RNA Therapeutics in Cardiovascular Disease

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Abstract: **Noncoding RNAs have been shown to exert important physiological and pathophysiological functions. Various studies suggest that modulating noncoding RNAs may provide a therapeutic option. Noncoding RNAs comprise small RNAs, mainly microRNAs, and long noncoding RNAs. MicroRNAs postranscriptionally regulate gene expression pattern by binding to the 3′untranslated region of a given target mRNA, thereby blocking protein translation or inducing its degradation. Long noncoding RNAs on the contrary have more diverse functions acting as epigenetic regulators, molecular scaffolds, or decoys. In this article, we summarize examples of microRNAs and long noncoding RNAs, which might be promising novel targets for treatment of cardiovascular diseases, such as heart failure, acute myocardial infarction, fibrosis, as well as atherosclerosis. Furthermore, we give insights into the available tools to inhibit or overexpress noncoding RNAs and discuss the challenges for translation. Strategies for improving RNA therapeutics and reducing toxicity, for example, by augmenting tissue specificity or cellular uptake will be discussed. (*Circ Res*. 2018;123:205-220. DOI: 10.1161/CIRCRESAHA.117.311311.)**

Key Words: heart failure ■ microRNAs ■ myocardial infarction ■ organ specificity ■ RNA, long noncoding

Although the main part of the genome is transcribed, only a limited amount of ≈2% are protein-coding exons. The remaining part of noncoding sequences comprise small and long noncoding RNAs (lncRNAs). Among others, small noncoding RNAs include mainly microRNAs (miRNAs). miRNAs are mostly 21 to 23 nucleotides in length and play important roles in many different cellular functions, like cell differentiation, proliferation, and survival.[1] By now, 1881 precursors and 2588 mature miRNAs are annotated in humans based on the latest release of miRBase. They function by binding to complementary target mRNAs in their 3′-untranslated region and induce mRNA translational inhibition or degradation. miRNAs are transcribed as long primary transcripts, named pri-miRNAs. Pri-miRNAs are processed in the nucleus by the drosha-DGCR8 DGCR (DiGeorge syndrome chromosome region) complex, leading to a 70-nucleotide-long premiRNA structure. This hairpin structure is then further processed in the cytoplasm leading to the ≈22-nucleotide long mature miRNA.[2] Because 1 individual miRNA modulates the expression level of several mRNAs and thereby specifically affects diverse biological processes in different tissues, it is not surprising that dysregulation of miRNAs has been discovered in many disease states like cardiovascular or metabolic disorders.[3,4]Thus, they became a promising target for the development of therapeutics. To date, there are 2 approaches to modulate miRNA function. One is to overexpress a specific miRNA by using miRNA mimics or viral vectors and the other one is to inhibit a certain miRNA preferentially by utilization of specific antisense oligonucleotides (ASOs) or by genetic knockout mouse models.

In contrast to small noncoding RNAs, lncRNAs comprise a more heterogeneous class of RNAs and are >200 nucleotides in length. To date, ≈60 000 lncRNAs are identified in the human genome.[5] lncRNAs can be transcribed from intergenic regions (long intervening noncoding RNAs), from intronic regions of protein-coding genes (intronic RNAs), or from the antisense strand of a specific gene.[6] Finally, back splicing of exons, thereby forming circular RNAs, can also generate lncRNAs.[7] The molecular mode of action of lncRNAs is diverse. They can have epigenetic regulatory activities thereby influencing transcription of target genes, they can function as molecular scaffolds to be part of protein complexes or act as decoys for specific target molecules to downregulate their function.[6,8] Furthermore, lncRNAs can interact with DNA, RNA, or even miRNAs influencing transcription and post-transcriptional gene regulation by modulating splicing or by sponging miRNAs. Such as miRNAs, many lncRNAs are dysregulated in different disease conditions.[6] Whether lncRNAs can be therapeutically targeted in a comparable manner as miRNAs needs to be investigated. To date, Gapmers or small interfering RNAs (siRNAs) were shown to be useful tools to induce lncRNA degradation thereby inhibiting their actions.

In this review, we discuss promises and challenges in using noncoding RNAs as therapeutic targets for the treatment of cardiovascular diseases. We provide an overview of important miRNAs and lncRNAs reported to have significant impact on the regulation of the cardiovascular system and in disease conditions. Furthermore, we will discuss the toxicological obstacles of systemic interference with noncoding RNAs and the current options to avoid unwanted side effects by modifying the delivery options of RNA therapeutics.

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Nonstandard Abbreviations and Acronyms

AAV	adeno-associated virus
ALT	alanine aminotransferase
AMI	acute myocardial infarction
ANRIL	antisense noncoding RNA in the INK4 locus
APF	autophagy-promoting factor
ApoE	apolipoprotein E
ASGPR	asialoglycoprotein receptor
ASO	antisense oligonucleotide
AST	aspartate transaminase
CARL	cardiac apoptosis-related lncRNA
Cebpb	CCAAT/enhancer-binding protein-β
Chast	cardiac hypertrophy-associated transcript
CHRF	cardiac hypertrophy-related factor
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9
DARPin	designed ankyrin repeat protein
Dlk1	δ-like 1
eGFP	enhanced green fluorescence protein
HDL	high-density lipoprotein
LDL	low-density lipoprotein
LNA	locked nucleic acid
lncRNA	long noncoding RNA
Malat-1	metastasis-associated lung adenocarcinoma transcript 1
MDRL	mitochondrial dynamic related lncRNA
MHC	myosin heavy chain
Mhrt	myosin heavy chain-associated RNA transcripts
MIAT	myocardial infarction-associated transcript
miRNA	microRNA
NF-κB	nuclear factor-κB
NRF2	nuclear factor (erythroid-derived 2)-like 2
PCSK9	proprotein convertase subtilisin/kexin type 9
Pdk4	pyruvate dehydrogenase lipoamide kinase isozyme 4
PHB2	prohibitin 2
scFvs	single-chain variable fragments
SENCR	smooth muscle and endothelial cell-enriched migration/differentiation-associated long noncoding RNA
SGK1	serum and glucocorticoid-regulated kinase 1
siRNA	small interfering RNA
Spry1	sprouty homologue 1
SREBF	sterol regulatory element-binding transcription factor
TAC	transverse aortic constriction
TGF-β1	transforming growth factor-β1
TRAF6	tumor necrosis factor receptor–associated factor 6

miRNA Therapeutics

Examples for Important miRNAs in the Cardiovascular System

Various miRNAs have been described to interfere or contribute to the development or progression of cardiovascular diseases, like acute myocardial infarction (AMI), fibrosis, heart failure, and atherosclerosis (Figure 1). The following chapter will provide examples of miRNAs regulating cardiovascular diseases, which might be interesting and promising therapeutic targets. More detailed information on miRNA functions is given in the recent overviews.[3,4,9–11]

Acute Myocardial Infarction

miR-15, miR-24, miR-34, miR-92a, miR-199a, miR-320, and miR-590 are examples of miRNAs regulating cardiac remodeling on myocardial infarction. The miR-15 family is regulated in the infarcted region of the heart in response to ischemia/reperfusion injury in mice and pigs and regulates hypoxia-induced cardiomyocyte cell death.[12] Inhibition of miR-15 family members promotes postnatal cardiac regeneration.[13] Mechanistically, the pyruvate dehydrogenase lipoamide kinase isozyme 4, which is a key regulator of mitochondrial function and is decreased during hypertrophic remodeling, and checkpoint kinase 1 have been identified as miR-15 family member targets.[12,13] MiR-34 family members are not only induced by ischemia but also by aging and trigger cardiomyocyte cell death, impair DNA damage control, and promote telomere erosion, thus acting as a critical regulator of cardiac repair and regeneration.[14–16] With regard to the mechanism, the aging-related induction of cardiomyocyte cell death is directly associated with the miR-34a regulated repression of the target gene *PNUTS* (protein phosphatase 1 nuclear-targeting subunit). PNUTS, on the contrary, regulates telomere shortening, DNA damage response, and cardiomyocyte apoptosis resulting in an improvement of functional recovery after AMI in mice.[14] MiR-92a is upregulated in ischemic tissue as well but exhibits anti-angiogenic properties by repressing its target genes *integrin α5* and the *deacetylase sirtuin 1* and thereby inhibits ischemia-induced angiogenesis.[17–19] In addition to miR-92a, miR-24 does also target sirtuin 1 and was shown to act as a critical regulator of endothelial cell apoptosis and angiogenesis after AMI.[20]

Fibrosis

Several miRNAs are implicated in cardiac fibrosis either by reducing the extent of injury, for example, by targeting cardiomyocyte death or angiogenesis after myocardial infarction, or by directly interfering with the fibrotic response itself.[21] Among the latter, miR-29 and miR-21 are the most prominent examples. MiR-29 overexpression reduces fibrosis by targeting several matrix proteins.[22] MiR-21 is highly expressed in cardiac fibroblasts on AMI or transverse aortic constriction (TAC) and augments the profibrotic ERK-MAP kinase signaling pathway, for example, by inhibiting Spry1 (sprouty homologue 1).[23] This mechanism regulates fibroblast survival and growth factor secretion apparently controlling the extent of interstitial fibrosis and cardiac hypertrophy.[23] Although these effects were first not recapitulated in miR-21–/– mice,[24] further studies confirmed that genetic and pharmacological inhibition of miR-21 reduced cardiac fibrosis in other models.[25–28]

Hypertrophy and Heart Failure

Prominent examples of miRNAs described in heart failure include the myomiRs miR-1, miR-133, miR-208, and miR-499, as well as miR-25 and miR-212/132. Improving the contractility of cardiomyocytes by modifying intracellular calcium level might be an effective therapy against heart failure. In line, inhibition of miR-25 restored cardiac function by improving

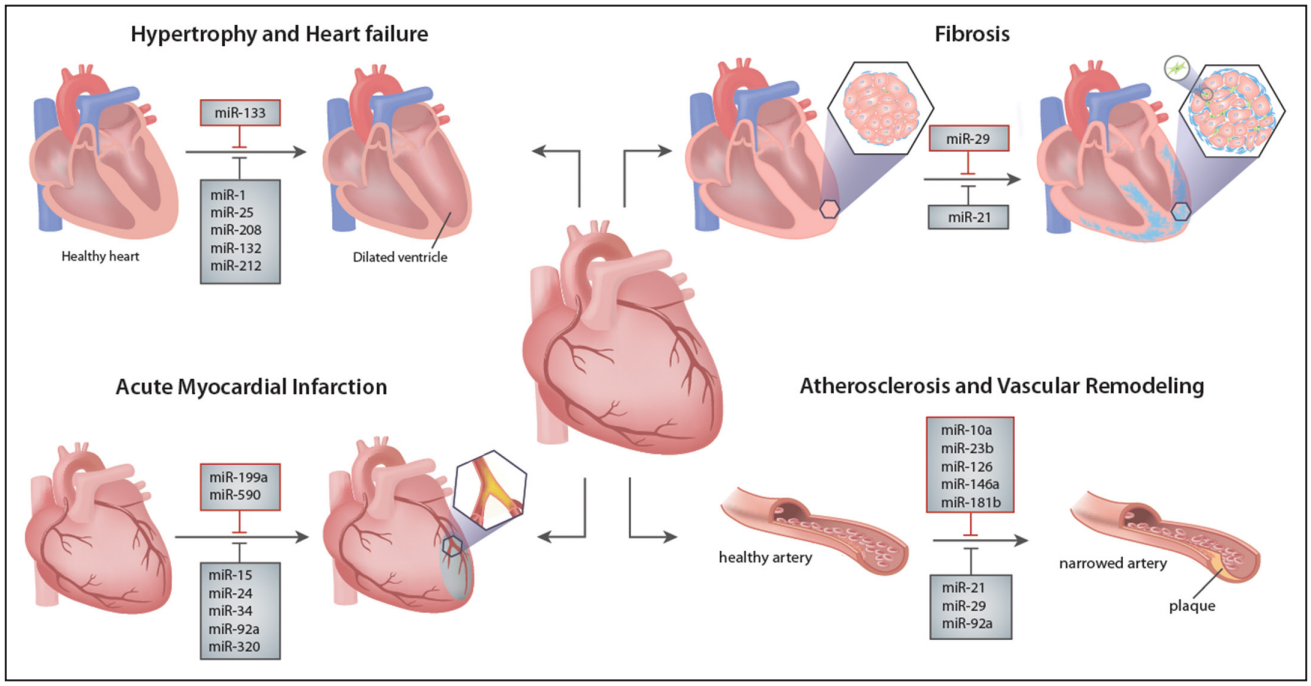

Figure 1. microRNA (miRNAs) function in the cardiovascular system. Summary of miRNAs that might be considered as therapeutic targets for the treatment of cardiovascular diseases. Red boxes represent a beneficial effect by miRNA overexpression; black boxes, a beneficial effect by miRNA inhibition. Schematic representation of (**A**) hypertrophy and heart failure, (**B**) fibrosis, (**C**) acute myocardial infarction, and (**D**) atherosclerosis and vascular remodeling. Magnification in **B** represents an increased infiltration of fibroblasts (green cells) and accumulation of extracellular matrix (blue fibers). Atherosclerosis and vascular remodeling is represented by an occluded cardiac artery (in yellow; **D**).

