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Review

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Biomaterials to Facilitate Delivery of RNA Agents in Bone Regeneration and Repair

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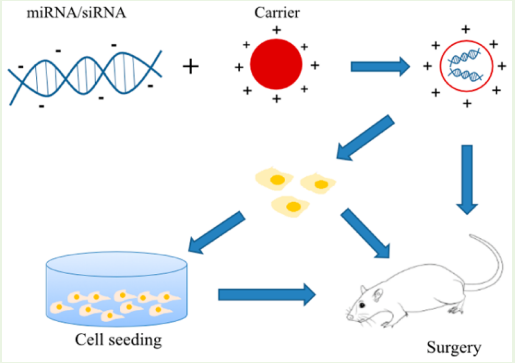
ABSTRACT: Bone healing after traumatic injuries or pathological diseases remains an important worldwide problem. In search of safer and more effective approaches to bone regeneration and repair, RNA-based therapeutic agents, specifically microRNAs (miRNAs) and short interfering RNA (siRNA), are beginning to be actively explored. In this review, we summarize current attempts to employ miRNAs and siRNAs in preclinical models of bone repair. We provide a summary of current limitations when attempting to utilize bioactive nucleic acids for therapeutic purposes and position the unique aspects of RNA reagents for clinical bone repair. Delivery strategies for RNA reagents are emphasized and nonviral carriers (biomaterial-based) employed to deliver such reagents are reviewed. Critical features of biomaterial carriers and various delivery technologies centered around nanoparticulate systems are highlighted. We conclude with the authors' perspectives on the future of the field, outlining main critical issues important to address as RNA reagents are explored for clinical applications.



KEYWORDS: *microRNAs, bone rengeneration, RNA reagents, scaffold, carrier*

1. INTRODUCTION

Bone healing after a traumatic injury or pathological diseases remains an important worldwide problem. Each year, ~6.2 million bone fracture cases are reported in the United States alone, where 5−10% result in nonunion or delayed union.[1,2] Bone tissue possesses the ability to remodel damaged skeleton via intramembranous and endochondral pathways without leaving a scar tissue.[3,4] In situations of impaired self-repair, bone grafting is the preferred treatment; annually, more than 2 million bone grafting are performed worldwide.[5] Bone grafting involves removal of live tissue from another site (most commonly from iliac crest) to fill a defect.[6] Without immunological rejection, autografts provide the best osteoconductive (scaffold), osteogenic (cells), and osteoinductive (growth factors, GFs) properties that constitute the three essential elements of bone regeneration.[7] However, as high as 30% complication rates were reported at the harvest site, such as excess hematoma formation, blood loss, increased risk of deep infection, and sometimes chronic pain. As an alternative to bone grafts,[8] biomimetic bioactive devices are being pursued to safely repair bone tissue by tapping into the cellular and molecular biology of bone regeneration and repair.

Bone healing comprises of an inflammatory phase, two phases of repair involving soft and hard callus formation, and finally remodelling. At the cellular level, inflammatory cells, vascular cells, osteochondral progenitors, and osteoclasts play key cells in the injury response.[9] At the molecular level, the process is driven by pro-inflammatory cytokines, GFs, and angiogenic and pro-osteogenic factors that are secreted by local cells or released from extracellular matrix (ECM).[10,11] Their activities can control the cellular migration, proliferation and differentiation, collagen synthesis, and angiogenesis.[12] The exact concentration, timing, and spatial location of GFs play essential roles during the healing process.[11,13,14]

Involvement of GFs in bone regeneration makes them natural candidates for therapeutic agents. The US Food and Drug Administration has already approved BMP-2 and BMP-7 (rhOP-1) for selected clinical applications and other GFs have undergone or are currently undergoing clinical trials.[15−17] However, there are concerns with localization of GFs and high protein doses needed (evident in preclinical studies and early clinical trials) as well as their short in situ residence time.[18,19] Conventional delivery of GFs may result in undesirable tissue responses, such as bone resorption and local inflammation.[20,21] High doses have been a concern because of the post-treatment side effects such as swelling, ectopic bone formation, tumor formation, and seroma after BMP-2 in spinal fusion therapy.[22,23] The cost of the treatment is also concerning in the case of GF therapies.[24] Gene therapy can be an alternative approach to avoid the limitations associated with protein therapy. Genes are

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Table 1. Specific miRNAs Involved in Osteogenesis in Vitro[a]

miRNA	study outcome	carrier	scaffold	ref
miRNA-20a	sustained and controlled release from the hydrogels over a period of 3−6 weeks; osteogenic differentiation was enhanced	PEI 25	PEG hydrogel	63
miRNA-29a	contributes to osteogenesis; SOX9 down-regulates its transcription; overexpression of miRNA-29a strongly inhibits the expression of chondrocyte-specific markers during in vitro chondrogenic differentiation of MSCs	Oligofectamine	NO	110
miRNA-29b	AuNPs enters efficiently the miRNA to cytoplasm; NP/miRNA system promotes osteoblast differentiation and mineralization	PEI- Au nanoparticles, Lipofectamine	NO	111
miRNA-31	overexpression of miRNA-31 inhibits the osteogenesis of MSCs; miR-31 regulates the osteogenesis of MSCs by targeting SATB2	Lipofectamine	NO	112
miRNA-125b	regulatory factor of osteoblastic differentiation by directly targeting Cbfβ & indirectly acting on Runx2 during early stage of osteoblastic differentiation	Lipofectamine	NO	113
miRNA- 133a	enhancement of Runx2 and osteocalcin expression; increase in ALP and calcium deposition	nHA particles	COL/nHA scaffold	91
miRNA-138	inhibition of osteogenic differentiation of MSCs & suppresses the phosphorylation of FAK, ERK1/2, and Runx2	Oligofectamine	NO	114
antimiRNA-138	high transfection efficiency; increase in the osteogenesis of MSCs	CS/TPP/HA nanoparticles	NO	115
miRNA-140-5p	inhibition of miR-140-5p expression in MSCs upregulates BMP2	DharmaFECT	NO	116
miRNA-146a	important role in skeletogenesis by down regulation of SMAD2 and SMAD3 function	DharmaFECT	NO	117
miRNA-148b	MSCs become susceptible to osteogenic factors; rapid and robust induction of bone related markers	Human MSC Nucleofection kit	PEG-NB hydrogel	118
miRNA-154-5p	mechanical stress promotes osteogenic differentiation of murine ADSCs; under mechanical stress, miRNA-154-5p inhibits Wnt/PCP pathway and prevents osteogenic differentiation of ADSCs	lentivirus	NO	119
miRNA-194	regulates STAT1 expression; overexpression promotes the nuclear translocation of Runx2	lentivirus	NO	120
miRNA-218	improves osteogenic differentiation of hASCs; directly targets the SFRP2 and DKK2; enhances Wnt/β-catenin signaling activity	lentivirus	NO	121
miRNA-222-3p	promotes osteoblast-specific gene expression, ALP activity, and matrix mineralization	lentivirus	NO	122
miRNA-302a	induction of BMP-2-Runx2 signals in preosteoblasts; promotion of osteoblast differentiation by targeting COUP-TFII miRNA	Lipofectamine RNAiMAX	NO	123
miRNA-489	MSCs are becoming susceptible to osteogenic factors; rapid and robust induction of bone related markers	Human MSC Nucleofection kit	PEG-NB hydrogel	118

