REVIEW ARTICLE

An integrated hypothesis for miR-126 in vascular disease

Authors

Bo Yu[1], Yinghua Jiang[2], Xiaoying Wang[2], Shusheng Wang[1,3*]

Affiliations

[1]Department of Cell and Molecular Biology, Tulane University, 2000 Percival Stern Hall, 6400 Freret Street, New Orleans, LA, 70118, USA

[2]Department of Neurosurgery

[3]Department of Ophthalmology, Tulane University School of Medicine, 1430 Tulane Avenue, SL-69, New Orleans, LA 70112, USA

Correspondence

Shusheng Wang

Email: swang1@tulane.edu

Abstract

microRNA miR-126 was among the early discovered miRNAs that are expressed specifically in the vasculature and have critical functions in vascular development. Recent studies have started to unveil potentially important function of miR-126 in vascular diseases, including atherosclerosis, coronary artery disease, stroke and diabetic vasculopathy. The action of miR-126 reflects its function in angiogenesis and inflammation. The expression of miR-126 is downregulated in a variety of vascular diseases, and miR-126 overexpression appears to beneficial for most vascular disease models. In the minireview, we summarize the historic and current research regarding miR-126 function and mechanisms in the vascular system, its link to long noncoding RNAs (lncRNA), as well as the potential of miR-126-based therapeutics for vascular diseases. To explain the seemingly conflicting function of miR-126 from different studies, an integrated hypothesis is proposed that miR-126 has strand- and cell type-specific functions in angiogenesis and inflammation, making it beneficial in many different vascular disease models.

Introduction

The vascular system consists of vessels, including arteries, veins and lymphatic vessels, that carry blood and lymph through the body. Any dysfunctions in vascular system could lead to vascular diseases, such as ischemic stroke, atherosclerosis, coronary artery disease and pulmonary arterial hypertension. In fact, almost all diseases, including cancer and retinopathies, have vascular abnormalities. Vascular diseases could pose severe threat to globe health by causing disability or death in affected individuals. Excessive or insufficient angiogenesis has been associated with vascular diseases. Drugs that target tumor or ocular angiogenesis has been used clinically to treat cancer and retinopathies.

MicroRNAs (or miRNAs) are endogenous small noncoding RNAs that regulate gene expression post-transcriptionally, leading to mRNA degradation or translational inhibition. Dozens of miRNAs have been identified to be associated with angiogenesis and/or vascular disease [1,2]. Among them, miR-126 is among the first discovered vascular miRNAs that have important function in angiogenesis and vasculature integrity [3-5]. miR-126 contains two mature miR-126-3p and miR-126-5p strands, resides within intron of *EGFL7* gene and is conserved cross different species from human, mouse to zebrafish. In humans, a long noncoding RNA (lncRNA) lncEGFL7OS is located next to miR-126, transcribed in the opposite strand of miR-126 gene [Fig.1] [6]. The physiological expression of miR-126 is enriched in endothelial cell (EC) and hematopoietic cell lineages [3,7]. Gene knockout studies in mice and knockdown study in zebrafish have established an indispensable role for miR-126 in angiogenesis and vascular integrity. The angiogenic action of miR-126 is mediated by promoting MAP kinase and PI3K signaling in response to VEGF and FGF, through targeting negative regulators of the pathways, including the Sprouty-related protein (Spred)-1 and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2/p85-β) [3-5]. Recent studies also confirmed a critical role of miR-126 in modulating lymphatic vascular development, suggesting that miR-126 is important for both blood vessel and lymphatic vessel development [8,9]. Besides, emerging studies have indicated important functions for miR-126 in vascular diseases, or diseases with vascular contributions [10]. While the majority of the studies suggest a beneficial role for miR-126 in disease models, conflicting results also exist, which will be the main focus for the minireview.

