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microRNA Regulation of Skeletal Development

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Abstract

Purpose of review—Osteogenesis is a complex process involving the specification of multiple progenitor cells and their maturation and differentiation into matrix-secreting osteoblasts. Osteogenesis occurs not only during embryogenesis, but also during growth, after an injury, and in normal homeostatic maintenance. While much is known about osteogenesis associated regulatory genes, the role of microRNAs, which are epigenetic regulators of protein expression, is just beginning to be explored. While miRNAs do not abrogate all protein expression, their purpose is to finely tune it, allowing for a timely and temporary protein down-regulation.

Recent findings—The last decade has unveiled a multitude of microRNAs that regulate key proteins within the osteogenic lineage, thus qualifying them as ‘osteomiRs’. These miRNAs may endogenously target an activator or inhibitor of differentiation, and depending on the target, may either lead to the prolongation of a progenitor maintenance state or to early differentiation. Interestingly, cellular identity seems intimately coupled to the expression of miRNAs, which participate in the suppression of previous and subsequent differentiation steps. In such cases where key osteogenic proteins were identified as direct targets of miRNAs in non-bone cell types, or through bioinformatic prediction, future research illuminating the activity of these miRNAs during osteogenesis will be extremely valuable.

Summary—Many bone related diseases involve the dysregulation of transcription factors or other proteins found within osteoblasts and their progenitors, and the dysregulation of miRNAs, which target such factors, may play a pivotal role in disease etiology, or even as a possible therapy.

Keywords

Osteoblast; Osteogenesis; microRNA; neural crest; Runx2; skeletal defect

Introduction

Birth defects that affect the skeleton account for 5% of all infant deaths. In survivors, skeletal defects can result in lifelong burdens ranging from stunted growth and

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Conflict of Interest

Steven R. Sera and Nicole I. zur Nieden declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

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malformations to poor bone density and the need for surgery. The key to understanding normal and abnormal bone formation, in addition to finding possible treatments for bone disorders, lies in studying the genetic regulation of osteogenesis. Recent advances in genomics have led to the discovery of endogenous small RNA molecules, known as microRNAs (miRNAs, miRs), which regulate genetic expression at the transcript level through degradation or a translational halt of the target transcript. Understanding how miRNAs contribute to the regulation of osteogenesis may provide insights into the molecular causality of skeletal defects and disorders.

Faulty osteogenesis as the root cause of skeletal defects

Osteogenesis describes the process of bone formation during early development, bone homeostasis in adults, and bone remodeling after an injury. Osteoblasts, the cells that secrete a bone-specific extracellular matrix (ECM) which later becomes calcified and mineralized (Figure 1), is derived from progenitor cells of a distinct germ layer origin in the embryo. Vertebrae and some craniofacial bones are derived from paraxial mesoderm, while the appendicular skeleton, or long bones, are derived from lateral plate mesoderm [1]. The majority of the craniofacial bones and cartilage however, are derived from cranial neural crest cells (NCCs).

Independent of their origin, bone progenitor cells condense at sites of future skeletal element formation. These condensations are composed of loosely packed mesenchymal cells, which during endochondral ossification, differentiate into chondrocytes, undergo hypertrophy, apoptosis, and are later replaced by osteoblasts [1] (Figure 1). Recent lineage tracing experiments ended a decade long debate whether the cartilage anlage was necessary for mesodermal progenitors to differentiate into osteoblasts. This was demonstrated by showing that a subset of collagen X expressing hypertrophic chondrocytes survive and can directly transdifferentiate into osteoblasts [2,3,4,5] (Figure 1), a hypothesis that had long been fueled by the fact that these transitory chondrocytes begin to express Runt-related factor 2 (*Runx2*) [6,7,8], a master osteogenic transcription factor (TF) [9•]. In contrast, during intramembranous ossification or dermal bone formation, progenitor cells of neural crest cell origin differentiate directly into osteoblasts (reviewed in [10]) (Figure 1). In cases where mesenchyme cells commit directly to the osteogenic lineage, they first form pre-osteoblasts, which have the ability to proliferate expansively [11]. Osteocytes, the terminally matured osteoblasts, are fully surrounded by ECM and communicate with each other through dendritic processes to sense mechanical stimuli and regulate mineral homeostasis [12•].

Dysregulation of any event during osteogenesis can result in a wide array of skeletal disorders and diseases. While cleft lip and palate may be visibly morphologically apparent immediately at birth [13], others, such as osteogenesis imperfecta (OI) or brittle bone disease, presents a variety of symptoms from loose joints to weak bones, which cause dysmorphism and premature death later in life [14]. Some rarer diseases, such as fetal skeletal dysplasia, exhibit stunted bone growth, poor mineralization, and limb agenesis [15]. The underlying causes may be genetic, including mutations in genes such as collagen and fibroblast growth factor receptors, or may be environmental such as in the case of phosphate or Vitamin D metabolism deficiency [15].

Transcriptional control of osteogenesis

The etiology of skeletal malformations is often associated with the misregulation of the genes coding for ECM proteins or master transcription factors. In osteogenesis, master lineage regulatory TFs include *Runx2*, also known as Core-binding factor $\alpha 1$, and Osterix (*Osx*) [16]. RUNX2 controls the promoters of all major osteoblast ECM genes, including *Coll1* (type I collagen), *Spp1* (osteopontin), *Ibsp* (bone sialoprotein, BSP) and *Bglap* (osteocalcin, OCN), the transcription of which contributes to the establishment of an osteoblast phenotype [9,17]. Consequently, disrupted *Runx2* results in a complete lack of bone [6]. Furthermore, mutations in *RUNX2* that diminish protein activity can lead to cleidocranial dysostosis [18], a disease characterized by absent parietal bones and delayed skull ossification [19].

As a central mediator of osteogenesis, RUNX2 executes signals from the Wnt, bone morphogenetic protein (BMP) and fibroblast growth factor (FGFs) signaling pathways [16,20,21,22]. In *Runx2* expressing cells, *Osx* is responsible for their final commitment to mature osteoblasts [23]. OSX works partially through activating the Wnt signaling pathway [24], downstream of which transcription of *Runx2* is activated [25]. Thus, it appears that *Runx2* and *Osx* work in a feed-forward loop that implements the osteogenic phenotype [26].

microRNAs in disease etiology and development

The molecular underpinnings of osteogenic gene expression have been extensively studied in a variety of model organisms [26], but only recently have we started to explore the critical roles that miRNAs play in their regulation. After the first miRNA, lin-4, was discovered in *Caenorhabditis elegans* [27], it was subsequently found that miRNAs were highly conserved throughout the animal kingdom, as well as in plants [28,29,30]. Furthermore, genetic studies in animal models demonstrated that miRNAs play crucial roles in animal development [31,32], and disease etiology [33,34]. Instead of representing all-or-nothing on/off switches, miRNAs fine-tune gene expression by dampening protein expression epigenetically through binding to the 3' UTR of mRNA transcripts [35]. Accelerated deadenylation and subsequent decapping destabilizes mRNAs [36], ultimately reducing protein abundance.

