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REVIEW

MicroRNAs Involved in the Regulation of Angiogenesis in Bone Regeneration

Sepanta Hosseinpour[1] · Yan He[1] · Ashwin Nanda[1] · Qingsong Ye[1]

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Abstract

MicroRNAs (miRNAs) as a newly founded and thriving non-coding endogenous class of molecules which regulate many cellular pathways after transcription have been extensively investigated in regenerative medicine. In this systematic review, we sought to analyze miRNAs-mediated therapeutic approaches for influencing angiogenesis in bone tissue/bone regeneration. An electronic search in MEDLINE, Scopus, EMBASE, Cochrane library, web of science, and google scholar with no time limit were done on English publications. All types of original articles which a miRNA for angiogenesis in bone regeneration were included in our review. In the process of reviewing, we used PRISMA guideline and, SYRCLE's and science in risk assessment and policy tools for analyzing risk of bias. Among 751 initial retrieved records, 16 studies met the inclusion criteria and were fully assessed in this review. 275 miRNAs, one miRNA 195~497 cluster, and one Cysteine-rich 61 short hairpin RNA were differentially expressed during bone regeneration with 24 predicted targets reported in these studies. Among these miRNAs, miRNA-7b, -9, -21, -26a, -27a, -210, -378, -195~497 cluster, -378 and -675 positively promoted both angiogenesis and osteogenesis, whereas miRNA-10a, -222 and -494 inhibited both processes. The most common target was vasculoendothelial growth factor-signaling pathway. Recent evidence has demonstrated that miRNAs actively participated in angio-osteogenic coupling that can improve their therapeutic potentials for the treatment of bone-related diseases and bone regeneration. However, there is still need for further research to unravel the exact mechanisms.

Keywords MicroRNA · AngiomiR · Angiogenesis · Bone angiogenesis · Bone regeneration · Bone tissue engineering

Abbreviations

AMPK	AMP-activated protein kinase
HIF	Hypoxia-inducible factor
DKK2	Dickkopf2
DC-STAMP	Dendritic cell-specific transmembrane protein
Fbxw7	F-box WD-40 domain protein
NICD	Notch intercellular cytoplasmic domain
P4HTM	Prolyl 4-hydroxylase possessing a transmembrane domain
PKC	Protein kinase C-α
Runx2	Runt-related transcription factor 2
SFRP1	Secreted frizzled-related protein 1
TAK1	Transforming growth factor-activated kinase 1
VEGF	Vasculoendothelial growth factor

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✉ Qingsong Ye
a.ye@uq.edu.au

Sepanta Hosseinpour
sp.hosseinpour@uqconnect.edu.au

Yan He
h.he@uq.edu.au

Ashwin Nanda
a.nanda@uq.edu

[1] School of Dentistry, The University of Queensland, Herston, Brisbane, QLD 4006, Australia

Introduction

Bone regeneration is a complex process and includes numerous biological mechanisms, angiogenesis being one of the important process amongst them [1]. Angiogenesis and vascularization, controlled by various local cytokines and growth factors, are closely associated with the chemo-attraction, proliferation, and differentiation of osteoprogenitor cells [2]. In fact, osteogenesis and angiogenesis are coupled

in the formation of new vessels from the existing vessels after activation of pre-osteocytes and endothelial progenitor cells [3]. This biological duets are regulated by auto/paracrine cascade of factors which could be produced by endothelia, osteoblasts and their precursor cells [4]. In an ideal situation, synchronous occurrence of angiogenesis and osteogenesis leads to a complete rehabilitation of the bone defect with its original biomechanical and morphological properties [5]. However, these self-repairing mechanisms are not able to reconstruct all bony defects especially large critical-sized defects [6]. Moreover, some pathologic conditions such as avascular necrosis and vascular function deficiencies can negatively affect the repair [7]. In this regard, many novel strategies for improving angiogenesis and vascularization in bone regeneration such as bio-functionalization of scaffolds [8], growth factor delivery [9], and targeting signaling pathways via microRNAs (miRNAs) [10] have been investigated.

MicroRNAs as short single strand, non-coding nucleotide series (21–22 nucleotides) can regulate post transcriptional gene expressions [11]. More than 28,645 miRNAs were reported by http://www.miRNAbase.org and many have been associated with various diseases [12]. Upregulation of suppression of miRNAs can simultaneously regulate the secretion of several endogenous growth factors [13]. Recently, Nakasa et al. [10] reviewed the application miRNAs as a new therapeutic modality which is effective in treatment of bone fracture, osteoporosis, and other bone-related diseases. In addition, clinical application of miRNA also revealed positive impact in reducing the hepatitis C virus RNA level in chronically infected patients [14].

Several miRNAs are detected for therapeutic trials in bone diseases. For instance, systemic administration of miRNA-214 inhibitor in osteoporosis represented significant increase of bone mineral density in animal models [15]. MiRNA-92a inhibitor showed an enhancement in callus formation and treatment of non-union bone defects [16]. Moreover, down-regulation of miRNA-31 resulted in greater osteogenesis and less remaining scaffold by its antagonist observed in rats' critical-sized bone defects [17, 18].

Among the recognized miRNAs, there are 33 miRNAs that have been reported to have the angiogenic impacts [19]. In 2006, Poliseno et al. [20] presented the association of miRNAs and angiogenesis in human umbilical vein endothelial cells (HUVECs) for the first time. Vascular endothelial response to angiogenic induction is modulated with certain miRNAs (miRNA-17, -93, -126, -221/222, and -214) [21]. All recognized miRNAs fundamentally function in either pro-angiogenic or anti-angiogenic pathways [22]. MiRNA-21, -155, and -126 are involved in the vascular diseases and miRNA-221/222, miRNA-130a, miRNA-378, miRNA-27 have been implicated for endothelial cell functioning [23]. Understanding the complex network of miRNAs, their targets, and certain gene expressions will provide a strong tool to develop new therapeutic modalities not only to improve angiogenesis but also to couple this process with other regenerative mechanisms such as bone regeneration. In this systematic review, we aimed to seek current miRNAs-mediated therapeutic approaches to influence angiogenesis in bone regeneration.

Materials and Methods

Protocol and Characteristics of Included Studies

For this systematic review, we used the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA statement) guidelines [24].

Study type All in vitro, in vivo, and clinical investigation studies, which administrated microRNAs or their inhibitors as a therapeutic agent for coupling angiogenesis and osteogenesis in bone tissue, were included in this review. Systematic and literature reviews, letter to editors, book chapters, conference papers, note and thesis were omitted. All documents' publication stage was at final or in press stage at the time of searching.

