Circulation Research

REVIEW

Preclinical and Clinical Development of Noncoding RNA Therapeutics for Cardiovascular Disease

Cheng-Kai Huang, Sabine Kafert-Kasting, Thomas Thum

ABSTRACT: RNA modulation has become a promising therapeutic approach for the treatment of several types of disease. The emerging field of noncoding RNA-based therapies has now come to the attention of cardiovascular research, in which it could provide valuable advancements in comparison to current pharmacotherapy such as small molecule drugs or antibodies. In this review, we focus on noncoding RNA-based studies conducted mainly in large-animal models, including pigs, rabbits, dogs, and nonhuman primates. The obstacles and promises of targeting long noncoding RNAs and circRNAs as therapeutic modalities in humans are specifically discussed. We also describe novel ex vivo methods based on human cells and tissues, such as engineered heart tissues and living myocardial slices that could help bridging the gap between in vivo models and clinical applications in the future. Finally, we summarize antisense oligonucleotide drugs that have already been approved by the Food and Drug Administration for targeting mRNAs and discuss the progress of noncoding RNA-based drugs in clinical trials. Additional factors, such as drug chemistry, drug formulations, different routes of administration, and the advantages of RNA-based drugs, are also included in the present review. Recently, first therapeutic miRNA-based inhibitory strategies have been tested in heart failure patients as well as healthy volunteers to study effects on wound healing (NCT04045405; NCT03603431). In summary, a combination of novel therapeutic RNA targets, large-animal models, ex vivo studies with human cells/tissues, and new delivery techniques will likely lead to significant progress in the development of noncoding RNA-based next-generation therapeutics for cardiovascular disease.

Key Words: animal models ■ cardiovascular diseases ■ nucleic acids ■ nucleosides ■ nucleotides ■ therapeutics

It is well known that <2% of the human transcriptome encodes protein-coding RNAs, whereas the majority are noncoding RNAs (ncRNAs), including ribosomal RNA, tRNA, microRNA (miRNA, or miR), long noncoding RNA (lncRNA), circular RNA (circRNA), and other small RNAs.[1,2] Over the past 2 decades, there has been increasing evidence that ncRNAs act as key players in the onset and progression of cardiovascular diseases (CVDs).[3–5] As the ncRNA research field has progressed, researchers have developed complex tools to modulate these ncRNAs with the aim of establishing novel, next-generation strategies to combat CVDs.[6] For example, some of the first miRNA or lncRNA targets identified in cardiac remodeling were miR-21 and the lncRNA *Chast*.[7,8] Therefore, ncRNA-orientated next-generation drugs might offer a novel therapeutic option for CVDs, for which innovations have been scarce in the last few decades.

CVDs are the main cause of death in both Europe and the United States, according to Atlas (European Society of Cardiology) and the Centers for Disease Control and Prevention (CDC, United States).[9,10] One of the drawbacks to develop new therapeutic innovations is that most observations have only been made in in vitro systems or small animal models (eg, rodents) but have not yet been replicated or have failed to be replicated in larger animal models. Indeed, rodents exhibit several fundamental differences in certain cardiovascular

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Nonstandard Abbreviations and Acronyms

ABCA1	ATP-binding cassette transporter A1
AF	atrial fibrillation
Arl2	ADP-ribosylation factor-like 2
ASO	antisense oligonucleotide
ATP	atrial tachypacing
CVD	cardiovascular disease
EHT	engineered heart tissue
hiPSC	human-induced pluripotent stem cell
LNA	locked nucleic acid
lncRNA	long noncoding RNA
MI	myocardial infarction
ncRNA	noncoding RNA
SREBP	sterol-response element-binding protein
VLDL	very low density lipoprotein

physiology elements, such as heart weight, heart rate, blood pressure, and the coronary artery system, in comparison to larger animals and humans that may influence experimental conclusions.[11,12] Excitingly, several RNA-based drugs targeting CVD have already been approved by the US Food and Drug Administration, and the pharmaceutical development of ncRNAs is also currently under way.[13–15] To progress in clinical application, proof of concept and safety evaluation in large animal models, such as pigs and nonhuman primates, are helpful and often necessary steps before the start of first-in-human trials. Thus, in the present review, we focus on ncRNA-targeting therapeutic studies mainly performed in large animals and current clinical trials.

