MicroRNAs and micromanaging the skeleton in disease, development and evolution

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Abstract

MicroRNAs (miRNAs) are short, non-protein-encoding RNAs that effect post-transcriptional gene regulation by targeting messenger RNAs. miRNAs are associated with specific human diseases and help regulate development. Here we review recent advances in understanding the roles of miRNAs in skeletal malformations, including cleft palate, and in the evolution of skeletal morphologies. We propose the hypothesis that evolutionary variation in miRNA expression patterns or structural variation in miRNA binding sites in messenger RNAs can help explain the evolution of craniofacial variation among species, the development of human craniofacial disease and physiological changes leading to osteopenia that increases with ageing.

Keywords: zebrafish • craniofacial development • microRNA • miR-140 • osteopenia • osteoporosis • medaka • ageing • skeleton evolution

Introduction

Decades of genetic analysis have shown that the proper development of an organ system, such as the vertebrate skeleton, requires precise execution of various genetic pathways. Small RNAs have recently been recognized to function as important modulators of gene regulation. MicroRNAs (miRNAs) function in post-transcriptional modulation of genetic pathways that regulate various developmental and physiological processes. In vertebrates, miRNAs often act as subtle negative regulators of gene translation by recognizing and binding to complementary sites in the 3’ untranslated regions (UTRs) of target genes (reviewed in [1]). The developing skeletal system expresses several different miRNAs, often with precise temporal and cell-type specificity, and therefore it is likely that miRNAs play a significant role in sculpting skeletal form. A central question is the role miRNAs play in the evolution of new skeletal forms and in the origin of skeletal diseases, including bone loss disease, or osteopenia, that almost universally accompanies the ageing process.

The skeleton in evolution, health and disease

The skeletons of various vertebrate species are variations on a theme and even populations within a species often have subtle differences in bone shapes — compare, for example limb proportions of Athabascans inhabiting the frigid Alaskan tundra to the Masai living in the hot Serengeti Plain of Kenya [2]. Likewise, a rich
The vertebrate skeletal system is composed of cartilage and bone. Cartilage is made of cells surrounded by extracellular matrix rich in proteoglycans and staining positive for Alcian Blue, while bone...
Endochondral ossification and intramembranous ossification. (A) 12-day-old G. aculeatus pharyngeal skeleton stained with Alcian Blue for cartilage ossification and Alizarin Red for bone shows intramembranous and endochondral ossification. Italic bold font labels intramembranous ossification and roman font indicates endochondral ossification. The arrow indicates the endochondral ossification centre of the ceratohyal cartilage. (B) Endochondral ossification of ceratohyal of 11 day old spotted gar. Green, Col II staining includes the perichondrium, which surrounds the cartilage core; red, Col X staining in chondrocytes of the ceratohyal; blue, nuclear stain. Abbreviations: bb, basibranchial; bh, basihyal; bsr, branchiostegal ray; cc, chondrocytes; ch, ceratohyal; cl, cleithrum; cs, scapulocoracoid; den, dentary; ed, endoskeletal disc; ent, entopterygoid; hs, hyosymplectic; mx, maxilla; me, Meckel's cartilage; op, opercle; pa, pharyngeal arch; pc, perichondrium, pmx, premaxillary; pq, palatoquadrate; ptp, pterygoid process; ra, retroarticular and te, teeth.

is a calcium phosphate matrix staining positive for Alizarin Red (Fig. 2A). Cartilage and bone both develop from mesenchyme derived from neural crest, paraxial mesoderm, or lateral plate mesoderm. During embryogenesis, some bones form by endochondral ossification and others by intramembranous ossification [14–16] (Fig. 2A). In the endochondral ossification, mesenchymal cells differentiate into chondrocytes that form a condensation with the shape of the future cartilage element. Chondrocytes differentiate, become hypertrophic and deposit extracellular matrix. Hypertrophic chondrocytes terminally differentiate and undergo apoptosis. At the periphery of the condensation, osteoblasts develop from the perichondrium surrounding the cartilage (Fig. 2B) and secrete bone, which merges with the cartilage matrix. As the matrix thickens and calcifies, osteoblasts trapped by the extracellular matrix differentiate into osteocytes, which maintain the bone. In contrast, in intramembranous ossification, mesenchymal cells bypass the cartilage model and differentiate directly into osteoblasts, which secrete extracellular matrix that becomes mineralized, thus forming bone.

