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Intracellular Delivery Strategies for MicroRNAs and Potential Therapies for Human Cardiovascular Diseases

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Abstract

MicroRNAs (miRNAs) effectively regulate gene expression in cultured cells and human disease models, and such regulation can be blocked with antibodies against miRNAs if miRNA-associated adverse effects occur. Promising findings using miRNAs to prevent disease progression in animal studies give hope to patients with disorders caused by dysregulated gene expression, such as cardiovascular diseases. Inflammatory cell infiltration, endothelial cell dysfunction, and angiogenesis are common pathologies of cardiovascular diseases. Accumulating data suggest that miRNA-mediated inhibition of gene expression can drive these pathologies in cardiac tissue or vasculature. It is often desirable to deliver exogenously prepared miRNAs or antibodies against miRNAs to target genes or miRNAs in specific cell or tissue types. Because naked miRNAs or antibodies against miRNAs are often unstable in the circulation, investigation has focused on their packaging and efficient delivery to diseased organs.

MicroRNAs (miRNAs) are noncoding single-strand RNAs of ~22 nucleotides in length that, together with short interfering RNAs (siRNAs), are key components of the RNA interference pathway. miRNAs are often transcribed from introns of protein-coding transcripts or from introns and exons of noncoding RNAs. By binding to the 3'-untranslated region (3' UTR) of the target mRNA, miRNAs inhibit expression of the target gene, and their distribution is often tissue- and cell-specific (1–4). In the cardiovascular system, for example, some miRNAs are abundant in cardiac muscle (3), arterial smooth muscle cells (SMCs) (4), or endothelial cells (ECs) (1,2). It is common for one miRNA to target multiple genes or for one gene to be regulated by multiple miRNAs, because of imperfect base pair matches between miRNAs and target sequences (5). Therefore, deletion (knockdown) of unintended target genes and the associated side effects are cause for concern.

Many of the 800 currently known miRNAs are associated with various cardiovascular diseases. miRNA-mediated gene transcription regulation can be beneficial or detrimental to the cardiovascular system. For example, the cardiac muscle-specific miR-1 and miR-133 prevent hypertrophy, and overexpression of miR-133 in cultured cardiomyocytes inhibits hypertrophy (6). Human atherosclerotic lesions show low abundance of miR-10a. Knockdown of miR-10a in human aortic ECs increases the abundance of inflammation- or migration-promoting factors, such as nuclear-localized p65 [a nuclear factor- κ B subunit], cytokines, chemokines, and adhesion molecules. In contrast, miR-10a overexpression reduces the basal abundance of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (7), molecules that mediate inflammatory cell adhesion and initiate atherogenesis. Balloon-catheter angioplasty, a technique used to dilate arterial blockage that is often followed by

arterial wall restenosis, results in the reduced abundance of miR-143 and miR-145 in rats (4,8). Introduction of miR-145 in injured rat carotid arteries inhibits neointimal lesion formation (9). miR-155, which is present in ECs and vascular SMCs, targets the mRNA encoding the angiotensin II, type 1, receptor (AT1R), which reduces AT1R abundance and, consequently, impairs angiotensin II signaling and associated increases in blood pressure (10). In contrast, other miRNAs play detrimental roles in cardiovascular diseases. For example, miR-23a, miR-23b, miR-24, miR-195, and miR-214 promote hypertrophy, and forced or transgenic expression of these miRNAs induces hypertrophy in cultured cardiomyocytes and pathological cardiac growth and heart failure in vivo (11). miR-21, which is present in SMCs (2), ECs (12), cardiomyocytes (13), and cardiac fibroblasts (14), shows increased abundance in men with cardiovascular diseases, such as cardiac hypertrophy (15). Cardiac stress leads to increased abundance of miR-21, enhancing signaling through the ERK (extracellular signal-regulated kinase)–MAPK (mitogen-activated protein kinase) pathway and resulting in fibroblast proliferation and fibrosis. Decreased miR-21 abundance reduces cardiomyocyte size and heart weight, whereas miR-21 silencing prevents cardiac hypertrophy and reverses cardiac remodeling in response to stress (16). miR-122 plays a role in cholesterol metabolism, and its silencing decreases expression of genes involved in cholesterol biosynthesis and triglyceride metabolism; increases hepatic fatty acid oxidation; and reduces plasma cholesterol concentrations, hepatic fatty acid synthesis, and cholesterol synthesis (17–19).

