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***ORIGINAL ARTICLE***

# The Role of Angiogenesis-Inducing microRNAs in Vascular Tissue Engineering

May-Hui Ding, BS, Eloy G. Lozoya, Rene N. Rico, and Sue Anne Chew, PhD



Angiogenesis is an important process in tissue repair and regeneration as blood vessels are integral to supply nutrients to a functioning tissue. In this review, the application of microRNAs (miRNAs) or anti-miRNAs that can induce angiogenesis to aid in blood vessel formation for vascular tissue engineering in ischemic diseases such as peripheral arterial disease and stroke, cardiac diseases, and skin and bone tissue engineering is discussed. Endothelial cells (ECs) form the endothelium of the blood vessel and are recognized as the primary cell type that drives angiogenesis and studied in the applications that were reviewed. Besides ECs, mesenchymal stem cells can also play a pivotal role in these applications, specifically, by secreting growth factors or cytokines for paracrine signaling and/or as constituent cells in the new blood vessel formed. In addition to delivering miRNAs or cells transfected/transduced with miRNAs for angiogenesis and vascular tissue engineering, the utilization of extracellular vesicles (EVs), such as exosomes, microvesicles, and EVs collectively, has been more recently explored. Proangiogenic miRNAs and anti-miRNAs contribute to angiogenesis by targeting the 3′-untranslated region of targets to upregulate proangiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and hypoxia-inducible factor-1 and increase the transduction of VEGF signaling through the PI3K/AKT and Ras/Raf/MEK/ERK signaling pathways such as phosphatase and tensin homolog or regulating the signaling of other pathways important for angiogenesis such as the Notch signaling pathway and the pathway to produce nitric oxide. In conclusion, angiogenesis-inducing miRNAs and anti-miRNAs are promising tools for vascular tissue engineering for several applications; however, future work should emphasize optimizing the delivery and usage of these therapies as miRNAs can also be associated with the negative implications of cancer.

**Keywords:** microRNA, angiogenesis, vascular tissue engineering, blood vessel, endothelial cells, mesenchymal stem cells

### Impact Statement

Recent review articles have discussed the utilization of proangiogenic microRNAs (miRNAs) in angiogenesis and osteogenesis coupling and for bone tissue engineering applications. In this review, we discuss proangiogenic miRNAs that have been applied in vascular tissue engineering for different applications (i.e., not only for bone tissue engineering but also for peripheral arterial disease and stroke, cardiac diseases, and skin tissue engineering). This work provides an overview of the current state of utilization of proangiogenic miRNAs and emphasizes the importance of accuracy and dose of administration in designing future applications as these miRNAs can also be associated with the negative implications of cancer.

## Angiogenesis

Neovascularization or new blood vessel formation is critical for the survival of cells and for tissue to function adequately as oxygen and nutrient diffusion are limited to ~150–200 μm.[1] Blood vessels are formed through the migration and proliferation of endothelial cells (ECs) and the formation of tubular structures, which are modulated by different growth factors, cytokines, adhesion molecules, integrins, and enzymes.[2] Neovascularization can occur by (1) angiogenesis, which is the formation of new blood vessel from preexisting blood vessels, and (2) vasculogenesis, which is the formation of new blood vessel *de novo* by self-assembly of endothelial or endothelial progenitor cells.[3,4]

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There are two types of angiogenesis: (1) sprouting angiogenesis, a better understood process where sprouts of ECs add blood vessels to tissue areas devoid of blood vessels, and (2) intussusceptive angiogenesis, a more recently discovered process in which blood vessels form through a splitting process.[5] Both angiogenesis and vasculogenesis occur in embryonic development; however, in adults, transient formation of blood vessel occurs only in certain situations such as in the placenta during pregnancy and mainly by angiogenesis.[6] Angiogenesis is vital in adults during wound healing and postinjury regeneration, and also occurs during tumor growth. In contrast to tumor angiogenesis, which produces a large number of immature and disorganized vessels, reparative angiogenesis produces the functional and interconnected vessels fundamental in tissue regeneration.[7,8]

Angiogenesis in tissue regeneration has positive implications in myocardial ischemia and the engineering of three-dimensional (3D) tissue engineering applications such as for bone tissue. The lack of vasculature during the bone regeneration process can decrease bone formation and mass and formation of fibrous tissue.[2] Thus, angiogenesis is vital in bone tissue engineering to ensure that the bone repair with tissue-engineered systems is not impaired. Like in bone tissue engineering, it is also important to prevascularize other tissue engineering constructs, such as skin, before implantation to ensure better success of the application *in vivo*. Without the integration of blood vessels, the size and complexity of tissue engineering constructs and their subsequent success may be hindered from the lack of nutrient and oxygen supply that blood vessels deliver. The reduction of the delivery of oxygen can affect cells and tissue during wound healing and cardiac diseases such as myocardial infarction (MI), resulting in activation of complex signaling pathways to overcome the hypoxia at the impaired environment, to restore oxygen and nutrient homeostasis.[9] Thus, applications that can help induce angiogenesis during these processes and diseases are vital.

## The Overexpression of microRNAs for Vascular Tissue Engineering

microRNAs (miRNAs) are short ($\sim$22 nucleotides in length) noncoding single-strand RNAs that act as post-transcriptional gene regulators by binding complementary sequences typically in the 3′-untranslated region (UTR) of target messenger RNAs (mRNAs). miRNAs have been shown to play essential roles in diverse biological processes, including osteogenesis and angiogenesis, by regulating different cellular activity such as development, differentiation, proliferation, metabolism, and apoptosis.[10] miRNA binding to the 3′-UTR of mRNAs results in either the degradation of the target mRNA or inhibit translation of the mRNA into the protein of interest.[11,12] By repressing the inhibitors of angiogenesis, miRNAs can upregulate the expression of angiogenesis-inducing growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), or transcription factors, such as hypoxia-inducible factor-1 (HIF-1), which can enhance angiogenesis.

Extensive research has been focused on delivering growth factors for tissue engineering and regeneration. However, shortcomings associated with delivering growth factors in the protein form, such as the short half-life of protein and cost and difficulty of manufacturing these recombinant proteins, have led to the search of other methods of delivering bioactive factors for tissue engineering. By controlling the production of the bioactive factors *in vivo* via gene delivery (i.e. plasmid DNA, siRNA or miRNA) the proteins can be delivered in a more biologically active form[13] with more precise post-translational modification and tertiary structure formation[14,15] compared to the exogenous growth factors that may be altered through the delivery process.

Compared to plasmid DNA or siRNA, which regulates one specific mRNA, miRNAs can regulate multiple mRNAs or genes. The regulation of angiogenesis involves many different genes, and thus, the focus on a single target is generally insufficient or inefficient.[16] Instead of utilizing plasmid DNA or siRNA to regulate one specific mRNA, miRNA-based therapies can be used to influence a regulatory sequence resulting in regulation of a whole pathway or multiple pathways. Therefore, the overexpression of miRNAs to regulate *in vivo* production of their target genes vital in vascular tissue engineering may be a promising alternative to current traditional treatments of delivering growth factors as recombinant protein or delivering the plasmid encoding these proteins. Table 1 provides a summary of the difference between these different bioactive factors (i.e., protein, plasmid DNA, siRNA, and miRNA).

In this review, we discuss miRNAs that can induce angiogenesis, which have been utilized in applications where vascular tissue engineering is needed (i.e., ischemic diseases, cardiac diseases, and skin and bone tissue engineering). We discuss the different miRNAs or anti-miRNAs, nonviral or viral gene delivery vectors, biomaterial scaffolds or injection methods, targeting agents, and cell types used in these applications. We also discuss the role of mesenchymal stem cells (MSCs) in these applications, particularly their role in paracrine signaling of secreted growth factors or cytokines and contribution as constituent cells in the new blood vessels that are formed. We also discuss the utilization of extracellular vesicles (EVs), such as exosomes, microvesicles, or EVs collectively, for angiogenesis and vascular tissue engineering. Figure 1 depicts the different applications of these angiogenesis-inducing miRNAs and anti-miRNAs in vascular tissue engineering.

Frohlich published an article on miRNAs that have been identified to be involved in the coupling of osteogenesis and angiogenesis,[17] and Hosseinpour *et al.* evaluated miRNAs used in the regulation of angiogenesis in bone regeneration in 2019.[18] Urbich *et al.* reviewed miRNA in vascular diseases, inflammation, and angiogenesis, which was published much earlier (i.e., 2008).[19] In this work, we focused on discussing publications that not only have identified the proangiogenic miRNAs but also have applied them to a model or a disease that requires aid in vascular tissue engineering, and not just limited to bone tissue engineering applications. Furthermore, we also discussed the role MSCs play in angiogenesis, as there is conflicting evidence on their contribution in blood vessel formation, and also discuss the more recent application of EVs in angiogenesis for vascular tissue enginering.

