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MicroRNA Delivery for Regenerative Medicine

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Abstract

MicroRNA (miRNA) directs post-transcriptional regulation of a network of genes by targeting mRNA. Although relatively recent in development, many miRNAs direct differentiation of various stem cells including induced pluripotent stem cells (iPSCs), a major player in regenerative medicine. An effective and safe delivery of miRNA holds the key to translating miRNA technologies. Both viral and nonviral delivery systems have seen success in miRNA delivery, and each approach possesses advantages and disadvantages. A number of studies have demonstrated success in augmenting osteogenesis, improving cardiogenesis, and reducing fibrosis among many other tissue engineering applications. A scaffold-based approach with the possibility of local and sustained delivery of miRNA is particularly attractive since the physical cues provided by the scaffold may synergize with the biochemical cues induced by miRNA therapy. Herein, we first briefly cover the application of miRNA to direct stem cell fate via replacement and inhibition therapies, followed by the discussion of the promising viral and nonviral delivery systems. Next we present the unique advantages of a scaffold-based delivery in achieving lineage-specific differentiation and tissue development.

Graphical Abstract

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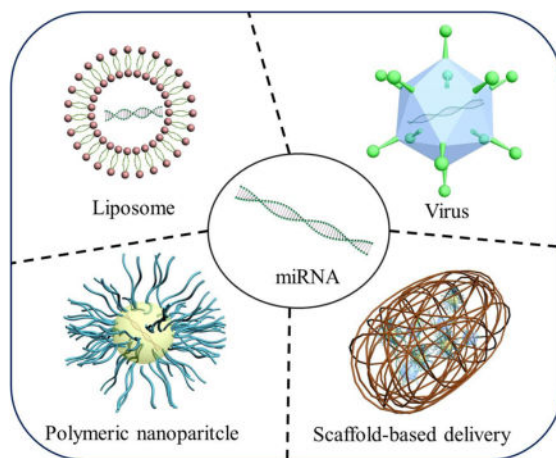
Keywords

MicroRNA; Non-viral vector; Regenerative medicine; Scaffold; Stem cells; Tissue engineering; Viral vector

1. Introduction

Regenerative medicine aims to restore normal functions of damaged cells, organs, or tissues [1]. It holds promise in treating trauma, burns, degenerative diseases, and other maladies that cause tissue or organ failures. Cytokines and growth factors are widely used to augment the cell-mediated tissue regeneration. However, protein delivery suffers from the inherent disadvantages of limited stability, high cost, and short half-life [2]. Gene therapy offers an alternative [3–5]. Instead of using plasmid DNA to produce the growth factors, new development of microRNA (miRNA) has attracted much interest because it can influence a wide range of cell functions including the control of proliferation, differentiation, apoptosis and other metabolic processes by down-regulating or up-regulating the expression of their target genes [6–9]. There are over 1000 miRNAs in humans and they regulate over 30% of genomic RNAs [10, 11].

As a post-transcriptional gene regulator, miRNA is small non-coding single-strand endogenous RNA with length of 20–24 nucleotides (nt) [12]. It is derived from the precursor transcript called primary miRNA (pri-miRNA) which contains stem-loop structures with length of hundreds to thousands nt. Pri-miRNA is further processed in the nucleus by DGCR8 and ribonuclease Drosha into 70 to 100 nt long hairpin structure, called pre-miRNA [13–15]. The pre-miRNA is then translocated into the cytoplasm by a shuttle system containing the Exportin 5 and its cofactor Ran. In the cytoplasm it is further processed by Dicer, an RNase III-like enzyme, into approximately 22 nt double-strand miRNA duplex (Fig. 1) [16, 17]. As the mature miRNA strand loads onto the miRNA-induced silencing complex (miRISC), the passenger strand (also denoted as miRNA*) is released and subsequently degraded [18, 19].

The mature miRNA strand in the miRISC can recognize the target mRNA by binding of the seed sequence (position 2 to 8 from the 5′ end) of the mature miRNA strand to the 3′ untranslated region of mRNA. This specific interaction generally results in deadenylation, decapping, translation inhibition and eventually degradation of the target mRNA [20, 21]. However, in rare cases, the translation could be promoted rather than repressed by the miRNA [22]. Unlike the short interfering RNA (siRNA), complementarity between miRNA and mRNA does not require perfect pairing, which means a single miRNA could simultaneously recognize hundreds of target mRNAs and an mRNA could be regulated by multiple miRNAs [23]. The complex miRNA regulation network has profound impact on the expression of an abundance of proteins, and eventually important ramifications on many developmental and cellular processes.

Recently, the identification of stem-cell-specific miRNA has brought the stem cell and miRNA fields together [6, 7, 24]. It suggests that miRNA therapy could impact regenerative medicine. A great obstacle to the progression of miRNA therapy is delivery of miRNA to the desired cell types, tissues or organs. Because of the negative charge of miRNA, it cannot penetrate the cellular membrane by passive endocytosis. Furthermore, rapid clearance of naked miRNA in the blood stream renders its systemic delivery highly inefficient. So the successful application of miRNA largely depends on the development of effective delivery platforms [25]. The aim of this review is to capture the state-of-art of miRNA delivery systems and highlight their design consideration from the regenerative medicine perspective.

2. MicroRNA therapeutic approaches

It is now clear that miRNA plays an important role in directing stem cell fate by fine-tuning the levels of various factors. *Ex vivo* manipulation of the miRNA level in stem cells is a viable strategy for regenerative applications. Depending on the expression status of the target miRNA, the miRNA therapy could be separated into miRNA replacement therapy which up-regulates miRNA expression and miRNA inhibition therapy which down-regulates miRNA expression.

2.1. miRNA replacement therapy

The levels of one or more miRNAs of stem cells will change significantly during differentiation. Overexpression of the target miRNA would be a viable strategy to enhance this differentiation process. The miRNA replacement therapy could be performed in two ways. The first one is delivery of miRNA mimics that are double-strand oligonucleotides containing the same sequence as the mature endogenous miRNA. Because the miRNA mimics possess the same structure with the miRNA duplex, they enter the miRISC complex and affect the target mRNA [26]. Although single-strand RNA molecules could also work as miRNA mimics, the high potency of double-strand miRNA mimics (100 to 1000 fold higher than single-strand miRNA mimics) [27] makes them much better candidates.

However, subtle difference in physico-chemical properties between DNA and RNA renders the optimization of miRNA delivery not so straightforward; one cannot assume that an efficient DNA delivery system is also efficient for miRNA delivery. The persistence length of DNA is about 50 nm [28], making a typical pDNA of several Kb a flexible molecule

amenable to condensation by polycations. In contrast, the persistence length of RNA is about 70 nm (260 nt) [29], and it is also a stiffer molecule more resistant to efficient condensation. So just like siRNA polyplexes, miRNA polyplexes tend to be larger and have fewer options of cellular uptake; for instance, they are not typically internalized via the clatherin-mediated endocytic pathway [30–32].

The second mode of miRNA activation is delivery of synthetic miRNA precursor mimics or miRNA-expressing DNAs which could be incorporated into the viral vectors [33]. This strategy possesses the advantage of sustained generation of miRNA, which is especially important for regeneration application. However, since pri-miRNA is processed in the nucleus, nuclear targeting is required for delivery of pri-miRNA. On the other hand, delivery of pri-miRNA might saturate the RNA machinery, resulting in off-target effects that are undesirable [34].

2.2. miRNA inhibition therapy

In contrast to the miRNA replacement therapy, the miRNA inhibition therapy aims to block the miRNA repression of protein expression. Several methods have been developed to inhibit miRNA functions through disruption of the miRISC complex (Fig. 2). The most straightforward method is to use anti-miRNA oligonucleotide (AMO), which is complementary to the miRNA mature strand, to inhibit interactions between miRISC and its target mRNA. To achieve effective inhibition, several independent chemical modifications have been adopted to improve the affinity and stability of AMOs [35, 36].

AntagomiRs are the first miRNA inhibitors demonstrated to work in mammals [37]. They contain 2′-O-methyl-modified ribose sugars (2′-OMe), a terminal phosphorothioate linkage instead of a natural phosphate linkage, and a cholesterol group at the 3′ end. Although AntagomiRs have shown higher stability than AMOs and effective inhibition of specific miRNA in several tissues, the high dose required for effective inhibition hinders their application.

Locked nucleic acid (LNA) antisense nucleotides are modified with an extra bridge connecting the 2′ oxygen and 4′ carbon (Fig. 3) [38]. The locked ribose conformation increases the resistance to many endonucleases and the RNA melting temperature significantly. Lower dose of LNA-anti-miRs required for miRNA inhibition makes them more suitable for regenerative medicine applications.

Peptide nucleic acids (PNAs) are synthetic polymers with a structure similar to that of nucleic acids and function like nucleic acids with high specificity, and additional benefit of stability [39, 40]. Different from RNA, the backbone of PNA is N-(2-aminoethyl)-glycine. Because of the non-charge nature of PNAs, transfection agents are usually not required for delivery of PNAs. Cell penetrating peptides can be linked with PNAs to enhance their delivery using standard peptide chemistry.

