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## MicroRNAs in skeletogenesis

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### Abstract

MicroRNAs (miRNAs) are a class of highly conserved small noncoding RNAs that negatively regulate gene expression by imperfectly base pairing to the 3'-untranslated region of their target mRNAs, leading to mRNA degradation or translational inhibition. The emerging field of miRNA biology has begun to unravel roles for these regulatory molecules in a variety of biological processes. This review concentrates on the roles of miRNAs in skeletogenesis as well as in skeleton-related disease processes. Before describing these data, we present a brief review of the biogenesis and action of miRNAs, the approaches to miRNAs study, and miRNAs as global regulators of development. We finish by emphasizing that the study of the biological functions of miRNAs in skeletogenesis and dysplasia represents an entirely new avenue in the exploration of bone and cartilage biology, and large gaps remain in our knowledge of miRNAs in skeletogenesis *in vivo* and in our knowledge of the molecular events underlying miRNA-mediated musculoskeletal disorders.

### Keywords

MicroRNAs; Skeletogenesis; Bone; Cartilage; Arthritis; Implants; Review

## 2. INTRODUCTION

MicroRNAs (miRNAs) are a class of short (~20-24nt) non-coding single-stranded RNA molecules that play an important role in regulating cellular gene expression. They function at the post-transcriptional level, by binding to and inhibiting mRNA. The first miRNA, *lin-4* and its target, *lin-14* were identified in 1993 as regulators of early larval development in *C. elegans*. Since then, the number of miRNAs to be identified or predicted has grown to >600, and it is thought that the human genome may code for 800-1000 miRNAs (1-4). miRNAs are currently thought to target the expression of approximately one-third of all mammalian genes (5). Of particular interest, miRNAs have been found to play important roles in mediating the fundamental biological processes of cell proliferation and differentiation in a variety of tissues types. In mammals, miRNAs have been found to play an important role in

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the development of a broad range of tissues and organ systems including the heart, skin, skeletal muscle, brain, neurons, kidneys, lung, adipocytes, and hematopoiesis (6-13). Recent evidence has suggested that miRNAs may also play a significant role in bone and cartilage development. In this review, we summarize what is currently known about the roles of miRNAs in the skeletogenesis and musculoskeletal disorders, including arthritis.

### 3. MICRORNAS—HOW DO THEY WORK?

The expression of mature miRNA from a primary genomic template has been well described (Figure 1A). Long primary genomic transcripts (pri-miRNAs) are processed in the nucleus by the enzymes Drosha and DGCR8, resulting in the excision of a stem loop structure. The 60-80-nucleotide stem-loop product, also known as precursor miRNA (pre-miRNA) is exported from the nucleus by Exportin 5. In the cytoplasm, precursor miRNA undergoes processing by the RNase III enzyme Dicer, which generates a short RNA duplex. This is then processed into the single-stranded mature miRNA, which is incorporated into an RNA-induced silencing complex (RISC), an RNA-protein complex that includes members of the Argonaute protein family (Figure 1A).

MicroRNAs suppress target gene expression by initially binding to the 3'-untranslated region (3'-UTR) of mRNA before directing the repression of translation and/or mRNA degradation (Figure 1B). This requires that the 3'-UTR contains at least one specific ~7nt sequence that displays at least partial complementarity to a "seed site," located within the 5' region of the miRNA. Binding with full complementarity is required for mRNA cleavage while partial complementarity results in repression of translation. However, binding with either full or partial complementarity to the seed site is not always sufficient for gene repression; indeed a multitude of other factors, such as the relative position of the seed site binding sequence within the 3' UTR, are likely involved (14). Following the binding of a particular mRNA by RISC, the whole complex is thought to be relocated to a cytoplasmic focus known as a processing body (P-body), while translation initiation is inhibited (15,16).

