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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 Serengetti Plain of Kenya [2]. Likewise, a rich

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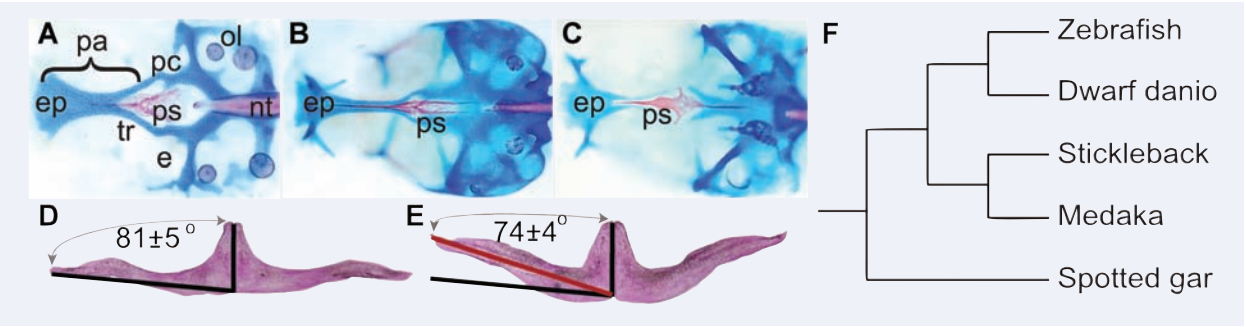

Fig. 1 Closely related species and populations within a species often display subtle morphological differences in skeletal systems. Such morphological variation probably results from slight differences in the timing or location or intensity of the action of specific genes. (**A**–**C**) Skeletal variation in the palate and neurocrania of three species of teleost fish. (A) *Danio rerio* (zebrafish), (**B**) *Gasterosteus aculeatus* (stickleback) and (**C**) *Oryzias latipes* (medaka). Note species-specific differences in the shapes of the palate, consisting of ethmoid plate and parasphenoid. (**D**–**E**) Anterior view of the premaxilla bone showing variation in shape between *D. rerio* (zebrafish) (**D**) and *D. nigrofasciatus* (dwarf danio) (**E**). The angle of zebrafish premaxilla bone is 81 ± 5° ($n = 44$) for *D. rerio* while the angle of *D. nigrofasciatus* is 74 ± 4° ($n = 35$). (**F**) Evolutionary relationships of some fish discussed in this review, including the basally diverging non-teleost *Lepisosteus oculatus* (spotted gar). Abbreviations: e, eye; ep, ethmoid plate; nt, notochord; ol, otolith; pa, palate; pc, parachordal; ps, parasphenoid and tr, trabeculum.

diversity of craniofacial forms equip various species of cichlid fish in the Great Rift Lakes of Africa to a diet of hard-shelled benthic mollusks, or plankton in the water column, or scales on exclusively the left side of other fish [3]. Different but closely related species of teleost fish [4, 5] often show subtle differences in the craniofacial skeleton (Fig. 1). How do such differences arise during development? What evolutionary forces promote the fixation of these genetic differences over many generations? And might the same mechanisms that differ between species to effect different skeletal features also differ between individuals or even change during a person's lifetime to contribute to skeletal disease?

Arguments concerning the genetic mechanisms that lead to the evolution of morphological differences like those shown in Fig. 1 or to skeletal differences among individuals are currently contentious – is evolution primarily due to genetic change in protein coding regions or in non-coding regulatory regions (see, *e.g.* [6, 7]. An additional hypothesis is that the subtle kinds of morphological changes that result in ecologically important skeletal variation can also occur by variation at regulatory sites embedded in non-coding regions of transcripts, for example in miRNA binding sites in messenger RNAs.

In addition to differences in skeletal form, human populations exhibit variation in susceptibility to skeletal disease, including osteopenia, osteoporosis and osteoarthritis. Osteopenia is a reduction in bone mineral density. About 34 million American women and 12 million American men have osteopenia. Osteopenia often leads to osteoporosis, a disease characterized by low bone mass, bone deterioration, bone fragility, increased susceptibility to fracture and slow healing of bone fracture [8]. Osteoporosis is a threat to the health of about 44 million Americans. Of Americans over 50 years old, 55% already have osteoporosis [9]. Osteoporosis increases the lifetime risk of fractures to about 50% in women and about 20% in men. As the population ages, the number of hip fractures worldwide is predicted to increase from 1.7 million in 1990 to 6.3 million in 2050 [10]. After hip fracture due to bone loss, half of all patients fail to recover their previous mobility and independence, and in the first year after the fall, more than 25% die [11]. A better understanding of the genetic mechanisms of osteopenia and osteoporosis should help lead to improved therapies for the prevention and treatment of bone loss diseases.