Usually found throughout intergenic regions of the genome, miRNA genes often cluster together, such that they can be transcribed as poly-cistronic transcripts [37] (Figure 2). Polymerase II transcription generates a stem-loop structure containing the primary miRNA (pri-miRNA), which can range in size from hundreds of nucleotides up to kilobases [38]. The pri-miRNA then adopts a secondary stem loop structure, which undergoes nuclear cleavage by a multiprotein complex. The core components of this complex are the RNase III enzyme Drosha and the dsRNA-binding domain protein DGCR8 (DiGeorge syndrome chromosomal [or critical] region 8) [39]. The resulting hairpin shaped pre-miRNA, which is about 65 nucleotides long, contains a 2-nt 3' overhang that is recognized by exportin-5, allowing nuclear export via a Ran-GTP-dependent mechanism [40]. microRNAs, which are encoded in introns and are part of the primary mRNA transcript, are processed by the

spliceosome, which also produces a pre-miRNA that is then shuttled into the cytoplasm for further processing via the same mechanism [41,42].

Next, the pre-miRNA is cleaved by cytoplasmic Dicer to produce the mature ~19–25 nt miRNA duplex [43]. The miRNA strand with the lower relative thermodynamic stability of base-pairing at its 5' end is then loaded onto an Argonaut protein and incorporated into the RNA-induced silencing complex (RISC). This results in a mature miRNA, which is then directed to the 3' UTR of the target mRNA [44]. Based on partial complementarity, the mRNA targets are then blocked from being translated [45] or directed to the cellular 5'-to-3' mRNA decay pathway. There, mRNAs are first deadenylated, decapped, and ultimately degraded by the cytoplasmic 5'-to-3' exonuclease XRN1 [46,47,48].

Based on these mechanisms, the regulatory nature of miRNAs is multifold. Due to the partial complementarity described above, one miRNA may have several target sites in the 3'UTR of a single mRNA, and one 3'UTR has tens to hundreds of different binding sites for different miRNAs. Thus, multiple co-expressed miRNAs may act in concert to ensure that the expression of a specific mRNA is repressed as efficiently as possible. In addition, because one miRNA has multiple distinct mRNA targets, a down-regulated miRNA may offset the up-regulation of another in the control of a common target mRNA. Given these considerations, it appears that the complexity of osteogenesis is compounded by miRNA expression as well as by any feedback regulation that may exist between the microRNAs and their targets, a few examples of which will be discussed in this review.

Of the hundreds of miRNAs confirmed to exist, a subset of them associated with terminal osteoblast differentiation and acquisition of osteogenic identity from already committed progenitors, have been designated 'ostemiR' [49•]. This review discusses the role of microRNAs during early and late specification of progenitor cells with a particular emphasis on osteogenic potential in the craniofacial skeleton. In addition to known 'ostemiRs' (Table 1), we also discuss miRNAs that silence proteins associated with osteogenesis in non-bone tissues.

microRNAs that control osteoblast identity and homeostasis

Osteoblasts secrete a variety of unique proteins that comprise an extremely specialized ECM. A subset of these serve as a scaffold upon which mineral is deposited in a final maturation step, while others impart structural flexibility to withstand compressive and tensile stress. Any microRNA targeting the non-collagenous glycoproteins and proteoglycans such as osteonectin, BSP and OCN, which are all implicated in calcification of the ECM [50], would execute an important control over matrix mineralization. Consequently, the osteonectin-targeting miR-29a and -29c appear to play a role in osteoblast maturation and indeed their expression levels increase during late osteogenesis [51]. Similarly, miR-125b, which directly targets the OCN mRNA, *Bglap*, is highly expressed in primary human osteoblasts isolated from human trabecular bone, and thus is implicated in normal bone homeostasis [52].

In bone, vitamin D bound to its receptor, VDR, participates in the mineralization process, and disruption to the VDR pathway can lead to mineralization defects such as those found in Rickets [53]. The importance of miR-125b in osteoblast identity is further illustrated in the fact that exogenous miR-125b blocked differentiation, while in contrast, its inhibition indirectly yielded higher ALP activity [54]. Furthermore, in breast and prostate cancer, which often metastasize to bone [55], down-regulation of miR-125b results in increased expression of its target *Vdr*, [56] again demonstrating the importance of miR-125b in osteogenesis. While the only currently reported miRNAs directly targeting the bone-type *Alpl* are miR-204/211 [57], 16 additional conserved miRNA binding sites are predicted in its 3'UTR (microrna.org). Since expression of ALP not only maintains, but also initiates matrix mineralization, thusly associating with earlier stages of osteogenesis, the miRNAs post-transcriptionally regulating this enzyme may be expressed by mesenchymal stem cell as was indeed confirmed for miR-204 [58•].

Once fully embedded in the matrix, osteoblasts take on new functions as they terminally differentiate into the osteocytes, which senses and responds to mechanical stimulation in a bone-anabolic manner. This response is mediated by cyclooxygenase-2 (COX2) [59,60], which produces proliferation-stimulating prostaglandins [61]. While no *Cox2* regulating microRNAs have been identified in the context of osteoblasts or mechanical loading, miR-101a and miR-199a down-regulate *Cox2* in early mouse embryos during implantation [62]. Identified in several miRNA screens, miR-199a associates with osteoblast differentiation [63,64], suggesting that its identification as an 'ostemiR' may be linked to its control of *Cox2*. Additionally, while not directly linked to *Cox2*, miR-218, miR-191*, miR-3010a and miR-33 were recently identified in MC3T3-E1 osteoblastic cells to be responsive to mechanical strain [65].

miRNAs expressed at the preosteoblast stage

Prior to matrix calcification, proliferating osteoprogenitors secrete OPN [66], encoded by *Spp1*. While several microRNAs have been associated with *Spp1* regulation (i.e. miR-541 [49•]; miR-21 [67]), no miRNA directly silencing *Spp1* has been identified in osteoblasts or their precursors. However, miR-299-5p targets *Spp1* in breast cancer cells [68] and miR-127-5p targets *Spp1* in chondrocytes [69] and thus they may also do so in pre-osteoblasts.