### 4. APPROACHES TO MICRORNA STUDY

The discovery of novel miRNAs and insights into their function generally follow a logical sequence of study with different experimental approaches making contributions at various stages. The initial prediction of miRNA sequences within the genome has been performed bioinformatically by screening for hairpin stem-loop structures characteristic of pre-miRNAs (2,17). However, predicted miRNAs must then be further supported with cloning or expression evidence before they can be included in miRBase, a database that catalogues all known miRNA (1).

An investigation often begins by screening for miRNAs which exhibit a tissue-specific pattern of expression or which are differentially expressed during different stages of various biological processes. Microarray technology has been implemented to analyze the expression of hundreds of miRNA simultaneously (18,19). The results of this analysis can then be confirmed via various techniques including real-time RT-PCR with miRNA-specific primers, *in situ* hybridization using locked-nucleic acid (LNA) probes, and the repression of reporter transgenes (20-22). The biological function of miRNA can also be confirmed

through the cellular overexpression of miRNA, either through direct transfection of antisense miRNA or via the construction of a miRNA expression vector (23-29). Similarly, the repression of miRNAs can be accomplished through the transfection of 2'-O-methyl antisense oligoribonucleotides (30), or through a vector expressing siRNA that targets the hairpin portion of the miRNA precursor.

miRNA target prediction is critical to explaining the biological function of miRNAs. Multiple resources are available for algorithms that compare miRNA and 3'-UTR mRNA sequences, thus identifying a group of potential target genes (31). Although different algorithms can often result in divergent results, it has been suggested that at least a certain class of conserved miRNA targets can be confidently predicted. Once a list of predicted targets is acquired, individual targets can be confirmed by observing the repression of reporter transgenes in a miRNA overexpression model.

## 5. MICRORNAS AS GLOBAL REGULATORS OF DEVELOPMENT

miRNAs play an important role in a variety of biological processes, including development and differentiation in a variety of organisms and tissue types. Indeed, one of the first insights into the significance of miRNA function was reached through the disruption of the Dicer gene. Dicer is encoded at a single locus and is required for miRNA processing; Dicer ablation results in a significant reduction in the levels of nearly all mature miRNAs (32-34). Dicer mutants were found to have profound developmental defects across a whole range of animal and plant species, including *Arabidopsis*, *Drosophila*, *C. elegans*, and zebrafish (32,35-39). In mice, Dicer ablation resulted in embryonic lethality by day 7.5 (32).

More recently, individual miRNAs have been found to play important roles in the development of various tissues and organs. For example miR-1, which is widely conserved during evolution, is essential for proper muscle development in *Drosophila* (40,41). miR-1, which targets the genes Hand2 and HDAC4, is expressed downstream of muscle differentiation regulators SRF, MyoD and Mef2 (13). miR-1 has also been implicated in muscle-related disease processes, such as cardiac hypertrophy (42-44).

miR-143 has been found to stimulate adipogenesis; inhibition of miR-143 resulted in the lowered expression of adipocyte-specific genes (8). miR-221 and miR-222 inhibit angiogenesis by targeting the stem cell factor receptor c-Kit, and have been implicated in melanoma progression (45,46). miR-124a and miR-124b stimulate neuronal differentiation by targeting lin-28 (47,48).

## 6. MICRORNAS IN SKELETOGENESIS

A recent study demonstrated that functional miRNAs are essential for normal skeletal growth *in vivo* (49). Mice whose Dicer was differentially disrupted in chondrocytes (by site-specific Cre recombination under the control of a Col2a1 promoter) displayed significant skeletal growth defects. Analysis of the growth plates revealed decreased chondrocyte proliferation and accelerated hypertrophic differentiation, resulting in a reduced number of proliferating chondrocytes, an expanded hypertrophic region, and significant defects in

skeletal development. This suggests that at least several miRNAs may be involved in inhibition of the chondrogenic differentiation pathway.