[a]The data are derived from cell culture studies where a specific miRNA was delivered with a viral or non-viral carrier. The cells that were tested were MSCs and human amniotic-derived stromal cells (hADSC).

Table 2. Specific miRNAs Involved in Bone Regeneration and Repair in Vivo[a]

miRNA	study outcome	carrier	scaffold	in vivo model	ref.
miRNA-26a	improves vascularization and bone regeneration; HP-HA-PEG system improves miRNA-26a expression	siPORT NeoFX	HP-HA-PEG hydrogel	calvarial bone defect in mouse	94
	actions through targeting Gsk-3β to increase osteoblastic activity; long-term delivery for higher expression of multiple osteogenic genes	PLGA microspheres	PLLA scaffold	subcutaneously in mouse	95
antimiRNA-31	increase in the expression of osteogenic genes in vitro; robust new bone formation in vivo; miRNA-scaffold system improved (~60%) in vivo bone formation	lentiviral	poly(glycerol sebacate) scaffold	cranium bone defect in rat	124
miRNA-34a	modulator of osteoblastic differentiation of MSCs; targets JAG1-ligand for Notch 1; controls both hMSCs proliferation and osteoblast differentiation	Lipofectamine	3D-spheroid HA/TCP scaffold	heterotopic model in mouse	98
miRNA-103a	mechanosensitive miRNA that regulates osteoblasts differentiation and bone formation by targeting Runx2	Lipofectamine	NO	hindlimb unloading model in mouse	125
miRNA-135	upregulation during osteogenesis of rat ADSCs; overexpression promotes bone formation	Lipofectamine	poly(sebacoyl diglyceride)	calvarial bone defect in rat	126
antimiRNA-138	enhancement in the in vitro osteogenesis and in vivo bone formation	Lipofectamine	cell sheet	subcutaneously in mouse	
miRNA-148b miRNA-196a miRNA-29b miRNA-26a	miRNA-148b & -196a showed more osteoinductive effects; co-transduction of hASCs with miRNA-148b accelerates bone formation in vivo in 12 weeks	baculovirus	PLGA scaffold	calvarial bone defect in mouse	127
miRNA-199a-5p	improves osteogenic differentiation of MSCs via HIF1α-Twist1 pathway and promotes in vivo bone regeneration	Lipofectamine Chitosan/ agomiR	NO	tibia defect in rat	64
miRNA-216a	promotes osteogenic differentiation of hASCs in vitro and bone formation in vivo	Lipofectamine	HA/TCP scaffold	subcutaneously in mouse	128

[a]The data are derived from animal models where a specific miRNA was delivered into a bone defect with a viral and non-viral carrier. The cells that were tested were MSCs and human amniotic-derived stromal cells (hADSC).

commonly delivered within a plasmid DNA (pDNA) that can be designed to promote a signaling mechanism supportive of regeneration, or to suppress mediators inhibiting bone formation. Several regenerative genes have been explored to-date, mainly based on GFs (e.g., BMPs, PDGF, and FGFs) and transcription factors associated with bone/cartilage formation Runx2/Cbfa1 and Osterix.[25] Gene delivery has the flexibility to express proteins locally, focally and intracellularly, as needed. It eliminates any

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Table 3. Specific siRNA Targets Involved in Osteogenesis in Vitro[a]

siRNA	study outcome	carrier	scaffold	ref
Noggin	sustained and controlled release from the hydrogels over a period of 3−6 weeks; enhancement of osteogenic differentiation	PEI 25	PEG hydrogel	63
	>98% intracellular uptake of MC3T3-E1 cells after 48 h; reduction in the use of rhBMP-2 by knockdown BMP-2 antagonists	Lipofectamine	fibrin hydrogel	51
	stimulation of BMP signaling by downregulating Noggin; promotion of osteogenesis	lentiviral particles	Chitosan/Chondroitin sulfate (Apatite-coated) scaffold	92
Sox9	major regulator of direct osteogenesis; leads to indirect or direct suppression of Runx2	neon transfection system	NO	129
VEGF	hypoxic conditions can stimulate cell proliferative response; activation of PI3K/Akt plays a vital role in inducing proliferation, osteogenesis, and angiogenesis	Lipofectamine	natural bone-derived scaffold	93
RANK	in vitro delivery of siRNA from MBG for over 3−4 days; >70% intracellular uptake; complexes successfully inhibit the expression of RANK	mesoporous bioactive glass nanospheres (MBG)	NO	72
PTX3	PTX3 is not directly influences osteoblast or osteoclast differentiation; exogenous PTX3 indirectly affects osteoclast differentiation by increasing RANKL production of precursor osteoblasts	Lipofectamine RNAiMAX	NO	130
HDAC8	inhibition of HDAC8 by HDAC inhibitor promotes the level of (H3K9Ac) that enhances the expression of osteogenic genes	lentiviral/ Lipofectamine	NO	131
LSD1	successful down regulation of LSD1 and differentiation of hMSCs; presence of HGF could promote the differentiation of hMSCs	gold nanorods	NO	76
Simvastatin	estrogen receptor (Era) has a crucial role in simvastatin-induces osteogenic gene expression and mineralization	Lipofectamine RNAiMAX	NO	132

[a]Data are derived from cell culture studies where a siRNA against a specific target was delivered with a viral or non-viral carrier.