Osteoporosis has a major genetic component, but is also affected by the environment [12]. For example, obesity, diet and weight-bearing exercise have major effects on bone mineral density, but family and twin studies reveal high heritability for osteopenia and osteoporosis [13]. Thus, susceptibility to osteoporosis, like many other common human diseases, has both genetic and environmental risk factors. We review here the evidence that miRNAs are involved in skeletal development and suggest the possibility that environmental factors may act, in part, to alter bone density *via* a miRNA-mediated process.

This review poses the following questions: (*i*) What roles do miRNAs play in skeletal development? (*ii*) To what degree are miRNAs involved in the fine-tuning of skeletal structure that occurs in adaptive evolution? (*iii*) What might be the roles of miRNAs in skeletal diseases of ageing? To approach these questions on development, evolution and disease, we first briefly overview skeletal development and our current understanding of miRNA biology, then focus on new discoveries of miRNA action in skeletal system development and finally discuss the hypothesis that variation in miRNA biology may contribute to the evolution of skeletal system diversity and human skeletal disease.

An overview of skeletal development

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

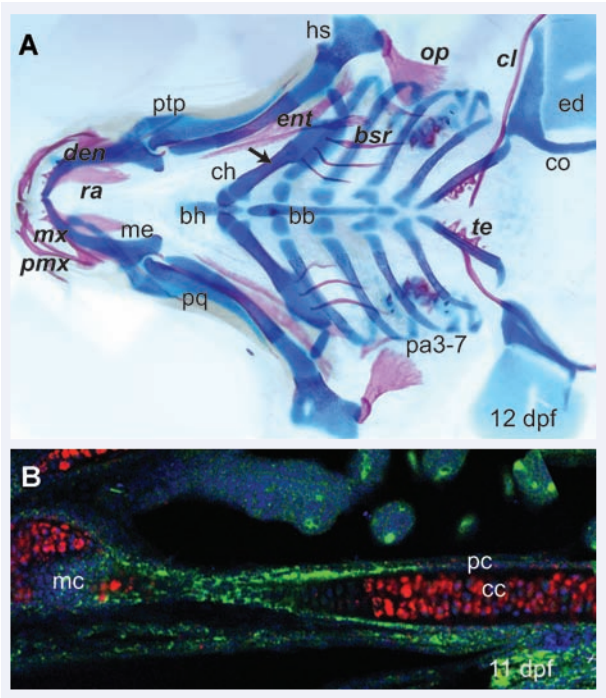

Fig. 2 Endochondral ossification and intramembranous ossification. (**A**) 12-day-old *G. aculeatus* pharyngeal skeleton stained with Alcian Blue for cartilage and Alizarin Red for bone shows intramembranous and endochondral ossification. Italic bold font labels intramembranous ossification and roman 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; co, 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 skeleton, bone forms on a cartilage model, as in bones of limbs in tetrapods and paired fins in fish. 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 miRNAMap 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 knockout 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

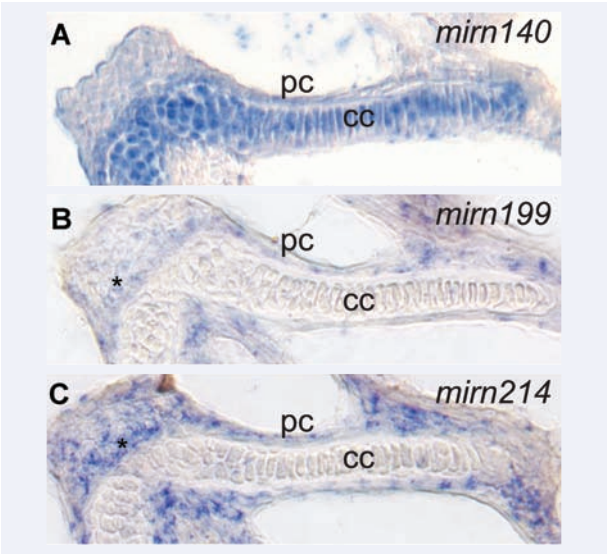

Fig. 3 Skeletal miRNAs are expressed in discrete patterns. Conventional *in situ* hybridization to pre-miRNAs in 3dpf zebrafish larvae shows that *mirn140*, *mirn199* and *mirn214* are expressed in the ceratohyal cartilage, confirming results with LNAs [77]. In both skeletal elements, *mirn140* is expressed in the chondrocytes while *mirn199* and *mirn214* are expressed in the perichondrium and surrounding mesenchyme cells. Abbreviations: cc, chondrocytes; mc, mesenchyme and pc, perichondrium.