The non-collagenous component of the ECM constitutes only a small portion. In fact, over 90% of the secreted ECM proteins are collagenous fibrils, primarily those of type I collagen. Since collagen is so abundant, it is not surprising that perturbation of collagen production is a root cause of OI [70,71]. Any miRNA regulating collagen genes may thus not only be important for normal osteogenesis, but their misregulation could potentially be implicated in the etiology of OI. For example, miR-29b directly regulates *Col1a1*, *Col1a2*, and *Col3a1* in hepatic cells [72], but this has not yet been replicated in cells of the osteogenic lineage. Secretion of ECM proteins decreases in association with prolonged differentiation, and the function of miR-29b in osteoblasts could be to suppress the expression of collagen proteins allowing the collagen fibril matrix to mature for mineral deposition [73]. Consequently, the

expression of miRNAs similar to miR-29b, would be expected to be higher as osteoblasts transition to the calcification step.

Rat mesenchymal stem cells overexpressing miR-21 exhibit enhanced performance in a fracture healing model *in vivo* [67]. The pro-osteogenic effect of miR-21 may occur through an indirect regulation of *Col1a1*, since miR-21 indirectly causes elevated expression of *Col1a1* and in turn deposition of type I collagen, in a murine model of lung fibrosis [74]. Interestingly, recombinant COL1A1 positively regulates miR-21 expression, illustrating an unknown mechanistic feedback loop between protein and miRNA that likely prevents complete transcript shut-off [74]. miR-21 is also increased by the pro-osteogenic TGF β and BMPs, but surprisingly not through transcriptional control, but rather through enhanced processing of the pri-miRNA [75•]. The designation of miR-21 as an ‘ostemiR’ is however controversial, because miR-21 is upregulated in most types of cancer [76].

Another ECM protein produced in pre-osteoblasts is fibronectin, an adhesion protein which binds to integrins to influence cell proliferation and tissue development [77,78]. Although miR-200b and miR-377 regulate fibronectin in kidney proximal tubular cells and in diabetic neuropathy respectively, a role for these miRNAs in osteogenesis remains to be determined [79,80]. Interestingly, miR-377 is expressed during osteogenic differentiation of human dental pulp stem cells [81], and is upregulated in response to contact of human osteoblast-like MG-63 cells with osteo-inductive biomaterials used for surgical bone restoration [82,83].

microRNAs associated with the mesenchymal state and mesenchymal commitment

Mesenchymal stem or stromal cells (MSCs) have the potential to generate skeletal as well as connective tissue [84]. Markers that define MSC identity have been agreed on by the International Society for Cellular Therapy to include the surface expression of CD90, CD73 and CD105 [85]. In terms of microRNAs however, none are known to directly target the corresponding mRNAs during osteogenesis. The only existing evidence comes from cancer cell lines, in which miR-422 targets *Nt5e* (codes for CD73) [86] and miR-370 negatively regulates *Eng* (codes for CD105) [87].

Another surface glycoprotein shared by MSCs is CD44, which functions in adhesion and migration, and binds hyaluronan and OPN [88]. In both human renal and prostate cancer cells, *CD44* has been identified as a direct target of miR-34a [89], a miRNA that promotes osteogenesis in human adipose-derived stem cells [90]. In addition, the 3' UTR of *CD146*, which has been associated with a higher potential for osteogenic differentiation [91], is directly targeted by miR-329 in endothelial cells [92]. Also in endothelial cells, expression of *Vcam1*, which encodes the cell-cell adhesion molecule CD106 and reduces the migratory ability of MSCs [93], is controlled by miR-126 [94]. However, again, direct regulation has yet to be confirmed in osteoblasts or their precursors. Potentiating the MSC state is miR-140-5p, which inhibits osteogenic lineage commitment and is commonly enriched in undifferentiated human MSCs from various tissue sources [95]. In zebrafish, injection of miR-140-5p phenocopies *Bmp2* repression, resulting in aberrant embryonic bone

development (short stature, curved trunk, craniofacial malformations), and confirming a direct relationship between the two [96,97].

For expression of pre-osteoblastic matrix genes to occur, which turn MSCs into pre-osteoblasts and later mature osteoblasts, the master TFs RUNX2 and OSX must be genetically activated. miR-125b, a miRNA identified in MSCs throughout many genetic screens [98,81], is predicted to target *Osx*. However, a relationship has not been confirmed beyond the finding of reduced *Osx* mRNA [99]. In contrast, miR-143 [100,101], miR-145 [100], miR-214 [102] and miR-637 [103] suppress osteogenic differentiation by directly targeting *Osx*. Due to the ability of miR-322 to directly target *Tob2*, which normally helps to degrade *Osx*, *Osx* mRNA is stabilized, allowing osteoblast differentiation to occur [104].

Genetic manipulation of *Runx2* *in vivo* indicated that its expression is both necessary and sufficient for mesenchymal cell differentiation towards the osteoblast lineage [105,106]. Discovered as endogenous attenuators of *Runx2* expression, which prevent cells from differentiating into osteoblasts, the inhibition of miR-23a, miR-34c, miR-628-3p miR-137, miR-204, miR-205, miR-338-3p, miR-433, miR-375, and miR-135 promoted osteoblast differentiation along with an increase in bone specific markers [107,58•, 108,109,110,111,112,113,114,115]. A recent publication bioinformatically identified additional microRNAs predicted to target *Runx2* [116]. These include miR-141, miR-200a and -200b, whose expression is expected to be down-regulated as differentiation progresses, but remain high in mesenchymal cells thus representing MSC markers. Alternatively, a microRNA may qualify as an ‘ostemiR’ if its expression is up-regulated and it targets inhibitors of RUNX2, as is the case for miR-129-5p, which targets the signal transducer and activator of transcription 1 [117] that normally sequesters RUNX2 in the cytoplasm to prevent its nuclear activity [118].

In the nucleus, RUNX2 may regulate transcription not only of genes, but also of miRNAs, such as the pro-osteogenic miR-690 [64] and miR-1192 [119]. In contrast, RUNX2 represses the promoter of the miR23a~27a~24-2 cluster [112]. The consequence of this negative feed forward loop regulation is to cause depression of special AT-rich sequence-binding protein 2 (*Satb2*), a scaffold protein that increases RUNX2 activity to promote differentiation. This is an interesting example of a cluster, in which all three miRNAs share *Satb2* as a common target, but only one of them, miR-23a, targets *Runx2*, the attenuation of which seems to fine tune the pace of progression of the osteoblast phenotype rather than switching it off completely.