calcium handling by derepressing the calcium uptake pump SERCA2a (sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a).[29] However, in contrast in another study, inhibition of miR-25 by antagomiRs sensitized the murine myocardium to heart failure, mediated by the reexpression of the embryonic transcription factor Hand2 (heart- and neural crest derivatives-expressed protein 2), thus exacerbating cardiac remodeling in a TAC model.[30] Furthermore, decreased expression levels of miR-133 and miR-1, transcribed from the same genomic cluster, have been observed in mouse and human models of cardiac hypertrophy. Corresponding in vitro overexpression of miR-133 or miR-1 inhibited cardiac hypertrophy, whereas suppression of miR-133 by antagomiRs caused marked and sustained cardiac hypertrophy.[31] Three miR-133 target genes were identified, which are potentially involved in the effect on cardiac hypertrophy, namely *RhoA* (a GDP-GTP exchange protein), *Cdc42* (a signal transduction kinase), and *Nelf-A/WHSC2* (a nuclear factor).[31] Additionally, miR-1/133 regulates cardiac muscle repolarization, and reduction of miR-1/133a dosage induced a long-QT phenotype in mice, especially at low heart rates.[32] In contrast to miR-1 and miR-133, miR-212 and miR-132 expression is upregulated by hypertrophic stimuli in cardiomyocytes and thus is sufficient to drive the hypertrophic growth of cardiomyocytes by the direct regulation of the transcription factor FoxO3 (Forkhead box protein O3) that has antihypertrophic and proautophagic properties.[33] Finally, miR-208 is encoded by an intron of the *αMHC* gene and was shown to be involved in cardiomyocyte hypertrophy, fibrosis, and expression of βMHC (myosin heavy chain-β) in response to stress and hypothyroidism.[34]

Atherosclerosis and Vascular Remodeling

In atherosclerotic disease conditions, a central role is described for the flow-regulated miRNAs miR-92a, miR-126, miR-146, and miR-181.[35] Furthermore, miR-10a and miR-23b have been shown to be atheroprotective.[36,37] A screen for aberrantly expressed miRNAs in the vascular walls on balloon injury identified miR-21 as an important regulator of neointima lesion formation, cell proliferation, and apoptosis.[38] Further analysis identified *PTEN* and *Bcl-2* as target genes that are involved in miR-21–mediated cellular effects. The age-regulated miRNA miR-29 downregulates extracellular matrix proteins like collagens and thereby sensitizes the aorta for the formation of aneurysms in advanced age.[39,40] Under disease conditions, miR-29 is upregulated, leading to a downregulation of its collagen target genes. Inhibition of miR-29 abrogates aortic dilation and protects from vessel rapture. Furthermore, the intronic miRNAs miR-33a and miR-33b, coexpressed with the sterol regulatory element-binding proteins SREBF (sterol regulatory element-binding transcription factor) 1 and SREBF2, regulate cholesterol, fatty acid, and triglyceride homeostasis, and, therefore, genetic deletion was shown to increase circulating HDL (high-density lipoprotein) cholesterol levels.[41] Endothelial miR-126 expression maintains a proliferative reserve in endothelial cells through suppression of the Notch1 inhibitor Dlk1 (δ-like 1 homolog) and thereby prevents atherosclerotic lesion formation.[41] Finally, miR-146a[42] and miR-181b[43] exert anti-inflammatory functions by directly targeting the 3′-untranslated region of TRAF6 (tumor necrosis factor receptor–associated factor 6) and importin α3, subsequently inhibiting NF-κB (nuclear factor-κB) activity.[42,43] This

regulation is highly relevant given that NF-κB is a regulator of proinflammatory chemokine expression that mediates the adhesion of monocytes to endothelial cells. In addition, the differential expression of miR-10a contributes to the regulation of proinflammatory endothelial phenotypes that might influence the development of atherosclerosis.[44]

All described examples for the function of miRNAs in the cardiovascular system highlight their potential as valuable targets for the development of therapeutic strategies to treat different cardiovascular diseases.

miRNA Inhibitors for the Treatment of Cardiovascular Diseases

As highlighted above, miRNAs are described to regulate postinfarction angiogenesis and remodeling, cardiac fibrosis and hypertrophy, arrhythmias, atherosclerosis, and metabolic diseases.[3,4,10,21,34] The function of miRNAs was mostly studied either by using genetically modified mice, RNA therapeutics, or viral vectors to inhibit or overexpress specific miRNAs. ASOs or siRNAs were mainly used to inhibit a certain miRNA. Both substances are chemically modified for a better stability against RNases mostly by using phosphorothioate backbones. In addition, RNAs were modified to decrease the likelihood of triggering an innate immune response, to lower the incidence of off-target effects and to improve pharmacodynamics by enhancing cellular uptake through the conjugation of cholesterol. Antisense molecules perfectly match in a complementary fashion to the target miRNA and prevent base pairing with the given mRNA targets, thereby blocking the inhibitory function of miRNAs. miRNA inhibitors are also known as antimiRs and can be divided into different groups depending on their chemical modifications.

Antisense Oligonucleotides

AntagomiRs and locked nucleic acid (LNA) antimiRs are the most prominent examples in this category of antimiRs. AntagomiRs are 3′cholesterol-conjugated, 2′-O-Me, 2′-fluoro or 2′O-methoxyethyl oligonucleotides fully complementary to the mature miRNA sequence with additional phosphorothioate backbone linkages, in which sulfur replaces one of the nonbridging oxygen atoms in the phosphate group.[45] The phosphorothioate backbone increases stability by providing nuclease resistance, and supports binding to plasma proteins (especially albumin), leading to reduced renal clearance and improved pharmacokinetic properties.[46] It was shown that the addition of cholesterol enhances cellular uptake of antagomiRs,[45] whereas adding 2′-O-Me, 2′-fluoro or 2′-methoxyethyl improves binding affinity to the target miRNA and reduces off-target effects.

The development of LNA-modified antimiRs has further complemented the oligonucleotide chemistry area. LNAs are chemically locked by a bridge that connects the 2′-oxygen and the 4′-carbon in a ribonucleotide mimicking C3′-endo conformation. Specific patterns of deoxyribonucleotide and locked ribonucleotide mixmers were used and have shown promising results in different in vivo models, including nonhuman primates, and in clinical trials.[47,48] LNA-based antimiRs have a length of ≈15 to 16 nucleotides with high affinity to their target miRNA. Moreover, a shorter LNA-based antimiR version was developed, also called 8-mer LNA-based antimiR or tiny LNAs.[49] Tiny LNAs bind only the seed region of a given miRNA having the advantage to simultaneously target whole miRNA families with overlapping functional activities. Thus, a combined targeting of a whole miRNA family might potentiate the beneficial effect in certain disease conditions. One example is a tiny LNA against the seed region of miR-15 family members, including miR-15a, 15b, 16 to 1, 16 to 2, 195, and 497, which was more efficient in the derepression of downstream targets than the conventional LNA-based antimiR version targeting only 1 specific miRNA family member.[12] The uptake of LNAs in cardiac tissue was comparable for both short and long LNAs, indicating that the length of antimiRs does not necessarily influence cellular uptake.[12] Tiny LNAs, however, do not seem to be always as efficient as longer oligonucleotides. Thus, Thum et al[25] showed that miR-21 inhibition by antagomiR treatment reduces cardiac fibrosis and hypertrophy, whereas tiny LNAs directed against miR-21 did not exert beneficial effects.

Two further strategies were developed to improve the functionality of LNA antimiRs. One novel class of bicyclic RNA analogues are selenomethylene LNAs that display high affinity, improved metabolic stability, and increased potency for miR-21 inhibition in cancer cell lines in vitro.[50] The other strategy is termed small RNA zipper. Small RNA zippers are based on LNAs and are designed to be complementary to the second half sequence of a miRNA molecule and the first half of another with a nucleotide gap to provide space allowing the formation of a stable structure.[51] Small RNA zippers were successfully used to inhibit miR-221 and miR-17 in breast cancer cell lines in vitro.[51] But for both new inhibitory strategies, no approaches are published to date on their potential application for the treatment of cardiovascular diseases.

Finally, peptide nucleic acids are ASOs in which the phosphate–sugar polynucleotide backbone is replaced by a flexible pseudopeptide polymer to which the nucleobases are linked.[52] The phosphodiester backbone is substituted by repetitive units of N-(2-aminoethyl) glycine to which the purine and pyrimidine bases are attached via a methyl carbonyl linker. Peptide nucleic acids show a high affinity to their target sequence and are resistant to DNases and proteinases.[52] The successful use of peptide nucleic acids against miRNAs in vivo was, for example, shown by inhibiting miR-155 in B cells in mice.[53]

siRNAs and Other Inhibitory Strategies

Alternatively to ASOs, siRNAs are used to block miRNAs. siRNAs are RNA duplexes that are mostly chemically modified in a similar fashion to antimiRs to improve the nuclease stability and cellular uptake.[54] siRNA-mediated knockdown of a target miRNA is achieved by binding to the loop region of the miRNA. In the study by Li et al,[55] for example, miR-181a was downregulated by siRNAs and led to decreased arrhythmogenic effects of skeletal myoblast transplantation in rats with myocardial infarction.

Another targeting strategy includes miRNA target site blocker. They bind in a complementary manner to the miRNA target site of an mRNA, thereby preventing miRNAs from gaining access to that site.[56] This strategy allows the protection of specific miRNA targets instead of influencing all targets in parallel. For example, in the study by Messina et al,[57] the use of target site blockers showed selective impairment of

the ability of miR-155 to target the Cebpb (CCAAT/enhancer-binding protein-β) transcript in the infantile hypothalamus.

Finally, miRNA function can be modulated or reduced by miRNA sponges that are produced from transgenes within cells. Sponge RNAs contain 4 to 10 complementary binding sites to an miRNA of interest introduced in the 3′-untranslated region of the RNA. The binding site can be chosen to bind to the miRNA seed regions or can be based on a specific mRNA target sequence, thereby competing with the binding of a given miRNA to its targets.[57] Because miRNA sponges need a transgene construct to be expressed, delivery of sponge constructs to tissue in living animals is feasible with the use of viral vectors. For example, Carè et al[31] used an adenoviral eGFP (enhanced green fluorescence protein) sponge to inhibit miR-133 in cardiac myocytes in vivo in a mouse model of cardiac hypertrophy. However, miRNA sponges have the disadvantage that high endogenous miRNA concentrations within a cell have to be bound by excessive concentrations of the sponges.

Many studies in the cardiovascular research field highlight especially the potential of above described antimiRs to efficiently inhibit miRNA functions in the vascular wall and in heart tissue in mice and in few cases in larger animal models, such as pigs. The following chapter will discuss the therapeutic application of the different types of antimiRs and potential challenges especially in preclinical and clinical development.

Therapeutic Application of AntagomiRs

AntagomiRs were successfully used to target miR-21, which reduced the extent of interstitial fibrosis and cardiac hypertrophy after TAC in mice.[23] Furthermore, application of antagomiRs against miR-92a was shown to augment neovascularization after hindlimb ischemia and improve the recovery of heart function after myocardial infarction in mice.[17] A single injection of antagomiR-92a encapsulated in microspheres prevented adverse infarct remodeling in a percutaneous pig model of reperfused AMI.[19] AntagomiRs directed against miR-92a further prevent endothelial dysfunction and atherosclerosis in mice and rats.[58,59] MiR-25, which shares the seed sequence with miR-92a, is upregulated in the failing heart, and its inhibition by antagomiRs (300 µg per mouse; diluted in in vivo-jetPEI solution) improved cardiac function, improved survival, and halted established heart failure in a mouse model.[29] Although subsequent studies with tough decoys confirmed the protective effects of miR-25 inhibition,[60] another study showed that antagomiR-25 applied by intraperitoneal injection at a concentration of 80 mg/kg induced spontaneous cardiac dysfunction.[30] It is unclear whether the different effects might be attributed to the different formulations and concentration of antagomiR-25. Another example relates to the inhibition of miR-320 by antagomiRs, which improves heart function after ischemia/reperfusion injury.[61] Finally, inhibition of the miR-212/132 by antagomiRs rescues cardiac hypertrophy and heart failure induced by TAC in mice.[33]

Therapeutic Application of LNA AntimiRs

LNA-based antimiRs are efficient inhibitors of miRNAs, which were also applied in several disease models. LNAs against miR-29 reduce aneurysm formation by augmenting matrix synthesis and maintaining the structural integrity of the vascular wall.[39,40] Furthermore, therapeutic inhibition via LNA-based antimiRs against miR-208a improves cardiac function and survival during heart failure.[62] Inhibition of the entire miR-34 family[15] or preferential inhibition of miR-34a alone[14,16] reduced cell death and fibrosis after myocardial infarction and thereby improves recovery of heart function. One study directly compared antagomiR- and LNA-based miR-34a inhibitors side by side in a mouse model of chronic myocardial infarction, and both showed beneficial effects.[14] The significance of these data, however, is limited because of the fact that different concentrations of the antimiRs were used and a dose-response curve was not provided.[14] LNA-based antimiRs against miR-34a were further suggested to extend the regeneration window in postnatal mice by enhancing cardiomyocyte proliferation.[16] Finally, the abovementioned beneficial effect of antagomiR-based inhibition of miR-92a was also confirmed by using LNA-based antimiRs in a pig ischemia/reperfusion model.[18]

Challenges of AntimiR Application

Although first therapeutic benefits by miRNA inhibition in different mouse and some large animal models are documented as summarized above, targeting heart tissue is still more challenging in comparison with other organs, such as kidney or liver.