Several genetic pathways help regulate the developmental programs that specify the type, size and shape of each skeletal element. For instance, Sox9 and Runx2 transcription factors together regulate differentiation of cartilage and bone [17–21]. The Bmp, Hh and Fgf signalling pathways sculpt skeletal morphogenesis [22–27] and extracellular matrix components like collagens, Sparc and glycosaminoglycans influence cell signalling and cell shape [28–32]. Modulation of the Edn1 pathway or changes in Hox gene expression alters bone morphologies along the dorsal/ventral and anterior/posterior axes, respectively ([33–40]. In principle, any or all of these genetic pathways are potentially subject to miRNA modulation and thus provide numerous genetic levers to understand the roles of miRNAs in skeletal development, morphological evolution and skeletal disease.

The biogenesis of miRNAs

MiRNAs are a group of small regulatory RNAs that generally attenuate gene function by inhibiting the production of proteins and were first discovered in the nematode Caenorhabditis elegans [41–43]. miRNAs are involved in many developmental signalling pathways and in housekeeping regulation for organ physiology [41–43]. Consistent with their broad involvement in regulation, thousands of miRNAs have been recognized across the genomes of viruses, plants, fungi and animals [44–49], with at least 542 human miRNAs deposited in the miRNome database [50]. Genes encoding miRNAs are scattered across genomes in intergenic or intragenic regions, in UTRs or in translated sequences of protein-coding genes, and they can be oriented either in sense or in antisense orientation with respect to their primary transcript and host gene (see [51]). Previous studies revealed that most miRNA genes are transcribed by RNA polymerase II, with 5’ caps and 3’ polyA tails, like most protein coding genes [52].

The pathway of miRNA biosynthesis has been well explored. (See Table S1 for nomenclature rules for intermediates in the pathway.) In the canonical pathway, an mRNA-like primary transcript (pri-miRNA) forms a stem-loop secondary structure in the nucleus. The Drosha-DGCR8 complex, an RNase III machine, digests the pri-miRNA into a free hairpin structure called a pre-miRNA [53, 54]. Some miRNA genes, called mirtrons, are embedded in introns and are spliced out directly from their host gene transcript into a pre-miRNA, thus avoiding the Drosha processing step [55]. Exportin5, a nuclear envelope protein, transports free pre-miRNA hairpins into the cytoplasm [56]. Dicer, a cytoplasmic RNase III enzyme, then processes the pre-miRNA by cutting off the loop region and releasing mature miRNA duplexes, which can diffuse in the cytoplasm and bind to mRNA targets [57, 58].
Mature cytoplasmic miRNA duplexes are the ultimate regulatory components of the system, and they carry both a stable passenger miRNA strand and an unstable guide miRNA strand [59, 60]. In the cytoplasm, the miRNA duplex interacts with a group of proteins to form an RNA-protein complex called miRNA-induced silencing complex (miRISC) [61]. In the miRISC, the miRNA guide strand recognizes its binding site in the 3′ UTR of target mRNAs. miRNA target sites do not need to be perfectly complementary to the miRNA for binding and target regulation. Perfect binding of miRNAs usually leads to target degradation, and incomplete complementarity often inhibits target translation either by interfering with 5′ cap recognition, by causing message deadenylation, or by blocking translation elongation [62–64]. Because of their short sequence and ability to act as regulators even without a perfect match, miRNAs can have many predicted targets (see prediction tools in Table S2). It is easy to understand how miRNAs could have a profound influence on evolution because each one of the hundreds of miRNAs can potentially regulate hundreds of target genes.