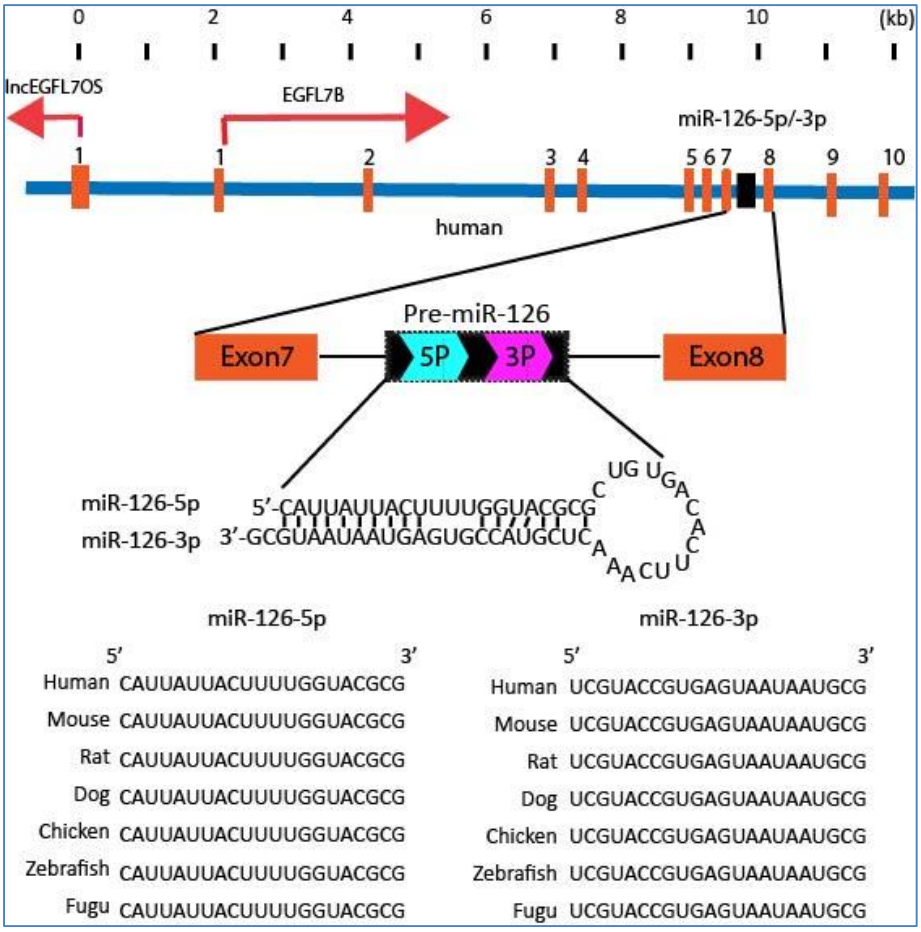

Fig.1: Schematics of miR-126 gene locus in human genome and its evolutionary conservation. miR-126 is localized in the intron 7 of EGFL7 and is near to lncEGFL7OS that is transcribed in the opposite direction of EGFL7/miR-126 gene. miR-126-5p and -3p conservation across multiple species was shown.

Dis-regulation and beneficial role of miR-126 in vascular diseases

In adults, the vasculature is normally quiescent and functions to maintain oxygenized and nutritional blood flow. Maintaining vascular integrity, including the blood-brain barrier (BBB) and blood-retinal barrier (BRB), is of utmost importance. This involves mechanisms opposite to vascular angiogenesis and inhibition of vascular inflammation. However, physiological angiogenesis (such as during menstrual cycle) and pathological angiogenesis (such as in wet age-related macular degeneration, wound healing and tumorigenesis) both occur in adults. It would be preferable to inhibit pathogenic angiogenesis but to maintain physiological angiogenesis or promote reparative (regenerative) angiogenesis. Therefore, the requirement of angiogenesis in adult is context-dependent in adults. For context-dependent example, it would be beneficial to prevent early BBB integrity disruption after ischemic stroke, but to promote late cerebrovascular angiogenesis at later stage [11]. To evaluate miR-126 in

vascular diseases, it would be critical to consider the nature and the stage of the diseases.

miRNAs can be secreted through microparticles (or exosomes) into body fluids and blood circulation and act as disease biomarkers. Circulating miR-126 has been shown to be down-regulated in a variety of vascular or related diseases, including ischemic stroke [12], coronary artery disease (CAD) [13,14], acute myocardium infarction (AMI) [15], chronic kidney disease [16,17], end-stage renal disease [18], heart failure [19] and type 2 diabetes [20]. However, in an independent study, circulating miR-126 level was not significantly changed in CAD patients, but inversely correlated with low-density lipoprotein (LDL) cholesterol level [21]. In another study, miR-126 level in the plasma was not different between diabetic and nondiabetic patients, but its expression in microparticles was significantly lower in diabetic patients [22]. miR-126 expression was also significantly lower in the vitreous body, proliferative membrane tissues and plasma of proliferative diabetic retinopathy (PDR) patients, which is in reverse to the expression of VEGF [23]. EC or endothelial progenitor cell (EPC) dysfunction plays an important role in vascular diseases, including atherosclerosis and diabetic vasculopathies. miR-126 was down-regulated in the EPCs derived from CAD and type II diabetes patients, which may contribute to the plasma miR-126 changes in these diseases [24,25]. Plasma miR-126 level was downregulated in pre-atherosclerosis [26], but increased and correlated with the presence and severity of cerebral atherosclerosis, suggesting a stage-dependent regulation of miR-126 expression in atherosclerosis [27].

