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Review

Mimic miRNA and Anti-miRNA Activated Scaffolds as a Therapeutic Strategy to Promote Bone, Cartilage, and Skin Regeneration

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Abstract: MiRNA-based therapies represent an innovative and promising strategy applicable to various medical fields, such as tissue regeneration and the treatment of numerous diseases, including cancer, cardiovascular problems, and viral infections. MiRNAs, a group of small non-coding RNAs, play a critical role in regulating gene expression at the post-transcriptional level and modulate several signaling pathways that maintain cellular and tissue homeostasis. The clinical trials discussed in the review herald a new therapeutic era for miRNAs, particularly in tissue engineering, using synthetic exogenous mimic miRNAs and antisense miRNAs (anti-miRNAs) to restore tissue health. This review provides an overview of miRNAs' biogenesis, mechanism of action, regulation, and potential applications, followed by an examination of the challenges associated with the transport and delivery of therapeutic miRNAs. The possibility of using viral and non-viral vectors that protect against degradation and ensure effective miRNA delivery is highlighted, focusing on the advantages of the emerging use of 3D biomaterial scaffolds for the delivery of mimic miRNAs and anti-miRNAs to facilitate tissue repair and regeneration. Finally, the review assesses the current landscape of miRNA-activated scaffold therapies on preclinical and clinical studies in bone, cartilage, and skin tissues, emphasizing their emergence as a promising frontier in personalized medicine.

Keywords: mimic miRNA; anti-miRNA; viral vectors; non-viral vectors; 3D scaffold; bone regeneration; cartilage regeneration; wound healing; skin regeneration

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1. MiRNA Biogenesis

Heritable changes that are not encoded by DNA are referred to as epigenetics. Epigenetics involves factors that induce biological changes in genomes. Epigenetic mechanisms, specifically alterations in microRNAs (post-transcriptional regulators of gene expression), DNA methylation, and modifications in histones (chromatin proteins on which DNA coils to compact), have the potential to modify genome function as a result of exogenous environmental factors [1–3].

MiRNAs (or microRNAs) are endogenous, double-stranded [4], noncoding RNAs of approximately 20–22 nucleotides with one or more mismatches and are capable of modifying gene expression at the transcriptional and post-transcriptional levels throughout life or in disease states. MiRNAs control the activity of approximately 30 percent of all mammalian protein-coding genes and are highly conserved across species [5].

The expression level of one miRNA can regulate the expression of hundreds of messenger RNAs (mRNAs) due to the complementarity, while more than one miRNA can control

the same mRNA simultaneously. There are three mechanisms by which miRNAs influence the expression level of target proteins: by overlapping with target mRNA complementary sequences, miRNAs can (a) repress target mRNA translation or (b) induce target mRNA degradation; or ultimately miRNA can (c) destabilize target mRNA through shortening the poly(A) tail [6].

MiRNAs regulate gene expression without inducing changes in the DNA sequence. MiRNAs can determine the selective "turning on" or "turning off" of genes, allowing mRNA to adapt, in a short time, to environmental changes. An miRNA can 'regulate' the availability of many proteins and consequently induce systemic action.

The majority of studies on miRNA-target interaction focus on the complementary miRNA–mRNA target interaction that occurs at 'seed' sequences at the 3′ end or, rarely, at the 5′ end, followed by the target mRNA degradation. However, Lytle et al. have brought new insight into the investigation of miRNA interaction, proposing that miRNAs might bind to any mRNA target position, even in the 5′ UTR site [7].

Additional mechanisms of action of miRNAs include the targeting of gene promoters and also a decoy activity that interferes with the function of regulatory proteins [8,9]. Additionally, miRNAs can regulate gene expression at the transcriptional level by binding directly to DNA regulatory elements [10]. For about two decades, it has been discovered that miRNAs also act as controllers of DNA methylation through targeting DNA methyltransferase enzymes [11]. The current understanding of these RNA molecules represents only the tip of the iceberg because miRNAs are implicated in a plethora of functions, including cell development, proliferation, differentiation, apoptosis, stress resistance, fat metabolism, survival, tumorigenesis, and metastasis [12,13].

In the past decade, the therapeutic potential of miRNAs has been demonstrated in the treatment of various disease states where miRNAs play a critical role, including cancer, cardiovascular disease, diabetes, mental disorders, and viral infections [14–16].

Although epigenetic factors can lead to numerous pathologies, it must be emphasized that these factors are usually crucial in adaptive processes to ensure the survival of the individual and the species. For this reason, the decision to interfere with their activity needs to be supported by a deep understanding of biogenesis and the action mechanism [17].

In addition to regulating mRNA and protein levels, although most miRNAs have an intracellular presence, miRNAs play an intercellular signaling role. A large percentage of miRNAs migrate into body fluids: blood, urine, saliva, seminal fluid, and breast milk. Circulating miRNAs (cmiRNAs), to protect themselves from digestion by RNases, travel in body fluids bound to Argonaute 2 proteins (90%) or packaged in microvesicles [18]. Many authors suggest that miRNAs, through exosome-mediated intercellular communication, play an essential role as biomarkers in cancer [19–23], neurology, cardiovascular disease [24–26], bone diseases [27–30], and infectious diseases [31,32].

In comparison with conventional small molecule therapeutics, miRNAs can be engineered to modify the expression of any mRNA (and thus protein) of interest, with the advantage of being able to act on pharmacologically inaccessible targets, such as proteins lacking enzymatic function or with a conformation inaccessible to conventional drugs [33].

Aberrant expression of miRNAs is implicated in the onset of numerous pathologies. Consequently, the use of miRNAs as therapeutic agents is likely the next frontier in treatment options for human and veterinary diseases.

In mammalian cells, miRNAs are transcribed by the RNA polymerase-II gene that forms the hairpin loop architecture, which undergoes processing by an enzyme called Drosha to form short 20–22 nucleotide miRNA. One of the two strands is selectively loaded into an Argonaute protein to form the miRNA-induced silencing complex (miRISC) and actively repress gene expression, while the other strand is ejected from the complex and degraded [4]. The two strands of miRNA are denoted in the literature as miR (5p) or miR* (3p), where miR or the 5p strand is complementary to mRNA (mostly complementary to 3′ UTR of mRNA) and actively induces translational arrest and silencing of genes, while the miR* or 3p strand acts as an miRNA inhibitor (anti-miRNA) and alters the expression

of endogenous miRNA. Selection of the right strand within the miRISC is crucial for minimizing off-target effects. Kadekar et. al. has elegantly designed an asymmetric miRNA strategy that selectively enhances the selection of miR strands within the miRISC and thus suppresses off-target effects [34].

MiRNAs, in addition to their role in regulating mRNA fate, may regulate homeostatic pathways by controlling target mRNA gene expression levels, and these mRNAs, in turn, can influence the expression modulation of miRNAs [35]. Next, there exists the regulation of competing endogenous RNAs (ceRNAs) that contain miRNA-binding elements (MREs). MREs allow ceRNAs to compete on miRNA binding sites and thus establish a sort of ceRNA-dependent regulatory crosstalk [36]. An miRNA that shows a multifunctional role in the control of gene signaling and possesses the characteristics to modulate its expression level during biogenesis as well as after maturation is miR-155. Endogenous tuning of miR-155 expression depends on the type of cell in which it is produced, the tissue surrounding the cell, and the external signaling [37]. The regulation of miR-155 expression is controlled during biogenesis by multiple endogenous signaling pathways [38]. Several transcription factor binding sites have been identified in the miR-155 gene (BIC gene), including nuclear factor kappa B (NF-κB), SMAD4, interferon-sensitive response elements (ISREs), interferon regulatory factors (IRFs), and AP-1 [39]. In particular, miR-155 is an NF-κ B-dependent miRNA, and the increased expression of miR-155 inhibits the NF-κ B signaling pathway by reducing apoptosis, inflammation, and oxidative stress [40]. MiR-155 expression regulates the general inflammatory response [38] and the adaptive and innate immune response [41]. MiR-155 is particularly sensitive to many inflammatory stimuli, such as toll-like receptor (TLR), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, interferons, pathogen-associated molecular patterns, and damage-associated molecular patterns [42]. In addition, miR-155 biogenesis is induced by inflammatory stimuli including the lipopolysaccharide LPS (Figure 1) [43].