miRNAs and genome evolution

Support is emerging for the notion that miRNAs are closely associated with evolutionary novelty and some suggest this link to be causative. For example, one burst of new miRNAs occurred at about the time of the origin of vertebrate characters and another increase occurred as placental mammals evolved [65, 66, 131]. Once new miRNAs have integrated into genetic regulatory networks, their primary sequences tend to remain highly conserved and miRNAs are rarely lost secondarily [62–64]. This conservation over time suggests that substantial selective pressure preserves miRNAs. miR-140, miR-199 and mir-214 (discussed in greater detail below), all appeared at the base of the vertebrate radiation [65] and are expressed strongly and specifically in developing skeletal systems. Because the evolution of a mineralized endoskeleton is one of the novel features that characterize vertebrates, we suggest that miRNAs participated in the origin of the vertebrate skeletal system and today contribute to its development, its morphological diversity and its function in health and disease.

miRNAs and the micromanagement of development

The development of morphologies, the evolution of morphological diversity and the prevention of bone deterioration diseases all require the precise regulation of protein levels during crucial processes such as cell fate specification, cell differentiation, cell proliferation, cell migration and stem cell maintenance. Although miRNAs are likely to provide a widely used post-transcriptional developmental control mechanism and recent reports estimate that the hundreds of miRNAs in the human genome may together regulate about 30% of human genes [67, 68], the functional significance of miRNA utilization remains largely under-explored.

To understand the aggregate roles of miRNAs in development, researchers constructed knockout situations for the miRNA-processing enzyme Dicer in mice and fish. Because the maturation of all miRNAs requires Dicer, embryos lacking this enzyme should be deficient in all miRNAs at once and thus, Dicer knockdown should reveal the functions of at least early-acting miRNAs. The knockout of zygotic (but not maternal) Dicer in mouse caused homozygous mutant embryos to die before the establishment of the body plan during gastrulation. This result suggests that Dicer function is necessary for the maturation of miRNAs essential for normal embryonic patterning, morphogenesis and maintenance of embryonic stem cells in mouse embryos [69, 70].

Zebrafish mutants lacking both the maternal and the zygotic function of dicer have relatively normal axis formation and can differentiate multiple cell types, but mutants showed abnormal morphogenesis during gastrulation and irregularities in the development of the brain, somites and heart [71]. Collectively, the mouse and zebrafish studies reveal first, that miRNAs as a group play important developmental roles, and second, that different vertebrate species vary in their apparent reliance on miRNAs as developmental regulators.

While it is useful to learn the phenotype that results from the knockdown of all miRNAs in all embryonic cells, this sledgehammer approach results in embryonic defects at the earliest embryonic stage that requires any miRNA; thus, the functions of miRNAs that act later in development, such as those acting during skeletogenesis, are likely to remain undetected by this procedure.

Micromanaging skeletal system development

The first in vivo evidence that miRNAs regulate skeletal development came from the knockdown of Dicer specifically in cartilage cells expressing the collagen gene Col2a1 [72]. Embryos lacking Dicer function in cartilage cells display severe skeletal defects and premature death due to progressive reduction in chondrocyte proliferation and precocious differentiation to hypertrophic chondrocytes. The acceleration of chondrocyte differentiation in cells lacking miRNAs suggested that one or more miRNAs inhibit action of one or more genes that normally slow chondrocyte maturation. Despite this clear effect, analysis showed that the level of many miRNAs in Dicer-deficient chondrocytes continued at 30–40% of control levels. The function of these residual miRNAs may have obscured a true null effect that might result in substantially more severe phenotypes. Analysis of over 4000 predicted miRNA targets in microarray expression profiling failed to show significant reduction of messenger RNA levels in Dicer-deficient chondrocytes, consistent with the expectation that miRNAs act primarily post-transcriptionally [72–75]. These experiments provided
important proof that miRNAs are important for embryonic skeletal development. Further investigations suggested that skeletal miRNAs may act by a pathway independent of Pthrp and Ihh, two signalling molecules that regulate skeletal maturation [24, 72], and thus, the experiments may suggest a new, miRNA-based mechanism for chondrocyte maturation.