MiRNA sponges are DNA sequences with multiple binding sites to the miRNA. They are usually expressed by plasmid DNAs encoding the miRNA sponges to saturate the miRISC complex. Different miRNA binding sites could be introduced into miRNA sponges to inhibit

a family of miRNAs. Viral vectors could be used to deliver miRNA sponges to a number of hard-to-transfect cells. However, before applying miRNA sponges clinically, the expression of transcripts must be precisely controlled to avoid the unwanted side effects [41, 42].

The miRNA inhibition strategies mentioned above always sequester all the functions of target miRNA. Because a single miRNA could regulate expression of many genes, this inhibition strategy may lead to unwanted side effects. So miRNA masks are developed to minimize the off-target effects. MiRNA masks are designed to selectively block specific mRNA pathway, consequently only the specific protein expression is inhibited. But the clear profile of miRNA target genes is required.

3. Therapeutic prospect of microRNA in regenerative medicine

Since the discovery of microRNAs (lin-4 and let-7) playing a crucial role in the development of C. elegans, their role in the mammalian system has been expanding into development, metabolism, proliferation, and apoptosis [43]. More importantly, microRNA plays an important role in the differentiation and proliferation of stem cells. Unlike siRNA, miRNA is an endogenous RNA. So either activation or inhibition strategy can be used to up-regulate or down-regulate miRNA expression during the development and differentiation process to achieve therapeutic effects.

3.1 MiRNA in bone regeneration

There are two fundamentally distinct processes for bone formation. Direct differentiation of mesenchymal stem cells (MSCs) into osteoblasts produces intramembranous bones such as calvarial bone. Differentiaton of MSCs toward chondrocytes via endochondral ossification produces a cartilaginous template, which contributes to the longitudinal growth of long bones [44]. Depletion of miRNAs by conditional deletion of the miRNA-processing endoribonuclease Dicer enzyme reveals the role of miRNAs in these two processes [45, 46].

MiRNAs are involved in multiple pathways for promoting osteoblast differentiation [47]. Runx2 is a transcription factor essential for osteoblastogenesis. The miRNA cluster 23a~27a~24–2 could repress the osteoblast differentiation by targeting the SATB2 protein, a co-regulator of Runx2. Conversely, Runx2 could inhibit expression of those miRNAs by a feedforward mechanism to de-repress SATB2, resulting in promotion of osteoblast differentiation [48]. However, miR-27a in the cluster could downregulate Hoxa10, an activator of Runx2. It further results in repression of Runx2. During the mineralization of bone formation, miR-23a could repress Runx2 to attenuate osteoblast maturation. A second miRNA regulatory loop is induced by the BMP2-Runx2 pathway. In the BMP2-induced osteogenesis of ST2 stromal cells, Runx2 increases the expression of miR-2861~miR-3960 cluster by binding to their promoter. MiR-2861 and miR-3960 maximize Runx2 activity to drive differentiation by targeting two Runx2 inhibitors, Hdac5 and Hoxa2, respectively [49]. Thus, fine-tuning the miRNA levels spatially and temporally could be a delicate way for bone regeneration. To illustrate the importance of miRNA in osteogenesis, introduction or inhibition of several miRNAs including miR-31 [50], miR-26a [51, 52] and miR-29b [52] for calvarial defect repair has been evaluated *in vivo*.

In addition to intramembranous bone formation, miRNA also participates in the endochondral ossification process [53]. *In vitro* studies have identified several miRNAs related to the MSC chondrogenic differentiation, including negative regulators miR-145 and miR-449a [54, 55] and positive regulator miR-23b [56]. MiR-449a could repress expression of SOX9, the essential transcription factor for chondrogenesis, leading to delayed progression of chondrogenesis while positive regulator could promote chondrogenic differentiation of human MSCs by inhibiting protein kinase A (PKA) signaling [55].

Expression of miR-140 is predominantly limited to cartilage [57]. The positive function of miR-140 in craniofacial development and endochondral bone formation has been proved both in mouse and zebrafish models [58, 59]. MiR-140 represses two negative effectors including histone deactylase 4 (HDAC4; a known inhibitor of chondrocyte hypertrophy) and a splicing factor which reduces BMP signaling [59].

3.2 MiRNA in wound healing

The wound healing process in adults is a highly orchestrated physiological process involving keratinocytes, fibroblasts, endothelial cells, macrophages and platelets [60]. It is regulated by a complex signaling network of molecules including several miRNAs [61]. It can be divided into three overlapping phases: inflammation, proliferation and remodeling. The migration, proliferation and differentiation of keratinocytes in the proliferation phase are the key steps in wound healing. The keratinocytes fill in the gap created by the wound and restore the integrity of the skin [62]. Inhibition of miR-198, which could switch off the expression of follistatin-like 1 protein, has proved useful to enhance the keratinocyte migration and proliferation [63]. Due to the complexity of wound healing process, simply activation or inhibition of miRNA expression may not be enough. For instance, although miR-21 could promote keratinocyte migration and re-epithelialization [64], overexpression of miR-21 would also induce inhibition of epithelialization and granulation tissue formation in a rat wound model [65].

The maturation phase is important for wound contraction and scar formation. It is characterized by ECM adjustment, remodeling of collagen from type III to type I, and the replacement of granulation tissue by scar tissue. MiR-29b is a positive effector in this process by directly targeting ECM genes, such as fibronectin, collagen type I, and collagen type III. Topical application of miR-29b to mouse wound has proved effective to improve the collagen type III/I ratio, increase the activity of matrix metalloproteinase 8, and enhance scarless wound healing [66].

3.3. MiRNA in skeletal muscle and cardiac regeneration

Although skeletal muscle has a remarkable capacity to respond to pathological stress and regenerate upon injury, severe traumatic injuries associated with significant muscle tissue loss and gaps could not be repaired endogenously, such as volumetric muscle loss (VML) injuries. Similar to the wound healing process, skeletal muscle repair can also be roughly divided into three stages: demolition stage, repair stage and remodeling stage [67]. During this process, satellite cells (resident mononuclear stem cells) serve as the dominant contributor to muscle repair [68]. *In vivo* study establishes that miR-206 promotes satellite

cell differentiation during skeletal muscle regeneration in response to injury [69], via a mechanism of repressing a set of negative regulators including Pax7, Notch3, and Igfbp5 [70]. Besides, overexpression of miR-206 could upregulate the expression of various growth factors by inhibition of connexin 43 (CX43) and histone deacetylase 4 (HDAC4) [71]. MiR-26a also promotes differentiation of myoblasts [72]. It targets the transcription factors Smad1 and Smad4, which are critical for the TGF-b/BMP pathway. Inhibition of miR-26a after injury could prevent myogenesis and delay muscle regeneration [73].

Unlike skeletal muscle, repair of cardiac damage through myocardial regeneration is limited because of the low proliferative rate of cardiomyocytes during adult life [74]. MiRNA can activate cardiomyocyte proliferation and promote mammalian cardiac repair *in vivo* [75–77]. The miR-15 family, including miR-195, miR-15a, miR-15b, miR-16 and miR-497, plays key role of postnatal cardiomyocyte mitotic arrest by targeting multiple cell cycle regulators, including the checkpoint kinase 1 (Chek1) [78]. Through large scale screening of miRNA, miR-590 and miR-199a have been identified as the promotors of cardiomyocyte proliferation in both neonatal and adult animals [79]. They also show the ability to stimulate remarkable cardiac regeneration in the murine myocardial infarction model.

Another way to repair cardiac damage is to stimulate the differentiation of cardiac stem cells (CSCs) into cardiomyocyte [75]. MiR-499 is reported as a negative regulator of Sox6 and Rod1 and promotor of CSC differentiation [80, 81]. Most importantly, miR-499-overexpressed hCSCs show enhanced cardiac differentiation and regeneration abilities. Injection of these cells into the infarcted mouse heart leads to a 2.1-fold greater regenerated myocyte mass and better preservation of the left ventricular (LV) function than injection of control hCSCs.

3.4. MiRNA in angiogenesis

Angiogenesis is one of the most important parameters to optimize in tissue engineering such as bone repair and wound healing [52, 82]. During angiogenesis, endothelial cells are induced to proliferate, migrate out of an existing vessel, differentiate, and assemble to form branches of tubules capable of carrying blood and its constituents. Mice with endothelial-selective Dicer inactivation have an impaired angiogenic response to limb ischemia, proving that miRNAs are important for angiogenesis [83–85]. Overexpression of miR-503 in endothelial cells results in impaired cell proliferation, migration, and ability to form networks by targeting the cell division cycle 25 homolog A and cyclin E1 [86]. Corresponding *in vivo* study shows that local miR-503 inhibition could correct diabetes mellitus-induced impairment of post-ischemic angiogenesis and recover the blood flow in diabetic mice. Cardiac endothelial cells enriched miR-24 could inhibit transcription factor GATA2 and the p21-activated kinase PAK4, resulting in endothelial cell apoptosis, impaired endothelial capillary network formation, and cell sprouting [87]. Blocking of endothelial miR-24 leads to improved myocardial angiogenesis and cardiac function in the mouse myocardial infarction model [88].

Due to the pathogenic relationship between vascularity and tumor, the use of miRNA to promote angiogenesis might increase the risk of oncogenesis [89]. For instacne, the miR-17–92 cluster (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92–1) improves

angiogenesis both *in vitro* and *in vivo* [90]. However, this cluster has also been identified as tumor-promoting miRNA oncomiR-1. Therefore, the use of miRNA for angiogenesis must be judiciously applied [91].