Participants All in vitro cellular studies (such as various types of mesenchymal stem cells, HUVECs, etc.), in vivo animal studies (such as rats, mice, canines, primates, etc.), and human studies were included.

Interventions Studies investigating microRNAs or their inhibitors as therapeutic agents on cells/animals/humans for both angiogenic and osteogenic improvements were included in this study. Investigations which assessed only one of these processes (angiogenesis OR osteogenesis) were excluded. Moreover, angiogenesis evaluations of bone marrow derived stem cells for other therapeutic approaches other than bone regeneration such as cardiac muscle repair or cancer treatment were excluded. Assessments of microRNAs for determining the pathway, diagnosis, and prognostic research were also excluded.

Outcome measures Various osteogenic and angiogenic differentiation assessments were used including alkaline phosphatase assay (ALP), alizarin red staining (ALS), microarray analysis, reverse transcription-quantitative PCR (RT-qPCR), enzyme-linked immunosorbent assay (ELISA), western blot analysis, etc.

Information Sources

MEDLINE, Scopus, EMBASE, Cochrane library, Web of Science, and Google Scholar were the information sources. In addition, the following journals were searched manually to retrieve any in press or non-indexed articles; *Annual*

review of biomedical engineering, Biomaterials, Acta biomaterialia, and *Tissue engineering journal*.

Search, Study Selection and Data Collection

An electronic search was conducted in all aforementioned databases without any time limits. Articles were limited to English language, published and in press studies. Published papers on angiogenesis in bone regeneration which administered microRNAs or their inhibitors as therapeutic agents were found using the following keywords alone or ensemble: ("MicroRNAs"[Mesh], "Bone Regeneration"[Mesh], "Neovascularization, Physiologic"[Mesh], "miRNA", and "angiogenesis"). In addition, manual search of the articles in the selected journals were done.

Two independent experienced reviewers analyzed all retrieved articles using keywords, titles, and abstracts. After initial assessment, included studies were analyzed based on their full text. In the case of disagreement between two reviewers, a third expert person involved to resolve it by discussing. Finally, authors confirmed all included records. All steps were performed based on the guidelines of the PRISMA statement.

Data Items

All included articles were summarized. Key information was extracted and listed as follows: (a) first author and publication date, (b) study type (in vitro/in vivo), (c) sample type (cell/animal/human), (d) defect type, (e) evaluated microRNA or inhibitor, (f) target genes or molecules, (g) delivery method, (h) assessments, and (i) outcomes.

Risk of Bias in Individual Studies

Articles were categorized according to their type (in vitro/in vivo). In vitro studies were analyzed by the science in risk assessment and policy (SciRAP) (http://www.scirap.org/) and for by two independent reviewers. SciRAP was modified according to our study and questions in Table 1 in supplementary, were answered for each study. In vivo studies were evaluated according to the Systematic Review Centre for Laboratory animal Experimentation (SYRCLE)'s risk assessment tool for animal intervention studies to assess methodological quality and to identify aspects of bias that impacting on the animal experiments (Table 2 in Supplementary) [25]. The responses were no, yes, or unclear. Then, all studies were divided into three groups based on their risk of bias; low risk if all criteria were met, medium risk if two criteria were not met, or high risk if more than two criteria were not met. Both assessments were performed in reviewed articles where both in vitro and in vivo data were provided.

Results

Study Selection

Figure 1 illustrated the PRISMA flowchart of our search strategy in this study. In total, 751 records were retrieved from all data bases that screened by two independent reviewers. Then, 341 duplicate and non-relevant articles were omitted. After reviewing all titles and abstracts of remaining 410 articles, 361 records were removed based on the inclusion criteria and keywords. 49 full-texts were analyzed, 33 of which were excluded due to lack of information and diagnostic concept of study instead of therapeutic approach. Further exclusion of article was performed because miRNAs were not reported on either angiogenic or osteogenic impacts. Studies that investigated the small interfering RNAs (siRNA) instead of miRNA were excluded from current review. Finally, 16 studies (six in vitro and ten both in vitro and in vivo) were included and assessed in this study. Six of the included studies reported on the in vitro evaluation of miRNA on angiogenesis and osteogenesis. Ten studies disclosed information about both in vitro and in vivo comparison of the miRNA.

Characteristics of Included Records

We summarized all pathways and targets of the evaluated miRNAs in Fig. 2. In this review, 275 miRNAs, one miRNA 195~497 cluster, and one Cysteine-rich 61 short hairpin RNA which have differentially expressed during bone regeneration with 24 predicted targets reported in 16 studies were analyzed. Among them only 12 miRNAs have influenced (positively or negatively) both osteogenesis and angiogenesis.

In included 6 in vitro articles, 11 miRNA variants were studied including miRNA-9, miRNA-10a, miRNA-20a, miRNA-29b and c, miRNA-146b, miRNA-195, miRNA-210, miRNA-378, miRNA-497, and miRNA-675 [26–31]. Table 1 summarized all data extracted from these studies and further detailed results were mentioned in the ensuing paragraphs. Human mesenchymal stem cells (hMSCs) [26, 27, 31], mouse-derived stem cells (mouse UVECs, bone marrow MSCs, and MC3T3-E1) [29, 30], and human umbilical vein endothelial cells (HUVECs) [28] were studied in selected six articles. In addition, in most of the studies, transfection were done by transfection agents or Lipofectamine [26–28, 30, 31]; only in one study lentiviral vector was administered to transfect the cells [29]. VEGF signaling was targeted in three studies [27, 29, 31]; Wnt and MAPK signaling [26], AMPK signaling [28], and Wnt/β-catenin pathway [27] were aimed in other articles.

