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microRNA function in Craniofacial Bone Formation, Regeneration and Repair

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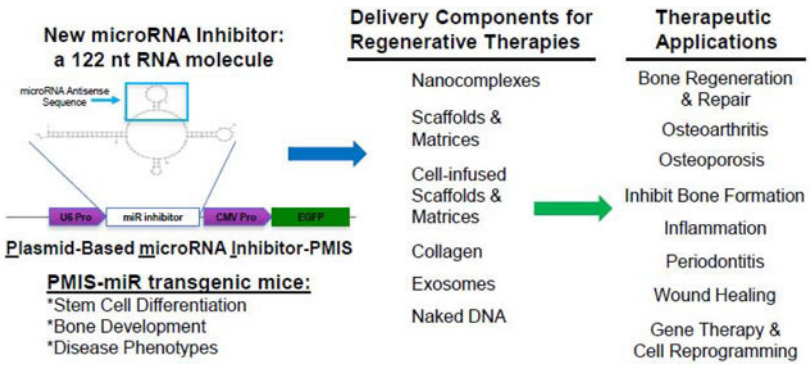
Abstract

Bone formation in the craniofacial complex is regulated by cranial neural crest (CNC) and mesoderm-derived cells. Different elements of the developing skull, face, mandible, maxilla (jaws) and nasal bones are regulated by an array of transcription factors, signaling molecules and microRNAs (miRs). miRs are molecular modulators of these factors and act to restrict their expression in a temporal-spatial mechanism. miRs control the different genetic pathways that form the craniofacial complex. By understanding how miRs function *in vivo* during development they can be adapted to regenerate and repair craniofacial genetic anomalies as well as bone diseases and defects due to traumatic injuries. This review will highlight some of the new miR technologies and functions that form new bone or inhibit bone regeneration.

Graphical Abstract



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Keywords

microRNA therapeutic; microRNA Inhibitor system (PMIS); Bone repair; Bone regeneration; microRNA mouse models; Bone development

INTRODUCTION

Bone formation during early development can be by two processes;1) intramembranous bone formation or 2) endochondral ossification [1–3]. Cranial neural crest (CNC) cells migrate from the neural tube into the anterior region of the skull to form some of the bones and cartilages of the face and anterior skull. The cartilages and bones in the posterior region of the skull are derived from prechordal mesoderm cells. These cells condense and either differentiate into osteoblasts (which directly form bone by intramembranous bone formation or differentiate into chondrocytes (which form cartilage and bones by endochondral bone formation). Thus, CNC-derived cells involved in intramembranous bone formation form the anterior region of the skull, face, mandible and maxilla. The mesoderm-derived cells form the posterior regions of the skull by endochondral ossification [3–7].

The molecular mechanisms that control craniofacial growth that gives rise to the different vertebrate head sizes and morphology include signaling and growth factors [3,8–19]. In particular, the Wnt, Fgf, Bmp, Shh, and Tgf-β signaling pathways control the early patterning and growth of the craniofacial skeleton by regulating the migration, proliferation, differentiation and transformation of cells derived from the mesoderm and cranial neural crest [20–29]. These factors and pathways interact and intersect to control development of the brain and skull [7,9,10,13,14,30–32]. Furthermore, early signals by these pathways especially the Wnt pathway emanating from the pharyngeal endoderm and epithelium, appear to regulate patterning of the developing skeleton [19,33–35]. Tissue-tissue interactions that give rise to cell fate decisions are fundamental to the development of head structures, especially for the patterning and morphogenesis of craniofacial organs including teeth [36–38]. These early developmental cues drive the morphogenesis and patterning of perinatal craniofacial tissues. Hippo signaling has also been shown to regulate prenatal and post-natal craniofacial development and growth [39,40]. Recently, microRNAs (miRs) have emerged as new modulators of craniofacial bone development and maintenance.

miRs are short non-coding RNA molecules approximately 22 nucleotides long. miRs bind to complementary targets on the 3’ untranslated region (UTR) of messenger RNAs (mRNAs), attenuating mRNA translation via either mRNA strand degradation or sequestration [41]. Through this mechanism, miRs play a broad role in the regulation of mRNA translation and have been demonstrated to play a significant part in an array of biological processes [42–44].

As we understand the mechanisms and functions of miRs they act as global modulators of multiple gene expression pathways. It is not simply one miR regulates one gene, network and/or process. In our research we find that miRs are regulators of one or more genetic pathways controlling specific tissue, organ or cellular processes. The temporal and spatial levels of miR expression change either moderately or dramatically depending on the tissue during development. Almost all cells will express low levels of specific miRs, but there

appears to be a threshold of miR expression for them to be effective modulators of cellular processes. The complexity of miR biogenesis, processing and degradation during developmental time points mediates the fine-tuning of gene expression.

There are many cell-based studies on the role of miRs in osteogenesis that have identified several genetic pathways for the differentiation of cells into osteoblasts and osteocytes. We have found that *in vitro* based miR expression and profiling differ from the *in vivo* state. The process of using osteogenic differentiation media in different types of cell cultures can result in miR expression profiles that are different from *in vivo* bone development. Many cell types can be manipulated to express miRs using osteogenic media and many miRs can induce the bone-forming program in cells under osteogenic conditions. This review will focus on miR mouse and rat models for craniofacial bone development, regeneration and repair.

I. NEW TECHNOLOGY TO INHIBIT MICRORNAS *IN VIVO* AND AS THERAPEUTIC REAGENTS

Plasmid-Based microRNA Inhibitor System (PMIS)

The problems with current miR inhibition methods are that chemically modified anti-miR oligonucleotides (AMOs) and locked-nucleic acids (LNAs) bind miRs transiently and inefficiently, do not remain in dividing cells and require repeated large doses of oligos in cells to be effective and they have severe off-target effects. In previous preclinical and clinical trials with LNAs, antagomirs, AMOs and other miR inhibitors they were toxic, non-specific and caused multiple adverse effects in animals and humans. The sponges and decoys also suffer from a lack of stability, inefficient binding of the miR, lack of specificity and require toxic delivery systems.

To circumvent these major problems, we developed a new method of miR inhibition, the Plasmid-Based miRNA Inhibition System (PMIS), to allow for the simultaneous knockdown of homologous miR families *in vitro* and *in vivo* [45–47]. The PMIS inhibitor is composed of native, unmodified nucleic acids that enables the development of stably expressing cells (lentivirus) and animal models for the study of genome-wide miR family inhibition to identify miR targets and cellular processes. The PMIS can be used to knockdown miRs during embryonic development to determine their effect on stem cells, cell proliferation and differentiation as well as developmental processes. The PMIS system represents a major paradigm shift in miR biotechnology that allows researchers to finally dissect the role of identical miRs expressed in clusters and on multiple chromosomes. The PMIS can distinguish between and differentially inhibit miRs with only one nucleotide change in their seed sequence. The unique structure of the PMIS-miR complex is bound by factors of the RNA-Induced Silencing Complex (RISC), making it very stable, efficient, with a high specificity and affinity for specific miRs and is not toxic in animals and cells (Fig. 1)[45]. The PMIS transcript is expressed using the U6 Polymerase III promoter to control PMIS-miR transcript expression levels in the cell. We found that using the CMV Polymerase II promoter that PMIS-miR expression levels were exceedingly high and interfered with cellular processes. The U6 promoter expresses PMIS-miR constructs at lower levels with increased functionality. Furthermore, we engineered the PMIS miR inhibitor to bind to the

RISC proteins and it is extremely stable in the cell. This miR inhibitor works in transgenic animals (*in vivo*) as well as xenograft animal models without adverse side effects, it has great promise as a therapeutic molecule in clinical applications including bone repair and regeneration.

We are currently working on inducible PMIS-miR constructs, but we find that the specificity of the PMIS-miR is very efficient and while it is expressed in every cell, not every cell expresses the targeted miR, so only those cells/tissues with high levels of targeted miR expression are affected during development. Furthermore, not every cell/tissue expresses the same genes at high levels, thus we see differential gene expression with and without inhibiting specific miRs. Clearly, we observe other cell/tissue developmental defects in our PMIS-miR mice depending on the miR that is inhibited and specific genes targeted by the miR. It has been reported many times that conditional ablation of a miR cluster for tissue-specific knockouts have no effect on development. This is because the tissue specific Cre knocks out the miR too late in development to observe a defect. The specificity of the PMIS system during embryonic development provides a new mechanism to study miR function, especially at time points that miRs play critical roles in developmental processes.