## Targets and Mechanisms

Table 2 summarizes the direct targets and mechanisms for the proangiogenic miRNAs and anti-miRNAs that were reviewed in this article. The VEGF pathway is the primary hormonal pathway controlling angiogenesis. Among the different isoforms of VEGF, VEGF-A is the central isoform

TABLE 1. DIFFERENCES BETWEEN THE APPLICATION OF TRADITIONAL APPROACHES VERSUS MICRORNAS FOR TISSUE ENGINEERING

| | *Protein* | *Plasmid DNA* | *siRNA* | *microRNA* |
|---|---|---|---|---|
| Target | Delivers one specific protein | Regulates the expression of one specific mRNA | Regulates the expression of one specific mRNA | Regulates the expression of multiple mRNAs |
| Stability/half-life | Short half-life | Longer half-life | Longer half-life | Longer half-life |
| Manufacturing | High cost and is more difficult to manufacture | Lower cost and less difficult to manufacture | Lower cost and less difficult to manufacture | Lower cost and less difficult to manufacture |
| Protein delivery or production | Exogeneous protein delivery | Protein produced *in vivo*, proteins can be delivered in a more biologically active form with more precise post-translational modification and tertiary structure formation. | Protein production is prevented<br>This could lead to upregulation of the production of other proteins which will be produced *in vivo*, proteins can be delivered in a more biologically active form with more precise post-translational modification and tertiary structure formation. | Protein production is prevented<br>This could lead to upregulation of the production of other proteins which will be produced *in vivo*, proteins can be delivered in a more biologically active form with more precise post-translational modification and tertiary structure formation. |
| Size | Can be large | Usually large | Small | Small |



that regulates angiogenesis and EC growth.[20] VEGF has also been associated with increased cell viability, proliferation, and decrease apoptosis in MSCs.[21,22] VEGF functions by binding to VEGF receptors (VEGFR), which in turn activates multiple downstream pathways, including Ras/Raf/MEK/ERK, PI3K/Akt, or Src/FAK signaling pathways, which lead to angiogenesis[23] through the regulation of the fate of ECs.[24] There are several miRNAs (i.e., miRNA-34, miRNA-195, and miRNA-377) that directly target the 3′-UTR of *VEGF* and suppress its expression (Table 2). To enhance angiogenesis, the inhibitors of these miRNAs can be used for therapeutic applications. miRNA-378 targets the 3′-UTR of *VEGF*, competing with miRNA-125a, an inhibitor of VEGF expression. By competing for the same seed site, miRNA-378 can strengthen the expression of VEGF.[25]

It has been depicted that the levels of circulating growth factors such as VEGF are not downregulated in MI and peripheral arterial disease (PAD), so impairment of angiogenesis could be due to downstream signaling instead of decrease in the expression of these growth factors.[24] The 3′-UTR of phosphatase and tensin homolog (*PTEN*) is the target for several miRNAs (i.e., miRNA-21, miRNA-26a, and miRNA-130a) (Table 2). Overexpression of PTEN inhibits the PI3K/AKT pathway, which is important in enhancing angiogenesis through regulating EC survival, migration, and capillary-like structure (CLS) formation.[26] By suppressing the expression of PTEN with a miRNA, the signaling of the PI3K/Akt pathway can be activated. Although PTEN is a well-known negative regulator of the PI3K/AKT pathway, it has also more recently been

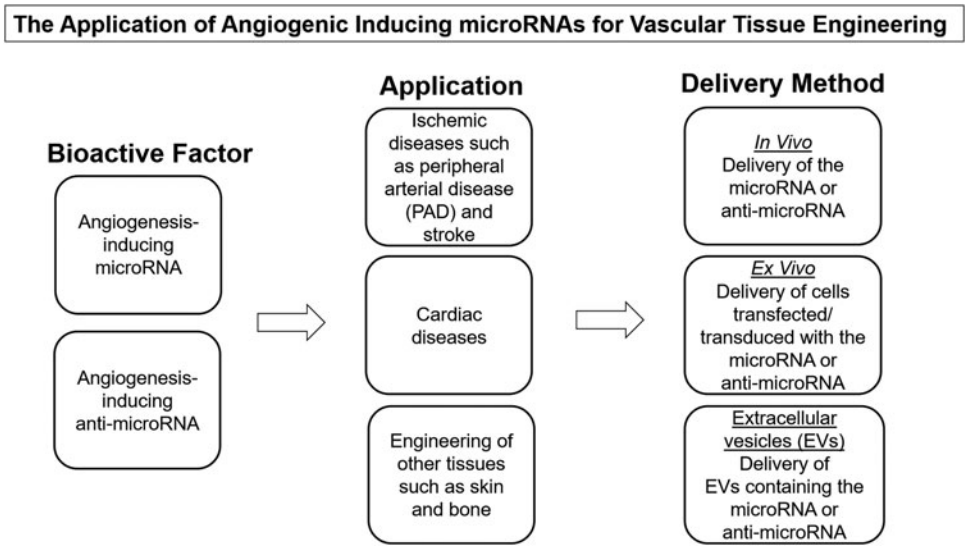


**FIG. 1.** Schematic summarizing the applications of proangiogenic microRNAs or anti-microRNAs for vascular tissue engineering.

Table 2. The Target and Mechanism of the Proangiogenic microRNAs and Anti-microRNAs Used for Vascular Tissue Engineering

| *miRNA* | *Mechanism* |
|---|---|
| miRNA-21 | miRNA-21 targets the 3′-UTR of *PTEN* and suppresses its expression.[27] PTEN downregulates PI3K/AKT and Ras/Raf/MEK/ERK signaling pathways, and also leads to the inhibition of HIF-1α and VEGF expression.[34,136] miRNA-21 also targets 3′-UTR of *SPRY1* and suppresses its expression.[137] SPRY1 inhibits the proangiogenic factors, VEGF, FGF, and EGFL7, through the Raf/MEK/ERK signaling pathway.[137,138] |
| miRNA-26a | miRNA-26a targets the 3′-UTR of PTEN and suppresses its expression.[139] PTEN downregulates PI3K/AKT and Ras/Raf/MEK/ERK signaling pathways, and also leads to the inhibition of HIF-1α and VEGF expression.[34,136] |
| miRNA-31 | miRNA-31 targets the 3′-UTR of *FIH1* and suppresses its expression.[32] FIH1 inhibits the transcriptional activity of HIF-1, a proangiogenic factor involved in angiogenesis.[33,34] |
| Anti-miRNA-34 | miRNA-34 targets the 3′-UTR of *VEGFs*, vinculin, protein *O*-fucosyltransferase, Notch1, and semaphorin 4B, which are important for angiogenesis and cardiac function.[57] |
| Anti-miRNA-92a | miRNA-92a targets the 3′-UTR of *ITGA5*.[40,41] Suppression of ITGA5 leads to the downregulation of eNOS, which is important for vascular tone and postnatal neovascularization.[42,43] |
| miRNA-93 | miRNA-93 targets the 3′-UTR of *CDKN1A* and suppresses its expression.[53] CDKN1A (p21) counteracts actions of VEGF and MAPK.[140] |
| miRNA-125a | miRNA-125a targets the 3′-UTR of *DLL4* and suppresses its expression.[37] DLL4 is an angiogenic inhibitor and a ligand of the Notch signaling pathway that inhibits endothelial tip cell formation in angiogenesis.[37,38] |
| miRNA-126 | miRNA-126 targets the 3′-UTR of *Spred-1*[141,142] and *PI3KR2* (p85-beta)[141] and suppresses their expression. Spred-1 and PI3KR2 suppress VEGF and FGF[142] and VEGF-dependent PI3K/AKT and Ras/Raf/MEK/ERK signaling.[141] miRNA-126 also targets the 3′-UTR of *PTPN9* and suppresses its expression.[51] PTPN9 dephosphorylates VEGFR2, thus inactivating it.[143] |
| miRNA-130a | miRNA-130a targets *PTEN* and suppresses its expression.[26] PTEN downregulates PI3K/AKT and Ras/Raf/MEK/ERK signaling pathways, and also leads to the inhibition of HIF-1α and VEGF expression.[34,136] |
| miRNA-132 | miRNA-132 targets 3′-UTR of *RASA1* and suppresses its expression.[124] RASA1 is a negative regulator of vascular sprouting and vessel branching through the inactivation of the Ras/Raf/MEK/ERK pathway. |
| Anti-miRNA-135a-3p | miRNA-135-3p targets the 3′-UTR of *HIP1* and regulates the VEGF-HIP1-p38k signaling axis for angiogenesis.[24] |
| Anti-miRNA-143 | miRNA-143 targets the 3′-UTR of *IGF-IR*, a receptor that inhibits the production of nitric oxide and angiogenesis.[44] NO is a multifunctional factor that maintains vascular homeostasis, protects injured vessels, and regulates cell growth.[39] |
| miRNA-145 | miRNA-145 primary target gene is *KLF4*, which is associated with SMC contractile phenotype[73] |
| miRNA-146a | miRNA-146a targets the 3′-UTR of *NF2* and suppresses its expression. NF2 is an inhibitor of PAK1,[81] which is a component of the Rac-PAK signaling pathway that is important for lumen formation and angiogenesis in EC function.[144] |
| miRNA-181b-5p | miRNA-181b targets the 3′-UTR of *TRMP7*.[122] The suppression of TRMP7 promotes the early stages of angiogenesis, cell proliferation, migration, adhesion, and tube formation, through the Ras/Raf/MEK/ERK pathway.[145,146] |
| Anti-miRNA-195 | miRNA 195 targets the 3′-UTR of *VEGF* and suppresses its expression.[71] VEGF is a key angiogenic mediator.[71] |
| miRNA-199-3p | miRNA-199-3p targets the 3′-UTR of *Sema3A* and suppresses its expression.[123,147] Sema3A suppresses the formation of tip cell filopodia,[123,147] which are important for vessel sprouting formation, and migration of ECs.[148] |
| Anti-miRNA-200b | miRNA-200b targets v-Ets erythroblastosis virus E26 oncogene homolog 1 (*ETS1*) and suppresses its expression.[132] ETS1 is a master regulator of EC gene transcription[149] that is downstream of VEGF in the PI3K/AKT and p38 MAPK signaling pathways.[150] |
| Anti-miRNA-210 | miRNA-210 targets the 3′-UTR of Ephrin-A3 (*EFNA3*) and suppresses its expression.[151] EFNA3 is a glycosylphosphatidylinositol-anchored membrane protein that is vital for stimulation of tubulogenesis and formation of capillary-like structures, crucial functions in angiogenesis. |
| Anti-miRNA-222 | miRNA-222 targets the *c-kit receptor*[152] and *STAT5A*.[153] The c-Kit receptor is expressed on ECs and is the receptor for the angiogenic activity of SCF.[152] STAT5A activates bFGF and IL-3, which activates vascular EC morphogenesis in the STAT5A signaling pathway.[153] |
| Anti-miRNA-377 | miRNA-377 targets the 3′-UTR of *VEGF*, resulting in suppression of VEGF.[58] VEGF is a key angiogenic mediator.[71] |
| miRNA-378 | miRNA-378 targets the 3′-UTR of *VEGF* and competes with miRNA-125a, which inhibits VEGF expression. By competing for the same seed site, miRNA-378 can strengthen the expression of VEGF.[59] |
| Anti-miRNA-493 | miRNA-493 targets the 3′-UTR of macrophage MIF, an angiogenic regulator, which inhibits angiogenesis by inhibiting the of PI3K/Akt and Ras/Raf/MEK/ERK pathways.[52] It is involved in EC proliferation migration and tube formation.[154] |