MiRNAs have also been knocked out in aggregate specifically in the developing limb and its skeleton, resulting in embryos with delayed differentiation of the endochondral skeleton, twisted long bones and digit anomalies [70]. Driving Dicer loss only in cells of the limb’s zone of polarizing activity, a signalling centre that controls anterior-posterior patterning of the limb, showed that digit abnormalities arise from decreased cell numbers in the developing handplate rather than from a defect in limb bud patterning. Differences in cell number such as this are important for the evolution of animal form: for example, variation in chondrocyte number rather than differences in primary patterning is what makes a bat’s wing – with its very long digits – different from the forelimb of a mouse [76]. So although many possible mechanisms could affect the number of cells in the limb skeleton, variation in miRNA quantity or variation in miRNA affinity for target binding sites in messenger RNAs that regulate chondrocyte number provide a hypothetical but testable mechanism for generating skeletal variation among species and perhaps skeletal robustness over a person’s lifetime.

Tissue specific Dicer knockout gives insight into the importance of miRNAs in development, but this technique also has limitations. The method does not identify which miRNA(s) regulate skeletal morphogenesis or the identity of the miRNA targets. Aggregate loss of miRNAs could also mask pairs of antagonistic miRNAs. For these reasons, it is imperative to identify and analyse the functions of individual candidate miRNAs during skeletal development.

Candidate miRNAs controlling skeletal development

MiRNAs expressed in skeletal cells are candidates for playing a role in skeletal development. Mature miRNAs are too short to detect expression by conventional in situ hybridization methods. Therefore, large-scale surveys of miRNA expression patterns [77, 78] have generally used locked nucleic acid (LNA) oligonucleotide probes. LNA probes bind RNAs tightly [79, 80], but are expensive. Conventional in situ hybridization costs less than LNAs and, although it cannot detect mature miRNAs, it can detect expression patterns of primary transcripts and pre-miRs [81].

A sweeping survey of 115 miRNAs identified several miRNAs that are specifically expressed in the developing skeletal system of zebrafish, including mirn140, mirn199, mirn214 and mirn27b [77]. Chondrocytes, but not the perichondrium, of the pharyngeal arches, head skeleton, and fin skeleton express Mirn140 (Fig. 3A). In contrast, the perichondrium and surrounding mesenchyme, but not the chondrocytes, of the eye capsule, the endochondral disc of the pectoral fin and the pharyngeal arch skeleton express both mirn199 and mirn214 (Fig. 3B and C). These cell-type specific expression patterns suggest that these miRNAs play a role in the embryonic development of specific parts of the zebrafish craniofacial and appendicular skeleton.

Based on expression analyses, miRNAs are likely to also regulate skeletal development in amniote embryos. The expression patterns of 117 miRNAs are available for chick embryos at the Geisha expression database (see Table S2 and reference [78]). At least mirn106, mirn128, mirn135, mirn140, mirn200, mirn216, mirn217, mirn218 and mirn223 are expressed in developing pharyngeal and/or limb cartilage in chick embryos. Comparisons of the expression patterns of orthologous miRNAs in chicken and zebrafish show similarities and differences that will be discussed in detail below. Expression patterns such as these can provide the first step in a mechanistic analysis of miRNA function because miRNAs must be co-expressed with their targets.

The function of mirn140 in skeletal development: a case study

Skeletogenic cells, including precursors of the palate (defined as the skeletal elements situated in the roof of the mouth), express mirn140 (miR-140) in fish, chicken and mouse [77, 81–84]. This result
suggests that mirn140 may modulate signalling during palatogenesis across vertebrate species. But how does miR-140 act? Overexpression analysis showed that miR-140 caused a cleft lip and cleft palate phenotype in zebrafish [81] (Fig. 4). If miR-140 acts by diminishing the expression of a target gene, then a mutation in the miR-140 target should also have cleft lip and cleft palate. In mouse [85–87] and in zebrafish [81], knockout of components of the PDGF (platelet derived growth factor) signalling pathway can cause cleft lip and cleft palate. Sequence comparisons showed that among PDGF ligands and receptors, only the receptor Pdgfra had miR-140 binding sites in the 3′ UTR that were conserved across vertebrate phylogeny. In all species analysed, neural crest cells express Pdgfra, hence both expression analysis and mutant phenotypes are consistent with the hypothesis that miR-140 modulates Pdgfra levels. Transcripts containing the 3′ UTR of pdgfra fused to the coding sequence for enhanced green fluorescent protein (eGFP) were translated less effectively than normal in miR-140-injected embryos and more efficiently than normal in miR-140 antisense-treated embryos [81]. This result shows that the 3′ UTR of pdgfra is a target of miR-140 and suggests a mechanism for the disrupted palate phenotypes in miR-140 injected zebrafish.

