RNA Therapeutics for Treatment of Cardiovascular Diseases
Promises and Challenges

Tina Lucas, Stefanie Dimmeler

Recent studies suggest that the majority of the human genome is transcribed, but only 2% account for protein coding exons. The remaining noncoding sequences include small noncoding RNAs, particularly microRNAs, which are 22 nucleotides in length. MicroRNAs posttranscriptionally control gene expression by targeting multiple mRNAs. By binding to the 3′UTR (untranslated region) of mRNAs, microRNAs induce degradation of the targeted mRNA or they block protein translation. Thereby, one microRNA is able to repress up to hundreds of genes in parallel. In addition to it, long noncoding RNAs (lncRNAs) with a length of >200 nucleotides comprise a more heterogeneous class of noncoding RNAs that include, for example, intergenic lncRNAs, antisense transcripts, and enhancer RNAs. LncRNAs primarily act as epigenetic and transcriptional regulators but may also have additional functions as microRNA sponges or regulators of splicing. Finally, alternative splicing can lead to the formation of circular RNAs, which are highly stable RNAs that may act as microRNA sponges. Among the noncoding RNAs, many of them were described to control the cardiovascular system and may comprise novel therapeutic targets for the treatment of cardiovascular diseases. However, targeting coding RNAs is far more advanced in comparison to noncoding RNAs and has Food and Drug Agency–approved products. The tools to target coding mRNAs include different flavors of antisense oligonucleotides (ASO). Silencing can be achieved by morpholinos or GapmeRs with phosphothioate-modified oxygen and 4′ nucleotides to increase stability and limit immunogenicity. That are chemically modified, for example, by 2′-O-methyl nucleotides to increase stability and limit immunogenicity. Locked nucleic acids (LNAs) are RNA nucleotides with an extra bridge connecting the 2′ oxygen and 4′ carbon in the ribose sugar, which increases the melting temperature and, thereby, augments the efficiency (for summary, see Figure 1). Most clinical studies used such agents to target genes expressed in liver to either treat liver or metabolic diseases (for review, see Sehgal et al1). For example, siRNA-mediated silencing of PCSK9, an enzyme that regulates cholesterol transport and is highly expressed in liver tissue, was recently reported to efficiently reduce low-density lipoprotein cholesterol and was safely used in a phase I clinical trial. Thus, one may consider using these or other approaches also for targeting the noncoding genome.

But, despite the enthusiasm for such novel approaches, the development of RNA therapeutics for cardiovascular disease is challenging, and hurdles for pharmaceutical development of RNA therapeutics are chemistry, toxicity, and delivery. This perspective provides a short overview about the therapeutic potential of targeting the noncoding genome and discusses the challenges in translating these novel findings into the clinic.

MicroRNA Therapeutics

Among noncoding RNAs, microRNAs are best studied and were shown to control postinfarction angiogenesis and remodeling, cardiac fibrosis and hypertrophy, arrhythmias, atherosclerosis, and metabolic disease.2 Most therapeutic approaches to block microRNA function in experimental models depend on the use of ASO, which are modified for better stability and enhanced cellular uptake. In that case, different types of molecules have been used (for review, see van Rooij and Kauppinen3), which include phosphothioate-modified antisense RNAs that are linked to cholesterol for enhanced uptake of the oligonucleotide (so-called antagoniRs) or LNA DNA mixmers (often called LNA-anti-miRs). Many studies have shown that different types of anti-miRs efficiently inhibit microRNA functions in the vascular wall and in heart tissue in mice, and some studies have also shown efficiency in larger animal models, such as pigs. Anti-miRs and antagoniRs can be administered intravenously, intraarterially, or subcutaneously and were shown to induce long-term suppression for up to 6 weeks. Repetitive dosing may be used in case one single injection is not efficient enough. Most studies used repetitive dosing of 0.5 to 25 mg/kg bodyweight LNA-based anti-miR or 8 to 80 mg/kg bodyweight antagoniRs to suppress microRNAs in the heart or the vasculature in mice, but some microRNA may be easier to be targeted (Online Table). The concentration of most anti-miRs used to efficiently repress microRNAs in the cardiovascular system is higher than what is needed for targeting organs, such as liver or kidney. In clinical trials, anti-miRs directed against the liver-enriched miR-122 were applied at concentrations between 3 and 7 mg/kg (5 weekly injections),4 but improved formulations for siRNAs delivery resulted in
Effective concentrations of <0.01 mg/kg that are required to target liver-expressed genes. If concentrations >10 mg/kg are required to target microRNAs in the cardiovascular system, the therapeutic window may overlap with a dose range, possibly leading to toxic and unspecific effects of delivered RNAs. Safety concerns include hepatotoxic or nephrotoxic effects because ASO including LNA-anti-miRs highly accumulate in these organs. Liver and kidney toxicity, however, shows a surprising sequence-specific variability. A second concern relates to ASO-induced activation of the immune response. Some ASO can be proinflammatory by nonspecifically stimulating the immune system via toll-like receptors. But specific, sequence-independent modifications have been shown to prevent those effects. These findings highlight the fact that in addition to the specific sequence of a certain ASO, also the sequence-independent modifications need to be carefully chosen and well studied. Phosphorothioate backbones were also reported to activate platelets, but we could not reproduce such findings with LNA-based anti-miRs directed against miR-92a (data not shown).