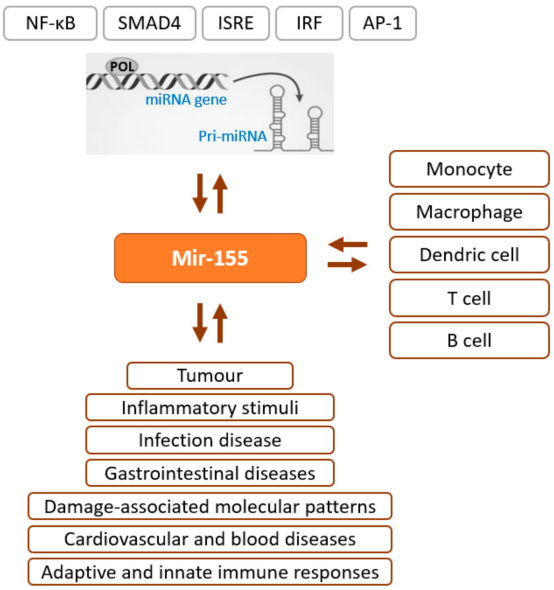

Figure 1. The miR-155 mutual regulatory crosstalk. The NF-κB, SMAD4, ISRE, IRF, and AP-1 transcription factors regulate the miR-155 transcription via POL II in a reciprocal regulatory loop. As shown in the lower part of the figure, there is reciprocal regulatory crosstalk between miR-155 and the listed pathological processes. The cells in which miR-155 activity plays a critical regulatory role are listed on the right.

In consideration that upregulations or downregulations of the miRNA expression levels exert a highly influential role in homeostatic control and beyond, it is hoped for the success of an miRNA-based therapy involving the use of both anti-miRNA and mimic miRNA in either inhibiting the endogenous miRNA activity or augmenting the endogenous

miRNA effect [44]. In the second approach, the activity of the endogenous miRNA is enhanced by an exogenous mimic miRNA (mimic miRNA) [45]. Anti-miRNA and mimic miRNA have similar physicochemical properties but distinct functions. Mimic miRNA leads to degradation/inhibition of the mRNA [44].

2. Epigenetics–miRNA Regulatory Loop

MiRNAs can be classified as either 'intragenic' or 'intergenic', depending on whether the miRNA is located in a region of the genome that is transcribed by a gene. In silico analysis revealed that approximately 31 per cent of miRNAs are intergenic, while the remaining ones are intragenic concerning the main regulated gene [46]. Various studies suggest that intragenic miRNA expression shifts can arise from changes in the expression of host genes in which the miRNA is encoded [47,48].

Depending on the physiological state of the organism or various external factors, the regulation of miRNA expression can occur at the transcriptional (first level) or post-transcriptional level (second level). The transcriptional regulation can occur by changes in miRNA DNA methylation or by transcription factors (TFs) binding to the promoter region of the miRNA. These changes may serve as a regulatory mechanism for miRNA expression levels. It has been established that methylation of the miR-210 gene promoter can suppress the expression of this miRNA, which is an important step in the process of angiogenesis [49].

At the post-transcriptional level, changes in miRNA processing or variations in miRNA stability induce modifications in the microRNA expression level (Figure 2) [47].

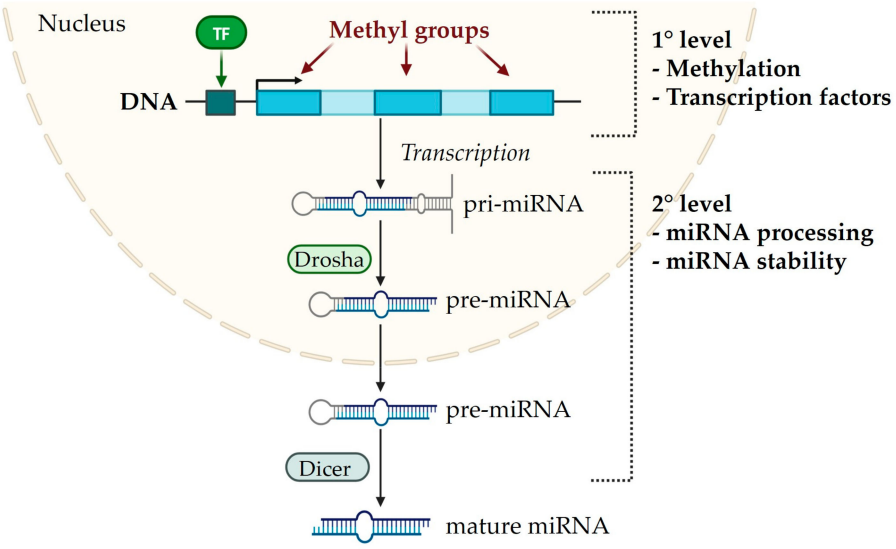

Figure 2. Transcriptional and post-transcriptional regulation of microRNA expression. The figure illustrates the transcriptional regulation of miRNA expression levels (1° level): by transcription factors binding to the miRNA promoter (miRNA upregulation) or by methylation of the miRNA-encoding DNA (miRNA downregulation). The post-transcriptional regulation of miRNA expression levels (2° level) depends on changes in miRNA processing by the endoribonucleases DROSHA and DICER, and on changes in miRNA stability that contribute to the accumulation of miRNAs in the cell. Figure adapted from Gulyaeva et al., J. Transl. Med. 2016 [47].

3. Emerging Clinical Application of Mimic miRNA and Anti-miRNA

Since Victor Ambros discovered in 1993 that microRNAs are essential molecules for gene regulation, scientific research has focused on designing miRNA-based therapies for the treatment of a wide range of diseases [50]. In terms of biotechnology initiatives, RNA-based therapy has represented a significant business niche for the last 20 years, serving as an area for new drug design. The goal of miRNA-based therapies includes increasing endogenous miRNA levels (with exogenous miRNA) when it acts as a disease

suppressor, or reducing endogenous miRNA levels (with anti-miRNA) when it acts as a disease inducer [44]. Anti-miRNAs are potent specific mechanisms for the in vivo silencing of miRNAs over-expressed in disease states. They are efficient, long-lasting, and specific in targeting the miRNAs (complementary miRNAs) for which they are designed [44].

The biological significance of miRNA silencing using anti-miRNAs was first studied for miR-122, gaining a small nucleic acid patent that showed 90 percent identity similarity to the complementary sequence of miRNA-122 [51,52]. In mice, intravenous administration of anti-miR-122, anti-miR-16, anti-miR-192, and anti-miR-194 induces silencing of the corresponding miRNAs in various organs, highlighting the value of anti-miRNAs as a therapeutic strategy [51].

Endogenous miRNA levels can also be reduced by the use of miRNA sponges, which are RNA constructs containing several miRNA binding sites [53].

To deliver mimic miRNA/anti-miRNA, it is necessary to protect these nucleic acids from blood destruction, bring the mimics closer to the target cells, allow their uptake into the cells, avoid immunogenic reactions, and possibly evaluate biocompatible and biodegradable materials to be used as mimic miRNA/anti-miRNA substrates [54].

The workflow for the clinical application of endogenous mimic miRNAs and anti-miRNAs starts with deciding whether to transfect free miRNAs or to use a delivery system for the targeted delivery of miRNA. The direct administration of free miRNAs in the body faces several challenges. Firstly, miRNAs are negatively charged, so they have a limited ability to cross the cell membrane. In addition, unmodified free anti-miRNAs and mimic miRNAs are rapidly degraded and eliminated in the bloodstream by numerous nucleases [55]. Finally, miRNAs can be immunotoxic by activating interferons or Toll-like receptors (TLRs) [56] and neurotoxic by inducing neurodegeneration through TLRs [57,58].

The next step is choosing the type of vectors to deliver miRNAs into the cells. In this scenario, the miRNA coupled to the carrier delivery system must allow passage across the cell membrane, mask the negative charge of free miRNAs, and protect from RNA degradation mediated by RNase. For this purpose, both viral and non-viral miRNA transporting vectors are used (Figure 3), each having advantages and disadvantages.