II. FUNCTIONS OF MICRORNAS IN CRANIOFACIAL BONE FORMATION AND DEVELOPMENT

The tissue-specific *Dicer, DGCR8* and *Ago2* knockouts in mice have shown miRs are involved in bone development [48–54] and the developing tooth [48,55,56]. miRs are post-transcriptional regulators that repress gene expression by regulating the translation of mRNA and promoting the degradation of targeted mRNAs and are thought to regulate tooth and bone development by repressing the transcription factors and signaling factors involved in these processes [57].

Murine models for miR-17–92 regulation of bone formation

miRs come in a variety of genomic contexts, both intragenic and intergenic [58,59], and can be isolated or grouped into polycistronic clusters, as is the case with the widely-studied miR cluster, *miR-17–92* [60,61]. *miR-17–92* is a cluster of six highly conserved miRs from four different families located on chromosome 13 in humans and chromosome 14 in mice [62]. However, identical miRs to the *miR-17–92* cluster are also expressed on chromosomes X and 7 in humans and chromosomes X and 5 in mice [45]. Recent studies have implicated the *miR-17–92* cluster in the development of orofacial and craniofacial defects [63,64]. We knocked down all three clusters on these chromosomes using *PMIS-miR-17–18, PMIS-miR-19–92* and *PMIS-miR-17–92* mice to dissect the functions of identical miRs within the *miR-17–92* clusters and found each contributed to craniofacial bone development in different aspects of growth [46]. Craniofacial defects were identified in all of the PMIS transgenic mice at three weeks of age by High-Resolution X-Ray Microtomograph scans (uCT scans). Specific measurements from the uCT scans identified defects in total cranial length (rhinion to opisthion, rh-op), neurocranial length (nasion to opisthion, na-op), nasal bone length (rhinion to nasion, rh-na), palate length (prosthion to anterior nasal spine, pr-pns), frontal bone length (nasion to bregma, na-br), and parietal bone length (bregma to

lambda, br-l). We report defects in the width and length of cranial structures in all PMIS mice compared to WT (Fig. 2) [46]. Other defects identified included suture, mandibular condyle growth defects and microcephaly [46].

Murine models for miR-26b regulation of bone formation

Recent studies have reported that *miR-26b* functions as an odontogenic regulator [65,66]. A *miR-26b* transgenic over-expression (OE) mouse is similar to the *Lef-1* general knockout mouse as *miR-26b* regulates *Lef-1* expression [65]. These mice lack teeth, including molars and incisors, and full body uCT images reveal skeletal and bone defects in these mice. The cranial base and cranial breath measurements are essentially identical in the *miR-26b OE* and WT mice. However, the *miR-26b OE* mice have a shorter nasal bone, snout, frontal bone, parietal bone, cranial breath, and cranial base length compared to WT mice. The cranial base angle and ramus height are also decreased in the *miR-26b OE* mice compared to WT mice. The *miR-26B OE* mice have a decreased mandibular length compared to WT mice [65]. It appears that *miR-26b* targets *Lef-1* and Wnt signaling to control bone growth and formation.

Murine models for miR-200 regulation of bone formation

The *miR-200c/141* knockout mice have defects in mandibular bone formation (a decrease in alveolar bone) linked to a *Noggin* regulatory pathway and *Bmper* expression [66]. *miR-200c* directly targets and inhibits *Noggin* expression (a Bmp inhibitor) resulting in an increase in Bmp activity and cell differentiation [66]. However, the lack of *miR-200c* increases *Noggin* expression and decreases Bmp activity resulting in a bone developmental defect in these mice. The *PMIS-miR-200c* mice also have a decrease in ossification of craniofacial bones, maxilla/palatine bones and facial bone [47]. The molecular mechanism of *miR-200c* in osteogenic differentiation includes a *Sox2*-mediated Wnt signaling and *Klf4* pathway. Conversely, the inhibition of *miR-200a* (*PMIS-miR-200a*) in mice results in an increase in bone density (Fig. 3). Through multiple validation experiments and gene expression analyses we have identified multiple *miR-200* regulated pathways (Fig. 3). These *in vivo* results demonstrate the effect the *miR-200* family has on gene regulatory pathways, gene networks and cellular processes.

Murine models for miR-23–24-27 regulation of bone formation

The *miR-23–24-27* cluster has been shown to regulate osteoblast differentiation through several genetic pathways. A report using a novel *miR-23a* cluster knockdown mouse model demonstrated a role for this cluster in maintaining stage-specific *HoxA* factor expression during osteogenesis [67]. *HoxA5* and *HoxA11* are targets of the *miR-23a* cluster and these *Hox* genes regulate bone-specific gene expression through chromatin modifications. The *miR-23a* cluster knockdown increased cortical and trabecular bone mass [67]. These researchers used an inducible anti-*miR-23a CI* knock-in mouse to show regulation of the PRC2 complex and repressive H3K27me3 deposition contrasting with BAF-linked activating H3K27ac chromatin modifications promote osteoblast differentiation by this miR cluster [67]. Other researchers have used miR decoys for *miR-23a*, *miR-27a* and *miR-24–2* to knockdown these miRs in mice to show regulation of osteocyte differentiation [68]. The knockdown of *miR-23a* or *miR-27a* decreased osteocytes but knockdown of *miR-24–2* had

no effect. In contrast over-expression of the *miR-23a* cluster in mice demonstrated low bone mass associated with a decrease in osteoblasts but an increase in osteocytes [68]. It was suggested that the *miR-23a* cluster regulates osteocyte differentiation by modulating the TGF-ß signaling pathway.

Murine models for miR-34 regulation of bone formation

A *miR-34c* over-expression (OE) mouse demonstrated that *miR-34c* can regulate bone remodeling by affecting both osteoblasts and osteoclasts leading to increased bone resorption [69]. The defect in osteoblast differentiation by *miR-34c* OE was due to targeting *Satb2* and *Runx2*. However, *miR-34c* also targets Notch signaling in osteoblasts and *miR-34c* regulates bone homeostasis by regulating multiple targets in osteoblasts and osteoclasts [69].

Murine models for miR-214 regulation of bone formation

miR-214 OE under the control of the osteoclast specific *Acp5* promoter in mice demonstrated a role for this miR in bone formation. The increase in *miR-214* decreased *Pten* levels, increased osteoclast activity and reduced bone mineral density [70]. The expression of *miR-214* was upregulated in the process of m-CSF and RANKL induced osteoclastogenesis, thus inhibition of *miR-214* levels reduced osteoclastogenesis.

Murine models for miR-335 regulation of bone formation

A transgenic mouse over-expressing *miR-335* using the osterix promoter revealed higher bone mass and increased bone formation in the transgenic mouse compared to wildtype [71]. Mechanistically in these mice *Runx2* and *Osx* were upregulated, while the Wnt antagonist Dickkopf-1 was down-regulated. The researchers suggest that application of *miR-335* modified bone marrow stem cells could be used in craniofacial bone regeneration [71].

A common theme in these critical mouse models is that several miRs can regulate identical and intersecting pathways required for bone formation and bone remodeling. The use of animal models for studying miR regulation of bone formation is critical to understanding how miRs affect osteogenesis. We follow a validation process to determine miR targets in mouse tissues and cell-based assays (Fig. 4). The role of miRs in bone homeostasis will be discussed in the following section.