MiRNA Studies in Large Animals

MiRNAs are a class of ncRNAs with short (≈18–22 nucleotides) and highly conserved sequences that predominantly exist in eukaryotes. Functionally, miRNAs are involved in various gene regulatory mechanisms including mRNA degradation and translational repression via the RNA-induced silencing complex.[16] Since miRNAs are highly conserved, exhibit short sequences, and are highly abundant, they became the first class of ncRNAs studied in large animal models (Tables 1 and 2; Figure 1) and, recently, clinical trials (Table 3; Figure 1).

Pig Studies

Pigs are a popular model for mimicking human heart disease, especially for myocardial infarction (MI) studies, since the porcine heart shares many similarities with the human heart, including heart weight, blood pressure, and heart rate.[11,43,44]

One of the first studies that investigated miRNA therapeutics in large animal models targeted the miRNA miR-92a. MiR-92a is ubiquitously expressed and has multiple functions in the body, including the modulation of angiogenic pathways.[29] In a mouse model, it was shown that miR-92a was upregulated after cardiac ischemic injury. Silencing miR-92a by 2'-O-methyl (2'-O-Me)-modified antagomir-92a significantly enhanced angiogenesis in vitro and in vivo. Furthermore, the inhibition of miR-92a in a MI mouse model reduced the infarct size and improved certain cardiac functions.[29] Meanwhile, in an ischemia-reperfusion injury pig model, there was a reduction in infarct size, less cardiomyocyte apoptosis, and better myocardial function after the inhibition of miR-92a expression.[18] The downregulation of miR-92a also increased capillary density and reduced cardiac inflammation; however, this study focused only on the short-term (three or seven days) effect of antagomir-92a treatment. To study more long-term effects and overcome the potential off-target issues of a systemic miR-92a blockade, Bellera et al[19] delivered antimiR-92a encapsulated in bioabsorbable and biocompatible microspheres via intracoronary injections in a MI pig model. The microsphere-antimiR-92a was detected mainly in the capillaries of the anterior myocardial wall and surprisingly showed no distribution to remote organs. Regarding the long-term effects of microsphere-antimiR-92, the treatment also induced angiogenesis 1 month following MI induction. This data revealed that a drug meant to inhibit miRNAs may have higher specificity and a greater long-term effect when modified with proper physical protections or conjugation chemistries. A miR-92a inhibitor was further tested in 2 phase I clinical trials (Table 3) and was named MRG-110 (miRagen Therapeutics, Inc, NCT03603431 and NCT03494712).[17] MRG-110 is expected to accelerate wound healing by improving blood flow via its proangiogenic properties. Indeed, Gallant-Behm et al[17] demonstrated in a pig model that the administration of antimiR-92a inhibitors significantly increased blood flow and revascularization in peri-wound areas. The results of these phase 1 studies have not yet been published.

Another mechanism regulated by miRNAs and often contributing to CVD is mitochondrial dysfunction.[45] MiR-15b has been shown to be involved in mitochondrial dysfunction by targeting Arl2 (ADP-ribosylation factor-like 2). In a primary rat cardiomyocyte model, both cellular atrial tachypacing (ATP) levels and Arl2 mRNA expression decreased following miR-15b overexpression, while miR-15b inhibition reversed this phenotype.[30] Hullinger et al[20] further applied locked nucleic acid (LNA)-modified antimiR-15b to a MI pig model and showed that miR-15b inhibition restored porcine cardiac function. In addition to a 16-mer antimiR, researchers also developed a short 8-mer antimiR-15b and found that it efficiently suppressed miR-15b expression and also enhanced cardiac function. Interestingly, there were differences between the 2 oligonucleotide inhibitors. For example, treatment with a 16-mer (but not an 8-mer)

