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Exploring microRNAs in craniofacial regenerative medicine

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Abstract

microRNAs (miRs) have been reported over the decades as important regulators in bone development and bone regeneration. They play important roles in maintaining the stem cell signature as well as regulating stem cell fate decisions. Thus, delivering miRs and miR inhibitors to the defect site is a potential treatment towards craniofacial bone defects. However, there are challenges in translation of basic research to clinics, including the efficiency, specificity, and efficacy of miR manipulation methods and the safety of miR delivery systems. In this review, we will compare miR oligonucleotides, mimics and antagomirs as therapeutic reagents to treat disease and regenerate tissues. Newer technology will be discussed as well as the efficiency and efficacy of using these technologies to express or inhibit miRs in treating and repairing oral tissues. Delivery of these molecules using extracellular vesicles and nanoparticles can achieve different results and depending on their composition will elicit specific effects. We will highlight the specificity, toxicity, stability, and effectiveness of several miR systems in regenerative medicine.

Introduction

Currently, there is a growing demand for effective remedies to deal with craniofacial bone defects that occur due to aging, traumatic injuries, disease, and birth abnormalities. To this day, autografts remain the gold standard for treating bone defects, but are met with shortcomings that include severe supply limitations and donor site morbidity [1]. Alternatively, allografts and xenografts can be used as substitutes, but are ultimately rejected by the host immune system [2]. Tissue engineering, however, can circumvent these complications by its potential to harness the repair mechanisms of the host organism for bone regeneration. Previous studies have documented successful reprogramming of multipotent progenitor cells into tissue specific lineages by the administration of bioactive

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molecules [3,4]. In tissue engineering, specific biomaterials and cell types are deliberately selected in order to recapitulate tissue and extracellular matrices that were lost or damaged due to trauma [5]. The interaction between cells and biomaterials is a critical factor for facilitating appropriate cell–cell communication in recapitulating tissue specific niches.

Traditionally, tissue engineering has relied on the presence of proteins such as bone morphogenic proteins, fibroblast growth factors, human recombinant bone morphogenic proteins, or parathyroid hormone to facilitate osteogenesis in synthetic bone grafts [6–14]. However, these drugs are noted to have several complications. They are generally unstable, expensive, require large amounts of administration, and have numerous side effects, including tumorigenesis, inappropriate formation of adipose tissue, ectopic bone formation, and inflammatory responses [15–21]. Thus, these proteins generally have specific targets and as such, are limited in efficiency. On the other hand, microRNAs (miRs) have demonstrated an ability to efficiently regulate multiple cell signaling pathways through downstream cascading effects [22,23]. Previous studies have revealed that miR's can stimulate cellular proliferation, differentiation, and growth without the addition of specific proteins [24,25].

miRs are small, non-coding regulatory RNAs that bind to their specific binding sites in the 3′UTR of mRNAs and thus inhibit the expression of their target genes [26]. They have been reported to control several developmental processes, as well as diseases and regeneration processes [27].

At present, miRs have also been largely related to maintaining the stem cell signature, as well as the stem cell fate decision towards different lineages, which makes them a potential tool to correct craniofacial bone defects. As the research knowledge about the roles of miRs in craniofacial development are defined, it is possible to treat congenital craniofacial defects and injuries in facial and skull structures with new therapeutic approaches. The translation of basic knowledge to clinics requires precise miR over expression and/or inhibition methods, as well as a safe and efficient delivery system. The current challenge for over expressing or inhibiting miRs *in vivo* is the design of miR over expression constructs or mimics and miR inhibitors or antagomirs. Furthermore, dosage control which largely affect the efficacy and specificity of the miRs to regulate downstream targets. The delivery method as well as the scaffold are also important.

In this review, we will discuss the current knowledge of the role of miRs in maintaining stem cell signatures and regulating stem cell fate decisions. We will compare the efficiency and efficacy of current and newer miR technologies as therapeutic methods to treat disease and regenerate tissues. We will also highlight the specificity, toxicity, stability, the effectiveness of several new systems to deliver these molecules into the specific tissue. This review will also cover eight different biomaterials that have been used in conjunction with miR gene therapy approaches for promoting osteoblastic differentiation and bone regeneration.

