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# Vitamin D and MicroRNAs in Bone

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## Abstract

MicroRNAs (miRNAs) are short noncoding RNAs that orchestrate complex posttranscriptional regulatory networks essential to the regulation of gene expression. Through complementarity with messenger RNA (mRNA) sequences, miRNAs act primarily to silence gene expression through either degradation or inhibited translation of target transcripts. In this way, miRNAs can act to fine-tune the transcriptional regulation of gene expression, but they may also play distinct roles in the proliferation, differentiation, and function of specific cell types. miRNA regulatory networks may be particularly important for signaling molecules such as vitamin D that exert pleiotropic effects on tissues throughout the body. The active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) functions as a steroid hormone that, when bound to its nuclear vitamin D receptor, is able to regulate target gene expression. However, recent studies have also implicated $1,25(OH)_2D$ in epigenetic regulation of genes most notably as a modulator of miRNA function. The current review details our understanding of vitamin D and miRNAs with specific emphasis on the implications of this interaction for biological responses to vitamin D in one of its classical target tissues, i.e., bone.

### Keywords

miRNA; vitamin D; VDR; osteoblasts; bone

## I. INTRODUCTION

To accomplish complex cellular and physiological functions, higher eukaryotic organisms are obliged to coordinate expression of multiple genes, including messenger RNA (mRNA) transcripts and proteins, for generalized housekeeping functions as well as specific functions in specialized tissues. It is now clear that to achieve this requires not only complex control of genome coding sequences but also an extensive system of noncoding (nc) regulatory sequences. This is highlighted by the fact that similar levels of protein-coding genes are present within lower organisms such as *Caenorhabditis elegans* (*C. elegans*) compared to humans, despite the fact that humans have a substantially larger genome.[1] Integrated transcriptome and proteome analyses indicate that only 40–45% of the transcriptome for various organisms is actually translated.[2–4] Such a disparity may be the result of differences

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in ribosome efficiency and/or posttranscriptional mechanisms, but also occurs as a consequence of the abundance of ncRNA produced and because mRNA is subject to multilevel, posttranscriptional regulation. These events are largely mediated by regulatory networks established by RNA-binding proteins (RBPs) and specific noncoding RNA species such as small microRNAs (miRNAs). In essence, RBPs and miRNAs are to posttranscriptional gene regulation what transcription factors are to transcription (Fig. 1).

The discovery of single-stranded endogenous miRNA molecules occurred in the Ambros lab in 1993 during studies of developmental timing events in *C. elegans*.[5] Subsequently, it became apparent that miRNAs are well conserved in all eukaryotic organisms and regulate a diverse array of cellular and physiological processes. Some miRNAs are differentially expressed in cancer cells and may act as oncogenes or tumor suppressor genes,[6] and some miRNAs may act as secreted biomarkers for diseases such as breast cancer with the potential to assist in disease diagnosis, prediction, and treatment.[7] However, miRNAs also play an essential role in normal physiology by facilitating gene expression in complex tissue systems. Prominent among these is the skeleton, where the integrated activity of multiple cell types and genes is essential for optimal mechanical and metabolic function. For example, miRNA-145 (miR-145) was recently reported to play a functional role in human articular chondrocytes[8] and during chondrogenic differentiation of murine mesenchymal stem cells (MSCs).[9] The role of miRNAs in coordinating the activities of bone-forming osteoblasts and bone-resorbing osteoclasts has also been reviewed extensively.[10,11] Less clear is how the activities of such miRNAs are integrated with the established transcriptional activities of hormones and growth factors associated with the skeleton. This review addresses this by focusing on the interaction between miRNAs and vitamin D, an important modulator of bone cells and a key factor in the optimization of bone mineralization and skeletal function.

## II. MIRNA BIOGENESIS AND REGULATORY ACTION

In the nucleus, long [>100 nucleotide (nt)] primary miRNA precursors (pri-miRNA) are cleaved by the double-stranded RNA–specific ribonuclease Drosha/Pasha microprocessor complex to a precursor miRNA (pre-miRNA) that is usually 70–100 nt long containing only the stem loop (Fig. 1). The pre-miRNA is then actively exported out of the nucleus into the cytoplasm, where the loop portion is cleaved by the enzyme RNase III Dicer (Dicer) to generate an approximately 22 nucleotide–long miRNA duplex. This transient miRNA duplex then undergoes strand selection, and one strand (referred to as the passenger or miR*) is degraded, while the other mature strand (referred to as the guide or miR) is incorporated into the RNA-induced silencing complex (RISC) to interact with the target mRNA transcripts (Fig. 1). The most crucial component for target specificity is the seed region that comprises nucleotides 2–8 at the 5’ end of the mature miRNA guide strand.[12]

Short hairpin miRNAs and miRNA genes are often located intergenically in the genome, within introns of ncRNAs (such as ribosomal RNA) or protein-coding genes in a single or clustered format. In the case of miRNAs located intronically, expression usually involves co-transcription with the host gene, but miRNA expression may also be regulated independent of the host gene by control of pre-mRNA splicing or by independent regulation of the miRNA gene.[13–15] In the case of miRNAs linked to regulation of the host gene, the spliced “mirtrons” are exported out of the nucleus and then cleaved by Dicer and incorporated into RISC.[16] For independent miRNA genes, transcription by RNA polymerase II/III leads to a pri-miRNA with secondary and stem loop structures whose expression can be controlled by upstream regulatory elements much like protein-coding genes (reviewed in Ref. 17).

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miRNAs are known to regulate the posttranscriptional silencing of genes by at least two mechanisms: (i) degradation of target mRNA transcripts; (ii) repression of mRNA translation via (partial) complementary base-pair binding to the three prime untranslated (3'UTR) or exonic regions with retainment of the RISC (reviewed in Ref. 18) (Fig. 1). Besides contextual features associated with the complementarities between miRNA and mRNA, several other factors may influence miRNA action such as impaired processing, methylation, gene polymorphisms, gene amplification, and deletion of Dicer.[6] Although the majority of miRNAs target the 3'UTR or exonic regions at distinct miRNA response elements (MREs), there have been reports of gene promoter regions as viable nonconserved target sites.[19] This mode of regulation generally targets histone modification and DNA methylation to control target gene expression. In order to engage their biological functions, miRNAs maneuver in a combinatorial fashion targeting a single mRNA with numerous MREs, and the magnitude of shared or competing MREs among transcripts may offer another level of co-regulation or antagonism.[20]