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

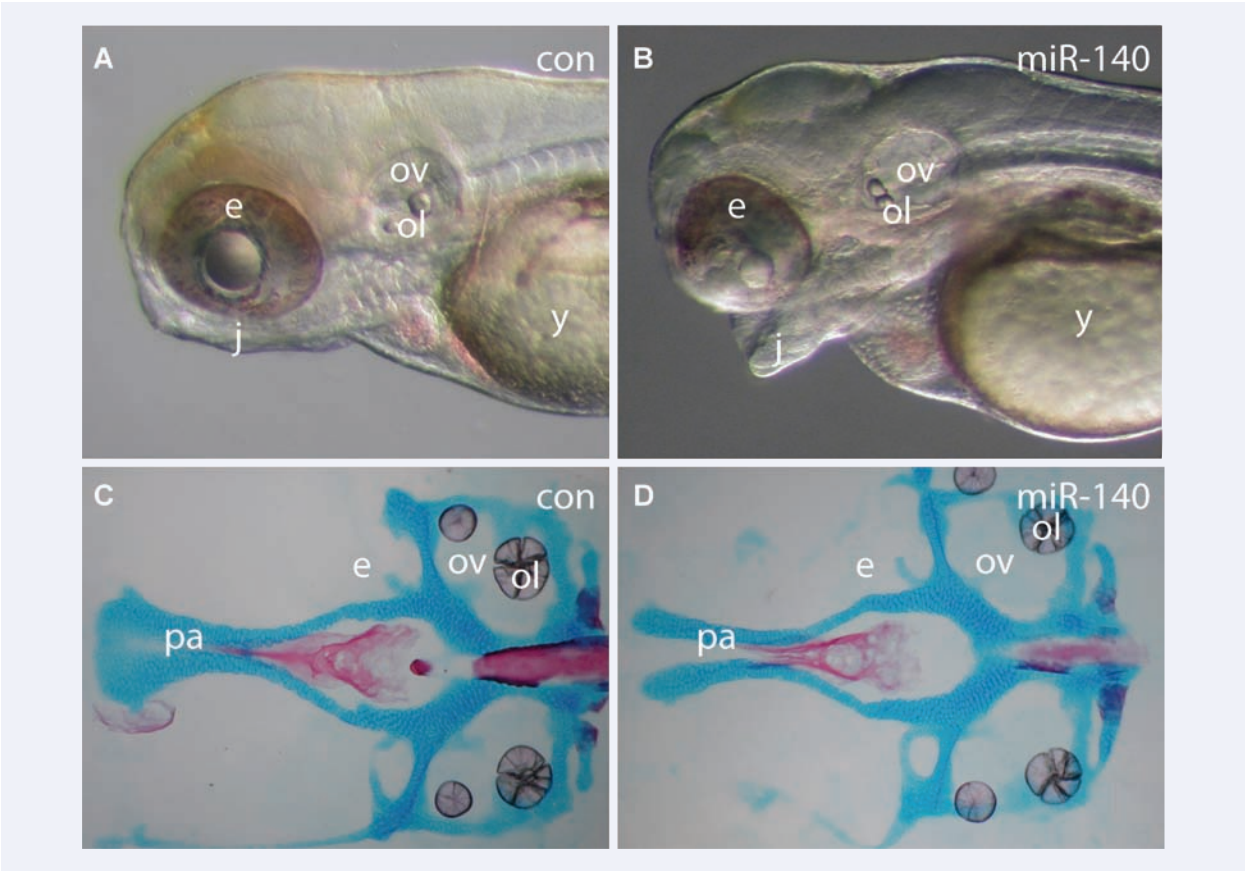

Fig. 4 Overexpression of miR-140 causes cleft palate. (**A**) Whole mount of 3 dpf control zebrafish larva. (**B**) Whole mount of 3 dpf larva over-expressing miR-140. Note protruding lower jaw. (**C**) Palate (neurocranium) of control larva. (**D**) Cleft palate of larva over-expressing miR-140. Abbreviations: con, control; e, eye; j, jaw; miR-140, overexpression construct for miR-140; ol, otolith; ov, otic vesicle; pa, palate and y, yolk.