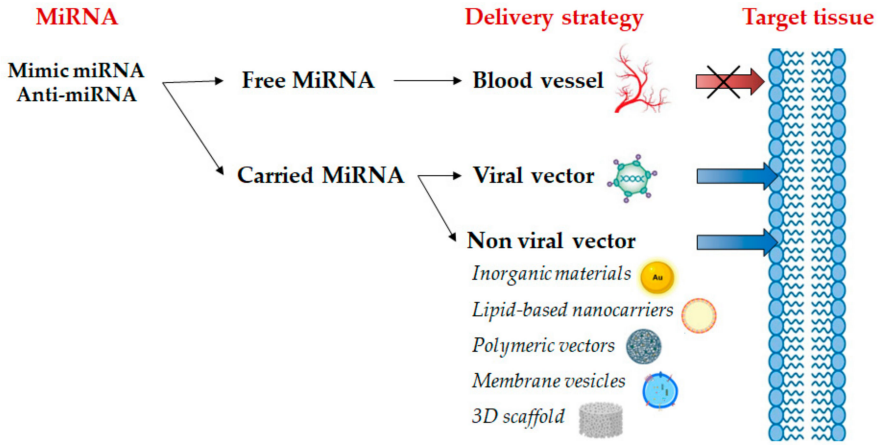

Figure 3. Exogenous mimic miRNA and anti-miRNA delivery strategies to reach the target tissue. Mimic miRNAs can be introduced into the organism as free miRNAs or as miRNA coupled to a carrier. The fate of the first is different from that of the second. Free miRNAs are introduced into the bloodstream directly. These miRNAs are not protected from the action of RNases and do not cross the cell phospholipid barrier. MiRNAs coupled to carriers are apt to cross the cell phospholipid membrane. MiRNAs coupled to carriers are distinguished depending on the type of vector into two main types: miRNA coupled to viral vectors and miRNA coupled to non-viral vectors. The principal viral vectors are adenovirus, adeno-associated virus, lentivirus, and retrovirus. Non-viral vectors are further subdivided into distinct delivery-based categories, utilizing lipid-based nanocarriers, polymers, inorganic materials, vesicles derived from cell membranes, and 3D scaffolds.

Clinically tested miRNA biopharmaceutical products are primarily administered by cutaneous or intravenous injection, and for respiratory diseases, by inhalation [59].

The choice of vector for miRNA delivery is critical to promote the entry of miRNAs into the target cells [60,61]. In recent decades, a variety of viral delivery vehicles have emerged, as they can be adapted for specific transgenes, various treatments, and different cell types (ref). Adenoviruses (ADVs), adeno-associated viruses (AAVs), retroviruses, and lentiviruses are the main viral vectors used for miRNA or anti-mRNA delivery, which are effective in ensuring cell entry and have a high transfection efficiency [62]. Viral vectors are generally modified at specific sites in the genome to prevent replication, thus ensuring their safety [63]. Genetically modified viruses can efficiently deliver oligonucleotides into various tissue types and ensure high levels of gene expression for prolonged periods [64]. However, viral vectors carry many disadvantages, including elevated cytotoxicity, low loading capacity, virus-dependent recombination, strong immunogenicity, problems related to biodistribution, high manufacturing costs, and difficulties in large-scale production [65]. For this reason, several non-viral vectors have been designed and constructed as a viable alternative to viral vectors due to their low toxicity and high biocompatibility [66].

Non-viral vectors are promising for miRNA delivery as (a) miRNA-mediated translational arrest occurs in the cytoplasm and nuclear transport is not required, (b) non-viral delivery systems display low toxicity and immunogenicity, and (c) facilitate the delivery of large amounts of cargo [63]. In addition, the production cost for the non-viral delivery system is low, and the formulation is controlled reproducible, and less complicated when compared to viral vectors [67]. However, non-viral vectors have a lower transfection efficiency than their viral counterpart. Some of the most versatile non-viral delivery systems are lipid-based nanocarriers, polymeric micelles, inorganic nanomaterials, membrane vesicles, and three-dimensional (3D) scaffolds that are developed for the efficient delivery of miRNAs into target cells [68].

Lipid-based nanocarriers are the most widely used non-viral vectors among different delivery systems. Conventionally, cationic lipids are widely used for complexing anionic RNA molecules that form stable lipoplexes and mitigate enzymatic degradation. Cationic lipids also have a high affinity for the cell membrane, and endosomal membrane and are easy to produce [64]. The most widely used commercially available lipid cationic vectors for miRNA delivery are Lipofectamine™ RNAiMAX [69], Lipofectamine™ 2000 [70], and siPORT™ NeoFX™ [71]. Despite their high transfection efficiency in vitro, their performance in clinical trials is less satisfactory. This is attributed to the rapid opsonization of cationic lipids leading to rapid clearance by the reticuloendothelial system (RES) [72]. This drawback is addressed by the utilization of ionizable lipids in the nanoformulation. The lipid-based nanoparticles for RNA delivery currently in clinical use are composed of phospholipids, ionizable lipids, cholesterol, and PEGylated lipids, where PEGylated lipids provide enhanced systemic circulation time, while ionizable lipids play a crucial role in disrupting endosomes and protecting RNA degradation from endonucleases, thus facilitating efficient cytosolic transport [73]. The liposomal formulations with ionizable lipids remain neutral during circulation, thus preventing opsonin binding and overcoming rapid RES clearance, and upon endocytosis followed by endosomal trafficking, they become cationic and facilitate fast endosomal leakage. Yan et. al. have provided a comprehensive review of lipid-based nanovesicles for the systemic delivery of RNA [74].

Polymeric delivery systems mainly use cationic polymers, including polyethyleneimine (PEI), poly(lactide-co-glycolide) (PLGA), and polyamide dendrimers (PAMAM) [75], where the positively charged amine groups bind the negative phosphate group of the miRNAs, protecting them from degradation and enabling cellular uptake [76]. Compared to lipid nanocarriers, polymeric vectors usually have lower toxicity but also a lower transfection efficiency [64,68].

Inorganic materials are less widely used for miRNA transport than lipids and polymers [63]. Currently, the most studied inorganic compounds are gold nanoparticles (AuNPs) [77], Fe_3O_4-based iron oxide nanoparticles [78], and silica-based nanoparticles [79].

These inorganic complexes, with appropriate synthesis and functionalization technology, exhibit unique optical, magnetic, and electrical properties, strong loading capacity, mechanical stability, controllable size, and porosity [57].

Membrane vesicles are also increasingly being considered as non-viral vehicles for miRNA-based therapies due to their high biocompatibility, low cytotoxicity, and low antigenicity. This category mainly includes exosomes which are extracellular vesicles involved in intercellular communication allowing the transport of biomolecules (including miRNAs) through the bloodstream [64,80].

A promising non-viral vector delivery strategy is to embed miRNA in a biodegradable 3D matrix that can be surgically inserted into affected tissue to provide continuous miRNA release [81,82] (Figure 4).