A single miRNA may target several genes and may therefore provide a very effective strategy for promoting multiple biological functions.[21] However, only a small minority of the 18,226 miRNAs across 140 species have so far been characterized in any detail. In humans, to date, over 1700 miRNAs have been identified with the potential to target 40–60% of genes based on computational predictions (miRBase Release 18).[22] Importantly, the regulation exerted by a miRNA is reversible, since feedback/forward regulatory loops have also been shown to relay modifying effects.[23] Although the precise functions and bona fide target genes of most miRNAs are unknown, they are known to engage in many different cellular and physiological roles including tissue homeostasis, developmental timing, patterning and embryogenesis, proliferation, differentiation, stem cell progenitor commitment, and antiviral responses (reviewed in Refs. 24 and 25). Large-scale comparisons of specific cancer genomes and transcriptomes have revealed rearrangements in noncoding genes similar to those in coding genes, suggesting that critical changes in miRNA profiles may be causal for cancer resistive states.[26]

## III. NUCLEAR RECEPTORS AND MIRNAS

Several reports have documented the influence of miRNA expression and biogenesis by steroid nuclear receptors (NRs) and, conversely, the modulation of NRs by miRNAs (reviewed in Refs. 27 and 28). NRs can influence pri-miRNA expression or pri-/pre-miRNA biogenesis, with the estrogen receptor (ER)α being the most well characterized NR to date (reviewed in Refs. 27 and 29). Besides ERα, other NRs such as the androgen receptor are known to upregulate miRNAs that increase cellular proliferation, inhibit apoptosis, and influence epigenetic gene regulation in a number of prostate cancer cell lines by targeting multiple mRNA transcripts.[30,31] Because some miRNAs are encoded by genes, their transcription can be controlled by NRs and co-regulatory complexes via host gene promoter/enhancer regions. Several labs have shown that the ERα binds to potential cis-regulatory elements in the vicinity of the miR-21 gene in breast cancer cells.[32] ERα can transrepress miR-221 and −222 by recruiting the NR co-repessor proteins NCoR and SMRT to the upstream promoter regions of the host genes for these miRNAs, and this may play a pathological role in tamoxifen-resistant breast cancers.[33] Also, some ER-positive breast cancers are enriched for miRNAs (e.g., miR-200 and let-7 families) following estradiol-mediated induction of an epithelial phenotype.[27] Estradiol-bound ERα is known to affect the biogenesis of miRNAs by inhibition of Drosha,[29,34] but it can also enhance the nuclear transport[35] and subsequent maturation of pre-miRNA by induction of Dicer[36] and Ago2.[37]

The principal mode of action of miRNAs in modulating NR signaling entails the targeting of co-modulators or NR-regulated transcripts themselves and has, for the most part, been

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studied in the context of ERα signaling (reviewed in Ref. 28). The 3'UTR regions of most NRs are variable in length and contain conserved miRNA sites that may be responsible for the stability of the NR mRNA in response to auto-regulatory influences of the NR cognate hormones themselves in a cell-specific manner.[38] For example, miRNAs can cause ERα mRNA degradation by targeting MREs located throughout the ERα transcript, or affect mRNA stability by targeting NR co-regulators as observed in breast cancer.[39,40] ERα is one of the first miRNA-targeted NRs to be studied where its 3'UTR harbors 14 evolutionary conserved MREs.[28] Besides miRNA target sites, NR 3'UTRs are often long and contain AU-rich elements that may also influence mRNA stability.[38] Furthermore, NR signaling can be indirectly altered by miRNAs that interact with NR-target genes. The remainder of this review will focus on the miRNA interactions of another member of the steroid hormone family with established NR signaling function, namely, vitamin D.

## IV. MIRNAS THAT TARGET VITAMIN D SYNTHESIS, METABOLISM AND SIGNALING SYSTEMS

Vitamin D is recognized as an essential dietary nutrient that plays a pivotal role in bone metabolism and mineralization, although it can also act in an extra-skeletal fashion to influence the immune system and cell proliferation and differentiation (reviewed in Refs. 41–45). Following dietary absorption or endogenous synthesis, vitamin D is metabolized in the liver to 25-hydroxyvitamin D (25(OH)D),[46] the major serum form of vitamin D. Circulating 25(OH)D is then further metabolized in the kidney by the mitochondrial enzyme 25-hydroxyvitamin D-1α-hydroxylase (encoded by the gene *CYP27B1*) to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). Catabolism of 25(OH)D and $1,25(OH)_2D$ is known to occur via another enzyme, vitamin D-24-hydroxylase, encoded by the gene *CYP24A1*.[46] Active $1,25(OH)_2D$ functions as a steroid hormone by binding to its cognate NR, the vitamin D receptor (VDR) (reviewed in Refs. 41, 45, 47, and 48). The ability of the $1,25(OH)_2D$-VDR complex, incorporating co-modulator (co-activator and co-repressor) proteins, to regulate gene transcription has been well documented and involves interaction with DNA vitamin D response elements (VDRE) at single gene loci or as part of networks of genes.[47–49] However, recent studies have shown that $1,25(OH)_2D$ can induce epigenetic responses via the regulation of histone-modifying enzymes,[50] whereas epigenetic mechanisms have been shown to corrupt $1,25(OH)_2D$-mediated transcriptional regulation in cancer cells.[51] Although $1,25(OH)_2D$ is also known to influence expression of miRNAs in various cellular settings (Table 1), its effects on specific facets of miRNA biogenesis or promoter co-modulator machinery have yet to be fully documented. Likewise, the mechanisms by which miRNAs refine VDR signaling at various steps during chromatin remodeling are also unclear, but potential effects may be mediated via the VDR, its co-modulators, metabolic enzymes, and transport proteins (Table 2). These different components of interaction between $1,25(OH)_2D$ and miRNAs are discussed in more detail in the next three sections of this review.