3.5. MiRNA in neurogenesis

The poor regenerative potential of the nervous system has stimulated various research strategies to induce neuronal regeneration using reprogrammed neuronal cells. The main obstacle in neuronal reprogramming is the low neuroblasts differentiation yield of adult stem cells. MiRNA plays essential roles in neurogenesis [92–94], particularly the ones (miR-9, -124, -128, -132, -134, -138) that are specifically or richly expressed in the brain and spinal cord [95]. Among them, miR-124 and miR-9/9* could induce direct conversion of fibroblasts into neuronal-like cells [96–98]. Upon neuronal differentiation, BAF53a (subunit composition of ATP-dependent BAF chromatin remodeling complex) is replaced by its homolog BAF53b. Ectopic expression of miR-124 and miR-9* induces the down-regulation of BAF53a, allowing the incorporation of BAF53b and neurogenesis. MiR-124 could also inhibit small carboxy-terminal domain phosphatase 1 (SCP1; also known as CTDSP1 [99], a component of the RE1-silencing transcription factor (REST)) and polypyrimidine tract binding protein 1 (PTBP1; a repressor of neuron-specific splicing) [100] while miR-9 targets TLX, a highly conserved orphan nuclear receptor that is critical for neural stem cell self-renewal [101]. Notch signaling is one of the key pathways regulating neuronal development and expansion of neural progenitors. MiR-124 also targets several components of the Notch signaling cascade including Notch ligand *Jag1* [102] and the Notch down-stream effector *Sox9* [103] to increase neurogenesis. In addition, miR-9 regulates the Hes gene family members [104].

Repair of the central nervous system is much more challenging than that of the peripheral nervous system. It is largely due to the failure of remyelination by oligodendrocytes, which do not proliferate in response to injury [105]. Microarray analyses have identified a series of microRNAs that are developmentally regulated during maturation of oligodendrocytes [106]. MiR-219 and miR-338 discovered in this study promote oligodendrocyte differentiation by repressing transcription factors Sox6 and Hes5, the antagonists of oligodendrocyte maturation [107].

4. MicroRNA delivery systems for regenerative medicine

It is now widely recognized that effective delivery system is the key to miRNA development. Primary stem cells are more difficult to transfect than immortalized cell lines, therefore requiring a more efficient miRNA delivery system for regenerative medicine applications. Tissue regeneration or development can also be a lengthy process. Besides the obvious requirements of low cytotoxicity and high transfection efficiency, an ideal miRNA delivery system for regenerative medicine should be able to deliver the miRNA at specific tissue or organ in a local and sustained manner.

In the context of this review, miRNA delivery systems could be simply divided into systemic or scaffold-mediated delivery. Systemic or bolus gene delivery systems for DNA or siRNA delivery have been well studied and expertly reviewed in the past two decades

[34, 113–115]. Numerous viral and non-viral vectors with high transfection efficiency, good biocompatibility, and even high targeting efficiency have been developed. Those vectors might be directly adaptable for miRNA delivery. However, despite the promise of systematic delivery systems they are intended mostly for cancer therapy or genetic diseases [26, 27, 116], and may not meet the requirement of high and sustained miRNA levels for regenerative medicine applications.

Scaffold-mediated delivery by encapsulating or immobilizing the miRNA in or onto the tissue engineering scaffold addresses such requirement for regenerative medicine applications. Scaffold-mediated delivery offers a three-dimensional (3D) distribution of miRNA at target tissue for more controlled, localized transfection. Furthermore, the miRNA could be released in accordance with the degradation of the scaffold to achieve a transgene expression kinetics matching the profile of tissue regeneration. By carefully choosing the scaffold fabrication and sterilization process, loss of miRNA activity could also be minimized. It is an emerging approach with limited report, but the promise is stimulating chemical, biochemical, and mechanical innovations to design miRNA-functionalized scaffolds for tissue regeneration.

4.1. Systemic miRNA delivery systems

4.1.1. Viral vectors—Various viral vectors can now transfect the majority of cell types [116, 117]. The major drawback of miRNA is its relative short half-life due to the susceptibility to degradation by RNase. They only exert a transient silencing effect because miRNA concentration decreases with cell division, although gene construct design can mitigate this transient effect. The high transduction efficiency and sustained transgene expression of viral vectors render them attractive for miRNA delivery both *in vitro* and *in vivo* (Table 2). Safety issues such as potential immunogenicity and insertional mutagenesis remain the main barriers for clinical translation of viral vectors [116, 118].

Adeno-associated virus (AAV) is a non-enveloped virus from the Parvoviridae family with single-strand DNA genomes of 4.7 kb. To date, 12 primate serotypes (AAV1-12) have been identified [119]. AAV vectors are the smallest known viral vectors. However, they are ideal for the delivery of miRNA because of smaller size of miRNA compared to pDNA. Furthermore, their non-pathogenicity in humans is attractive. Because rAAV9 vector has shown high affinity for myocardium, it is frequently used for delivering miRNA-related therapeutic molecules to the heart [120]. Mauro Giacca *et al.* [79] used rAAV9 to deliver miR-590 and miR-199a effectively to the heart of neonatal mice by both intraperitoneal and intracardiac injection. The former resulted in ~240-fold increase of miR-590 expression and 3.5-fold increase of miR-199a expression comparing to control rAAV9 vector. The latter was even more effective, which led to over 1000-fold increase of miR-590 expression and 13-fold increase of miR-199a expression. One single intracardiac injection of rAAV9-miR-590 or rAAV9-miR-199a could boost the normally ineffective myocardial repair and restore the cardiac function. The left ventricular ejection fraction (LVEF) increased from 38% to 58% (rAAV9-miR-590) or 52% (rAAV9-miR-199a), fractional shortening (LVFS) increased from 19% to 31% (rAAV9-miR-590) or 26% (rAAV9-miR-199a), and end-systolic anterior wall thickness (LVAW) increased from 0.75 mm to 1.12 mm (rAAV9-

miR-590) or 0.95 mm (rAAV9-miR-199a) after 60 days. The infarct size also decreased from 32% LV to 14% LV (rAAV9-miR-590) or 13% LV (rAAV9-miR-199a) after 60 days.

Retroviruses are enveloped viruses that carry two copies of the single-strand RNA genome of ~10 kb. Upon cell entry, the RNA is copied by the reverse transcriptase enzyme into double-strand DNA. It stably integrates into one of the host chromosomes, guaranteeing long-term expression of inserted therapeutic genes. Viral protein synthesis is not required for retroviral entry and genome integration, therefore all viral genes can be replaced with foreign sequences [121, 122].

Retrovirus vectors are frequently used for cellular reprogramming because of their ability to simultaneously deliver four full-length genes (i.e., either Oct4-Sox2-c-Myc-Klf4 or Oct4-Sox2-Nanog-Lin28) into targeted somatic cells. So they have also been used to deliver miRNA with or without those genes to generate induced pluripotent stem cells (iPSCs). Using a retrovirus vector as a carrier, Ye *et al.* [123] delivered miR-138 together with Oct4, Sox2, and Klf4 (OSK) to OG-mouse embryonic fibroblast (MEFs) to promote the iPSCs generation. The delivery of miR-138 reduced the level of p53 by 60% and promoted the reprogramming with 3 fold higher efficiency than using OSK alone. Moreover, single miRNA delivery by retrovirus vector may be enough to achieve cellular reprogramming. For instance, human embryo stem cell-like pluripotent stem cells can be generated by retroviral delivery of miR-302s into human cancer cell lines including Colo and PC3 cells at extremely high transfection efficiency (~100%) [124]. Retrovirus-based constructs can also deliver multiple miRNAs. To study the importance of miRNA gene cluster miR-106b~25 (miR-106b, miR-93, and miR-25) in neurogenesis, researchers delivered a retrovirus vector encoding the cluster to neural stem/progenitor cells (NSPCs) [125]. As a result, the expression of miR-106b, miR-93, and miR-25 was increased by 16, 11, and 30 fold, respectively, compared with empty retrovirus. In addition, Tuj1-positive (a marker of neurons) cells in the NSPCs infected by miR-106b~25 retroviruses were increased by 2.6 fold comparing with the NSPCs infected by empty retrovirus, indicating that delivery of miR-106b~25 could enhance neurogenesis effectively.

Lentivirus forms a subgroup of retrovirus that can integrate the exogenous gene into the host genome [126], enabling a stable miRNA expression and a long-term silencing effect. Unlike retroviruses, lentiviruses favor integration within introns of active transcriptional units, which limits their potential to cause insertional oncogenesis [127][66], so they are more frequently used in miRNA delivery for regenerative medicine research in recent years.