**Improvement of the Efficiency and Specificity of Anti-miRs**

Because of the modest efficiency of anti-miRs to target cardiovascular microRNAs, the development of strategies to reduce the therapeutic dose by specifically enhancing the uptake of biologically active anti-miRs to cells that contribute to cardiovascular pathologies is warranted (Figure 2). The development of cell type-specific enrichment of anti-miR uptake may additionally reduce adverse effects in case that microRNAs are ubiquitously expressed and exhibit diverse functions in different tissues. For example, microRNA inhibitors that increase cardiomyocyte proliferation and, thereby, augment cardiac regeneration, such as inhibitors of miR-15 or miR-34 family members, might lead to more tumor growth because these microRNAs are tumor suppressive. Thereby, the development of strategies for cell- or tissue-specific targeting of microRNA inhibitors may circumvent putative unwanted effects in other tissues.

Catheter-based intracoronary or retrograde delivery of anti-miRs was shown to enhance the efficiency to suppress microRNAs in heart tissue. However, catheter-based delivery of anti-miRs to the heart was found to not fully preventing systemic inhibitory effects of the delivered anti-miRs. Future studies, therefore, may address whether optimizing the catheter-based application, for example, also by intramuscular injection of anti-miRs, as it has been done for cell therapy, may provide a more specific enrichment to the heart. Drug-eluting stents or balloons may additionally be used to deliver anti-miRs or other RNA therapeutics to the vascular wall (Online Table). Alternative strategies may include local activation of anti-miRs by light, as it was shown in first proof-of-concept studies in primary human endothelial cells. But its feasibility in inlying organs, such as the heart, needs to be studied.

In addition, several molecular targeting strategies have been experimentally used, including microspheres or lipid nanoparticle-based delivery of RNAs or cell type-specific delivery by viral vectors. Intracoronary administration of antagoniR-92a encapsulated in specific microspheres (9 μm poly-d,l-lactide-co-glycolide) was shown to preferentially target the vasculature and augment cardiac function in pigs. But specific strategies for enriched delivery of ASO to the cardiovascular system are sparse. In contrast, for targeting the liver, nanoparticle containing ionizable amino lipids have become the leading delivery technology for siRNA, with several products in clinical trials.
siRNAs induced hepatocyte gene silencing at doses as low as 0.005 mg/kg bodyweight in animal models. Additionally, conjugation of ASO to N-acetyl galactosamine (GalNAc, GN3), a high-affinity ligand for the hepatocyte-specific asialoglycoprotein receptor, enhanced the potency by >10-fold compared with unmodified ASO. Similar efforts to augment the uptake to other cells might be possible and would improve the therapeutic usage of anti-miRs in cardiovascular diseases.

Overexpression of MicroRNAs

In contrast to inhibition of microRNAs, its overexpression has been even more challenging. RNA mimics, which are double-stranded RNA fragments mimicking endogenous microRNAs, were preferentially used to augment miRNA concentrations in the cardiovascular tissue in mouse models. For example overexpression of miR-126-5p mimics provided antiatherosclerotic effects by enhancing endothelial cell proliferation, and miR-181b mimic treatment was shown to improve glucose levels and insulin responsiveness in high-fat diet–fed animals. But for both studies, high concentrations of mimics were necessary with numerous serial injections to achieve significant effects (Online Table).