miRNA, microRNA; UTR, untranslated region; PTEN, phosphatase and tensin homolog, HIF-1, hypoxia-inducible factor-1; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; EGFL7, epidermal growth factor-like protein 7; FIH1, factor-inhibiting HIF-1; ITGA5, integrin subunit alpha5; eNOS, endothelial nitric oxide-synthase; DLL4, delta-like 4; VEGFR, vascular endothelial growth factor receptor; HIP1, huntingtin-interacting protein 1; IGF-IR, insulin-like growth factor 1 receptor; NO, nitric oxide; KLF4, Krüppler-like factor 4; SMC, smooth muscle cell; NF2, neurofibromin 2; PAK1, p21-activated kinase-1; EC, endothelial cell; STAT5A, signal transducer and activator of transcription 5A; SCF, stem cell factor; bFGF, basic fibroblast growth factor; IL, interleukin; MIF, migration inhibitory factor.

established as a negative regulator of the Ras/Raf/MEK/ERK pathway. Therefore, the inhibition of PTEN by a miRNA will also activate the signaling of the Ras/Raf/MEK/ERK pathway. Besides controlling EC contribution to angiogenesis, PTEN suppression can also control the fate of induced pluripotent stem cells (iPSCs). Di Bernardini *et al.* demonstrated that miR-21 targets the 3′-UTR of *PTEN*, thereby regulating the PI3K/Akt pathway for iPSC differentiation into ECs.[27]

In addition to the Ras/Raf/MEK/ERK and PI3K/AKT pathways, another pathway important for EC migration and proliferation through VEGF and VEGFR is the VEGF-huntingtin-interacting protein 1 (HIP1)–p38 signaling pathway.[24] Icli *et al.* demonstrated that miRNA-135 regulates the p38 MAPK signaling pathway in response to VEGF stimulation, but not the ERK1/2 or Akt pathways.[24] This is because miR-135-3p targets the 3′-UTR of *HIP1* and regulates the VEGF-HIP1–p38 signaling axis.[24] Anti-miRNA-135-3p can prevent the inhibition of HIP1, activating the VEGF-HIP1-p38k. Therefore, p38 MAPK signaling resulting from VEGF stimulation contributes to EC migration and proliferation.[24]

Hypoxia-inducible factor family of proteins is another important mediator of angiogenesis, and, in particular, HIF-1, which is made from the HIF-1α and HIF-1β subunits.[9] At normoxia, HIF-1α levels are maintained at a low level. However, during hypoxic conditions, the degradation of HIF-1α is inhibited, and heterodimerization with HIF-1β is constitutively expressed, allowing translocation into the nucleus. Then, the HIF-1α/β dimer binds to hypoxia response elements, and this results in the transcription of target genes involved in angiogenesis.[28] HIF-1α also plays a role in activating the transcription of many angiogenesis-related genes, including VEGF.[29,30] HIF-1α can be induced by hypoxia or loss of PTEN.[31] Thus, besides inhibiting the PI3K/AKT and Ras/Raf/MEK/ERK signaling pathways, the overexpression of PTEN can also decrease the expression of both critical angiogenic modulators, HIF-1α and VEGF. miR-31 targets the 3′-UTR of factor-inhibiting HIF-1 (*FIH1*) and suppresses its expression.[32] FIH1 inhibits the transcriptional activity of HIF-1.[33,34] Therefore, the overexpression of miR-31 leads to a decrease in FIH1 levels, which in turn will increase HIF-1 levels.

Besides VEGF, fibroblastic growth factor (FGF) is another growth factor that contributes to angiogenesis.[20] The main signaling pathway for FGF is through its association with FGFR-1.[23] FGF levels can be regulated by proangiogenic miRNAs such as miRNA-21 and miRNA-126, which target the 3′-UTR of *SPRY-1* and *Spred-1*, respectively. The suppressed expression of these targets by the miRNAs increases the expression of FGF. miRNA-222 targets the 3′-UTR of the signal transducer and activator of transcription 5A (*STAT5A*), which activates FGF. By inhibiting miRNA-222, STAT5A suppression by miRNA-222 can be avoided, which will lead to increased FGF levels.

Another pathway involved in vasculature and angiogenesis is the Notch signaling pathway. Notch signaling decreases the formation of tip cells and reduces the density of vascular network.[35,36] Tip cells are ECs that are located at the very tip of angiogenic sprouting. Besides binding to the 3′-UTR seed region of *VEGF*, miRNA-125a also targets the 3′-UTR of delta-like 4 (*DLL4*) and suppresses its expression.[37] DLL4 is an angiogenic inhibitor and a ligand of the Notch signaling pathway that inhibits endothelial tip cell formation in angiogenesis.[37,38]

Nitric oxide (NO) is a multifunctional factor that maintains vascular homeostasis, protects injured vessels, and regulates cell growth, making it crucial for angiogenesis.[39] The production of NO can be regulated by miRNAs as well. NO is generated by NO synthase (NOS), including endothelial NOS (eNOS). miR-92a targets the 3′-UTR of integrin subunit alpha5 (*ITGA5*).[40,41] Suppression of ITGA5 leads to the downregulation of eNOS, which is important for vascular tone and postnatal neovascularization.[42,43] miR-143 targets the 3′-UTR of insulin-like growth factor (*IGF*) 1 receptor, a receptor that inhibits the production of NO and angiogenesis.[44]

## Applications

### *Vascular disease and tissue engineering*

Vascular tissue engineering is needed for many applications, including cardiac diseases, skin tissue engineering in burn victims or in wound healing, and the regeneration of 3D thick tissues such as bone. The delivery of ECs for therapeutic vascularization or tissue engineering is often applied to increase tissue perfusion. However, the utilization of miRNA to enhance this process can be very promising to further augment the blood vessel formation and improve the function of the tissue of interest. Table 3 summarizes the miRNAs and anti-miRNAs that have been investigated for different applications to induce angiogenesis for new blood vessel formation. Devalliere *et al.* showed that ECs transfected with miRNA-132 to a mouse abdominal wall subcutaneous pocket can improve EC transplantation and vascularization.[45] Small-diameter vascular grafting (diameter 6 mm) can be challenging due to thrombosis and restenosis after graft implantation.[46,47] Wen *et al.* demonstrated miRNA-145 can be utilized to regulate smooth muscle cell (SMC) phenotype and proliferation, and thus may be promising for the regeneration of small-diameter blood vessels.[73]

Ischemia results from inadequate blood supply due to the blockage or damage of blood vessels, which can result in insufficient supply of oxygen to a certain part of the body such as the heart, brain, or limbs. Ischemic stroke results from the blockage of blood vessels in the brain. Even after timely treatment of the acute phase of ischemic stroke, patients will suffer long-term neurological deficits.[48] Thus, in ischemic stroke patients, blood flow restoration is a key treatment[49] as neovascularization has been shown to result in positive neurobehavioral outcome after stroke.[50] Qu *et al.* investigated the utilization of miRNA-126 on ischemic stroke using a mouse middle cerebral artery occlusion model.[51] They demonstrated that the overexpression of miRNA-126 resulted in not only increased angiogenesis but also neurogenesis, which improved neurobehavior. Li *et al.* investigated the inhibition of miRNA-493 in a rat middle cerebral artery occlusion model, which showed increased capillary density and decreased focal infarct volume, and as a result, improved neurologic deficit.[52]