Two miRNAs that directly target *Satb2*, miR-34b and miR-34c, were shown to affect osteoblast proliferation and differentiation *in vivo* [113]. SATB2 itself has recently been implicated as a major osteogenic TF, since *Satb2*^{-/-} mice exhibit craniofacial abnormalities [120]. *Satb2* is expressed in cells of the osteoblast lineage in developing mice [120], and was reported to function both upstream and downstream of RUNX2 and OSX [120,121,122,123]. Due to its participation in regulatory feedback loops together with RUNX2 and miRNAs, this implicates *Satb2* in the acquisition of an osteoprogenitor fate from both the NC and the mesoderm derived progenitor cells.

One such feedback loop comprises miR-31, whereby in bone marrow MSCs, RUNX2 occupies and activates transcription from the miR-31 promoter, which in turn lowers *Satb2* mRNA and protein expression [122]. While a more recent paper suggests the same feedback loop directs dental follicle cells towards osteogenesis [124], neither of the two studies provides evidence for the direct binding of miR-31 to the 3'UTR of *Satb2*. Other studies place SATB2 upstream of RUNX2 protein and under inflammatory conditions, TNF α -activated miR-33-5p can reduce RUNX2 by directly targeting *Satb2* [123].

Due to the negative regulation of their target transcripts, it would be expected that any miRNA targeting *Satb2* directly would be down-regulated to induce osteogenic commitment. miR-205, for instance exhibits this expression pattern during bone marrow MSC differentiation, and its inhibition promotes osteogenesis [114]. However, this is another example of a miRNA for which *Satb2* regulation is predicted, but has not yet been experimentally confirmed.

MicroRNAs involved in neural crest induction and differentiation

Some osteomiRs participate in the direct conversion of a mesenchymal cell into an osteoblast, such as in the cases of intramembranous ossification or in the perichondrium. Yet others, which are not discussed here, may modulate endochondral bone formation through tuning chondrogenic differentiation from mesenchymal cells. In addition, microRNAs may also take part in the newly discovered transdifferentiation of hypertrophic chondrocytes into osteoblasts [2,3,4,5].

The general importance of microRNAs in the specification of neural crest cell (NCC) derived bone in the skull, which is formed via intramembranous ossification, became apparent in conditional knockouts of *Dgcr8* (which processes nuclear miRNAs) in cranial NCCs [125,126]. Although the role of individual miRNAs during NCC development has not been thoroughly analyzed, miRNAs likely contribute to the intricate, multi-step process of craniofacial cartilage and bone formation. This developmental process is controlled by a number of transcription factors that interact in a so-called gene-regulatory network (GRN), governed by feedback loops and repetitive gene expression (Table 2, see also [127]). However, virtually nothing is known about the miRNAs governing most of the genes in the GRN that regulate NCC specification and development.

The classical theory of NCC specification proposes a partition of a dorsal subset of neuroepithelial cells from the neural tube as the source for NCCs [128]. More recently, it has been proposed that specification occurs during gastrulation and is initiated by neural plate border specifier genes such as *Pax7* [129,130]. Originally identified as being required for muscle cell differentiation, homeostasis, and repair [131], *Pax7* is targeted directly by miR-431 in muscle satellite cells, the overexpression of which increases myogenic differentiation [132]. Thus, miss-expression of miR-431 could negatively impact NCC development.

Once the expression of neural plate border specifiers is initiated, *Pax7* engages with other border specifiers such as *Gbx2*, *Zic1/3*, and *Tfap2*, in a mutual cross regulatory network

(reviewed in [127•]). Only one silencing miRNA has been identified each for *Zic3* and *Tfap2* mRNAs, namely miR-564 [133] and miR-214 [134], respectively. Since the cross-regulation of border specifier genes ensures the stabilization of NCC identity, allowing for continued expression of this gene set through subsequent developmental stages, it is highly likely that the misexpression of regulatory miRNAs would disrupt this network. In addition, the neural plate border genes not only control genes within their own network, but also inhibit neural transcription factors (and vice versa) to sharply define cellular identity in the border region [135]. Hence, overexpression of the neural specifiers *Sox2/3*, which can occur through an absence of microRNA control (i.e. miR-126 [136]), would result in the repression of NCC identity.

Presumptive NCCs reside at the junction between the neural plate and the preplacodal ectoderm, and begin to express of a set of NCC specifier genes, among them *Snai1/2*, *FoxD3*, and *Sox10* [126•]. The only miRNA identified to regulate *Foxd3*, miR-429, is currently only known to participate in skin pigmentation [137]. In human hepatocellular carcinoma, FOXD3 activates miR-137 transcription, which decreases proliferation and migration in tumor cells [109]. The role of miR-137 in hepatocellular carcinoma may mirror a similar role in NCCs to slow down proliferation and migration, and ultimately maintain or expand the NCC pool.

Following specification, NCCs undergo delamination and leave the neural tube, where they migrate dorsolaterally and differentiate into diverse cell types. This progression requires frequent switches between epithelial and mesenchymal states [138,139,140] similarly observed in cancer metastasis, and involves the same reiterated use of a TF machinery including TWIST1 and MSX1/2 [141,142,143,144]. Interestingly, *Twist1*, which is downregulated by miR-1271 in pancreatic cancer cells [145], is a NCC specifier gene [127•], that is also necessary for maintaining NCCs after migration, facilitating the formation of craniofacial bone [146]. In turn, *Msx1/2* are expressed at the plate border as well as in migrating NCCs, which later re-express *Msx2* as well as *Dlx5/6* as they transition into mesenchymal osteoprogenitors [147,148]. Due to their co-regulation of RUNX2 [149], deficiencies in *Msx1/2* cause severe cranioskeletal abnormalities, and deletion of both *Msx1* and *Msx2* cause late gestation lethality [150]. miR-322 indirectly increases *Msx2* expression [104] and therefore it is possible that this regulatory mechanism occurs during the migration of NCCs. In turn, the *Dlx5* silencing miR-141 and miR-200a [148] may control both the early induction as well as the differentiation of NCCs.