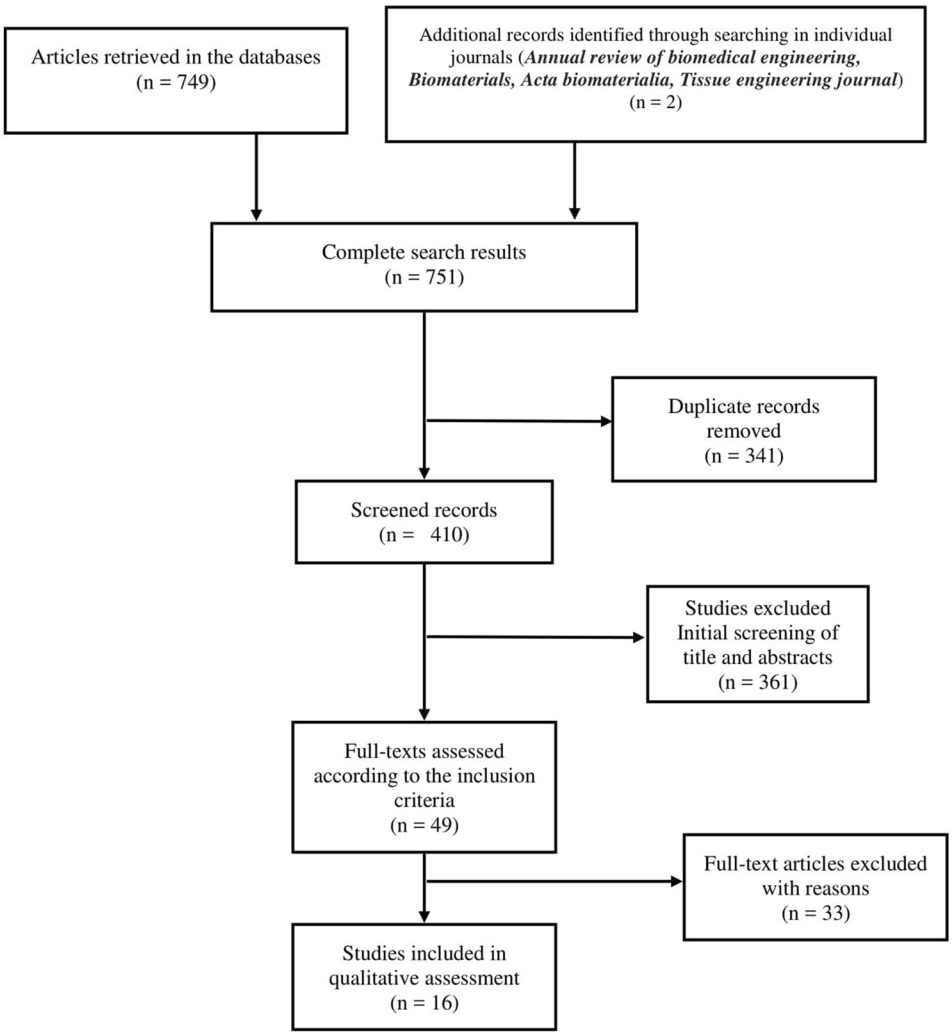

Fig. 1 The PRISMA flow diagram of search strategy administered in this systematic review. NCBI PMC, Pubmed, Embase, Scopus, Cochrane library, web of science, and google scholar databases were searched (with different combination of these keywords; ("MicroRNAs"[Mesh], "Bone Regeneration"[Mesh], "Neovascularization, Physiologic"[Mesh], "miRNA", and "angiogenesis") with the limitation of English language and no restricted time

Ten studies that assessed the miRNAs angiogenesis therapeutic in bone tissue and stem cells both in vitro and in vivo were included in this study (Table 4) [16, 32–40]. In these studies, influence of 264 miRNAs, one miRNA 195~497 cluster [37], and one Cysteine-rich 61 short hairpin RNA [36] were evaluated by microarray analysis, ALP activity, alizarin red staining, RT-PCR, WBA, tube formation assay, dual-luciferase reporter assay, sequential fluorescent labeling, relative tartrate-resistant acid phosphatase (TRAP) activity, DAPI, fluorescence microscopy, confocal microscopy, flow cytometry, μ-computed tomography (CT) analysis, and histological analysis. In the in vitro part of records, six articles applied transaction agents (Lipofectamine, siPORT NeoFX, and Attractene), three studies used lentiviral vector [32, 35, 36], and the other two used miRNA Nanocapsules in combination with strontium-substituted hydroxyapatite (SrHA) [34] and polyethylenimine [41] functionalized graphene oxide (GO) complex [33]. The most common types of stem cells in the articles were bone marrow MSCs and preosteoblastic cell lines (MG-63 and MC3T3-E1) and most of them had mice origin, following by human [34, 36, 39], and dog [32]. In three studies both mice and human originated stem cells were used [35, 37, 40]. Mice was the most common animal model, followed by rats [35, 39], rabbit [34], and dog [32].

Risk of Bias

In Vitro Part

After analyzing all studies according to SciRAP guidelines, we realized that all studies used control group for their comparisons except one study [34]. All other criteria and responses were summarized in Table 1. Although all of them clearly expressed cell lines and culture media, some of them did not mention the number of cell passage [16, 34–36, 38, 39] and cell density applied [27, 30–32, 36, 37, 39]. In addition, most of them did not mention the way of contamination control in their studies [16, 26, 27, 30, 31, 34, 37, 39, 40]. One of the major concerns about the in vitro findings is the source of

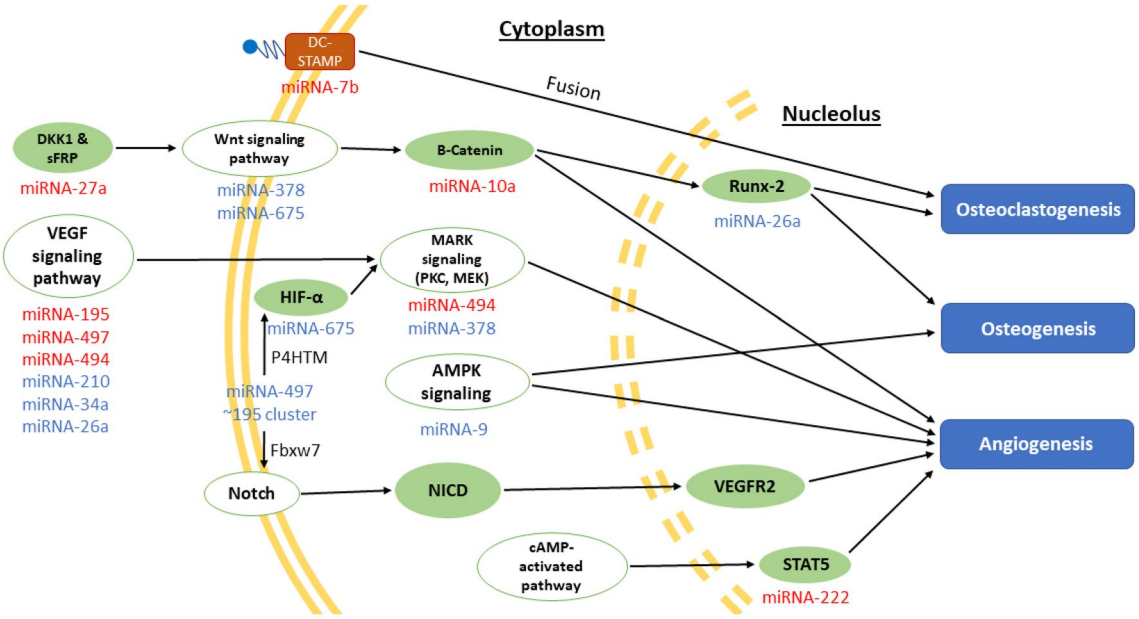

Fig. 2 Schematic summary of various miRNAs impacts on different pathways in the reviewed articles. Outlined text boxes show signaling pathways, and solid text boxes represent target subjects that being influenced. In addition, red text means inhibitory impact, and blue one show upregulatory effect

studied cells. Same types of microRNAs can act differently in various species [42–44]. Therefore, it may happen that the evaluated microRNA in for instance mouse MSCs may have different effect on human MSCs. In addition, concentration of miRNA therapy or the efficacy of transfection were not assessed in these studies except two [30, 33] which may influence the results.