Regulation of stem cell self-renewal and differentiation by microRNAs

Mesenchymal stem cells (MSCs) are recognized as self-renewed and multipotent with the capability to differentiate into osteoblasts, chondroblasts, adipocytes and endothelium-producing cells [28]. These cell types are required for tissue regeneration especially when treating craniofacial defects. Over the years, specific miRs have been identified that not only maintain the MSC feature, but also regulate their differentiation into desired lineages [29,30]. Other than the intrinsic regulation, MSCs have the ability to produce extracellular vesicles (EVs) including exosomes, in order to effect other cells through their paracrine activity [31–33]. A selected pattern of miRs can be shuttled in EVs produced by MSCs and delivered to other cells [34]. Thus, delivery of miRs that stimulate osteogenic lineages into the defect site with therapeutic approaches can promote osteogenic differentiation of endogenous MSCs and thus induce bone regeneration.

mirs maintain the MSC signature

It has been reported that MSC's have their own miR expression signatures. Although different miR expression patterns are detected between intracellular and the EV environment of human embryonic stem cell derived mesenchymal stem cells (hES-MSCs), a high level of the *let-7* family of miRs is predominant in both contexts. Intracellular miRs in hES-MSCs also include *miR-199a-3p*, *miR-29a*, *miR-21*, *miR-152*, *miR-143*, *miR-221*, *miR-103*, *miR-100*, *miR-24* and *miR-125b* [35]. In addition, adult MSCs derived from different tissues share a core signature in their transcriptomes but also exhibit secondary signature expression profiles between different origins [34,36,37]. This indicates that MSCs have a characteristic gene and miR expression profile that help maintain the MSC signature.

mirs regulate osteogenic differentiation of MSCs

miRs are recognized to regulate differentiation of MSCs. The regulation of osteogenic differentiation by miRs in MSCs have been widely studied over the decades. Most of these studies are done *in vitro* with either primary MSCs of different origins, or potential differentiated stable cell lines. It has been reported that specific miRs target to osteogenic genes including *BMP2*, *DKK1* and *SMAD* genes, as well as signaling pathways involved in the osteogenic process (Table 1). Several miRs/miR clusters have roles in regulating bone formation *in vivo*. *miR-140*, *miR-17-92*, *miR-452*, *miR-199/214*, *miR-26b*, *miR-135a* and *miR-200c* have been reported to positively regulate osteogenesis *in vivo*, while *miR-200a*, *miR-29cb2* and *miR-378* are negative regulators [38–48]. While the whole *miR-23a-27a-24-2* cluster is negatively regulating bone formation *in vivo*, *miR-23* and *miR-27* individually promote osteogenesis [49,50].

microRNA delivery systems

Recent developments in the field of gene therapy have utilized a miR-based approach to treat a wide range of diseases. However, the application of gene therapy suffers from issues related to a lack of proper delivery methods to get genes and miRs into the cells. A practical and safe gene delivery system is a crucial factor required for the success of miR-based

gene therapies. Currently, there are two approaches to express miRs: Oligonucleotide-based approaches and Plasmid DNA-based approaches.

Oligonucleotide-based approach

This approach uses chemically modified double-stranded RNA molecules that mimic the activities of select miRs. These miR mimics can be transfected and potentially interact with the RISC complex [69]. Like endogenous miRs, the miR mimics can function in a variety of ways, including translational repression, mRNA cleavage and deadenylation. The disadvantages of these mimics include cell toxicity, repeated applications of high concentrations of oligonucleotides, the transfection efficiency of mimic miRs is low and the formation of a functional new miR from the complementary strand has limited the use of this approach [70].

In cells, tissues, and cell lines most of the functional testing for miR activity (miR inhibition) has used chemically modified anti-miR oligonucleotides (AMOs) and locked-nucleic acids (LNAs) inhibitors that 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. The sponges and decoys also suffer from a lack of stability, inefficient binding of the miR, lack of specificity and require toxic delivery systems. The biggest issue is the off-target effects of using large quantities of these miR oligo inhibitors in cells that cannot distinguish between a one nucleotide difference in miR families such as *miR-23a* and *miR-27a*. Therefore, many reports of genes and pathways affected in different types of cells by these miRs have not been rigorously validated. It is well-known that every miR targets multiple mRNAs, which can inhibit gene networks and have large biological consequences [71]. The current issues of using *miR-23* mimics and LNA-23 miR inhibitors is highlighted in a current report stating that *miR-23a* and *miR-23b* target different transcripts, even though they have identical seed regions and only differ by one nucleotide outside of the seed region (Table 2) [72]. We suggest that these results are due to the nonspecific and off-target effects of the oligo-based mimics and inhibitors. Furthermore, they report using the LNA-23 miR inhibitor did not increase their target gene expression [72]. However, *miR-24* has a completely different seed sequence and flanking sequences compared with *miR-23* and *miR-27* (Table 2). Therefore, the targets of *miR-24* should have no overlap with the other miRs in this cluster.