III. THE ROLES OF MIRS IN INFLAMMATION, BONE MAINTENANCE, REGENERATION AND REPAIR

miRs in bone homeostasis: a balance of formation and resorption

miRs are actively involved in the bone homeostasis by regulating the balance of osteogenesis and osteoclastogenesis. This regulation has been investigated using different cell lines and *in vivo* models, including bone homeostasis under estrogen deficiency, mechanical loading and corticosteroid stimulation. miRs regulate bone loss by directly and indirectly modulating the inflammation that subsequently activates osteoclastogenesis through the RANKL-RANK-OPG system. miRs may directly regulate the RANKL/OPG balance to adjust

osteoclastogenesis. The upregulation and activation of RANKL accelerates the osteoclastic differentiation and bone resorption, while downregulation of RANKL diminishes osteoclastogenesis. For example, *miR-29a* and *miR-146a* regulate the RANKL-RANK-OPG system by directly targeting *RANKL* and *Smad4*, resulting in the inhibition of bone resorption in ovariectomized (OVX) mice [72,73]. Inhibition of these miRs are shown to relatively improve bone mass by overcoming osteoclastogenesis [74]. miRs also modulate osteoblastogenesis by directly targeting osteogenic signal pathways to inhibit bone formation. *miR-139*, *−145, −26a, −451*, and *−100* have been found to target osteogenic signals, including *Wnt/β-catenin*, *Notch*, *BMP/Smads*, and *FGF-21*, which accelerating osteoporosis in the OVX murine models [75–81]. Similarly, some miRs upregulated the activities of *Wnt-β/catenin* and *Bmp/Smads* and promoted osteogenic differentiation by targeting the inhibitory regulator of these pathways. For examples, *miR-199a* [82,83], *miR-219a* [84], and *miR-208a* [85] have been reported to improve osteogenesis and bone formation by directly targeting an adipogenesis promoter, *TET2* (Tet Methylcytosine Dioxygenase 2), *Rorβ* (retinoic acid-related orphan receptor), a transcriptional enhancer of inhibitor HBP1 of the Wnt pathway, and a Bmp ligand Activin A receptor type I (*ACVR1*). In addition, *miR-23b* [86] and *miR-103a* [87] directly target the osteogenic transcription factor, *Runx2*, to inhibit bone formation, which accelerates osteoporosis in the OVX model.

Anti-inflammatory Therapeutic Potential of miRs in Periodontitis and Bone Loss

The exaggerative inflammatory response plays key roles in the pathogenesis of periodontitis, including activated alveolar bone loss and inhibited bone formation. Thus, by manipulating the miRs that actively participate in periodontitis, an epigenetic approach is an innovative therapeutic tool for periodontitis by fine-tuning the inflammatory response. Although numerous miRs are involved in the pathogenic progress of periodontitis, and both *miR-146a* and *miR-200c* have been reported to negatively feedback to *IL-6*. *miR-146a* also negatively affects *IL-1β* and *TNF-a*, and *Hey2* expression [88]. However, *miR-146a* has been reported to be upregulated in gingival tissue of periodontitis and in gingival cells under LPS stimulation, which activates inflammation [89]. In the mouse model of *P. gingivalis* (PG) infection-induced experimental periodontitis, injection of a *miR-146a* mimic exhibited protective function on periodontitis associated bone loss [90]. In comparison to *miR-146a*, although it has smaller variation in periodontitis patients, *miR-200c* was found to be significantly down-regulated in the gingival tissues of periodontitis patients [91]. *miR-200c* reduced *IL-8* expression by targeting the inhibitor of nuclear factor kappa B kinase subunit beta targeting (IKBKB) in the NF-kB signal pathway [92]. Our previous studies have confirmed that *miR-200c* directly targets the 3'UTRs of *IL-6*, *IL-8* and *CCL-5* [93]. Reporter gene analysis showed that *miR-200c* targets the 3'UTR of the interferon related developmental regulator1 (*Ifrd1*). We have observed that a local application of plasmid DNA encoding *miR-200c* effectively suppressed *IL-6*, *IL-8*, *Ifrd1*, and *NF-kB* in rat gingival tissues. In a rat model of periodontitis induced by PG-LPS, we observed that the local application of *miR-200c* significantly down regulated the proinflammatory cytokines and alveolar bone loss stimulated by of LPS injection.

Therapeutic function of miRs in bone healing and regeneration.

Several approaches have been attempted to explore the therapeutic function of miRs on enhancing bone formation and regeneration. The majority of the reported studies of miR-driven bone regeneration were cell-based. Both mesenchymal stromal cells from bone marrow (BMSCs) and adipose tissues (ADSCs) have served as progenitor cells of bone forming cells. Either mimics and/or antagomir inhibitors of specific miRs were transfected into BMSCs or ADSCs to increase osteogenic differentiation. The mimics of *miR-375* [94,95], *miR-101* [57], miR-450b [96], *miR-135* [97], *miR-218* [98], *miR-335* [99,100], *miR-199a* [83], and *miR-99a* [101] have reported to regenerate bone tissue by targeting the inhibitors or antagonist of osteogenic signaling *BMP*, *IGF,* and *WNT,* including *IGFBP3*, *EZH2* (the enhancer of zeste homolog 2), *Hoxa2*, *DKK1*, *HIF1a*, and *KDM6B*. In addition, the antagomirs or inhibitors of miRs that target osteogenic differentiation also have shown the osteogenic capabilities and bone regeneration after transfection into BMSCs or ADSCs. The antagomirs to *miR-133a* [102], *miR-137* [103,104], and *miR-31* [105,106] improve *Runx2* and *Bmp* signaling in the progenitor cells. The antagomirs of *miR-146a* (74), *miR-21* [107], and *miR-221* [108] increase osteogenic differentiation by rescuing *Smad* signaling. Inhibitors of *miR-138* [109–111] and *miR-124* [112] also demonstrated the enhancement of osteogenic differentiation by regulating *FAK* and *Dlx* signaling. Cells transfected with miR mimics and antagomirs also exhibited the capabilities to regenerate bone tissues at ectopic sites of murine models and restore calvarial bone defects and long bone fractures at murine models.

Section III Summary

miRs	genes/pathways targeted	effect on bone growth/regeneration
miRs-29a, −146a	RANKL-RANK-OPG	inhibition of bone resorption (OVX model)
miRs-139, −145, −26a, −451 and −100	osteogenic signals	increase osteoporosis (OVX model)
miRs-199a, −219a, −208a	osteogenic signals	improve osteogenesis
miRs-23b, −103a	RUNX2	inhibit bone formation (OVX model)
miRs-146a, −200c	inflammation, *IL6, IL-1B, TNF-a*	inhibition of pro-inflammatory cytokines
miR-200c	inflammation, *IL-6, IL-8, CCL-5*	inhibition of pro-inflammatory cytokines
miRs-375, −101, −450b, −135, −218, −335, −199a, −99a	BMP, IGF, WNT	regenerate bone tissue, increase osteogenic differentiation
miRs-133a, −137, −31	*RUNX2, BMP*	bone progenitor cell differentiation
miRs-146a, −21,−221	*SMADs*	osteogenic differentiation
miRs-138, −124	*FAK, DLX*	increase osteogenic differentiation

IV. MICRORNA DELIVERY APPROACHES FOR THERAPEUTIC TREATMENTS

General strategies of miR-based therapeutic approaches

Depending on the expression strategy of the target miR, the miR therapies are divided into two categories: 1) miR activation or gain-of-function therapy which up-regulates exogenous miR expression and 2) miR inhibition or loss-of-function therapy which down-regulates endogenous miR expression [113,114]. Overexpression of the target miR also can be achieved through the delivery of either miR mimics that are double-strand oligonucleotides or miR-expressing viral vectors. The double-stranded miR mimics are designed either to target a single mRNA or a group of multiple miR units target different mRNAs [115,116]. Viral vector-mediated miR over expression enables the sustained generation of miRs, which is especially attractive for regenerative medicine applications. Instead of using a viral vector for over expressing miRs, our group recently reported that plasmid DNA-mediated sustained expression of *miR-200c* was a viable and translational way for craniofacial and periodontal bone regeneration [117–119]. In contrast to the miR activation therapy, the miR inhibition therapy aims to block the endogenous miR. So far, the most straightforward technique is to use anti-miR oligonucleotides and several different methods have been developed to inhibit miR functions. The major limitation of the anti-miR oligonucleotides is their short half-life because of the susceptibility to degradation. Therefore, the oligonucleotide has a transient effect because its concentration decreases quickly with cell division [113]. Our newly developed plasmid-based miR inhibitor system (PMIS) can largely address this challenge by sustained production of miR inhibitor molecules to inhibit miR family members in both cells and mice [120].