PAD is an atherosclerotic disease that affects the arteries of the limb[53] and induces tissue hypoperfusion, and eventually results in critical limb ischemia.[54] Shu *et al.* demonstrated that overexpression of miRNA-93 can help enhance

Table 3. Proangiogenic microRNAs or Anti-microRNAs That Have Been Investigated for Different Vascular Tissue Engineering Applications

| *miRNA* | *Transfection agent* | *Biomaterial vehicle/injection* | *Cell type* | In vivo *model* | *Outcome* | *References* |
|---|---|---|---|---|---|---|
| Vascular tissue engineering | | | | | | |
| miRNA-21 | Lipofectamine RNAiMAX | Matrigel | Pluripotent stem cells (iPSCs)<br>Paracrine signaling and differentiation into vascular lineage | Mouse subcutaneous model<br>Delivery of cells transfected with miRNA (*ex vivo*) | Induced capillary formation | 27 |
| Anti-miRNA-92a | GeneTrans II® | Intravenous injection (systemic) | HUVECs | Mouse hind limb ischemia model<br>Delivery of anti-miRNA (*in vivo*) | Increased number of capillaries and smooth muscle actin-positive arterioles, reducing toe necrosis | 40 |
| miRNA-93 | Lipofectamine RNAiMax | Intramuscular injection (local) | EA.hy926 endothelial cells | Mouse hind limb ischemic model<br>Delivery of miRNA (*in vivo*) | Enhanced capillary density, decreasing muscle necrosis | 53 |
| miRNA-126 | Lentivirus | Lentiviral vector brain injection (local) | HUVECs | Mouse middle cerebral artery occlusion model<br>Delivery of miRNA (*in vivo*) | Increased vessel length and diameter, improving neurobehavioral recovery | 51 |
| miRNA-132 | Complexed with spermidine and then loaded into PLGA nanoparticles with surface-bound cRGD | Collagen fibronectin gel (local) | HUVECs | Mouse abdominal wall subcutaneous pocket model<br>Delivery of cells transfected with miRNA (*ex vivo*) | Increased vessel density/ total lumen area, increased microvessels per square millimeter, and increased smooth muscle alpha-actin (SMA) vessels. | 45 |
| miRNA-145 | VAPG peptide-modified TMC-*g*-PEG-VAPG | PELCL electrospun membranes (local) | SMCs | *In vitro* only | Decreased expression of KLF4, increased expression of α-SMA and myocardin, increased cellular uptake of SMC, and modulated SMC phenotype | 73 |
| Anti-miRNA-493 | Lipofectamine 2000 | Intracerebroventricular injection (local) | RBMECs | Rat middle cerebral artery occlusion model<br>Delivery of miRNA (*in vivo*) | Increased capillary density in the IBZ and decreased focal infarct volume, improving neurologic deficit | 52 |
| Cardiac disease | | | | | | |

(*continued*)

TABLE 3. (CONTINUED)

| *miRNA* | *Transfection agent* | *Biomaterial vehicle/injection* | *Cell type* | In vivo *model* | *Outcome* | *References* |
|---|---|---|---|---|---|---|
| Anti-miRNA-34 family | None | Subcutaneous injection (systemic) | HEK 293T and H9c2 cells for Luciferase-reporter assay | Mouse MI and pressure overload models<br>Delivery of miRNA (*in vivo*) | Increased angiogenesis and increased capillary density, improving cardiac function | 57 |
| Anti-miRNA-92a | GeneTrans II | Intravenous injection (systemic) | HUVECs | Mouse acute MI models<br>Delivery of miRNA (*in vivo*) | Enhanced perfused lectin-positive vessels and smooth muscle actin-positive arterioles, improving cardiac function | 40 |
| miRNA-126 | Lentivirus | Re-thoracotomy and intramyocardial injection (local) | Mouse BMMSCs<br>Paracrine signaling, not clear if they participated in differentiation into vascular lineage | Mouse acute MI model<br>Delivery of cells transfected with miRNA (*ex vivo*) | Increased number of mature microvessels and microvessel density, improving myocardial function | 56 |
| miRNA-146a | siLentFect™ | Intramyocardial injection (local) | Human BMMSCs<br>Paracrine signaling and differentiation into vascular lineage | Rat I/R injury animal model in the coronary artery<br>Delivery of cells transfected with miRNA (*ex vivo*) | Increased formation of vascular branches, increased number of branching points, and reduced fibrosis, improving cardiac functions | 81 |
| Anti-miRNA-377 | Lentivirus | Border area of the left ventricle injection (local) | Rat BMMSCs<br>Paracrine signaling | Rat MI model<br>Delivery of cells transfected with miRNA (*ex vivo*) | Enhanced tube formation, increased vessel density and reduced fibrosis, improving myocardial function | 58 |
| miRNA-378 | Lipofectamine 2000 | None | Rat BMMSCs<br>Differentiation into vascular lineage | *In vitro* only | Increased expressions of VEGF α VEGF-A, PDGF-B, and TGF-B1 in hypoxic-ischemic conditions | 59 |
| Skin tissue engineering | | | | | | |
| Anti-miRNA-92a | Dharmafect | Intradermal injection (systemic) | None | Mouse diabetic db/db and normal pig wound models<br>Delivery of anti-miRNA (*in vivo*) | Increased number and size of blood vessels in the mouse diabetic model, increased granulation tissue formation, angiogenesis, and tissue perfusion, improving wound healing | 62 |

(*continued*)

TABLE 3. (CONTINUED)

| *miRNA* | *Transfection agent* | *Biomaterial vehicle/injection* | *Cell type* | In vivo *model* | *Outcome* | *References* |
|---|---|---|---|---|---|---|
| Anti-miRNA-135a-3p | Lipofectamine 2000 | Intradermal injection (local) | HUVECs | Mouse diabetic db/db model of dermal wound healing<br>Delivery of miRNA (*in vivo*) | Increased granulation tissue thickness and angiogenesis, improving diabetic wound healing | 24 |
| Bone tissue engineering | | | | | | |
| miRNA-26a | siPORT NeoFX | Thiol-modified analog of heparin with thiol-modified hyaluronan and poly(ethylene glycol) diacrylate HP-HA-PEG (Glycosil$^{TM}$) (local) | Human BMMSCs<br>Unclear if paracrine signaling or differentiation into vascular lineage | Mouse calvarial bone defect<br>Delivery of cells transfected with miRNA | Increased density of blood vessels and regulated endogenous angiogenesis–osteogenesis coupling, improving bone regeneration | 12 |
| Anti-miRNA-92a | Lipofectamine 2000 | Intravenous injection through the tail vein (systemic) or injection at the fracture site (local) | Primary osteoblasts, MC3T3–E1, and ATDC5 cells | Mouse femoral bone fracture model<br>Delivery of anti-miRNA | Increased blood vessel volume, number of CD31+ blood vessels, and CD31+ capillary invasion in cartilage, and higher ratio of vessel area, increasing fracture healing | 41 |
| Anti-miRNA-195 | Lipofectamine 2000 | None | BMMSCs<br>Paracrine signaling | *In vitro* only | miRNA (not the anti-miRNA) decreased endothelial vessel growth in a chicken embryo CAM angiogenesis assay and osteogenic differentiation | 71 |
| Anti-miRNA-222 | None | Atelocollagen (local) | None | Rat femoral refractory fracture model<br>Delivery of anti-miRNA | Increased capillary density, accelerating bone healing through enhanced osteogenesis, chondrogenesis, and angiogenesis | 70 |

VAPG, Val–Ala–Pro–Gly; TMC-*g*-PEG-VAPG, trimethyl chitosan-*g*-poly(ethylene glycol) cRGD; cyclic RGD; PELCL, poly(ethylene glycol)-*b*-poly(L-lactide-*co*-ε-caprolactone); BMMSC, bone marrow-derived mesenchymal stem cell; HUVEC, human umbilical vein endothelial cell; RBMEC, rat brain microvascular endothelial cell; SMC, smooth muscle cell; iPSC, induced pluripotent stem cell; I/R, ischemia/reperfusion; PDGF-B, platelet-derived growth factor-β; TGF-B1, transforming growth factor-β1; CAM, chorioallantoic membrane; MI, myocardial infarction; α-SMA, alpha-smooth muscle actin; PLGA, poly(lactic-co-glycolic acid); IBZ, ischemic boundary zone.

the proliferation, migration, and tube formation of ECs, ameliorating ischemia in the mouse hind limb for PAD therapy.[53] Bonauer *et al.* also utilized the mouse hind limb ischemia model to test anti-miRNA-92a by ligation of the superficial and deep femoral artery and vein.[40] They determined that the inhibition of miRNA-92a enhanced the growth of blood vessels, resulting in significant reduction in toe necrosis.

Below we discussed other specific applications where the overexpression or inhibition of miRNA to generate and engineer blood vessels has been employed.