Table 1. Modulation of miRNA Expression in Different Large Animal Models

Experimental Model	Therapeutic Target	Therapeutic Approaches	Disease Model	Mechanisms	Reference
Pig	miR-92a	antimiR	Excisional wound	*ITGA5* de-repression	17
	miR-92a	antimiR	IRI	Improved cardiac function	18
	miR-92a	antimiR	MI	Long-term recovery	19
	miR-15b	antimiR	IRI	*PDK4/SGK1* de-repression	20
	miR-208a	antimiR	IRI	Stress-dependent	21
	miR-199a	AAV6-mediated overexpression	MI	Activation of several heart development markers (*GATA4*)	22
	miR-132	antimiR	MI	FoxO3 de-repression	23
Dog	miR-328	AV-mediated overexpression	AF	*CACNA1C/CACNB1* de-repression	24
		antimiR			
	miR-206	LV-mediated overexpression	AF	*SOD1* de-repression	25
		antimiR			
Rabbit	miR-1	LV-mediated overexpression	AF	*KCNE1/KCNB2* de-repression	26
		antimiR			
Nonhuman primate	MiR-33a/b	antimiR	Dyslipidemia	*ABCA1* de-repression	27
	MiR-33a/b	antimiR (8-mer)	Obesity	*ABCA1* de-repression	28

AF indicates atrial fibrillation; AV, adenovirus; IRI, ischemia-reperfusion injury; LV, lentivirus; and MI, myocardial infarction.

antimiR increased left ventricular end-diastolic pressure, whereas treatment with only the 8-mer antimiR significantly reduced infarct size.[20] These data indicated the importance of designing miRNA inhibitors to achieve an efficient therapeutic response.

Importantly, the pharmacological effects of antimiRs might be influenced by the disease condition. For instance, the cardiac-enriched miR-208a is encoded from the intron of the *α-MHC* gene and has been reported to be responsible for cardiac hypertrophy and fibrosis.[46] Montgomery et al[32] further demonstrated that the inhibition of miR-208a improved cardiac function in a hypertension-induced heart failure rat model. Eding et al[21], however, showed that differentially expressed downstream genes modulated by antimiR-208a are different in TAC and MI rat models, and a similar stress-dependent antimiR effect was also observed in a pig MI model. These results, therefore, suggested that the disease type and severity of a disease should be considered in the preclinical development of a miRNA drug.

Another miRNA, miR-132, was shown to be crucially involved in cardiac growth and autophagy.[40] Indeed, miR-132 is both necessary and sufficient for driving pathological cardiomyocyte growth, a hallmark of adverse cardiac remodeling. Recently, the safety, tolerability, favorable pharmacokinetics, dose-dependent pharmacokinetic/pharmacodynamic (PK/PD) relationships, and the high clinical potential of an antimiR-132 treatment in pigs following myocardial infarction has been documented.[23]

It is known that the adult mammalian heart has no significant regenerative capacity following injury, causing massive cardiomyocytes loss and subsequently leading to cardiac dysfunction and heart failure. Based on a whole-genome miRNA library screening that compared postnatal day 1 and day 7 rodent hearts, miR-199a was identified and suggested to promote the cardiomyocyte cell cycle re-entry both in vitro and in vivo. The overexpression of miR-199a increased cardiomyocyte proliferation and preserved cardiac function after inducing MI in mice.[31] The same group next overexpressed miR-199a in pigs after MI via the intramyocardial injection of adeno-associated virus-containing miR-199a.[22] Indeed, the overexpression of miR-199a in pig hearts post-MI improved cardiac contractility, increased muscle mass, and reduced scar size; however, 70% of the adenoassociated virus-miR-199a treated pigs (7 out of 10) died from sudden cardiac death 7 to 8 weeks after virus injection. Further histological analysis revealed that a small group of cells expressing cell proliferation markers (eg, Ki67) and early heart development markers (such as GATA4) were infiltrating the infarcted myocardium. These cells were poorly differentiated, highly proliferating, and immature premyocytes that likely induced the observed ventricular fibrillation and sudden cardiac death of the pigs.[22] Overall, this miR-199 pig study impressively demonstrated the power of miRNAs in achieving biological effects in the heart and highlighted the need for the careful preclinical characterization and off-target effect prediction of miRNA-based drugs before clinical testing.