Due to the limited information available on miRNAs that regulate NCC development, we conducted an elementary search for predicted miRNAs using the published GRN and the miRanda algorithm (microRNA.org). Additional helpful hypothetical lists of candidate miRNAs with potential involvement in NC development can be found in a recent review [151]. In total, 201 candidate miRNAs were identified (Table 2), 126 of which (62.7%) targeted more than one GRN gene and 25 of which targeted more than 5 genes (Figure 3). Four of these miRNAs are predicted to silence 10 of the 22 GRN genes across multiple steps of NCC development (Figure 3, Table 2). These, we consider the most powerful miRNAs as they may possess the potential to completely block the NC developmental program. Of the 120 candidate miRNAs that specifically target NC specifiers, 8 target 5 or more mRNAs,

possibly explaining the co- and inter-regulation of neural crest specifier genes that is necessary to maintain the NC in an undifferentiated state [152]. Intriguingly, seven of these overlap with miRNAs that regulate multiple stages, except for miR-489, which seems specific to neural crest specifiers.

One of the miRNAs potentially regulating 9 different GRN genes is miR-1192, a miRNA induced by RUNX2. This interesting coincidence suggests that once a cell has committed to a specific lineage, it ensures it does not revert back in developmental time. Moreover, 15 of the candidate GRN miRNAs target *Runx2*, *Osx* and/or *Satb2*, which could be interpreted as a safeguard that helps lock-in a specific cell fate. For instance, considering that miR-204 expression would block the neural plate border state and simultaneously prevent *Runx2* expression, it may therefore be expressed by NCCs to preserve NCC identity. A slightly different scenario may occur with miR-217, which targets both border and NC specifier genes. Here, additional targeting of *Runx2* may take place in a more mature osteoblast to block all prior cell states.

Conclusion

This review discussed the different stages of osteogenesis bringing together different miRNAs and their targets, the disruption of which may potentially cause severe malformations or deformities. Interestingly, cellular identity is intimately coupled to the expression of miRNAs suppressing previous and subsequent differentiation steps. miRNAs may promote osteogenic differentiation and matrix mineralization through targeting genes with functions in osteoclastogenesis and chondrogenesis and/or signaling, and thus may also qualify as ostemiRs. Although some miRNA target identification has occurred in non-bone cell types, or by bioinformatic prediction, future research illuminating the activity of these miRNAs during (NC-) osteogenesis will be extremely valuable.

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Figure 1. Osteogenesis in the long bones and from cranial neural crest cells

In the lateral mesoderm-derived long bones, osteoblasts are created through three different processes: i) in the perichondrium, they are directly differentiated from mesenchymal cells, ii) in trabecular bone, they are also created from mesenchymal cells, but with influence from hypertrophic chondrocytes, iii) trabecular osteoblasts, according to recent lineage tracing evidence, may also derive from transdifferentiating hypertrophic chondrocytes [2,3,4,5]. In contrast, neural crest-derived craniofacial bone is differentiated from cranial neural crest cells that specify at the neural plate border, which later delaminate and become migratory. Through intramembranous ossification, these crest-derived progenitors first differentiate into mesenchyme which directly converts into pre-osteoblasts without a cartilage intermediate. Developmental trajectory is denoted by encircled numbers.

Figure 2. microRNA biogenesis and target recognition

miRNAs encoded in intergenic regions can be transcribed between exons or as polycistronic miRNAs by RNA polymerase II/III [37]. Single transcribed miRNAs are spliced out of the exons by the spliceosome into a hairpin structure known as pre-miRNAs, while polycistronic miRNAs are known as pri-miRNAs before being processed to single pre-miRNAs by DROSHA and DGCR8 proteins [41,42]. The pre-miRNAs are then exported from the nucleus via Exportin-5 [40]. The hairpin loop of the pre-miRNA is then cleaved off by Dicer, forming a miRNA duplex [43], and loaded onto the RNA induced silencing complex (RISC) via Argonaute proteins [44,45]. The miRNA along with RISC is then loaded onto the target mRNA transcript and can halt transcription [45], or cause degradation by exonuclease activity [46–48].

Figure 3. MicroRNAs potentially implicated in neural crest osteogenesis

Candidate miRNAs predicted to target neural crest GRN genes categorized according to the potential stages of neural crest development they may regulate. Candidate microRNAs that are predicted to target genes in the neural crest gene regulatory network (adopted from [127•]) were assembled with the miRanda algorithm available at microRNA.org [153] and categorized according to the expression of their targets during neural crest development. Based on mouse miRNAs, only conserved microRNAs with a good miRSVR score were taken into account.

Table 1
List of known ostemiRs with confirmed target mRNAs

Runx2, *Osx* and *Satb2* targeting miRNAs were categorized under mesenchyme although their expression as well as that of their targets may be implicated in multiple stages of osteogenesis. This list also includes a *Smad5* targeting miRNA, which is implicated in bone-anabolic BMP signaling, although it is not directly discussed in the text.

Cell Type	miRNA	Direct Target	Reference
Osteoblast	miR-29a, miR-29c	<i>Sparc</i>	[51]
	miR-138	<i>Bglap</i>	[154]
	miR-125b	<i>Bglap</i>	[52]
	miR-125b	<i>Vdr</i>	[56]
	miR-204, miR-211	<i>Alpl</i>	[57]
	miR-101a, miR-199a	<i>Cox2</i>	[62]
	miR-135	<i>Smad5</i>	[115]
Pre-osteoblast	miR-299-5p	<i>Spp1</i>	[68]
	miR-127-5p	<i>Spp1</i>	[69]
	miR-29b	<i>Colla1</i> , <i>Colla2</i> , <i>Col3a1</i>	[72]
	miR-200b	<i>Fibronectin</i>	[79]
	miR-377	<i>p21 - activated kinase</i>	[80]
Mesenchyme	miR-422	<i>Nte5</i>	[86]
	miR-370	<i>Eng</i>	[87]
	miR-34a	<i>CD44</i>	[89]
	miR-329	<i>CD146</i>	[92]
	miR-143	<i>Osterix</i>	[100,101]
	miR-145	<i>Osterix</i>	[100]
	miR-214	<i>Osterix</i>	[102]
	miR-637	<i>Osterix</i>	[103]
	miR-322	<i>Tob2</i>	[104]
	miR-23a, miR-34c, miR-628-3p, miR-133, miR-137, miR-204, miR-205, miR-338-3p, miR-375, miR-433	<i>Runx2</i>	[58,107,108, 110,111,112,114]
	miR-129-5p	<i>Stat1</i>	[118]
	miR-34b, miR-34c	<i>Satb2</i>	[113]

Table 2

Candidate miRNAs hypothetically implicated in the regulation of neural crest development

Candidate mRNAs were classified according to their role in neural crest development as described in [127•]. microRNAs targeting the GRN genes were identified using the miRanda algorithm at microrna.org [153]. Note that *Dlx5*, *Msx1* and *Msx2* have been implicated both as neural plate border specifiers (NPBS) and later during osteoprogenitor differentiation. miRNAs regulating both *FoxD3* and *Sox10* are in blue.