Vitamin D metabolites such as 25(OH)D and $1,25(OH)_2D$ are transported in serum primarily via binding to vitamin D binding protein (DBP). Currently, there are no published findings on miRNAs that modulate DBP expression, although software such as Targetscan, which predicts the enrichment of over-represented miRNA targets within a gene set using statistical computations, can help to implicate biological roles for specific miRNAs and miRNA-regulated genes under study. The basic premise of software such as Targetscan is to search for conserved 8mer and 7mer sites in the seed region of the miRNA. The output is a context score based on seed-pairing stability and target-site abundance. Using this strategy, a single miR, miR-4528, is predicted to be a putative regulatory miRNA for DBP expression (Table 2). In contrast to DBP, multiple miRNAs are predicted to target the vitamin D–

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activation enzyme CYP27B1, but only one of these has been validated experimentally. Studies in *Mycobacterium leprae (M. Leprae)*–infected monocytes have shown that miR-21 acts to suppress expression of CYP27B1, with concomitant inhibition of downstream antibacterial responses that are normally induced by intracrine vitamin D signaling.[52] MiR-21 is ubiquitously expressed in a variety of cell types and other validated targets for this miRNA such as Pdcd4, PTEN and Bcl-2 were not monitored in the M. Leprae study, and may thus also influence vitamin D antibacterial responses.[53] In a similar fashion to CYP27B1, multiple miRNAs have also been predicted to target the vitamin D–catabolic enzyme CYP24A1 but this has only been validated experimentally for miR-125b. Studies using ovarian granulosa and breast cancer cells have shown that over-expression or antisense knockdown of miR-125b respectively suppresses and enhances expression of 24-hydroxylase protein.[54] Further analyses in the same report showed that expression of miR-125b was decreased in human breast tumor tissue relative to normal tissue, suggesting that this may be a mechanism for the dysregulated 24-hydroxylase activity that has been previously reported for breast cancer cells.[55]

In addition to its effects on vitamin D catabolism, miR-125b has also been shown to target the VDR itself (Table 2 and Fig. 2). Decreased expression of miR-125b in melanoma cell lines has been shown to enhance responses to $1,25(OH)_2D$ in these cells,[56] whereas increased miR-125b has been implicated in the resistance to $1,25(OH)_2D$ described in MCF-7 breast cancer cells by suppressing endogenous levels of VDR protein.[57] Other miRNAs predicted to target VDR have yet to be validated, although one of these, miR-326, is elevated in peripheral blood lymphocytes of type 1 diabetic patients (T1D).[58] In view of the proposed role for vitamin D in protecting against autoimmunity,[59] it is tempting to speculate that miR-326 may act to impede the postulated immunomodulatory effects of $1,25(OH)_2D$ in preventing inflammatory, autoimmune activity. Beyond the VDR protein itself, it is important to recognize that VDR responses require specific co-regulators that direct $1,25(OH)_2D$-mediated expression of target genes,[47,49,60,61] and these accessory proteins are also likely to be regulated by miRNAs. Targeting of NR co-regulatory proteins by miRNAs has been described previously,[27,28] and specific miRNAs have been predicted to affect VDR co-activators such as SNW1 (see Table 2). However, to date there have been no experimentally validated studies to assess miRNA actions on VDR accessory proteins.

## V. REGULATION OF MIRNAS BY VITAMIN D

The most well documented link between vitamin D and miRNAs concerns the impact of vitamin D metabolites on the expression of specific miRNAs, most notably in the setting of cancer or immunomodulation (Table 1 and Fig. 2). For example, the $1,25(OH)_2D$-VDR regulation of miRNAs in myeloid[62] and prostate cancer[63] cells has been reported as a novel mode of action for the anti-proliferative and differentiation effects of $1,25(OH)_2D$ with respect to cancer therapy. Interestingly, acute myeloid leukeamia and prostate cancer have a strong epidemiological association with vitamin D deficiency,[62–65] underlining the potential importance of miRNA regulation as a mechanism for vitamin D– mediated prevention of some neoplasms. As part of its immunmodulatory activity, $1,25(OH)_2D$, has been shown to promote the immaturity of dendritic cells (DCs) by inducing downregulation of co-stimulatory molecules and interleukin (IL)-12 with enhanced expression of IL-10 production resulting in decreased T-cell activation and responsiveness.[66] Interestingly, these $1,25(OH)_2D$-induced changes in DC phenotype have also been linked to alterations in miRNA expression, notably enhanced expression of miR-378, although the cause and effect relationship for these changes has yet to be proven.[67]

To date, only one report has documented the effects of circulating vitamin D status [serum concentrations of 25(OH)D] on miRNA expression. In this case, the authors described

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changes in peripheral blood mononuclear cell mRNA and miRNA in pregnant women with high or low serum concentrations of 25(OH)D.[68] The authors reported differential expression of 11 miRNAs according to vitamin D status, with these miRNAs being involved in a wide range of cellular functions, including organ and system development (e.g., angiogenesis), and inflammatory and metabolic processes (e.g., carbohydrate/lipid metabolism). Although no direct cause-and-effect relationship was established in the study, data suggest that the B cell CLL/lymphoma 11A (BCL11A) zinc finger protein may be a critical protein since it was a predicted transcript target for most of the differentially regulated miRNAs, and its mRNA levels were differentially expressed in high and low serum concentrations of 25(OH)D. Furthermore, downregulation of BCL11A has been implicated to play a functional role during hematopoietic cell differentiation.[69] Vitamin D deficiency has been linked to pregnancy health complications such as gestational diabetes, preeclampsia, and bacterial vaginosis.[70] Although many of these responses may involve transcriptional regulation,[71] it appears that variations in vitamin D–mediated miRNA expression may also influence pregnancy health outcomes at a posttranscriptional level.

The complex sets of hormone-activated miRNA interactions described above most likely involve an extensive network of feedback and feed-forward regulatory loops. Most of the studies involving $1,25(OH)_2D$-treated cells were performed at time points of one to six days, suggesting that those miRNAs found to be influenced by $1,25(OH)_2D$ may have been affected indirectly. This possibility is supported by studies of whole-genome VDR binding analyses. Three major chromatin immunoprecipitation sequencing (ChIP-Seq) studies using human lymphoblastoid,[72] monocytic,[73] and a mouse pre-osteoblast cell lines[74] have revealed direct VDR gene targets throughout the genome, but no obvious miRNA genes or coordinates were reported.[75] On the basis of these observations, it is possible that the majority of miRNAs that are regulated by vitamin D might be indirectly regulated via modulation of: (i) co-transcribed host genes, (ii) secondary transcription factors or co-modulators part of a feedback loop mechanism, and/or (iii) miRNA biogenesis.