AntimiRs in general can be administered intravenously, intraperitoneally, or subcutaneously and with an efficient long-term inhibition for several weeks. Most studies used single or repetitive dosing of ≈0.5 to 25 mg/kg body weight LNA-based antimiR in mice or rats (Table). Higher concentrations were needed for antagomiRs (8–80 mg/kg body weight) to efficiently suppress miRNAs in the heart or the vasculature (Table). Interestingly, the efficient dose and dosing scheme (number of repeated injections) is likely dependent not only on the chemistry but also on the target sequence. The efficient inhibition of miR-92a in the heart, for example, could be achieved in mouse studies using rather low concentrations, such as 0.5 mg/kg LNA-92a or 8 mg/kg antagomiR-92a.[18] In contrast, targeting other miRNAs, such as miR-34a or miR-29, in the same laboratory required much higher dosing concentrations even though the target tissue was the same (5 mg/kg LNA-based antimiRs, 20 mg/kg antagomiRs).[14,40]

In comparison, in the first clinical trial, antimiRs directed against the liver-enriched miR-122 were applied at concentrations between 3 and 7 mg/kg body weight (5 weekly injections),[74] and improved chemical formulations for siRNA delivery resulted in effective concentrations of <0.01 mg/kg bodyweight that are required to target liver-expressed genes.[75] In general, several aspects seem to influence the effective dose of different miRNA inhibitors. The key points are certainly bioavailability and biodistribution, mechanism of cellular uptake, the expression level of the targeted miRNA and its target genes, and, as mentioned above, the sequence of the miRNA, as well as the specific sequence of the inhibitor itself. Bioavailability was improved by reducing the clearance from the circulation because of the development of new chemical modifications. As a consequence, antimiR activity is no longer limited to tissues with high accumulation like liver and kidney

Table. Examples of Delivery Strategies and Selective Targeting of RNA Therapeutics to Improve Tissue Enrichment

Delivery Strategy	miRNA Chemistry	Dosing	Application	Citation
Packaging strategies	AntagomiR-92a encapsulated in microspheres	0.1 mg/kg bw, single intracoronary injection, pig	Myocardial infarction	Bellera et al[19]
	Nanoparticle packed with miR-126-5p mimics	100 μg per injection, IV every 3 days for 4 wk, mouse	Atherosclerosis	Schober et al[41]
	Lipofectamine mixed with miR-181b mimics	1 nmol mimic, 2 injections per wk for 4 wk or 1 injection per wk for 12 wk, IV, mouse	Atherosclerosis	Sun et al[43]
	Lipid nanoparticle (cationic lipopolyamine) loaded with LNA-antimiR-145	2 mg/kg bw, 3 injections (IV) for 5 wk, rat	Pulmonary hypertension	McLendon et al[63]
Receptor-mediated uptake/aptamers	Transferrin receptor aptamer linked to premiR-126	50–500 nmol/L	Human endothelial cells, breast cancer cells	Rohde et al[64]
	Gapmers against apolipoprotein C or transthyretin linked to galactosamine (hepatocyte-specific receptor)	3–10 mg/kg bw (apolipoprotein C), 6–20 mg/kg bw (transthyretin), single injection, mouse	Liver	Prakash et al[65]
	E-selectin targeting multistage vector microparticle combined with nanoparticle packed with miR-146a and miR-181b	15 μg per mouse	Atherosclerosis	Ma et al[66]
Local activation	Light-inducible caged antagomiR-92a	2 μg in 25 μL, 3 treatments (1× topical, 2× ID injection), mouse	Diabetic wound healing	Lucas et al[67]
Vector-based enrichment	AAV encoding miR-590 and miR-199a	1×10^{11} viral genome particles per mouse	Myocardial infarction	Eulalio et al[68]
	Lentivirus Si-mlincRNA-p21	1×10^{8} UT/mL in 20 μL per mouse	Atherosclerosis	Wu et al[69]
	Adenovirus-driven expression of siRNA-MDRL or MDRL-lncRNA	2×10^{11} moi virus, catheter-based injection, mouse	Myocardial infarction	Wang et al[70]
	Adenovirus-driven expression of CARL	2×10^{11} moi virus, catheter-based injection, mouse	Myocardial infarction	Wang et al[71]
	AAV9-driven expression of miR-1	5×10^{11} viral genomes, IV injection, rat	Cardiac hypertrophy	Karakikes et al[72]
Delivery by devices	Catheter-based intracoronary delivery of LNA-antimiR-92a	0.03/0.15 mg/kg bw single intracoronary infusion, pig	Myocardial infarction	Hinkel et al[18]
	LNA-antimiR-21–eluting stent	5 mg/kg bw, rat	Restenosis	Wang et al[73]

AAV indicates adeno-associated virus; bw, body weight; CARL, cardiac apoptosis-related long noncoding RNA; IV, intravenous; LNA, locked nucleic acid; lncRNA, long noncoding RNA; MDRL, mitochondrial dynamic related long noncoding RNA; miRNA, microRNA; and siRNA, small interfering RNA.

but can be also observed in other target organs. However, measuring the uptake of ASO alone may not be necessarily related to their biological activity.[76] ASOs are mainly taken up by endocytotic pathways, which can be divided in productive and nonproductive pathways. Productive pathways lead to the binding of the ASO to the target, whereas uptake by nonproductive pathways might lead to accumulation in late endosomes or lysosomes.[77] Although the mechanisms underlying the route toward a production versus nonproduction have not been fully established, first studies suggest that the 1 key ESCRT-I (endosomal sorting complexes required for transport I), especially its component *TSG101*, is a major regulator of nonproductive uptake of anti–miR-21.[78] Inhibition of *TSG101* improved productive uptake in vitro and in vivo. The mechanism of uptake is most likely cell specific depending on surface protein expression and binding abilities.[77] In addition, the effective dose might depend on the expression level of the miRNA of interest and its target genes in a given cell type. To date, knockdown efficiency was mainly tested by isolating RNA from whole tissue that might give misleading information. Measuring miRNAs by polymerase chain reaction may lead to an overestimation of the inhibitory effects because the polymerase chain reaction can be directly influenced by the presence of the antimiR in the tissue extract. Northern blot and particularly measuring target derepression should, therefore, be used to confirm the extent of inhibition. Moreover, cellular heterogeneity may provide obstacles on the true assessment of the biological implications of miR inhibition. Especially for the heart, which is composed of cardiomyocytes, endothelial cells, fibroblast, and immune cells, miRNAs and their targets can be differentially expressed in individual cell types, which may preclude to detect effects in whole heart tissue. It may be warranted to assess the effects of antimiR treatments on selected cell types. Of note, expression levels of both miRNAs and their targets can significantly change under disease conditions, which should also be considered.

AntimiR Toxicity

Increasing doses of ASOs or antimiRs augment the risk of the occurrence of toxicities. In general, toxicity observed by

oligonucleotide treatment can be divided into 2 major classes: hybridization-dependent and hybridization-independent toxicity. Hybridization-dependent toxicity is because of the sequence of the oligonucleotide and is induced either by the modulation of the target itself (on-target effects) or by unspecific binding to similar nucleotide sequences (off-target effects). The risk of direct target-related toxicity can be evaluated and reduced by considering known information about the target, such as mode of action and expression profile and level during preclinical and clinical development. Off-target effects might mainly occur by targeting miRNAs of the same family because of the homology of the seed sequence or by unspecific interaction with DNA, RNA, or proteins. Even though antimiRs targeting the 3′-region of the miRNA have been tested, the results are controversially discussed, and thus, antimiRs specific to the seed region seem to be still the most effective choice.[49] Moreover, targeting of a whole miRNA family, for example, by using tiny LNAs, may be beneficial but may also not be suitable for all miRNAs, for example, as shown by the controversial findings on miR-21.[25] Off-target effects could be further induced by hybridization to double-stranded DNA regions, potentially inducing site-specific mutagenesis by triplex formation. Even though this event is possible, testing of various oligonucleotides for their potential to induce genotoxicity by standard tests like AMES and micronucleus testing revealed negative results and thus no evidence for genotoxicity.[79] However, genotoxicity testing of oligonucleotides with new modifications is still recommended.

Hybridization-independent toxicity is mainly because of the backbone and modifications of the oligonucleotide. RNA oligonucleotides in general have been shown to activate the immune system by inducing TLR (Toll-like receptor) signaling.[80] Complement activation in nonhuman primates treated with phosphorothioate-modified oligonucleotides has been observed especially at high plasma concentrations.[81–83] These effects can be avoided by slow infusion rather than bolus injection to reduce the peak plasma levels. Of note, similar complement activation induced by oligonucleotide treatment was not observed in human serum.[84] Phosphorothioate-modified oligonucleotides have been further shown to affect blood coagulation by binding to platelets, thus leading to platelet activation, aggregation, and thrombus formation in vitro and in vivo.[85] Recently, it was demonstrated that the insertion of LNA modifications could markedly reduce platelet activation by preventing the binding of the ASOs to platelet proteins.[86] Nevertheless, adequate immunologic tests should be included in the preclinical and clinical program during therapeutic development. A specific issue of some LNA-modified oligonucleotides is an increase of liver transaminases ALT (alanine aminotransferase) and AST (aspartate transaminase) even observed at low dosing levels in mice, which indicate potential hepatotoxicity.[87] However, because several LNA-based oligonucleotides did not show any signs of hepatic toxicity in different in vivo models, hepatotoxicity seems not to be a general phenomenon but rather a sequence-specific issue. Interestingly, specific trinucleotide motifs have been identified, which seem to be a predictive indicator for potential hepatotoxic sequences by increasing p53 and NRF2 (nuclear factor [erythroid-derived 2]-like 2) pathway activation in vivo.[72]

The risk of hybridization-independent toxicities might additionally be reduced by lowering the effective dose and by improving the targeting to the tissue of interest. Current strategies to reduce potential toxicological side effects by improving delivery strategies of antimiRs enhancing tissue-specific uptake of antimiRs or by local activation of antimiRs will be discussed in detail in the last chapter of this article and are summarized in Figure 2, as well as in the Table.[88]

miRNA Overexpression to Restore miRNA Function in the Cardiovascular System

In contrast to miRNA inhibition, the establishment of suitable approaches to induce miRNA overexpression is even more challenging. To augment a certain miRNA in vivo, synthetic RNA duplexes, named miRNA mimics, are mostly used. miRNA mimics are double-stranded RNA fragments mimicking the sequence of endogenous miRNAs to replace or augment miRNA concentrations in tissues. Similar to antimiRs, miRNA mimics need to be chemically modified in terms of stability and cellular uptake. The guide strand of the miRNA mimic is the actual functional strand, identical to the miRNA of interest. A 2′-F modification was shown to protect the guide strand against exonucleases without affecting target binding. The opposite strand is less stable and can be linked to cholesterol for better cellular uptake. With the exception of the linked cholesterol, this strand remains unmodified to allow rapid degradation. As described for antimiRs, there are several studies showing therapeutic benefits of miRNA mimics for treatment of cardiovascular diseases (Table).[88] Overexpression of miR-126-5p mimics, for example, provides antiatherosclerotic effects by enhancing endothelial cell proliferation,[41] and miR-181b mimic treatment was shown to improve glucose levels and insulin responsiveness in high-fat diet–fed animals.[43] Furthermore, systemic overexpression of the 2 miRNAs, miR-146a and miR-181b, inhibits the activation of NF-κB and atherosclerosis through cell-specific mechanisms in the vascular endothelium.[66] The muscle-specific miR-1 is a key regulator of cardiac hypertrophy, and adeno-associated virus (AAV) serotype 9–mediated therapeutic cardiac-targeted delivery of miR-1 reduced cardiac hypertrophy and halted pathological remodeling.[89] Finally, augmentation of miR-590-3p and miR-199a-3p expression levels by using miRNA mimics was shown to induce cardiomyocyte proliferation and thereby stimulates cardiac regeneration after AMI.[68] Of note, in all previously mentioned studies, high concentrations of miRNA mimics with numerous serial injections were necessary to achieve significant effects. A reason for the high dosage might be the fast degradation of unformulated RNA mimics by endogenous, extracellular RNases.