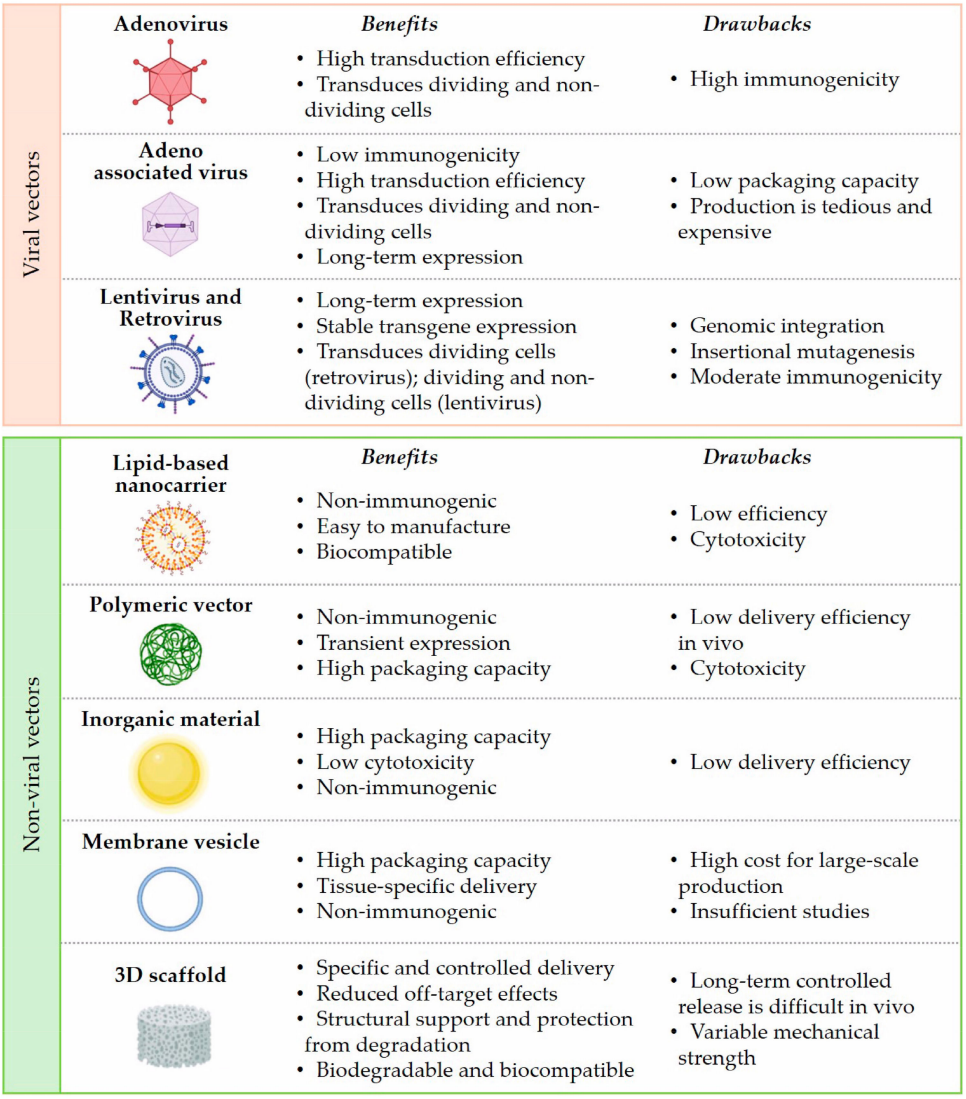

Figure 4. Viral and non-viral vectors for miRNA delivery. The figure shows the main advantages and disadvantages of viral and non-viral vectors used for miRNA delivery: adenoviruses, adeno-associated viruses, lentiviruses, and retroviruses (viral vectors); lipid-based nanocarriers, polymeric vectors, inorganic materials, membrane vesicles, and 3D scaffolds (non-viral vectors). Figure adapted from Dasgupta et al. Methods and Protocols, 2021 [64].

An important question to be addressed involves determining the beneficial dose to achieve the desired effect while minimizing adverse off-target effects. MiRNA-based therapies are dose-dependent, with a risk of adverse effects. Furthermore, the dosage can affect genes not purposely targeted by the handled miRNA. Additionally, overdosing on

an miRNA can exclude endogenous miRNAs from free RNA-induced silencing complexes (RISCs), activating toxic off-target effects [83,84].

It is essential to highlight that miRNAs, as epigenetic modulators, regulate genes affected by exposure to toxic substances and environmental changes [85]. The miRNAs' capacity as gene regulators makes restoring their expression level a remarkably attractive therapeutic device when the variation is part of the pathological disease changes. However, as miRNAs act against numerous genetic targets, off-target effects can hardly be avoided [86,87]. An episode of catastrophic side effects has been observed with miR-34a in cancer therapies, where severe immune-related side effects led to the death of patients [88].

Currently, miRNA-based therapies are in preclinical or clinical studies and constitute a focus for patent rights (Table 1).

Table 1. Current miRNA-based therapies under study. The table shows, starting from the first column on the left, biopharmaceutical products, treatment, synthetics-produced miRNA, and pharma companies.

Drug	Disease	MiRNA	Pharma Company
Miravirsen	Hepatitis C virus	miR-122	Santaris Pharma
MRX34	Different types of cancers	miR-34a	miRNA Therapeutics
RG-101	Viral diseases	miR-122	Regulus Therapeutics
RGLS4326	Polycystic kidney disease	miR-17	Regulus Therapeutics
MGN-1374	Post-myocardial infarction	miRNA-15/195	miRagen therapeutics
MGN-2677	Vascular disease	miR-143/145	miRagen therapeutics
MGN-4220	Cardiac fibrosis	miR-29	miRagen therapeutics
MGN-4893	Abnormal red blood	miR-451	miRagen therapeutics
MGN-5804	Cardiometabolic disease	miR-378	miRagen therapeutics
MGN-6114	Peripheral arterial disease	miR-92	miRagen therapeutics
MGN-9103	Chronic heart failure	miR-208	miRagen therapeutics
Cobomarsen	Cutaneous T-cell lymphoma	miR-155	miRagen therapeutics
MRG-107	Amyotrophic lateral sclerosis	miR-155	miRagen therapeutics
MRG-110	Ischemia	miR-92a	miRagen therapeutics
Remlarsen	Fibrosis	miR-29	miRagen therapeutics

MiRNA treatments, considered biopharmaceutical treatments, are the next-generation therapy option for many types of cancer [89].

The first miRNA-based biopharmaceutical product in phase II clinical trials is Miravirsen: a locked nucleic acid (LNA) for miR-122 for the treatment of hepatitis C virus infection. LNAs, new customized RNA analogs with therapeutic value [90], as well as anti-miR (specific miRNA inhibitors), represent a potential new class of drugs [91]. LNA, also recognized as a bridged nucleic acid (BNA), commonly termed inaccessible RNA, is a modified RNA nucleotide. This modification changes the ribose component with an additional bridge linking the 2′ oxygen and 4′ carbon. The bridge "locks" the ribose into the 3′-endo (North) conformation, a characteristic frequently observed in A-form duplexes. Compared to nucleic acids, LNA offers improved biostability, offering protection against enzymatic degradation [92].

On the other hand, miRNA-based cancer therapy faces significant challenges, such as poor stability, rapid elimination in the blood, restricted penetrability, possible immune system activation, and unwanted adverse effects [54,93]. In the case of melanoma miRNA treatment, the MRX34 liposomal injection of microRNA by Therapeutics Inc. has been retired due to adverse effects [88].

As of the latest PubMed data from 2015 to 2024, around 1200 scientific manuscripts on "miRNA-based therapies" have been published.

4. Mimic miRNA and Anti-miRNA Scaffold Delivery for Tissue Regeneration

MiRNAs are master regulators of tissue development, homeostasis, and tissue repair, as these nucleic acid drugs become activated during injury or infection and thus modulate several types of gene expression and reset healing and regeneration. Therefore, therapeuti-

cally modulating miRNA expression is a promising approach for tissue engineering and regenerative medicine applications [94]. Recent advances in molecular biology, nanotechnology, and biomaterials have made it possible to tailor new delivery systems for targeted or local delivery of therapeutic miRNA at the desired site [95].

The ability of miRNAs to regulate key cellular processes that define the cellular phenotype makes synthesized miRNA an excellent tool for fine-tuning gene expression and restoring tissue homeostasis. Distribution of miRNAs through scaffolds—in cells of interest—is preferable to systemic distribution because less expensive RNA oligonucleotides are required and off-target effects on non-targeted cells or tissue can be averted [96]. Furthermore, using 3D scaffolds as delivery vehicles allow space–time control and overcomes the biological and mechanical barriers that impede stable and efficient miRNA delivery to the target location [65]. For this reason, the strategy of miRNA-activated scaffolds has emerged in recent years particularly in the field of regenerative medicine and tissue engineering to restore the function and structure of damaged or dysfunctional tissues [65].

Mimic miRNAs and anti-miRNAs are both used in tissue regeneration therapy [97]. These synthetic molecules can be delivered in scaffolds to turn on or off gene expression in a specific tissue, depending on the intended application. Specifically, mimic miRNAs suppress target protein expression by degrading mRNA or inhibiting mRNA translation. On the contrary, as already described, anti-miRNA leads to an increase in mRNA and protein expression [65].

The idea of miRNA-activated scaffolds derives from the late 1990s "gene-activated matrices" (GAM) in which the combination of gene vectors expressing target proteins and a suitable biodegradable matrix/scaffold showed great potential for tissue regeneration [98–100]. More recently, this concept has been applied to miRNA delivery.

MiRNA-activated scaffolds result from the combination of miRNA—delivered by a vector (viral or non-viral)—with three-dimensional (3D) structural biomaterials. This system enables localized and controlled delivery of miRNAs and prevents systemic spread and off-target effects, thus maximizing the miRNA therapeutic function. In addition, the 3D scaffold microenvironment can offer physical protection against degradation, preventing miRNAs' rapid clearance once released into the target tissue. This allows the maintenance of a higher miRNA concentration in the injection site and sustained release to the target tissue and thus prolongs miRNA therapeutic efficacy, addressing a critical need in regenerative medicine and tissue engineering applications [100].