NPBS/osteo	NPBS		Transitional NPBS		Neural Crest Specifiers (NCS)				NCS/Migration		Migratory Crest	
Dlx5	Zic1	Zic3	Pax7	Tfap2a	Foxd3	Snail	Snat2	Sox9	Myb	Twist1	Ets1	Ebf1
miR-124	miR-17	miR-33	miR-1	miR-15a	miR-101a	let-7a (2)	miR-1	miR-30a	miR-15a (2)	miR-25	miR-1 (2)	miR-21
miR-129-5p	miR-20a	miR-103	miR-7a	miR-15b	miR-101b	let-7b (2)	miR-9 (2)	miR-30b	miR-15b (2)	miR-31	miR-33	miR-124
miR-141	miR-20b	miR-107	miR-7b	miR-195	miR-129-5p	let-7c (2)	miR-23a	miR-30c	miR-16 (2)	miR-32	miR-124	miR-186 (2)
miR-200a	miR-23a	miR-1192 (3)	miR-16	miR-497	miR-150	let-7d (2)	miR-23b	miR-30d	miR-96	miR-33	miR-125a-5p	miR-299
miR-203	miR-23b	miR-129-5p (3)	miR-18a			let-7e (2)	miR-30a	miR-30e	miR-101a	miR-92a	miR-125b-5p	miR-326
miR-296-3p	miR-31	miR-136	miR-18b	Tfap 2c		let-7f (2)	miR-30b	miR-101a	miR-101b	miR-92b	miR-129-5p	miR-328
miR-376b	miR-93	miR-144	miR-22	miR-10a		let-7g (2)	miR-30c	miR-101b (2)	miR-103	miR-127	miR-135a	miR-330
miR-543	miR-106a	miR-154	miR-23a	miR-10b		let-7i (2)	miR-30d	miR-1192	miR-107	miR-132	miR-135b	miR-340-5p
miR-590-3p	miR-106b	miR-155 (2)	miR-23b	miR-22	miR-186	miR-30a	miR-30e	miR-124	miR-1192 (2)	miR-137	miR-139-5p	miR-431
Msx1	miR-1192 (2)	miR-181a	miR-96	miR-136	miR-216a	miR-30b	miR-33	miR-125a-3p	miR-124	miR-181a	miR-144	miR-590-5p
	miR-214	miR-181b	miR-129-5p	miR-137	miR-216b	miR-30c	miR-103	miR-128	miR-128	miR-181b	miR-146a	miR-592
	miR-217	miR-181c	miR-129-5p	miR-141		miR-30d	miR-107	miR-129-5p (2)	miR-130a (2)	miR-181c	miR-146b	Rarg
	miR-29b	miR-320 (2)	miR-181d	miR-153	miR-340-5p	miR-30e	miR-122	miR-144 (2)	miR-130b (2)	miR-181d	miR-148a	
miR-29c	miR-328	miR-183	miR-133b	miR-186	miR-374	miR-34a	miR-124 (2)	miR-145	miR-134	miR-182	miR-148b	
miR-101a	miR-381	miR-186 (2)	miR-134	miR-200a	miR-381 (2)	miR-34b-5p	miR-129-5p	miR-155	miR-136	miR-18a	miR-23b	
miR-101b	miR-485	miR-190	miR-143	miR-200b	miR-410	miR-34c	miR-141	miR-184	miR-144	miR-18b	miR-155	miR-31
miR-144	miR-487b	miR-190b	miR-150	miR-203		miR-98 (2)	miR-142-3p	miR-186 (3)	miR-150 (2)	miR-205	miR-181a	miR-148a
miR-216b	miR-494	miR-191	miR-183 (2)	miR-204	miR-486	miR-153	miR-181a	miR-335-5p	miR-153	miR-212	miR-181b	miR-148b
miR-340-5p	miR-495	miR-193	miR-195	miR-211	miR-488	miR-200b	miR-181b	miR-342-3p	miR-155 (2)	miR-326	miR-181c	miR-152
miR-374	miR-543	miR-193b	miR-206	miR-214		miR-340-5p (2)	miR-181c	miR-381	miR-181a (2)	miR-330	miR-181d	miR-183
miR-376c	miR-761	miR-200b (2)	miR-223	miR-224 (2)	miR-543	miR-381	miR-181d	miR-384-5p	miR-181b (2)	miR-361	miR-185	miR-485
miR-410		miR-200c (2)	miR-320	miR-290-5p	miR-590-3p	miR-384-5p	miR-200a	miR-425	miR-181c (2)	miR-363	miR-193	
miR-494	Gbx2	miR-205	miR-326	miR-292-5p	miR-592	miR-410 (2)	miR-200b (2)	miR-489	miR-181d (2)	miR-367	miR-193b	
miR-495	miR-24	miR-217	miR-330	miR-329	miR-876-5p	miR-429	miR-200c (2)	miR-495 (2)	miR-182	miR-381	miR-199a-5p	