## VI. MIRNA REGULATION OF OSTEOBLASTS AND THE SKELETAL ENVIRONMENT

The role that individual and clusters of miRNAs play in mesoderm-type lineage commitment, osteoblastic differentiation, osteoegenesis, bone remodeling, and bone pathologies has been investigated using a variety of cell types and animal model systems (reviewed in Refs. 10 and 76; Table 3). Most investigations thus far have focused on the role of miRNAs as modulators of osteoblast bone formation. Manipulation of miRNA activity using anti-miRs, miRNA expression vectors, and *in vivo* targeting has enabled better understanding of the biological functions of miRNAs across all stages of osteoblastogenesis. These approaches have successfully shown that osteoblastic miRNAs target an eclectic group of proteins from integral gap junction channels to chromatin remodeling components to dictate cellular functions. Specifically, recent data suggest that miRNAs play a critical role in mediating skeletal development,[77,78] osteoclast activity,[79] osteogenic lineage progression,[80] osteogenic differentiation of adipose tissue–derived stem cells[81] and MSCs,[82] and osteoblastic differentiation[83–86] under both normal and disease conditions.[87,88] These events involve highly regulated processes influenced by a large number of factors such as steroid hormones,[29,88] integrin and β-adrenergic receptors,[89,90] viruses,[91] splicing,[92] stress,[93–97] and locally produced growth factors[98] affecting miRNA function through either cis- or trans-acting regulatory elements or by affecting their biogenesis (reviewed in Ref. 76).

By far the most well studied facet of miRNA activity with respect to osteoblast function has been the regulation of Runx2, bone morphogenic protein (BMP), and Wnt signaling

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pathways. Stein and colleagues showed that miRNAs that positively and negatively regulate the expression of Runx2, a key transcription factor associated with fate determination, potently affect skeletal morphogenesis and osteoblastogenesis.[99] Positive regulation of Runx2 is achieved by miRNAs that target Runx2 inhibitors such as histone deacetylase 5 (HDAC5),[87] whereas the direct targeting of Runx2 message results in negative regulation occurring in regulatory loops.[80,100] Regulation of the BMP-2/Smad signaling pathway by miRNA-135 and −26a leads to inhibition of osteoblastogenesis.[81,101] Wnt signaling is a positive regulator of osteoblast differentiation and miR-29a, which is upregulated during osteoblast differentiation, and can activate Wnt signaling by targeting Wnt inhibitors.[102] Furthermore, miR-29a can become activated by Wnt signaling and participate in a positive regulatory loop to fine-tune factors that promote osteoblast differentiation.[103]

Bone tissue arises from MSCs differentiated toward the osteoblast lineage by genetic and epigenetic mechanisms. Many miRNA experiments have utilized either bone marrow– or adipose tissue–derived human stromal stem cells to assess miRNA function on self-renewal and lineage determination for tissue regeneration. The first paper linking miRNAs to osteoblast formation showed that miR-125b inhibited BMP-4-induced osteoblastic differentiation by regulating cell proliferation in mouse ST2 MSCs, via targeting of the receptor tyrosine kinase Erb2.[85] Subsequently, miR-204 and 211 were shown to inhibit osteogenesis in human MSCs to promote adipogenesis by targeting Runx2.[100] Later, it was demonstrated that miR-637 can target the key osteogenic transcription factor osterix (osx) to enhance adipogenesis of human MSCs both *in vitro* and *in vivo*.[104] Within human MSCs, both miR-128 and −20a are known to regulate osteogenic differentiation by targeting focal adhesion kinase[105] and BMP signaling.[82]

The effects of miRNAs on a wide range of osteogenic functions in committed osteoprogenitors, osteoblasts, and an osteocytic cell lines have been extensively studied (Table 3). In mice, deletion of the mature miRNA-generating Dicer in committed osteoprogenitors manifests into embryonic lethality,[77] highlighting the importance of miRNAs during skeletal development. In general, miRNA targets may have either positive or negative effects on bone cell differentiation (Table 3). MiR-2861 was recently shown to target and degrade HDAC5, a suppressor of Runx2, thereby positively regulating osteoblast differentiation in mice and contributing to primary osteoporosis in humans.[87] Recently, a network connecting Runx2, Satb2 (i.e., a Runx2 stabilizing factor), and the miR-23a/27a/24-2 cluster was shown to regulate osteoblast differentiation,[84] in which Runx2 was shown to repress the miRNA cluster that degrades the chromatin regulator Satb2 to mediate murine bone formation. MiR-335-5p was found to positively modulate osteogenic differentiation by specifically downregulating the Wnt antagonist dickkopf-related protein 1 (DKK1) in mice.[106] Additionally, miR-29b was characterized as a positive osteoblast differentiation regulator by targeting inhibitors of osteoblast differentiation that include matrix maturation and mineralization targets.[77,86] Conversely, miRNAs 133 and 135 target Runx2 and Smad5 to decrease osteoblast formation in murine C2C12 and MC3T3-E1 precursor cell lines.[80,101]

## VII. ROLE OF VITAMIN D-MEDIATED MIRNAS IN OSTEOBLASTS

Despite the wealth of information linking osteoblast regulation to miRNA activity,[107,108] the known actions of vitamin D as a regulator of osteoblast function,[45] and the recent descriptions of miRNA-vitamin D interactions described above, relatively little is known about the role of miRNAs as mediators of vitamin D responses in bone. Osteoblast differentiation is mediated by a number of local and systemic factors and is a key step in skeletal development and acquisition of bone mass. Active $1,25(OH)_2D$ can both positively and negatively regulate the expression of osteoblastic markers of mineralization depending on the stage of the osteoblast differentiation and adaptive responses to calcium

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malabsorption.[109–111] In recent studies, we characterized miRNAs that were specifically regulated by 1,25$(OH)_2$D in primary cultures of human osteoblasts (HOBs).[112] DNA array analyses showed that miR-637 and miR-1228 expression were significantly elevated in HOBs via unique mechanisms following treatment with 1,25$(OH)_2$D. Expression of miR-637 was induced by 1,25$(OH)_2$D independently of its host gene, DAPK3, via VDR binding to a specific vitamin D response element (VDRE) located within an intronic region of DAPK3 that also contains the miR-637 gene. Thus, with respect to regulation by VDR, miR-637 effectively functions as an independent target gene for 1,25$(OH)_2$D. By contrast, 1,25$(OH)_2$D-mediated induction of miR-1228 was associated with transcriptional upregulation of its host gene LRP1, mediated via binding of liganded VDR to consensus VDRE within the proximal promoter of LRP1. Subsequent studies showed that miR-637 and miR-1228 were able to downregulate the target proteins type IV collagen, alpha 1 chain (COL4A1), and BMP-2 induced kinase (BMP2K), respectively (Table 1 and Table 3). Each of these targets was repressed via a distinct mechanism. In the case of COL4A1, this involves degradation of COL4A1 mRNA by miR-637. In the case of BMP2K, miR-1228 acts to inhibit protein translation.