Despite the beneficial effect caused by overexpression of a given miRNA, supraphysiological levels of a specific miRNA because of systemic miRNA mimic activity might cause adverse effects. This could be particularly the case in an organ, in which a given miRNA is not endogenously expressed.[90] Additionally, it needs to be mentioned that miRNA mimics can potentially activate the immune response. They exert their proinflammatory activity by nonspecifically stimulating the immune system via toll-like receptors in a sequence-independent manner.[80] Several approaches to overcome these

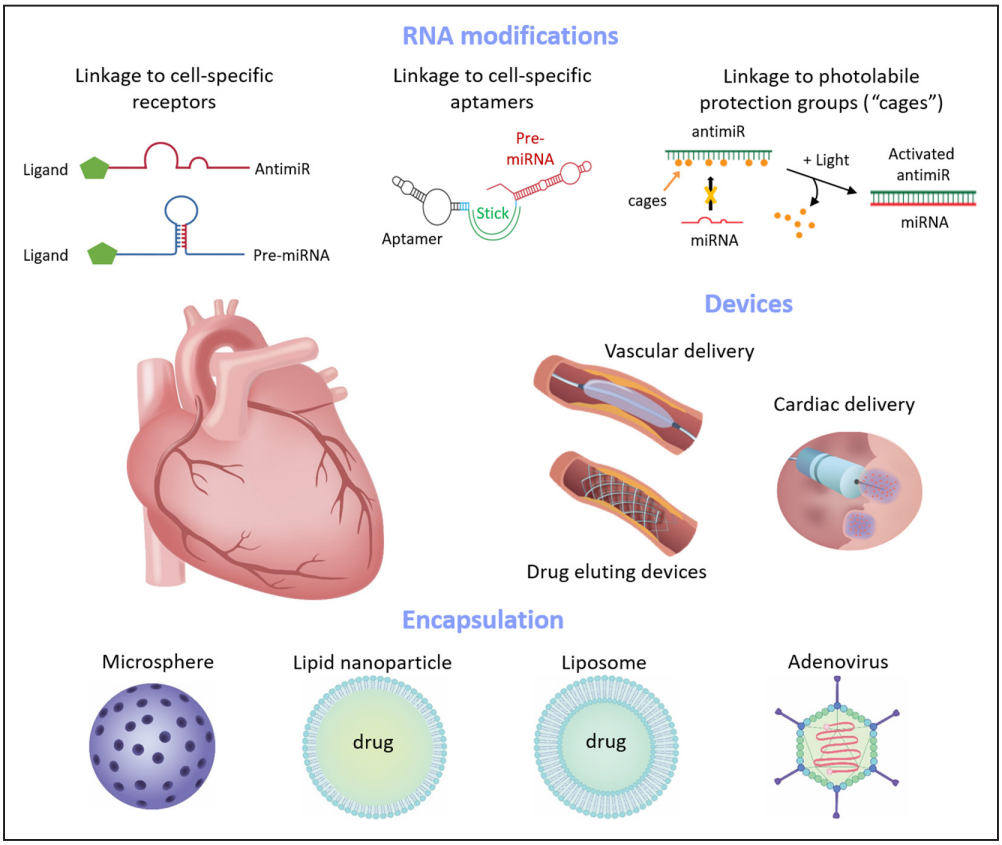

Figure 2. Delivery strategies to improve therapeutic uptake of RNA therapeutics in cardiac tissue. RNA therapeutics can be chemically modified, applied by devices or encapsulated to improve target specificity or cellular uptake to reduce unwanted side effects and toxicology.

problems are currently under investigation to achieve better cellular uptake and tissue specificity (see Delivery Options to Improve the Efficiency and Specificity of Noncoding RNA Therapeutics section).

The use of lentiviruses or adenoviruses promises an efficient expression of miRNAs. However, targeting of the cardiovascular system, particularly the endothelium, is not well established (see Delivery Options to Improve the Efficiency and Specificity of Noncoding RNA Therapeutics section). In addition, lentiviruses or adenoviruses have the disadvantage that only the precursor is overexpressed leading to the generation of 2 miRNAs. Because increasing evidence suggests that the star strand may not necessarily be degraded but may have important specific functions,[41] the overexpression of both strands in an uncontrolled manner may lead to an unanticipated response.

miRNA mimics can also be linked to aptamers, which are RNA molecules that can bind to specific receptors and thereby allow a cell type-specific uptake. The options for improving miRNA expression will be discussed in more detail in the last chapter of this article and are summarized in Figure 2, as well as in the Table.[88]

lncRNA Therapeutics

Example for Important lncRNAs in the Cardiovascular System

First interest in lncRNAs was raised by genome-wide association studies implicating SNPs (single nucleotide polymorphisms) adjacent to coding genes that predict cardiovascular events.[6] Subsequent RNA sequencing provided first evidence that lncRNAs are indeed regulated in human cardiac diseases. It was further reported that the expression profile of lncRNAs is more predictive than the profile of mRNAs or miRNAs to discriminate failing hearts of different pathologies and are markedly altered in response to disease conditions, indicating an important pathophysiological role of lncRNAs in the cardiovascular system.[91] In the following chapter, we will summarize the functional relevance of the best-described lncRNAs in cardiovascular diseases. More detailed information on the role of lncRNAs is given in the recent review articles.[6,92–95]

Myocardial Infarction

Expressed by chromosome 22q12.1, the lncRNA MIAT (myocardial infarction-associated transcript) was identified as a risk allele for myocardial infarction,[96] but the exact function of MIAT in heart tissue is still not unraveled. However, there is evidence that MIAT functions as a molecular sponge for miR-150[97] and as a target gene regulator of the fibrosis-related actors miR-24, furin, and TGF-β1 (transforming growth factor-β1).[98] Furthermore, MIAT is involved in the pathology of diabetic retinopathy, highlighting a role of this lncRNA in pathological angiogenesis by influencing endothelial cell proliferation, migration, and tube formation capacity.[99] Mechanistically, it was shown that MIAT functions as a competing endogenous RNA and forms a feedback loop with vascular endothelial growth factor and miR-150-5p to regulate endothelial cell functions.[99]

Moreover, the lncRNAs CARL (cardiac apoptosis-related lncRNA), MDRL (mitochondrial dynamic related lncRNA), and APF (autophagy-promoting factor) regulate cardiomyocyte cell death by inhibiting specific miRNAs[70,71,100] and thereby have a supportive healing effect on myocardial infarction.

Cardiac Hypertrophy and Fibrosis

The lncRNA CHRF (cardiac hypertrophy-related factor) acts as an miRNA sponge in the context of cardiac hypertrophy. CHRF is able to directly bind miR-489 and to regulate MyD88 expression and hypertrophy.[101] Global lncRNA expression profiling indicated that several other lncRNA transcripts are deregulated during pressure overload–induced cardiac hypertrophy in mice.[102] The lncRNA Chast (cardiac hypertrophy-associated transcript), for example, was identified to be upregulated in cardiomyocytes in vivo in TAC-operated mice. Chast negatively regulates pleckstrin homology domain-containing protein family M member 1 (opposite strand of Chast), thus inhibiting cardiomyocyte autophagy and thereby driving hypertrophy.[102] Han et al identified a cardiac-specific lncRNA named Mhrt (myosin heavy chain-associated RNA transcript). During pathological stress conditions like pressure overload–induced hypertrophy, Mhrt expression is repressed, and restoring the physiological expression level provides cardioprotective effects. Mechanistically, Mhrt interferes with the chromatin remodeling factor Brg1 and thereby regulates its target genes, such as Myh6, Myh7, and osteopontin.[103] Finally, a recent article describes the role of the lncRNA *Meg3* in cardiac fibrosis.[104] Inhibition of *Meg3* reduced cardiac fibrosis and diastolic dysfunction by downregulating matrix metalloproteinase-2 in cardiac fibroblast.[104]

Atherosclerosis, Angiogenesis, and Vascular Remodeling

One of the most prominent lncRNAs is encoded by the chromosome 9p21 locus, which is associated with several cardiovascular diseases, like carotid artery plaque, stroke, aneurysms, peripheral artery disease, heart failure, and cardiovascular mortality. Several functional studies identified differential expression of this lncRNA named ANRIL (antisense noncoding RNA in the INK4 [inhibitor of CDK4] locus), as well as neighboring protein-coding genes at the INK4/ARF (ADP-ribosylation factor 1) locus.[105] ANRIL is expressed in different cell types important in atherogenesis, and several studies showed that it influences cell viability, proliferation, adhesion, and apoptosis. ANRIL acts by binding to polycomb group proteins[106] thus epigenetically regulating target gene expression.

Variations of the lncRNA H19 at chromosome 11p15.5 have been associated with coronary artery disease,[107] although detailed functional analysis is still lacking. Under physiological conditions, H19 expression is downregulated postnatally and is specifically expressed in heart and skeletal muscle tissue in adult mice, as well as in several tumors.[6] However, H19 expression is strongly induced on vascular injury[108] and in human atherosclerotic plaques,[109] indicating an important function under these disease conditions. A potential underlying mechanism of H19 action might be the role as sponge for let-7 family members as described in skeletal muscle differentiation in vitro,[110] which may prevent let-7 to exert its inhibitory effects on specific target genes. However, this still needs to be confirmed in cardiovascular cell types.

Wu et al identified lincRNA-p21—a direct transcriptional target of p53—as a key regulator of cell proliferation and apoptosis in atherosclerosis. lincRNA-p21 expression was strongly downregulated in atherosclerotic plaques of ApoE (apolipoprotein E)−/− mice and in patients with coronary artery disease. Mechanistically, it was shown that lincRNA-p21 indirectly regulates p53 expression by a positive feedback loop thus changing the expression pattern of several p53 target genes.[69]

The Malat-1 (metastasis-associated lung adenocarcinoma transcript 1) lncRNA is hypoxia induced and regulates endothelial cell function and vessel growth.[111] Furthermore, Malat-1 expression is highly upregulated under diabetic conditions leading to endothelial dysfunction and diabetic retinopathy. Thereby, Malat-1 upregulation represents a critical pathogenic mechanism for diabetes mellitus-induced microvascular dysfunction.[112]

Two more lncRNAs, namely linc00323-003 and MIR503HG, were also shown to be hypoxia induced in endothelial cells and contribute to angiogenesis. For both lncRNAs, it was shown that they inhibit the expression of the key endothelial transcription factor GATA2 (GATA-binding protein 2) and thereby potentially controlling cell proliferation and tube formation capacity.[113]

RNA sequencing of human coronary artery smooth muscle cells revealed the vascular cell-enriched lncRNA SENCR (smooth muscle and endothelial cell-enriched migration/differentiation-associated lncRNA) that was shown to be an inhibitor of smooth muscle cell migration and a stabilizer of the smooth muscle cell contractile phenotype.[114]

Taken together, not only small noncoding RNAs, such as miRNAs, but also lncRNAs play an important role in the regulation of fundamental processes in the cardiovascular system and are thus promising targets for the development of new therapeutic approaches.

lncRNA Inhibition for the Treatment of Cardiovascular Diseases

Consistent to the inhibition of miRNAs by antimiRs, lncRNA function can be blocked by antisense-based strategies or by shRNAs, which comprise siRNAs, modified ASOs, and gapmers. Although siRNAs and ASOs preferentially target cytoplasmic lncRNAs, gapmers can enter the nucleus and target nuclear-enriched lncRNAs by introducing ribonuclease H-dependent cleavage of the RNA target.[6,115,116] Because lncRNAs are preferentially located within the nucleus, to date, antisense LNA gapmers are the most widely used. Such gapmers are chimeric ASOs that contain a central block of deoxynucleotide monomers sufficiently long enough to induce the important step for the RNase H cleavage. Beyond this, the central block is flanked by 2′-O–modified ribonucleotides to protect from nuclease cleavage. Similar to other ASOs, a phosphorothioate backbone is often used to stabilize the gapmers for therapeutic in vivo approaches.