The selection of the appropriate scaffold is crucial for efficient tissue implantation. First, very general properties such as biocompatibility, bioresorbability, and ease of fabrication must be met; equally important for clinical use are the ease of sterilization, stability, and long-term storage [65]. The choice of scaffold must also take into account the physiochemical properties of the building block, the kinetics of RNA release, and the interaction with the target tissue, which vary depending on the type of biomaterial [100]. Recent advances in regenerative medicine have led to the development and testing of several biomaterials as novel delivery platforms for miRNA-activated scaffold therapies, including hydrogels, collagen, nanohydroxyapatite scaffolds, electrospun fibers, and microspheres [64].

The use of 3D scaffolds enables the delivery of miRNAs to the target site in two different ways, either "cell-free" or "cell-mediated". Cell-free delivery is achieved by introducing an miRNA with a delivery vector (viral or non-viral) into the scaffold and then implanting the miRNA-loaded scaffold directly into the site of interest. Once positioned, the scaffold serves as a depot for the miRNA–vector complexes, which are released slowly to the surrounding tissue and transfect the resident cells in the vicinity and modulate the gene expression. This modality enables tissue repair or regeneration without the need for direct cell implantation, taking advantage of the intrinsic properties of the scaffold and the therapeutic role of miRNA [65,100]. In contrast, cell-mediated delivery results from the in vitro pre-transfection of a cell population with the miRNA of interest using viral or non-viral vectors, then harvesting and seeding the modified cells onto the scaffold. The cell-activated scaffold is then implanted in vivo that regulate the tissue regeneration. Typically,

this strategy requires an additional period of in vitro culture before cell implantation. Using this cell-mediated approach, the cell population is the only one modified by the miRNA and thus responsible for the therapeutic outcome after tissue implantation (Figure 5) [65,100].

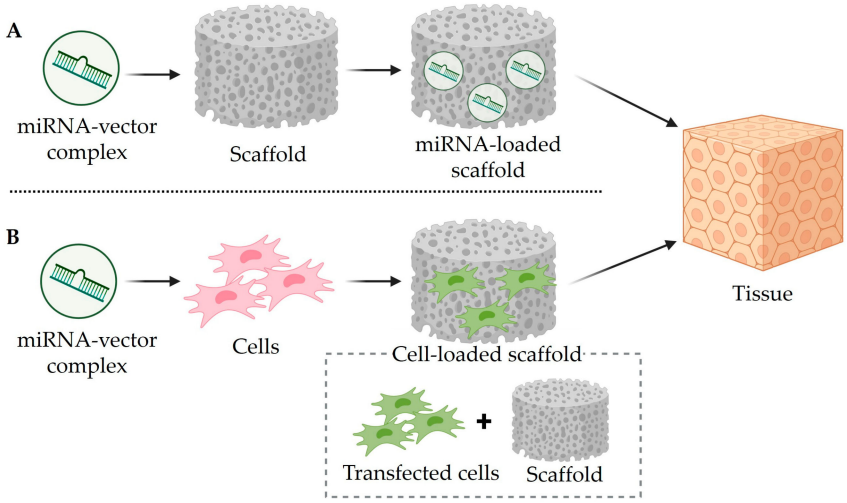

Figure 5. Two strategies for miRNA-activated scaffold delivery. (**A**) "Cell-free" delivery is obtained by adding the exogenous miRNA–vector complex into the scaffold; after in vivo tissue implantation, a host cell population encounters the miRNA-loaded scaffold, takes up the complexes, and becomes transfected. (**B**) "Cell-mediated" delivery is achieved by pre-transfecting a cell population with the miRNA–vector complex in vitro, then seeding the transfected cells onto the scaffold, which is then implanted in the target tissue.

Currently, both strategies for miRNA delivery are used, but most scientific groups studying miRNA-activated scaffolds work with cells pre-transfected with miRNAs, loaded into the scaffolds, and implanted at the target site (cell-mediated strategy), rather than with miRNAs delivered locally directly from the scaffolds (cell-free strategy) [96].

An important cell population that is used for the cell-mediated delivery of miRNA-activated scaffolds in the tissue engineering field is mesenchymal stem cells (MSCs). MSCs are multipotent stromal cells of adult or fetal origin that can self-renew, proliferate, undergo multilineage differentiation, and replace dead cells in the body [101]. Bone marrow and adipose tissue are the richest sources for isolating adult MSCs, but they can be found in almost all postnatal tissues. Fetal MSCs are present in fetal, perinatal, or neonatal tissues such as cord blood, umbilical cord, and placenta [102]. MSCs are considered interesting candidates for cytotherapy in a variety of diseases and for regenerative medicine as they can be easily isolated and cultured in vitro and can migrate to sites of inflammation, secrete immunomodulatory factors, and thus create a regenerative microenvironment [101]. MSCs can differentiate into multiple cell lineages such as osteoblasts, chondrocytes, and adipocytes [103]. The differentiation of MSCs is a complex process involving the expression of several genes which are widely regulated by miRNAs. MiRNAs have been shown to both positively and negatively regulate multiple functions and properties of MSCs (e.g., stemness maintenance, self-renewal, and differentiation potential). This regulation can be exploited for therapeutic strategies in tissue regenerative medicine [104].

To date, miRNA-activated scaffolds, administered via MSCs or directly cell-free, for tissue repair are gaining increasing interest, with studies targeting bone, liver, cardiac, cartilage, neurons, epithelia, and other tissues [105]. The following section of this review provides an overview of the principal miRNA-activated scaffold strategies used in bone, cartilage, and skin regeneration (Table 2).

Table 2. Overview of miRNA-activated scaffolds for tissue regeneration. The table summarizes the main miRNA-activated scaffold strategies described in the text for bone, cartilage, and skin regeneration. The columns of the table list in order are the type of scaffold, therapeutic miRNA, in vivo and in vitro studies (appearing in the table underlined), target tissue, reference, and advantages and disadvantages of the biomaterial scaffold type. Acronyms: ADSCs, adipose-derived stem cell; GelMA, gelatin methacrylate; FB/HA, fibrin/hyaluronan; GA, gelatin–alginate; nHap, nanohydroxyapatite; coll-nHap, collagen–nanohydroxyapatite; GCMA, gelatin–chitosan methacrylate; B, bone; C, cartilage; S, skin.

Scaffold	MiRNA	In Vitro and In Vivo	Tissue	Ref	Advantages and Disadvantages
Collagen-based hydrogel	miR-148b	BMSCs; rat calvarial defect	B	[106]	Advantages: biodegradable, highly biocompatible, easily modifiable, and versatile. Disadvantages: poor mechanical strength and stiffness; variability of isolated collagen.
	miR-210 and anti-miR-16	HMSCs; rat calvarial defect	B	[107]	
	anti-miR-133a	rat calvarial defect	B	[108,109]	
	miR-34a	MSCs; rat tibia	B	[110]	
	anti-miR-221	hMSCs	C	[111]	
Other hydrogel	miR-20a	hMSCs; rat calvarial defect	B	[112]	Advantages: high biocompatible, controlled biodegradation rate. Disadvantages: variable mechanical strength.
	chol-miR-26a	hMSC	B	[113]	
	exo-miR-26a	rat calvarial defect	B	[114]	
	miR-29b	mouse subcutaneous implantation	B	[115]	
	miR-221	mouse osteochondral defect	C	[116]	
	miR-99a-3p	murine models of osteoarthritis	C	[117]	
	miR-223 5p	macrophages J774A.1	S	[118]	
	miR-17-5p	diabetic mouse	S	[119]	
Hydroxyapatite	miR-21/124	MC3T3-E1 and 4B12; mouse bilateral cranial defect	B	[120]	Advantages: bioactive, biocompatible, osteoconductive, non-toxic, and non-inflammatory. Disadvantages: brittle, slowly degradable.
	anti-miR-221	rat calvarial defect	B	[121]	
Calcium phosphate	anti-miR-31	rat critical-sized bone defect; canine medial orbital wall defect	B	[122,123]	Advantages: excellent biocompatibility, good, osteoconductivity, adequate mechanical strength. Disadvantages: slowly degradable, brittle, non-resorbable, poor mechanical properties.
	miR-200c	BMSCs; rat calvarial defect	B	[124]	

4.1. MiRNA-Activated Scaffold and Bone Regeneration

Bone is a unique tissue with excellent regenerative properties that can repair small fractures or minor bone defects. However, in cases of large fractures or inadequate vascularization, self-healing is often delayed, or impossible, and therapeutic intervention is required. In the last few decades, advances in tissue engineering have led to a shift away from the use of prostheses or grafts towards the use of mechanisms capable of stimulating endogenous wound repair and tissue regeneration [96]. Endogenous bone regeneration is the result of (1) biodegradable scaffolds, (2) multipotent stem cells, mainly bone marrow-derived MSCs or umbilical cord-derived MSCs, and (3) bioactive molecules as miRNAs or anti-miRNAs.