Table 4. Specific siRNA Targets Employed for Bone Repair in Animal Models

siRNA	role/study outcome	carrier	scaffold	in vivo model	ref
Noggin	efficient gene knockdown with minimal toxicity, and osteogenesis promotion in vitro and bone regeneration promotion in vivo	stereosomes and Lipofectamine	methacrylated glycol chitosan hydrogel	calvarial defect in mouse	133
Cbfa-1	NPs easily enter the hMSCs in vitro and can differentiate into chondrocytes; high markers expression in mature chondrocytes	PLGA−PEI particles	NO	subcutaneous injection in mouse	134
Plekho 1p	presence of CH6 improves in vitro osteoblast-selective uptake of the siRNA and promotion of bone regeneration in vivo	CH6-Lipid nanoparticles	NO	injection to ovariectomized rat	46
siCkip-1 siFlt-1	upregulation of osteogenic and angiogenic genes. Promotion of bone regeneration in vivo	Lipofectamine	chitosan sponge	calvarial defect in rat	52
CTRP3	CTRP3 is a negative regulator of RANKL and acts as an inhibitor of NFATc1 activation through the AMPK pathway	Lipofectamine	NO	calvarial defect in mouse	135

issues related to contamination of a protein preparation with incorrectly folded and possibly antigenic species. An additional advantage is the ability to sustain protein production in situ for a longer time. Gene delivery is likely to result in lower levels of therapeutic proteins so that it may reduce protein exposure to the body (lower undesirable side effects) as well as reduced cost.[26,27] The use of pDNA is now well-established for bone repair, with promising preclinical studies elucidating the factors responsible for its successful application.[28]

More recently, RNA-based approaches have been providing a new path for bone diseases. Being chemically close to DNA and sharing the same limitations as DNA delivery, RNA agents will rely on decades of development work on DNA delivery technology for successful translation to clinical settings. Two types of RNA molecules are now actively explored to modulate bone repair, micro-RNAs (miRNA) and short interfering RNAs (siRNA). Mature miRNAs are non-protein-coding small (20−24 nucleotide) RNAs that bind to RNA-Induced silencing complex (RISC), which then bind miRNA at the 3′ untranslated region to reduce or inhibit the translation.[29−31] Several studies suggest a strong connection between the presence of specific miRNAs and regulation of various osteogenesis steps, acting as both inhibitors of osteogenesis and promoters of osteoblast differentiation. Table 1 provides a summary of miRNAs currently explored for stimulation of in vitro osteogenic differentiation. The importance of miRNAs have been initially identified from cell culture and mutagenesis models,[32,33] but recent activity is beginning to validate their therapeutic utility in preclinical animal models (Table 2). The latter includes studies where specific miRNAs were directly delivered to a bone repair site to modulate cell fate at the site, or when cells modified with specific miRNAs are implanted in bone repair models. It has been possible to identify both inhibitory and stimulatory miRNAs on osteogenesis and even silence inhibitory miRNAs to obtain a stimulation of bone induction.[34] While one can envision direct delivery of RNA-based agents to modulate cell fate, one can also deliver pDNA expression vectors for in situ synthesis of miRNAs or anti-miRNAs.

Double-stranded siRNAs, on the other hand, are synthetic entities that can target specific miRNAs and inhibit their translation after binding by pair-specificity on miRNA. Tables 3 and 4 summarize, respectively, recent siRNA targets employed for in vitro stimulation of osteogenic differentiation and bone repair in animal models. The early activity on siRNA delivery in animal studies, which involved specific siRNA against Plekho1 (casein kinase-2 interacting protein-1), GNAS1 and PDH2 combination,[28] were recently expanded with siRNAs against numerous new protein targets.

Scientists have established a potential utility of circulating miRNA as biomarkers for diseases affecting bone (e.g., cancer)

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that will help with early detection and selection of treatment. In the field of bone regeneration, successful application of miRNAs or siRNA is still limited and is in early preclinical trials.[35,36] The aim of this review is to highlight and review recent developments for RNA-mediated bone repair and to identify current limitations encountered in the field. We here review the main carries used for RNA delivery and the specific RNA reagents explored, including in vitro and preclinical assessment in animal models. The literature research was conducted by using online databases and referenced papers was selected from the pool of publications generated by the keywords "microRNA", "siRNA", "scaffolds", and "bone regeneration".

2. BIOMATERIAL CARRIERS IN INTRACELLULAR RNA DELIVERY

Successful use of nucleic acids requires carriers that facilitate cellular entry of nucleic acids into target cells (Figure 1). In the absence of a carrier, the nucleic acid is likely to be degraded in biological fluids by nucleases before it reaches surface of target cells. It will also have a low chance of undergoing cellular uptake due to electrostatic repulsion at the cell membrane. In spite of their high efficiency, clinical application of viral expression systems (vectors) is limited due to toxicity and immunogenicity issues. Naturally derived and synthetic biomaterials, when combined with nucleic acids, can create nanoparticles (NPs) suitable for cellular uptake, which could be further aided by the presence of targeting moieties or excess cationic charge for binding to cell membrane. Being synthetic, nonviral carriers offer excellent molecular tunability (facile chemistry), large scale production, stability for long-term storage, and reconstitution.[37,38] Nonviral carriers can also provide optimal unpacking for robust transfer and dissociation of the genes as required. Although cytotoxicity on host cells is an important concern, lack of long-term immune response or little chance of oncogenic transformation are the key reasons for their pursuit for clinical applications. With pDNA delivery, access to the nucleus is paramount and complexes have to overcome the passage of nuclear membrane. However, the miRNA knockdown by siRNA and miRNA regulation of biochemical pathways can occur in the cytoplasm,[39−41] making effective delivery more feasible. Smaller RNA-based agents requires lower amount of carrier also, which reduces the cost and toxicity associated with carriers used with pDNA delivery studies.[42] Below, we critically evaluate different types of carriers used to deliver RNA agents in bone regeneration.