Although miR-126 knockout is detrimental for vascular development, an overall beneficial role for miR-126 overexpression has been confirmed in a variety of vascular disease models. With regard to cardiovascular disease, *miR-126*$^{-/-}$ mice exhibited impaired neovascularization after experimental MI [3]. EC-specific *miR-126* knockout (KO) in mice showed decreased cardiac function and increased cardiac hypertrophy, fibrosis and inflammatory gene expression after ischemic stroke, while EC-derived exosome from wild-type, but not KO mice, decreased cardiomyocyte hypertrophy *in vitro* [28]. miR-126 was substantially downregulated in angiogenic early growth cells (EOCs) from chronic heart failure (CHF) patients, and miR-126 mimic transfected EOC cells improved neovascularization and cardiac function after myocardial infarction (MI) *in vivo* [29]. In pulmonary arterial hypertension (PAH) patients, downregulation of miR-126 was associated with right ventricle failure, and intravenous injection of miR-126-5p mimic improved cardiac vascular density and function in monocrotaline-induced PAH rats [30]. EPC-derived exosomes loaded with miR-126 significantly promoted thrombus organization, recanalization and resolution in an animal model of venous thrombosis by targeting protocadherin-7 [31]. In a high-fat diet-fed *apolipoprotein E* (*ApoE*)$^{-/-}$ CAD mouse model, overexpression of miR-126 using agomiR-126 decreased coronary atherosclerotic plaques by targeting S1PR2 and relieving inflammation, while miR-126 knockdown by antagomir-126 had the

opposite effect [32]. In the *ApoE*$^{-/-}$ atherosclerosis model, miR-126-5p, but not miR-126-3p, maintained a proliferative reserve in ECs through suppression of the Notch1 inhibitor delta-like 1 homolog (Dlk1) and thereby preventing atherosclerotic lesion formation [33]. In a relevant research, miR-126-3p expressing endothelial microparticles reduced vascular smooth muscle proliferation and limited neointima formation by targeting LRP6 in a carotid artery injury model [34]. Intravenous delivery of agomiR-126 reduced the release of inflammatory cytokines IL-6, TNF-a and CD68 in *ApoE*$^{-/-}$ mice [35].

With regard to miR-126 in diabetic endothelial cell dysfunction, endothelial microparticle-mediated delivery of miR-126 promoted vascular EC repair via Spred-1 in a endothelium denudation mouse model [22]. Restoration of miR-126 expression in EPCs from diabetes mellitus promoted proliferation, migration and inhibited apoptosis of EPCs through targeting Spred-1 [25]. Down-regulation of miR-126 preceded microangiopathy in a mouse model of type 2 diabetes, and overexpression of miR-126 improved angiogenic potential of high glucose treated EC cells [36].

miR-126 also has important function related to diseases in the brain and/or retina. A genetic variant regulating mi-126 has been associated with sight threatening diabetic retinopathy [37]. *miR-126*$^{-/-}$ mice showed defective postnatal retinal vascular development and remodeling, which is partially rescued by genetic knockout of its target gene Spred-1. Surprisingly, either silencing miR-126-3p by LNA-antimiR or overexpressing miR-126-3p by miRNA mimic repressed choroidal neovascularization (CNV) in laser-injury wet age-related macular degeneration (AMD) model [38,39]. This was attributed to cell type- and strand-specific function of miR-126, which will be discussed in more detail later. miR-126 also regulates BBB or BRB function. miR-126-3p mimic or miR-126 expressing plasmid attenuated hemorrhage-induced BBB or ischemia-induced BRB disruption respectively, which is associated with upregulation of its target VCAM-1 [40,41]. In a rat model of contusion spinal cord injury model, miR-126 promoted angiogenesis and inhibited leukocyte extravasation and inflammation via targeting Spred-1, PI3KR2 and VCAM-1 [42]. In a mouse middle cerebral artery occlusion (MCAO) model, lentivirus-mediated miR-126 overexpression promoted angiogenesis and neurogenesis and reduced brain atrophy through targeting PTPN9 by both miR-126-3p and miR-126-5p, which leads to subsequent activation of ERK and AKT pathway [43]. We speculate that miR-126 is an important endogenous modulator to preserve vascular homeostasis by maintaining vascular barrier integrity and promoting reparative angiogenesis after stroke.