MiRNAs can stimulate osteogenesis either on their own or in conjunction with other bioactive agents. Different miRNAs have been identified that play a role in osteogenic differentiation by targeting transcription factors or positive/negative regulatory genes associated with osteogenesis. Numerous miRNAs have been used in scaffold-based tissue engineering and tested in vivo, mainly in a mouse model of a cranial or calvarial bone defect [121,125–127].

The expression profile of miRNAs during osteogenesis is usually determined by microarray or by next-generation sequencing (NGS) followed by validation of the results through quantitative reverse transcription-polymerase chain reaction (RT-qPCR) in vitro on cell cultures (mainly MSCs) [128,129]. To identify these miRNAs, osteogenesis

is usually induced by osteogenic induction media containing mainly dexamethasone, β-glycerophosphate, and l-ascorbic acid [130]; otherwise, osteogenesis can be induced with bone morphogenetic protein 2 (BMP-2) [131]. MiRNAs upregulated during osteogenesis, acting as positive regulators, are commonly used as mimic miRNAs to modify stem cells in vitro, inducing osteogenic differentiation. In contrast, miRNAs that are downregulated during osteogenesis, and thus acting as negative regulators, are used as anti-miRNAs.

Several identified miRNAs, including miR-20a [132], miR-450b [133], miR-148b [106], and miR 26a [127,134], and anti-miRNAs, such as anti-miR-221 [111], and anti-miR-335 [125], promote the differentiation of MSCs into the osteoblastic lineage via two cellular pathways: transforming growth factor-beta (TGF-β)/BMP and Wingless/Int-1 (Wnt)/β-catenin [96]. MiR-125a-3p regulates the osteoblastic differentiation of human adipose-derived MSCs by targeting SMAD4 and JAK1 mRNAs [104]. MiR-26a on the other hand modulates bone formation in osteoporotic models by repressing the Tob 1 gene, a negative regulator of the BMP/Smad pathway [135].

Activation of these signaling leads to the upregulation of Runt-related transcription factor 2 (RUNX2), which is the major transcription factor driving osteogenic differentiation. RUNX2 plays an essential role in the regulation of genes promoting osteogenesis, bone formation, and extracellular matrix (ECM) biosynthesis [136].

Therapeutic approaches using miRNAs in bone regeneration may involve the incorporation of miRNA into biologically inactive, biocompatible synthetic materials: scaffolds. Scaffolds are porous polymers that can act as substrates to allow the exchange of miRNA with neighboring cells, thereby activating the formation of new bone. The majority of scientific publications on miRNA-based scaffolds for bone regeneration report the use of collagen-based hydrogel scaffold, another natural and synthetic hydrogel scaffold, hydroxyapatite (Hap)-based scaffolds, and calcium phosphate scaffolds. Figure 6 schematizes the diverse biomaterials employed as scaffolds for bone tissue engineering and the relevant miRNAs loaded into each scaffold type.

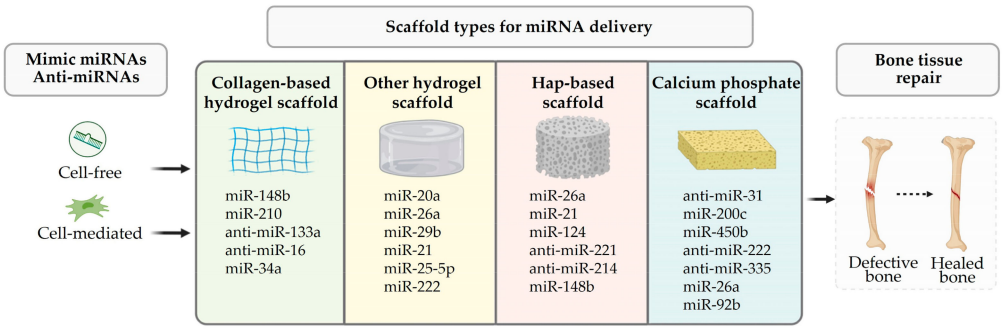

Figure 6. Scaffold types and miRNAs used for bone tissue regeneration. The figure illustrates mimic miRNAs/anti-miRNAs used in bone regeneration. The cell-free mimic miRNAs/anti-miRNAs are directly incorporated into the scaffold. In contrast, cell-mediated mimic miRNAs/anti-miRNAs are delivered into MSCs and then the transfected cells are incorporated into the scaffold. The main types of scaffolds, collagen-based hydrogel, other natural/synthetic hydrogel, Hap-based, or calcium phosphate, are subsequently implanted into the damaged bone.

- Collagen-based hydrogel scaffolds

Hydrogels are 3D structures formed by the cross-linking of hydrophilic polymer chains. Hydrogels have attracted attention in bone tissue engineering for their good biocompatibility and porous structure, similar to the ECM. Hydrogels also possess a soft texture that can minimize the inflammatory response when in contact with adjacent cells and tissues. Collagen is one of the most widely used hydrogel scaffolds for miRNA delivery in bone tissue engineering, as it is the major organic component of the bone extracellular matrix [137]. Collagen exhibits excellent biocompatibility, the ability to combine with other materials, high porosity, low antigenicity, ease of processing, hydrophilicity, and

excellent absorbability [138]. Collagen is also an optimal supporting structure for cell attachment and development [139]. These properties make collagen a desirable biomaterial for tissue engineering applications. Nevertheless, collagen's sensitivity to pH, temperature, ionic strength, and poor mechanical properties hinder the realization of its full potential. Consequently, collagen-based composite hydrogels and bioinks are developed that display excellent stability and bioprinting resolution [140,141]. The 3D-printed scaffolds are then loaded with miRNA-transfected cells and applied to bone defective sites that augment bone regeneration [142,143].

Moncal et al. developed a novel cell-mediated miRNA delivery system using collagen-infilled 3D-printed scaffolds [106]. In this study, hydroxypropyl cellulose-modified silver nanoparticles are used to deliver miR-148b to rat bone marrow stem cells (BMSCs); the transfected cells are then loaded into a collagen gel, which are subsequently used to fill a 3D-printed polymer scaffold. The results of the study demonstrate the ability of miR-148b-enriched scaffolds to effectively modulate the osteogenic differentiation of BMSCs by increasing osteogenic gene expression (including RUNX2) and by repairing critical-sized calvarial bone defects in a Fischer rat model.

Not only mimic miRNAs but also anti-miRNAs are delivered using collagen-based scaffolds. To provide an example, anti-miR-133a delivery with collagen-nanohydroxyapatite (coll-nHap) scaffolds enhances RUNX2 activity and increases bone repair after cell-free implantation in rat calvarial defects (7 mm in diameter) [108,109].

The same group of researchers investigating anti-miR-133 conducted another study in which an miR-210 mimic, an activator of both angiogenesis and osteogenesis, and anti-miR-16, as miR-16 is known to inhibit both pathways, were co-administered with a collagen-nanohydroxyapatite scaffold system [107]. In vitro, the treatment significantly improved the angiogenic–osteogenic coupling of human bone marrow-derived MSCs (hMSCs). In vivo, the potential of this dual miRNA-loaded scaffold to rapidly accelerate bone repair was evaluated in a rat calvarial defect model, showing increased bone volume and blood vessel recruitment. Liu et al. have shown that the delivery of miR-34a in a collagen hydrogel promotes the osteogenic differentiation of MSCs and induces ectopic bone formation and promotes bone healing in irradiated rat tibias [110].

- Other natural and synthetic hydrogel scaffolds

To generate a microRNA-activated scaffold, it is possible to use other natural hydrogels such as hyaluronic acid (HA), alginate, chitosan, gelatin, and fibrin, or one can also use synthetic hydrogels such as polycaprolactone (PLC), polylactic acid (PLA), polyoxyethylene (PEO), polyvinyl alcohol (PVA), and polyethylene glycol (PEG) [144].