Plasmid DNA-based approach

Plasmid DNA-based approaches have been introduced to overcome a short-term unstable expression of mimic miRs. This approach can be accomplished by inserting the miRs precursor sequence downstream of RNA polymerase II or III in a vector system [73,74]. Currently, plasmid DNA expression of a pre-miR is an attractive approach because they are easy to prepare and transfect, have biochemical simplicity, and more stable when compared with the oligonucleotide-based approach [70]. A study comparing the transient transfection of chemically synthesized miR mimics to lentiviral or plasmid transfection of miRs demonstrated that using miR mimics results in non-specific gene expression profiles and accumulation of high molecular weight RNA species [75]. The miR mimics also caused cell death and toxicity [75]. In another study it was shown that miR mimics have side-effects

and off-target effects that reduce specificity [76]. Therefore, a promising alternative is to use a plasmid DNA-based approach for expression of the pre-miR.

Our group has developed the Plasmid-based microRNA Inhibitor System (PMIS) as a method to efficiently knock down miRs both *in vitro* and *in vivo* [27,41,42,45,47,55,56,77–84].

Delivery systems are also an important factor to enhance transfection efficiency. The simplest method to deliver miRs and/or miR inhibitors is to directly transfect cells with naked plasmid DNA. Some studies have reported the successful transfection using exogenous naked pDNA injections in high concentrations [85,86]. However, the limitation is the transfection efficacy of naked pDNA due to the large size of the phosphate group and the cells being hydrophilic in nature. Therefore, current research has developed gene delivery systems to increase their transfection efficiency. Currently, classification of gene delivery systems can be divided into two groups: Viral vectors and non-viral vector carriers.

Viral transfection vector carriers

Viral-based gene therapy was first introduced by Rosenberg et al. [87] who successfully inserted an exogenous gene into tumor-infiltrating lymphocytes using retroviral-mediated gene transduction for treating melanoma patients. This led to the development of other viral transfection vector carriers including adenovirus and lentivirus-mediated gene transduction [88]. Although, viral based gene therapy has a high transduction efficiency, the major challenges of this approach are complicated side effects due to immunogenicity and toxicity.

Non-viral transfection carriers

A practical and safe gene delivery system is the key to success for plasmid DNA encoding miR-based gene therapies. Considering the safety issues with viral vectors, non-viral gene delivery systems are preferred. Advantages of a non-viral delivery system are easier preparation processes and stability of the nanoparticle [89]. Examples of non-viral vector carriers commonly used are lipofectamine and polyethylenimine (PEI).

Lipid-based nanocarriers are the most used nonviral gene delivery systems *in vitro*. Besides numerous *in vitro* studies, lipid-mediated miR delivery is also feasible for *in vivo* applications based on recent findings.

Numerous cationic natural and synthetic polymers have been widely studied and show great promise for both plasmid DNA and RNA gene delivery [90]. Compared with 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 [91]. Among the currently reported polymer-based vectors, high molecular weight branched polyethylenimine (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. However, PEI nanoparticles are extremely toxic to cells, resulting in inflammation and cell death.

Chitosan (CS) is one of the most studied natural polymeric gene carriers derived from partial deacetylation of chitin [92,93]. 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 [92–94].

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 [95–97]. Among the gene vectors being considered to date, the 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 [98]. Similar to CaPs, nano-sized CaCO3/DNA co-precipitates were also studied for gene delivery because of their high biocompatibility and inducible biodegradability [99]. Our unpublished data indicated that the $CaCO_3$-based approach has much higher efficiency than PEI (25KD) at the same culture conditions with 10% serum presence to deliver plasmid DNAs to many different primary cell types, including primary human stem cells and tumor cell lines with significantly lower cytotoxicity. Importantly, $CaCO_3$-mediated miR delivery also showed great efficiency *in vivo* using different rodent models. Moreover, as an alternative to CaP-based biomaterials, CaCO3-based nanoparticles have shown some unique advantages, e.g. biocompatibility, biodegradation, and osteoconductivity for bone regeneration [100,101]. Therefore, the $CaCO_3$-based approach shows great promise for bone tissue engineering and translational applications.