How miR-126 manages to have diverse functions during development and in different vascular diseases models is still a mystery. The underlying mechanisms could be multifold, involving several cell types. (1) miR-126 promote EC angiogenesis, survival and vascular regeneration through Spred-1, PI3KR2 and other target genes [3-5,44]; (2) Overexpression of miR-126 represses the expression of its target VEGF-A, which are usually highly expressed in non-EC cells,

therefore indirectly preventing the activation of quiescent EC cells; (3) miR-126 secreted through microparticles could limit the proliferation and migration of other cell types, such smooth muscle cells; (4) miR-126 could reduce inflammation by targeting VCAM-1 in multiple cell types [45,46]. Downregulation of VCAM-1 by miR-126 could enhance EC barrier function, prevent trans-endothelial leukocyte trafficking and inflammatory recruitment; (5) miR-126-3p and miR-126-5p have different functions when they are overexpressed. However, the distinct function of miR-126-3p and 5p in vascular diseases has not been sufficiently addressed in most studies.

Is miR-126 pro-angiogenic or anti-angiogenic?

Although miR-126 overexpression is beneficial in most vascular disease models, controversy exists on whether miR-126 is pro-angiogenic or anti-angiogenic.

In vivo evidence supports miR-126 is pro-angiogenic, at least during vascular development. miR-126 resides in the intron of *EGFL7* gene. *miR-126*$^{-/-}$ mice display similar vascular abnormalities to the *Egfl7*$^{-/-}$ mice, including edema, defective cranial vessel and retinal vascularization, raising a question as to which molecule is responsible for the observed phenotype [3,47]. Shortly after the report of the *miR-126*$^{-/-}$ mouse phenotype, floxed alleles of *Egfl7* (*Egfl7*$^{\Delta/\Delta}$) and *miR-126* (*miR-126*$^{\Delta/\Delta}$) were generated [5]. *Egfl7*$^{\Delta/\Delta}$ mice, in which *Egfl7* is knocked out miR-126 is not affected, were phenotypically normal. However, *miR-126*$^{\Delta/\Delta}$ mice, in which *Egfl7* is normally expressed but miR-126 is knocked out, recapitulated numerous embryonic and postnatal vascular phenotypes in the previously reported *Egfl7*$^{-/-}$ mice. These results clearly indicate that miR-126 is required for angiogenesis and maintenance of vascular integrity in mice, although it cannot rule out a role for EGFL7 in angiogenesis since the *in vivo* functions of *Egfl7* could be masked by its paralog *Egfl8*. Since *miR-126*$^{-/-}$ mice are partially embryonic lethal, few studies were focused on the KO mouse phenotypes in adult. In general, adult *miR-126*$^{-/-}$ mice exhibited impaired neovascularization and decreased cardiac function, and increased fibrosis and inflammation after MI or ischemic stroke [3,28]. The impaired angiogenesis in *miR-126*$^{-/-}$ mice was also supported by aortic ring assay and *in vivo* Matrigel assays [3]. Regarding miR-126 overexpression studies, *in vivo* mouse models have not been reported. The data from the *in vitro* studies appear to be more complicated, most depending on the mature miRNA strands, cell types and methods used, as detailed below.