MiR-26a and miR-20a are the most cited examples of miRNAs for bone regeneration delivered through hydrogel-based scaffolds. Nguyen et al. demonstrate the ability of miR-20a to differentiate hMSCs incorporated into PEG hydrogels [112]. MiR-20a promotes osteoblastic differentiation of hMSCs by targeting PPARγ (Peroxisome Proliferator-Activated Receptor γ), Bambi, and Crim1, which in turn upregulate BMPs and RUNX2 [132]. These PEG hydrogel scaffolds encapsulated with hMSCs and miR-20a display excellent bone regeneration in critical-sized calvarial defects in rats [145].

In 2021, Gan et al. reported a system formed by cholesterol-modified miR-26a (Chol-miR-26a) conjugated to an injectable PEG hydrogel [113]. MiR-26a promotes the osteogenic differentiation of hMSC by directly interfering with glycogen synthase kinase 3β (GSK3β) which is well-characterized as a negative regulator of the β-catenin pathway [146]. Kuang et al. utilized exosomes extracted from bone marrow-derived MSCs as a carrier for miRNA-26a (Exo@miR-26a) delivery. The Exo@miR-26a was encapsulated within a gelatin–chitosan methacrylate (GCMA) composite gel that not only upregulated osteogenic gene and angiogenesis but also regulated osteoclast-related cascade during the bone remodeling process in a rat calvarial defect model [114]. Pan et al. designed a 3D bio-printed miR-29b activated matrix using a gelatin–alginate hydrogel system and miR-29b gold nanoparticle complex with controlled scaffold degradation and miRNA release profile for osteoinduction in subcutaneous implantation model in mice [115].

As shown in Figure 4, several miRNAs have been delivered via hydrogel-based scaffolds in bone regeneration, including miR-21 [147], miR-29b [115], miR-25-5p [148], and miR-222 [149].

- Hydroxyapatite-based scaffolds

Hydroxyapatite (Hap, $Ca_5(OH)(PO_4)_3$) is a bioactive, nontoxic, osteoconductive ceramic of great importance for bone scaffolds because of its similarity to the mineral part of the natural bone and its ability to form chemical bonds with living tissues [150]. Hap can act as a scaffold alone or in combination with tricalcium phosphate (TCP) or other biomaterials (hydrogels or collagen), as Hap increases the strength of composite scaffolds. Hap material may also be used in the nano-Hap (nHap) form combined with collagen or chitosan to form a composite scaffold for miRNA release [96,142,143].

Researchers use Hap-based composite scaffolds to deliver different types of miRNAs for bone regeneration. Marycz et al. developed nHap/iron oxide nanoparticle scaffolds functionalized with miR-21/124 for efficient bone regeneration [120]. Sadeghi et al. incorporated nano-Hap into electrospun Polycaprolactone (PCL) scaffolds to deliver MSCs transfected with anti-miRNA-221 [121]. In contrast, Wang et al. used Hap by itself as a porous Hap scaffold for the delivery of miRNA-26a [70].

Other miRNAs, such as anti-miR-214 [151] and miR-148b [106], have been delivered using HA-based composite scaffolds for bone regeneration.

- Calcium phosphate scaffolds

Calcium phosphate scaffolds (CPS) are composed of a material suited for bone regeneration to actively promote osteogenesis because of their stable properties, biocompatibility, and chemical similarity to bone minerals [152]. CPS has been developed using biphasic calcium phosphate (a combination of Hap and tricalcium phosphate [153]), but CPS can also incorporate collagen, glycosaminoglycan, natural polymer (e.g., fibrin), and synthetic polymers (e.g., polyglycolic acid and polylactic acid) [154]. The CPS types most commonly used in bone regeneration are HA, beta-tricalcium phosphate (β-TCP), and combinations of Hap and beta-TCP [142].

Deng et al. conducted studies with β-TCP scaffolds [122,123]. Porous β-TCP is a synthetic and biodegradable ceramic material that has been extensively studied for the repair of bone defects [155]. It has good osteoconductive properties due to its high porosity and interconnectivity, which can facilitate intercellular communication between osteogenic cells residing in the lacunae [156]. β-TCP scaffolds were used by Deng et al. to deliver anti-miR-31 in a mouse model and subsequently in a canine model. It was shown that miR-31 suppresses osteogenesis by targeting SATB2, which in turn represses the expression of several HOX genes that reduce RUNX2 expression. Therefore, inhibition of miR-31 with anti-miRNA-31 increases the expression of RUNX2 [157].

In the first study, adipose-derived MSCs (ADMSCs) were transfected with lentivirus (Lenti)-anti-miR-31, then combined with β-TCP scaffolds and finally implanted in vivo into a rat critical-sized bone defect [123]. The results show that ADMSCs cultured in vitro with Lenti-anti-miR-31 significantly increase the expression of mRNA and osteogenic proteins and that these modified cells significantly improve the repair of bone damage when implanted in vivo with β-TCP scaffolds.

In the second study, conducted in canine models, porous β-TCP scaffolds seeded with canine autologous anti-miR-31-modified BMSCs showed a significant ability to repair medial orbital wall defects in dogs (10 mm in diameter) [122].

In a recent study of 2021, Remy et al. developed engineered synthetic bone grafts combining a 3D-printed biodegradable β-TCP with the osteoinductive miR-200c [124]. This structure improves the transfection efficiency of miR-200c in both rat and human BMSCs and enhances the osteogenic differentiation of BMSCs in vitro. In addition, scaffolds incorporating miR-200c significantly improve bone regeneration in rat critical-sized calvarial defects.

Many other research groups have investigated a combination of TCP scaffolds with different miRNAs, including miR-21 [147], miR-450b [133], anti-miR-222 [158], anti-miR-335 [125], miR-26a [127], and miR-92b [159]. Almost all the above studies demonstrate improved bone healing using miRNA-activated CPS scaffolds [142].

4.2. MiRNA-Activated Scaffold and Cartilage Repair

The need for cartilage repair has increased significantly over the last decade. Adult articular cartilage does not heal spontaneously after injury; several factors, including the avascular composition of cartilage tissue, low cell density, and slow nutrient diffusion, make even small cartilage defects difficult to repair [65]. Today, cartilage degeneration due to trauma and age is a major cause of morbidity, particularly in humans, and is associated with enormous costs to health and social systems [160]. Consequently, there is a need to develop interventions that can help to prevent these disorders and therapies that can effectively treat them.

To date, developing optimal repair and reconstruction strategies for damaged cartilage remains an unmet clinical need [161]. However, the development of tissue engineering strategies that combine a cell source such as MSCs, a biomaterial scaffold (mainly hydrogel), and bioactive molecules such as miRNA seems to be particularly encouraging [65].

MSCs have been identified as an attractive cell source for cartilage regeneration due to their ease of isolation, regenerative potential, and chondrogenic differentiation [162].

Recently, strategies have emerged to engineer MSCs by silencing anti-chondrogenic factors and suppressing the function of proteins that negatively affect chondrogenesis or whose expression inhibits chondrogenic potential [163–166]. Several molecules have been identified as negative modulators of chondrogenic differentiation, including transcription factors, proteins, and miRNAs. The idea of generating MSCs without anti-chondrogenic factors represents an approach both to study the function of a gene or microRNA in the context of chondrogenesis and to provide a new therapeutic tool to improve cartilage repair [167].

Although considerable efforts have been made to elucidate the miRNAs involved in cartilage development, disease, and repair, and although scaffolds for cartilage repair have been developed, very few studies describe the therapeutic potential of miRNA-activated scaffolds in this tissue [168].

The most interesting results are provided by Lolli et al. who demonstrated the potential therapeutic application of miRNA-based delivery systems in a series of in vitro and in vivo studies aimed at silencing miRNA-221, a known inhibitor of the chondrogenesis process [116]. Lolli et al. first characterized miR-221 as a novel anti-chondrogenic miRNA and found that silencing miR-221 by transfecting cultured hMSCs with anti-miR-221 induces chondrogenic differentiation in vitro and in vivo without exposure to the chondrogenic inducer TGF-β [116]. Subcutaneous implantation of anti-miR-221 transfected hMSCs is sufficient to repair an osteochondral defect in mice by promoting the production of newly formed tissue expressing type II collagen [169]. miR-140 is another important microRNA that promotes chondrogenesis by the upregulation of SOX9 and ACAN proteins and modulates chondrogenic differentiation in cartilaginous tissues in mice [170].