Chemically modified anti-miRs

Tremendous efforts have been reported to develop chemical modification techniques that improve the performance of anti-miRs (synthesized oligonucleotides) through enhancing cellular uptake, binding affinity, and nuclease resistance [114]. For example, phosphorothioate linkage is the most commonly used internucleotide modification on synthesized oligonucleotides. This modification delays plasma clearance and enhances cellular uptake through the nonspecific binding to serum albumin and membrane proteins [121,122]. AntagomiRs containing a terminal phosphorothioate linkage, a cholesterol conjugation, and 2’-O-methyl-modified ribose sugars (2-OMe), are the first miR inhibitors reported to work in many different tissues in mammals [123]. By using this technique, some specific antagomiRs have been developed to modulate bone mass through systemic injection methods. One group reported that the blocking of *miR-148a* by *antagomiR-148a* through tail vein injection significantly increased bone formation while suppressing bone resorption in ovariectomized (OVX) mice [124]. Similarly, hind limb-unloading-induced bone loss in mice was partly prevented by injection of *antagomiR-103a* since *miR-103* was a mechanosensitive miR in bone tissue [87]. Moreover, aging-related bone loss was mitigated in rats through the injection of *antagomiR-31a-5p* into the femoral bone marrow cavity [125]. Although antagomiRs have shown encouraging results in several different tissues and animal models, the safety concerns caused by high dose requirements prevent their

applications [113,114]. Additionally, other chemical modification strategies have been reported with promising applications, e.g., peptide nucleic acids (PNAs) [126] and locked nucleic acids (LNAs) [127].

Viral vectors

The high transfection efficiency and sustained gene expression of viral vectors make them the most effective techniques so far for miR delivery both *in vitro* and *in vivo*. Retrovirus, lentivirus, adeno-associated virus (AAV), and baculovirus are the most frequently used viral vectors for gene therapy [128–130]. Retroviruses carrying two copies of the single-strand RNA genome can stably integrate into the host chromosomes, guaranteeing long-term expression of inserted therapeutic genes [131]. However, the inability of transducing nondividing cells and the safety concern caused by genome integration at an undesired location significantly impede the application of retrovirus vectors in regenerative medicine [114]. Lentivirus is a subgroup of retrovirus is a more promising vector for *in vivo* miR delivery because it can not only transfect nondividing cells [130] but also prefer to integrate within introns of active transcriptional units, thereby reducing the potential to cause insertional oncogenesis [132,133]. Therefore, lentivirus-mediated miR delivery has been used to improve bone formation in many reports. For example, lentivirus-mediated *miR-29a* over expression can protect rats against glucocorticoid-Induced bone loss and fragility [134]. Lentivirus- mediated *miR- 26a* over expression in bone marrow mesenchymal stem cells (BMSCs) significantly improved murine cranial bone regeneration [135]. Additionally, lentivirus-mediated *anti-miR-31* to inhibit the expression of *miR-31* in BMSCs also can repair critical-sized calvarial defects in rats [105]. AAV vectors are emerging as the most promising gene therapy tool for clinical applications because they have several advantages: 1) small size (only ~4.7kb DNA genome; 2) infection of both dividing and nondividing cells; 3) nonpathogenicity in humans, and; 4) relatively low immunogenicity [113,114,129,136]. A recent study reported that either systemic or direct joint administration of an rAAV9 vector carrying an artificial-miR that targets shn3 (*rAAV9-amiR-shn3*) in mice markedly improved bone formation via augmenting osteoblast activity [137]. Baculoviruses are insect viruses that can transfect a wide variety of mammalian stem cells at high efficiency [138]. Importantly, these baculovirus vectors are relatively safe for clinical applications because they lack the capabilities of replication and gene integration in mammalian cells [139]. One study reported the aberrant elevated expression of *miR-214* in the BMSCs isolated from OVX rats. To suppress the miR levels, the authors constructed hybrid baculovirus vectors expressing miR sponges to bind *miR-214*. Notably, transplantation of the *miR-214* sponges in OVX-BMSCs effectively healed the critical-size bone defects and ameliorated the bone quality in OVX rats while the OVX-BMSCs ectopically expressing Bmp2 failed to heal the defects [140].

Lipid-based delivery system

Lipid-based nanocarriers are the most commonly used nonviral gene delivery systems *in vitro*. Besides numerous *in vitro* studies, lipid-mediated miR delivery is also feasible for *in vivo* application based on recent findings. One study reported that tail vein injection of a *miR-451a* mimic containing liposome (Invivofectamine® 3.0) significantly enhanced osteoblastogenesis, reversed OVX-induced bone loss, and improved bone strength [141].

However, so far most of the lipid-mediated miR applications are relying on *in vitro* stem cells (e.g., BMSCs) transfection first and then transplantation of these miR-expressing cells *in vivo* [142]. These "ex-situ" strategies instead of direct administration of liposome/miR to "in situ" bone defects are largely because the nonspecific distribution and low stability of liposomes in tissue fluid will severely limit their efficacy *in vivo* [114,142,143]. To address this challenge, one seminal study developed an innovative bone targeting delivery system comprising 1,2-dioleoyl-3-trimethylammonium-propane-based (DOTAP) liposome linked with (AspSerSer)6, which selectively bind to osteoblast-mediated mineralizing nodules and amorphous calcium phosphate [144]. Through systemic injection of this (AspSerSer) 6-liposome-containing casein kinase-2-interacting protein-1 (Plekho1) siRNA, bone formation, bone microarchitecture, and bone mass were all significantly improved in both healthy and osteoporotic rats [144]. Soon after, this exciting bone formation surface-targeting delivery system, (AspSerSer)6-liposome, was successfully applied to deliver *antagomiR-214* [145] and *agomiR-33–5p* [146] to improve bone formation/mass in OVX [145] and unloading [146] mice models, respectively. In addition to targeting bone formation surface, a novel eight repeating sequences of aspartate (D-Asp8) peptide-conjugated DOTAP-based liposome was developed to deliver miR to bone resorption surfaces [147] because of the ability of D-Asp8 for selectively binding to highly crystallized hydroxyapatite (HA), which is the characteristic of bone resorption surfaces [148]. They demonstrated that D-Asp8 conjugation promoted the enrichment of *antagomir-148a*/liposome after intravenous injection and the subsequent down-regulation of *miR-148a* expression in osteoclasts *in vivo*, resulting in significantly reduced bone resorption in OVX-induced osteoporotic mice [147].

Polymer-based delivery system

Numerous cationic natural and synthetic polymers have been widely studied and shown great promise for both plasmid DNA and RNA gene delivery [149]. Compared to lipid vectors, one obvious advantage of polymer-based delivery systems is that they are more flexible and versatile through variation in polymer molecular weight, structure, composition, and conjugation [113]. Among the currently reported polymer-based vectors, high molecular weight branched polyethyleneimine (PEI, 25KD) is still the gold standard and has been most widely used in both preclinical studies and clinical trials because of its relatively high nucleic acid transfer efficiency. Recently, we successfully delivered plasmid DNA containing *miR-200c* into primary human periodontal ligament fibroblasts and bone marrow MSCs. *miR-200c* delivered using PEI effectively inhibited IL-6, IL-8, and CCL-5 in periodontal ligament fibroblasts and enhanced osteogenic differentiation of human bone marrow MSCs *in vitro* [119]. However, the significant safety concern derived from PEI's high cytotoxicity and non-degradability prevent its applications in regenerative medicine [150]. Many efforts have been reported to reduce PEI's cytotoxicity while improving the gene transfer efficiency. One strategy is to crosslink low molecular weight PEI via a disulfide linkage [149]. Another promising strategy is to combine with other inorganic nanoparticles. For example, a PEI-capped gold nanoparticle (AuNPs) was developed for *miR-29b* delivery. These AuNPs not only didn't show obvious cytotoxicity on hMSCs but also they were more efficient in improving osteoblastic differentiation compared to Lipofectamine RNAi MAX/*miR-29b* complexes [151]. Similarly, PEI combined with iron oxide magnetic nanoparticles (MNPs) indicated moderate cytotoxicity and high uptake

efficiency (~75%). MNPs-PEI provided a long-last effect in delivering *miR-335* into hMSCs *in vitro* [152].