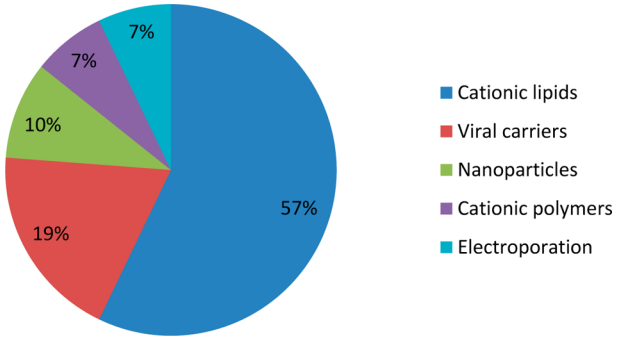

Figure 1. Summary of delivery systems used to deliver RNA agents. The percentages indicate the relative use of specific type of delivery system in the studies outlined in Tables 1−4.

2A. Cationic Lipids. Cationic lipids were the earliest materials explored in gene delivery.[43] They are composed of three structural domains; a cationic headgroup, a hydrophobic tail and a linker between these domains. Cationic headgroup is the specific component that interacts with nucleic acids, forming nanosized "lipoplexes" or cationic liposomes. These complexes are usually small enough (~100 nm) for cellular uptake and resilient enough to protect the payload against digestion.[44,45] Main cationic lipids used in therapeutic delivery and bone tissue engineering are shown in Figure 2.[46,47] Zhang et al. reported a comprehensive formulation for a bone-targeting liposomal system $(AspSerSer)_6$-DOTAP encapsulated with a siRNA specific for PleKho1.[47] $(AspSerSer)_6$ specifically target to osteogenic-linage of the cells, the osteoblasts at tissue level. Liang et al. reported aptamer-functionalized lipid NPs for osteogenic siRNA delivery. The integration of aptamer onto lipid NPs is to facilitate endocytic uptake.[46] Systemic delivery of

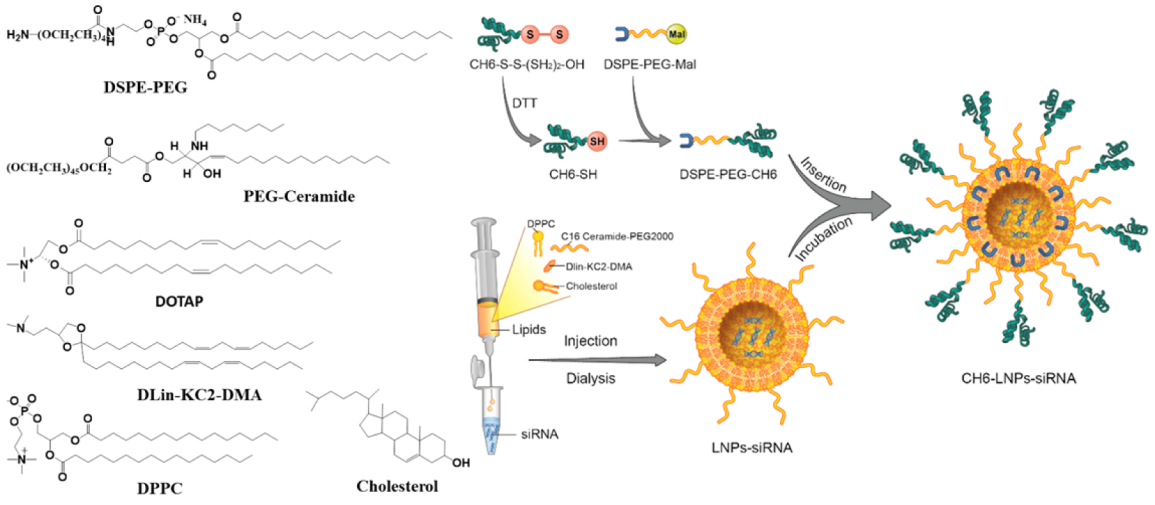

Figure 2. Chemical structure of cationic lipids and schematic of targeted NP preparation of described in this review. DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)]; PEG-ceramide, N-palmitoyl-sphingosine-1-succinyl[methoxy(polyethylene glycol)]; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DL-in-KC2-DMA, 2,2-dilinoley-4-(2-dimethylaminoethyl)- [1,3]-dioxolane; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. The schematic of NP formulation was adopted from ref 46.

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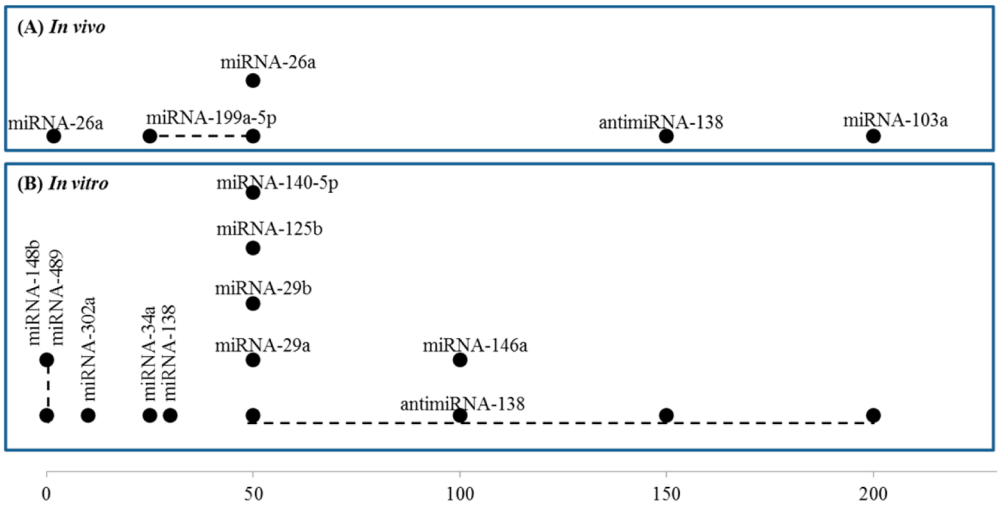