In 2023, Intini and co-authors published a new study in which a novel miRNA-activated scaffold was developed to enhance MSC chondrogenesis and cartilage regeneration through the administration of anti-miR-221. The composite scaffold containing type II collagen was prepared by lyophilization and then functionalized with enhanced transduction system (GET) nanoparticles (NPs) encapsulating the miR-221 inhibitor. Anti-miR-221-activated scaffolds were then cultured with hMSCs in vitro. The cells were successfully transfected. The innovative anti-miR-221 miRNA scaffold showed the ability to enhance chondrogenesis and further research is expected to investigate how to improve cartilage repair [111]. MiR-99a-3p has recently been identified as a potent microRNA for suppressing cartilage degeneration for osteoarthritis treatment. Yin et al. have recently demonstrated that the delivery of miR-99a-3p using adipose stem cell-derived exosomes

embedded within a PEG-hyaluronic acid hydrogel alleviates OA progression in the murine model and shows enhanced cartilage ECM production [117].

Simultaneous regeneration of articular cartilage and subchondral bone to treat osteochondral lesions caused by trauma or osteoarthritis is another challenge that is difficult to overcome. Agili-C™ (CartiHeal) has recently recieved FDA approval as an osteochondral plug for treating osteochondral defects [171]. Recently, Celik et. al. designed a 3D bio-printed heterotypic osteochondral interface using aspiration-assisted and microvalve-based bioprinting [172]. In this study, they induced osteogenic differentiation of adipose stem cell-derived spheroids using miR-148b delivery and chondrogenic differentiation by co-delivery of miR-140 and miR-21. They followed a scaffold-free bioprinting approach where miRNA-transfected spheroids were printed in double layers (in an alginate support bath) to explore the potency of these spheroids in reconstituting the osteochondral interface. Interestingly, the printed osteochondral interface through miRNA-induced differentiation exhibits distinct osteogenic and chondrogenic layers with better shape retention and higher cell proliferation [172].

4.3. MiRNA-Activated Scaffold and Wound Healing and Skin Regeneration

Newer strategies, including cellular and biological factor therapies and electromechanical stimulation, are being investigated for the treatment of chronic wounds. Nevertheless, healing is a complex process due to the unique and challenging environment of the biological wound. While current therapies, including skin substitutes, are improving skin healing, there is no evidence that these therapies can effectively restore normal skin structure and function [173,174].

The physiological process of wound healing involves a series of events: hemostasis, inflammation, proliferation, and ECM remodeling [175]. This process is mediated by keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets [176] and is regulated by a complex network of signaling molecules, including several miRNAs [71,177].

As in other areas of regenerative medicine, researchers are developing engineered miRNA delivery strategies to induce acute and chronic wound healing [178]. MiR-21 is one of the most studied microRNAs that promote wound healing by downregulating PTEN and RECK genes and activating the MAPK/ERK signaling pathway [179]. Other microRNAs such as miR-129 and miR-335 are also reported to accelerate wound healing by inhibiting MMP-9 expression through the Sp1 gene [180]. Due to their small size and long half-life, miRNAs provide a continuous and effective level of regulation of cellular behavior and can modify the bioactive environment of the skin [173].

In recent years, much progress has been made in the design of 3D biomaterial scaffolds to overcome problems associated with chronic wounds. These scaffolds provide an excellent microenvironment that ensures correct cell growth, proliferation, and differentiation at the lesion site where they are implanted. In addition, biomaterial scaffolds are of interest as mediators of scar formation, as they allow greater hydration of the epidermis covering the scar and minimize the risk of infection in the healing wound [173].

In particular, hydrogels have found application in the delivery of miRNAs for the treatment of chronic wounds as they exhibit desirable properties such as native tissue-like elasticity, they can provide a protective barrier, mimic the native ECM, and provide a humid environment [174]. Furthermore, hydrogel engineering for wound healing applications promotes the inhibition of bacterial growth, enhances re-epithelialization and vascularization, improves recovery of tissue function, and accelerates wound healing overall [174].

In a recent study by Salem et al. (2019), adhesive hydrogels containing hyaluronic acid (HA) nanoparticles coupled with an miR-223 5p mimic (miR-223*) are developed to control the polarization of tissue macrophages during the wound healing process [118]. MiR-223 is a regulator of the acute inflammatory response and is predominantly expressed in neutrophils and macrophages at skin wound sites [181]. MiR-223 also drives macrophage polarization toward the anti-inflammatory phenotype (M2), accelerating wound healing [182]. In vitro overexpression of miR-223* in macrophages J774A.1 shows increased

expression of the anti-inflammatory gene Arg-1 and decreased pro-inflammatory markers, such as TNF-α, IL-1β, and IL-6. Finally, histological evaluation and qPCR analysis after in vivo transplantation using a mouse model of acute excitotoxic injury show that the local release of miR-223* effectively promoted the formation of uniformly vascularized skin at the wound site, mainly driven by the M2 polarization of macrophages. Recently, Wei et. al. have designed a promising miR-17-5p loaded hydrogel developed by encapsulating miR-17-5p within an extracellular vesicle embedded within the gelatin methacrylate (GelMA)-derived hydrogel matrix for diabetic wound healing [119]. Here, the human umbilical-cord-derived MSCs are first transfected with lentivirus carrying miR-17-5p and EVs are extracted and encapsulated in the GelMA hydrogel. Administration of this bioactive wound dressing to a diabetic wound model repressed p21 expression and effectively facilitated diabetic wound healing by enhancing local angiogenesis and collagen deposition.

Collagen-based hydrogel is also an excellent biomaterial for skin wound healing. As an ECM-derived scaffold, collagen acts as a physical support to promote tissue organization and resist aggressive wound contraction and scar tissue formation [183]. The 3D collagen scaffolds enable the maintenance of the biological and structural integrity of the skin, providing mechanical support and protection from the external environment [184].

Monaghan et al. used four-armed cross-linked collagen type I polyethylene glycol-terminated succinimidyl glutarate (4S-StarPEG) encapsulated with miR-29b to enhance the wound healing process [183]. MiR-29b promotes wound healing by directly targeting ECM genes such as fibronectin, collagen type I, and collagen type III. This scaffold, once functionalized with miR-29b and applied to a rat excisional wound model, modulates the wound healing response by reducing collagen type I production (which is directly silenced by miR-29b), subsequently improving the collagen type III/I ratio, and increasing matrix metalloproteinase 8 activity.

5. Conclusions and Future Outlook

MiRNA mimic and anti-miRNA strategies are promising curative therapeutic strategies in tissue regeneration. In comparison with DNA-based gene therapy, miRNA-based therapy possesses the great benefit that miRNA does not integrate into the genome, risking mutation development, in addition, compared with protein-based therapy, miRNA has a longer lifetime. Strategic delivery of mimic miRNA and anti-miRNA through three-dimensional scaffolds offers spatial and temporal control, mitigating potential off-target effects and improving therapeutic outcomes. MiRNA-activated scaffolds hold promise for the precise regulation of gene expression within target tissues. To date, the main obstacle to the progression of therapeutic choice based on miRNAs and anti-miRNAs lies in the limited in vitro and in vivo knowledge of the mechanism of action of these small pleiotropic molecules. Substantial help in the intricate web of miRNA function remains the confluence of bioinformatics knowledge to identify the various miRNA binding targets and the associated biological pathways implicated. To date, pharmaceutical companies' investments in developing these therapeutic strategies are still in an early stage without clinical realization. The promise of scaffold-embedded mimic and anti-miRNA therapies represents an attractive frontier in regenerative medicine; however, to claim that these strategies today are already a triumph is premature. We are confident that the use of miRNA mimic and anti-miRNA activated scaffolds as a therapeutic strategy to promote bone, cartilage, and skin regeneration options will allow us to benefit from their efficacy in several years, as there are still many unanswered questions about the required dosage and possible side effects. Surely, to date, the principal question on the collateral effect remains how to design oligonucleotides and delivery vehicles to minimize non-target cell impacts. However, this side effect is outweighed by the advantage that one miRNA with the ability to target multiple genes has the potential to regulate the entire signaling pathway in complex disease conditions where multiple target genes of the same miRNA are deregulated. In our opinion, one of the future outlooks of miRNA-activated scaffold research should be to align the miRNA release rate with the scaffold matrix degradation rate, because it would

be a useful dosage system, and because it would allow the monitoring of collateral effects over a definite time range. Furthermore, miRNA-based therapies may hold remarkable promise for personalized medicine and problems related to bone and cartilage senescence, and perhaps, why not, for tissue regeneration in aesthetic aging/senescence problems?

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