Gapmers have already been used in vivo for targeting coding RNAs, such as the liver-enriched PCSK9 (proprotein convertase subtilisin/kexin type 9) in nonhuman primates; however, a phase I clinical study was halted because of potential hepatotoxicity as a result of off-target effects.[117] In contrast, gapmers against hypoxia-inducing factor 1α did

not raise any safety concerns during the 1-year observation period.[118] Several publications have described effective inhibition of lncRNAs in the cardiovascular system in vivo. For example, pharmacological inhibition of the hypoxia-induced lncRNA Malat-1 by gapmer injection reduced endothelial cell proliferation and ischemia-induced revascularization.[111] Gapmers against Chast efficiently reduced cardiac hypertrophy,[102] and *Meg3* inhibition decreased cardiac fibrosis and improved diastolic performance after TAC.[104] Therefore, both therapeutic strategies may be used as potential therapeutic options to prevent cardiac remodeling.

The inhibition of lncRNAs by siRNAs was successfully shown for lincRNA-p21 and APF. As described above, lincRNA-p21 was discovered as a key regulator of cell proliferation and apoptosis by interfering with p53 transcriptional activity during atherosclerosis. Its inhibition by lentivirus-driven supply of siRNA against lincRNA-p21 results in neointima hyperplasia in a carotid artery injury model.[69] The lncRNA APF regulates autophagic cell death in vitro, and the inhibition via siRNAs reduced ischemia/reperfusion injury in mice.[100]

To date, high concentrations and repeated dosing of siRNAs and gapmers is needed to achieve sufficient inhibition of lncRNAs in vivo (4–20 mg/kg, several repetitive injections). Based on the chemistry of the inhibitor used, potential dose-dependent toxicities might be considered during preclinical and clinical testing. Most of these toxicities were already described in the section AntimiR Toxicity. In addition to general toxicities of RNAs, gapmers might induce hepatotoxicity in an RNase H1-dependent manner.[117] Reduction of RNase H1 levels before gapmers treatment markedly ameliorated the hepatotoxic events suggesting that hepatotoxicity of LNA-modified gapmers is a result of off-target RNase-dependent RNA degradation.

Another challenge for the development of lncRNA therapeutics is the fact that, unlike miRNAs, lncRNAs are not necessarily conserved among different species. Hence, animal studies focusing on the mode of action of a given lncRNA but also toxicity studies need to be carefully designed and critically analyzed before translating the results into the clinic. One option would be the use of the human-specific sequence in different toxicological animal models rather than the endogenously expressed sequence. Nevertheless, this has the disadvantage that only hybridization-independent toxicology of a given gapmer can be analyzed, while sequence-dependent side effects cannot be studied in more detail. Humanized models or organoid cultures may be more suitable models to study the function of human-specific lncRNAs. This step will be of high importance, because the function of lncRNAs can be complex by interfering with chromatin structures or epigenetic control mechanisms, and without defining the exact mechanism, unwanted side effects may become a drawback in terms of the development of lncRNA therapeutics.

A further challenge in the development of lncRNA therapeutics is the fact that lncRNAs are often preferentially expressed in the nucleus (as opposed to the cytoplasmic localization of most mRNAs and miRNAs) and may be more integrated in complex structures that are not easily accessible. Therefore, besides direct inhibition by antisense strategies, small molecules, which are designed to specifically interfere with conserved RNA structures and, for example, block RNA-protein complexes, may be helpful.

lncRNA Overexpression to Restore Their Function in the Cardiovascular System

To date, overexpression of lncRNAs to restore their function in an organism is achieved by viral vectors or by recently described synthetic modified RNAs. However, this approach may not be useful for those lncRNAs, which act in a local manner, for example, cis-regulatory lncRNAs, which directly interfere with expression of neighboring genes. Conventional overexpression from viral vectors or plasmids may not restore the function of such lncRNAs, because such overexpression strategies do not guarantee the appropriate localization of these lncRNAs. RNA-guided endogenous gene activation by CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9)–based transcription factors may circumvent this problem allowing the induction of the endogenous lncRNA as recently described by Maeder et al[119] in bacteria and human cells, but whether this approach is feasible in living animals needs to be elucidated. Furthermore, lncRNAs are often expressed in different transcript variants with possibly different functions and, like proteins, can be post-transcriptionally modified by methylation or editing. Particularly, editing is mostly found in primate Alu sequences, which are enriched in lncRNAs.[120] This in turn adds a new level of complexity to the development of lncRNA therapeutics to restore their function in vivo. Nevertheless, there are some positive examples published that show the successful overexpression of a specific lncRNA and their therapeutic potential. MDRL (mitochondrial dynamic-related lncRNA), for example, inhibits mitochondrial fission and apoptosis in cardiomyocytes in vitro, as studied by the transfection with adenoviral MDRL siRNA to inhibit MDLR function or by transfection with adenoviral MDRL to overexpress the lncRNA.[70] In vivo intracoronary delivery of adenovirus-expressed MDRL successfully reduced myocardial infarction after ischemia/reperfusion injury in mice. A related function and mechanism was also discovered by the same group for the lncRNA CARL. CARL suppresses mitochondrial fission and apoptosis by targeting miR-539 and PHB2 (prohibitin 2), and adenoviral overexpression reduced cardiac remodeling in mice.[71] But also here, long-term studies need to be done to proof the tolerance of lncRNA overexpression in living organisms.

Delivery Options to Improve the Efficiency and Specificity of Noncoding RNA Therapeutics

Although oligonucleotide-based therapeutics targeting short- and long noncoding RNAs have been extensively studied and, as described above, are promising tools for the treatment of several cardiovascular diseases, there are still challenges that need to be addressed. In principal, there are 2 major aims that need to be reached. The first aim is to reduce hybridization-independent toxicity by lowering the dose that is currently needed to efficiently inhibit noncoding RNAs in cardiovascular tissues. The second aim is to reduce the risk of toxicity

and sequence-specific side effects by targeting the antisense molecules to specific cell types or tissues. The improvement of delivery strategies to facilitate the cellular uptake or even provide cell-type specificity to reduce the risk of dose-dependent toxicities and off-target effects is the method of choice to overcome these obstacles. A ubiquitously expressed miRNA, for example, can exhibit diverse functions in different cell types, of which some may be beneficial and others detrimental. Thus, overexpression or inhibition of such an miRNA is therapeutically feasible only if it can be targeted to the relevant cell type. A prominent example for the need of cell type-specific enrichment of RNA therapeutics are miRNA inhibitors that increase cardiomyocyte proliferation thereby augmenting cardiac regeneration, such as inhibitors of miR-15 or miR-34 family members.[13,14] Although the augmentation of cardiomyocyte proliferation may provide a therapeutic benefit and might even allow for the long pursuit for regeneration of the heart, such a powerful miRNA inhibitor likely also induces proliferation in other cell types potentially facilitating tumor growth. Indeed, miR-34 and miR-15 are known tumor suppressors,[121,122] and miR-34 overexpression is tested as anticancer therapy.[123] In the following, we will provide an overview about current efforts to improve different delivery strategies.

Device-Based Approaches to Achieve Tissue Enrichment of RNA Therapeutics

Local delivery of RNA therapeutics by devices comprises a first attempt to augment the concentration in the heart or the vasculature. In a large animal model, Hinkel et al[18] tested the efficacy of LNA-92a administration in a pig ischemia/reperfusion model via systemic intravenous, intracoronary (antegrade), or retrograde infusion with concentrations of 0.03 mg/kg body weight (Figure 2; Table). Even though miR-92a expression was reduced by all 3 application routes in the heart, antegrade or retrograde infusion resulted in a significantly improved heart function and reduction in apoptosis and infarct size compared with intravenous infusion. Of note, the concentration of LNAs in this study is low (0.03 mg/kg body weight) in comparison with other LNA-based studies (0.5–25 mg/kg body weight),[40,62,124] and increasing the dose to 0.15 mg/kg (intracoronary application) did not further improve the therapeutic benefit. Catheter-based delivery of antimiRs to the heart, however, was not fully preventing systemic inhibitory effects of the delivered antimiRs because liver and kidney miR-92a expression was still suppressed. An alternative strategy may be to inject the RNA therapeutic intramuscularly because such a delivery strategy was shown to be more efficient for cell therapy. Furthermore, drug-eluting stents or balloons may additionally be used to deliver RNA therapeutics to the vascular wall. Indeed, Wang et al[73] achieved local miR-21 suppression by using anti-21–coated stents and reported less unwanted side effects in comparison with systemic miR-21 inhibition, whereas the therapeutic effect was comparable for both strategies.

Viral Vector-Mediated RNA Therapeutic Delivery

A further successful concept of tissue-specific enrichment of miRNAs or lncRNAs is the cell type-specific delivery by viral vectors (Figure 2; Table).[88] Eulalio et al,[68] for example, used in their study AAV serotype 9 for a prolonged overexpression of the 2 proproliferative miRNAs miR-590 and miR-199a in cardiomyocytes. Furthermore, to provide another example, the same strategy was also used successfully for restoration of miR-1 level to prevent cardiac hypertrophy.[89] AAV serotype 9 viruses are targeting preferentially cardiomyocytes and were repeatedly used for overexpression of miRNAs, shRNAs, and mRNAs in proof-of-concept studies in small animals.[31,55,68,89] However, the disappointing efficiency of AAV-mediated gene therapy in recent human trials (CUPID trial [Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease])[125] suggests that a further improvement of vector efficiency is necessary. In regard to the vasculature, the current tools are even less advanced. Although in principle vectors targeting endothelial cells have been developed,[126] these vectors target liver but not cardiac endothelial cells. A novel approach on virus-based tissue delivery was published recently. Herein, large polypeptides were covalently couple to the surface of fully assembled AAV particles via split intein-mediated protein trans-splicing. By this method, gene transfer to specific cell types was achieved by using scFvs (single-chain variable fragments) or DARPins (designed ankyrin repeat proteins) that display a high affinity to cell surface receptors selectively expressed on the surface of target cells. Importantly, in this study, protein trans-splicing–based AAVs exhibited significantly less gene transfer into target receptor-negative cells than AAVs displaying the same targeting ligand but coupled genetically.[127]

Modification of RNA Therapeutics to Achieve Tissue Enrichment

Another strategy either to achieve locally restricted activity or to improve tissue-specific uptake of RNA therapeutics is chemical modification of the ASO itself (Figure 2; Table). Light-inducible antimiRs against miR-92a were successfully used to treat impaired wound healing in diabetic mice.[67] Here, miR-92a was efficiently downregulated in murine skin by administration and activation of light-inducible antimiR-92a that was as efficient as the treatment with conventional, not activatable, antimiRs. Furthermore, miR-92a levels in other organs, like kidney or liver, were mostly unchanged, indicating an advantage of local activatable antimiRs over conventional antimiRs. Nevertheless, the feasibility of light induction of RNA therapeutics in internal and well-perfused organs, such as the heart, needs to be studied in future experiments.

Moreover, miRNAs can be linked to aptamers, which are RNA molecules that bind to specific receptors on the cell surface thereby allowing a cell-type specific enrichment of a given antimiR or miRNA mimic. Aptamer-mediated delivery of miRNAs was shown to date for endothelial cells and in tumor models. In 1 study, miR-126 was linked to an aptamer binding the ubiquitously expressed transferrin receptor.[64] However, evidence that this is feasible in the cardiovascular system in vivo is lacking to date. The use of the transferrin receptor miR-126 chimera or the combination of the delivery strategy with other endothelial- or tumor-specific aptamers may provide an interesting therapeutic option to treat vascular disease conditions or cancer. A combination of aptamer-mediated cell type-specific enrichment and microparticle-based enhanced cellular uptake of miRNA mimics (encapsulation of RNA therapeutics will be

discussed in the next paragraph) was shown by Ma et al[66] to treat atherosclerosis. In this study, porous silicon multistage vectors, which are micrometer-sized nanoporous microparticles, were loaded with the miRNAs miR-146a and miR-181b and were additionally linked to a thioaptamer binding E-selectin to deliver miRNAs specifically to activated endothelial cells.

Encapsulation of RNA Therapeutics

A promising option to improve cellular uptake of RNA therapeutics is encapsulation of the substances (Figure 2; Table). To date, several molecular targeting strategies have been experimentally tested. For example, intracoronary administration of antagomiR-92a encapsulated in specific microspheres (9 µm poly-D,L-lactide-co-glycolide) was shown to preferentially target the vasculature and augment cardiac functions in pigs.[19] A nanoparticle-based delivery strategy was shown for the overexpression of miR-126-5p via miRNA mimics. Augmenting miR-126 levels rescued endothelial cell proliferation and thereby limited atherosclerosis.[41] In another study, miR-181b mimics were mixed with lipofectamine before injection, and systemic delivery inhibited the formation of atherosclerosis through cell-specific mechanisms in the vascular endothelium.[43] The abovementioned study by Mo et al tested the concept of miR-146a and miR-181b overexpression to treat atherosclerosis by using an E-selectin–targeting multistage vector to inflamed endothelium that covers atherosclerotic plaque. Cy5-conjugated miR-146a and miR-181b were packaged in polyethylene glycol-polyethyleneimine nanoparticles and loaded into E-selectin–targeting multistage vector microparticles.[66] Finally, recently, Lesizza et al[128] used successfully synthetic lipid formulations equipped with hsa-miR-199a-3p and hsa-miR-590-3p mimics to improve cardiac function after AMI by a single intracardiac injection. Hence, a single administration of synthetic miRNA lipid formulations is sufficient to stimulate cardiac repair and restoration of cardiac function.