Despite mounting evidence that miRNAs play a significant role in embryonic development and other biological processes, the function of only a handful of miRNAs has been determined thus far. And of these miRNAs, only a small subset has been implicated in cartilage and/or bone development (Table 1). Using *in situ* hybridization of locked nucleic acid probes, it has been demonstrated that a high degree of tissue specificity exists for certain miRNAs, suggesting that these miRNAs may play a key role in tissue differentiation and development (22). In particular, miR-140 was found to be expressed exclusively in the cartilage tissue of embryonic zebrafish. Other miRNAs were also found to be highly (although not exclusively) expressed in the developing tissues of the zebrafish skeletal system, including miRNAs 146, 140\*, 199a, 199a\*, 145, as well as others that were ubiquitously expressed (22). For miRNAs 140, 199a, and 199a\*, this pattern of expression is conserved in medaka and chicken embryos (50). Although one study (51) did not detect high expression levels of these three miRNAs in mouse embryos, *in situ* staining of later-stage mouse embryos confirmed that miR-140 is expressed specifically in mouse embryonic cartilage tissue (52). Although the expression of miR-199a in mouse connective tissue has been established via small RNA library sequencing (53), the expression patterns of miR-199a and miR-199a\* in mouse embryos has yet to be confirmed through *in situ* hybridization.

Other studies involving differentiation of human mesenchymal stem cells into osteocytes and chondrocytes implicated a different subset of miRNAs. miR-638 and miR-663 were found to be upregulated in chondrocytes, while miR-24, let-7a, let-7b, let-7c, miR-138, and miR-320 were associated with osteocyte maturation (54). The transcription of many upregulated miRNAs was found to be under the control of platelet-derived growth factor (PDGF)-responsive transcription factors, suggesting that PDGF may mediate the differential regulation of miRNAs during osteogenesis (54). Interestingly, miR-140 has recently been found to target PDGF receptor alpha, thus acting as a negative regulator of PDGF during craniofacial development (55). However, it is unknown whether this is also significant for osteogenic differentiation.

Although miR-140 is unique as the only identified miRNA that is specifically expressed in cartilage, it remains unknown whether miR-140 plays any significant role in chondrogenic or osteoblastic differentiation. miR-140 has been shown to target HDAC4, a known corepressor of Runx2, which is an essential transcription factor for chondrocyte hypertrophy and osteoblast differentiation (52). However, HDAC4 is also targeted by various other miRNAs, including miR-1, which when overexpressed, has been shown to promote muscle differentiation without affecting osteoblastic differentiation (7,56). Thus, a single gene can be differentially regulated by various tissue-specific miRNAs, thus playing separate roles in different developmental pathways.

Recent findings from our lab have implicated miR-199a and miR-199a\* as having a significant role in the course of chondrogenic differentiation. Experiments involving real-time PCR have shown miR-199a\* to be dramatically downregulated in early

chondrogenesis, suggesting that miR-199a\* may play an inhibitory role in the early stage of chondrocyte differentiation. Overexpression of miR-199a\* precursor in a chondrogenic differentiation cell model resulted in the repression of early chondrogenesis markers (Lin *et al*, 2008, unpublished data). Interestingly, both miR-199a and miR-199a\* are predicted to target SMAD1, a downstream transcription factor of the bone morphogenic protein (BMP) signal transduction pathway. Thus, by the inhibition of SMAD1, miR-199a and miR-199a\* may act as important regulators of chondrocyte and/or osteoblast differentiation.

A greater number of microRNAs have been implicated in the regulation of osteoblast differentiation than chondrocyte differentiation. miR-26a, which promotes myogenesis by targeting a histone methyltransferase (57), has been identified as an inhibitor of late osteoblast differentiation (58). This suggests that miR-26a may act as a facilitator to maintain cells in one differentiation fate over another—silencing osteoblast differentiation genes while promoting the expression of myogenic factors. There is evidence to show that miR-26a inhibits osteoblast differentiation by targeting the important cell-signaling molecule, SMAD1 (58). Interestingly, SMAD1 interacts with the transcriptional repressor HOXA9, which binds to the OPN promoter and represses OPN gene expression (59,60). OPN is an intermediate marker of osteoblastic differentiation. Therefore, downregulation of SMAD1 by miR-26a would relieve the inhibition on HOXA9, thus increasing the inhibition of OPN.