In Vivo Part

All in vivo parts of studies were assessed based on the SYRCLE's risk assessment tool. These studies could not clearly express their randomization or blindness strategies (investigators or evaluators) (Table 2). Only two studies [32, 35], mention that the animals were being allocated randomly. Moreover, only Yoshizuka et al. and Chen et al. [36, 39] reported that they blinded the operators or assessors. In only one study, plain vehicle applied in vivo as a control and comparison with treated groups [37]. Moreover, there were no report of optimization or assessment of dosages and administration intervals for the therapeutic in vivo approaches. Various dosages and timing of application might meaningfully influence the results.

Discussion

MiRNAs and Their Targets

Osteogenesis in bone regeneration and remodeling is coupled with angiogenesis by osteo-angiogenic factors which is released by pre-osteoblast/osteoclast and osteoblast/clast cells [1]. It has been shown that miRNAs are involved in physiologic bone and vessel formation by targeting various transcription factors in stem cells, osteoblasts, osteoclasts, and chondrocytes [45, 46]. In addition, increasing evidence demonstrates miRNAs role in cardiovascular development and angiogenesis in tumor [47]. Table 3 is summarizing miRNAs that have "promoting" or "inhibitory" impacts on both angiogenesis and osteogenesis process according to the included studies.

Positive Regulatory Effect on Both Angiogenesis and Osteogenesis

MiRNA-7b This miRNA enriched in a wide variety of tissues for developmental and functional biological activities [48]. It can directly inhibit dendritic cell-specific transmembrane protein (DC-STAMP) which is needed for osteoclastic fusion [49]. It can also decrease number and formation

Table 1 Role of miRNAs in the included six in vitro investigations

Evaluated miRNA (s)	Cell type	Evaluated target(s)	Delivery method	Method of evaluation	Regenerative impacts	References
miRNA-378	hBMMSCs	Wnt and MAPK signaling	siPORT NeoFX transfection reagent	ALP staining Alizarin Red S ELISA RT-qPCR Matrigel™ Angiography WBA	↑ Angiogenic genes ↑ Osteogenic genes osteogenesis-angiogenesis coupling	[26]
miRNA-675	hMSCs	VEGF/HIF alpha signaling, Wnt/β-catenin pathway	Attractene transfection reagent	ELISA WBA Immunofluorescence analysis RT-qPCR	↑ hypoxia-mediated angiogenesis ↓ hMSCs stemness markers ↑ early osteoblast markers Activation of Wnt pathway	[27]
miRNA-9	MC3T3-E1** and HUVECs	AMPK signaling	Transfected using Lipofectamine	Transwell migration assay RT-PCR WBA ALP activity Alizarin Red S ELISA Tube formation assay	↑ cell migration ↑ VEGF expression ↑ VE-cadherin ↑ Osteoblastic differentiation of MC3T3-E1 ↑ HUVECs proliferation	[28]
miRNA-210	BMMSCs	VEGF signaling	Lentiviral vector	RT-qPCR WBA	↑ VEGF expression ↑ Osteoblastic differentiation ↓ Adipogenic differentiation	[29]
miRNA-10a	MC3T3-E1 and MUVECs	β-catenin	Transfected using Lipofectamine	MTT assay Transwell migration assay RT-PCR ALP activity Alizarin Red S Tube formation assay ELISA	↓ MUVECs proliferation and migration ↓VEGF expression ↓ VE-cadherin ↓ osteogenic differentiation of MC3T3-E1	[30]
miRNA-146b, miRNA-29b, miRNA-29c, miRNA-20a, miRNA-195, and miRNA-497	MC3T3-E1 And phMSC	VEGF signaling	Transfected using Lipofectamine	ALP activity Alizarin Red S RT-qPCR MicroRNA Array RRA Immunostaining CAMAA ELISA Cloning and luciferase assays	miRNA-195 and miRNA-497 ↓ Angiogenesis ↓ VEGF expression modulates osteogenic differentiation ↓ cell proliferation	[31]

ALP alkaline phosphatase, *AMPK* AMP-activated protein kinase, *CAMAA* chicken embryo chorioallantoic membrane angiogenesis assay, *ELISA* enzyme-linked immunosorbent assay, *hBMMSCs* human bone marrow mesenchymal stem cells, *HIF* hypoxia-inducible factor, *HUVECs* human umbilical vein endothelial cells, *MSC* mesenchymal stem cell, *MUVECs* mouse umbilical vein endothelial cells, *phMSC* primary human mesenchymal stem/stromal cells, *RRA* Resazurin reduction assay, *RT-qPCR* real time-quantitative polymerase chain reaction, *VEGF* vasculoendothelial growth factor, *WAB* Western blot analysis

**MC3T3-E1: Pre-osteoblast mouse cell line

Table 2 Role of miRNAs in the included records which had both in vitro and in vivo investigations

Study sections (in vitro/in vivo)	Evaluated miRNA	Evaluated target(s)	Delivery method	Cell type/animal model and defect type	Method of evaluation	Regenerative impacts	References
In vitro part	65 miRNAs	Especially DKK2, sFRP1	Transfected using Lipofectamine	Dog BMMSCs	ALP activity Alizarin Red S RT-PCR WBA Tube formation assay Dual-luciferase reporter assay Sequential fluorescent labeling	Targets DKK2 and SFRP1 ↑ Angiogenesis ↑ Osteogenesis	[32]
In vivo part	miRNA-27a	DKK2, sFRP1	Lentiviral vector	Dogs mandibular peri-implantitis	µCT analysis Histological analysis	↑ reosseointegration ↑ New bone formation	
In vitro part	miRNA-7b	DC-STAMP	PEI functionalized GO complex	Mice BMMs	Relative TRAP activity DAPI WAB Fluorescence microscopy Confocal microscopy Flow cytometry RT-qPCR ELISA	↑ Angiogenesis ↑ Osteogenesis ↓ Osteoclast fusion ↓ DC-STAMP	[33]
In vivo part	miRNA-7b	DC-STAMP	PEI functionalized GO complex	OVX mice Osteoporotic femoral bone	µCT analysis Immunofluorescence Microphil-perfused angiography Histological analysis	↑ PDGF-BB ↑ bone mineral density ↑ bone volume ↑ bone vascularization	
In vitro part	miRNA-21	NM*	Nanocapsules	MG-63**	MTT assay ALP activity ELISA RT-qPCR	↑ CD31 expression ↓ RANKL expression Synergetic effects with SrHA on osteogenesis	[34]
In vivo part	miRNA-21	NM*	Nanocapsules and SrHA coated Ti	Rabbits 4-mm femoral bone defect	BIC analysis µCT Analysis Histological analysis Immunohistochemistry	Synergetic effects with SrHA on osteogenesis ↑ osteogenesis-related genes ↑ New bone formation and bone remodeling	