MiR-637 is a primate-specific miRNA that was originally identified in the colorectal miRNAome.[113] Although it was discovered seven years ago, its biological role remains elusive. Interestingly, it was recently shown that endogenous miR-637 is downregulated in four hepatocellular carcinoma (HCC) specimens, suggesting a tumor suppressor role in specialized cancers,[114] whereby overexpression of miR-637 may block cell growth and induce apoptosis of HCC cells. More recent studies have highlighted a musculoskeletal function for miR-637, with expression being enhanced during adipocyte differentiation in human MSCs. Conversely, miR-637 expression appears to decrease osteoblast lineage commitment by targeting the early osteoblast-specific transcription factor osterix.[115] One of the potential targets for miR-637, COL4A1, is found primarily in the basal lamina of vessels in many distinct locations including osteons and bone marrow in human alveolar and calvarial bones, and dentin.[116–118] Primary HOBs and other osteoblastic cell lines express low levels of COL4A1 *in vitro*.[119] Recently, osteogenin, an ECM component of bone, was identified as a differentiation factor that initiates endochondral bone formation.[120] As it relates to our studies, both osteogenin and transforming growth factor beta (TGF-β)[121] were shown to avidly bind to COL4A1, which may modulate their local actions not only in nearby basement membranes, but in osteoblasts. It is unclear whether COL4A1 expression during osteoblastic differentiation acts as an inhibitor of matrix mineralization as seen for other collagenous and non-collagenous matrix proteins in bone. Recent studies have shown that miR-29b regulates osteoblast differentiation by suppressing several inhibitors of osteoblast differentiation, including col4a2,[77,86] which is the fibrillation partner of col4a1. Further studies are necessary to determine the importance of COL4A1 repression during vitamin D–induced osteoblastic differentiation.

Initial studies identified miR-1228 from a mammalian screen of small RNA libraries from *Rhesus macaque* and human brain tissues,[122] but its function has yet to be determined. The mature miR-1228 sequence is homologous to mouse miR-667,[112] with nearly all of the 19 nucleotides of the mature guide strand conserved in mouse miR-667 (78% similar), including perfect similarity of the crucial seed region. Currently, it is unclear whether mouse miR-667 is regulated by 1,25$(OH)_2$D in the same fashion as miR-1228. Rather than being a classical miRNA, miR-1228 is predicted to be a mirtron,[16] and not a miRNA gene. Spliced mirtrons are exported out of the nucleus and then cleaved by Dicer and incorporated into RISC. Our study showed that miR-1228 affects one of its targets, BMP2K, via repression of protein translation.[112] This mode of miRNA-translation repression is known to exist in other systems such as in *C. elegans*[123] and *Arabidopsis*,[124] and depends on a number of strand contextual features and can be distinguished from RISC endonuclease activity. There are

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other examples where miRNA silencing only decreases protein levels in human cells,[125] suggesting this process is viable in HOBs via 1,25$(OH)_2$D treatment. BMP2K was initially identified as being overexpressed in a Runx2-independent manner during BMP-2-induced differentiation of a prechondroblastic cell line toward an osteoblastic phenotype.[126] BMP2K is a protein kinase that contains a nuclear localization signal and glutamine-rich region similar to transcription factors. When BMP2K was stably expressed in murine osteoblastic cells, alkaline phosphatase activity and osteocalcin mRNA levels were decreased and mineral deposition was reduced. Thus, it was concluded that BMP2K plays an important role in attenuating osteoblast differentiation. Downregulation of BMP2K protein, via a miR-1228-dependent mechanism, by 1,25$(OH)_2$D may serve to abrogate this attenuation of osteoblast differentiation.

## VIII. CONCLUDING REMARKS

Our knowledge of how miRNAs can influence the skeletal environment through the actions of steroid hormones such as vitamin D, and through posttranscriptional regulation of gene expression, is slowly taking shape. This knowledge will be important, since miRNAs represent key pharmacological targets that can be manipulated to alter entire cellular programs. Nevertheless, a detailed understanding of how miRNAs regulate vitamin D metabolism, their impact on nuclear receptors such as the VDR, and resultant effects on 1,25$(OH)_2$D signaling is far from clear and provides an exciting area of future research. Elucidating the role of miRNAs during osteoblastogenesis will provide additional insights into the pathogenesis and potential treatment strategies toward vitamin D–associated bone disorders such as rickets and osteoporosis.

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## ABBREVIATIONS

| | |
|---|---|
| **1;25OH2D** | 1;25-dihydroxyvitamin D |
| **25OHD** | 25-hydroxyvitamin D |
| **VDR** | vitamin D receptor |
| **ERα** | estrogen receptor |
| **NR** | steroid nuclear receptor |
| **miRNA** | microRNA |
| **MRE** | miRNA response element |
| **RISC** | RNA-induced silencing complex |
| **3'UTR** | 3 prime untranslated region |
| **MSC** | mesenchymal stem cell |
| **HOB** | human osteoblast |
| **BMP** | bone morphogenic protein |
| **BMP2K** | BMP-2 induced kinase |
| **COL4A1** | type IV collagen; alpha 1 chain |