More recent evidence suggests that HOXA9 is itself under the regulation of miRNAs, particularly miR-126 (61). Although miR-126 has yet to be formally implicated in the osteoblast differentiation process, miR-126 and miR-26a have counteracting effects on HOXA9 expression and the summation of their activities may serve to fine tune HOXA9 expression levels. Thus the combined activity of multiple miRNAs may provide various stages of regulation along the same signal transduction pathway. Of note, miR-126 has been shown to downregulate VCAM-1 expression in TNF- $\alpha$ -stimulated endothelial cells (62). Since VCAM-1 is an important mediator of osteoblast adhesion to osteoclasts, the potential that miR-126 plays a role in bone formation and remodeling is significant (63).

Recent evidence has shown that miR-125b is also involved in osteoblastic differentiation. When compared to the control, miR-125b is inhibited in cells undergoing BMP-4 induced osteoblastic differentiation, suggesting an osteoblast inhibitory effect. Indeed, overexpression of miR-125b resulted in the repression, while inhibition of miR-125b resulted in the promotion of osteoblastic differentiation (64). miR-125b was also found to inhibit cell proliferation—confirming previous findings that this miRNA modulates proliferation in a variety of cell types, including human carcinoma cells through its target gene ErbB2 (65-67). Interestingly, suppression of ErbB2 via siRNA transfection also results in reduced osteoblastic differentiation. This suggests that by regulating a single target gene, such as one that regulates cell proliferation, a particular miRNA may have wide-ranging effects on many biological processes in multiple tissue types.

## 7. MICRORNAS AND IMPLANTS

A number of studies have examined the effect of various biocompatible materials on bone formation. Because miRNAs have been implicated in growth and differentiation, several labs have sought to determine the effect of biocompatible materials on microRNA expression profiles. Many of these studies have focused on bone grafting or artificial implants in the context of dental procedures. For example, the synthetic bone graft material calcium sulfate (CaS) has been found to upregulate miR-377, while downregulating miRNAs-22, 93, 27a, 31, and 16 (68). Many of these miRNAs target genes that are involved in regulating osteoblast activity, such as BMP1, BMP7, FGFR1, PTH, CALCA and GHRHR. Interestingly, homeobox regulatory genes such as HOXA13, DLX5, and SHOX were also found as potential targets. P-15, a collagen analog that is used for bone grafting, has been found to upregulate miR-26a, an inhibitor of late osteoblast differentiation (58,69). These studies suggest that miRNAs play an important role in bone healing and remodeling following surgical procedures involving implants.

## 8. MICRORNAS IN ARTHRITIS

The possible role of miRNAs in the disease processes of rheumatoid arthritis (RA) and osteoarthritis (OA) has only been recently explored. One study found a significant upregulation of miR-155 and miR-146a in synovial fibroblasts (RASFs) and synovial fluid derived from patients with RA (70). Furthermore, stimulation of cultured RASFs with proinflammatory mediators, such as TNF- $\alpha$ , IL-1 $\beta$  and LPS, markedly induced miR-155 and miR-146 expression levels (70,71). Similarly, proinflammatory ligands stimulated miR-155 expression in synovial fibroblasts derived from patients with OA. Additionally, both miR-155 and miR-146 expression were significantly higher in RA synovial tissues when compared to OA, which is consistent with previous work outlining the role of both miRNAs in immune system function (70-74).