Mesoporous silica nanoparticles (e.g. MCM-41 and SBA-15 MSNs), are emerging as multifunctional drug delivery carriers because they are capable 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 cm^3/g), tunable pore size (2-10 nm), high surface area (~900 m^2/g), good chemical and thermal stability, good biocompatibility, excellent surface functionality, which are all advantageous for various controlled release applications [102,103].

Heat-shrinking (HS) DNA nanoparticles are polyacridine PEG-peptide stable DNA nanoparticles that are fully transfection competent in mice [104]. These particles are modified with Lysine residues to achieve high affinity and compaction with a short 18 amino acid peptide. These polyacridine PEG-peptides are readily customizable into targeted carriers to prepare DNA nanoparticles for *in vivo* gene delivery [104]. Heat shrunken DNA nanoparticles were formulated by combining plasmid DNA with a high affinity DNA binding peptide modified with a single Cys, disulfide-linked, polyethylene glycol (PEG) chain. Heating the DNA to 100°C for 10 min results in partial DNA denaturation and increased DNA flexibility to increase folding. The addition of PEG-peptide to partially denatured plasmid DNA leads to rapid heat shrinking of DNA nanoparticles from 170 nm to 60 nm diameter. Heat shrunken DNA nanoparticles remain fully functional at mediating gene transfer *in vivo*. The PEG corona layer blocks protein mediated nanoparticle aggregation to allow DNA particles to traverse to the cell membrane. DNA nanoparticles are designed to shed PEG by disulfide bond reduction upon macropinocytosis. An advantage of this formulation is that the nanoparticle size is unchanged during long term storage, freeze-thaw, freeze drying & reconstitution, and during concentration.

Tissue engineering in craniofacial bone defects

Hydroxyapatite

Hydroxyapatite is the most prevalent nonorganic substance found in bone tissue and therefore serves as an effective biomaterial for bone regeneration [105–107]. Hydroxyapatite possesses high mechanical strength and exhibits no cytotoxicity [108], but since it is not readily absorbed by cells, it can hinder bone remodeling unless incorporated appropriately into scaffolds [109]. Since hydroxyapatite alone is brittle, it is generally added in composite with other biomaterials to reinforce its structural integrity [110]. Two studies have investigated bone healing by coculturing human mesenchymal stem cells in hydroxyapatite/tricalcium phosphate ceramic powder. Eskildsen et al. [111] found that inhibiting *miR-138* resulted in the up-regulation of osteoblastic genes *Runx2* and *Osx*, enhanced alkaline phosphatase (ALP) activity, matrix mineralization, and a notable increase in bone formation by 60%. Likewise, Chen et al. [112] concluded that the repression of *miR-34a* up-regulated key osteogenic markers such as ALP, osteopontin, osteonectin, with a 3.5-fold increase in bone formation. Hydroxyapatite has also been successfully implemented in polycaprolactone nanofibers, where Sadeghi et al. inhibited *miR-122* in rat mesenchymal stem cells. The study found that seeding such cells into the nanofiber scaffold led to statistically significant closures of critical sized bone defects in rats [113]. An *in vitro* study by Castaño et al. investigated the role of *miR-16* in osteogenesis by utilizing collagen-nanohydroxyapatite scaffolds that were soaked in anti-*miR-16* oligomer suspensions. They found that inhibiting *miR-16* in human mesenchymal stem cells resulted in overexpression of *Runx2* and *Ocn* by values of 6.29 (±2.3)-fold and 8.19 (±1.96)-fold, respectively, and an increase in calcium deposition by Alizarin Red staining [114]. Wang et al. investigated the role of *miR-26a* in osteogenesis and angiogenesis [115,116]. Using a porous hydroxyapatite scaffold and adipose-derived stem cells transfected with *miR-26a* mimics by lipofectamine, Wang et al. [115] observed the formation of new bone tissue with hematoxylin and eosin and Masson’s trichrome staining, and cited an increase in ALP activity, collagen secretion and matrix mineralization.