Despite of the positive examples mentioned above, specific strategies for heart tissue enrichment of noncoding RNA therapeutics, especially for ASOs, are still sparse. Targeting of hepatic tissue, on the contrary, can be achieved by LNP (lipid nanoparticle) containing ionizable amino lipids, which has become the leading delivery strategy for siRNA, with several products already in clinical trials. Intravenous application of LNP siRNAs induced hepatocyte gene silencing at doses as low as 0.005 mg/kg body weight in animal models.[75] The tumor-suppressive miRNA miR-34 is delivered via mimics encapsulated in a liposomal nanoparticle formulation with a diameter of ≈120 nm, called SMARTICLES, and is currently tested in phase I clinical trials. This delivery strategy was shown to be the best in combining efficacy, bio distribution, and safety in comparison with other packaging strategies.[129] Moreover, conjugation of ASOs to GN3 (N-acetyl galactosamine)—a high-affinity ligand for the hepatocyte-specific ASGPR (asialoglycoprotein receptor)—enhanced the potency of 6- to 10-fold compared with unmodified substances in mouse liver.[65]

Clinical Perspective

Targeting of noncoding RNAs may be a promising option to develop novel therapeutic strategies for cardiovascular diseases. Although the development of RNA therapeutics, such as ASO or siRNAs, revealed obstacles in the past, recent clinical trials suggest that the currently used chemical modifications and strategies for targeting have improved the efficiency and reduced risk for unwanted adverse effects. Recently, the results of the phase 2, multicenter, double-blind, placebo-controlled, dose-escalation trial ORION-1 (A Placebo-Controlled, Double-Blind, Randomized Trial to Compare the Effect of Different Doses of ALN-PCSSC Given as Single or Multiple Subcutaneous Injections in Subjects With High Cardiovascular Risk and Elevated LDL-C) were reported assessing the efficacy of LDL (low-density lipoprotein) lowering by siRNAs targeting PCSK9 (named inclisiran) in 501 patients with high cardiovascular risk.[130] Inclisiran is a long-acting siRNA that is conjugated to triantennary N-acetylgalactosamine carbohydrates, which bind to abundant liver-expressed ASGPRs, leading to inclisiran uptake into hepatocytes. The siRNA is modified with a combination of phosphorothioate, 2′-O-methyl nucleotide, and 2′-fluoro nucleotide modifications to improve molecular stability. Two doses of 300 mg of inclisiran resulted in a profound and long-term (≤240 days) suppression of PCSK9 and LDL cholesterol. Adverse events were mostly mild or moderate and were similar in placebo and treatment groups (both 76%).[130] Symptoms of immune activation, which is often a concern with RNA therapeutics, were rare, and no effects on platelet levels were observed. Transient elevations in hepatic enzyme levels were detected in 3 patients receiving inclisiran. Injection-site reactions occurred in 4% of the patients who received 1 dose of inclisiran and 7% of the patients who received 2 doses of inclisiran. These rates are similar to injection-site reactions observed after monoclonal antibody therapies against PCSK9 (eg, 5.9% in the ODYSEE trial [A Randomized, Double-Blind, Placebo-Controlled, Parallel-Group Study to Evaluate the Effect of Alirocumab (SAR236553/REGN727) on the Occurrence of Cardiovascular Events in Patients Who Have Recently Experienced an Acute Coronary Syndrome][131]) and are in sharp contrast to previous ASO therapies, which led to rates of ≈80% of injection-site reactions (eg, 76% with mipomersen).[132] Although this siRNA did not target an miRNA or lncRNA in the cardiovascular system, the study suggests that current-generation siRNAs seem to be much improved with respect to efficacy and safety.

With respect to antimiRs, only 1 clinical study is published to date showing that targeting liver miR-122 with LNA-based antimiRs (mirvarsen) is effective and safe.[74] In another clinical program, effects of an antimiR against miR-21 (RG-012) on renal dysfunction associated with Alport syndrome (kidney fibrosis) is tested. The substance was tested in a phase 1 multiple ascending dose trial in healthy volunteers, and patients are currently recruited in a placebo-controlled phase 2 trial (HERA study [Herceptin Adjuvant]; NCT02855268).

Conclusions

Despite these promising clinical data, particularly on the use of siRNAs for lipid lowering, targeting the cardiovascular system may be more challenging because of the higher doses required to reach therapeutically relevant concentrations in the heart. Strategies to improve delivery are under investigation and likely will be important for translation of the proof-of-concept studies.

Moreover, there is a need to better understand the biology and functions of noncoding RNAs when exploring their therapeutic effects. Particularly the lncRNA field is in its infancies, and little mechanistic insights are available, and only few genetic studies provide confirmatory evidence for their functional role in disease models. But even for miRNAs, which have a well-established mechanism of action, we need a deeper understanding of cell type-specific functions and target regulation. This will be particularly important when seeking for efficacy end points in early clinical trials. Obviously, this relatively new field of research is challenging, and we may stumble on the way to translation. However, "Challenges are meant to be met and overcome" (Liu Xiang) hopefully allowing the success of these novel attempts to combat cardiovascular disease.

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Disclosures

A. Bonauer and S. Dimmeler have a patent on the use of microRNA-92a for treating cardiovascular disease. S. Dimmeler has applied for a patent on hypoxia-regulated long noncoding RNAs, is a member of the scientific advisory board of miRagen Therapeutics, and has a research collaboration with Servier. The other authors report no conflicts.

References

1. Small EM, Olson EN. Pervasive roles of microRNAs in cardiovascular biology. *Nature*. 2011;469:336–342.
2. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*. 2004;5:522–531.
3. Thum T. MicroRNA therapeutics in cardiovascular medicine. *EMBO Mol Med*. 2012;4:3–14.
4. Boon RA, Dimmeler S. MicroRNAs in myocardial infarction. *Nat Rev Cardiol*. 2015;12:135–142.
5. Iyer MK, Niknafs YS, Malik R, et al. The landscape of long noncoding RNAs in the human transcriptome. *Nat Genet*. 2015;47:199–208.
6. Boon RA, Jaé N, Holdt L, Dimmeler S. Long noncoding RNAs: from clinical genetics to therapeutic targets? *J Am Coll Cardiol*. 2016;67:1214–1226.
7. Ulitsky I, Bartel DP. lincRNAs: genomics, evolution, and mechanisms. *Cell*. 2013;154:26–46.
8. Perry RB-T, Ulitsky I. The functions of long noncoding RNAs in development and stem cells. *Development*. 2016;143:3882–3894.
9. Thum T, Condorelli G. Long noncoding RNAs and microRNAs in cardiovascular pathophysiology. *Circ Res*. 2015;116:751–762.
10. van Rooij E, Olson EN. MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. *Nat Rev Drug Discov*. 2012;11:860–872.
11. Schober A, Weber C. Mechanisms of microRNAs in atherosclerosis. *Annu Rev Pathol*. 2016;11:583–616.
12. Hullinger TG, Montgomery RL, Seto AG, Dickinson BA, Semus HM, Lynch JM, Dalby CM, Robinson K, Stack C, Latimer PA, Hare JM, Olson EN, van Rooij E. Inhibition of miR-15 protects against cardiac ischemic injury. *Circ Res*. 2012;110:71–81.
13. Porrello ER, Mahmoud AI, Simpson E, Johnson BA, Grinsfelder D, Canseco D, Mammen PP, Rothermel BA, Olson EN, Sadek HA. Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. *Proc Natl Acad Sci USA*. 2013;110:187–192.
14. Boon RA, Iekushi K, Lechner S, et al. MicroRNA-34a regulates cardiac ageing and function. *Nature*. 2013;495:107–110.
15. Bernardo BC, Gao XM, Winbanks CE, Boey EJH, Tham YK, Kiriazis H, Gregorevic P, Obad S, Kauppinen S, Du XJ, Lin RCY, McMullen JR. Therapeutic inhibition of the miR-34 family attenuates pathological cardiac remodeling and improves heart function. *Proc Natl Acad Sci USA*. 2012;109:17615–17620.
16. Yang Y, Cheng HW, Qiu Y, Dupee D, Noonan M, Lin YD, Fisch S, Unno K, Sereti KI, Liao R. MicroRNA-34a plays a key role in cardiac repair and regeneration following myocardial infarction. *Circ Res*. 2015;117:450–459.
17. Bonauer A, Carmona G, Iwasaki M, et al. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. *Science*. 2009;324:1710–1713.
18. Hinkel R, Penzkofer D, Zühlke S, Fischer A, Husada W, Xu QF, Baloch E, van Rooij E, Zeiher AM, Kupatt C, Dimmeler S. Inhibition of microRNA-92a protects against ischemia/reperfusion injury in a large-animal model. *Circulation*. 2013;128:1066–1075.
19. Bellera N, Barba I, Rodriguez-Sinovas A, Ferret E, Asín MA, Gonzalez-Alujas MT, Pérez-Rodon J, Esteves M, Fonseca C, Toran N, Garcia Del Blanco B, Pérez A, Garcia-Dorado D. Single intracoronary injection of encapsulated antagomir-92a promotes angiogenesis and prevents adverse infarct remodeling. *J Am Heart Assoc*. 2014;3:e000946.
20. Fiedler J, Jazbutyte V, Kirchmaier BC, et al. MicroRNA-24 regulates vascularity after myocardial infarction. *Circulation*. 2011;124:720–730.
21. Thum T. Noncoding RNAs and myocardial fibrosis. *Nat Rev Cardiol*. 2014;11:655–663.
22. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA, Olson EN. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci USA*. 2008;105:13027–13032.
23. Thum T, Gross C, Fiedler J, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. *Nature*. 2008;456:980–984.
24. Patrick DM, Montgomery RL, Qi X, Obad S, Kauppinen S, Hill JA, van Rooij E, Olson EN. Stress-dependent cardiac remodeling occurs in the absence of microRNA-21 in mice. *J Clin Invest*. 2010;120:3912–3916.
25. Thum T, Chau N, Bhat B, Gupta SK, Linsley PS, Bauersachs J, Engelhardt S. Comparison of different miR-21 inhibitor chemistries in a cardiac disease model. *J Clin Invest*. 2011;121:461–462; author reply 462–463.
26. Gupta SK, Itagaki R, Zheng X, et al. miR-21 promotes fibrosis in an acute cardiac allograft transplantation model. *Cardiovasc Res*. 2016;110:215–226.
27. Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, Thannickal VJ, Kaminski N, Abraham E. miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. *J Exp Med*. 2010;207:1589–1597.
28. Zhang J, Jiao J, Cermelli S, Muir K, Jung KH, Zou R, Rashid A, Gagea M, Zabludoff S, Kalluri R, Beretta L. miR-21 inhibition reduces liver fibrosis and prevents tumor development by inducing apoptosis of CD24+ progenitor cells. *Cancer Res*. 2015;75:1859–1867.
29. Wahlquist C, Jeong D, Rojas-Muñoz A, Kho C, Lee A, Mitsuyama S, van Mil A, Park WJ, Sluijter JPG, Doevendans PAF, Hajjar RJ, Mercola M. Inhibition of miR-25 improves cardiac contractility in the failing heart. *Nature*. 2014;508:531–535.
30. Dirkx E, Gladka MM, Philippen LE, et al. Nfat and miR-25 cooperate to reactivate the transcription factor Hand2 in heart failure. *Nat Cell Biol*. 2013;15:1282–1293.
31. Carè A, Catalucci D, Felicetti F, et al. MicroRNA-133 controls cardiac hypertrophy. *Nat Med*. 2007;13:613–618.
32. Besser J, Malan D, Wystub K, Bachmann A, Wietelmann A, Sasse P, Fleischmann BK, Braun T, Boettger T. MiRNA-1/133a clusters regulate adrenergic control of cardiac repolarization. *PLoS One*. 2014;9:e113449.
33. Ucar A, Gupta SK, Fiedler J, et al. The miRNA-212/132 family regulates both cardiac hypertrophy and cardiomyocyte autophagy. *Nat Commun*. 2012;3:1078.
34. van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science*. 2007;316:575–579.
35. Boon RA, Hergenreider E, Dimmeler S. Atheroprotective mechanisms of shear stress-regulated microRNAs. *Thromb Haemost*. 2012;108:616–620.
36. Qin X, Wang X, Wang Y, Tang Z, Cui Q, Xi J, Li YS, Chien S, Wang N. MicroRNA-19a mediates the suppressive effect of laminar flow on cyclin D1 expression in human umbilical vein endothelial cells. *Proc Natl Acad Sci USA*. 2010;107:3240–3244.
37. Wang KC, Garmire LX, Young A, Nguyen P, Trinh A, Subramaniam S, Wang N, Shyy JY, Li YS, Chien S. Role of microRNA-23b in flow-regulation of Rb phosphorylation and endothelial cell growth. *Proc Natl Acad Sci USA*. 2010;107:3234–3239.
38. Ji R, Cheng Y, Yue J, Yang J, Liu X, Chen H, Dean DB, Zhang C. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. *Circ Res*. 2007;100:1579–1588.
39. Maegdefessel L, Azuma J, Toh R, Merk DR, Deng A, Chin JT, Raaz U, Schoelmerich AM, Raiesdana A, Leeper NJ, McConnell M V, Dalman RL, Spin JM, Tsao PS. Inhibition of microRNA-29b reduces murine abdominal aortic aneurysm development. *J Clin Invest*. 2012;122:497–506.