Due to the similarity between pigs and humans regarding their cardiovascular systems and physiology, (mini-) pigs can also be valuable models for atherosclerosis. Based on different genetic alterations, minipigs with constitutive and/or diet-dependent increases in serum cholesterol have already been generated and used in drug testing. For instance, strains with an altered LDL receptor gene or apolipoprotein E deficiency had increased serum cholesterol and developed atherosclerosis.[47,48]

Table 2. The Developmental Progression of ncRNA Studies in Different Models and Clinical Trials

ncRNA	In Vitro/Small Animal Model		Large Animal Model	Clinical Development
	Target Characterization	Proof of Therapeutic Concept		
miR-92a	Human endothelial cells[17,29]	Mouse[17,29]	Pig[17–19]	Yes
miR-15b	Primary rat cardiomyocytes[30]		Pig[20]	
miR-199a	Primary rat/mouse cardiomyocytes[31]	Mouse[31]	Pig[22]	
miR-208a		Rat[32]	Pig[21]	
miR-328			Dog[24]	
miR-206	Mouse[33]		Dog[25]	
	Rat[34]			
miR-1	Primary rat cardiomyocytes[35]	Rat[36]	Rabbit[26]	
miR-33	8 human cell lines and 2 mouse cell lines,[37]	Mouse[37–39]	Nonhuman primate[27,28]	Yes
	2 human cell lines and 1 mouse cell lines[38]			
miR-132	Primary rat/mouse cardiomyocyte and 2 mouse cell lines[40]	Mouse[23,40]	Pig[23]	Yes
lncRNA *CHROME*	3 human cell lines and primary human hepatocytes[41]		Nonhuman primate (observational)[41]	
lncRNA *H19*	Human aortic smooth muscle cells[42]	Mouse[42]	Pig (observational)[42]	

lncRNA indicates long noncoding RNA; and ncRNA, noncoding RNA.

The engineered heart tissue (EHT) made from miniature pigs carrying the hypertrophic cardiomyopathy mutation *MYH7 R403Q* has presented increased stiffness and impaired muscle relaxation.[49] Mentzel et al[50] investigated the miRNA profiles of diet-based obese minipigs and found several miRNAs to be potential biomarkers and therapeutic targets. In the future, the testing of ncRNA therapeutic efficacy in such disease models may provide important contributions to a mechanistic understanding and pharmaceutical exploitation of the respective RNA compounds.

Dog and Rabbit Studies

In contrast to pigs, dog hearts have abundant collateral coronary vessels and thus are not easily useable as a MI model.[11,44,51] In contrast, dog hearts have an electrophysiological system very similar to that of humans, are prone to develop atrial fibrillation (AF), and are thus often used as a preferable model for AF research.

There are a variety of methods to induce AF in dogs, including nicotine treatment and ATP.[24,25,52,53] In an ATP-induced AF-dog model, miR-328 was found to be upregulated; moreover, the overexpression of miR-328 via an adenoviral approach recapitulated AF phenotypes in healthy dogs. Additionally, computational prediction revealed that the calcium voltage-gated channel subunits α1c and β1 are 2 genes targeted by miR-328. Treatment with antimiR-328 significantly de-repressed the expression of *CACNA1C* and *CACNB1* and reversed AF.[24]

In addition, miR-206 was shown to participate in AF progression. MiR-206 is a muscle-enriched miRNA and is also required for the regeneration of neuromuscular synapses. The knockout of miR-206 in an amyotrophic lateral sclerosis mouse model accelerated the disease progression.[33] The miRNA profiling in an AF-dog model revealed that miRNA-206 was induced 10-fold compared to in the control group. Additionally, the inhibition of miR-206 by lentiviral-antimiR-206 injection attenuated the AF-induced symptoms.[25] Although neuronal regeneration induced by miR-206 indicated the essential role of miR-206 during muscle denervation and reinnervation,[33,34] the overexpression of miR-206 aggravated the AF-induced symptoms. These results highlight that miRNAs could possess different functions in different organs and sometimes exhibit species-specific effects.