To explore whether miR-424 could regulate the differentiation of human dental pulp cells (hDPCs) into vascular lineage, both miR-424 and anti-miR-424 were delivered into hDPCs by lentivirus vector with over 70% transfection efficiency [128]. As a result, the miR-424 level was elevated more than 3 fold in miR-424 overexpression cells relative to empty vector-transfected cells and reduced by nearly 70% in miR-424 knockdown cells, respectively. The protein expression of endothelial markers (vWF and KDR) was enhanced by miR-424 knockdown. This study proves that miR-424 plays a negative role in regulating endothelial differentiation of hDPCs; consequently the inhibition of miR-424 expression in hDPCs promotes endothelial differentiation. Transfection of mesenchymal stem cells

(MSCs) was also achieved by miR-1 lentiviral vector with efficiency over 90% [129]. Overexpression of miR-1 inhibited the Hes-1 by about 25% at day 1, over 50% at day 7 and over 60% at day 14, resulting in promoted differentiation of MSCs into the cardiac lineage. Lentivirus vectors were also used to deliver anti-miR-31 into adipose tissue-derived stem cells (ASCs) [130] or bone marrow stem cells (BMSCs) [50] for bone regeneration. The inhibition of miR-31 in BMSCs increased the expression of Runx2, Opn and Ocn nearly 3 fold from day 4 to 21, which indicated osteogenic differentiation [50]. Then the poly(glycerol sebacate) (PGS) scaffold seeded with transduced BMSCs was implanted into the 8 mm critical-sized calvarial defects in rats for bone regeneration. Micro-CT showed that knockdown of miR-31 could increase new bone formation; the ratio of new bone volume relative to the tissue volume (BV/TV) was significantly higher than the control group (22.18±3.39 % vs 41.82 ±6.54 %) at week 8 (Fig. 4A).

Besides the common viral vectors, baculoviral vector is being increasingly investigated among various viral gene delivery systems. Baculoviruses are insect viruses in nature that can transduce a wide variety of stem cells including ASCs and BMSCs at high efficiency [131]. Unlike retroviruses, baculoviruses neither replicate inside the transduced mammalian cells nor integrate into host chromosomes. Importantly, baculoviruses are non-pathogenic to humans and baculoviral DNAs degrade in the mammalian cells over time [132]. Although rarely reported as miRNA carriers, the high transfection efficiency and low toxicity of baculoviruses would render them promising miRNA vectors for regenerative medicine [133, 134]. Liao *et al* [51] transduced human adipose derived stem cells (hASCs) with baculovirus vectors expressing human miR-26a, miR-29b, miR-148b and miR-196a respectively to stimulate the osteogenesis, in which mature miRNA level of miR-26a, miR-29b, miR-148b and miR-196a increased 5 fold, 4.8 fold, 7.9 fold and 3.4 fold at day 1. However, Bac-miR148b and Bac-miR196a induced more calcium deposition and higher expression of osteopontin at day 14, revealing that miR-148b and miR-196a expression triggered more potent osteogenesis than miR-26a and miR-29b. Implantation of the hASCs transfected with miR-148b-expressing and bone morphogenetic protein 2 (BMP-2)-expressing baculovirus vectors (Bac-FLPo/Bac-FCBW) into critical-sized (4 mm in diameter) calvarial bone defects in nude mice accelerated and potentiated the bone healing and remodeling, filling ~94% of defect area and ~89% of defect volume of native calvaria-like flat bone in 12 weeks (Fig. 4B). This study shows that baculoviruses can deliver miRNA into stem cells efficiently *in vitro*.

4.1.2. Lipid-based delivery systems—Lipid-based systems are among the most commonly used non-viral vectors *in vitro* [35, 140]. In comparison to viral transduction, nonviral delivery systems such as lipid-based (lipoplex) and polymer-based (polyplex) systems impose a lower degree of genetic perturbation and may be more beneficial to the clinical application of stem cell differentiation. Many cationic lipid-based systems can be selected from a number of commercially available products (Table 3), including Lipofectamine® [139, 141, 142], siPORT™ [143], Oligofectamine™ [144], MaxSuppressor™ In Vivo RNA-LANCEr II [76], HiPerFect [77], Lullaby [145], DharmaFECT® [146], SilentFect™ [101] and NeuroPorter™ [147]. Many investigations

have validated the possible delivery of miRNA by lipoplexes. However, safe and efficacious delivery *in vivo* has yet to be achieved due to toxicity and nonspecific uptake.

Sundaram *et al.* [63] used DharmaFECT® to deliver miR-198 mimics to the keratinocytes. Delayed keratinocyte immigration and scratch-wound closure were observed, indicating the negative role of miR-198 in the wound healing process. Consequently, inhibition of miR-198 might offer a different strategy to improve non-healing chronic diabetic ulcers. Commercialized lipid-based transfection reagents have also been used to transfect stem cells. Gain- and loss-of-function studies were performed by transfecting dental pulp cells with miR-720 mimics and miR-720 inhibitor using Lipofectamine® RNAi-MAX [148], resulting in about 50% decrease of *NANOG* mRNA level and promoting odontogenic differentiation.

4.1.3. Polymer-based delivery systems—Numerous cationic polymers have been synthesized for DNA and/or siRNA delivery for different therapeutic purposes [157, 158]. Surprisingly, polymer-mediated miRNA delivery is rarely reported, especially in the field of regenerative medicine. Compared with lipoplexes, polyplexes enjoy a higher degree of versatility via variation in molecular weight, molecular structure, and composition. Development of controlled polymerization such as atom transfer radical polymerization (ATRP), reversible addition-fragmentation chain transfer polymerization (RAFT) and ring-opening polymerization (ROP) offers a powerful tool to synthesize gene carriers with controlled size, well-defined structure and diverse functionalities. For instance, stimuli-sensitive moities such as pH-sensitive, redox-sensitive, or enzyme-sensitive linkages can be incorporated into the gene carrier to facilitate site-specific degradation or intracellular unpacking [158]. A wide choice of functional groups also simplifies ligand conjugation for tissue- and cell-specific delivery [39].

Polyplexes rely on electrostatic interaction between the polycation and the nucleic acid to form a nanocomplex. Polyethylenimine (PEI) is one of the most widely used and studied polycations for gene delivery [159]. The 'proton sponge' effect of PEI causes influx of protons and water into the endosome, which could swell and eventually disrupt the endosome to release the polyplexes to cytoplasm. However, high cytotoxicity prevents its clinical application. A promising modification to lower the cytotoxicity is to crosslink low molecular weight PEI through a disulfide linkage [158, 160]. Further introduction of rabies virus glycoprotein (RVG) enabled the carrier to specifically target to the nicotinic acetylcholine receptors on neuronal cells [158]. *In vitro* delivery of miR-124a, a neuron-specific miRNA that can potentially promote neurogenesis, to Neuro2a cells, revealed efficient intracellular uptake as early as 1 h after transfection and 90% knockdown of luciferase reporter activity. For *in vivo* studies, mannitol was used to facilitate transport across the blood-brain barrier and overcome the size limitation of PEI vector. Analysis of fluorescence signals revealed considerable accumulation of Cy5.5-miRNA in the brain, which represented a promising delivery system for *in vivo* neurogenesis, although the functional activity of miR-124a to promote neurogenesis remained to be investigated.

Poly(lactide-*co*-glycolide) (PLGA) is a biodegradable, FDA-approved copolyester that has been widely used for drug delivery. It can encapsulate miRNA by physical entrapment. The

ability to protect nucleic acids from degradation and prolonged release render them attractive for regenerative medicine application. Surface modification of PLGA nanoparticles such as pegylation or ligand decoration provides further options to control their pharmacodynamics. Saltzman *et al.* [161] showed that PLGA nanoparticles formed by double emulsion could load modified miRNA inhibitor with a neutral backbone (PMO and PNA) with high efficiency while the encapsulate efficiency of ssDNA or DNA-cation polyplexes by the PLGA nanoparticles was quite low. Modification of the PLGA nanoparticles with cell-penetrating peptides could significantly improve their intracellular uptake efficiency. *In vitro* study showed that delivery of anti-miR-155 PNAs and PMOs to KB cells could achieve ~60% knockdown. *In vivo* study further demonstrated that PLGA nanoparticles encapsulating anti-miR-155 PNA could accumulate in the pre-B-cell tumors through enhanced permeability and retention (EPR) effect [39]. After treatment with 1.5 mg/kg anti-miR-155 PNA loaded in PLGA nanoparticles for 5 days, the pre-B cell tumors had an approximately 50% decrease in growth relative to the control.

Other than synthetic polymers such as PEI and PLGA, natural polymers including peptides and polysaccharides have also been reported as miRNA carriers. Chitosan (CS) is one of the most reported natural polymeric gene carriers [162–164]. It has beneficial qualities such as low toxicity, low immunogenicity, biodegradability, as well as pH-adjustable cationic charge density to form polyelectrolyte complexes with negatively charged nucleotides. Deng *et al.* [165] demonstrated that nanocomplexes formed by hyaluronic acid and chitosan (HA-CS NPs) could encapsulate not only the miR-34a mimics but also doxorubicin (DOX) effectively. Delivery of miR-34a mimics to the human breast cancer cells MDA-MB-231 resulted in more than 60% Bcl-2 knockdown. Co-delivery of DOX and miR-34a mimics led to lower IC_{50} than free DOX or HA-CS NPs loaded with DOX alone, indicating the synergistic effect between DOX and miR-34a on cell apoptosis. Not only triggering apoptosis, the HA-CS NPs-mediated miR-34a delivery could also inhibit the migration of breast cancer cells via targeting Notch-1 signal. *In vivo* study showed that the co-delivery of DOX and miR-34a could suppress the growth of MDA-MB-231 solid tumors over 25 days. This miRNA delivery system should also be effective for delivery of miRNA to stem cells.