Time-lapse video-microscopy of green fluorescent protein (GFP)-expressing transgenic cranial neural crest cells revealed that neural crest cells normally migrate over and in front of the eyes and past the optic stalk to occupy the location of the future palate on the oral ectoderm. Few neural crest cells, however, reach the oral ectoderm in either miR-140 injected embryos or in pdgfra mutants. Collectively, these results demonstrate that miR-140 exerts its effects on palatogenesis through Pdgfra. Loss-of-function analyses, however, were necessary to determine the normal role of miR-140 in palatogenesis.

Loss of miR-140 function elevates Pdgfra protein levels in embryos and alters palatal shape. The injection of embryos expressing EGFP in neural crest with antisense morpholino directed against miR-140 results in neural crest cells accumulating around the optic stalk, a source of the attractant ligand Pdgfaa, one of two ligands for Pdgfra in zebrafish (the other being Pdgfab).
which is not expressed in palate-forming cells). Only few crest cells migrate further to the oral ectoderm. Likewise, the injection of embryos with pdgfra mRNA that lacks the miR-140 binding site result in Pdgfra production that cannot be regulated by miR-140, and again results in the accumulation of neural crest cells around the optic stalk. These results suggest that miR-140 functions to attenuate Pdgf signalling at the optic stalk, allowing crest cells to migrate onward to the oral ectoderm. These findings suggest an ancient conserved regulatory interaction of miR-140 and Pdgfra in the development of the palatal skeleton [81].

Tissue culture experiments suggest an additional role of miR-140 in skeletogenesis [83]. Prehypertrophic chondrocytes express histone deacetylase-4 (Hdac4), which inhibits differentiation to hypertrophic chondrocytes, perhaps by regulating the osteogenic gene Runx2 [88]. When introduced into mouse 3T3 cells growing in vitro, a miR-140 mimicking siRNA down-regulated a co-transfected target construct bearing the Hdac4 3’ UTR [83]. The down-regulation of an inhibitor of chondrocyte differentiation would be expected to accelerate the formation of hypertrophic chondrocytes, but in Dicer-deficient mice, chondrocyte hypertrophy was stimulated rather than inhibited, suggesting that down-regulation of Hdac4 is not likely to be the mechanism for the observed aberrant skeletal development, with the caveat that Dicer knockdown might inhibit several antagonistic pathways. Furthermore, the zebrafish hdac4 gene does not have a good candidate target site for miR-140, suggesting that for zebrafish at least, hdac4 is unlikely to be an in vivo target for miR-140. Thus, it is as yet unclear if Hdac4 is a significant in vivo target of miR-140 in any vertebrate embryos [89].

miRNAs and Hox gene patterning of the axial skeleton

MiRNAs are not only implicated in skeletal morphogenesis, but are also likely to be involved in skeletal patterning. Several miRNA genes are located within Hox clusters (Fig. 5) and share the expression patterns of nearby Hox genes [90]. Furthermore, these miRNAs have predicted target sites in Hox gene 3’ UTRs [90]. This is significant because Hox-cluster genes help establish axial patterning of animal bodies, and loss-of-function mutations of Hox-cluster genes in flies and mice can result in the transformation of body regions to fates appropriate for more posterior regions [91–94]. In mouse, posterior transformations of vertebral segments in the axial skeleton and abnormalities in the appendicular skeleton of the limbs are among the most obvious mutant phenotypes in Hox gene mutants (reviewed by [92]). Three major families of miRNA genes, Mirn10, Mirn196 and Mirn615, are located in conserved sites within the Hox clusters of animal genomes [95] (Fig. 5).