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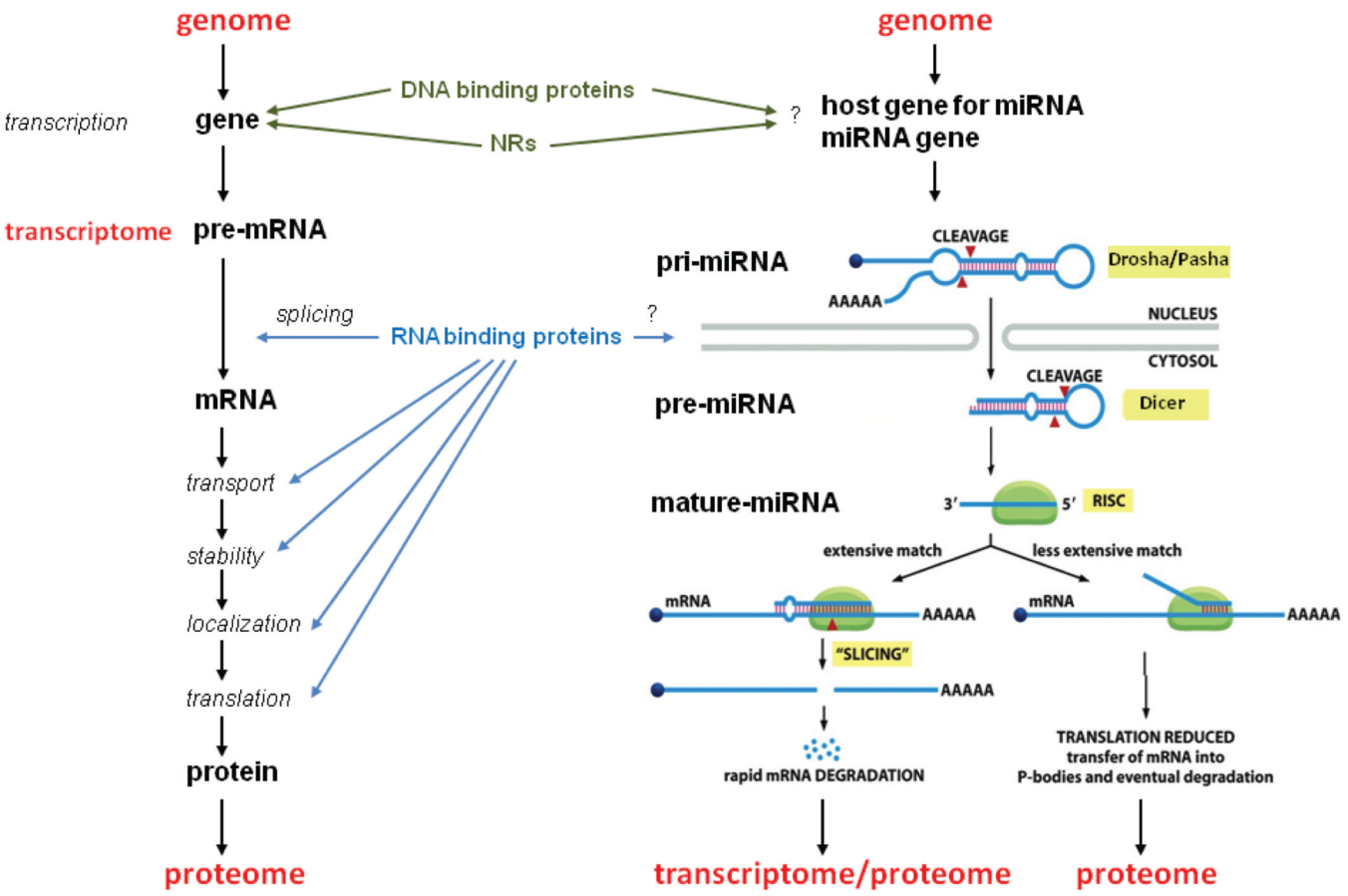


**FIG. 1.**

The steroid nuclear receptor and RNA binding protein mediation of transcriptional and posttranscriptional gene expression. A distinct set of proteins control most of the steps that link the genome to the proteome. Transcription factors, nuclear receptors (NRs), and accessory proteins regulate RNA transcription, with most events after this point being regulated by RNA binding proteins and ncRNA species. The canonical miRNA biogenesis pathway is conserved and processed by three major enzyme complexes (Drosha/Pasha, Dicer, and RISC; Reviewed in Ref. 15). RNA polymerase II or III induce the production of the primary miRNA transcript (pri-miRNA), which is then cleaved by Drosha/Pasha. A precursor hairpin pre-miRNA is then generated that is exported from the nucleus to the cytoplasm. The enzyme Dicer then cleaves the pre-miRNA to its functional mature strand. The mature miRNA is integrated into the RISC complex to silence target mRNAs via endonuclease cleavage ("slicing") or translational inhibition to modify the transcriptome and proteome.

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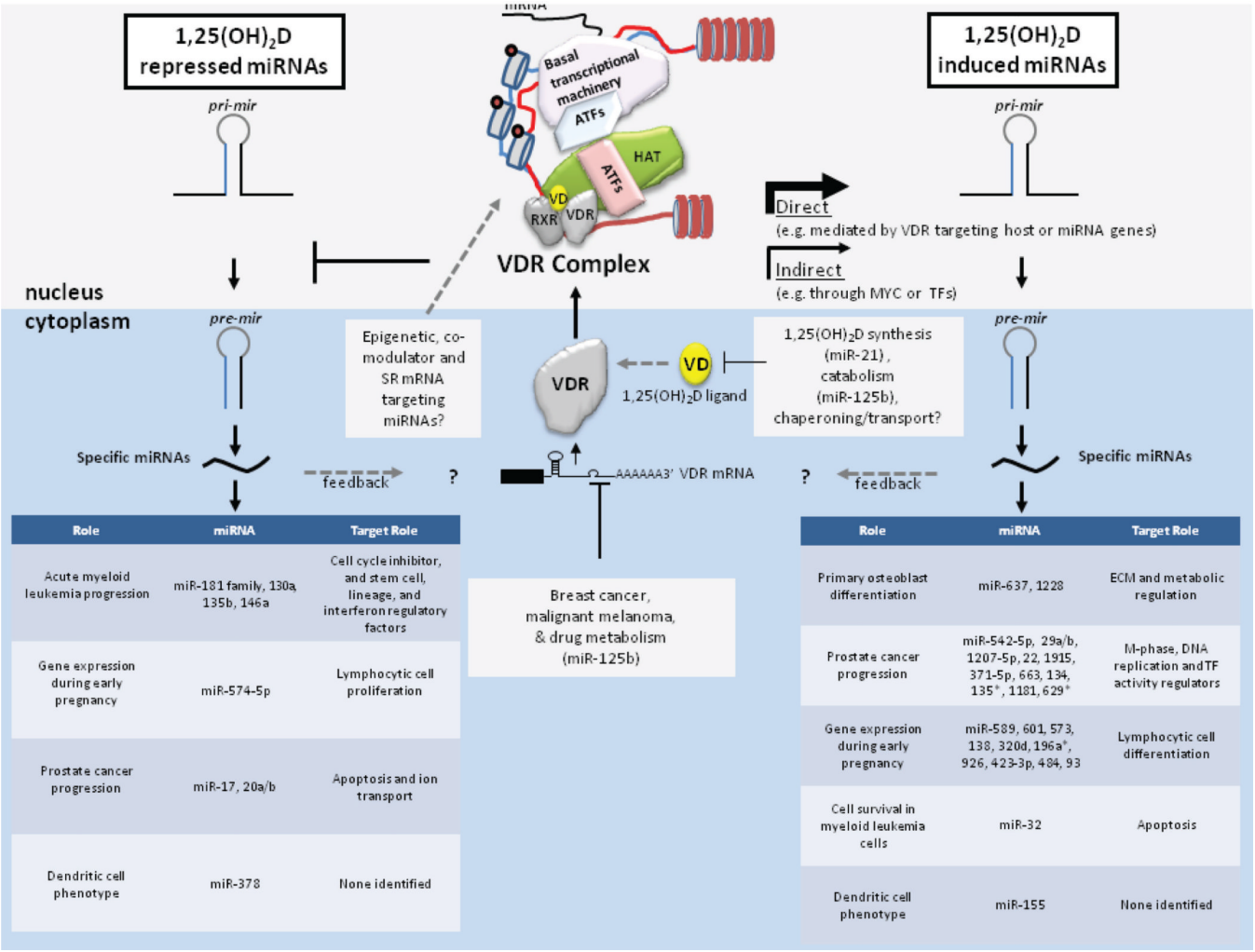