In an ATP-induced AF rabbit model, miR-1 was reported to promote cardiac arrhythmias and enhance calcium release by targeting several ion channel genes. These findings were also observed in mouse and rat models.[26,35,36] The inhibition of miR-1 via lentiviral-based antimiR-1 infections significantly prolonged the atrial effective refractory period and de-repressed potassium voltage-gated channel (KCN) E1 and B2 expression, 2 target genes of miR-1.[26]

Atherosclerosis studies have been performed in Watanabe heritable hyperlipidemic rabbits since their development/discovery in the 1970s. Meanwhile, 2 advanced strains were generated: one showing spontaneous coronary atherosclerosis (Watanabe heritable hyperlipidemic-CA) alone and the other showing myocardial infarction (Watanabe heritable hyperlipidemic-MI).[54,55] Despite certain differences from human pathophysiology, these animal models can be useful tools for the investigation of new drug candidates. However, there have so far been no reports on the profiles of the effect of miRNA, other classes of ncRNA, nor their inhibitors in Watanabe rabbits.

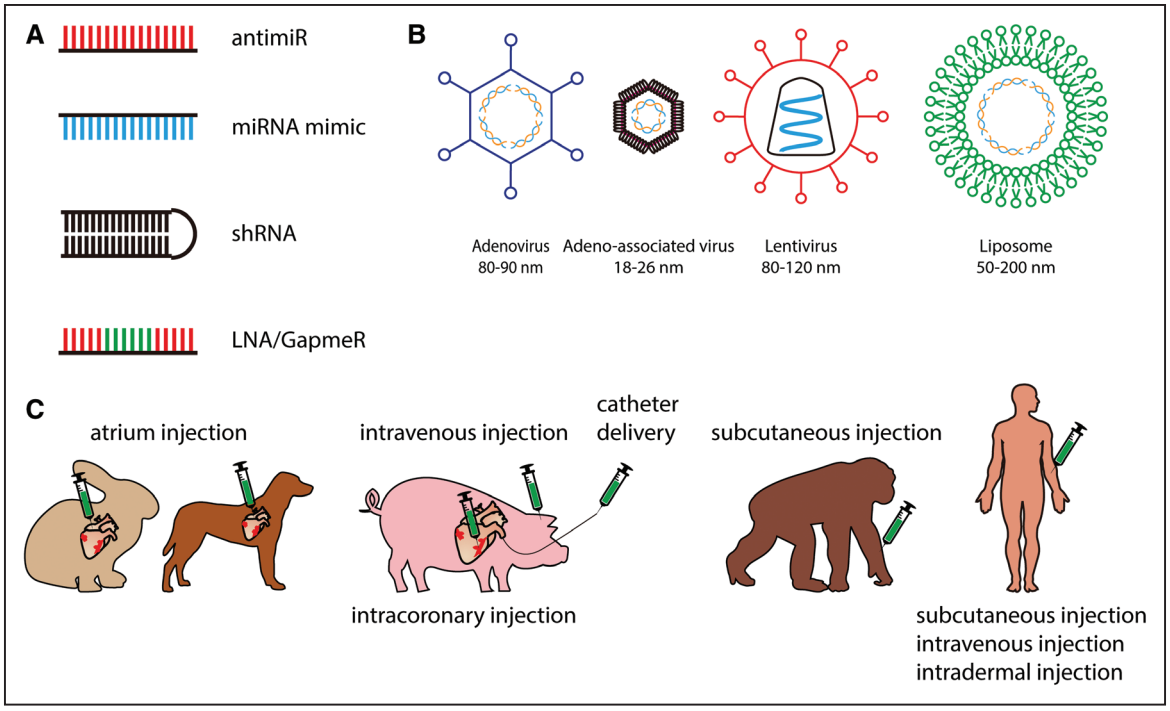

Figure 1. Scheme of oligonucleotide-based RNA delivery.
A, AntimiRs (miRNA inhibitors) can be modified with different chemical modifications, including locked nucleic acids (LNAs) and sugar backbone modifications (2'-O-Me, 2'-F/MOE, and 2'-O-MOE), while miRNA can also be enhanced via miRNA mimics. To inhibit mRNAs or long noncoding RNAs (lncRNAs), short hairpin RNAs (shRNAs), or LNA/GapmeR are commonly used. **B**, Adenovirus, adenoassociated virus (AAV), and lentivirus particles can be used as a vector to silence or overexpress target genes. In addition to viral-based delivery, liposomes or nanoparticles are another way through which to deliver antimiRs or miRNA mimics. **C**, Various delivery approaches can be applied in different species. For example, atrium injection is performed in rabbits and dogs with atrial fibrillation. For pigs, intravenous injection, catheter-based injection, and intracoronary injection are commonly used. Subcutaneous injection can be also used. Clinically, subcutaneous injection, intravenous injection, and intradermal injection are more attractive and easier delivery routes in humans.

Nonhuman Primate Studies