### *Cardiovascular diseases*

New blood vessel formation and regeneration are critical for the treatment of cardiovascular diseases.[24] Coronary artery disease is the most common cause of mortality worldwide, creating the need for artificial vascular grafts to aid in cardiovascular diseases.[55] However, these grafts remain a challenge because they can lead to thrombosis and restenosis after implantation,[46,47] and thus, other approaches to treat cardiac diseases are needed. In this study, we discuss the studies that have investigated the utilization of miRNA overexpression or inhibition in cardiac dysfunction.

MI, which is also known as a heart attack, is the most frequent cause of heart failure. Huang *et al.* used a mouse acute MI model to investigate the delivery of MSCs transfected with miRNA-126.[56] The MI model was induced by ligating the anterior descending artery of the animal. Huang *et al.*[56] demonstrated that myocardial function improved as the delivery of MSCs transfected with miRNA-126 increased the number of mature microvessels, and increased myocardial blood flow and microvessel density. Bonauer *et al.* also investigated the utilization of the inhibition of miRNA-92a for the treatment of MI.[40] They showed that the downregulation of miRNA-92a can lead to improved left ventricular systolic and diastolic function and reduced infarct size.

Bernardo *et al.* investigated the inhibition of miRNA-34 family members, which has been seen to be upregulated in the heart due to stress.[57] They saw that inhibition of miRNA-34 family members (i.e., miRNA-34a, miRNA-34b, and miRNA-34c) in mice subjected to MI or pressure overload through transverse aortic constriction resulted in attenuated pathological remodeling and improved cardiac function, respectively. Wen *et al.* used a mouse MI model to test the delivery of bone marrow-derived mesenchymal stem cells (BMMSCs) transfected with anti-miR-377 in cardiac repair.[58] They observed enhanced tube formation activity, increased vessel density, and reduced fibrosis, which led to improved myocardial function. Xing *et al.* investigated the utilization of miRNA-378 for cardiac repair.[59] They observed *in vitro* that overexpression of miRNA-378 in rat BMMSCs resulted in a larger number of vascular branches and increased expressions of VEGF-α (VEGF-A), platelet-derived growth factor-β (PDGF-B), and transforming growth factor (TGF)-β1 in hypoxic-ischemic condition.

### *Skin tissue engineering*

Skin engraftment can fail because of the delay or absence in vascularization.[60] Patients who are predisposed to the development of nonhealing skin ulcers face even more challenges in vascularizing skin substitutes, which will result in graft failure.[61] Tissue engineering strategies that utilize *in vitro* reconstructed skin may be a promising method to replace skin engraftment and may be utilized for the treatment of wounds in burn victims or nonhealing wounds in patients with vascular insufficiency, such as those with venous stasis or diabetes or in the elderly patients.[61] In addition, the use of proangiogenic miRNA delivery, as opposed to the traditional method to induce vascular tissue engineering (i.e., proangiogenic growth factor delivery, which is associated with short half-lives and instability *in vivo*), may be a promising skin tissue engineering approach.

Icli *et al.* investigated the inhibition of miRNA-135-3p through injection into mouse dermal wounds (i.e., 1 $cm^2$ dorsal full-thickness skin wounds), which can occur in patients with diabetes mellitus.[24] They saw that miRNA-135 was a pivotal regulator of pathophysiological angiogenesis and tissue repair, and that inhibition of miRNA-135 induced diabetic wound healing and increased angiogenesis. Gallant-Behm *et al.* delivered a synthetic anti-miRNA-92 (i.e., MRG-110) to a diabetic mouse skin wound and a normal pig skin wound model.[62] The inhibition of miRNA-92 resulted in the enhancement of angiogenesis and accelerated wound closure. Gallant-Behm *et al.*[62] demonstrated that MRG-110 had an addictive effect with recombinant human platelet-derived growth factor beta, which is the active ingredient for Becaplermin gel, an Food and Drug Administration-approved treatment for diabetic foot ulcers (Regranex). MRG-110 is currently being tested in two phase 1 clinical trials: a systemic and local administration trial (Clinicaltrials.gov reference numbers NCT03494712 and NCT03603431, respectively).[62]

### *Bone tissue engineering*

Compromised vascularity is a limiting factor in successful bone defect healing, regardless of the approach for bone tissue engineering.[63–65] Insufficient blood supply in the early phase of bone healing using a tissue engineering system results in inadequate cell integration and cell death in the system.[66] The shortcomings associated with bone tissue engineering systems, including only addressing osteogenesis, have hindered the use of bone tissue engineering constructs to replace current therapies. Thus, it is important to design a bone tissue engineering system that will address both osteogenesis and angiogenesis.

Several groups have shown the beneficial and synergistic effects of coupling angiogenesis with osteogenesis for optimal bone regeneration. VEGF, which is associated with angiogenesis, has been found to directly enhance the mRNA and protein expression of bone morphogenetic protein 2 (BMP-2), an osteogenic growth factor that induces osteogenic stem cell differentiation into osteoblasts.[67] Sipola *et al.*[68] and Peng *et al.*[69] have shown that VEGF and BMP-2 work synergistically in enhancing bone healing by demonstrating that VEGF antagonists (i.e., endostatin and soluble fms-like tyrosine kinase 1 [sFlt1], respectively) inhibit BMP-2-induced bone formation. Peng *et al.*[69] further showed that BMP-2-induced bone formation can be enhanced with exogenous VEGF delivery, which increased angiogenesis and also leads to acceleration in bone formation.

Instead of growth factor delivery for inducing angiogenesis, several groups have also explored the utilization of

proangiogenic miRNA to promote angiogenesis for bone tissue engineering. Murata *et al.* investigated the inhibition of miRNA-92a in a mouse hip and femoral fracture model.[41] They demonstrated that systemic and local administration of anti-miRNA-92a enhanced bone fracture healing through increased neovascularization. Li *et al.* investigated the local delivery of miR-26a using a biomaterial scaffold in a mouse calvarial bone defect.[12] They saw that miRNA-26a induces bone regeneration by regulating angiogenesis-osteogenesis coupling. Yoshizuka *et al.* investigated the inhibition of miRNA-222 in a rat femoral refractory fracture model.[70] They showed that besides being a negative modulator of angiogenesis, the inhibition of miRNA-222 could also enhance both osteogenesis and chondrogenesis, potentially making it a good candidate for endochondral ossification. Almeida *et al.* investigated the inhibition of miRNA-196 *in vitro*.[71] They observed that the downregulation of miRNA-196 resulted in MSC osteogenic differentiation, proliferation, and control of angiogenesis, and thus may be a promising tool for human bone defects.



## Delivery Vehicle

### *Biomaterial scaffolds*

In tissue regeneration applications, biomaterial scaffolds can play a vital role in providing a substrate for cells to adhere and colonize, while also functioning as a delivery vehicle for the cells and/or bioactive factor of interest, such as miRNAs or anti-miRNAs. These scaffolds can be made out of natural polymers, synthetic polymers, bioceramics, or hydrogels, depending on the targeting tissue of interest and the delivery cargo (example cells vs. only bioactive factors). The utilization of biomaterial scaffold delivery of cells transfected with miRNA or anti-miRNA can be superior to systemic delivery, as local biomaterial scaffold delivery will require smaller amounts of costly RNA oligonucleotides and adverse side effect on nontargeted sites can be avoided.[72] The incorporation of the miRNAs into a delivery system may result in the miRNA being exposed to a harsh environment during the fabrication process such as organic solvents or heat, and thus, researchers need to make sure that the miRNAs are stable after incorporation and are nonaffected by the fabrication process. The efficacy of these miRNAs is evaluated after incorporation into the biomaterials, and thus their stability and bioactivity are confirmed in the reviewed articles.

Devalliere *et al.* utilized a collagen-fibronectin-based scaffold to deliver miRNA-132 to mouse abdominal wall subcutaneous pockets.[45] They showed that the scaffolds were able to support EC engraftment, and when implanted, the vascularization site could be better controlled and the local delivery of miRNA resulted in biological effects in the area of interest. Natural polymers may be more conducive to EC engraftment and viability compared to synthetic polymer scaffolds.

Wen *et al.* used poly(ethylene glycol)-*b*-poly(L-lactide-*co*-ε-caprolactone) electrospun membranes to deliver miRNA-145.[73] The miRNAs, complexed with a nonviral vector, were incorporated into the scaffolds by mixing the complexes into a polymer solution before being electrosprayed. Electrospun materials are ideal for regeneration of small-diameter blood vessels, as they have high surface area and porosity that better mimic the extracellular matrix.[73] The membranes investigated by Wen *et al.*[73] were able to release the complexes for over 56 days.