Another type of nature-derived polymer, cell-penetrating peptide (CPP), has already been tested for stem cell transfection [166]. CPP could significantly improve cellular uptake of various therapeutic molecules both *in vitro* and *in vivo*. Suh *et al.* [167] reported that a non-toxic, arginine-rich CPP called low molecular weight protamine (LMWP) could effectively transfect miR-29b into hMSCs and induce osteogenic differentiation. The cell penetration nature of LMWP promoted cellular uptake as early as 0.5 h post-transfection. After 5 h of treatment, the transfection efficiency of miR-29b using LMWP was 6.5 fold higher than that using a commercialized cationic lipid: Lipofectamine® RNAi MAX. A panel of factors, including HDAC4, CTNNBIP1 and DUSP2 inhibits osteoblastic differentiation. Down-regulating these factors to 50%, 70% and 40%, respectively, by CPP-mediated delivery of miR-29b led to significant calcium deposition for up to 14 days. Not only the enhanced mineralization, but accompanied increase in osteogenic markers indicated the potency of miR-29b in enhancing osteogenesis.

4.2. Scaffold-based delivery systems

Proliferation and differentiation of cells depend on the exogenous signals including diffusible factors, extracellular matrix (ECM) proteins, cell-cell interactions, cell-substrate interactions, as well as biomechanical cues [168, 169]. An ideal regenerative medicine system should mimic the natural microenvironment and provide all those exogenous signals at the appropriate time and location to promote the functional tissue regeneration. By providing a structural support with optimized mechanical properties, surface nanotopography and surface chemistry, a scaffold plays an important role in regenerative medicine applications. However, a typical scaffold cannot provide the appropriate biochemical cues. Controlled release functions of drug and gene delivery incorporated into a scaffold design can overcome this limitation [170, 171].

4.2.1. Advantages of scaffold-based delivery systems—A major challenge of regenerative medicine is how to mimic the complex and dynamic microenvironment for tissue development. Although there are only limited reports on scaffold-based miRNA delivery, studies on using scaffolds for DNA/siRNA delivery have demonstrated the promise for regenerative medicine. First of all, gene transfer from a matrix offers more controlled, localized transfection compared with bolus delivery. By entrapping plasmid DNA in highly porous PLGA scaffold, Shea *et al.* showed that the pDNA released from the scaffold would be principally taken up by the surrounding cells at the implant site [172]. Secondly, delivery from scaffolds can achieve a more prolonged transgene expression over bolus delivery. By encapsulating siRNA/CPP complexes within PCLEEP fibers, Chew *et al.* [173] achieved sustained release of siRNA up to at least 28 days, which was 2 to 3 times longer than conventional bolus delivery. Finally, both the viral and non-viral vectors could be encapsulated into the scaffold to minimize the unwanted degradation or immune reaction [126, 174]. Immune response is the most important challenge for viral vectors. The scaffold could protect the vectors from neutralizing immune complexes and mitigate the immune response due to inflammatory cytokines released by immune cells. Sailaja *et al.* [175] showed that encapsulation of adenovirus vectors in alginate microspheres would greatly reduce the immune response of mice. As a result, high transfection efficiency was achieved while the bolus delivery of adenovirus vectors led to significantly reduced transgene expression in immunized animals primarily due to immune responses against the vectors. Chemical modification could also be incorporated into the scaffold to suppress the immune response. Ligands or antibodies that induce T-cell apoptosis through Fas signaling can be incorporated into the scaffold to provide local immunosuppression [174].

With all those advantages, scaffold-mediated gene delivery has been extensively reviewed previously [168, 170, 176, 177]. To date, scaffold-based miRNA delivery to stem cells remains an area of immense research opportunities. There are generally two ways to prepare scaffold-based miRNA delivery systems: one is encapsulation of miRNA into the scaffold, the other is immobilization of miRNA onto the scaffold surface.

4.2.2. Scaffold encapsulation—Among the materials used for scaffold construction, hydrogels are attractive because of their highly hydrated, tissue-like nature. By adding the miRNA before the crosslinking process, hydrogels encapsulated with miRNA could be

easily prepared since the mild crosslinking condition usually would not alter the bioactivity of miRNA. Both natural and synthetic polymers could be used as hydrogels for tissue engineering with distinct advantages. Hydrogels can be fabricated *ex vivo* for subsequent implantation, or can be formed *in situ* through minimal invasive surgery. Degradation of hydrogels can be tailored to occur through a variety of mechanisms including enzymolysis and hydrolysis. The mesh size, ion exchange, and the strength of crosslinking interactions could also influence the degradation rate of hydrogels. Since the release of encapsulated miRNA occurs through a combination of hydrogel degradation and payload diffusion, long-term controlled release could be achieved through manipulating the physicochemical properties of the hydrogels.

Monaghan *et al.* [66] used the four-arm poly(ethylene glycol)-terminated succinimidyl glutarate (4S-StarPEG) crosslinked collagen type I encapsulated with miR-29b for amelioration of the wound healing process. The release rate was tuned by the crosslink density in an inversely proportional manner. The miR-29b and collagen scaffold showed synergistic effect during the wound healing process. The collagen type III : collagen type I ratio is an important indicator of injury recovery because the ratio would decrease significantly after traumatic injury. Collagen alone could promote restoration of the natural balance between collagen type III and collagen type I. However, addition of miR-29b further pushed this ratio toward the value in normal tissues (Fig. 5). This ratio increased with time up to 14 days, suggesting the sustained release of miR-29b. *In vivo* study showed a dose-dependent increase of granulation tissue and decrease of wound contraction through delivery of miR-29b, indicating a better wound healing. Upon the delivery of miR-29b, expressions of a number of proteins have been regulated including the matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMPs). The increase of MMP-8 : TIMP-1 ratio was in accordance with the high collagen type III : collagen type I ratio in the wound healing process because MMP-8 would break down collagen type I.

Supramolecular hydrogels formed by dipeptide Gly-Ala linked with biphenyl-substituted tetrazole (Tet-GA) also have been used as matrix to entrap and deliver miRNA [178]. Tet-GA could form stable and biocompatible gel at neutral pH and entrap both miRNA and living cells in 3D manner. In comparison of 2D vs 3D configuration, transfection of HepG2 cells by naked miR-122 mimics was successful only in the hydrogel, showing ~80% transfection and a 10 fold increase of miR122 expression over the control.

Li *et al.* [52] used the commercialized hydrogel named HyStem-HP which contained thiol-modified HA, thiol-modified heparin, thiol-modified gelatin, and a thiol-reactive crosslinker to deliver miR-26a for bone regeneration. The authors proved that increasing endogenous miR-26a *in vivo* could augment bone repair through coordinating processes of endogenous angiogenesis and osteogenesis processes. Delivery of miR-26a through the hydrogel resulted in an 8-fold miR-26a level elevation compared with control at 24 h post-transfection. The level increased to 70 fold on day 11. In the *in vivo* study, hydrogels loaded with agomiR-26a hBMMSCs were implanted into 5-mm critical-sized calvarial bone defects in nude mice. Twelve weeks post-implantation analysis showed that the miR-26a delivery led to nearly complete repair of the defect while implantation of the control hydrogel only resulted in moderate bone regeneration (~40%) (Fig. 4C). At the same time, micro-CT angiography

analysis and immunofluorescence staining for CD31 showed that vascular volume of the treated group significantly increased. The levels of vascular endothelial growth factor (VEGF) and osteogenesis gene marker Runx2 of the treated group were also higher than that of the control group. These results confirmed that sustained delivery of miR-26a not only enhanced bone formation but also accelerated vascularization.

Hydrophobic polymers can also be used to encapsulate miRNA or siRNA [179]. This in principle can be done in the fabrication of fibrous scaffolds, which has been widely used as tissue grafts such as skin, vascular, cartilage, bone, and nerve grafts [180, 181]. Electrospinning is an effective and popular technique to produce fibers with diameter between hundreds of nanometers and several micrometers [180, 182]. The porous nature of fibrous scaffold provides a 3D matrix with high specific surface area for cell proliferation and nutrient transport. The topography of nanofiber could influence the migration and differentiation of stem cells [183–185]. It would be reasonable to hypothesize that a combination of nanofiber topography and miRNA delivery could modulate tissue development on a fibrous scaffold. However, despite many reports on the application of fibrous scaffolds for DNA or siRNA delivery, there has been no report on the use of miRNA entrapment for tissue engineering. Owing to the structural similarity between miRNA and siRNA, we can learn from the promising fibrous scaffold-based siRNA delivery systems discussed below.

SiRNA/polymer mixed solution has been used to encapsulate siRNA into the electrospun fibers by blend electrospinning technique [186, 187]. Chew *et al.* have used this technique to encapsulate siRNA/CPP complexes in electrospun PCLEEP fibers with uncompromised bioactivity [172]. A sustained release of siRNA was obtained for at least 28 days. Although the bolus delivery of siRNA could efficiently down-regulate the expression of collagen type I (COL1A1) at day 3, the COL1A1 level returned to normal at day 5. On the contrary, the scaffold-mediated delivery showed continuous down-regulation of COL1A1 level up to day 14. Transfection reagent (TransIT-TKO) and two CPPs (MPG and CADY) have been evaluated and TKO exhibited the best performance both *in vitro* and *in vivo*. The knockdown efficiency of PCLEEP fibers encapsulated with TKO/siCOL1A1 complexes was 50.5% at day 14, compared with 26.7% and 46.7% for siCOL1A1/MPG and siCOL1A1/CADY samples, respectively. Excessive deposition of collagen matrix would result in formation of fibrous capsule surrounding implants. *In vivo* study showed that the thickness of fibrous capsule formed around the PCLEEP fibers encapsulated with TKO/siCOL1A1 complexes was only about 60 μm at week 2 while that of the control sample was 80 μm. The fibrous capsule thickness further decreased to less than 30 μm at week 4.