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,

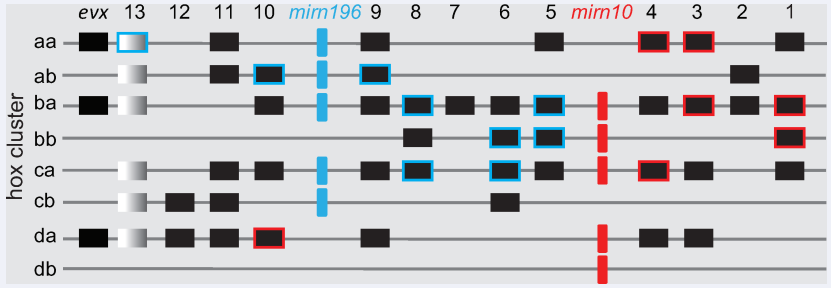

Fig. 5 *Hox* genes are predicted targets for *mirn10* and *mirn196*. Due to genome duplication and subsequent gene loss, zebrafish retains seven clusters of *hox* genes (gradient grey boxes) that include five copies of *mirn196* (blue boxes) and five copies of *mirn10* (red boxes). Although protein coding genes of the zebrafish *hoxdb* cluster were lost, this cluster retained a copy of *mirn10* [108]. The *mirn10* (gradient grey boxes with red surroundings) and *mirn196* (gradient grey boxes with blue surroundings) genes both have multiple *hox* genes as computationally predicted targets.

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 of 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 *mirn* 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 teleost 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 *Mirn*s 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 *Mirn*s 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 *mirn*s 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. This 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 osteoarthritis. 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].

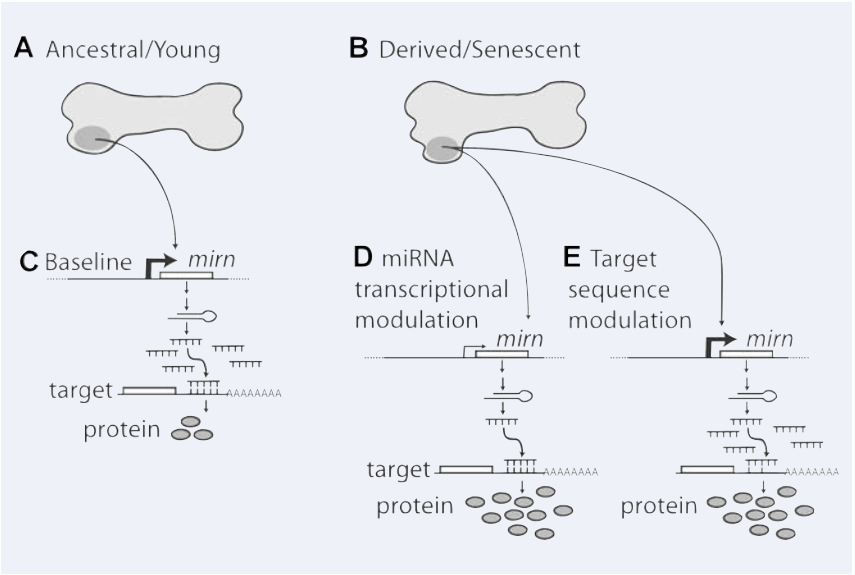

Fig. 6 A model for the roles of miRNAs in evolution and disease. (**A**) A representation of a skeletal element in an evolutionarily ancestral state, or a young or healthy condition. (**B**) A representation of a skeletal element in an evolutionarily derived state, or a senescent or diseased condition. (**C**) In the baseline condition, the *mirn* gene produces a given level of miR molecules, which bind to their target site with a certain affinity to allow a specific level of translation. (**D**) In the evolutionarily derived state, cis-acting enhancer mutations may decrease or increase (not shown) transcription of the *mirn* gene, which would lead to decreased (or increased, not shown) inhibition of the target mRNA and hence increased (or decreased, not shown) amounts of target protein, which could alter skeletal shape or function. Similar diminution of *mirn* expression could occur by stage specific changes in *mirn* transcription or epigenetic modifications. (**E**) Mutations accumulated during evolution could alter the miRNA recognition site on target mRNAs to increase or decrease binding, which could alter the amount of target protein produced compared to baseline. Altered protein levels could alter the morphologies or the relative rates of skeletal build-up or degradation by osteoblasts and osteoclasts.

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 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: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1582-4934.2009.00696.x
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