Table 2 (continued)

Study sections (in vitro/in vivo)	Evaluated miRNA	Evaluated target(s)	Delivery method	Cell type/animal model and defect type	Method of evaluation	Regenerative impacts	References
In vitro part	miRNA-34a	VEGF	Lentiviral vector	mMSCs, HUVECs	ALP activity Alizarin Red S Tube formation assay ELISA RT-qPCR	↓ Angiogenesis ↑ Osteogenesis ↓ HUVEC proliferation and angiogenesis ↑ mMSC proliferation and osteoblastic differentiation	[35]
In vivo part	miRNA-34a	VEGF	Lentiviral vector injection	Rats GIOFH	Histological analysis Immunohistochemistry	↑ osteogenesis-related genes ↑ CD31 expression	
In vitro part	CCN1 shRNA	miR-126 and PKC-a signaling pathway	Lentiviral vector	MG-63**	RT-qPCR WBA ELISA transwell migration assay luciferase assays CAMA	↑VEGF expression Activation PKC-a signaling	[36]
In vivo part	CCN1 shRNA	miR-126 and PKC-a signaling pathway	Lentiviral vector	C57BL/6J mice Collagen-induced arthritis	Matrigel plug assay μCT analysis	↑ bone mineral density ↑ bone volume ↑ trabecular bone ↓ cartilage erosion	
In vitro part	miRNA-497 ~195 cluster	Fbxw7, P4HTM	Transfected using Lipofectamine	Mouse and human BMECs	MiRNA Microarray assay Flow cytometry Three-point bending test luciferase reporter assay RT-qPCR WBA	miRNA-497~195 osteogenesis-angiogenesis coupling	[37]
In vivo part	miRNA-497 ~195 cluster	Fbxw7, P4HTM	Injection of aptamer-agomiR-195 and transgenic animal	Transgenic mice Aging bone	μCT analysis Immunofluorescence Immunohistochemistry	miRNA-195 ↑ vessel formation ↑ bone formation	
In vitro part	miRNA-494	VEGF, TAK1, SMAD9	Transfection reagent	C3H10T1/2***	Chondrogenic Differentiation assay RT-qPCR	↓ Angiogenesis ↓ Osteogenesis ↓ chondrogenesis	[38]
In vivo part	53 miRNAs	5438 target genes	-	OVX C57BL/6J mice Transverse femoral fracture	miRNA microarray analysis Pathway Enrichment Analysis RT-qPCR	11 novel miRNAs were identified to impair fracture healing.	

Table 2 (continued)

Study sections (in vitro/in vivo)	Evaluated miRNA	Evaluated target(s)	Delivery method	Cell type/animal model and defect type	Method of evaluation	Regenerative impacts	References
In vitro part	miRNA-222	STAT5A	Transfected using Lipofectamine	hBMMSCs	RT-qPCR ALP activity Alizarin Red S	↓ Runx2, COL1A1, and osteocalcin expression ↑ COL2A1, aggrican, and SOX9 expression	[39]
In vivo part	miRNA-222	STAT5A	atelocollagen	Rats Transverse femoral fracture	Radiographic evaluation Histologic analysis Immunohistochemistry	↓ Capillary density ↓ Fracture healing	
In vitro part	miRNA-92a	COL 1A2 and ANGPT1	Transfected using Lipofectamine	Mice primary osteoblasts	RT-qPCR	antimiRNA-92a No significant effect on COL 2, COL 10, and Sox9 ↑ angiogenesis-related genes	[16]
In vivo part	134 miRNAs	Inhibition targeting of miRNAs	LNA injection	C57BL-6NCrSlc mice Rib and femoral transverse fracture	µCT analysis Histological Analysis Luciferase assay	antimiRNA-92a ↑ Neovascularization ↑ callus volume ↑ Bone mineral Density ↑ CD 31 expression	
In vitro part	miRNA-26a	VEGF and Runx2	siPORT NeoFX transfection reagent	hBMMSCs and MC3T3-E1****	ALP activity ELISA RT-qPCR WBA Immunofluorescent microscopy alizarin red S	Osteogenesis–angiogenesis coupling ↑ angiogenic factors ↑ osteogenesis	[40]
In vivo part	miRNA- 26a	VEGF and Runx2	hydrogel	C57BL6J mice 3-mm calvarial defect	RT-qPCR WBA µCT Analysis	miRNA-26a ↑ vessel formation ↑ bone formation	

ANGPT1 angiopoietin1, *ALP* alkaline phosphatase, *BIC* bone-implant contact, *BMEKs* bone marrow endothelial cells, *BMMs* primary bone marrow monocytes/macrophages, *BMMSCs* bone marrow mesenchymal stem cells, *CAMA* chicken embryo chorioallantoic membrane assay, *COL1A1* collagen type 1 A1, *COL2A1* collagen type II A1, *CD* cluster differentiation, *CT* computed tomography, *Cyr61 or CCN1 shRNA* cysteine-rich 61 short hairpin RNA, *DAPI* 4',6-diamidino-2-phenylindole, dihydrochloride, *DKK2* dickkopf2, *DC-STAMP* dendritic cell-specific transmembrane protein, *ELISA* enzyme-linked immunosorbent assay, *Fbxw7* F-box WD-40 domain protein, *GIOFH* glucocorticoid-induced osteonecrosis of the femoral head, *GO* graphene oxide, *HUVECs* Human umbilical vein endothelial cells, *LNA* locked nucleic acid, *mMSCs* mouse mesenchymal stem cells, *OVX* ovariectomized, *P4HTM* prolyl 4-hydroxylase possessing a transmembrane domain, *PDGF-BB* platelet-derived growth factor-BB, *PEI* polyethylenimine, *PKC* protein kinase C-alpha, *Runx2* runt-related transcription factor 2, *RANKL* receptor activator of nuclear factor kappa-B ligand, *RT-qPCR* real time-quantitative polymerase chain reaction, *SFRP1* secreted frizzled-related protein 1, *SrHA* strontium-substituted hydroxyapatite, *TAK1* transforming growth factor-activated kinase 1, *Ti* Titanium, *TRAP* tartrate-resistant acid phosphatase, *VEGF* vasculoendothelial growth factor, *WBA* Western blot assay