40. Boon RA, Seeger T, Heydt S, Fischer A, Hergenreider E, Horrevoets AJG, Vinciguerra M, Rosenthal N, Sciacca S, Pilato M, van Heijningen P, Essers J, Brandes RP, Zeiher AM, Dimmeler S. MicroRNA-29 in aortic dilation: implications for aneurysm formation. *Circ Res*. 2011;109:1115–1119.
41. Schober A, Nazari-Jahantigh M, Wei Y, Bidzhekov K, Gremse F, Grommes J, Megens RTA, Heyll K, Noels H, Hristov M, Wang S, Kiessling F, Olson EN, Weber C. MicroRNA-126-5p promotes endothelial proliferation and limits atherosclerosis by suppressing Dlk1. *Nat Med*. 2014;20:368–376.
42. Li K, Ching D, Luk FS, Raffai RL. Apolipoprotein E enhances microRNA-146a in monocytes and macrophages to suppress nuclear factor-κB-driven inflammation and atherosclerosis. *Circ Res*. 2015;117:e1–e11.
43. Sun X, He S, Wara AKM, Icli B, Shvartz E, Tesmenitsky Y, Belkin N, Li D, Blackwell TS, Sukhova GK, Croce K, Feinberg MW. Systemic delivery of microRNA-181b inhibits nuclear factor-κB activation, vascular inflammation, and atherosclerosis in apolipoprotein E-deficient mice. *Circ Res*. 2014;114:32–40.
44. Fang Y, Shi C, Manduchi E, Civelek M, Davies PF. MicroRNA-10a regulation of proinflammatory phenotype in athero-susceptible endothelium in vivo and in vitro. *Proc Natl Acad Sci USA*. 2010;107:13450–13455.
45. Krützfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M. Silencing of microRNAs in vivo with "antagomirs". *Nature*. 2005;438:685–689.
46. Lennox KA, Behlke MA. A direct comparison of anti-microRNA oligonucleotide potency. *Pharm Res*. 2010;27:1788–1799.
47. Elmén J, Lindow M, Schütz S, Lawrence M, Petri A, Obad S, Lindholm M, Hedtjärn M, Hansen HF, Berger U, Gullans S, Kearney P, Sarnow P, Straarup EM, Kauppinen S. LNA-mediated microRNA silencing in non-human primates. *Nature*. 2008;452:896–899.
48. Elmén J, Lindow M, Silahtaroglu A, Bak M, Christensen M, Lind-Thomsen A, Hedtjärn M, Hansen JB, Hansen HF, Straarup EM, McCullagh K, Kearney P, Kauppinen S. Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. *Nucleic Acids Res*. 2008;36:1153–1162.
49. Obad S, dos Santos CO, Petri A, Heidenblad M, Broom O, Ruse C, Fu C, Lindow M, Stenvang J, Straarup EM, Hansen HF, Koch T, Pappin D, Hannon GJ, Kauppinen S. Silencing of microRNA families by seed-targeting tiny LNAs. *Nat Genet*. 2011;43:371–378.
50. Nahar S, Singh A, Morihiro K, Moai Y, Kodama T, Obika S, Maiti S. Systematic evaluation of biophysical and functional characteristics of selenomethylene-locked nucleic acid-mediated inhibition of miR-21. *Biochemistry*. 2016;55:7023–7032.
51. Meng L, Liu C, Lü J, et al. Small RNA zippers lock miRNA molecules and block miRNA function in mammalian cells. *Nat Commun*. 2017;8:13964.
52. Paulasova P, Pellestor F. The peptide nucleic acids (PNAs): a new generation of probes for genetic and cytogenetic analyses. *Ann Génétique*. 2004;47:349–358.
53. Fabani MM, Abreu-Goodger C, Williams D, Lyons PA, Torres AG, Smith KG, Enright AJ, Gait MJ, Vigorito E. Efficient inhibition of miR-155 function in vivo by peptide nucleic acids. *Nucleic Acids Res*. 2010;38:4466–4475. doi: 10.1093/nar/gkq160.
54. Behlke MA. Chemical modification of siRNAs for in vivo use. *Oligonucleotides*. 2008;18:305–319.
55. Li YG, Zhang PP, Jiao KL, Zou YZ. Knockdown of microRNA-181 by lentivirus mediated siRNA expression vector decreases the arrhythmogenic effect of skeletal myoblast transplantation in rat with myocardial infarction. *Microvasc Res*. 2009;78:393–404.
56. Choi WY, Giraldez AJ, Schier AF. Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. *Science*. 2007;318:271–274.
57. Messina A, Langlet F, Chachlaki K, Roa J, Rasika S, Jouy N, Gallet S, Gaytan F, Parkash J, Tena-Sempere M, Giacobini P, Prevot V. A microRNA switch regulates the rise in hypothalamic GnRH production before puberty. *Nat Neurosci*. 2016;19:835–844.
58. Iaconetti C, Polimeni A, Sorrentino S, Sabatino J, Pironti G, Esposito G, Curcio A, Indolfi C. Inhibition of miR-92a increases endothelial proliferation and migration in vitro as well as reduces neointimal proliferation in vivo after vascular injury. *Basic Res Cardiol*. 2012;107:296.
59. Loyer X, Potteaux S, Vion AC, Guérin CL, Boulkroun S, Rautou PE, Ramkhelawon B, Esposito B, Dalloz M, Paul JL, Julia P, Maccario J, Boulanger CM, Mallat Z, Tedgui A. Inhibition of microRNA-92a prevents endothelial dysfunction and atherosclerosis in mice. *Circ Res*. 2014;114:434–443.
60. Jeong D, Yoo J, Lee P, Kepreotis SV, Lee A, Wahlquist C, Brown BD, Kho C, Mercola M, Hajjar RJ. miR-25 tough decoy enhances cardiac function in heart failure. *Mol Ther*. 2017. http://www.ncbi.nlm.nih.gov/pubmed/29273502.
61. Ren XP, Wu J, Wang X, Sartor MA, Jones K, Qian J, Nicolaou P, Pritchard TJ, Fan GC. MicroRNA-320 is involved in the regulation of cardiac ischemia/reperfusion injury by targeting heat-shock protein 20. *Circulation*. 2009;119:2357–2366.
62. Montgomery RL, Hullinger TG, Semus HM, Dickinson BA, Seto AG, Lynch JM, Stack C, Latimer PA, Olson EN, van Rooij E. Therapeutic inhibition of miR-208a improves cardiac function and survival during heart failure. *Circulation*. 2011;124:1537–1547.
63. McLendon JM, Joshi SR, Sparks J, Matar M, Fewell JG, Abe K, Oka M, McMurtry IF, Gerthoffer WT. Lipid nanoparticle delivery of a microRNA-145 inhibitor improves experimental pulmonary hypertension. *J Control Release*. 2015;210:67–75.
64. Rohde JH, Weigand JE, Suess B, Dimmeler S. A universal aptamer chimera for the delivery of functional microRNA-126. *Nucleic Acid Ther*. 2015;25:141–151.
65. Prakash TP, Graham MJ, Yu J, et al. Targeted delivery of antisense oligonucleotides to hepatocytes using triantennary N-acetyl galactosamine improves potency 10-fold in mice. *Nucleic Acids Res*. 2014;42:8796–8807.
66. Ma S, Tian XY, Zhang Y, Mu C, Shen H, Bismuth J, Pownall HJ, Huang Y, Wong WT. E-selectin-targeting delivery of microRNAs by microparticles ameliorates endothelial inflammation and atherosclerosis. *Sci Rep*. 2016;6:22910.
67. Lucas T, Schäfer F, Müller P, Eming SA, Heckel A, Dimmeler S. Light-inducible antimiR-92a as a therapeutic strategy to promote skin repair in healing-impaired diabetic mice. *Nat Commun*. 2017;8:15162.
68. Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, Zacchigna S, Giacca M. Functional screening identifies miRNAs inducing cardiac regeneration. *Nature*. 2012;492:376–381.
69. Wu G, Cai J, Han Y, et al. LincRNA-p21 regulates neointima formation, vascular smooth muscle cell proliferation, apoptosis, and atherosclerosis by enhancing p53 activity. *Circulation*. 2014;130:1452–1465.
70. Wang K, Sun T, Li N, Wang Y, Wang JX, Zhou LY, Long B, Liu CY, Liu F, Li PF. MDRL lncRNA regulates the processing of miR-484 primary transcript by targeting miR-361. *PLoS Genet*. 2014;10:e1004467.
71. Wang K, Long B, Zhou LY, Liu F, Zhou QY, Liu CY, Fan YY, Li PF. CARL lncRNA inhibits anoxia-induced mitochondrial fission and apoptosis in cardiomyocytes by impairing miR-539-dependent PHB2 downregulation. *Nat Commun*. 2014;5:3596.
72. Burdick AD, Sciabola S, Mantena SR, Hollingshead BD, Stanton R, Warneke JA, Zeng M, Martsen E, Medvedev A, Makarov SS, Reed LA, Davis JW, Whiteley LO. Sequence motifs associated with hepatotoxicity of locked nucleic acid–modified antisense oligonucleotides. *Nucleic Acids Res*. 2014;42:4882–4891.
73. Wang D, Deuse T, Stubbendorff M, et al. Local MicroRNA modulation using a novel anti-miR-21-eluting stent effectively prevents experimental in-stent restenosis. *Arterioscler Thromb Vasc Biol*. 2015;35:1945–1953.
74. Janssen HLA, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, van der Meer AJ, Patick AK, Chen A, Zhou Y, Persson R, King BD, Kauppinen S, Levin AA, Hodges MR. Treatment of HCV infection by targeting microRNA. *N Engl J Med*. 2013;368:1685–1694.
75. Jayaraman M, Ansell SM, Mui BL, et al. Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo. *Angew Chem Int Ed Engl*. 2012;51:8529–8533.
76. Geary RS, Wancewicz E, Matson J, Pearce M, Siwkowski A, Swayze E, Bennett F. Effect of dose and plasma concentration on liver uptake and pharmacologic activity of a 2'-methoxyethyl modified chimeric antisense oligonucleotide targeting PTEN. *Biochem Pharmacol*. 2009;78:284–291.
77. Crooke ST, Wang S, Vickers TA, Shen W, Liang XH. Cellular uptake and trafficking of antisense oligonucleotides. *Nat Biotechnol*. 2017;35:230–237.
78. Wagenaar TR, Tolstykh T, Shi C, et al. Identification of the endosomal sorting complex required for transport-I (ESCRT-I) as an important modulator of anti-miR uptake by cancer cells. *Nucleic Acids Res*. 2015;43:1204–1215.
79. Berman CL, Barros SA, Galloway SM, Kasper P, Oleson FB, Priestley CC, Sweder KS, Schlosser MJ, Sobol Z. OSWG recommendations for genotoxicity testing of novel oligonucleotide-based therapeutics. *Nucleic Acid Ther*. 2016;26:73–85.
80. Peacock H, Fucini R V, Jayalath P, Ibarra-Soza JM, Haringsma HJ, Flanagan WM, Willingham A, Beal PA. Nucleobase and ribose modifications control immunostimulation by a microRNA-122-mimetic RNA. *J Am Chem Soc*. 2011;133:9200–9203.
81. Monteith DK, Geary RS, Leeds JM, Johnston J, Monia BP, Levin AA. Preclinical evaluation of the effects of a novel antisense compound targeting C-raf kinase in mice and monkeys. *Toxicol Sci*. 1998;46:365–375.