Long-term studies will be needed to exclude toxicity. One reason for the high dosage might be that unformulated mimics are easily degraded by endogenous, extracellular RNases. Therefore, liposomal encapsulation was tested to enhance cellular uptake and to avoid degradation. For targeting liver, this approach is currently tested in Phase I clinical trials. Thereby, the tumor suppressive microRNA, miR-34, is delivered via mimics encapsulated in a liposomal nanoparticle formulation called SMARTICLES. Alternatively, microRNAs can be loaded in microparticles for targeted delivery or linked to aptamers that are RNA molecules, which can bind to specific receptors and, thereby, allowing a cell type–specific uptake. Aptamer-based direct delivery of miRNAs was shown for endothelial cells and in tumor models, but evidence that this is feasible in the cardiovascular system in vivo is lacking to date. However, a combination of aptamer and microparticle-based delivery of endothelial protective microRNAs improved endothelial function and reduced atherosclerotic lesion formation in vivo. In this study, porous silicon multistage vectors, which are micrometer-sized nanoporous microparticles, were loaded with microRNAs and were linked to a thioaptamer binding E-selectin to...
deliver microRNAs to activated endothelial cells. The usage of adenovirus or lentiviruses is an additional possibility to enriched microRNA overexpression in a tissue or cell type-specific manner, for example, as shown for cardiomyocyte-selective AAV9 vectors.

**Targeting lncRNAs**

lncRNAs may be targeted by ASO as described for coding mRNAs. However, it must be mentioned that in contrast to microRNAs, lncRNAs are not necessarily conserved among different species. As a result, it makes it more difficult to learn from animal studies, and humanized models may be necessary to study the function of lncRNAs.

lncRNAs are often preferentially expressed in the nucleus (as opposed to the cytoplasmic localization of most mRNAs) and may be more integrated in complex structures that are not easily reachable. But, for nuclear-expressed and accessible lncRNAs, GapmeRs might be useful. Antisense LNA GapmeRs are potent ASO that induced degradation of RNase H–dependent mechanisms. Because RNase H–dependent degradation is preferentially effective in the nucleus, this approach directly targets nuclear lncRNAs, whereas siRNAs favorably function in the cytoplasm. LNA-GapmeRs have already been used for targeting coding RNAs, such as the liver-enriched PCSK9 in nonhuman primates; however, a phase I clinical study was halted. In contrast, GapmeRs against hypoxia-inducing factor 1α producing factor 1 were useful. Successful repression of specific lncRNAs in mouse models in vivo was shown by using GapmeRs against the lncRNA Malat-1 that was leading to inhibited ischemia-induced angiogenesis and GapmeRs against the lncRNA Chast that affected cardiac remodeling. For both studies, repetitive treatments and high concentrations were necessary to achieve efficient repression of the particular lncRNA. As for anti-miRs or RNA mimics, long-term studies need to prove the tolerance of GapmR treatment against lncRNAs.

Beside 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.

Overexpression of lncRNAs may be achieved by viral vectors or recently described synthetic modified RNAs. However, one has to consider that cis-regulatory lncRNAs, which directly interfere with expression of neighboring genes, may need to be expressed by the endogenous locus. RNA-guided gene activation by CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats/clusters of regularly interspaced short palindromic repeat–associated 9)-based transcription factors may circumvent this problem, allowing the induction of the endogenous lncRNA.

In conclusion, the development of RNA therapeutics particularly for targeting noncoding RNAs, but also for the coding genome, is attractive. For example, exon skipping may be useful to rescue genetically caused cardiomyopathies. The challenge of developing microRNA therapeutics may preferentially relate to the relative poor uptake of RNAs in the cardiovascular system, which needs to be improved to allow a safe and efficient therapeutic application.

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**Disclosures**

S. Dimmeler has patents on miRNAs and lncRNAs in cardiovascular disease. S. Dimmeler is member of the scientific advisory board of miRagen Therapeutics. The other author reports no conflicts.

**References**

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## RNA Therapeutics for Treatment of Cardiovascular Diseases: Promises and Challenges

### Online Table: MicroRNAs in cardiovascular disease. Examples of therapeutic modulation of microRNAs in experimental cardiovascular disease models. Concentrations refer to kg body weight unless otherwise indicated.