Figure 3. miRNA concentrations (nM in horizontal axis) delivered by nonviral carriers in vitro and in vivo. The dashed line between points indicates the test of different concentrations in the same study.

these carrier in an animal model selectively accumulated siRNA in osteogenic/osteoblast cells and subsequently depleted PleKho1, resulting in enhanced bone microarchitecture and tissue mass. Lipoplexes were also utilized to promote bone regeneration with the 'cell-sheet' technology, where regenerative repair is achieved with a dense sheet of cells with abundant endogenous ECM.[48] Yan et al. reported on the in vitro osteogenic differentiation of BMSC-sheet after transfection with antimiR-138 using Lipofectamine 2000.[49] The antimiR-138 delivery, by down-regulating endogenous miRNA-138 and activating extracellular signal-regulated kinases pathways, enhanced the expression of runt-related transcription factor-2 (RUNX2), osterix, osteocalcin and BMP-2 at miRNA and protein levels. In vivo results from these BMSC sheets were also exciting in immunocompromised mice for bone regeneration. Lipofectamine has been also used to enable RNAi knockdown of specific inhibitors of BMPs.[50] Lipofectamine mediated siRNA delivery to preosteoblast MC3T3-E1 cells through hydrogel surfaces substantially down-regulated inhibitory noggin miRNAs.[51] The Lipofectamine-based cationic liposomes were also incorporated into scaffolds that maintained the integrity of siRNAs for longer period. In a recent study, Jia et al. reported a porous chitosan scaffolds bearing Lipofectamine 2000/siRNA (siCkip-1 and siFlt-1) complexes.[52] The bioactivity of these scaffolds was studied by growing bone marrow MSCs; the loaded siRNAs remained intact for 2 weeks. The target genes were significantly silenced and upregulation of ALP activities, VEGF, and osteocalcin were clearly observed in MSCs as a result of siRNA delivery.

2B. Cationic Polymers. Cationic polymers are the most studied material in nonviral gene delivery because of their facile chemistry, cost-effectiveness, and safety profiles.[40,53] Multivalent electrostatic interaction between cationic amino groups of polymers and anionic phosphate groups of RNA molecules forms condensed polyionic complexes (polyplexes). These complexes enhance cellular uptake via interaction with anionic cell surface proteoglycans and increase nucleic acid half-life in cytoplasm.[54,55] Polymers are especially attractive to establish a scaffold matrix for regenerative medicine[56−62] and sustain local presence of RNA agents in scaffolds.[63] The scaffold-mediated delivery of RNA is relatively safer since only local tissue get exposed to RNA agents. In one study, Chen et al. reported a proof-of-concept for delivery of has-miR-199a-5p (agomir/pDNA) to human MSCs (hMSCs) using chitosan NPs.[64] Overexpression of miR-199a-5p enhances hMSCs differentiation whereas its inhibition reduces the expression of osteoblast-specific genes, ALP activity and mineralization. The NPs displayed a sustained release of payload, effective transfection of hMSCs and significant bone repair. Cationic polymers were also utilized to construct hydrogels, whose structural similarities to ECM to encapsulate stem cells within a 3D network makes them useful for tissue induction.[65,66] The hydrogels, along with RNA regulators, can be deployed to modulate cellular process such as osteogenic differentiation of encapsulated stem cells. Nguyen et al. reported 25 kDa PEI/siRNA complexes integrated into poly(ethylene glycol) (PEG) hydrogels for bone tissue engineering.[63] This hydrogel was effective for sustained and controlled release of encapsulated siRNAs over 3 to 6 weeks and maintaining the bioactivity of siRNAs intact. The prolonged delivery of siRNAs against noggin and miR-20a substantially enhanced the osteogenesis of encapsulated hMSCs.

2C. Inorganic NPs. Inorganic nanomaterials have been used as gene carriers due to unique features such as light scattering, localized surface plasmon resonance effect and photothermal effect.[67,68] In recent studies, mesoporous bioactive glass nanospheres and silica NPs were explored for siRNA delivery because of their unique bone-binding activity and degradability,[69,70] with specific application for treatment of osteoporosis.[71,72] The survival of mature osteoclasts, bone resorption and expression osteoclast-specific genes is primarily driven by the interaction of cytokines RANKL and its receptor RANK, present on the surface of osteoclast precursors.[73,74] To this end, Kim et al. has reported mesoporous bioactive glass as a potential RANK-siRNA carrier to macrophage RAW264.7 cells.[72] These NP sustained the release of the payload over a period of ∼4 days and exhibit knockdown of osteoclastogenesis-related gene, including c-fos, cathepsin-K, tartrate-resistant acid phosphatase (TRAP) and nuclear factor of activated T-cells cytoplasmic-1 (NFATc1). Gold NPs is another unique carrier for gene delivery.[68] Tunable size and optical properties based on the size along with outstanding biocompatibility makes gold NPs a good choice for diagnostic and therapeutic application.[75] Zhao et al. has reported on delivery of LSD1-siRNA and consequent impact in differentiation of hMSCs with gold NPs.[76]

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LSD1 maintains the pluripotency in embryonic and other stem cells (such as neural and leukemic stem cells) so that silencing of LSD1 can down-regulate stemless and up-regulate differentiation genes.[77] LSAD1-siRNA was successfully grafted onto gold NPs by coating with poly(sodium 4-styrenesulfonate) and poly allylamine hydrochloride.[76] The delivery of LSD1-siRNA to hMSCs significantly induced the differentiation of hMSCs into a hepatocyte lineage.