miRNA interference can involve direct binding to other miRNAs or blocking of or competing with the binding sites located at the 3'UTR of target transcript (18). Chemical modification of miRNAs increases the efficiency of miRNA interference by enhancing miRNA uptake by cells. One such chemical modification uses oligonucleotide 2'-O-methyl-group (OMe), which generates single-strand RNA analogs, also called antagomirs, that bind to complementary miRNAs (18). Intravenous injection of cholesterol-conjugated antagomirs against miR-122 decreases the abundance of endogenous miR-122 (20). A second chemical modification uses oligonucleotide 2'-O-methoxyethyl (MOE), which generates antagomirs with higher affinity and specificity to RNAs than their OMe analogs. miR-122 has been successfully silenced by intraperitoneal injection of 2'-MOE phosphorothioate-modified antisense oligonucleotides, which results in increased expression of miR-122 target genes (19). In diet-induced obese mice, blocking miR-122 reduces serum cholesterol by 35%. A third chemical modification entails the use of oligonucleotides with locked nucleic acids (LNA-antimiR). Intravenous administration of LNA-antimiR to nonhuman primates depletes miR-122 in liver, which results in a dose-dependent lowering of plasma cholesterol concentrations (17). Besides chemical modifications, there are also several miRNA delivery strategies. miRNAs may be packaged with liposomes decorated with targeting antibodies or integrin affinity peptides that recognize targeting cell surface receptors (21,22). miRNAs may also be delivered by using adeno-associated virus or lentivirus (23,24). Finally, transgenic animals overexpressing miRNAs have been generated (25).

Proangiogenic miR-132 induces neovascularization by blocking an inhibitor of the oncogenic protein Ras (p120RasGAP) in ECs (22). To target miR-132, anti-miRNA antagomirs have been mixed with liposomes bearing $\alpha_v\beta_3$ integrin-targeting cyclic RGD (Arg-Gly-Asp) peptides, which deliver antibodies against miR-132 into the endothelium in vivo. Targeting miR-132 in this manner increases Ras abundance in the endothelium, reduces tumor angiogenesis, and lowers the tumor burden in a mouse model of human breast carcinoma (22).

miR-33 is in intron 16 of sterol-regulatory element-binding factor-2 (SREBF-2), a transcription factor regulating the genes encoding ATP-binding cassette (ABC) transporters ABCA1 and ABCG1, which are cholesterol transporters (26). In human and mouse

macrophages and hepatocytes, miR-33 decreases the abundance of ABCA1, which attenuates cholesterol efflux to apolipoprotein A1. In mouse macrophages, miR-33 targets ABCG1 and reduces cholesterol efflux to high-density lipoprotein (HDL). Both ABCA1 and ABCG1 exhibit antiatherosclerotic effects by stimulating cholesterol efflux from cholesterol-laden macrophages (27). In mice fed a normal diet, a high-fat diet, or a rosuvastatin-supplemented normal diet, the hepatic abundance of miR-33 correlated positively with that of SREBF-2 and inversely with that of ABCA1. The abundance of miR-33 was reduced in livers and peritoneal macrophages of *Ldlr*^{-/-} mice on a high-fat diet. In *Apoe*^{-/-} mice, the abundance of miR-33 in macrophages correlated inversely with cellular cholesterol concentrations and ABCA1 abundance. Lentiviral delivery of miR-33 to mouse peritoneal macrophages and hepatocytes reduced the abundance of ABCA1 and ABCG1, whereas transfection of an antibody against miR-33 increased abundance of ABCA1 and ABCG1 in macrophages and that of ABCA1 in hepatocytes and amplified cholesterol efflux (24). Lentiviral delivery of miR-33 to mice repressed ABCA1 abundance in the liver and reduced blood HDL concentrations, whereas mice expressing antibodies against miR-33 demonstrated increased ABCA1 and plasma HDL.