β-tricalcium phosphate

β-tricalcium phosphate (β-TCP) has been widely used in several applications to regenerate bone in craniofacial defects. A major advantage of β-TCP is that it is capable of releasing large amounts of calcium and sulfate ions to catalyze new bone growth, while also maintaining its structural integrity [117,118]. However, β-TCP has limited osteoinductivity and is rather brittle alone, rendering it inapplicable to load bearing [119]. The mechanical strength and osteoconductivity of β-TCP are enhanced when it is reinforced with other bioceramics such as hydroxyapatite or polycaprolactone [120]. Recent studies have revealed that incorporating microRNAs into β-TCP scaffolds significantly improves bone regeneration. Remy et al. evinced an effective relationship between *miR-200c* and a β-TCP scaffold, and for the first time, utilized a hybrid process that incorporated both into one biomaterial. The research found that coating β-TCP in a collagen type I suspension with *miR-200c* plasmid DNA substantially regenerated calvaria bone defect in rats, and outperformed experimental controls [84]. Other studies have simply seeded transfected progenitor cells into β-TCP. For instance, Janko et al. transfected bone marrow mononuclear

cells to selectively inhibit *miR335* and then seeded them in β-TCP scaffolds. After eight weeks, there was a 40.9% recovery of bone tissue in the femoral defects of the Sprague-Dawley rats that had received the scaffold treatment [121]. Deng et al. inhibited *miR-31* in progenitor cells and reported a 35.42 ± 6.12% and a 41.82 ± 6.54% recovery of bone volume in craniofacial defects in rats and canines, respectively [122,123]. Another study, using micro-CT scans, determined a statistically significant increase in bone volume after coculturing bone marrow stem cells overexpressing *miR-26a* in calvaria bone defects of mice filled with β-TCP scaffolds [124].

Hydrogels

Since hydrogels are highly water absorbent and hydrophilic, they possess material properties very similar to cartilaginous and bony tissues [125]. Hydrogels comprising of natural materials including chitosan, collagen, alginate, fibrin, elastin, heparin, and hyaluronic acid are minimally cytotoxic, highly biodegradable, and versatile for filling in bone defect irregularities [125]. Yet, they are inadequate for load bearing [125]. Conversely, synthetic polymer hydrogels are stiffer and can withstand heavier loads [125]. However, they are found to be much less biodegradable and exhibit varying degrees of cytotoxicity [125]. Hydrogel synthesis therefore has tradeoffs between biocompatibility and structural rigidity, and oftentimes the best approach is to hybridize them with ceramic materials and biodegradable polymers that can both promote biological synergy and improve mechanical durability [126]. Hydrogel scaffolds have been documented as suitable environments for microRNA delivery systems and cell growth [3]. A study conducted by Li et al. utilized a hydrogel construct of a rather sophisticated composition (a thiol-modified analog of heparin with thiol-modified hyaluronan and poly(ethylene glycol) diacrylate, and Glycosil™), which was seeded with human bone marrow mesenchymal stem cells that were transfected with *miR-26a* oligomers. After twelve weeks, micro-CT scans revealed a complete recovery of calvaria defects in mice [127]. Another study conducted by Quereshi et al. compared Matrigel™, a thermo-reversible hydrogel, to polycaprolactone. Human adipocyte stem cells transfected to overexpress *miR-14* were seeded into both types of matrices and were given twelve weeks to grow in calvaria defects. The study found that the transfected cells in Matrigel™ outperformed polycaprolactone in terms of calcium deposition, and normalized fracture healing [128]. Lei et al. investigated neurogenesis and bone regeneration that was regulated by *miR-222*, and fabricated a hydrogel comprised of a Poly(lactic-co-glycolic acid) (PLGA) core and a poly(ethylene glycol)-poly(*N*-isopropylacrylamide) (PEG-PNIPAM) shell for localized nanoparticle delivery of *miR-222* mimics and Aspirin. Human bone marrow stem cells that overexpressed *miR-222* demonstrated an up-regulation of neural differentiation markers such as microtubule associated protein (MAP2), nerve growth factor (NGF) and neural/glial antigen (NG2) [129]. A 21.97% ± 3.99% recovery of bone volume was observed ten weeks after PLGA/PEG-PNIPAM hydrogels comprised of *miR-222* mimics and Aspirin were fitted into mandibular defects [129]. The study also discovered an interesting dynamic that coupled stable bone growth with innervation [129]. Hydrogels therefore possess versatile material properties that can be tailored accordingly to meet diverse tissue engineering criteria.