Yoshizuka *et al.* utilized atelocollagen to deliver MSCs transfected with anti-miRNA-222.[70] Atelocollagen is a type of collagen that is made by the elimination of telopeptide moieties (the antigenic sites of collagen), which decreases its immunogenicity, making it a safe biomaterial.[74] This material is a liquid at lower temperatures, but solidifies above 30°C, and thus can be used in an injectable delivery system to slowly release the drug of interest.[75]

Li *et al.* used a thiol-modified analog of heparin with thiol-modified hyaluronan and poly(ethylene glycol) diacrylate (Glycosil™) biodegradable[12] hydrogel to deliver MSCs transduced with miRNA-26a. Glycosil is available on the market from ESI·BIO and suitable for 3D cell culturing.[76]

### *Injection*

The majority of the reviewed applications were delivered through local injection at the site of interest instead of utilizing a scaffold. Direct injection of miRNA at the site of interest is beneficial because of its ease of administration and it is less invasive, compared to the implantation of a drug delivery device, which may require a surgery for placement of the material. Several groups delivered cells that were transfected with the miRNA or anti-miRNA *in vitro*, which were injected *in vivo* at the site of interest or systemically. Huang *et al.* injected miRNA-126 at 5 sites in peri-infarcted areas in a mouse acute MI model.[56] Shu *et al.* injected miRNA-93 intramuscularly into the gastrocnemius muscle in a mouse hind limb ischemia model.[53] Qu *et al.* injected lentivirus vector containing miRNA-126 stereotactically at 2 mm lateral to the bregma and 2.5 mm deep under the dura of a focal cerebral ischemia mouse model.[51] In the skin tissue engineering of a mouse diabetic wound healing model, Icli *et al.* delivered anti-miR-135a-3p intradermally.[24]

Besides delivering miRNA or anti-miRNA or cells transfected with miRNA or anti-miRNA through local injection, there were also groups that employed systemic delivery through intravenous injection of the miRNA or anti-miRNA. This method may be more easily administered; however, it may not be as efficient and may result in nontargeted side effects. Murata *et al.* investigated the systemic delivery of anti-miRNA-92a intravenously by tail vein injection and compared it to the local injection of anti-miRNA-92a at the fracture site in a mouse femoral bone fracture model.[41] They determined that both the local and systemic delivery enhanced bone healing. Bonauer *et al.* systemically delivered anti-miRNA-92a to mouse hind limb ischemia and acute MI models by intravenous injection.[40] Bernardo *et al.* delivered anti-miRNA-34 systemically by subcutaneous injection.[57]

### *Gene delivery vehicle*

Although small in size, the delivery of miRNA into cells *in vitro* or *in vivo* is neither very successful nor efficient without the aid of a gene delivery vector. miRNAs are negatively charged biomolecules due to their phosphate backbone. Thus, miRNA displays coulomb repulsion with

the cell membrane, which is also negatively charged due to the lycoproteins and proteoglycans on its surface.[77] Therefore, positively charged nonviral vectors or efficient viral vectors are needed to transfect or transduce, respectively, miRNA into cells.

Viral. The advantage of viral vectors is its efficient delivery; however, they are also associated with immunogenicity and limitations in insert size.[78] Viral vectors that are often used for gene delivery include lentivirus, retrovirus, adenovirus, adeno-associated virus, and baculovirus. For the miRNAs reviewed in this article, only lentivirus was utilized. Lentivirus is a subclass of retroviruses that can transduce both dividing and nondividing cells.[79] Using lentiviruses, gene expression can be prolonged because the gene is stably integrated into the host DNA. Huang *et al.*[56] and Qu *et al.*[51] utilized a lentivirus containing plasmid DNA for miRNA-126. Wen *et al.* used lentivirus as their gene delivery vector for anti-miRNA-377.[58]

Nonviral. Majority of the gene delivery vectors utilized with the miRNAs discussed in this review were nonviral vectors, which are easy to obtain and used following the manufacturers' protocols and without the need to insert the miRNA of interest into a virus.

The nonviral gene delivery vector that is most commonly utilized to transfect nucleic acids, including miRNAs, into cells is Lipofectamine, a nonviral lipid-based reagent supplied by Invitrogen. It is composed of synthetic cationic lipids mixed with cholesterol and other lipids.[80] The negatively charged miRNAs can form polyplexes with Lipofectamine through electrostatic interactions. The complexes can be transported into cells by endocytosis and the Lipofectamine can protect the miRNA from enzymatic degradation. In the delivery of angiogenesis-inducing miRNAs, both Lipofectamine 2000 (suitable for DNA and siRNA) and Lipofectamine RNAimax (designed specifically for siRNA and miRNA) have been used by many groups. A few groups also utilized other manufactured lipid-based nonviral vectors. Li *et al.* utilized siPORT NeoFx by Invitrogen, which was designed for the delivery of siRNAs, to deliver miRNA-26a.[12] Seo *et al.* utilized siLentFect™ by Biorad, which was also designed for the delivery of siRNA, to deliver miRNA-146a.[81] Gallant-Behm *et al.* used Dharmafect reagent by Horizon, which is a siRNA/miRNA lipid-based vector, to deliver synthetic miR-92a inhibitor (MRG-110),[62] and Bonauer *et al.* used GeneTrans II® transfection agent by MoBiTec GmbH to delivery anti-miRNA-92a.[40]

Wen *et al.* utilized Val–Ala–Pro–Gly (VAPG) peptide-modified trimethyl chitosan-*g*-poly(ethylene glycol) (TMC-*g*-PEG-VAPG) as their nonviral vector to deliver miRNA-145.[73] Chitosan is a cationic charged natural polysaccharide polymer, making it ideal to be used to complex with the negatively charged phosphate backbone of nucleic acids such as miRNA. The chitosan used in the study, TMC, is modified by methylation to improve its water solubility and cationic charge.[82,83] Wen *et al.*[73] saw that TMC-*g*-PEG-VAPG with relatively higher molecular weight of chitosan (50 kDa vs. 5 and 20 kDa) could significantly enhance cellular uptake in SMCs.

Devalliere *et al.* utilized poly(lactic-co-glycolic acid) (PLGA) polymer nanoparticles (NPs) to deliver their miRNA of interest.[45] The NPs were loaded with miR-132 and coated with cyclic RGD (cRGD) peptides. Before incorporating the miRNA into the NPs during the fabrication process, the miRNA was complexed with spermidine, a positively charged polyamine that can interact with the negatively charged miRNA. The NPs were able to prolong the release of the miRNA for several weeks. Compared to only using a polymer or lipid-based vector, such as Lipofectamine, the NPs served as depots, as they are in intracellular vesicular compartments, to slowly release the miRNA into the cytosol. Thus, the particles are able to better protect the miRNA from degradation. Devalliere *et al.*[45] demonstrated that the targeted PLGA NPs were good vehicles to enhance EC transplantation and vascularization because they were a safe and efficient vehicle.

### *Targeting agents*

Systemic delivery of bioactive factors can result in accumulation of drugs in the mononuclear phagocytic system cells in the liver, spleen, and bone marrow,[84] and also increase hematologic toxicities as a result of circulation time.[85] By using targeting agents for cells at the site of interest, biomaterials can be used more effectively and efficiently to deliver bioactive factors such as miRNA and decrease side effects to nontargeted cells.

Wen *et al.* utilized VAPG, a short peptide that mimics an elastin-derived peptide sequence to deliver miRNA-145.[73] This peptide sequence can increase the adhesion to vascular SMCs. Gobin and West suggested that this peptide binds to the β3 integrin subunits of the elastin receptor on SMCs.[86] They also showed that fibroblasts, ECs, and platelets do not adhere to VAPG, making this targeting agent specific for SMCs.

Devalliere *et al.*[45] coated PLGA NPs with cRGD peptides that target integrin αvβ3 expressed on human umbilical vein ECs (HUVECs).[45] This receptor is scarcely detectable in ECs lining quiescent vessels, but is highly expressed in proliferating endothelium. Devalliere *et al.*[45] demonstrated a 2.9-fold increase in uptake of the miRNA-loaded NPs in ECs with RGD peptides.

## Cells

### *Endothelial cells*

New vessels are usually formed by transplantation of either mature ECs or committed endothelial progenitor cells.[45] To make sure this process is successful and can enhance the transplantation of ECs, proangiogenic miRNA can be used to accelerate the rate of vessel formation and maturation.[45] The formation of blood vessels can be initiated *ex vivo* within a scaffold, which can then be implanted at the targeted site. This is followed by vessel maturation and inosculation *in situ.* In a hypoxic environment, angiogenesis occurs and ECs are the primary cells that interface with blood to sense the change in levels of oxygen and respond to a depleted oxygen level environment.[87] Because of the primary role of ECs in angiogenesis, many studies utilize ECs for *in vitro* experiments and implantation after transfection or transduction of the miRNA of interest. Li *et al.* utilized a different source of primary ECs, rat brain microvascular endothelial cells (RBMECs), to investigate the inhibition of miRNA-493.[52] Shu *et al.* utilized EA.hy926 ECs

with miRNA-93,[53] which are a human umbilical vein cell line.[88] EA-hy926 was created by fusing HUVECs with A549 cells, which are adenocarcinomic human alveolar basal epithelial cells.[88] Compared to HUVECs, which are primary cells, this cell line grows rapidly in culture and does not require supplementation of special growth factors, which can be costly.