The exact mechanism behind these findings is still unclear. Overexpression of miR-155 in RASFs resulted in the inhibition of MMP-3, a known contributor to cartilage destruction in RA. This suggests that miR-155 may serve to protect joint tissues from excessive inflammatory damage. However, since miR-155 is not predicted to directly target MMP-3, it is likely that the mechanism of action is indirect, perhaps through the regulation of LPS signaling (75). The mechanism for the role of miR-146 is better understood; miR-146 is known to target TRAF6 and IRAK1, which are key downstream molecules of TNF- $\alpha$  and IL-1 $\beta$  signaling (76). Thus, it is likely that both miR-155 and miR-146 serve to temper immune responses in rheumatoid arthritis by providing negative feedback through the targeting of key immune signaling pathway genes.

## 9. SUMMARY

Based on the literature to date, it is clear that miRNAs play a key role in bone and cartilage development. Although several miRNAs have been implicated in regulating chondro- and osteogenesis, much work remains to elucidate the complex interplay of different miRNAs and their gene targets, and the molecular events underlying miRNA-mediated skeletogenesis and musculoskeletal disorders, particularly with *in vivo* models. Nevertheless, the study of

miRNAs represents a novel and exciting avenue in the exploration of bone and cartilage biology.

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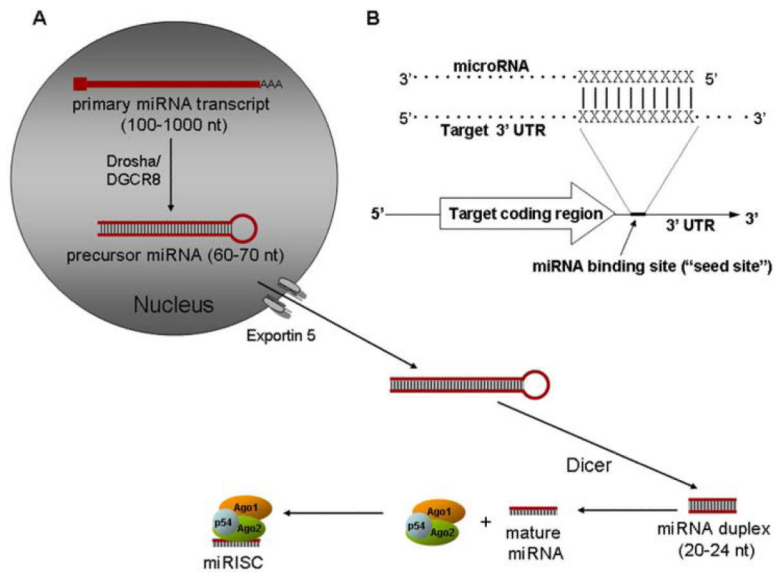
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**Figure 1.** Schematic of microRNA biogenesis. Panel A. Biogenesis of a miRNA-induced silencing complex (RISC). Panel B. Binding of microRNA to the 3'-UTR of mRNA. See text for details.

**Table 1**

## MicroRNAs in skeletogenesis

miRNA	Role in Differentiation	Expression in Embryonic Bone/Cartilage	Verified Targets	Citations
miR-140	Unknown	Exclusively expressed in zebrafish, medaka, chicken, and mouse	HDAC4	(22, 50, 52)
miR-140s	Unknown	Highly expressed in zebrafish		(22)
miR-199a	Inhibits chondrocyte <sup>1</sup>	Highly expressed in zebrafish, medaka, chicken, and mouse		(22, 50, 53)
miR-199as	Inhibits chondrocyte <sup>1</sup>	Highly expressed in zebrafish, medaka, chicken, and mouse	SMAD1 <sup>1</sup>	(22, 50)
miR-26a	Inhibits osteoblast		SMAD1	(57, 58)
miR-126	Unknown		VCAM1, HOXA9	(61, 62)
miR-125b	Inhibits osteoblast		ERBB2	(64)
miR-145	Unknown	Highly expressed in zebrafish		(22)
miR-146	Unknown	Highly expressed in zebrafish		(22)

VCAM1, vascular cell adhesion molecule 1; HOXA9, homeobox A9; ERBB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2

<sup>1</sup>Lin and Liu, 2008, unpublished data