Chitosan (CS) is one of the most studied natural polymeric gene carriers derived from partial deacetylation of chitin [153,154]. Chitosan-based gene carriers are especially attractive for regenerative medicine because of its high positive charge, excellent biodegradability, favorable biocompatibility, low toxicity, low cost, and low immunogenicity [153–155]. Additionally, chitosan has been widely studied in bone tissue engineering either as drug/gene carriers or scaffolds because of the pro-osteoblastic activity and inherent antimicrobial ability [156,157]. In one study, chitosan nanoparticles cross-linked by tripolyphosphate (TPP) were used to deliver *miR-199a-5p* agomiR plasmid. Their data indicated that the chitosan nanoparticles could stably overexpress *miR-199a-5p* in hMSCs *in vitro* and promoted both ectopic bone formation after implantation *in vivo* and bone defects repair delivered by hydrogel [158]. Chitosan/TPP/Hyaluronic Acid (HA) nanoparticles were also developed to deliver *antimiR-138* to rat bone marrow mesenchymal stem cells (rMSCs) [159]. These composite nanoparticles showed a high transfection efficiency (~70%) and significantly promoted rMSCs osteogenic differentiation *in vitro*. Moreover, some *in vivo* studies proved that it was feasible to effectively prevent the bone loss in OVX mice by systemic administration of *miR-34a* mimic [160], or *miR-182* inhibitor [161] using TPP/chitosan nanoparticles.

Inorganic nanoparticle-based delivery system

Calcium phosphates (CaP) have been used as gene carriers for decades through the way of DNA-calcium phosphate co-precipitation to introduce plasmid DNA into many cell types [162–164]. Among the gene vectors being considered to date, CaP nanoparticle is one of the most promising materials for dental and bone tissue regeneration applications by virtue of the excellent osteoconductivity, biocompatibility, and biodegradability [165]. Besides DNA, *e.g.,* pDNA encoding bone morphogenetic protein-2 (Bmp-2) [166], CaP nanoparticles also have successfully delivered miRs [167] and siRNAs [168] as well. The excellent osteoconductivity and biocompatibility of CaP nanoparticles as non-viral vectors are especially advantageous for bone and dental tissue engineering applications even though their transfection efficacy needs to be significantly improved compared to viral vectors [166,169].

Inspired by the rationale of co-precipitation of Ca(2+) with DNA, nano-sized CaCO(3)/DNA co-precipitates were also developed for gene delivery because of its high biocompatibility and inducible biodegradability [170]. Our unpublished data indicated that the CaCO3/PS has much higher (at least two times) efficiency than PEI (25KD) at the same culture condition with 10% serum presence to deliver plasmid DNA (eGFP) to mouse cranial osteoblasts with significantly lower cytotoxicity. Moreover, as an alternative to CaPO4-based biomaterials, CaCO3-based biomaterials have shown some excellent properties, e.g., biocompatibility, biodegradation, bioactivity, and osteoconductivity for bone implantation and regeneration [171,172]. Therefore, CaCO3-based materials show great promise for bone tissue engineering applications including as a non-viral gene delivery vector and scaffold materials.

Mesoporous silica nanoparticles (e.g., MCM-41 and SBA-15 MSNs), are emerging as a multifunctional drug delivery carriers because they are cable of absorbing/encapsulating large amounts of bioactive molecules through the hundreds of empty channels with a honeycomb-like porous structure (mesopores). MSNs have some unique features, e.g., large pore volume (~0.9 cm3/g), tunable pore size (2–10 nm), high surface area (~900 m2/g), good chemical and thermal stability, good biocompatibility, excellent surface functionality, which are all advantageous for various controlled release applications [173,174]. In addition to pDNA, one study indicated that MSNs could efficiently deliver siRNA, pDNA, and small anti-tumor drugs into cells without significant cytotoxicity when the surfaces of MSNs were noncovalent coated with PEI (10KD not 25 KD) [175]. More recently, a new technique was developed for functionalization of MSN surface with PEI through disulfide bonds which can achieve lysosomal delivery of chemotherapy drug (doxorubicin) and intracellular delivery of *miR-145* [176].

Section IV Summary

miRs	delivery approach/type of molecule	effect on bone growth/regeneration
miR-200c	viral vector/miR OE	bone regeneration
miR-148a	antagomirs/modified oligos	bone regeneration
miR-103a	antagomirs/modified oligos	inhibition of bone loss
miR-31a	antagomirs/modified oligos	inhibition of bone loss
miR-29a	viral vector/miR OE	protect against bone loss
miR-26a	viral vector/miR OE	improved bone regeneration
miR-31	viral vector/miR inhibition	increased bone formation
miR-214	viral vector/miR inhibition	increased bone formation
miR-451a	liposome/miR mimic	enhanced osteoblastogenesis
mIRs-214, −33	modified liposome/antagomir	improved bone formation
miR-148a	modified liposome/antagomir	reduced bone resorption (OVX)
miR-200c	polymer-based/miR OE	enhanced osteogenic differentiation
miR-199a	chitosan/antagomir	promoted ectoptic bone formation
miR-138	chitosan/antimir	increased osteogenic differentiation
miRs-34a, −182	chitosan/mimic-inhibitor	prevent bone loss (OVX)

V. SCAFFOLD-BASED LOCAL MIR DELIVERY SYSTEM

Stem cells for gene therapy

Currently, there are two gene therapy strategies in bone tissue engineering. The first one is a stem cell-based gene delivery which has been used in most reported pDNA or miR-based gene therapy studies. The second one is direct gene therapy or acellular gene delivery which delivers the therapeutics via viral or nonviral vectors to the injury sites to promote bone healing by recruiting endogenous reparative cells without using exogenous stem cells [177,178]. Compared to embryonic stem (ES) cells and induced pluripotent stem cells (iPSCs), which are pluripotential, the multipotent adult stem cells, especially several types of mesenchymal stem cells (MSCs), are the most widely used in dental and bone tissue

engineering due to the ethical and legal controversies with ES and iPSCs [179–181]. Almost half-century ago, bone marrow-derived MSCs (BMSCs) were identified and isolated from murine femur bones as an adherent fibroblast-like population with the capacity to self-renew and differentiate into at least three cell lineages, including bone, fat, and cartilage [182]. Since then, non–marrow tissue-derived MSCs were isolated and identified in almost all tissues, *e.g.,* adipose tissue, placenta, umbilical cord blood, dermis, and orofacial tissue [181,183] while most of the current studies are still using BMSCs in miR-based bone tissue engineering. However, it should be noted that craniofacial bones originate from two sources: most are of cranial neural crest origin while the parietal bones arise from the paraxial mesoderm. Therefore, BMSCs harvested from craniofacial bones exhibit distinct properties from long bone MSCs although overall, they are very similar. For example, craniofacial BMSCs are growing more rapidly with higher levels of alkaline phosphatase in cell culture, and forming more compact bone and less bone marrow upon transplantation *in vivo* [179–181]. While the BMSCs or other type of stem cells have been used for gene delivery and bone tissue engineering, very few studies have discussed if the origination of MSCs could affect tissue regeneration. Therefore, we need to investigate if it is appropriate to use long-bone derived MSCs to repair cranial bone defects as done previously in many cases. Moreover, too many procedures including stem cell isolation, culture, characterization, and gene transfection *in vitro* add extra safety concern and complexity that will prevent these approaches from clinical applications. Therefore, cell-free direct gene delivery is a more straightforward and translational strategy for regenerative medicine while it requires developing robust delivery system for local and sustained release of therapeutic molecules to promote, recruit and reprogram endogenous stem cells for tissue regeneration.