Hox-embedded miRNAs are highly conserved across species, suggesting that they could function in the Hox-mediated patterning of the axial skeleton. Mirn615 lies in the intron of Hox5 and is likely limited to mammals [96]; its functions have not yet been investigated, although it is expressed in at least two human colon adenocarcinoma cell lines and a human kidney epithelial line [97, 98]. Mirn10 was originally identified between Dfd (Hox4) and Scr (Hox5) in a fruitfly, a mosquito and a flour beetle [99]. It occupies orthologous genomic locations in sea urchin, amphioxus, fish and mammals [95] and acts in axial patterning by repressing hoxb1a and hoxb3a in the spinal cord, working cooperatively with hoxb4. When the Hox clusters duplicated from one to four in the genome duplication events that occurred at about the time of the vertebrate radiation (R1 and R2) [100–102] and once again just before the teleost radiation (R3) [103–107], these mir genes were duplicated with them, although some paralogs have become secondarily lost [95]. We had shown that the zebrafish possesses duplicates of three of the four tetrapod Hox clusters, but has a single Hoxd cluster [103]. Despite the loss of all protein-coding genes in the hoxdb cluster, the zebrafish lineage surprisingly retained a copy of mirn10 in the expected genomic location of the hoxdb cluster [108]. This unexpected finding suggests that selective pressure was greater for maintaining the duplicated mirn10 gene than for retaining duplicates of the hoxd protein coding genes. Interestingly, the pufferfish lineage lost all of the protein-coding genes in its hoxcb cluster, but even the mirnAs of the hoxcb cluster have disappeared in pufferfish, in contrast to the hoxdb cluster in zebrafish, [95, 108, 109]. As a result, both pufferfish and zebrafish have five mirn10 genes, but by different genomic
mechanisms. These curious results emphasize the evolutionary importance of miRNAs. It is possible that the zebrafish and pufferfish lineages both retained five copies of mirn10 after the telost genome duplication compared to the two copies present in mammals for stoichiometric reasons.

Moving along the Hox clusters, Mirn196 lies 5’ of Hox9 paralogs in tetrapods and teleosts [95], and in the sea lamprey, a basally diverging vertebrate, there are at least two paralogs of mirn196 [66, 95]. Mirn196 regulates the expression of Hoxb8 in mesoderm that is fated to form the forelimb skeleton of chicken and potentially mouse by facilitating cleavage of Hoxb8 mRNA, although the functional role of this interaction during skeletal morphogenesis remains unclear [110, 111].

The similarity of the expression patterns of Hox-cluster Mirn genes to Hox genes with their predicted targets and the tantalizing results so far available suggests that mis-regulation of Mirn10 and Mirn196 might result in skeletal defects similar to those induced by mutations in Hox genes. Because Hox-cluster miRNAs have multiple Hox genes as predicted targets, however, much work will be required to test any of these predictions. A solution to the problem will require more experiments conducted in developing embryos.

miRNAs and microevolution: a hypothesis

miRNAs are highly conserved in sequence and regulate spatial and temporal expression of other genes during development. The evolution of new miRNA families across species diversity (macroevolution) has accompanied explosions of evolutionary innovation, such as the origin of vertebrates [65, 66]. Evidence is sparse, however, about the functional roles of newly evolved Mirns in establishing morphological differences between individuals or populations within a species or between closely related species (microevolution).

Not only do the families of miRNAs found in different lineages differ, but the expression patterns for orthologous Mirns can differ across species. A comparison of the expression patterns of about 100 miRNAs in medaka fish and chicken with existing data for zebrafish and mouse showed that the timing and location of miRNA expression varies much more than miRNA structure [82]. Differences in expression can be associated with changes in miRNA copy number, genomic context, or both between species. For the skeletal system, seven of nine mirns exhibited delayed or different expression patterns in mouse and zebrafish (mirn27a, mirn27b, mirn140, mirn140*, mirn199a, mirn199a* and mirn214), while two that were expressed strongly in zebrafish showed weak expression or no expression in medaka fish, (mirn145 and mirn146). The degree to which these variations in expression pattern might account for developmental or morphological differences between species is an important topic for future investigation.