miR-126 is an endothelial cell-specific miRNA (2); its proangiogenic effects are partially mediated by targeting inhibitors of MAPK and phosphoinositide 3-kinase signaling in response to angiogenic factors, which promotes the activity of these signaling pathways (2,28,29). At the cellular level, miR-126 knockdown causes defects in EC proliferation, migration, tubule formation, and sprouting (2,28,29), which suggests that miR-126 may participate in the pathogenesis or progression of cardiovascular diseases. After myocardial infarction, neovascularization is essential to cardiac tissue regeneration, and miR-126-deficient mice display diminished angiogenesis and increased mortality after coronary ligation, a surgical procedure that induces myocardial infarction (29). Zernecke *et al.* (30) showed that apoptotic bodies from ECs, which are typically engulfed by phagocytes, contained mainly miR-126 as well as other minor miRNAs (Fig. 1A). Incubation of these apoptotic bodies with human umbilical vein ECs (HUVECs) resulted in transfer of miR-126 into recipient cells and production of the anti-inflammatory chemokine CXCL12 by HUVECs. Indeed, low circulating CXCL12 concentrations are associated with unstable coronary artery disease (31), but increased concentrations of circulating apoptotic bodies correlate with impaired EC function in coronary artery disease (32). These apoptotic bodies induce endothelial progenitor cell proliferation and differentiation in vitro (33). Bioinformatics analysis and overexpression of miR-126 in HUVECs revealed that miR-126 targeted the mRNAs encoding CXCL12, VCAM-1, sprouty-related protein 1, and the regulator G protein–signaling protein RGS16. RGS16 is a negative regulator of the CXCL12 receptor CXCR4 (34). In HUVECs, transfection of miR-126 or EC-derived apoptotic bodies increases CXCL12 abundance through inhibition of *RGS16* expression and concomitant enhancement of CXCR4-mediated signals. Although miR-126 reduces VCAM-1 abundance in ECs, which limits inflammatory cell infiltration (1), miR-126-induced increases in CXCL12 abundance in ECs enhanced CXCR4-mediated recruitment of progenitor cells, which contributes to vascular wall repair after injury (35). Thus, EC-derived apoptotic bodies could be used as efficient miRNA carriers to transfer miRNAs to target cells or tissues (Fig. 1A). A role of miR-126 and the efficacy of apoptotic body miRNA transfer were demonstrated in the *Apoe*^{-/-} mouse model of atherosclerosis. In *Apoe*^{-/-} mice fed a high-fat diet, intravenous injection of apoptotic bodies prepared from HUVECs increased the numbers of endothelial progenitor cells in the circulation and incorporation of endothelial progenitor cells into aortic root plaques in a CXCR4-dependent manner, which reduced the size, as well as the number, of macrophages and apoptosis in atherosclerotic lesions. In a collar placement-initiated mouse carotid artery neointima formation model (36), transduction of miR-126, either embedded in Pluronic gel or in apoptotic bodies, led to increased CXCL12 abundance in carotid walls and intimal SMCs and increased collagen

content, and to reduced macrophage content in lesions, apoptosis, RSG16 abundance, and neointimal formation. Consistently, when *Apoe*^{-/-} mice fed a high-fat diet were treated with miR-126-containing EC apoptotic bodies from patients with atherosclerosis (37), lesion size and macrophage content were reduced in a CXCR4-dependent manner (38). These observations confirmed that miR-126 confers protection against atherosclerosis, and that EC-derived apoptotic bodies may serve as carriers to deliver miR-126 to humans with atherosclerosis or other vascular diseases that are associated with EC dysfunction.

The findings of Zhang *et al.* (39) highlight the importance of miRNAs and miRNA delivery in human diseases. They found that miRNA abundance in human plasma changed during the progression of certain diseases, such as cancer or diabetes (40), which is consistent with other findings (41). To extend prior observational studies, this group demonstrated several properties and potential applications of miRNAs. First, the investigators showed that serum miRNAs were packaged into double-layered microvesicles, which are often shed from almost all cell types under normal and pathologic conditions (42) and interact selectively with target cells through receptors from the original cells (Fig. 1B). Second, microvesicles from human plasma and the human macrophage cell line THP-1 contained mainly miR-150, which targets the mRNA encoding c-Myb, a transcription factor that affects cell lineage commitment, proliferation, differentiation, and migration (43). Human blood cells or THP-1 cells also release such microvesicles into the culture media. miR-150 content in these microvesicles was increased after cells were stimulated with endotoxin, oleic acid, or palmitic acid, advanced glycation end products, or H₂O₂. Third, microvesicles transferred miR-150 to human microvessel ECs (HMECs) or other recipient cells in a temperature-, time-, and dose-dependent manner. Furthermore, one can prepare microvesicles from cells transfected with miR-150 to transfer miR-150 to other cells. In live animals, intravenous injection of THP-1–derived microvesicles—which are uniformly sized, exosome-like vesicles (42,44), unlike those from the plasma—can deliver miRNAs directly to the endothelium under physiological conditions. Fourth, microvesicles in plasma from patients with atherosclerosis contained increased miR-150. HMEC-1 cells treated with these human microvesicles also demonstrated miR-150 transfer, which reduced c-Myb abundance and increased migration and suggested that miRNA transfer by microvesicle can be universal (Fig. 1B). Consideration of these findings may be valuable with regard to generating large quantities of microvesicles containing miR-150, or any other miRNAs or antibodies against miRNAs of interest, and effectively delivering them to the affected endothelium in a convenient and cost-effective way. THP-1 cells may be a way to produce microvesicles containing a specific miRNA or antibodies against miRNA to target gene regulation in animals and in humans with cardiovascular diseases.

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Fig. 1.

Apoptotic bodies and microvesicles mediate miRNA transfer. **(A)** EC-derived apoptotic bodies contain mainly miR-126 and other minor miRNAs as indicated. These apoptotic bodies deliver miR-126 to ECs and SMCs to increase the abundance of the anti-inflammatory chemokine CXCL12 and the recruitment of endothelial and smooth muscle progenitor cells. **(B)** Microvesicles from THP-1 cells, macrophages, and plasma cells contain mainly miR-150 but also other minor miRNAs. These microvesicles use receptors from the original cells to target endothelial cells and to deliver miR-150, which reduces c-Myb abundance in endothelial cells and increases cell migration.