Scaffolds for gene delivery and regenerative medicine

In addition to stem cells, scaffolds are another critical component for the success of tissue engineering including miR-meditated bone and dental tissue regeneration [113,114,142,143]. Compared to systemic or bolus delivery, scaffold-based delivery provides more controllable, sustained, and local release of miRs to minimize the off-target side effects or immune reaction [113]. One basic rationale for developing a tissue engineering scaffold/biomaterial is to at least partially mimic the structure and functions of the targeting natural extracellular matrix (ECM), e.g., the bone matrix for bone tissue engineering. Ideally, these synthesized biomimetic biomaterials should not only provide cells with transient and sufficient physical support, e.g., topological and mechanical cues, via its three-dimensional (3D) structure but also instructional chemical signals, e.g., cytokines, growth factors, or miRs to guide these implanted exogenous cells or recruited endogenous cells to proliferate and differentiate until they produce native ECM [184,185]. Accordingly, collagen/gelatin, calcium phosphate/hydroxyapatite, and their composite materials, including hydrogels and 3D scaffolds, are the most used biomaterials for bone and dental tissue engineering because of the similar chemical components to the bone matrix. Additionally, some other natural and synthetic polymers, e.g., chitosan, alginate, polyethylene glycol (PEG), poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and their copolymer poly (lactic-co-glycolic acid) (PLGA), poly(hydroxyl butyrate) (PHB), and poly (caprolactone) (PCL), are also widely used for drug delivery and bone tissue engineering

largely because of their excellent biocompatibility and flexibility in composition and fabrication [184,185].

Hydrogels fabricated by either natural or synthetic polymers are attractive for tissue engineering applications because they are highly hydrated and similar to natural ECM. Therefore, a hydrogel can easily encapsulate cells and drugs in a mild condition and mix with them evenly for controlled release through tuning the degree of crosslinking. Moreover, injectable hydrogels are flexible and fit different geometrical deformities with minimal invasiveness which is especially advantageous for craniofacial bone tissue engineering applications [113]. Recently, an innovative PEG hydrogel was synthesized for simultaneous encapsulation of hMSCs and delivery of *siNoggin* and/or *miR-20a*. These locally and sustainably released RNAs significantly promoted osteogenic differentiation of hMSCs *in vitro* and subsequent bone repair in critical-sized rat calvarial defects after transplantation *in vivo* [186]. In another study, one commercialized hydrogel named HyStem-HP contained thiol-modified hyaluronic acid, gelatin, and heparin was used to deliver *miR-26a* for successful repair of mouse critical-sized calvarial bone defects by targeting both angiogenesis and osteogenesis [187]. Instead of relying on transplantation of exogenous stem cells for carrying miRs, one study reported that they developed a cell-free nanoparticle/hydrogel composite system for bone regeneration. Both stromal cell-derived factor-1α (SDF-1α) and chitosan/tripolyphosphate/hyaluronic acid/*anti-miR-138* nanoparticles were incorporated in chitosan/β-sodium glycerol phosphate hydrogel which sustained release to promote rat critical-sized calvarial bone regeneration by recruiting and differentiating the endogenous stem cells [188].

Another type of biomimetic scaffold/biomaterials that are promising for miR-based regenerative medicine are 3D nanofibrous scaffolds since they closely mimic the nanofiber structure of collagens, the main organic component of ECM. These biomimetic nanofibrous scaffolds have been widely studied in many types of tissues, e.g., skin, vascular, cartilage, bone, and nerve [185,189,190]. The porous nanofibrous scaffolds with the high surface area are valuable because they not only provide a 3D matrix for cell growth and nutrient transport but also enable a high dose of drug loading and local sustained release. Importantly, the topography of nanofiber could directly influence the migration/differentiation of stem cells [185,189,190] and facilitate endocytosis mediated gene transfer through modulating cell-matrix interactions [191,192]. To date, several techniques including electrospinning, phase separation, and self-assembly have been investigated to prepare nanofibrous scaffolds. Among these reported methods, the electrospinning technique has been most widely studied in many fields because it is facile and versatile [193–195]. Numerous studies have proved that electrospun nanofibers are versatile biomaterials for both viral and non-viral vector-mediated gene delivery, e.g., pDNA and siRNA, which were summarized in a recent review [196]. Additionally, a bilayer vascular scaffold was specially prepared via emulsion electrospinning of PEG-b-PLA-co-PCL (PELCL) and dual-power electrospinning of PCL and gelatin. The inner layer of PELCL loaded with complexes of *miR-126* in REDV peptide-modified trimethyl chitosan-g-PEG, modulated the response of vascular endothelial cells (VEC), while the outer layer of PCL/gelatin provided the mechanical stability. The bilayer vascular scaffold loaded with *miR-126* complexes were capable of accelerating VEC proliferation and improving endothelialization *in vivo* [197].

The major limitation of most electrospun scaffolds is the lack of macropores for cell growth and tissue formation due to their morphological structure of overlaid nanofiber mats [198]. Thus, it is critical to develop innovative strategies for producing biologically and clinically relevant 3D electrospun nanofibrous scaffolds with desired structural properties. To tackle this significant technical challenge, we and our collaborators have developed an innovative technique of thermally induced nanofiber self-agglomeration (TISA). The 3D electrospun PCL or PCL/PLA blend nanofibrous scaffolds are prepared using a TISA technique having high porosity of >95% as well as interconnected and hierarchically structured pores with sizes from sub-micrometers to ~300 μm. Compared to other 3D porous scaffolds, our 3D TISA nanofibrous scaffolds had higher elasticity, which enable them with press-fit ability suitable for irregular-shaped defects. Importantly, they favorably supported the growth and osteoblastic differentiation of hMSCs *in vitro* and both ectopic and cranial bone regeneration *in vivo* [199–202]. Another technique for the preparation of 3D nanofibrous scaffolds in our group is the thermally induced phase separation (TIPS) method with the porogen leaching technique (TIPS&P) [203–208]. One main advantage of these TIPS&P nanofibrous scaffolds fabricated either by PLLA or gelatin is that the macropore structure is well-defined with high interconnectivity and relatively strong mechanical properties. These biodegradable biomimetic 3D nanofibrous scaffolds are especially intriguing for bone tissue engineering with pro-osteoblastic activity and potent drug delivery capacity combined with versatile nanoparticles as we previously reported [209–216]. Furthermore, our group developed an innovative porogen-free TIPS technique for fabrication of 3D porous scaffolds with interconnected, hierarchically structured macropores, and biomimetic nanostructures. The porosity, pore size, and mechanical properties of these porous microspheres-aggregated 3D PCL scaffolds were highly tailorable and showed bone-like apatite forming ability and multiple drug loading and sustained release capacity. Importantly, the 3D nanofibrous scaffolds fabricated by TIPS&P combined with PLGA microspheres were successfully used for locally and controlled release of *miR-26a* polyplexes for heathy and osteoporotic cranial bone regeneration. This innovative two-stage release and cell-free strategy showed strong bone regenerative ability by harnessing endogenous reparative stem cells and signaling pathways [81]. Moreover, a new PLLA nanofibrous spongy microsphere-delivery system was fabricated and used as an injectable scaffold for both MSN-mediated fast release of IL-2/TGF-β and PLGA microsphere-mediated slow release of *miR-10a*/polyplexes. In a mouse model of periodontitis, this injectable and biomolecule-delivering system effectively rescued the periodontal bone loss through modulating regulatory T cells mediated immune response [217].

Perspective for biomaterials-based miRNA therapy

As we discussed above, most current miR-based bone regeneration studies rely on *ex vivo* stem cell transfection before their *in vivo* transplantation. On one side, this reminds us that stem cells play an important role in tissue repair by either differentiating the target cells or producing trophic and immunomodulatory factors [218]. On the other side, this also indicates that current conventional gene delivery vectors/scaffold systems are not efficient and safe enough to promote sufficient endogenous tissue regeneration using the direct gene therapy strategy. The cell-free strategy is less complicated, more straightforward, and translational than a cell-dependent strategy. This significant challenge urges the development

of innovative biomimetic tools for translational miR-based therapy. The exosome is emerging as a promising natural nanoparticle produced by all types of cells for gene delivery with excellent transfection efficiency, biocompatibility, and particular cell/tissue targeting ability. Importantly, exosomes similar to liposomes are capable of being engineered with extra targeting and functional moieties in addition to loading both small miR and large pDNA for customized drug delivery [219–221]. Aptamer, often termed ‘chemical antibodies’, developed by the groundbreaking technology systematic evolution of ligand exponential enrichment (SELEX), is another exciting cell targeting tool for gene therapy [222,223], and have shown promising functions in osteoblasts or MSCs-targeting delivery of nanoparticles/siRNA or miR for successfully improving bone formation in vivo [224,225].