Besides variation between species of vertebrates, variation also exists among individuals within populations. Tourette’s syndrome (TS), for example is a neuropsychiatric disease with a strong genetic component, and 2 of 174 TS patients, but 0 of 3600 control chromosomes, had sequence polymorphisms in the binding site for human miR-189 in the 3’ UTR of the mRNA for SLITRK1, which encodes a single-pass transmembrane protein [112]. Brain regions implicated in TS co-express MIRN189 and SLITRK1, consistent with the hypothesis that the miRNA regulates expression of the SLITRK1 gene. These results suggest that genetic variation in miRNA binding sites may play a role in psychiatric disease. In another example, a sequence in the serotonin receptor 1B (HTR1B) mRNA confers repression by miR-96, but a common human polymorphism changes a nucleotide critical for miR-96 binding. Loss of HTR18 function causes an aggressive phenotype in mice [113], and people with the miR-96 binding site on the HTR18 message reported more disorderly behaviour than individuals with the alternative allele. These studies show that variations in miRNA binding sites exist among individuals within populations and further suggest that these polymorphisms are linked to phenotypes that could be selected upon during evolution.

Because the nuanced differences in skeletal morphology that differentiate related populations are likely to be caused by subtle changes in intensity, duration, or spatial distribution of gene expression patterns, and because miRNAs provide that sort of variation in activity, it is likely that miRNAs may play an important role in microevolution of new morphologies [114]. We advance the specific hypothesis (Fig. 6) that diverging lineages can accumulate polymorphisms in Mirn expression patterns or in sequence variation in miR binding sites of skeletal development genes that alters the strength or quantity of miRNA binding to their targets, changing the activity of target genes. The altered gene activity, in turn, modifies cell proliferation, cell migration, cell differentiation or cell function in ways that cause the phenotypes of sister lineages to diverge in ways that adapt them to their environments. Experiments to test this hypothesis require well-understood developmental mechanisms of miRNA action coupled to studies investigating the variation of miRNA binding sites within target genes and mechanistic investigations of the consequences of such changes. Resolution of the problem, however, awaits these exciting prospects.

Skeletal miRNAs and skeletal disease

In addition to altering skeletal phenotypes across individuals, evidence suggests that miRNAs function in changes that occur within individuals over time. As people age, skeletons generally become less robust, culminating in osteopenias and osteoporosis, or bone grows in inappropriate places, resulting in osteoarthrits. The first evidence of miRNA involvement in the ageing process, once again, came from the nematode Caenorhabditis elegans, where reducing lin-4 activity shortens life span and over-expressing lin-4 extends life span [115]. The same may hold true for human beings, given the role of the human lin-4 ortholog miR-125b in osteoblast differentiation [116], skin diseases [117] and cancer survival [118].
Skeletal tissue can arise from mesenchymal stem cells, which can produce not only osteocytes and chondrocytes for bone and cartilage development, but also adipocytes for maintaining fat tissue [119]. As bone volume decreases in clinical osteoporosis, marrow adipose tissue increases, suggesting a reciprocal relationship between adipogenic and osteogenic potential in disease [120]. When mammalian mesenchymal stem cells age in culture, they experience a reduction in the spectrum of derivatives they can form [121]. Correlated with this age-related diminution of differentiative capacity, at least four miRNAs are significantly up-regulated in senescent relative to young mesenchymal stem cells (miR-371, miR-369-5P, miR-29c and miR-499) [119]. It is still unknown whether these or other miRNAs are up-regulated in ageing skeletal systems in older people and the identity of their targets, and these questions must be investigated. These results, however, suggest the hypothesis that osteopenias, osteoarthritis, or other skeletal conditions in older people may result at least in part by inappropriate changes in the differentiation potential of mesenchymal stem cells due to changes in the levels of specific miRNAs (Fig. 6).