Interestingly, over 20 miRNAs have been described the last two years to be involved in osteogenesis, where the majority of the studies examined the therapeutic applications of the new miRNAs. Most studies were reported with a single miRNA concentration (typically 50 nM, Figure 3) and this raises questions about the actual effectiveness of the miRNA since a dose−response relationship is paramount to fully assess the outcome of the therapy. Only limited number of studies had used scaffolds as miRNA reservoir and only one study used <50 nM miRNA dose. Due to the undesirable side effects of viral vectors, few studies examined the delivery of miRNA or siRNA with viral vectors in vitro or in vivo (Figure 4).

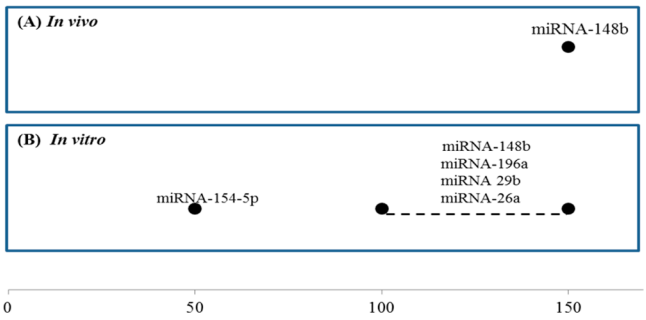

Figure 4. miRNA concentrations (multiplicity of infection − MOI- in horizontal axis) delivered by viral carriers in vitro and in vivo. The dash line between points indicates the test of different concentrations in the same study.

The application of siRNA compared to miRNA is still limited. The siRNAs that target Noggin are leading the field, but the functional dose needed for a successful application still remains to be determined (Figure 5).

3. SCAFFOLDS FOR BONE REGENERATION WITH RNA AGENTS

A major focus in bone tissue engineering is the development of implantable scaffolds that will closely imitate the natural tissue. Scaffolds intended for bone should be biocompatible, display controlled biodegradability, and appropriate pore size and should provide the right mechanical support. A highly porous scaffold (pore size >90%) has been shown to influence cell adhesion which promotes osteointegration, nutrient/GF transfer and vascularization.[78] The mechanical strength is an important consideration, because the scaffold tends to become mechanically fragile over time while undergoing degradation.[79] A scaffold can be further modified to mimic physiological aspects (i.e., cell adhesiveness) of native bone tissue matrix. A variety of biomaterials, either synthetic, natural or biomimetic, have been explored as 3D scaffolds for bone tissue repair because of their inherent bioactivity with the ability to promote cell adhesion, proliferation, and differentiation with no apparent cytotoxic effects.[80−83]

The incorporation of bioactive factors into scaffolds provides an additional dimension to modulate cellular responses to accelerate the formation of new tissue. The scaffold should protect the factors against extracellular barriers that could reduce their therapeutic efficacy, and display effective release levels for prolonged periods of time.[84] The factors are most commonly loaded into the bulk of a scaffold, either by mixing them into the scaffold material during fabrication or by simply adding (soaking) them into the scaffold postfabrication. Low affinity interactions between the factors and the scaffold will yield rapid release rates, while the release rates can decrease with stronger interactions with the scaffold, localizing the factors in the scaffold. The latter will provide more sustained modulation of local cellular responses, better controlling the desired tissue induction. Release from three-dimensional scaffolds that better mimics physiological environment of cells can yield greater encoded protein (with pDNA agents) than two-dimensional cell culture.[85,86] Following the early work with GFs and pDNAs, siRNA and miRNAs incorporation into scaffolds are now becoming extremely attractive for bone tissue engineering. The optimization of scaffold features is crucial to imitate the natural tissue by promoting osteoinduction, osteoconduction, and osteogenesis. Three main classes of biomaterials, naturally derived biomolecules, synthetic polymers, and ceramics, have attracted attention for early attempts to deliver RNA agents.

3A. Scaffolds from Natural Biomolecules. Natural biomaterial-based scaffolds (e.g., collagen, gelatin and chitosan) represent promising materials to mimic bone architecture. These scaffolds have been extensively used for bone regeneration due to good biocompatibility and osteogenic capabilities. Collagen and other natural polymers may exhibit low mechanical properties which can affect cellular infiltration and nutrient supply by reducing scaffold porosity. Moreover, low stiffness scaffolds lack the ability to provide clinically viable treatment for load-bearing long bone defects. To overcome those limitations, chemical cross-linking, or physical reinforcement has been explored to enhance the mechanical stiffness.

Collagen has been widely used for scaffolds because it is the primary ECM protein and major component of the organic phase of bone. The presence of a collagen scaffold can promote osteogenic differentiation of osteoblast and mesenchymal stem cells (MSCs).[87] Various physical forms of collagen type I (sponges, hydrogels, fibers, films) are used clinically because of its physiological compatibility, ready availability, and low cost.[88,89] However, studies have demonstrated that extraction and purification during native collagen processing can reduce the native's collagen cross-linking density with consequent impact on mechanical and degradation features of scaffolds.[90] These properties can be improved by varying the degree of chemical or physical (dehydrothermal treatment, ultraviolet irradiation, γ-irradiation) cross-linking among collagen fibers. A collagen-nanoHA (nHA) scaffold chemically cross-linked with EDC was examined for delivery of miRNA-133a complexes to hMSCs[91] [113]. The in vitro results showed an increase in the ALP activity and calcium deposition, as well as an increase of the osteocalcin expression over 28 days after cell seeding was higher in comparison to other groups.

Another natural polymer, chitosan (industrially sourced from crustacean shells) has been formulated into biocompatible, biodegradable, antibacterial, and nonallergic scaffold. Chitosan scaffolds with controlled pore sizes have been used as a drug reservoir that is able to deliver and release GFs and RNAs for bone regeneration in a controlled manner. A chitosan scaffold was used as a reservoir for siRNAs against casein kinase 2 interaction protein 1 (Ckip-1) and soluble VEGF receptor 1 (Flt-1).