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NPBS/osteo	NPBS		Transitional NPBS		Neural Crest Specifiers (NCS)				NCS/Migration		Migratory Crest	
	Zic1	Zic3	Pax7	Tfap2a	Foxd3	Snail	Snai2	Sox9	Myb	Twist1	Ets1	Ebf1
miR-590-5p	miR-141	miR-219	miR-335-5p	miR-346		miR-449a	miR-203	miR-590-3p	miR-195 (2)	miR-425	miR-200b (2)	
miR-1192	miR-185	miR-290-5p	miR-410	miR-370	Sox5	miR-449b	miR-206		miR-200b (2)	miR-491	miR-200c (2)	
Msx2	miR-200a	miR-292-5p	miR-431	miR-377	miR-21	miR-449c	miR-216b	Mye	miR-200c (2)	miR-543	miR-206 (2)	
	miR-203	miR-320 (2)	miR-485	miR-421	miR-96	miR-875-5p	miR-217	miR-34a	miR-203 (2)	miR-590-3p	miR-208a	
miR-1 (2)	miR-204	miR-340-5p	miR-488	miR-448	miR-129-5p		miR-218	miR-34b-5p	miR-208a		miR-208b	
miR-125a-5p	miR-211	miR-374 (2)	miR-490	miR-491	miR-132(2)	Sox10	miR-223	miR-34c	miR-208b	Twist2	miR-216b	
miR-125b-5p	miR-219	miR-376b	miR-495	miR-590-3p (2)	miR-181a	miR-125a-3p	miR-290-5p	miR-135a	miR-216b	miR-31	miR-218	
miR-135a	miR-221	miR-383	miR-539	miR-761	miR-181b	miR-154	miR-292-5p	miR-135b	miR-291a-3p	miR-124	miR-221 (2)	
miR-135b	miR-222	miR-410	miR-653		miR-181c		miR-340-5p (2)	miR-186	miR-294	miR-132	miR-222 (2)	
miR-186	miR-296-3p	miR-421	miR-1192		miR-181d		miR-374	miR-340-5p	miR-295	miR-133a	miR-299	
miR-204	miR-374	miR-429 (2)			miR-182		miR-376c (2)	miR-381	miR-296-3p	miR-133b	miR-320	
miR-206 (2)	miR-381	miR-451			miR-208a		miR-384-5p	miR-449a	miR-301a (2)	miR-138	miR-330	
miR-211	miR-494	miR-485			miR-208b		miR-410	miR-449b	miR-301b (2)	miR-143	miR-326	
miR-216b	miR-495	miR-495 (2)			miR-212 (2)	miR-383	miR-429 (2)	miR-449c	miR-302a	miR-145	miR-338-3p (2)	
miR-217	miR-592	miR-543			miR-218		miR-488		miR-302b	miR-212	miR-340-5p	
miR-340-5p (2)	miR-882	miR-551b			miR-223	miR-485	miR-489		miR-302d	miR-224 (2)	miR-351	
miR-351	miR-1192	miR-590-3p (2)			miR-328		miR-539		miR-322 (2)	miR-495	miR-365	
miR-376b					miR-485	miR-542-3p	miR-543 (2)		miR-338-3p	miR-490	miR-377	
miR-381					miR-495		miR-590-3p (2)		miR-340-5p (2)		miR-378	
miR-410 (2)					miR-499		miR-592		miR-374 (2)		miR-381	
miR-544					miR-590-3p				miR-381		miR-382	
miR-590-3p					miR-590-5p				miR-410 (2)		miR-410 (2)	
miR-653					miR-615-3p				miR-425		miR-421	
miR-758					miR-876-5p				miR-429 (2)		miR-429 (2)	
					miR-1192				miR-433		miR-488	
									miR-489		miR-491	
									miR-495 (2)		miR-495 (2)	
									miR-497 (2)		miR-876-5p	
									miR-499		miR-882	
									miR-503		miR-1192 (2)	

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NPBS/osteo		NPBS		Transitional NPBS		Neural Crest Specifiers (NCS)				NCS/Migration		Migratory Crest	
<i>Dlx5</i>		<i>Zic1</i>	<i>Zic3</i>	<i>Pax7</i>	<i>Tfap2a</i>	<i>Foxd3</i>	<i>Snaill</i>	<i>Snai2</i>	<i>Sox9</i>	<i>Myb</i>	<i>Twist1</i>	<i>Ets1</i>	<i>Ebf1</i>
miRNAs sharing 10 mRNAs										miR-543 (2)			
miRNAs sharing 9 mRNAs										miR-590-3p (2)			
miRNAs sharing 8 mRNAs										miR-592 (2)			
miRNAs sharing 7 mRNAs										miR-721 (2)			
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NPBS/osteo	NPBS	Transitional NPBS		Neural Crest Specifiers (NCS)				NCS/migration		Migratory Crest			
<i>Dlx5</i>	<i>Zic1</i>	<i>Zic3</i>	<i>Pax7</i>	<i>Tfap2a</i>	<i>Foxd3</i>	<i>Snaill</i>	<i>Snai2</i>	<i>Sox9</i>	<i>Myb</i>	<i>Twist1</i>	<i>Ets1</i>	<i>Ebf1</i>	
miR-124	miR-17	miR-33	miR-1	miR-15a	miR-101a	let-7a (2)	miR-1	miR-30a	miR-15a (2)	miR-25	miR-1 (2)	miR-21	
miR-129-5p	miR-20a	miR-103	miR-7a	miR-15b	miR-101b	let-7b (2)	miR-9 (2)	miR-30b	miR-15b (2)	miR-31	miR-33	miR-124	
miR-141	miR-20b	miR-107	miR-7b	miR-195	miR-129-5p	let-7c (2)	miR-23a	miR-30c	miR-16 (2)	miR-32	miR-124	miR-186 (2)	
miR-200a	miR-23a	miR-1192 (3)	miR-16	miR-497	miR-150	let-7d (2)	miR-23b	miR-30d	miR-96	miR-33	miR-125a-5p	miR-299	
miR-203	miR-23b	miR-129-5p (3)	miR-18a		miR-181a	let-7e (2)	miR-30a	miR-30e	miR-101a	miR-92a	miR-125b-5p	miR-326	
miR-296-3p	miR-31	miR-136	miR-18b		miR-181b	let-7f (2)	miR-30b	miR-101a	miR-101b	miR-92b	miR-129-5p	miR-328	
miR-376b	miR-93	miR-144	miR-22	miR-10a	miR-181c	let-7g (2)	miR-30c	miR-101b (2)	miR-103	miR-127	miR-135a	miR-330	
miR-543	miR-106a	miR-154	miR-23a	miR-10b	miR-181d	let-7i (2)	miR-30d	miR-1192	miR-107	miR-132	miR-135b	miR-340-5p	
miR-590-3p	miR-106b	miR-155 (2)	miR-23b	miR-22	miR-186	miR-30a	miR-30e	miR-124	miR-1192 (2)	miR-137	miR-139-5p	miR-431	
	miR-1192 (2)	miR-181a	miR-96	miR-136	miR-216a	miR-30b	miR-33	miR-125a-3p	miR-124	miR-181a	miR-144	miR-590-5p	
	miR-214	miR-181b	miR-125a-3p	miR-137	miR-216b	miR-30c	miR-103	miR-128	miR-128	miR-181b	miR-146a	miR-592	
Mx1	miR-217	miR-181c	miR-129-5p	miR-141	miR-320	miR-30d	miR-107	miR-129-5p (2)	miR-130a (2)	miR-181c	miR-146b		
miR-21	miR-320 (2)	miR-181d	miR-133a	miR-153	miR-340-5p	miR-30e	miR-122	miR-144 (2)	miR-130b (2)	miR-181d	miR-148a		
miR-29b	miR-328	miR-183	miR-133b	miR-186	miR-374	miR-34a	miR-124 (2)	miR-145	miR-134	miR-182	miR-148b		
miR-29c	miR-381	miR-186 (2)	miR-134	miR-200a	miR-381 (2)	miR-34b-5p	miR-129-5p	miR-155	miR-136	miR-18a	miR-152		
miR-101a	miR-485	miR-190	miR-143	miR-200b	miR-410	miR-34c	miR-141	miR-184	miR-144	miR-18b	miR-155		
miR-101b	miR-487b	miR-190b	miR-150	miR-203	miR-425	miR-98 (2)	miR-142-3p	miR-186 (3)	miR-150 (2)	miR-205	miR-181a		
miR-144	miR-494	miR-191	miR-183 (2)	miR-204	miR-486	miR-153	miR-181a	miR-335-5p	miR-153	miR-212	miR-181b		
miR-216b	miR-495	miR-193	miR-195	miR-211	miR-488	miR-200b	miR-181b	miR-342-3p	miR-155 (2)	miR-326	miR-181c		
miR-340-5p	miR-543	miR-193b	miR-206	miR-214	miR-489	miR-340-5p (2)	miR-181c	miR-381	miR-181a (2)	miR-330	miR-181d		
miR-374	miR-761	miR-200b (2)	miR-223	miR-224 (2)	miR-543	miR-381	miR-181d	miR-384-5p	miR-181b (2)	miR-361	miR-185		
miR-376c			miR-320	miR-290-5p	miR-590-3p	miR-384-5p	miR-200a	miR-425	miR-181c (2)	miR-363	miR-193		
miR-410	Gbx2	miR-205	miR-326	miR-292-5p	miR-592	miR-410 (2)	miR-200b (2)	miR-489	miR-181d (2)	miR-367	miR-193b		
miR-494	miR-24	miR-217	miR-330	miR-329	miR-876-5p	miR-429	miR-200c (2)	miR-495 (2)	miR-182	miR-381	miR-199a-5p		