Silk

In recent years, silk has emerged as an alternative biomaterial that can replace metal screws or substitutes such as poly-L-lactic acid, polyglycolic acid (PGA) and poly-lactic-coglycolic acid (PLGA) in fracture fixation [130]. It has also been found to be biodegradable with its resorption conducive to osteoid formation and bone healing [131–134]. *In vivo* studies have found that silk screws are self-tapping, and silk plates are adaptable to the natural curvature of bone, eliminating additional procedures that otherwise complicate orthopedic surgical implantations [130]. James et al. fabricated silk films using *Bombyx mori*. Human mesenchymal stem cells were transfected with either a nonspecific vector or *miR-214* inhibition oligomers, and then seeded onto the silk films. ALP staining, xylene orange staining for calcium deposition, and von Kossa staining suggested that inhibition of *miR-214* in stem cells expressed higher ALP activity levels, higher calcium depositions, and a consequential up-regulation of Runx2, Osx, ATF4, and osteocalcin [135]. The silk films were then manufactured into orthopedic screws and von Kossa stains suggested a relative increase in the optical density of the screws that housed anti-*miR-214* cells [135]. However, follow up *in vivo* studies are required to confirm the feasibility of silk screws and plates for bone regeneration as this biomaterial, in addition to microRNA incorporation, is still a very recent biomedical innovation for craniofacial reconstruction.

Polycaprolactone

Polycaprolactone (PCL) is another material used in tissue engineering due to its biocompatibility, biodegradability and high drug permeability [136]. Tahmasebi et al. fabricated a PCL/microRNA–gelatin nanofiber scaffold for culturing human induced pluripotent stem cells (hiPSCs), which encapsulated *miR-22* and *miR-126* mimics and was designed to sustain a controlled release of the microRNAs over a 14-day period. The study concluded that the hiPSC's, after two weeks of culture, exhibited statistically significant up-regulation of osteoblastic markers including ALP activity, Runx2, osteocalcin and osteonectin [137]. Hoseinzadeh et al. engineered PCL scaffolds that were hybridized with nanohydroxyapatite (nHA) to augment osteogenic differentiation of adipose-tissue derived stem cells. The stem cells were first transfected to repress *miR-221* and were then seeded into the scaffold. After three weeks of coculture, the transfected cells exhibited a statistically significant overexpression of Runx2, osteocalcin, and ALP activity [138]. However, the relationship between the microRNAs discussed and PCL scaffolds has yet to be confirmed by *in vivo* experiments.

Poly(lactic-co-glycolic acid)

Poly(lactic-co-glycolic acid) (PLGA), due to its biodegradability and biocompatibility, is an established biomaterial that has frequent tissue engineering applications with a documented history of successful brain implants in both mice and humans [139,140]. In the case study presented by Laio et al. disc shaped PLGA scaffolds that were seeded with human adipocyte mesenchymal stem cells were implanted into mice calvaria defects. The cells were transduced by baculovirus to stably express *miR-26a*, *miR-29b*, *miR-148b* and *miR-196a*. Stable overexpression of BMP-2 was another baculovirus vector that was incorporated. Interestingly, this study evinced a synergistic relationship between stable up-regulation of

a target microRNA and a target protein, namely *miR-148b* and BMP-2, that resulted in an impressive filling of 94% of the defect area and 89% of the defect volume twelve weeks postoperatively [141]. Qi et al. examined the osteoinduction of human adipose derived mesenchymal stem cells by *miR-181-a/b-1* that were seeded into electrospun nanofibrous bilayer scaffolds comprised of PLGA on the outer layer and polyplex/microRNA/gelatin on the inner layer. Cells were either transduced into stable expression and cultured on tissue culture polystyrene (TCPS) or were seeded into PLGA/gelatin scaffolds that encapsulated *miR-181a/b-1* mimics. Cells that were co-cultured in the PLGA scaffolds were reported to have a statistically significant increase in calcium secretion, ALP activity, Runx-2, collagen type I, osteocalcin and osteopontin than the TCPS control group [142]. However, the efficacy of *miR-181* and PLGA *in vivo* remains in question.