Section V Summary

miRs	scaffold type/cells	effect on bone growth/regeneration
miR-20a	PEG hydrogel/hMSCs	promoted osteogenic differentiation
mlR-26a	HyStemHP/exogenous stem cells	repair calvarial bone defects
anti-miR-138	chitosan nanoparticles/hydrogel	bone regeneration/endogenous cells
miR-126	PELCL/PCL/gelatin	improved endothelialization
miR-26a	PLGA nanosphere/3D PLLA scaffold	bone regeneration
miR-10a	PLGA microsphere	rescue bone loss

VI. CURRENT RESEARCH

Although genetically miR modified BMSCs and ADSCs have exhibited the therapeutic capabilities to generate bone tissue and facilitate bone formation and regeneration. The limited source of the cells and the biosafety of manipulating gene transfection *in vitro* sets up a huge barrier for their application in the clinic. Thus, the cell-free gene delivery of miRs has the advantage to provide a promising approach for bone regeneration. In comparison to the cell-based approaches, there are relatively few studies that use cell-free gene therapy of miRs for bone regeneration. The mature oligo *miR-26a* has been attempted to be delivered by nonviral nanoparticles for sustained release from PLGA microspheres [81]. *miR-26a* was reported to activate Wnt/β-catenin by targeting sclerostin domain containing 1 (SOSTDC1) [79]. The system has been demonstrated to successfully regenerate calvarial bone in a mouse model of critical-sized defects. *miR-200c* directly targets *Noggin*, an antagonist of Bmp signaling [66]. Our previous studies have demonstrated that *miR-200c* also directly targets *Sox2* and *Klf4* and activated Wnt signaling activities [117]. The expression of *miR-200c* was upregulated during *in vitro* osteogenic differentiation of human BMSCs. Inhibition of *miR-200c in vivo* using the PMIS system has exhibited significant downregulation of craniofacial bone development and the osteogenic differentiation of human BMSCs. We were surprised to observe that the naked plasmid encoding *miR-200c* can safely and effectively increase the transfection of *miR-200c in vitro* and *in vivo*. By over expressing *miR-200c* we effectively promoted the osteogenic differentiation of human BMSCs *in vitro*. Furthermore, the plasmid *miR-200c* incorporated collagen sponges has been shown to significantly improve bone formation and restore different sized calvarial defects in rats

[117]. Conversely, *miR-200a* OE delivered to skull sutures inhibited suture fusion and in *PMIS-miR-200a* mice calvarial bone development and bone density were increased in the transgenic mice (Fig. 3).

Our group has developed new technology and methods to inhibit miRs both *in vivo* and *in vitro* for therapeutic applications that can rapidly and efficiently repair and regenerate bone using several of the delivery methods outlined in this review. The PMIS system can be applied to also inhibit bone formation dependent on the miR that is targeted for inhibition. Experiments in dogs, rats and mice demonstrate that the PMIS is not toxic and highly effective. This is an exciting time for miR therapeutic research and their ability to regenerate bone and tissues.

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Highlights

- microRNAs regulate genetic pathways that form bone and inhibit bone regeneration
- New microRNA inhibition technology (PMIS) proven in transgenic mice models
- microRNAs regulate bone formation, inflammation, osteoporosis and periodontitis
- New microRNA inhibition technology (PMIS) more efficacious than oligonucleotides
- microRNA gene therapy approaches are compared to stem cell-based scaffolds

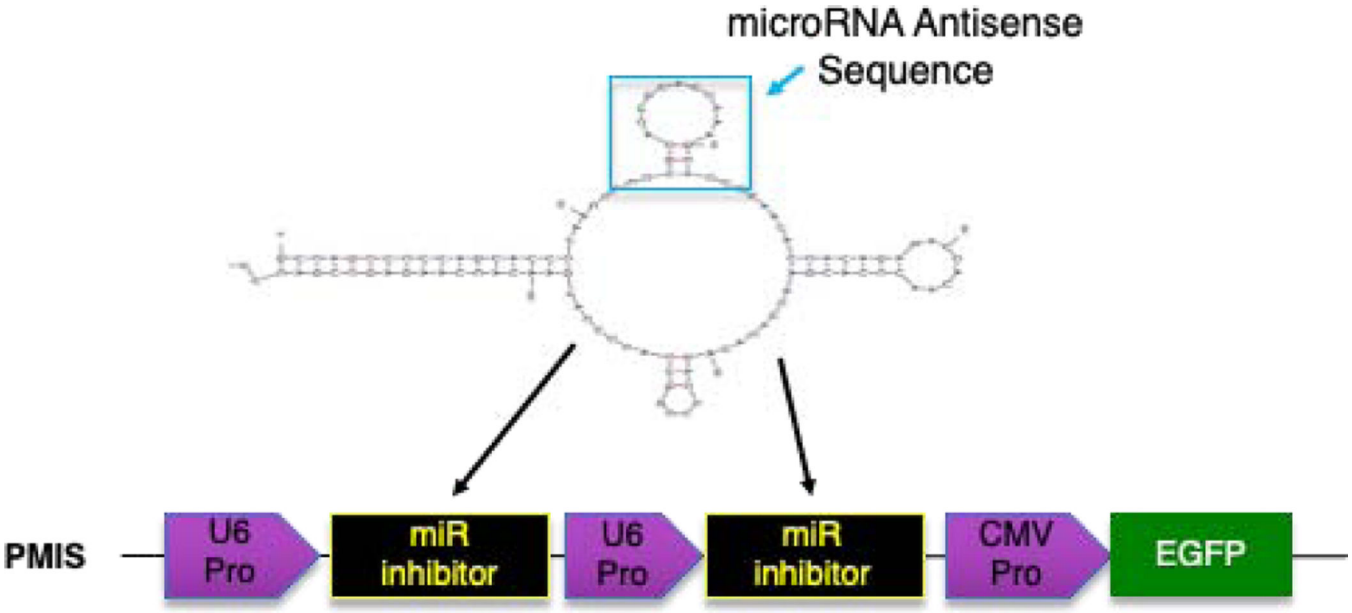
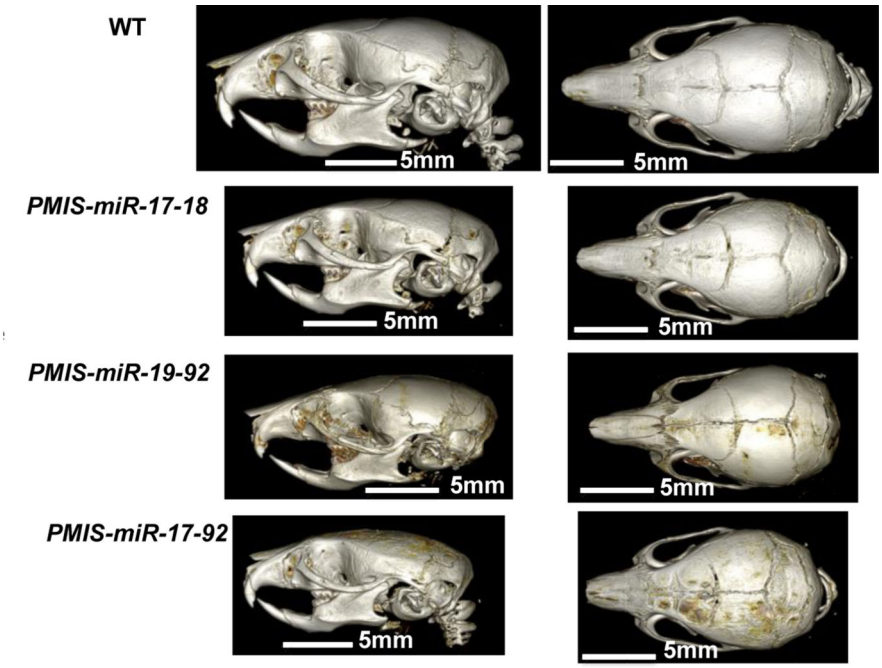


Figure 1. Schematic representation of the PMIS. A U6 promoter drives expression of the PMIS transcript.