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NPBS/osteo	NPBS		Transitional NPBS		Neural Crest Specifiers (NCS)					NCS/Migration		Migratory Crest
	Zic1	Zic3	Pax7	Tfap2a	Foxd3	Snail	Snai2	Sox9	Myb	Twist1	Ets1	Ebf1
Dlx5	miR-590-5p	miR-219	miR-335-5p	miR-346		miR-449a	miR-203	miR-590-3p	miR-195 (2)	miR-425	miR-200b (2)	
	miR-1192	miR-290-5p	miR-410	miR-370	Sox5	miR-449b	miR-206		miR-200b (2)	miR-491	miR-200c (2)	
Msx2		miR-292-5p	miR-431	miR-377	miR-21	miR-449c	miR-216b	Mye	miR-200c (2)	miR-543	miR-206 (2)	
		miR-320 (2)	miR-485	miR-421	miR-96	miR-875-5p	miR-217	miR-34a	miR-203 (2)	miR-590-3p	miR-208a	
miR-1 (2)	miR-204	miR-340-5p	miR-488	miR-448	miR-129-5p		miR-218	miR-34b-5p	miR-208a		miR-208b	
miR-125a-5p	miR-211	miR-374 (2)	miR-490	miR-491	miR-132(2)	Sox10	miR-223	miR-34c	miR-208b	Twist2	miR-216b	
miR-125b-5p	miR-219	miR-376b	miR-495	miR-590-3p (2)	miR-181a	miR-125a-3p	miR-290-5p	miR-135a	miR-216b	miR-31	miR-218	
miR-135a	miR-221	miR-383	miR-539	miR-761	miR-181b	miR-154	miR-292-5p	miR-135b	miR-291a-3p	miR-124	miR-221 (2)	
miR-135b	miR-222	miR-410	miR-653		miR-181c	miR-181a	miR-340-5p (2)	miR-186	miR-294	miR-132	miR-222 (2)	
miR-186	miR-296-3p	miR-421	miR-1192		miR-181d	miR-181b	miR-374	miR-340-5p	miR-295	miR-133a	miR-299	
miR-204	miR-374	miR-429 (2)			miR-182	miR-181c	miR-376c (2)	miR-381	miR-296-3p	miR-133b	miR-320	
miR-206 (2)	miR-381	miR-451			miR-208a	miR-181d	miR-384-5p	miR-449a	miR-301a (2)	miR-138	miR-330	
miR-211	miR-494	miR-485			miR-208b	miR-320 (2)	miR-410	miR-449b	miR-301b (2)	miR-143	miR-326	
miR-216b	miR-495	miR-495 (2)			miR-212 (2)	miR-383	miR-429 (2)	miR-449c	miR-302a	miR-145	miR-338-3p (2)	
miR-217	miR-592	miR-543			miR-218	miR-425	miR-488		miR-302b	miR-212	miR-340-5p	
miR-340-5p(2)	miR-882	miR-551b			miR-223	miR-485	miR-489		miR-302d	miR-224 (2)	miR-351	
miR-351	miR-1192	miR-590-3p (2)			miR-328	miR-489	miR-539		miR-322 (2)	miR-495	miR-365	
miR-376b					miR-485	miR-542-3p	miR-543 (2)		miR-338-3p	miR-490	miR-377	
miR-381					miR-495		miR-590-3p (2)		miR-340-5p (2)		miR-378	
miR-410 (2)					miR-499		miR-592		miR-374 (2)		miR-381	
miR-544					miR-590-3p				miR-381		miR-382	
miR-590-3p					miR-590-5p				miR-410 (2)		miR-410 (2)	
miR-653					miR-615-3p				miR-425		miR-421	
miR-758					miR-876-5p				miR-429 (2)		miR-429 (2)	
					miR-1192				miR-433		miR-488	
									miR-489		miR-491	
									miR-495 (2)		miR-495 (2)	
									miR-497 (2)		miR-876-5p	
									miR-499		miR-882	
									miR-503		miR-1192 (2)	

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NPBS/osteo		NPBS		Transitional NPBS		Neural Crest Specifiers (NCS)				NCS/Migration		Migratory Crest
<i>Dlx5</i>	<i>Zic1</i>	<i>Zic3</i>	<i>Pax7</i>	<i>Tfap2a</i>	<i>Foxd3</i>	<i>Snail</i>	<i>Snai2</i>	<i>Sox9</i>	<i>Myb</i>	<i>Twist1</i>	<i>Ets1</i>	<i>Ebf1</i>
									miR-543 (2) miR-590-3p (2) miR-592 (2) miR-721 (2)			