Chronic heart failure, subacute MI models, as well as models of atherosclerosis have also been studied in nonhuman primates[56,57]; however, due to ethical and financial issues, primates are not frequently used in cardiovascular research.

The transcription factor SREBP (sterol-response element-binding protein) regulates genes involved in cholesterol biosynthesis, such as ABCA1 (ATP-binding cassette transporter A1). A loss of ABCA1 expression can cause Tangier disease, which is characterized by a low level of circulating HDL.[58] Najafi-Shoushtari et al and Rayner et al showed that the human SREBP genome locus transcribes not only mRNA but also 2 miRNAs, miR-33a and miR-33b. MiR-33 inhibits the expression of ABCA1, which leads to circulating HDL-C reduction and, therefore, the silencing of miR-33 increased HDL-C expression in a mouse model.[37–39] Despite the promising results of developing miR-33 as a therapeutic target against dyslipidemia and atherosclerosis, its clinical progress is limited. MiR-33b, which is encoded from the *SREBP1* gene locus, only exists in large animals and not in mice. This difference may also significantly affect the results studied using knockout mouse models or insulin response experiments in mice.[59] To solve this issue, Rayner et al[27] injected 2'-fluoro/-O-methoxyethyl (2'-F/MOE)-modified antimiR-33a/b subcutaneously to treat African green monkeys (*Chlorocebus aethiops*) with dyslipidemia. They found the same results as observed in the mouse model: the knockdown of miR-33a/b increased ABCA1 expression and plasma HDL-C levels. Interestingly, beyond cholesterol metabolism, they also found genes involved in fatty acid oxidation and biosynthesis to be regulated. These effects resulted in the reduction of plasma VLDL (very low density lipoprotein) triglyceride levels, a new finding that was not observed in the mouse model.[27]

Another study employed subcutaneous administration of short seed-targeting 8-mer antimiRs in obese African green monkeys.[28] In this study, the de-repression of several miR-33 target genes, including ABCA1m were observed, plasma HDL-C levels were elevated, and no adverse effects were noticed.[28] These 2 studies performed in nonhuman primates provided evidence that the inhibition of miR-33a/b to raise plasma HDL-C levels could be a promising therapeutic strategy for the treatment of dyslipidemia.