*NM: not mentioned in the study

** MG-63: human osteosarcoma cell line

***C3H10T1/2: murine mesenchymal *cell line*

**** MC3T3-E1: pre-osteoblast mouse cell line

Table 3 Impact of evaluated microRNAs on angio-osteogenesis

Promoting effects on angio-osteogenesis	Inhibitory effects on angio-osteogenesis
miRNA-7b	miRNA-10
miRNA-9	miRNA-222
miRNA-21	miRNA-494
miRNA-26a	
miRNA-27a	
miRNA-210	
miRNA-378	
miRNA-195~497 cluster	
miRNA-675	

of osteoclasts [33]. Dou et al. [33] also represented that miRNA-7b delivery by GO-PEI increased ALP, Runx-2, and PDGF-BB expression after 3 days [33]. Although DC-STAMP is assumed to be essential for cells fusion, it is not essential for osteoclast differentiation. Therefore, it can be explained that the miRNA-7b inhibitory effect on osteoclastic fusion and maintaining platelet-derived growth factor-BB secretion that has enhanced new vessel and bone formation. On addition note cytoskeleton and focal adhesion staining demonstrated that this process is inhibited by miRNA-7b. Quantitative PCR and ELISA indicated that ALP, Runx-2, and platelet-derived growth factor-BB were expressed higher in the cells treated with miRNA-7b which was representing its potential effects on their pathways [33]. However, the study regarding this part of the field is in progress and more data are yet to yielded.

MiRNA-9 It has been shown that miRNA-9 been involved in the neural injury repair [50] and also can improve angiogenesis of tumor cells [51]. Qu et al. [28] demonstrated that miRNA-9 upregulated the amount of Runx-2 and osterix which are the early markers of osteogenic differentiation. Positive regulatory effect of miRNA-9 on adenosine monophosphate-activated protein kinase (AMPK) and its signaling pathway was validated with protein and molecular assessments [28]. In addition, it has been found that miRNA-9 can enhance the expression of cyclin D1 and progression of cell cycle in HUVECs. MiRNA-9 facilitated angiogenesis via improving endothelial progenitor cell migration and chemotaxis that have been shown by Transwell migration assay, VEGF and VE-cadherin upregulation [28]. This signaling path acts as an important role in pro-inflammatory activities [52], osteoblastic differentiation [53], and is essential for angiogenesis especially after hypoxia stress in endothelial cells. Therefore, it seems both osteogenesis and angiogenesis regulations underlie on this AMP-activated signaling. Other studies have also detected miRNA-9 role in tumor necrosis factor-α (TNF-α) and JAK-STAT pathways [51], which are regulating endochondral ossification and endothelial cell migration and angiogenesis.

MiRNA-21 MiRNA-21 increased the expression of CD-31, COL 1, Runx-2, OCN, and OPN according to Geng et al. [34] that demonstrated its osteogenic–angiogenic coupling ability. However, they did not validate the specific targets that cause these effects. MiRNA-21 can promote mineralization via Smad7-Smad1/5/8-Runx2 pathway [54]. In addition, miRNA-21 directly targets RhoB which is responsible to induce assembly of actin fibers in endothelial cells [55]. Geng et al. [34] also detected that miRNA-21 can promote osteoclastic differentiation in vitro and increase RANKL expression in vivo that can accelerate bone resorption. Sprouty 1 and FasL are the main targets in this process [56]. In the rabbits' femoral defect, bone-implant contact (BIC) evaluation exhibited higher level in miRNA-21 treated group in comparison with uncoated Ti. Moreover, miRNA-21 improved CD-1, COL 1A1, Runx-2, OPN, OCN, and CD-31 in vivo [34]. These findings demonstrated miRNA-21 potentials for improving orthopedic or dental implants.

MiRNA-26a MiRNA-26a is a promoter of osteogenic differentiation in mesenchymal stem cells [40]. In the comparison of miRNA-21, -26a, -29b, miRNA-26a exhibited greater expression in regenerated bone than the others [40]. In addition, it induced 2.4-10 folds more expression of Runx-2 and BMP-2 (early osteogenic markers) and 8.5 folds increase of OCN (late osteogenic marker), VEGF, and Ang-1 (angiogenic markers) which proved its ability for coupling osteo-angiogenesis. Several studies revealed its significant role in bone regeneration [57], and vessel formation [58]. MiRNA-26a majorly targets GSK3β to activate Wnt signaling in BMMSCs; however, it targets Smad-1 in adipose derived MSCs and interfering with BMP pathway [59] and osteogenic differentiation which revealed miRNA-26a' various impacts on different stem cells. In addition, BMP/Smad-1 signaling is regulated by miRNA-26a for angiogenesis in endothelial cells [60] which can regulate angiogenesis, osteogenesis, and other hard tissue formation including odontogenesis [61] that can be considered in further investigations.

MiRNA-27a Wu et al. [32] presented that miRNA-27a can be delivered by BMMSCs loaded β-TCP to treat peri-implantitis after dental implant placement. They concluded that miRNA-27a improve both angiogenesis and osteogenesis with direct inhibition of Dickkopf2 (DDK2) and secreted frizzled-related protein 1 (sFRP1) which are antagonists of Wnt [62]. Direct targeting of DKK-2 and SFRP-1 via miRNA-27a also confirmed by luciferase reporters assay [32]. In addition, they showed that tumor necrosis factor-α

(TNF-α) downregulates miRNA-27a expression and inhibits bone formation [32]. TNF-α also plays an important role in peri-implantitis due to its role in inflammation [63]. Therefore, these potentials make miRNA-27a as a good choice for treating or preventing peri-implantitis and also fabrication of dental implants.