Other polymers

There has been reported success of incorporating therapeutic microRNAs for bone regeneration in novel biomaterials. One such is poly(glycerol sebacate) (PGS), which is an electrospun and crosslinked nanofiber composed of sebacic acid, glycerol and gelatin that is cured in a vacuum under high heat for 1–2 days [143]. PGS is biocompatible and biodegradable, and random cross-linking gives it similar material properties to Vulcan rubber. Some of its limitations, however, deal with an undefined branch structure, low molecular weight, and a high tendency of premature crosslinking [144]. Deng et al. [145] investigated the osteogenic regulation of *miR-31* in biomaterial scaffold comprised of PGS, and transfected rat bone marrow stem cells with lentivirus to stably express either non-specific oligomers, *miR-31*or anti-*miR-31*. The cells were then seeded into disc shaped PGS scaffolds that filled calvaria bone defects in rats. The study found that the PGS scaffolds composed of anti-*miR-31* transformed cells demonstrated a 60% recovery of bone in the calvaria defect eight weeks postoperatively [145]. Poly(sebacoyl diglyceride)(PSeD) was designed as an improvement to PGS with notable advantages including a longer shelf life, a better-defined structure with more free hydroxyl groups and a higher molecular weight, all while preserving the biodegradability and biocompatibility of its predecessor [144]. In two different experiments, Xie et al. examined the delivery of lentiviral transduced rat adipose derived mesenchymal stem cells in PSeD scaffolds. Both studies reported that ~50% of bone volume was recovered in 8 mm rat calvaria defects in mesenchymal stem cells transformed with *miR-135* and anti-*miR-146a*, respectively [146,147]. Additional studies have also engineered scaffolds that were of complex mixtures of the biomaterials discussed above. Moncal et al. fabricated polymeric scaffolds that were comprised of a sophisticated combination of PCL, PLGA, and nanohydroxyapatite, which were then filled with a collagen type I gel to encapsulate rat bone marrow stem cells that were transfected *with miR-148b* by silver nanoparticles. The study found that after eight weeks of implantation into rat calvaria defects, micro-CT scans detected a 78.1 ± 20.8% recovery of bone volume and a 34.7 ± 8.9% normalized bone mineral density in the collagen scaffolds that cultured *miR-148b* overexpressing cells [148] (Table 3).

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Abbreviations

ALP	alkaline phosphatase
EVs	extracellular vesicles
MSCs	mesenchymal stem cells
PCL	Polycaprolactone
PEG	polyethylene glycol
PEI	polyethylenimine
PGS	poly(glycerol sebacate)
PLGA	Poly(lactic-co-glycolic acid)
PMIS	Plasmid-based microRNA Inhibitor System
PSeD	Poly(sebacoyl diglyceride)
TCP	tricalcium phosphate

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Perspectives

- The direct delivery of miRs to cells and tissues has the advantage to provide a promising approach for bone and tissue regeneration. PMIS-miR gene therapy works both *in vivo* and *in vitro*, and is specific with no off-target effects, no toxicity and is efficient and effective.
- We have shown that highly purified naked plasmid DNA encoding PMIS-miR inhibitors can safely and effectively increase the expression of PMIS transcripts *in vitro* and *in vivo*. We have used the PMIS system to generate mouse models to study the *in vivo* role of miRs
- The PMIS transcripts expressed from the plasmid DNA can transform neighboring cells by extracellular vesicles containing the PMIS miR inhibitor transcript. Extracellular vesicle expansion of the PMIS transcripts allows for rapid PMIS expression during tissue regeneration. Experiments in dogs, rats and mice demonstrate that the PMIS is not toxic and highly effective.

Table 1.

miRs that regulate osteogenic differentiation of MSCs.

miR	MSC types	Effect on osteogenic differentiation	Targeting gene/pathway	Reference
miR-23a	MC3T3-E1	Suppression	*Satb2*/TGF-β signaling	[50,51]
	HOS		*GJA1*	[52]
miR-27a	MC3T3-E1	Suppression	*Satb2*	[51]
	MC3T3-E1		*Prdm16*/TGF-β signaling	[50]
miR-200a	hPDLSC	Suppression	*ZEB2*/NF-κB	[53]
	hBMMSC		*GLS*	[54]
miR-200c	hBMMSC	Promotion	*Klf4*, Sox2/Wnt signaling	[55]
	HEPM		*IL-6, IL-8, CCl-5*	[56]
miR-889	hBMMSC	Suppression	*WNT7A/Wnt* signaling	[57]
miR-4286	hBMMSC	Promotion	*HDAC3*	[58]
miR-503	Rat MSC	Promotion	*Smurf1*	[59]
miR-483-3p	hFOB1.19	Promotion	*DKK2*/Wnt signaling	[60]
miR-424	WJSC	Promotion	-	[61]
miR-10b	hADSC	Promotion	*SMAD2*/TGF-β signaling	[62]
miR-378	hMSC	Suppression	*WNT6, WNT10A*/Wnt signaling	[48]
miR-375-3p	MC3T3-E1	Suppression	*LRP5*, β-catenin/Wnt signaling	[63]
miR-100-5p	hBMMSC	Suppression	*BMPR2*/BMPR2-SMAD1/5/9 signaling	[64]
miR-29b	Primary fetal rat calvaria osteoblasts MC3T3-E1	Suppression	*COL1A1, COL5A3, COL4A2*	[65]
miR-29a	HUVEC	Promotion	-	[66]
miR-16-2	hBMMSC	Suppression	*WNT5A/Wnt* signaling	[67]
miR-22	hBMMSC	Suppression	*YWHAZ*	[68]

Table 2.