### *Smooth muscle cells*

Besides ECs, vascular smooth muscle (SMC) cells also play a vital role in the normal stability and formation of blood vessels. For successful new blood vessel formation, the contractile and spindle-shaped phenotype need to be maintained.[89–91] However, in pathological or injury conditions, SMCs may be compromised and lead to hyperplasia and restenosis.[92] Wen *et al.* investigated the transfection of miRNA-145 released from an electrospun scaffold, into SMC.[73] They showed that miRNA-145 modulated the SMC phenotype during vascular regeneration. Besides SMCs and ECs, pericytes are also important cells in blood vessels. They are fibroblast-like cells that are found wrapped around 22–99% of EC surfaces in arterioles, capillaries, and venules. Thus far, to the best of our knowledge, there are no applications of angiogenesis-inducing miRNA or anti-miRNA with pericytes as their target cells.[93]

### *Mesenchymal stem cells*

Although human ECs are widely used to improve the vascularization of skin substitutes, unlike keratinocytes or fibroblasts, they can produce an immune response.[94,95] Autologous ECs can avoid immunological rejection, but this can be difficult, especially for patients who are prone to nonhealing wounds.[61] Thus, other cell sources are needed for new blood vessel formation for vascular tissue engineering.

MSCs are employed in many tissue engineering applications because they are known to differentiate into multiple lineages, including osteoblasts, chondrocytes, adipocytes, tenocytes, and myocytes.[96] MSCs are promising cells for these applications because they can be easily isolated and expanded *in vitro* and have immunologic privilege properties, making them suitable for xenogeneic implantation. Therefore, these characteristics make MSCs an ideal candidate for cell therapy in vessel formation for cardiac and vascular diseases, as well as skin and bone tissue engineering.

However, the differentiation capability of MSCs into the endothelial lineage for vascularization still remains unclear,[97] and there are conflicting findings in the role of MSCs in angiogenesis. MSCs have been shown to be located in the perivascular region of blood vessels, indicating their contribution to new blood vessel formation, but it is unclear if they serve as the progenitor cells that differentiate into ECs to form the blood vessels.[97] Several groups have shown that VEGF stimulation can result in the differentiation of MSCs into ECs. Oswald *et al.* showed that treatment of MSCs over the course of a week upregulated EC surface markers and formed CLS in Matrigel.[98] Similarly, Jazayeri *et al.*[99] and Liu *et al.*[100] observed similar results with MSCs exposed to VEGF with other growth factors like IGF and FGF.

In contrast, there are also groups such as Roobrouck *et al.*[101] and Fan *et al.*[102] that have indicated VEGF treatment did not result in expression of EC surface markers and did not form CLS in Matrigel. Furthermore, Au *et al.* showed that MSCs were not able to differentiate into ECs and could not form conduit for blood flow by themselves, and instead, acted as perivascular precursor cells to support and provide stability to ECs.[103] Thus, there are arguments indicating that MSCs do not differentiate into ECs or serve as vascular progenitor cells, but may function to secrete proangiogenic factor to recruit and induce ECs to form blood vessels instead.

Many studies focus on understanding the role of growth factors in inducing MSCs into the angiogenic lineage for vascularization. In this work, we are interested in understanding the role of MSCs in blood vessel formation with the application of miRNAs that have the ability to induce angiogenesis as the bioactive factor for the differentiation of these cells.

MSCs have been delivered for cardiac repair by many groups, but some studies have shown that there is not much incorporation of these cells into vascular capillaries, suggesting that MSCs play a different role in helping the repair process.[104] In cardiac injury, MSCs have been utilized by implantation of the MSCs for the secretion of soluble factors, such as growth factors and cytokines, which can induce angiogenesis in a paracrine manner.[105–108] Several groups have utilized MSCs in combination with proangiogenic miRNA for cardiac repair. Huang *et al.* demonstrated that the overexpression of miRNA-126 in mouse BMMSCs in a mouse acute MI model promoted angiogenesis by secreting VEGF and bFGF for paracrine signaling.[56] This resulted in the increase of microvessel formation at the ischemic tissue.

Li *et al.* determined that the delivery of miRNA-26a to a mouse bone calvarial defect model increased EC distribution and density of blood vessels, but it was unclear if the overexpression of miRNA-26a in the BMMSCs resulted in the induction of ECs or differentiation of MSCs into ECs.[12] Kinnaird *et al.* demonstrated that MSCs play the role of secreting cytokines such VEGF and bFGF rather than being the cells that are incorporated into new or remodeling blood vessels.[106] The injected, labeled MSCs were not incorporated into the blood vessels, suggesting their role confined to paracrine signaling. Kinnaird *et al.* showed that the MSC secretion of cytokines enhanced proliferation and migration of ECs.[104] Almeida *et al.* demonstrated that miRNA-195 decreased both osteogenesis and the paracrine effect on angiogenesis by downregulating VEGF mRNA and protein expression, and thus, the inhibition of miRNA-195 may be a promising tool for human bone defects.[71] Wen *et al.* showed that inhibition of miRNA-377 resulted in tube formation of human umbilical vein ECs and paracrine MSC-induced angiogenesis[58]

In addition to contributing to paracrine signaling in cardiac repair, MSCs have also been shown to transdifferentiate into cardiomyocytes as well as vascular cells.[109,110] Seo *et al.* demonstrated that overexpression of miRNA-146a increased the secretion of VEGF for paracrine signaling in angiogenesis, evidenced by the increased formation of vascular branches in human BMMSCs in ischemic/reperfusion injury.[81] Xing *et al.* showed that BMMSCs transfected with miRNA-378 had the capacity to form blood vessels and differentiate into the vascular lineage, observed by the larger number of vascular branches.[59] MSCs have demonstrated to be associated with low survival and apoptosis in hypoxic

environment.[111] However, miRNA-378 has been seen to enhance MSC proliferation and decrease apoptosis.[59]

Di Bernardini *et al.* investigated the overexpression of miRNA-21 in iPSC differentiation into ECs by the paracrine secretion of TGF-β2 to neighboring cells.[27] Embryonic stem cells are a promising pluripotent cell source that can differentiate into ECs.[112] However, they have conflicting ethical and immunological issues that make them difficult to employ,[27] and thus, iPSCs, somatic cells that are reprogrammed to an embryonic-like, pluripotent state, may be a more viable replacement cell source for tissue engineering applications.

### *Delivering cells*

For most applications reviewed in this article, the miRNA or anti-miRNA was delivered *in vivo* in the animal model chosen. However, there were some groups that delivered cells transfected with the miRNA or anti-miRNA through a scaffold or local injection. Di Bernardini *et al.* delivered iPSCs transfected with miRNA-21 in a mouse subcutaneous model.[27] Devalliere *et al.* delivered HUVECs transfected with miR-132 embedded in a collagen fibronectin gel to a mouse abdominal wall subcutaneous pocket.[45] Li *et al.* delivered human BMMSCs transfected with miRNA-26a by the implantation in a thiol-modified analog of heparin with thiol-modified hyaluronan and poly(ethylene glycol) diacrylate HP-HA-PEG (Glycosil) hydrogel in a mouse calvarial bone defect.[12] BMMSCs transfected or transduced with miRNAs or anti-miRNAs have also been used *in vivo* for cardiac disease models.[56,58,81] These cells are usually delivered locally or through intramyocardial injection.

## Extracellular Vesicles

Recently, the intercellular transfer of EVs has emerged as an up-and-coming tool in tissue engineering and, specifically, angiogenesis. EVs, which include exosomes, microvesicles, and apoptotic bodies, are heterogeneous plasma membrane vesicles that are secreted from most cells under both normal and stressed conditions.[113,114] Unlike living cells or protein, EVs mediate intercellular communication through the transport of biological information, including nucleic acids, lipids, and proteins, to target recipient cells, triggering downstream signaling events.[114,115] EVs hold advantages to traditional synthetic delivery vehicles due to their high biocompatibility and limited immunogenicity.[116]

The effect of EVs on angiogenesis is dependent on the EV content and surface molecule expression, which can be modulated by the stimulus that initially induces the EV.[117] The functional cargo of EVs that have been shown to stimulate angiogenesis include miRNAs, lipids, and proteins, including transcription factors.[117] EVs, likely composed of both exosomes and microvesicles, are isolated from the supernatants of cultured cells. The intercellular transport of angiogenesis-inducing miRNAs has been studied with EVs collectively or with isolated exosomes and isolated microvesicles, which are summarized in Table 4.