**FIG. 2.**

Interactions between vitamin D receptor (VDR) signaling and miRNA actions. Active $1,25(OH)_2D$ in conjunction with the VDR transcriptional machinery. The VDR complex that includes $1,25(OH)_2D$ (VD)VDR, retinoid X receptor (RXR), activating transcription factor (ATF), histone acetylase transferase (HAT), and the basal transcriptional machinery can either suppress or induce miRNAs, utilizing either direct transcriptional regulation via host or miRNA gene promoter sequences, or via indirect regulation utilizing other transcription factors. Conversely, miRNAs may act to regulate vitamin D action, synthesis, and metabolism, or be themselves influenced by vitamin D hormone receptor signaling in dynamic feedback mechanisms. For individual references (see Table 1).

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## TABLE 1

Overview of vitamin-D referenced miRNAs and functions

| miRNA | Vitamin D regulation | Biological/disease function | Putative target(s) | Refs.; notes |
|---|---|---|---|---|
| miR-125b | Unknown | Overexpression decreases VDR levels in MCF-7 breast cancer cells, abolishing the anti-proliferative effects of 1,25D | VDR (mRNA/protein) | 57, 127; *lipopolysaccharide* is known to down regulate miR-125b |
| | Unknown | Lower expression in 1,25D–responsive melanoma cell lines regulates proliferation; epigenetic modulation affects outcome | VDR (mRNA) | 56, 128; expressed variably in melanoma cell lines |
| | Unknown | Decrease levels in breast cancer tissues may be a cause for increased catabolism of 1,25D | CYP24A1 (mRNA/protein) | 54 |
| miR-326 | Upregulated in blood lymphocytes of type 1 diabetic (TD1) patients | May target the VDR in T1D for immune regulation | VDR (*in silico* prediction) | 58 |
| miR-27b, mmu-miR-298 | Unknown | May be involved in drug metabolism through CYP3A4 regulation | VDR (mRNA/protein) | 129, 130; regulated by diet and carcinogen exposure |
| miR-21 | Unknown | May target multiple genes associated with the vitamin D–dependent antimicrobial pathway during lepromatous leprosy | CYP27B1, IL1B (mRNA) | 52 |
| miR-181 family | Downregulated by 1,25D (1–100 nM; 48 h) | Potentiates the proliferation of myeloid leukemia cells (HL60/U937 lines) | p27Kip1 (mRNA/protein) | 62 |
| (I) miR-589, 601, 573, 138, 320d, 196a*, 926, 423-3p, 484, 93 (II) miR-574-5p | (I) Downregulated in low (<25 ng/ml) 25D serum (II) Upregulated in low 25D serum complications | May control gene expression in maternal peripheral blood associated with early pregnancy | (I) BCL11A (mRNA) and many others (*in silico* prediction) | 68 |
| miR-181 family, 130a, 135b, 146a | Down regulated by 1,25D (100 nM; 72 h) and phorbol 12-myristate 13-acetate (20 nM; 72 h) | May play a role in the monocytic differentiation of acute myeloid leukemia (AML) cell lines and patient subtype cells | KLF4, MAFB, IRF8, HOXA10, MCL1 (mRNA) | 64 |
| (I) miR-542-5p, 29a/b, 1207-5p, 22, 1915, 371-5p, 663, 134, 135*, 1181, 629* (II) miR-17, 20a/b | (I) Upregulated by 1,25D (100 nM; 48 h) (II) Down regulated by 1,25D (100 nM; 48 h) | Controls the differentiated phenotype in the prostate cancer cell line LNCaP | (I) M-phase, DNA replication, and transcription factor activity regulators (II) Apoptosis and ion transport regulators | 63, 131 |
| miR-32 | Upregulated by 1,25D (1–10 nM; 48 h) | May promote cell survival in human myeloid leukemia cells | Bim (mRNA/protein) | 65 |
| miR-98 | Upregulated by 1,25D | Regulates antitumor activity in prostate tumor LnCaP cells | CCNJ | 132 |

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| miRNA | Vitamin D regulation | Biological/disease function | Putative target(s) | Refs.; notes |
|---|---|---|---|---|
| (I) miR-378<br>(II) miR-155 | (I) Upregulated by 1,25D (10 nM; one to six days)<br>(II) Downregulated by 1,25D | May help define the 1,25D–modified regulatory immature dendritic cell phenotype | None identified | 67, 131 |
| miR-22 | Upregulated by 1,25D (dose/time depedent) | May play a role in the anti-proliverative effects of 1,25D (primary human colon cancer cells, and SW480-ADH and HCT116 cells) | NELL2, OGN, HNRPH1, RERE, and NFAT5 | 133 |
| miR-498 | Upregulated by 1,25D (array, 100 nM, six days; 10–100 nM, 24 h) | May be a mediator for the anti-tumor activity of vitamin D in ovarian cancer cells | hTERT | 131 |
| miR-637, miR-1228 | Upregulated by 1,25D (6 h, 10 nM) | Regulation of osteoblastic cell differentiation | COL4A1, BMP2K | 112 |