4.2.3. Surface immobilization—Since the fabrication and sterilization process might inactivate miRNA, surface immobilization after scaffold fabrication offers an alternative for scaffold-mediated delivery. Additionally, the surface immobilization is especially suitable for bone implants and intravascular stents made from metals such as titanium (Ti) and stainless steel. Both the nonspecific interactions including hydrophobic/ionic interaction and specific interactions such as antigen-antibody and biotin-avidin interactions have been used to immobilize nucleic acid for substrate-based gene delivery. Lyophilization is the simplest way to immobilize nucleic acid or nanocomplex onto the scaffold surface. Both miR-29b

and antimiR-138 lipoplexes (Lipofectamine® 2000) have been immobilized on microporous titanium oxide surface by lyophilization [188]. Through this technique, 1000 nm thick lipoplexes could be coated onto Ti surface and the transfection efficiency for MSC could reach up to 90%. Leveraging on the osteogenic potential of miR-29b and antimiR-138, Ti surface functionalized with these lipoplexes could be used as bone implant to enhance osteogenesis. The expression levels of osteogenic genes including ALP, BMP, OCN, COL1, OSX and Runx2 of MSC transfected by antimiR-138 and miR-29b functionalized surfaces were much higher than the control up to day 14. The enhanced collagen secretion and bone extracellular matrix (ECM) mineralization also proved that the Ti implants functionalized with miR-29b and antimiR-138 enhanced the osteogenic differentiation of MSC *in vitro*.

Ionic interaction is another technique to immobilize nucleic acids onto a surface due to their anionic sugar back bone. Poly(caprolactone) (PCL) fiber could be first coated with polydopamine (PD) to allow subsequent immobilization of siRNAs [189]. After adsorption of RE-1 silencing transcription factor (REST) siRNA onto PD-modified electrospun PCL nanofibers (PD-PCL scaffold), sustained REST knockdown in neural stem/progenitor cells (NPCs) was achieved up to 5 days. Comparing with the 2D PD-modified PCL film (PD-PCL film), the PD-PCL scaffold exhibited higher cumulative siRNA release and higher REST knockdown efficiency. Most importantly, siREST PD-PCL scaffold showed not only enhanced NPC neuronal commitment than the fiber control, but also significantly higher commitment than siREST PD-PCL film. It suggests that siREST together with the nanofiber topography has a positive influence on the neurogenesis of the seeded NPCs.

Layer-by-layer (LbL) technology is another approach to immobilize genes onto the scaffold surface through ionic interaction. Because of the small size of miRNAs/siRNAs, formation of multilayered polyelectrolyte films by LbL self-assembly of siRNA and polycations would result in inefficient ionic interaction and high initial burst release [190]. An alternative is to form multilayered polyelectrolyte films by siRNA/polycation polyplexes and polyanions [191, 192]. Dimitrova *et al.* [192] used PEI/siRNA polyplexes and HA/CS complexes to form multilayered polyelectrolyte films on the polylysine (PLL) and polyglutamic acid (PGA) films. With the degradation of HA by hyaluronidase, siRNA could be sustainably released from the film and efficiently inhibited HCV replication and infection in hepatocyte-derived cells over 12 days. Presumably LbL immobilization of polyplexes instead of naked siRNA would be advantageous because of higher transfection efficiency of the former. Although immobilization has proved effective in most studies, Shea *et al.* [193] showed that immobilization of PEI/DNA polyplexes onto the HA-collagen hydrogels with or without the biotin-neutravidin binding would not make a significant difference in transfection efficiency.

Antigen-antibody interaction was proved to be an efficient way to immobilize adenovirus vectors and plasmid DNA, especially for the gene-eluting stent application [194–196]. Probably due to complexity and high cost of antibody introduction, this approach has yet to be applied for miRNA delivery.

4.3. Scaffold properties

The properties of a scaffold may also affect the potency of scaffold-based gene delivery systems. Since the stiffness [197], nanotopography [198] and surface chemistry [175, 199]

of scaffolds could have significant influence on cell behavior, a combination of those factors could potentially enhance the transfection efficiency of miRNA delivery systems. Mooney *et al.* showed that the stiffness of a scaffold could impact the DNA/PEI polyplex transfection efficiency [197]. Increase of scaffold stiffness would enhance the cell endocytosis of polyplexes, facilitate unpacking of the pDNA, and ultimately increase the transfect efficiency. The effect of scaffold stiffness depends on the cell type [200]. Increase in scaffold stiffness would increase the transfection efficiency to fibroblasts as their cell area and nuclear aspect ratio would change significantly with substrate stiffness. However, the transfectability of BMSCs and myoblasts were not so sensitive to scaffold stiffness. In the case of scaffold-based delivery systems, the influence of scaffold stiffness might be more complicated. Segura *et al.* [201] tried gene delivery to MSC through matrix metalloproteinase (MMP)-degradable HA hydrogel scaffolds. The study showed that softer hydrogel (0.1 vs 1.7 kPa) would increase the transfection efficiency. The mechanism underlying this phenomenon may be multi-factorial and warrants further studies. For instance, the higher crosslinking density used to create the tougher hydrogel may also decrease the hydrogel degradability and the subsequent DNA/PEI polyplex release.

Structure and nanotopography of a scaffold would also influence the transfection efficiency of gene delivery systems. Chew *et al.* [189] showed that dopamine-modified PCL nanofibers would achieve higher REST knockdown in NPC than dopamine-modified PCL nanofilms. Shea *et al.* [202] fabricated macroporous PEG hydrogel by first embedding gelatin microspheres into the gel during the hydrogel formation, followed by dissolution of the gelatin microspheres to create interconnected pores. The lentivirus encapsulated inside the hydrogel maintained its activity during the whole fabrication process. They have proved that increase of the macroporosity would increase cell penetration and HT-1080 cells would be transfected throughout the hydrogel, while non-macroporous PEG hydrogels could only transfect cells in the adjacent host tissues. Most importantly, in the *in vivo* study, delivery of lentivirus encoding for VEGF increased vascularization throughout the macropores of the hydrogel.

Using the self-assembled monolayer (SAM) technology, Shea *et al.* showed that the surface chemistry has a significant influence on the transfection efficiency of the surface-immobilized DNA nanocomplexes [199, 203]. Generally, the scaffold with a hydrophilic surface would result in higher transfection efficiency than one with a hydrophobic surface. Transfection efficiency of anionic carboxylic-acid-terminated SAM was two times higher than the hydroxyl-terminated SAM. Introduction of oligo(ethylene glycol) groups to the carboxylic acid-terminated SAM would further increase the transfection efficiency to five fold. Not only the surface functional groups, the proteins immobilized on the scaffold surface would also alter the endocytosis pathway and increase the transfection efficiency. PEI/DNA polyplexes immobilized on fibronectin-coated scaffold exhibited improved transfection efficiency and their endocytic pathway was also changed from clathrin-mediated endocytosis to caveolae-mediated endocytosis [204]. Interestingly, bolus delivery of PEI/DNA polyplexes to NIH/3T3 cells seeded on the same surface would not exhibit the same phenomenon.

5. Closure

MiRNA delivery has proved a promisng approach to direct stem cell fate. Manipulating specific miRNA levels inside stem cells through replacement therapy or inhibition therapy, a number of studies have demonstrated success in augmenting osteogenesis, reducing fibrosis, and improving wound healing among many other tissue engineering applications. MiRNA for regenerative medicine is still an evolving topic. However, evidence so far suggests that it will be a fertile research direction.

Like in many other gene therapies, effective miRNA delivery holds the key to eventual clinical translation. Although viral vectors pose long term safety concerns, the approval of adeno-associated viral vectors (AAV) for treating patients with familial hyperchylomicronemia in Europe shows that viral delivery is a viable approach for applications with a high benefit-to-risk ratio. Since AAV-mediated RNA interference therapy has proved effective in many studies, there is reason to believe that this approach would warrant investigation. Baculoviral vector might also offer opportunities for safe and effective miRNA delivery.

While nonviral vectors may be safer, their intrinsic limitations of low and transient transgene expression must be overcome for many tissue engineering applications. The issue of low transfection efficiency may or may not be fatal since the interference of certain pathways upstream may already have a profound effect on phenotypic development of stem cells. The issue of transient expression may be compensated by innovations of controlled release technologies. For example, sustained and local delivery of miRNA polyplexes or lipoplexes by hydrogels to the seeded or encapsulated cells can lead to prolonged miRNA expression. Scaffolds-based miRNA delivery also presents another opportunity to rescue the nonviral approach.

Although scaffolds-based miRNA delivery for regenerative medicine is still in its infancy, it is eliciting excitement. Tissue engineering scaffolds encapsulated with miRNA not only can function as a substrate for cell delivery, proliferation, differentiation, and tissue development but also provide essential and complementary factors for stem cells to differentiate towards specific linkage. Reports on scaffold-based miRNA delivery have shown particular promise for osteogenesis as well as wound healing. As innovations developed for RNAi therapy inspire miRNA delivery, we should see increasing application of miRNA therapy impacting regenerative medicine.

