibility to osteoporosis.

Besides playing a role in osteoporosis, miRNAs are associated with destructive joint diseases such as OA and rheumatoid arthritis (RA). miR-140 has been shown to be highly expressed in normal human articular cartilage, and the expression is reduced in OA (120). Disruption of miR-140 predisposed to age-related OA and, conversely, its over-expression in chondrocytes protected from OA, indicating that miR-140 prevents development of the disease by a mechanism that may involve regulation of ADAMTS5 (121). MiR-146a has been associated with both OA and RA (122, 123, 124). It is a negative

regulator of inflammatory and innate immune responses and was recently discovered to inhibit osteoclastogenesis (125, 126). These studies suggest that miRNAs play important roles in the articular cartilage pathology and skeletal homeostasis *in vivo*.

miRNA targeting represents a novel therapeutic opportunity for treatment of osteoporosis and arthritis. Down-regulated miRNAs could be restored by over-expression using stable vector transfection or transient transfection by double-stranded miRNAs (ds miRNA). By artificial expression of a miRNA that is up-regulated during osteoblastic differentiation and osteogenesis, restoration of down-regulated miRNAs may be a potential treatment strategy in osteoporosis. This approach was applied to suppress bone and cartilage destruction in RA. *In vivo* administration of double-stranded miR-146a prevented joint destruction in arthritic mice, thus demonstrating potential as a therapeutic target for bone destruction in RA (125, 126).

Anti-miRs were used to investigate the *in vivo* function of miR-2861 in osteoporosis in mice. Anti-miR-2861 was injected into the tail vein in sham operated or ovariectomised (OVX) mice. Inhibition of miR-2861 by anti-miR resulted in significantly reduced BMD, the bone loss being most severe in OVX mice (63). Another approach is to combine miRNA technology with stem cell therapy. MSCs are successfully used as vehicles in gene therapy in skeletal repair purposes (127). We recently demonstrated enhanced *in vivo* bone formation by functional inhibition of miR-138 in hMSCs using an LNA-modified anti-miR oligonucleotide (77). The approach in using MSCs as vehicles to deliver miRNA mimics or anti-miRs may serve as a potential tool in future bone regeneration applications.

Future challenges

miRNA based therapy is successfully used in several diseases and an increasing number of reports have been published with evidence of their potential use in skeletal conditions such as osteoporosis and arthritis. Although studies have shown promising results in using miRNAs in skeletal therapy, further investigation is required to better understand the advantages and limitations of this approach. In order to develop effective and safe delivery methods, localisation of ds miRNAs or anti-miRs uptake after systemic delivery should be identified. Designing cell- or tissue-specific administration systems to avoid off-target or even opposing effects in non-targeted tissues remains to be a challenge. Optimisation of delivery dose as well as potential use, whether as a combination of several miRNAs as a cocktail or with other biological agents, needs to be carefully investigated for each application.

Conclusions and perspectives

miRNAs are increasingly recognised as important regulatory molecules of a large number of biological

functions. Understanding their expression profiles and dynamic regulation may be the key to enhance osteoblastic differentiation and bone formation in the treatment of pathological bone diseases. Recent advances in miRNA research have provided new perspectives on the regulation of skeletal development. Moreover, understanding the function of miRNAs and their association with the molecular pathogenesis of various diseases, including OA and osteoporosis, has provided novel insights into the development of therapeutic treatments. Using miRNAs as therapeutic targets by manipulating the miRNA levels to promote osteoblast differentiation may well become a powerful tool in the development of new therapeutic approaches. However, numerous questions including potential off-target effects and efficient delivery *in vivo* need to be solved before using miRNAs in bone disease therapeutics.

Declaration of interest

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