MiRNA-195 and miR-497~195 Cluster MiRNA-195 and -497 belong to miRNA-15 family which regulate many activities including angiogenesis [64]. In fact, miRNA-195 is placed close to miRNA-497 (10 kilo base pair distance) in chromosome seventeen and both of them can impair osteogenic differentiation, and cells proliferation [65]. Yang et al. [66] demonstrated that miR-497~195 cluster is highly expressed in specific types of endothelial cells which are essential neovascularization and promote Notch and HIF-1α levels in preventing the aging of mouse bone tissue. Prolyl 4-hydroxylase possessing a transmembrane domain (P4HTM) which can increase stability of HIF-1α directly inhibited by this miRNA cluster. It also inhibits Fbxw7 which regulate Notch, type H vessel, and bone formation. Their results exhibited that miRNA-195 can increase vessel and bone formation in aged animal models. MiRNA-195 regulates the angiogenesis especially in endothelial cells via targeting SMAD-5 and homeobox A10 genes which are described as the regulators of angiogenesis and osteogenesis [67]. On the other hand, Almeida et al. [67] showed that miRNA-195 transfection can reduce SMAD-5 and homeobox A10 mRNAs. They also have found that mouse MSCs that were cultured in conditioned medium (+extracellular VEGF) formed significantly lesser number of vessels after transfected with miRNA-195 which depict its inhibitory effect on VEGF signaling pathway. In addition, they reported that ALP staining in miRNA-195 and miRNA-497 transfected cells decreased to 21% and 60%, respectively, in comparison to control. These findings were in agreement with ALP, Runx-2, and osterix (early osteoblastic markers) reduction in the treated group. These discrepancy may explain with various microenvironments and cells which these studies used for their investigations. For instance, Yang et al. [66] applied miRNA on endothelial cells (in vitro and in vivo), but Almeida et al. performed all procedures on pre-osteoblasts cells in vitro. However, according to in vivo findings, this cluster has a beneficial impact on bone and vessel formation which confirmed the general angiogenesis–osteogenesis coupling effect of miR-497~195 cluster.

MiRNA-210 Therapeutic application of miRNA-210 in osteonecrosis patients showed its involvement in related regulatory procedures [68]. Liu et al. in [29] detected that miRNA-210 time-dependently promoted VEGF expression in rat BMMSCs. Moreover, RT-qPCR results indicated significant increase of osteoblastic differentiation markers' (ALP and osterix) expressions compared with controls. On the other hand, miRNA-210 inhibited PPAR$^{\gamma}$ which represents its inhibitory impact on adipogenic differentiation. They proposed that this may occurred through VEGF signaling regulation. In addition, Mizuno et al. [69] explained osteogenic ability of miRNA-210 with its inhibitory impact on TGF-α pathway. However, the exact mechanisms are still unknown and should be more investigated.

MiRNA-378 This miRNA involved in several biological metabolic processes. MiRNA-378 inhibited cell apoptosis and promoted tumor proliferation and angiogenesis by interfering with Fus-1 expression [70]. Furthermore, miRNA-378 targets PI3 K/Akt pathway to accelerate osteoblastic differentiation in high-glucose condition [71]. Zhang et al. [72] indicated its osteo-angiogenesis coupling ability by evaluating Runx-2, OCN, ALP, and VEGF markers which were significantly upregulated after miRNA-378 transfection. However, they did not approve which pathway was exactly targeted by this miRNA.

MiRNA-675 Recently, a cross-talk between HIF-1α and miRNA-675-5p was detected in glioma, hypoxic condition, and hypoxia-mediated angiogenesis [73]. In addition, inhibition of miRNA-675 under hypoxia condition represented decreased amount of hypoxia-inducible factor-1 (HIF-1) protein and mRNA, and also CD-44, -73, and -90 expression (stemness markers) which indicated the impact of miRNA-657 on the hypoxia-mediated angiogenesis. MiRNA-675 not only involved in angiogenesis, but it may also be able to modulate the β-catenin/Wnt signaling. In fact, Costa et al. [27] represented that transfection of miRNA-675 can induce the expression of *ALPL, BGLAP,* and *SSP1* (early and late osteoblast genes) after a week and also downregulate CD-90 and other stemness markers of hMSCs. It seems that miRNA-675 influenced several signaling pathways which are important for angiogenesis–osteogenesis coupling. However, further in vivo studies are required to approve its exact role.

Negative Regulatory Effect on Both Angiogenesis and Osteogenesis

MiRNA-10a MiRNA-10a involved in pathogenesis of many diseases including cardiac and kidney injuries [74], and inhibiting tumor angiogenesis [75]. Li et al. [30] demonstrated that BMP-2 induced osteogenic differentiation significantly downregulated miRNA-10a expression. On the other hand, miRNA-10a overexpression suppress β-catenin and canonical Wnt pathways and therefore decrease Runx-2, osterix and distal-less homeobox 5 genes expression which offer therapeutic potential of this miRNA for bone disease [76]. In fact, they have found that miRNA-10a reduced β

catenin mRNA in addition to its mRNA (transcriptional effect) which propose many targets that may involve in this process such as TGF-β/Smad2/STAT3/STATS pathway [77]. Moreover, the anti-angiogenic effect of this miRNA may be regulated by β-catenin pathway, but more investigations are needed to unravel this mechanism.

MiRNA-222 MiroRNA-222 inhibitor significantly promoted osteogenesis, angiogenesis, and chondrogenesis and improved bone fracture healing [78]. MiRNA-222 overexpression downregulated Smad-5 and Runx-2 protein levels, and also regulated osteoclastogenesis [69]. Although Yu et al. [79] had applied computational analysis to determine miRNA-222 targets (BMP-2, Runx-2, and osteocalcin), these targets have not been validated. Yoshikuza et al. [78] used miRNA-222 inhibitor for treating refractory bone fractures and exhibited significant improvement in neovascularization. They suggested that this miRNA may negatively modulate angiogenesis with targeting signal transducer and activator of transcription 5 (STAT-5) and c-Kit receptors according to other studies [20]. However, these targets were also not validated in their study.

MiRNA-494 MiRNA-494 hampered VEGF in vitro, and vessel formation in vivo [80]. He et al. [81] have determined that miRNA-494 might target nitric oxide signaling (angiogenesis-related pathway) and suppress it 2 weeks after bone fracture which leads to an impaired fracture healing in aged mice. Smad-9 and transforming growth factor-activated kinase-1 are the main potential targets according to their target analysis. In addition, pathway analysis revealed that miRNA-494 inhibits chondrogenic differentiation via targeting retinoid acid receptor signaling [82]. According to the in vitro assessments, miRNA-494 inhibits TAK-1, SMAD-9, and VEGF which are participate in TGF-β signaling, retinoid acid receptor activation, and nitric oxide signaling [38]. Therefore, it seems inhibition of this miRNA have the potentials for therapeutic bone-related disease applications.