Comparison of *miR-23-27-24* sequences

	Sequences	
miRs	***Mus musculus* (mmu)**	***Homo sapiens* (hsa)**
miR-23a-3p	AUCACA**U**UGCCAGGGAUUUCC	AUCACAUUGCCAGGGAUUUCC
miR-23b-3p	AUCACA**U**UGCCAGGGAUUACC	AUCACAUUGCCAGGGAUUACC
miR-24-1-3p	UGGCUCAGUUCAGCAGGAACAG	UGGCUCAGUUCAGCAGGAACAG
miR-24-2-3p	UGGCUCAGUUCAGCAGGAACAG	UGGCUCAGUUCAGCAGGAACAG
miR-27a-3p	UUCACA**G**UGGCUAAGUUCCGC	-
miR-27b-3p	UUCACA**G**UGGCUAAGUUCUGC	-
miR-27a-5p	-	AGAGCUUAGCUGAUUGGUGAAC
miR-27b-5p	-	AGGGCUUAGCUGCUUGUGAGCA

* The difference between two sequences from the same species are shown in red.

* The one nucleotide difference in the seed sequence osf *miR-23* and *miR-27* is shown in bold.

* *miR-24-1* and *miR-24-2* share the same 3p sequence, which terms *miR-24-3p* based on the database.

Table 3.

Biomaterials used in the application of specific miRs for bone formation and regeneration.

Biomaterial	miRs	Main Observation	*In vivo* testing?	Reference Index
Hydroxyapatite	*anti-miR-16*	Statistically significant upregulation of osteoblastic markers	no	[114]
	miR-26a	Notably enhanced bone growth in calvaria defect	yes	[115]
	anti-miR-34a	3.5-fold increase in formation of new bone	yes	[112]
	anti-miR-138	60% increase in new bone growth	yes	[111]
	anti-miR-221	Hard tissue formation detected and partial recovery of defect	yes	[113]
β-Tricalcium Phosphate	*miR-26a*	Notably enhanced bone growth in calvaria defect	yes	[124]
	anti-miR-31	35.42 ± 6.12 % and 41.82 ± 6.54% increase in bone volume, respectively	yes	[122,123]
	miR-200c	Statistically significant increase in new bone formation	yes	[84]
	anti-miR-335-5p	40.9% recovery of femoral bone defect	yes	[121]
Hydrogel	*miR-26a*	Complete restoration of calvaria bone defect	yes	[127]
	miR-148b	32.53 ± 8.3% recovery of calvaria bone defect	yes	[128]
	miR-222/ Aspirin	21.97 ± 3.99% recovery of bone volume in mandibular defect	yes	[129]
Silk	*anti-miR-214*	Statistically significant upregulation of osteoblastic markers	no	[135]
Poly-caprolactone	*miR-22*	Statistically significant upregulation of osteoblastic markers	no	[137]
	miR-126	Statistically significant upregulation of osteoblastic markers	no	[137]
	anti-miR-221	Statistically significant upregulation of osteoblastic markers	no	[138]
Poly (lactic-co-glycolic acid)	*miR-148b/BMP-2*	94% filling of area and 89% volume recovery in calvaria bone defect	yes	[141]
	miR-181a/b-1	Statistically significant upregulation of osteoblastic markers	no	[142]
Poly(glycerol sebacate)	*anti-miR-31*	41.82 ± 6.54 % recovery of bone volume in calvaria bone defect	yes	[145]
Poly(sebacoyl diglyceride)	*miR-135*	50.53 ± 4.45% increase in bone volume of cranial bone defect	yes	[146]
	anti-miR-146a	49.8 ± 5.49% increase in bone volume of cranial bone defect	yes	[147]
Collagen	*miR-148b*	78.1 ± 20.8% area recovery of calvaria bone defect	yes	[148]