Most published research supports a proangiogenic role for miR-126 in ECs (Fig. 2A). Knockdown of miR-126-3p by Morpholino or anti-miR significantly decreased human umbilical vein EC (HUVEC) proliferation, migration and vascular tube formation in Matrigel through targeting Spred-1 and PIK3R2 in the ERK and PI3K/AKT pathways [3-5]. Further study showed that inhibition of miR-126-3p by locked nucleotide acid (LNA)-anti-miR-126-3p, but not LNA-anti-miR-126-5p repressed angiogenesis in a HUVEC-fibroblast coculture angiogenesis model [38]. However, overexpression of miR-126-5p but not -3p by

miRNA mimic enhanced EC proliferation, migration and angiogenesis in the EC-fibroblast coculture model [33,38]. These different responses could be attributed to the different endogenous concentration of miR-126-5p and miR-126-3p, and therefore their sensitivity to additional overexpression/ knockdown manipulation in the cells. A recent study showed that miR-126-5p expressed in retinal ganglion cells promote retinal endothelial survival through regulating SetD5 [48]. Similar miR-126 function was reported in EPCs, with miR-126 mimic promoting EPC proliferation, migration and differentiation, while miR-126 inhibition having the opposite function [49]. In addition, inhibition of miR-126-3p by antagomir increased HUVEC apoptosis through targeting PIK3R2, which as rescued by miR-126-3p agomiR [44]. However, there was also a study showing that overexpression of either miR-126-3p or miR-126-5p using lentivirus promoted HUVEC proliferation, migration and tube formation in the Matrigel, while inhibition of miR-126-3p or -5p using miRNA sponge in lentivirus has opposite function [43].

Some studies suggest an anti-angiogenic role for miR-126 through studies mostly in cancer cells (Fig. 2B). A recent study found that overexpression of miR-126-3p in human microvascular ECs repressed cell migration and tube formation in the Matrigel, consistent with downregulation of VEGF, VEGFR2 and Spred-1 mRNA [39]. Whether miR-126 has distinct functions in micro-vessels needs further verifications. miR-126-3p overexpression by miRNA mimic also repressed CNV *in vivo* [38,39]. This could be attributed to the regulation of its target gene VEGF in non-EC cells, including retinal pigment epithelial (RPE) cells [22]. Of note, an inverse relationship between miR-126 and VEGF has been observed in EC, tumor and RPE cells [38,50]. Indeed, miR-126 expression is suppressed in most of the cancer models studied, and has been proved to be anti-angiogenic during tumorigenesis, likely by inhibiting cancer cell proliferation, as well as controlling EC recruiting to metastatic cancer cells through targeting PIK3R2 and proangiogenic genes including VEGF, Insulin Growth Factor Binding Protein 2 (IGFBP2) protein and c-Mer tyrosine kinase (MERTK) [51,52].