82. Levin AA. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. *Biochim Biophys Acta*. 1999;1489:69–84.
83. Henry SP, Beattie G, Yeh G, Chappel A, Giclas P, Mortari A, Jagels MA, Kornbrust DJ, Levin AA. Complement activation is responsible for acute toxicities in rhesus monkeys treated with a phosphorothioate oligodeoxynucleotide. *Int Immunopharmacol*. 2002;2:1657–1666.
84. Henry SP, Jagels MA, Hugli TE, Manalili S, Geary RS, Giclas PC, Levin AA. Mechanism of alternative complement pathway dysregulation by a phosphorothioate oligonucleotide in monkey and human serum. *Nucleic Acid Ther*. 2014;24:326–335.
85. Flierl U, Nero TL, Lim B, et al. Phosphorothioate backbone modifications of nucleotide-based drugs are potent platelet activators. *J Exp Med*. 2015;212:129–137.
86. Sewing S, Roth AB, Winter M, Dieckmann A, Bertinetti-Lapatki C, Tessier Y, McGinnis C, Huber S, Koller E, Ploix C, Reed JC, Singer T, Rothfuss A. Assessing single-stranded oligonucleotide drug-induced effects in vitro reveals key risk factors for thrombocytopenia. *PLoS One*. 2017;12:e0187574.
87. Swayze EE, Siwkowski AM, Wancewicz EV, Migawa MT, Wyrzykiewicz TK, Hung G, Monia BP, Bennett CF. Antisense oligonucleotides containing locked nucleic acid improve potency but cause significant hepatotoxicity in animals. *Nucleic Acids Res*. 2007;35:687–700.
88. Lucas T, Dimmeler S. RNA therapeutics for treatment of cardiovascular diseases. *Circ Res*. 2016;119:794–797.
89. Karakikes I, Chaanine AH, Kang S, Mukete BN, Jeong D, Zhang S, Hajjar RJ, Lebeche D. Therapeutic cardiac-targeted delivery of miR-1 reverses pressure overload-induced cardiac hypertrophy and attenuates pathological remodeling. *J Am Heart Assoc*. 2013;2:e000078–e000078.
90. van Rooij E, Kauppinen S. Development of microRNA therapeutics is coming of age. *EMBO Mol Med*. 2014;6:851–864.
91. Yang KC, Yamada KA, Patel AY, Topkara VK, George I, Cheema FH, Ewald GA, Mann DL, Nerbonne JM. Deep RNA sequencing reveals dynamic regulation of myocardial noncoding RNAs in failing human heart and remodeling with mechanical circulatory support. *Circulation*. 2014;129:1009–1021.
92. Sallam T, Sandhu J, Tontonoz P. Long noncoding RNA discovery in cardiovascular disease: decoding form to function. *Circ Res*. 2018;122:155–166.
93. Hennessy EJ. Cardiovascular disease and long noncoding RNAs: tools for unraveling the mystery lnc-ing RNA and phenotype. *Circ Cardiovasc Genet*. 2017;10:e001556.
94. Li Y, Maegdefessel L. Non-coding RNA contribution to thoracic and abdominal aortic aneurysm disease development and progression. *Front Physiol*. 2017;8:429.
95. Viereck J, Thum T. Long noncoding RNAs in pathological cardiac remodeling. *Circ Res*. 2017;120:262–264.
96. Ishii N, Ozaki K, Sato H, Mizuno H, Saito S, Takahashi A, Miyamoto Y, Ikegawa S, Kamatani N, Hori M, Saito S, Nakamura Y, Tanaka T. Identification of a novel non-coding RNA, MIAT, that confers risk of myocardial infarction. *J Hum Genet*. 2006;51:1087–1099.
97. Zhu XH, Yuan YX, Rao SL, Wang P. LncRNA MIAT enhances cardiac hypertrophy partly through sponging miR-150. *Eur Rev Med Pharmacol Sci*. 2016;20:3653–3660.
98. Qu X, Du Y, Shu Y, Gao M, Sun F, Luo S, Yang T, Zhan L, Yuan Y, Chu W, Pan Z, Wang Z, Yang B, Lu Y. MIAT is a pro-fibrotic long non-coding RNA governing cardiac fibrosis in post-infarct myocardium. *Sci Rep*. 2017;7:42657.
99. Yan B, Yao J, Liu JY, Li XM, Wang XQ, Li YJ, Tao ZF, Song YC, Chen Q, Jiang Q. lncRNA-MIAT regulates microvascular dysfunction by functioning as a competing endogenous RNA. *Circ Res*. 2015;116:1143–1156.
100. Wang K, Liu CY, Zhou LY, Wang JX, Wang M, Zhao B, Zhao WK, Xu SJ, Fan LH, Zhang XJ, Feng C, Wang CQ, Zhao YF, Li PF. APF lncRNA regulates autophagy and myocardial infarction by targeting miR-188-3p. *Nat Commun*. 2015;6:6779.
101. Wang K, Liu F, Zhou LY, Long B, Yuan SM, Wang Y, Liu CY, Sun T, Zhang XJ, Li PF. The long noncoding RNA CHRF regulates cardiac hypertrophy by targeting miR-489. *Circ Res*. 2014;114:1377–1388.
102. Viereck J, Kumarswamy R, Foinquinos A, et al. Long noncoding RNA Chast promotes cardiac remodeling. *Sci Transl Med*. 2016;8:326ra22.
103. Han P, Li W, Lin CH, et al. A long noncoding RNA protects the heart from pathological hypertrophy. *Nature*. 2014;514:102–106.
104. Piccoli MT, Gupta SK, Viereck J, Foinquinos A, Samolovac S, Kramer FL, Garg A, Remke J, Zimmer K, Batkai S, Thum T. Inhibition of the cardiac fibroblast-enriched lncRNA Meg3 prevents cardiac fibrosis and diastolic dysfunction. *Circ Res*. 2017;121:575–583.
105. Holdt LM, Teupser D. Recent studies of the human chromosome 9p21 locus, which is associated with atherosclerosis in human populations. *Arterioscler Thromb Vasc Biol*. 2012;32:196–206.
106. Holdt LM, Hoffmann S, Sass K, et al. Alu elements in ANRIL non-coding RNA at chromosome 9p21 modulate atherogenic cell functions through trans-regulation of gene networks. *PLoS Genet*. 2013;9:e1003588.
107. Gao W, Zhu M, Wang H, Zhao S, Zhao D, Yang Y, Wang ZM, Wang F, Yang ZJ, Lu X, Wang LS. Association of polymorphisms in long noncoding RNA H19 with coronary artery disease risk in a Chinese population. *Mutat Res*. 2015;772:15–22.
108. Kim DK, Zhang L, Dzau VJ, Pratt RE. H19, a developmentally regulated gene, is reexpressed in rat vascular smooth muscle cells after injury. *J Clin Invest*. 1994;93:355–360.
109. Han DK, Khaing ZZ, Pollock RA, Haudenschild CC, Liau G. H19, a marker of developmental transition, is reexpressed in human atherosclerotic plaques and is regulated by the insulin family of growth factors in cultured rabbit smooth muscle cells. *J Clin Invest*. 1996;97:1276–1285.
110. Kallen AN, Zhou XB, Xu J, Qiao C, Ma J, Yan L, Lu L, Liu C, Yi JS, Zhang H, Min W, Bennett AM, Gregory RI, Ding Y, Huang Y. The imprinted H19 lncRNA antagonizes let-7 microRNAs. *Mol Cell*. 2013;52:101–112.
111. Michalik KM, You X, Manavski Y, Doddaballapur A, Zörnig M, Braun T, John D, Ponomareva Y, Chen W, Uchida S, Boon RA, Dimmeler S. Long noncoding RNA MALAT1 regulates endothelial cell function and vessel growth. *Circ Res.*. 2014;114:1389–1397.
112. Liu JY, Yao J, Li XM, Song YC, Wang XQ, Li YJ, Yan B, Jiang Q. Pathogenic role of lncRNA-MALAT1 in endothelial cell dysfunction in diabetes mellitus. *Cell Death Dis*. 2014;5:e1506.
113. Fiedler J, Breckwoldt K, Remmele CW, Hartmann D, Dittrich M, Pfanne A, Just A, Xiao K, Kunz M, Müller T, Hansen A, Geffers R, Dandekar T, Eschenhagen T, Thum T. Development of long noncoding RNA-based strategies to modulate tissue vascularization. *J Am Coll Cardiol*. 2015;66:2005–2015.
114. Bell RD, Long X, Lin M, Bergmann JH, Nanda V, Cowan SL, Zhou Q, Han Y, Spector DL, Zheng D, Miano JM. Identification and initial functional characterization of a human vascular cell-enriched long noncoding RNA. *Arterioscler Thromb Vasc Biol*. 2014;34:1249–1259.
115. Wahlestedt C, Salmi P, Good L, et al. Potent and nontoxic antisense oligonucleotides containing locked nucleic acids. *Proc Natl Acad Sci USA*. 2000;97:5633–5638.
116. Mangos MM, Min KL, Viazovkina E, Galarneau A, Elzagheid MI, Parniak MA, Damha MJ. Efficient RNase H-directed cleavage of RNA promoted by antisense DNA or 2'F-ANA constructs containing acyclic nucleotide inserts. *J Am Chem Soc*. 2003;125:654–661.
117. Burel SA, Hart CE, Cauntay P, et al. Hepatotoxicity of high affinity gapmer antisense oligonucleotides is mediated by RNase H1 dependent promiscuous reduction of very long pre-mRNA transcripts. *Nucleic Acids Res*. 2016;44:2093–2109. doi: 10.1093/nar/gkv1210.
118. Krieg AM. Targeting LDL cholesterol with LNA. *Mol Ther Nucleic Acids*. 2012;1:e6.
119. Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK. CRISPR RNA-guided activation of endogenous human genes. *Nat Methods*. 2013;10:977–979.
120. Batzer MA, Deininger PL. Alu repeats and human genomic diversity. *Nat Rev Genet*. 2002;3:370–379.
121. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA*. 2005;102:13944–13949.
122. Hermeking H. The miR-34 family in cancer and apoptosis. *Cell Death Differ*. 2010;17:193–199.
123. Agostini M, Knight RA. miR-34: from bench to bedside. *Oncotarget*. 2014;5:872–881.
124. Daniel JM, Penzkofer D, Teske R, Dutzmann J, Koch A, Bielenberg W, Bonauer A, Boon RA, Fischer A, Bauersachs J, van Rooij E, Dimmeler S, Sedding DG. Inhibition of miR-92a improves re-endothelialization and prevents neointima formation following vascular injury. *Cardiovasc Res*. 2014;103:564–572.
125. Greenberg B, Butler J, Felker GM, Ponikowski P, Voors AA, Desai AS, Barnard D, Bouchard A, Jaski B, Lyon AR, Pogoda JM, Rudy JJ, Zsebo KM. Calcium upregulation by percutaneous administration of gene therapy in patients with cardiac disease (CUPID 2): a randomised, multinational, double-blind, placebo-controlled, phase 2b trial. *Lancet (London, England)*. 2016;387:1178–1186.

126. Buchholz CJ, Friedel T, Büning H. Surface-engineered viral vectors for selective and cell type-specific gene delivery. *Trends Biotechnol*. 2015;33:777–790.
127. Muik A, Reul J, Friedel T, Muth A, Hartmann KP, Schneider IC, Münch RC, Buchholz CJ. Covalent coupling of high-affinity ligands to the surface of viral vector particles by protein trans-splicing mediates cell type-specific gene transfer. *Biomaterials*. 2017;144:84–94.
128. Lesizza P, Prosdocimo G, Martinelli V, Sinagra G, Zacchigna S, Giacca M. Single-dose intracardiac injection of pro-regenerative microRNAs improves cardiac function after myocardial infarction. *Circ Res.*. 2017;120:1298–1304.
129. Bader AG. miR-34 - a microRNA replacement therapy is headed to the clinic. *Front Genet*. 2012;3:120.
130. Ray KK, Landmesser U, Leiter LA, Kallend D, Dufour R, Karakas M, Hall T, Troquay RPT, Turner T, Visseren FLJ, Wijngaard P, Wright RS, Kastelein JJP. Inclisiran in patients at high cardiovascular risk with elevated LDL cholesterol. *N Engl J Med*. 2017;376:1430–1440.
131. Robinson JG, Farnier M, Krempf M, et al; ODYSSEY LONG TERM Investigators. Efficacy and safety of alirocumab in reducing lipids and cardiovascular events. *N Engl J Med*. 2015;372:1489–1499.
132. Raal FJ, Santos RD, Blom DJ, Marais AD, Charng MJ, Cromwell WC, Lachmann RH, Gaudet D, Tan JL, Chasan-Taber S, Tribble DL, Flaim JD, Crooke ST. Mipomersen, an apolipoprotein B synthesis inhibitor, for lowering of LDL cholesterol concentrations in patients with homozygous familial hypercholesterolaemia: a randomised, double-blind, placebo-controlled trial. *Lancet (London, England)*. 2010;375:998–1006.

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