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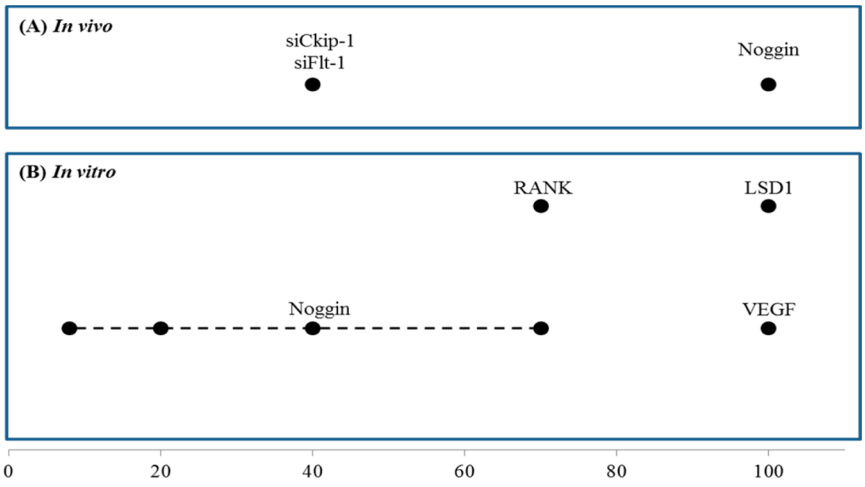

Figure 5. siRNA concentrations (nM in horizontal axis) delivered by nonviral carriers in vitro and in vivo. The dashed line between points indicates the test of different concentrations in the same study.

The two siRNAs could be maintained in scaffolds for >2 weeks and improve the MSCs proliferation in vitro. Implantation of the chitosan scaffold loaded with two therapeutic molecules exhibited a synergetic outcome that promoted significant defect restoration in a rat calvarial defect model, compared to matrix loaded with Ckip-1 siRNA.[52] However, chitosan scaffolds may not mimic the mechanical properties of native tissue and there are some concerns regarding its cytotoxicity. Chitosan scaffolds have been accordingly modified with various supporting materials and biological factors. Fan et al. fabricated chitosan/chondroitin sulfate scaffolds with a biomimetic apatite coating. This platform was able to deliver and slowly release BMP-2 and transfected with noggin shRNA (short hairpin RNA) adipose derived stem cells (ASCs). This system allowed the considerable increase of osteogenesis compared to the other groups of scaffolds loaded with BMP-2 or control cells alone.[92]

Fibrin-based matrices are another alternative given their innate similarity to the initial hematoma formed at a wound site. They could provide a stimulatory milieu for high levels of cell proliferation, a uniform 3D distribution of cells, and excellent adhesion to surrounding tissue with minimal toxicity. Fibrin was previously used for bone regeneration in delivery of BMP-2 protein and nucleic acids. Recently, Kowalczewski et al. reported successful delivery of siRNA or siRNA complexes to MC3T3-E1 preosteoblasts via surface-mediated delivery to cells in contact with fibrin hydrogels. After 3 days, ~80% of both the free and complexed siRNA was released. The MC3T3-E1 preoblasts attached to a fibrin hydrogel showed up-regulations of noggin miRNA expression levels in response to increasing amounts of rhBMP-2 which can lead to ectopic bone formation.[51]

Finally, Zhou et at. employed human bone-derived scaffolds and bone marrow-derived MSCs (BMMSCs) in a hypoxic environment can induce the production of angiogenic and osteogenic factors. Seeding of transfected BMMSCs with siRNA which targets VEGF in scaffolds and receiving hypoxia treatment was able to stimulate both osteogenic and angiogenic responses because bone-derived scaffolds alone does not possess sufficient osteogenesis. Human bone delivered scaffolds have been extensively described but it is not clear if the proposed system may be a promising approach for bone regeneration.[93]

3B. Synthetic Polymers. Biodegradable synthetic scaffolds, such as PEG, poly(lactic-*co*-glycolic acid) (PLGA), poly(lactic acid) (PLA), and poly(glycerol sebacate) (PGS), have been deployed for drug delivery systems because of their predictable degradation features. Although most scaffolds are synthesized a priori and implanted along with bioactive agents, Nguyen et al. explored the possibility of fabricating a 3D PEG scaffold along with therapeutic agents for the controlled delivery of siRNA and miRNA. In this study, siRNA against noggin (siNoggin) and/or miRNA-20a were mixed with acrylate- and thiol-terminated PEG macromers in aqueous media at physiological conditions. Cross-linking of the polymer chains were expected to form an interpenetrating network and entrap the bioactive molecules to sustain a controlled release. The delivery of siRNA or miRNA using in situ forming PEG hydrogels was prolonged and enhanced the osteogenic differentiation of encapsulated hBMSCs. This efficient approach to deliver of macromolecules could be applied for bone regeneration.[63] To improve binding and release properties of synthetic scaffolds, one can incorporate biofunctional features; Li et al. were able to release agomiR-26a from a PEG scaffold incorporating heparin with thiol-modified hyaluronan. By "bioactive" hydrogel showed significantly improved vascularization and bone regeneration, which resulted in the complete repair of the defect in calvarial bone defect model at 3 month-post implantation.[94]

In a separate approach, to create multiphasic scaffolds, Zhang et al. encapsulated miRNA-26a polyplexes (using a hyper-branched PEG−H_2O−PEI copolymer as a carrier) in PLGA microspheres, which were then incorporated into a cell-free PLA scaffold. The burst release of polyplexes was decreased by increasing the polymer molecular weight used to fabricate the PLGA microspheres (as expected). Incorporating of polyplexes/microspheres in PLLA scaffolds allowed the transfection of endogenous cells in vivo and enhanced bone regeneration in a critical-sized calvarial bone defect in osteoporotic mice.[95] Compared to the study of Li et al. where miR-26a enhanced osteogenic differentiation of BMSCs, Zhang et al. study was able to deliver miR-26a and enhance multiple osteogenic genes both in vitro and in vivo, and thus enhanced the repair of calvarial bone defect without adding cells.