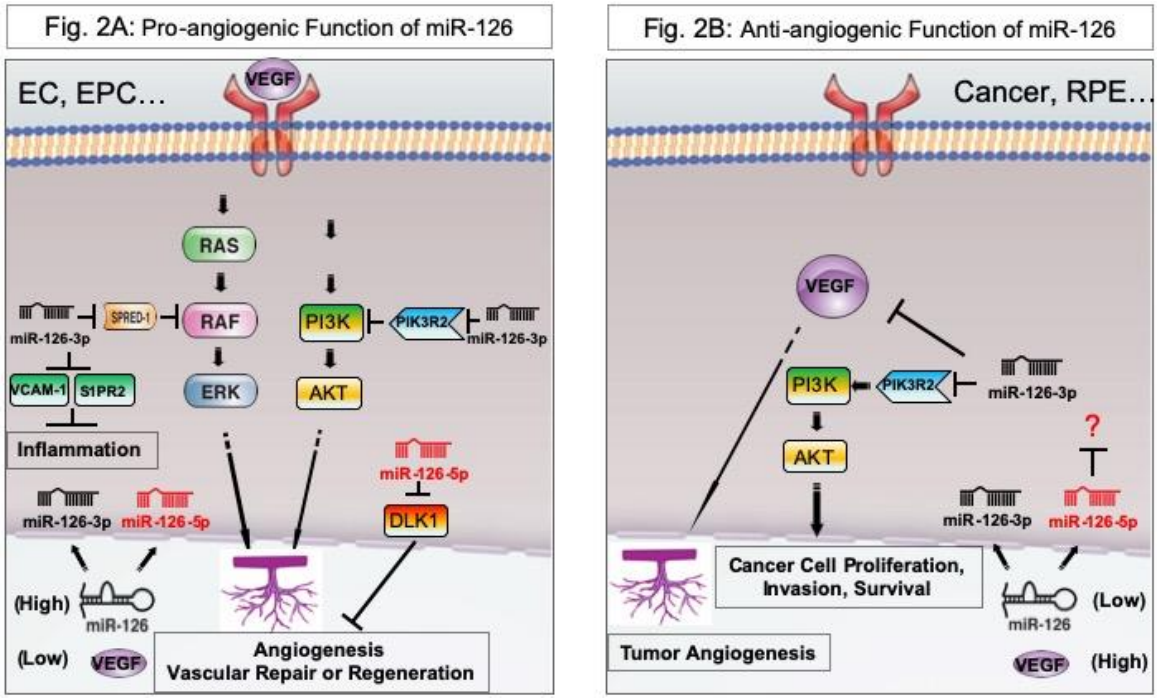

Fig.2: Mechanisms of miR-126 pro- and anti-angiogenic function in different cell-types. A: In ECs or EPCs, where miR-126 expression is high but VEGF expression is low, miR-126-3p promotes angiogenesis, vascular repair and regeneration through targeting Spred-1, PIK3R2 in the ERK and AKT pathways respectively. miR-126-5p enhances angiogenesis through targeting DLK1. miR-126 also represses inflammation by targeting VCAM-1 and S1PR2, therefore helping maintaining vascular integrity; **B**: In cancer or RPE cells, where miR-126 expression is low and VEGF expression is high, miR-126 overexpression inhibits tumor angiogenesis through targeting VEGF, and inhibits cancer cell proliferation, invasion and survival by targeting PIK3Rs in the PI3K-AKT pathway.

Linking Long non-coding RNAs (lncRNAs) to miR-126

Emerging studies have linked lncRNAs to miR-126. LncEGFL7OS has been recently shown to be transcribed at the EGFL7/miR-126 locus in human [6]. It regulates angiogenesis by controlling miR-126 and EGFL7 expression through interacting with MAX transcription factor. Inverse relationship has been found between LncRNA HOTAIR and miR-126-5p in Parkinson's disease (PD) cells and in PD mice [53]. HOTAIR silencing increased PD cell proliferation and survival by sponging miR-126-5p. Through similar mechanism, decreased HOTAIR expression led to miR-126 upregulation and enhanced angiogenesis during burn wound healing [54]. LncRNA PVT1-5 promoted cancer cell proliferation in lung cancers through miR-126/SLC7A5 pathway by sponging miR-126 [55].

An integrated hypothesis on miR-126 in vascular disease

The current consensus is that miR-126 is downregulated in a variety of vascular diseases and cancers, and overexpression of miR-126 (-3p and/or -5p) is beneficial for most vascular diseases, including coronary artery disease, atherosclerosis, diabetic vasculopathies, wet AMD, as well as tumorigenesis. The beneficial effects likely result from diverse mechanisms of miR-126 function. The expression profile of miR-126

and its multiple target genes is likely different depending on cell or tissue types, which could dictate its function under different conditions. Our integrated hypothesis is that miR-126 has strand- and cell type-specific function, making it both pro-angiogenic and anti-angiogenic depending on scenarios **(Fig. 2). (1) (Fig. 2A)** In EC or EPC cells, where miR-126 expression level is high, miR-126 is required for maintaining angiogenesis and vascular integrity. Specifically, miR-126-3p enhances angiogenesis and vascular repair or regeneration by targeting Spred-1 and PIK3R2 in the ERK and AKT pathways respectively. miR-126-5p promotes angiogenesis by targeting DLK1. In addition, miR-126-3p also suppresses inflammation by targeting VCAM-1 and s1PR2. Together, these could explain the beneficial effect of miR-126 in vascular diseases where miR-126 is downregulated. **(2) (Fig. 2B)** In cancer cell or RPE cells, where miR-126 expression level is low and VEGF level is high, overexpression of miR-126 could impact potentially different set of targets. miR-126-3p can target VEGF to repress tumor angiogenesis. Paradoxically, it represses PI3K-AKT signaling by targeting PIK3R2, therefore repressing cancer cell proliferation, invasion and survival. The specific function of miR-126-5p in cancer cells is currently unclear.

Future Perspectives

Current research established miR-126 as a potential biomarker and therapeutic target for vascular diseases and cancer. Future studies should further dissect the strand- and cell type-specific function of miR-126 in different disease-related conditions. For therapeutic development, miR-126-3p and/or -5p mimics, miR-126 expressing viruses, or miR-126 containing exosomes [56], could be tested in different disease models for efficacy and safety. Besides, lncRNAs could be used to modulate miR-126 expression and function in disease settings. Despite existing challenges, a measured optimism exists for miR-126-based therapy in vascular diseases.

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