The inhibitor complex contains a stem loop that comprises the antisense miR of choice. The 21–22 nucleotide antisense sequence of each miR is cloned into this site in the stem loop. EGFP expression is used to determine expression. More than one miR inhibitor can be constructed in the same plasmid.



μCT measurements of mouse heads (mm)		WT	PMIS-miRs		
			17-92	17-18	19-92
Total cranial length	rh-op	21.81	16.57	20.75	17.42
Neurocranial length	na-op	15.9	12.41	15.34	12.8
Nasal bone length	rh-na	6.54	4.66	5.98	4.99
Palate length	pr-pns	11.12	8.54	10.85	8.91
Frontal bone length	na-br	7.18	4.95	7.1	5.43
Parietal bone length	br-l	7.95	3.67	4.02	3.55
Neurocranial breadth	Immed. superior to base of zygomatic proc.	10.36	8.63	10.12	9.05
Relative neurocranial breadth	breadth/length	0.6515	0.6954	0.6597	0.7070
Relative frontal length	frontal L/total cranial L	0.3292	0.2987	0.3421	0.3117
Relative parietal length	parietal L/total cranial L	0.3645	0.2214	0.1937	0.2037
Relative nasal bone length	nasal bone L/total cranial L	0.2998	0.2812	0.2881	0.2864
Relative palate length	palate L/total cranial L	0.5098	0.5153	0.5228	0.5114

Figure 2. miRs within the *miR-17–92* cluster differentially regulate craniofacial development.

WT, *PMIS-miR-17–18*, *PMIS-miR-19–92* and *PMIS-miR-17–92,* 3 week-old heads were analyzed by μCT. In-depth measurements were obtained for different aspects of craniofacial growth. Quantitative measurements of total cranial length and breadth of the PMIS transgenic mice are shown compared to WT, N=3.

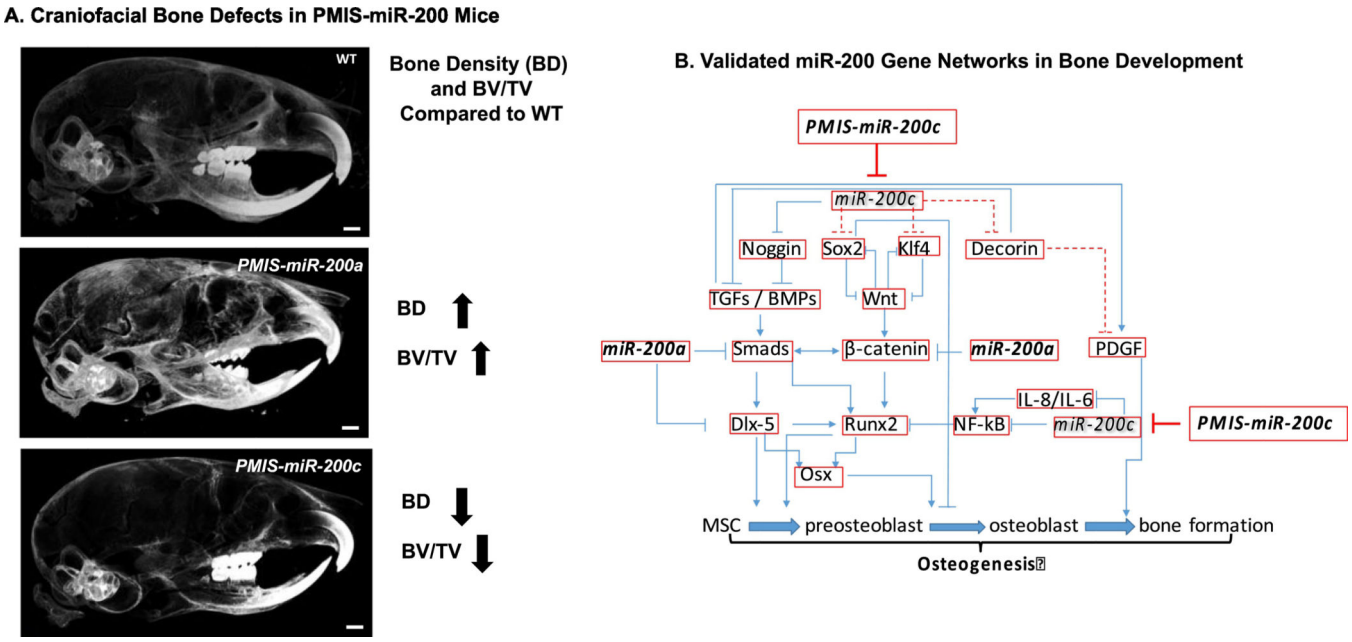


Figure 3. The *in vivo* role of the *miR-200* family in craniofacial bone development.

A. Bone density in 4 week old WT, *PMIS-miR-200a* and *PMIS-miR-200c* mice heads. BD, bone density; BV/TV, bone volume/tissue volume. **B.** Gene networks involved in bone formation regulated by the *miR-200* family.

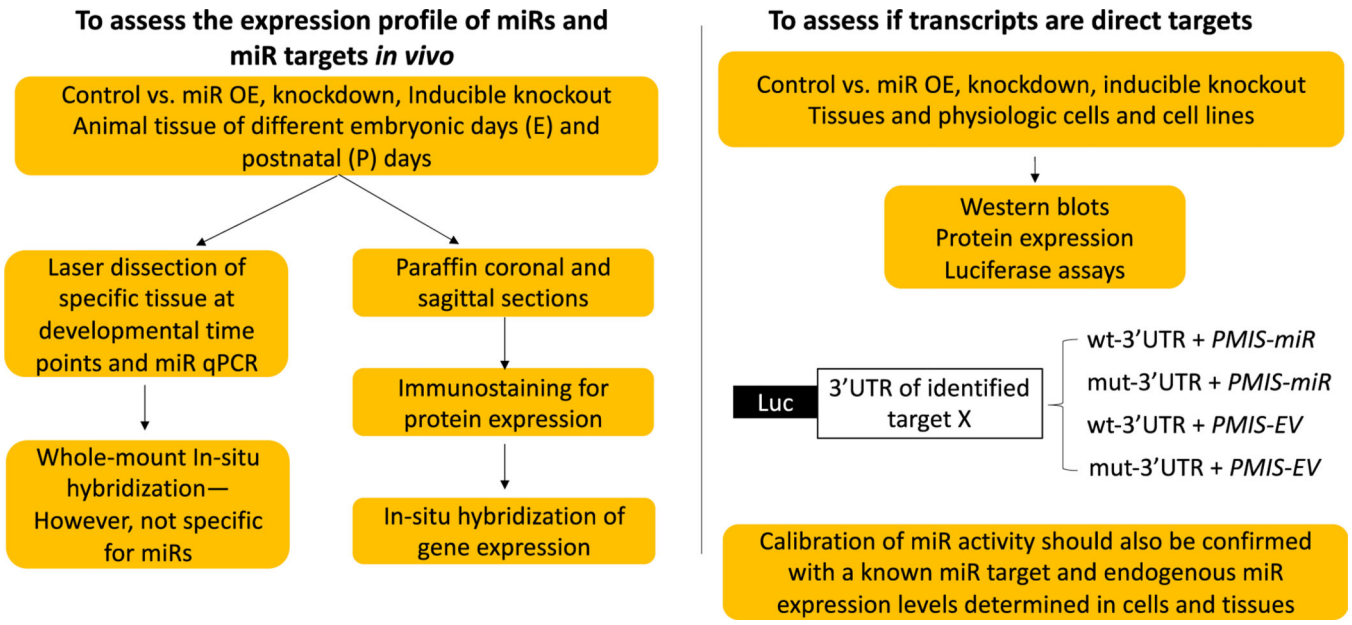


Figure 4. Validation Workflow of *in vivo* RNA-Seq., scRNA-Seq. or miR Microarray potential targets (compare Control vs miR knockdown, miR OE or inducible (conditional) miR inactivation)

• Note- In situ hybridization for gene and miR transcripts may not yield accurate information as miRs and miR targets may not be degraded upon miR inhibition or over expression (OE), respectively.