Acknowledgments

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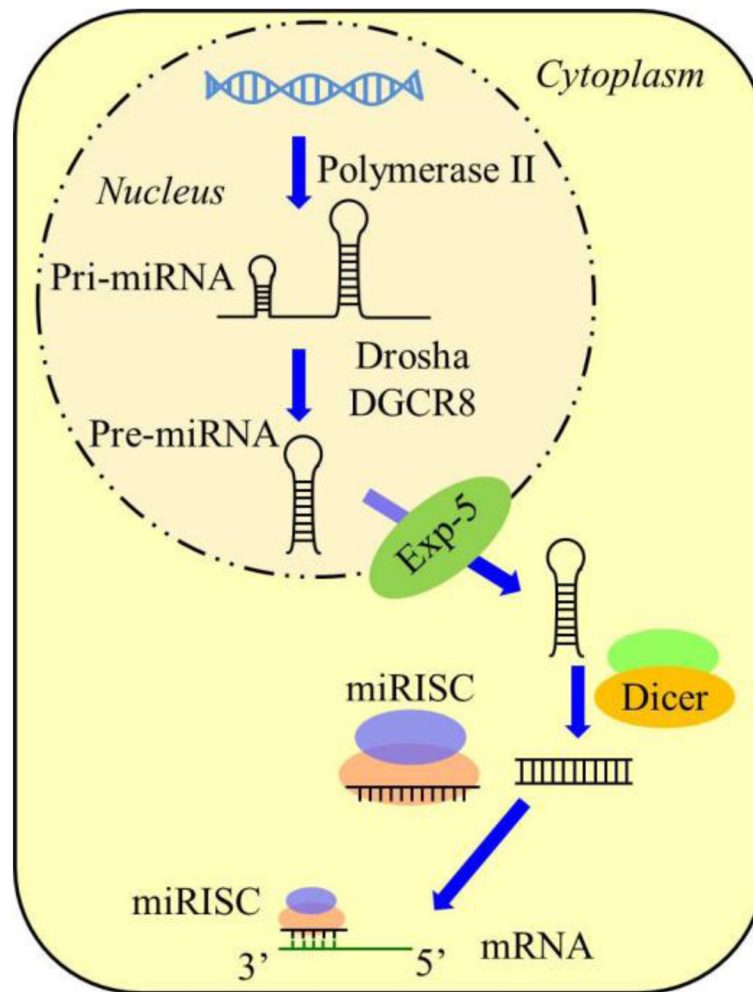
Figure 1.
Schematic representation of the biogenesis of miRNA. Following loop formation, the pri-miRNA is processed by DGCR8 and Drosha into pre-miRNA. The pre-miRNA is exported from the nucleus by Exportin 5 before maturation by Dicer. Then the miRNA duplex is unwound and the mature strand remains within the miRISC complex which further binds to the 3′ untranslated region of mRNA.
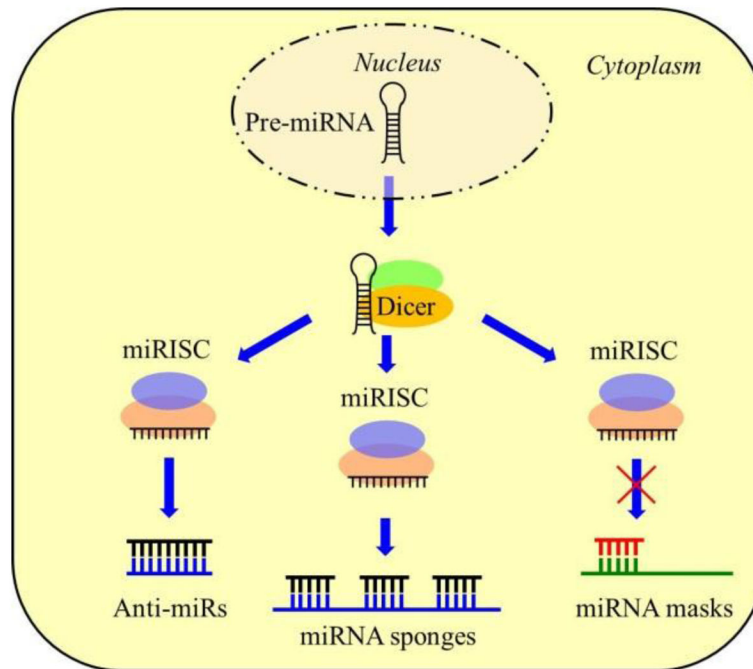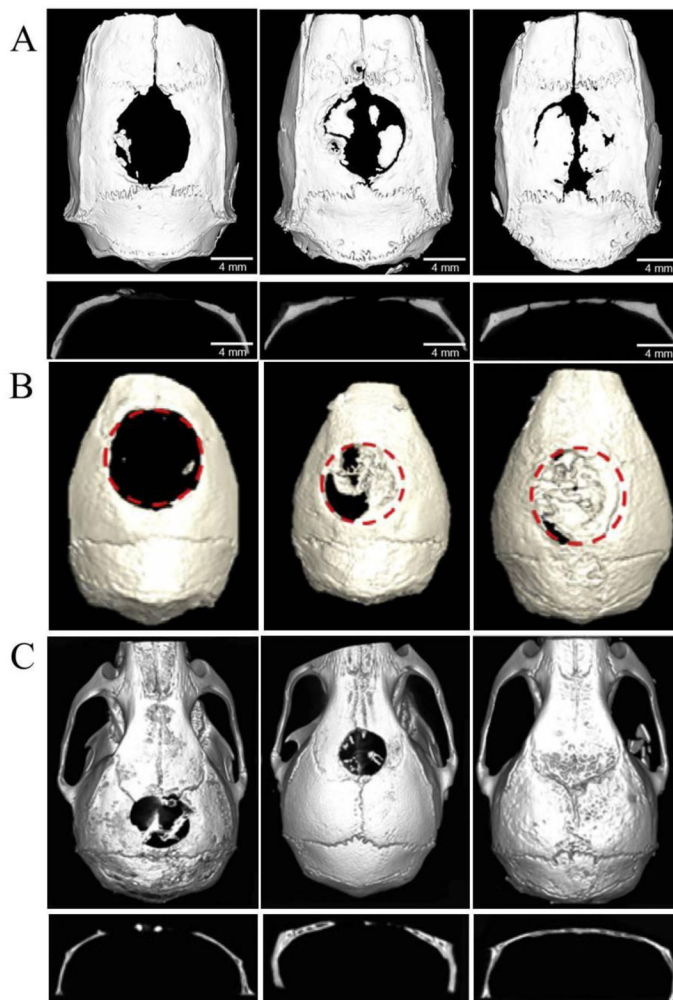
Figure 2.
Strategies of miRNA inhibition therapy. Anti-miRs contain sequences that are complementary to the miRNA mature strands and act as competitive inhibitors. MiRNA sponges are DNA sequences with multiple binding sites to the miRNA. They could inhibit a panel of miRNAs. MiRNA masks could selectively block specific mRNA pathway.
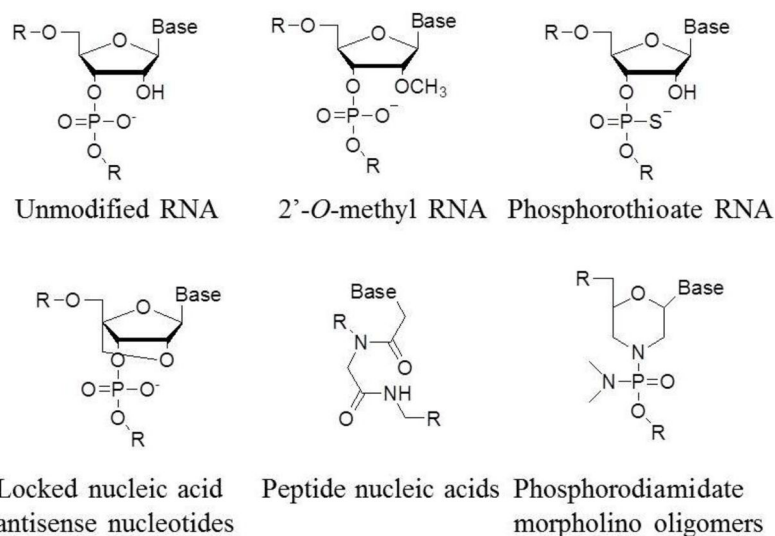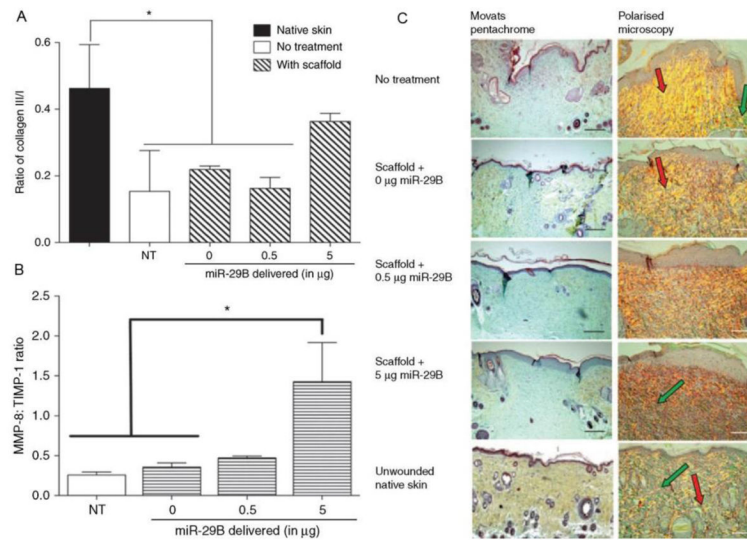
Figure 3.
Chemical modifications of AMOs. 2′-*O*-methyl RNA is modified by changing the 2′ hydroxy group of the ribose into methoxy group; phosphorothioate RNA introduces the sulfur substitution of a non-bridging oxygen to make a phosphorothioate linkage between nucleotides; locked nucleic acid links 2′, 4′ of the ribose by methylene bridge to form a bicyclic nucleotide; peptide nucleic acids (PNAs) substitute a peptide bond at the 1′ amide linkage to the base. Phosphorodiamidate morpholino oligomers (PMOs) contain morpholine rings instead of deoxyribose rings that are linked through phosphorodiamidate groups instead of phosphates.