### *Exosomes*

Exosomes are generally a homogenous population with a cup-like shape and size ranging from 30 to 120 nm in diameter.[118] Exosome formation is derived from the endosomal system by intraluminal budding of endosomal compartments that form intraluminal vesicles (ILVs) in intracellular multivesicular bodies, which are mediated by the endosomal sorting complexes required for transport machinery.[119] The ILVs are released as exosomes to the extracellular environment after multivesicular bodies are fused with the plasma membrane.[120]

Recent studies have depicted that exosomes derived from adipose-derived stem cells (ADSC) are key regulators in angiogenesis through the delivery of proangiogenic miRNA. ADSCs have been found to promote angiogenesis and directly differentiate into ECs.[121] Yang *et al.* showed that ADSC exosomes overexpressing miRNA-181b-5p increased migration distance and tube length in RBMECs *in vitro*.[122] In addition, miRNA-199-3p elevated in ADSC exosomes increased proliferation and migration of endothelial tip cells.[123] Liang *et al.* demonstrated that miRNA-125a transferred by ADSC exosomes modulated angiogenesis through promoting tube formation and endothelial tip cell formation.[37]

Exosomes derived from BMMSCs have also shown to promote angiogenesis, mainly when the recipients are ECs. Liu *et al.* used BMMSC exosomal-transferred miR-130a in lithium-incorporated bioglass ceramics to enhance PTEN and AKT expression, factors that stimulate critical steps in angiogenesis, in HUVECs.[26] Ma *et al.* also utilized exosomes from BMMSCs for the delivery of miR-132 to recipient HUVECs to stimulate angiogenesis *in vivo* and *in vitro*.[124] Exosomal delivered miRNA-132 increased tube length and mesh number in HUVECs *in vitro*.[124] In an MI model, mice were subcutaneously injected with HUVECS pretreated with miR-132 exosomes, which resulted in an increased number of blood vessels and capillary density in the infarct area.[124]

Exosomes are widely present in biological fluids such as blood, urine, bronchoalveolar lavage fluid, breast milk, synovial fluid, pleural effusions, and ascites.[125] Exosomes derived from the coronary serum of MI patients, carrying knockdown miRNA-143, have been seen to promote cell proliferation and tube formation in HUVECs.[44]

### *Microvesicles*

Microvesicles are generally more heterogenous and larger in size, ranging from 100 to 1000 nm in diameter, than exosomes and are formed by the outward blebbing of the plasma membrane.[126,127] Microvesicle formation has been seen to occur particularly in the lipid-rich microdomains in the membrane.[128,129] Although less extensively studied in the transfer of miRNAs for angiogenesis, microvesicles have shown to also promote angiogenesis. In Kang *et al.*, the delivery of miRNA-31 through microvesicles released from ADSCs to HUVECs was able to enhance sprouting outgrowth in *ex vivo* mouse aortic rings and induce vascular formation in mice *in vivo*.[32]

### *EVs collectively*

EVs collectively carrying proangiogenic miRNA have also been studied for angiogenesis, although not extensively as isolated exosomes. Wang *et al.* found that miRNA-210 enriched in BMMSC extracellular vehicles was necessary to promote angiogenesis *in vivo* and *in vitro*.[130] Specific knockdown of miRNA-210 in MSC-EV induced capillary-

Table 4. Proangiogenic microRNAs and Anti-microRNAs Derived from Extracellular Vehicles That Have Been Utilized for Angiogenesis

| *miRNA* | *Application* | *Extracellular vehicle* | *Biomaterial vehicle/injection* | *Cell type* | *Outcome* | *References* |
|---|---|---|---|---|---|---|
| Exosomes | | | | | | |
| miRNA-125a | *In vitro* only | Exosome | None | Exosome derived from human-ADSCs<br>Recipient:<br>HUVECs | Promoted tube formation and endothelial tip cell formation | 37 |
| miRNA-130a | *In vitro* only | Exosome | Li-BCG | Exosome derived from rat BMMSCs<br>Recipient:<br>HUVECs | Enhanced expression of proangiogenic genes (PTEN and AKT) and inhibition inhibited tube-like structures and branch nodes | 26 |
| miRNA-132 | Mouse MI model | Exosome | Intramyocardial injection | Exosome derived from mouse BMMSCs<br>Recipient:<br>HUVECs | Increased number of blood vessels and capillary density in infarct area | 124 |
| Anti-miRNA-143 | *In vitro* only | Exosome | None | Exosome derived from the coronary serum of MI patients<br>Recipient:<br>HUVECs | Promoted cell proliferation and tube formation | 44 |
| miRNA-181b-5p | *In vitro* only | Exosome | None | Exosome derived from rat ADSCs<br>Recipient:<br>RBMECs | Increased migration distance and tube length in BMECs. Upregulated protein expression of hypoxia-inducible factor 1a and vascular endothelial cell growth factor | 122 |
| miRNA-199-3p | *In vitro* only | Exosome | None | Exosome derived from human ADSCs<br>Recipient:<br>human peripheral mononuclear cells (endothelial tip cells) | Increased proliferation and migration in endothelial tip cells | 123 |
| Microvesicles | | | | | | |
| miRNA-31 | Mouse subcutaneous model | Microvesicle | Matrigel implantation (local) | Microvesicle derived from human ADSCs<br>Recipient:<br>HUVECs | Induced functional vascular formation | 32 |
| EVs collectively | | | | | | |
| miRNA-21 | *In vitro* only | EV, production stimulated by collagen biomaterial | None | EV derived from CD34+ cells<br>Recipient:<br>HUVECs | Promoted greater capillary-like formation, increase in HUVEC tubule network formation | 131 |
| Anti-miRNA-210 | Mouse peri-infarction model | EV | Matrigel injection (local) | EV derived from mouse BMMSCs<br>Recipient:<br>HUVECs | Increased capillaries in mouse peri-infarct regions | 130 |

Li-BCG, lithium incorporated bioglass ceramics; ADSC, adipose-derived stem cell; EV, extracellular vesicle.

like tube formation and newly formed vessels in recipient HUVECs *in vitro*. Compared to miRNA-210 silenced in MSC-EV, control MSC-EV showed more capillaries in mouse peri-infarct regions *in vivo*. McNeill *et al.* used EVs collectively, which were derived from CD34+ cells to deliver miRNA-21 to HUVECs.[131] The production of these EVs containing miRNA-21 was stimulated by collagen biomaterial. They demonstrated that this resulted in the greater capillary-like formation and increase in HUVEC tubule network formation.

### *Gap junction*

Besides, through EVs, paracrine signaling has been studied in co-cultured cells. Fan *et al.* discovered interestingly, the transfer of miRNA-200b, from osteogenic cells to vasculogenic cells co-cultured together.[132] Rat BMMSCs are able to form cell-cell interaction with HUVECs through gap junctions. The loss of miRNA-200b in the BMMSCs results in the upregulation of VEGF-A, leading to increased osteogenic differentiation. The increased levels of miRNA-200b in HUVECs result in reduced angiogenic potential. The transfer is triggered by TGF-β that is released from HUVECs and bound to receptors on BMMSCs.

## Conclusion and Future Directions

Proangiogenic miRNAs and anti-miRNAs can be utilized as therapeutic molecules for vascular tissue engineering in ischemic diseases, such as in the limbs and the brain (i.e., stroke), cardiac disease, as well as skin and bone tissue engineering. Many studies directly inject miRNAs or anti-miRNAs systemically or locally at the site of interest. However, there are also some studies that apply biomaterials to control the release of these bioactive factors. Although ECs, such as HUVECs, are usually the main cells studied and utilized for these applications, several projects also investigated the use of MSCs. These multipotent cells, when transfected/transduced with proangiogenic miRNAs or anti-miRNAs, can secrete cytokines or growth factors to neighboring cells in paracrine signaling and/or themselves differentiate into vascular cells.

Most applications utilized the delivery of miRNA or anti-miRNA themselves, but some applications relied on the delivery of cells that have been transfected *ex vivo* with miRNA or anti-miRNA, which were then delivered to the site of interest by implantation in a scaffold or local injection. Aside from the use of viral or nonviral vectors to deliver miRNA, some groups have investigated the delivery of miRNAs through EVs, including exosomes, microvesicles, or EVs collectively. As naturally occurring vesicles in the body, EVs are associated with high biocompatibility and limited immunogenicity. The production of the miRNAs by EVs can also be controlled by biomaterials.[131]

VEGF is the central molecule in the control of angiogenesis, and its expression and subsequent binding to the VEGFR receptor utilize several signaling pathways, such as PI3K/AKT, Ras/Raf/MEK/ERK, and p38 MAPK pathways, to transduce its signal for angiogenesis. Several miRNAs target VEGF directly and using an inhibitor of the miRNA (anti-miRNA) can be an angiogenic therapy for vascular tissue engineering. There are several miRNAs that target PTEN, a negative regulator of both the PI3K/AKT and Ras/Raf/MEK/ERK pathways. Thus, the suppression of PTEN expression by miRNA will lead to increased signaling of VEGF through these pathways. Other critical pathways regulated by miRNAs or anti-miRNAs to enhance angiogenesis include the Notch signaling pathway and the pathway of NO production. HIF-1α and FGF are other molecules that are also critical in angiogenesis, and their expression levels can also be controlled by miRNAs or anti-miRNAs.

Although the role of miRNAs and anti-miRNAs to control the production and signal transduction of proangiogenic factors that therapeutically induce angiogenesis for vascular tissue engineering is promising, there are limitations and risks, including the development of hemangioma and reoccurrence of tumors for patients after radiotherapy.[133] Many miRNAs investigated for angiogenesis have been studied for the purposes of antiangiogenic therapies in cancer, and this implies the risk of negative implications in cancer of the utilization of proangiogenic miRNA for vascular tissue engineering.

For example, PTEN is a tumor suppressor that is associated with negative outcomes in cancer. However, this molecule is also targeted by many pro-miRNAs (i.e., miRNA-21, miRNA-26a, and miRNA-130a) and inhibition of this gene may affect its vital role as a tumor suppressor if not executed correctly. Another example is HIP-1, which is expressed in tumor cells and is a marker of relapse in prostate cancer patients in whom the overexpression of HIP-1 can lead to tumor formation.[134,135] This protein can be upregulated by anti-miRNA-135a-3p and has been suggested for therapeutic intervention for angiogenesis.[24] Thus, the accuracy and dose of administration are vital to avoid these side effects and should be a focus for future applications and the utilization of biomaterials can contribute to the local and targeted delivery of these promising therapeutic factors.

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