<table>
<thead>
<tr>
<th>Disease indication</th>
<th>Micro-RNA</th>
<th>Chemistry</th>
<th>Dosing</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>miR-21</strong></td>
<td>2’OMe-miR-21 Anti-miR-21-Eluting Stent</td>
<td>30 µg per carotid artery (rats) Amount coated on stent was similar to a systemic dose of 5 mg/kg (rats)</td>
<td>PMID:17478730 PMID:26183619</td>
<td></td>
</tr>
<tr>
<td><strong>miR-92a</strong></td>
<td>AntagomiRs LNA-antimiRs AntagomiRs</td>
<td>16 mg/kg i.v., two injections week 4 and week 9 after HFD (mice) 0.5 mg/kg, two injections (mice) 8 mg/kg i.v., three injections (rats)</td>
<td>PMID:24255059 PMID:25020912 PMID:22890560</td>
<td></td>
</tr>
<tr>
<td><strong>miR-29</strong></td>
<td>LNA-antimiRs LNA-antimiRs</td>
<td>20 mg/kg i.v. single injection (mice) 10 mg/kg i.v. single injection (mice)</td>
<td>PMID:21903938 PMID:22269326</td>
<td></td>
</tr>
<tr>
<td><strong>miR-92</strong></td>
<td>AntagomiRs LNA-antimiRs PLGA copolymer loaded with antagomir-92a</td>
<td>8 mg/kg, 5 injections i.v. (mice) 0.03 / 0.15 mg/kg single intracoronary infusion (pigs) 0.1 mg/kg, single intracoronary infusion (pigs)</td>
<td>PMID:19460962 PMID:23897866 PMID:25240056</td>
<td></td>
</tr>
<tr>
<td><strong>miR-15-family</strong></td>
<td>8-mer LNA-antimiRs (tiny-miR)</td>
<td>0.5 mg/kg (mice) 0.518-5.18 mg/kg i.v. (pigs, only inhibition was studied)</td>
<td>PMID:22052914</td>
<td></td>
</tr>
<tr>
<td><strong>miR-320</strong></td>
<td>AntagomiR</td>
<td>80 mg/kg i.v. (mice)</td>
<td>PMID:19380620</td>
<td></td>
</tr>
<tr>
<td><strong>miR-34</strong></td>
<td>AntagomiRs LNA-antimiRs 8-mer LNA-antimiR</td>
<td>8 mg/kg i.v. (mice) 5 to 20 mg/kg i.v. (mice) 25 mg/kg s.c. loading dose, 3 time per week 10 mg/kg</td>
<td>PMID:23426265 PMID:23426265 PMID:23047694</td>
<td></td>
</tr>
<tr>
<td><strong>miR-21</strong></td>
<td>AntagomiRs Tiny LNA-antimiR (8 mer)</td>
<td>80 mg/kg, 3 injections jugular vein (mice) 25 mg/kg, 3 injections (mice) (no functional benefit)</td>
<td>PMID:19043405 PMID:20978354</td>
<td></td>
</tr>
<tr>
<td><strong>miR-208</strong></td>
<td>LNA-antimiR</td>
<td>25 mg/kg, every 2 weeks</td>
<td>PMID:21900086</td>
<td></td>
</tr>
<tr>
<td><strong>miR-132</strong></td>
<td>Antagomirs</td>
<td>80 mg/kg, retro-orbital injection day 0 and day 1</td>
<td>PMID:23011132</td>
<td></td>
</tr>
</tbody>
</table>
## microRNA Overexpression

<table>
<thead>
<tr>
<th>Disease</th>
<th>miRNA</th>
<th>Method</th>
<th>Dose/Condition</th>
<th>PMID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherosclerosis</td>
<td>miR-126-5p</td>
<td>Nanoparticle carrier system packed with miR-126-5p mimics</td>
<td>100 µg injection (i.v.) every 72 h for 4 weeks</td>
<td>PMID:24584117</td>
</tr>
<tr>
<td></td>
<td>miR-181b</td>
<td>Lipofectamin mixed with miR-181b mimic</td>
<td>1 nmol mimic mixed with lipofectamine 2000 (30 µl) in total volume of 200 µl, twice a week for 4 weeks or once a week for 12 weeks (i.v.)</td>
<td>PMID:24084690</td>
</tr>
<tr>
<td></td>
<td>miR-146a and miR-181b</td>
<td>Thioaptamer targeting E-selecting linked to porous silicon multistage vector system loaded with mature miRs</td>
<td>15 µg miRs, Biweekly injections (i.v.) for 12 weeks</td>
<td>PMID:26956647</td>
</tr>
<tr>
<td>Myocardial Infarction</td>
<td>miR-590-3p</td>
<td>AVV9</td>
<td>1x10^{11} viral genome particles per animal</td>
<td>PMID:23222520</td>
</tr>
<tr>
<td></td>
<td>miR-199a-3p</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PLGA: poly-d,-lactide-co-glycolide; i.v.: intravenous, s.c.: subcutaneous