The adipocyte/osteocyte balance that is abnormal in osteoporosis may be regulated, at least in part, by miRNAs. In a gene expression profiling study of human multipotent adipose tissue-derived stem cells, which are capable of become adipocytes or osteoblasts, most of the genes repressed during fate commitment had the potential of being regulated by miRNAs [122]. Bone morphogenetic protein-2 (BMP2) stimulates bone development by activating BMP receptor, which activates SMAD1, which regulates transcription of osteogenic genes such as alkaline phosphatase, Col1a1, Runx2 and Osterix [123], thereby promoting osteoblast proliferation and differentiation [124]. miR-26a inhibits the differentiation of adipose tissue-derived stem cells towards an osteogenic fate by inhibiting SMAD1 translation [125], and thus, overexpression of miR-26a could be an unrecognized contributor to osteopenia, a hypothesis that should be tested.

SMADs may also be involved miRNA biogenesis because BMP signalling stimulates pre-miR-21 production post-transcriptionally, at least in cardiac cells [126]. In addition, expression profiling of miRNAs during BMP2-induced bone development of mesenchymal cells in culture showed that BMP2 down-regulates miRNAs that inhibit osteogenic genes [127]. Thus, pathogenic over- or under-activity of miRNAs targeting osteogenic genes or the inappropriate action of BMPs on miRNA biogenesis may unfavourably influence bone development, leading to osteopenia or, reciprocally, hyperossification disease.

MiRNA-based therapies for human disease provide promise for the future. Strategies include antisense oligonucleotides directed towards specific miRNAs to prevent them from associating with their endogenous targets or synthetic miRNAs directed towards the 3’ UTRs of pathogenic targets. The use of LNAs seems especially promising for therapies because of their high biostability, low toxicity and reasonable biodistribution after in vivo administration [80, 128]. For example, mice or monkeys treated intravenously with LNA or with antagomirs (cholesterol-conjugated 2-O-methyl oligonucleotides) to silence miR-122, a liver-expressed miRNA that targets cholesterol synthesizing genes, led to reduced plasma cholesterol [129, 130]. These new results suggest that the identification of miRNAs involved in skeletal diseases and their targets have the potential to lead to novel miRNA-based therapies for osteopenias, osteoporosis, osteoarthritis and other skeletal diseases that accompany old age.
Conclusions

This review has pointed out what is known about the role of miRNAs in development, evolution and disease. Because the field is so new, many of the reviewed works point to hypotheses that demand testing. Despite the identification of miRNAs expressed with exquisite precision in skeletal tissues, we still know little about how any of them work to affect skeletal development. While miRNAs are highly conserved in structure over evolutionary time, bursts of new miRNAs are associated with explosions of evolutionary diversity, and apparently functional variations in miRNA binding sites exist as polymorphisms within populations, no miRNAs have been directly implicated in adaptive morphological change in skeletal or other body systems. Finally, although miRNAs can phenocopy human skeletal disease in model organisms, none has yet been directly linked to human skeletal disease. The promise of miRNA mimics or inhibitors as systemic therapies is so new, many of the reviewed works point to hypotheses that demand testing. Despite the identification of miRNAs expressed with exquisite precision in skeletal tissues, we still know little about how any of them work to affect skeletal development. While miRNAs are highly conserved in structure over evolutionary time, bursts of new miRNAs are associated with explosions of evolutionary diversity, and apparently functional variations in miRNA binding sites exist as polymorphisms within populations, no miRNAs have been directly implicated in adaptive morphological change in skeletal or other body systems. Finally, although miRNAs can phenocopy human skeletal disease in model organisms, none has yet been directly linked to human skeletal disease. The promise of miRNA mimics or inhibitors as systemic therapies of disease promises should propel the intensive investigation of the roles of miRNAs in skeletal disease.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 MicroRNA nomenclature
Table S2 Selected online resources for microRNAs

This material is available as part of the online article from: [link]

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