Figure 4.
Bone regeneration through different miRNA treatment. (A) Coronal and sagittal views of the harvested skulls by micro-CT at week 8 post-implantation (left: PGS scaffold, middle: anti-miR-negative/BMSCs/PGS, right: anti-miR-31/BMSCs/PGS). Adapted and reprinted with permission from Ref. [50]. (B) Micro-CT of nude mice at week 12 after surgery. (left: hASCs/scaffolds, middle: Bac-FLPo/Bac-FCBW/hASCs/scaffolds, right: Bac-FLPo/Bac-FCBW/Bac-miR-148b/hASCs/scaffolds). Adapted and reprinted with permission from Ref. [51]. (C) Micro-CT of live mice at week 12 after surgery. (left: BMSCs/hydrogel, middle: miR-negative/BMSCs/hydrogel, right: miR-26a/BMSCs/hydrogel). Adapted and reprinted with permission from Ref. [52].

Figure 5.

(A) Delivery of miRNA-29b increased the ratio of collagen type III-like fibers to collagen type I-like fibers within the wound bed. (B) Transfection of miRNA-29b could increase the ratio of MMP-8 to TIMP-1 in excised tissue at day 28. NT indicates an excisional wound with no treatment applied. (C) The first column showed Russel-Movat's pentachrome staining of wound bed sections at day 28. The granulation tissue is represented by blue/cyan and the original skin collagen represented by yellow. The second column shows the polarized light images of picrosirius red-stained wound bed sections. The presence of collagen type III-like fibers could be seen as green stained fibers and the collagen type I-like fibers as yellow and red-stained fibers. Collagen type-I like fibers are highlighted with red arrows within some sections and collagen type-III like fibers are highlighted with green arrows. Scale bar = 100 μm. Adapted and reprinted with permission from Ref. [133].

Table 1

Representative *in vivo* studies of miRNA therapy for regenerative medicine

Target miRNA	Means of delivery	Therapeutic approaches	Observations	Application	Ref.
MiR-31	Poly(glycerol sebacate) scaffold	Inhibition	Percentage of new bone area after 8 weeks increased from 35% to 61%	Osteogenesis	[50]
MiR-148b	Baculovirus	Replacement	Percentage of new bone area after 12 weeks increased from 61% to 94%	Osteogenesis	[51]
MiR-26a	HyStem-HP™ hydrogel	Replacement	Percentage of new bone area after 12 weeks increased from 40% to ~100% and accelerated vascularization	Osteogenesis and angiogenesis	[52]
MiR-138	Hydroxyapatite-tricalcium phosphate scaffold	Inhibition	Total bone volume increased 2.2-fold in implants treated with the antimiR-138 compared with miR control	Osteogenesis	[108]
MiR-29b	Atelocollagen/PEO scaffold	Replacement	MMP-8: TIMP-1 ratio 3-fold higher than treated with scaffold alone	Promoting wound healing	[66]
MiR-21	Direct injection of plasmid expressing miR-21	Replacement	Wound contraction increased over 30% compared to untreated wound	Promoting wound healing	[109]
MiR-1, miR-133; miR-206	Atelocollagen	Replacement	The number of centronucleated myofibres in the miRNA group increased 136% than that in the control group; capillary density in the miRNA group increased over 30% than that in the control group	Myogenesis and angiogenesis	[110]
MiR-302	MaxSuppressor™ In Vivo RNA-LANCEr II	Replacement	Fibrosis size of hearts decreased over 60% after 50 days compared to treated with control miRNA	Cardiomyogenesis	[76]
MiR-590; miR-199a	Adeno-associated virus	Replacement	Infarct size of miRNA treated group decreased about 50% at 12 days and 60 days after myocardial infarction	Cardiomyogenesis	[79]
MiR-34a	RiboJuice™	Inhibition	Infarct cell death in heart treated with anti-miR-34a decreased 75 % compared to control group after myocardial infarction	Cardiomyogenesis	[77]
MiR-132	PLGA polymer nanoparticles	Replacement	Over 10-fold increase in the number of microvessels per square millimeter compared to negative control	Angiogenesis	[111]
MiR-134	Adeno-associated virus	Replacement	13% reduction of total dendritic length compared to control vector	Dendritogenesis	[112]

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Table 2

Representative viral miRNA delivery vectors for regenerative medicine

Viral vectors	Target miRNA	Cell type	Therapeutic approaches	MiRNA level variation	Potential application	Ref.
Adenovirus	MiR-375	Alveolar epithelial cell	Replacement	~17 fold increase	Lung recovery	[135]
Adeno-associated virus	MiR-26a	Myoblast	Inhibition	About 75% knockdown	Myogenesis	[73]
Retrovirus	MiR-138	MEFs	Replacement	>1000 fold increase	Cellular reprogramming	[123]
Retrovirus	MiR-205	Hair follicle stem cells	Inhibition	Nearly 100% knockdown	Skin recovery	[136]
Retrovirus	MiR-106b; miR-93; miR-25	NSPCs	Replacement	miR-106b: ~18 fold; miR-93: ~15 fold; miR-25: ~30 fold	Neurogenesis	[125]
Lentivirus	MiR-424	hDPCs	Inhibition	70% knock down	Osteogenesis	[128]
Lentivirus	MiR-31	Bone marrow stromal stem cells	Inhibition	~90% knock down	Osteogenesis	[51]
Lentivirus	MiR-346	hBMSCs	Replacement	5.5 fold increase	Osteogenesis	[137]
Lentivirus	MiR-153; miR-181a/a*; miR-324-5p/3p	Human neural stem cell	Replacement	10~300 fold increase	Neurogenesis	[138]
Lentivirus	MiR-1	MSCs	Replacement	~300 fold increase	Cardiogenesis	[129]
Lentivirus	MiR-143; miR-145	Corneal epithelial progenitor cells	Replacement	MiR-143: 2487 fold increase; miR-145: 876 fold increase	Vasculogenesis	[139]
Baculovirus	MiR-26a; miR-29b; miR-148b;miR-196a	hASCs	Replacement	miR-26a: ~5 fold; miR-29b: ~5 fold; miR-148b: ~8 fold increase; miR-196a: ~3 fold increase	Osteogenesis	[134]

Table 3

Representative lipid-based miRNA delivery systems for regenerative medicine

Transfection reagents	Target miRNA	Cell type	Therapeutic approaches	MiRNA level variation	Potential application	Ref.
HiPerFect	MiR-146b-5 p; miR-23b; miR-99a	Human neural stem cells	Replacement	MiR-146b-5p: ~65 fold increase; miR-23b: 30 fold increase; miR-99a: 25 fold increase	Neurogenesis	[149]
DharmaFECT®	MiR-198	keratinocytes	Replacement	>3 fold increase	Promoting wound healing	[63]
SiPORT™ NeoFX™	MiR-21	MSCs	Replacement	~600 fold increase	Osteogenesis	[150]
SiPORT™ NeoFX™	MiR-335-5p	Primary calvarial osteoblasts	Replacement	Transfection efficiency ~40%	Osteogenesis	[151]
SiPORT™ NeoFX™	MiR-17	human periodontal ligament tissue-derived mesenchymal stem cells	Replacement	>4000 fold increase	Osteogenesis	[152]
SiPORT™ NeoFX™	MiR-1; miR-499	human cardiomyocyte progenitor cells	Replacement	miR-1: increase to 8 Ct; miR-499: increase to 12 Ct	Cardiomyogenesis	[153]
Lipofectamine® 2000	MiR-1	ESC	Inhibition	~90% down regulation of ESC differentiation	Osteogenesis	[154]
Lipofectamine® 2000	MiR-375	3T3-L1 adipocyte	Inhibition	~80% knockdown	Adipogenesis	[155]
Lipofectamine® RNAi-MAX	MiR-720	hDPCs	Replacement	~180 fold increase	Osteogenesis	[148]
Lipofectamine® RNAiMAX	MiR-682	Myogenic progenitor cell	Inhibition	~50% knockdown	Myogenesis	[156]