3C. Ceramics and Composite Scaffolds. The ability of creating a stable bond between the host bone and implanted synthetic scaffold is important and ceramic scaffolds with

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bioactive inorganic materials such as hydroxyapatite (HA) and tricalcium phosphate (TCP) with similar chemical and crystallographic structure to the bone's inorganic phase, have been explored to create osteoconductive and osteoinductive capabilities.[96] The presence of HA enhances cell adhesion sites for a higher cell attachment.[97] A study by Chen et al., explored the delivery of hMSC transfected with either pre-miR-34a or anti-miRNA-34a that were loaded on HA/TCP scaffold and then implanted subcutaneously in NOD/SCID immune deficient mice for 8 weeks. The hMSC formulated normal lamellar bone and the amount of bone formed was enhanced in implants containing cells transfected with anti-miR-34a. Moreover, overexpression of miR-34a decreased bone formation by 2-fold compared with pre-miRNA controls.[98]

The brittle nature and poor fidelity of ceramics could be overcome by formulating HA into porous scaffolds composed of other biomacromolecules, such as collagen and ECM proteins.[99,100] A recent study by Castan˜o et al. looked at the influence of collagen-nanoHA (nHA) scaffolds loaded with nHA/Dy547 miR mimic/antagomiR particles on hMSCs for bone tissue applications. Their results suggest that Dy547-nanomiR mimic/antagomiR particles resulted in higher cellular internalization in monolayer hMSCs with limited cytotoxicity. Furthermore, the scaffolds were able to maintain the silencing of their respective targets throughout the time period assessed.[101] Col-HA scaffolds have been also synthesized that mimic the hierarchical structure of bone at different length scales. This approach involves self-assembly of collagen fibers and the in situ apatite precipitation. The presence of bone-like apatite layers enhances the ability to bond with the host bone and improves osteoconductivity.[102] Simulated body fluid (SBF), in particular, developed by Kokubo with ion concentration similar to that of human extracellular fluids has been used for HA formation on collagen scaffolds.[103−105] Collagen-HA scaffolds are mostly prepared by lyophilization (freeze-drying) technique in order to achieve high levels of pore size (>98%) and interconnected porosity. The pore size can be controlled by controlling the final freezing temperature in the chamber of the freeze-dryer, whereas the distribution of the pore size is controlled via the cooling rate.[106−109]

4. FUTURE PERSPECTIVES

The delivery of nucleic acid-based agents for bone regeneration is a rapidly growing area because of limitations of excessive doses employed with protein therapeutics. siRNAs and miRNAs are promising new agents that can silence and/or enhance the expression gene(s) of interest. Although a large number of bone-related miRNAs and siRNAs have been identified, and their therapeutic potentials were demonstrated in vitro and in vivo, they still face the usual safety issues (and perhaps new unexpected ones) and therapeutic efficacy barriers that need to be overcome for clinical utility. The molecular stability remains a challenge for the RNA agents, because without chemical or structural modifications they may be rapidly degraded and no longer bind to intended targets. In addition, little is known about the clearance of delivery systems (biomaterials) in vivo; the emphasis has been naturally on therapeutic outcomes in the reported studies, rather than the biodistribution and subsequent metabolism of the administered agents. It is difficult to reproduce or predict these issues in any culture system and efforts in preclinical animal models are minimal in this regard.

For effective therapeutic use, the carrier systems must be carefully designed to avoid degradation of the RNA cargo in serum, to identify and seek the target cells and tissues, and to present the RNA agents at the right location (usually intracellular) in free form for specific interactions. A variety of nonviral as well as viral carrier systems are currently evaluated for their ability to deliver osteogenic molecules. Despite the better delivery efficiency of viral vectors, their use might not be justifiable (from safety perspective) in nonlife threatening bone diseases. There is much room for improvement in designing effective nonviral carriers that will offer better therapeutic profile, since only a handful of biomaterials have been tested in preclinical models to-date. The cost issues related to development of nonviral delivery systems is likely to be relatively minimal, since they will be more amenable to scale-up and large-scale production. Systematic studies exploring correlations for in vitro−in vivo efficacy profiles are missing and will benefit the field tremendously. One usually operates with the assumption that most efficacious system in vitro will also be efficacious in animal models, but this assumption needs to be validated, considering its important consequences. The majority of the studies that have been reviewed in this report employed limited in vivo studies, which limits critical comparison among different systems. The reported studies examined (i) different types of miRNAs or siRNAs at different concentrations, (ii) different delivery systems with different base materials, and (iii) the complex/scaffold systems at different anatomical sites. Accordingly, it is difficult to come to a conclusion on the most promising therapeutic system to enhance bone formation.

Although scaffolds for bone regeneration have initially served as space fillers, the current scaffolds are usually multifunctional, where the space filling functionality is supplemented with features that directly address cellular events in osteogenesis (such properties as attachment, proliferation and differentiation of the pool of osteogenic progenitor cells). A good understanding of delivery requirements for RNA agents can further contribute to design of scaffolds, engineering the material composition and physicochemical properties to control the release of therapeutic agents at the right rate, as well as sequentially and/or simultaneously in the case of multiple agents. A scaffold that will closely imitate the native bone tissue biologically and structurally need to provide a guide to surrounding cells to stimulate their adhesion, migration, proliferation and differentiation. In this sense, the inclusion of polyplexes in 3D scaffolds may better mimic the events at repair site and facilitate to further regeneration of this complex tissue. Although the major focus of RNA agents has been the delivery of osteogenesis-inducing agents, it might be possible to deliver RNAs for other cellular events, such as the control of infiltrating cell pool or angiogenesis induction.

As the field moves toward combinational therapeutics, new improved tissue-inducing systems are bound to be introduced for bone regeneration based on RNA agents. This will make it possible to achieve clinical success for indications lacking an effective intervention.

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Notes

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COMMON ABBREVIATIONS

BMP: bone morphogenetic protein
BMSC: bone marrow stromal cells
ECM: extracellular matrix
GF: growth factor
HA: hydroxyapatite
miRNA: microRNA
MSC: mesenchymal stem cells
NP: nanoparticle
pDNA: plasmid DNA
siRNA: short interfering RNA

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