Other miRNAs

MiRNA-34 This family of miRNAs especially miRNA-34 b and c has been detected as a functional factor in murine osteoblasts [83]. However, findings regarding osteogenic impacts of miRNA-34 are controversial [84]. Chen et al. [85] determined its inhibitory effect on osteoblastic differentiation of human MSCs and bone formation in vivo. They suggested that this effect may be caused by influencing the Notch pathway. On the contrary, another research showed that miRNA-34 improved osteogenic differentiation by positive targeting Notch pathway. Zha et al. [35] also demonstrated that the transfection of miRNA-34a into rats via lentiviral vectors promoted the osteogenesis in dexamethasone inhibited bone formation. In addition, they have found that it negatively regulated VEGF expression and angiogenesis. It seems that Sirtuin-1 and Cyclin-dependent kinase 4 which are essential in cell cycle are the potential targets for this effect [86]. The discrepancy between different studies cannot be explained until the exact mechanism of action of miRNA-34 will be determined.

MiRNA-92a MiRNA-92a belongs to the miRNA-17 family which involved in the developmental procedures of vertebrate and highly expressed in endothelial cells [87]. Murata et al. investigated 134 miRNAs from the blood sample of four trochanteric fractures patients with microarray analysis. Only miRNA-92a represented significant reduction after fracture compared with healthy individuals in the first 24 h. In the next steps, they applied antimiRNA-92a and miRNA-92a in mice with femoral fracture and the molecular and μCT assessments revealed better fracture healing after injection of antimRNA-92a. On the other hand, luciferase reporter test in vitro did not indicate the specific target of miRNA-92a [16]. This miRNA targets aggrecanase-1 and 2 [88] which participate in chondrogenic differentiation, hosphatase and tensin homolog in AKT signaling, and integrin subunits α5 [89] that is involved in angiogenesis.

MiR-126 Cysteine-rich 61 or CCN-1 dose-dependently reduced miRNA-126 expression in vitro and in vivo. Chen et al. [90] transfected miRNA-126 into osteoblasts and they have found that this miRNA can decrease CCN-1 level. Chen et al. [90] revealed that inhibition of miRNA-126 with CCN-1 in the PKC-signaling pathway can increase angiogenesis. Luciferase reporter assay conducted to assess the direct target of miRNA-126 and represented VEGF gene in the PKC pathway as its main target. In animal models, CCN-1 due to its inhibitory effect on miRNA-126 increased amount of bone formation and density [36]. They also showed that miRNA-126 directly bind to VEGF gene and downregulate its expression in pre-osteoblasts. Endothelial cells suppress vascular cell adhesion molecule 1 (VCAM-1) by expressing miRNA-126 [91]. Therefore, these can explain animal findings that demonstrated CCN-1 due to its inhibitory effect on miRNA-126 increased amount of bone formation and density [36].

Delivery Methods

Although miRNAs application for bone regeneration revealed several advantages such as efficacy at low dose, extended half-life (in comparison to growth factor or plasmid DNA delivery) [92], finding a safe and successful delivery method limited its success. Various vectors, mainly categorized in two viral or non-viral delivery systems were

proposed in the recent literature to facilitate miRNAs transduction to cells [93].

Viral Vectors

Lentiviruses represented lesser risk of mutagenesis compared with other viral vectors such as retroviruses [94] which may explain current trend in using lentiviral vectors. In this review, four studies used lentiviral vectors [29, 32, 35, 36]. These studies did not report any adverse effects including immune reactions on cells or animal models that were transfected with these vectors. However, there are several concerns relating to these systems such as biodistribution, high costs, and mass-producing problems.

Non-viral Vectors

In this review, cationic lipids including Lipofectamine, siPORT NeoFX, and Attractene [16, 26–28, 30, 32, 37–40] were the most common non-viral delivery system especially for in vitro studies. The key aspect for these delivery systems are their structural versatility to adapt for various cargos and target cells [95]. However, these advantages should be balanced by their reported toxicity and inflammatory impacts. Another positive charged carrier is polyethylenimine (PEI) polymers which applied with graphene oxide in Dou et al. study [33]. This complex strongly binds to nucleic acids and represented effective delivery (escaping from lysosomes) in the cells [96]. Major concerns for this application are PEI cytotoxicity and preservation of miRNAs because of their instability. Geng et al. [34] demonstrated a novel miRNA Nanocapsules which are able to deliver in vivo and in vitro. They showed that application of this delivery system on the surface of titanium can effectively deliver miRNA-21 and improve osteo-angiogenesis, but in their study, they did not evaluate transfection efficiency of their delivery systems. Mixing atecollagen and miRNA inhibitor also used in refractory bone fractures, but due to limited information provided, its pros and cons should be assessed in future research.

Aptamers which can selectively bind to target cells [97] also applied for delivering miRNAs into osteoporotic bone tissues [37]. This delivery system previously used for ocular and hematological malignancies. Therefore, it may provide a promising vehicle for systemic delivery of miRNAs in bone diseases, but more investigations are required for especially for balancing its cost-efficiency.

Locked nucleic acid (LNA)-stabilized oligonucleotide technology has been adopted for inhibition of miRNA-92a in bone fracture healing, and unravel LNA-based antisense potentials as a therapeutic modality for bone defects [16]. In clinic, dosage and side-effects of systemic application of LNA are concerning issues [98]. Therefore, local administration and determining optimum dosage should be addressed in future investigations.

Li et al. [40] in vivo part, thiol-modified hyaluronan, heparin, and gelatin in combination with polyethylene glycol and hydrogel as a local delivery system of miRNA-26a into the defect site. Although this sustained delivery system did not assess for the exact miRNA-releasing amount, via fluorescent labeling, it has been shown that this system maintained the local concentration of miRNA for a long time in vivo and also protected these molecules from degradation enzymes and proteins.

In conclusion, miRNAs coordinate in a broad spectrum of complex biological mechanisms. Previous studies have made major progress in understanding these processes in bone regeneration and remodeling. According to included studies in this review, miRNA-7b, -9, -21, -26a, -27a, -210, -378, -195~497 cluster, and -675 positively promote both angiogenesis and osteogenesis, whereas miRNA-10, -222, and -494 inhibited both processes. However, there are several major issues that prevent therapeutic approaches of miRNAs especially in clinics. Each miRNA usually has numerous targets. In fact, bioinformatics analysis demonstrated that they can modulate hundreds of targets individually or by influencing regulatory loops and networks [99]. For instance, miRNA-92a which can influence bone healing and neovascularization, also have a proto-oncogenic capability [100]. Moreover, they may influence different cells in different ways. Therefore, developing smart delivery systems that can provide miRNAs in the right location and time is really needed. Not only developing an efficient and safe delivery system for prolong releasing of miRNAs for bone regeneration is required, but also balancing the cost and benefit of these therapeutic approaches should be considered to develop further clinical applications.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest related to this study.

Research Involving Human and Animal Participants Due to the essence of this study, as a review article, there were not any human or animal participants, but we included the studies which all of them have ethical approval.

Informed Consent This is not applicable for this study.

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