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## TABLE 2

Predicted/validated MREs in vitamin D-related mRNA targets

| Component | Refseq ID | 3'UTR (nt) | [1]Targeting miRNAs |
|---|---|---|---|
| DBP | NM_000583 | 202 | miR-4528 |
| CYP27B1 | NM_000785 | 808 | [3]**miR-21**, 1972, 4530, 575, 4676-5p, 1207-5p, 3064-3p, 4727-5p, 4688 |
| VDR | NM_000376 | 3209 | miR-125a-5p, **125b**, 351, 670, 4319, 124, 124ab, [2]326, 506, 23abc, 23b-3p, 93, 93a, 105, 106a, 291a-3p |
| CYP24A1 | NM_000782 | 1323 | **125b**, 125a-5p, 4319, 30, 384-5p, 144 |
| hnRNPC (VDR co-repressor) | NM_001077442 | 2086 | miR-455-5p, 499-5p (2x), 144, 208ab, 30, 384-5p |
| SNW1 (VDR co-activator) | NM_012245 | 487 | miR-520d-5p, 524-5p, 659, 512-3p, 548n, 451b, 4307 |

[1]TargetScan (www.targetscan.org) was used to generate the miRNA list. Only conserved mammalian miRNAs are shown. The bolded underlined miRNAs are experimentally validated. TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA.[12] Identified are sites with mismatches in the seed region that are compensated by conserved 3' pairing.

[2]miR-326 was identified as a poorly conserved VDR-binding miRNA using TargetScan.

[3]miR-21 is located upstream of the 3'UTR.

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## TABLE 3

Cellular, homeostatic, and pathological roles of miRNAs in the skeletal environment

| miRNAs | Target(s) | Biological function | Refs.; notes |
|---|---|---|---|
| **Multipotent stem cells** | | | |
| Clusters 1 and 2 (e.g., let-7, miR-24) | None validated (several predicted across cell lineages) | Cluster 1 and 2 osteogenic miRNAs are regulated in a PDGF manner | 98 |
| miR-125b | ErbB2 | Downregulated during ob commitment in murine stromal-derived ST2 cells | 85 |
| miR-26a | Smad1 | Restricts osteogenic differentiation of hASCs by targeting Smad1 | 81 |
| miR-637 | Osx | Enhances adipogenesis of hMSCs and in nude mice | 115 |
| miR-138 | PTK2 | Inhibits hMSC osteogenic differentiation *in vitro*/*in vivo* targeting the FAK/ERK pathway | 105 |
| miR-146a | IRAK1 | Blocks osteogenic differentiation of hASCs by inhibiting NF-κB activation | 134 |
| (I) miR-148b<br>(II) miR-27a, miR-489 | (II) GCA | (I) Promotes osteogenic differentiation of hMSCs<br>(II) Inhibits differentiation | 107 |
| miR-196a | HoxC8 | Promotes osteogenic differentiation of hASCs by inhibiting a transcriptional repressor | 135 |
| miR-199a, miR-346 | LIF | Promotes osteogenic differentiation of hMSCs | 108 |
| miR-210 | ActR1B | Promotes ob differentiation by inhibiting the TGF-β/activin signaling pathway in mouse ST2 cells | 136 |
| miR-3960 | Hoxa2 | Promotes osteoblastogenesis by targeting Hoxa2 repressor Runx2 (regulatory feedback) in mousST2 cells | 137 |
| miR-20b | PPARγ<br>Bambi<br>Crim1 | Promotes osteogenesis in hMSCs by activating the BMP/Runx2 pathways | 138 |
| miR-204, 211 | Runx2 | Inhibits osteogenesis and promotes adipogenesis of MSCs and BMSCs | 100 |
| **Osteoblast progenitors, pre-osteoblasts, and osteoblasts** | | | |
| miR-23a/27a/24-2 cluster | SATB2 | The miR cluster expression is negatively regulated by Runx2 by targeting Satb2 to promote ob differentiation in murine cells | 84 |
| miR-27 | APC | Promotes hFOB differentiation by activating the Wnt signaling pathway | 139 |
| miR-637, 1228 | Col4a1, BMP2K | Regulated by 1,25D and promotes ob differentiation by targeting ECM and metabolic targets in primary hOBs | 112 |
| miR-29a | Osteonectin (ON), sFRP2, kremen2, Dkk1 | Promotes hFOB differentiation by activating the canonical Wnt signaling pathway in a positive feedback loop | 102, 103 |
| miR-29b | Kremen, HDAC4, TGF-β3, ACVR2A, CTNNBIP1, DUSP2, Col1a1, Col5A3, Col4A2 | Promotes murine ob differentiation by influencing matrix maturation and mineralization stages | 77, 86 |

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| miRNAs | Target(s) | Biological function | Refs.; notes |
|---|---|---|---|
| miR23a, 30c, 34c, 133a, 135a, 137, 204, 205, 217, 338 | Runx2 | Decreases osteogenic maturation in mouse MC3T3-E1 cells | 80 |
| miR-125b | ErbB2 | Inhibits ob differentiation by BMP-4 in ST2 cells | 85; earliest bone miRNA paper |
| (I) miR-133<br>(II) miR-135 | (I) Runx2<br>(II) Smad5, Runx2 | Decreases ob formation in C2C12 and MC3T3-E1 cells | 80, 101 |
| miR-135b | None identified | Prevents osteogenic differentiation of unrestricted somatic stem cells | 140 |
| miR-141, 200a | Dlx5 | Promotes pre-osteoblastic differentiation in MC3T3-E1 cells | 141 |
| miR-155 | None identified | May be involved in RANKL signaling within osteoclasts (OC); identified in Dicer deficient OCs | 142 |
| miR-206 | Cx43 | Inhibits osteoblastic differentiation both *in vitro* (C2C12) and *in vivo* | 83 |
| miR-208 | Ets1 | Inhibits pre-osteoblastic differentiation as Ets1 transactivates key osteogenic factors in murine obs | 143 |
| miR-335-5p | Dkk1 | Promotes ob (terminal) differentiation by activating Wnt signaling in MC3T3-E1 and MLO-Y4 cells | 106 |
| miR-378 | GalNT-7 | Inhibits MC3T3-E1 cell differentiation by decreasing ECM nephronectin glycosylation and production | 144 |
| **Pathological Role** | | | |
| miR-2861 | HDAC5 | Promotes ob differentiation in ST2 cells, and pre-miR-2861 mutations cause primary osteoporosis in humans | 87 |
| miR-214 | ATF4 | Inhibits bone formation; upregulated during age-related osteoporosis in humans | 145 |

Human adipose–derived stem cells (hASCs), human mesenchymal stem/stromal cells (hMSCs), human fetal obs (hFOBs; mesenchymal precursor), murine fetal liver–derived stromal cell line (ST2), mesench6ymal precursor cell line (C2C12), bone marrow stromal cells (BMSCs)