LncRNA and circRNA Studies in Large Animals

LncRNAs are another class of ncRNAs with longer (>200 nucleotides) but less conserved sequences.[60] Having

Table 3. Clinical Trials With ncRNA-Based Therapeutics

Targeted miRNA	Developmental Drug	Chemistry/ Mechanism	Indication	Sponsor/Collaborators	Clinical Trial Identifier	Phase
miR-92a	MRG-110	LNA antimiR	Wound healing	miRagen Therapeutics, Inc	NCT03603431	Phase I
					NCT03494712	Phase I
miR-16	Mesomir	TargoMir	Malignant pleural mesothelioma	Asbestos Diseases Research Foundation/EnGeneIC Limited	NCT02369198	Phase I
miR-34a	MRX34	miRNA mimic	Cancer/melanoma (advanced)	Mirna Therapeutics	NCT01829971	Phase I
					NCT02862145	Phase II
miR-122	Miravirsen	Various	Hepatitis C	Various	NCT01646489	Phase I
	RG-101				NCT00979927	Phase I
	Other				NCT00688012	Phase I
					NCT01200420	Phase II
miR-155	Cobomarsen (MRG-106)	LNA antimiR	Blood cancer (eg, chronic lymphocytic leukemia)	miRagen Therapeutics, Inc	NCT02580552	Phase I
					NCT03713320	Phase II
					NCT03837457	Phase II
miR-21	RG012	AntimiR	Alport syndrome	Sanofi Genzyme	NCT03373786	Phase I
					NCT02855268	Phase II
miR-29b	Remlarsen (MRG-201)	miRNA mimic	Cutaneous fibrosis	miRagen Therapeutics, Inc	NCT02603224	Phase II
miR-132	CDR132L	AntimiR	Heart failure	Cardior Pharmaceuticals GmbH	NCT04045405	Phase Ib

LNA indicates locked nucleic acid; and ncRNA, noncoding RNA.

various biological functions, lncRNAs are certainly promising therapeutic targets; however, translational studies in animals are difficult with this class of ncRNA due to their poor sequence conservation between species.[61,62] Thus, only well-conserved lncRNAs seem promising as translationally relevant disease targets for new therapies. Indeed, the number of conserved lncRNAs is still quite limited.[8,63,64] As the degree of DNA/RNA sequence conservation among different species is commonly used to predict the biological functions of the species,[65,66] it explains why lncRNA-targeting experiments are not frequently performed in large animals. Studies have begun to identify novel un-annotated lncRNAs in different large animal models. Kern et al analyzed lncRNAs from three farm animals (chicken, cattle, and pigs) and found that half were not annotated in NCBI or other databases. As expected, the lncRNAs from these species were less conserved. Interestingly, researchers also found that many have locus-conserved transcripts (a transcript with a diverged sequence but the same genomic position as its neighboring genes), which might indicate similar biological functions between themselves.[67] In dogs, Béguec et al analyzed the lncRNA profile of 26 different tissue types and developed a tool called FEELnc.[68] Surprisingly, around 900 lncRNAs (10%) were highly conserved to human transcripts, including well-known *HOTAIR*, *MALAT1,* and *NEAT1*.[68,69] In addition to these specific species, large-scale lncRNA analysis in >7 divergent species, from zebrafish to humans, was also reported.[70,71]

The dynamic expression of lncRNAs in the progression of heart disease is also important. In a porcine ischemic heart model, RNA-seq was performed to compare the expression of lncRNAs between healthy and ischemic zones of the heart. Four hundred fifty lncRNAs were identified that were not previously annotated and were differentially regulated after ischemic injury. Among these novel lncRNAs, transcripts that are transcribed antisense to myocardial transcription factors, such as *GATA4*, *GATA6,* and *KLF6,* were identified and observed to potentially have important biological functions in the heart.[72] An experimentally validated database (a heart disease-related, noncoding RNA database, HDncRNA) developed by Wang et al contains around 2000 lncRNAs that are associated with heart diseases in 6 species, including humans, rodents, pigs, calves, and dogs. This database is equipped with a web-based interface that allows users to easily search for lncRNA candidates, directing them to the original relevant publications.[73] Recently, Wu et al[74] analyzed the lncRNA-mRNA network in carotid atherosclerotic rabbit models and discovered several novel lncRNAs involved in the disease progression of atheroscelrosis.

In spite of the obstacles mentioned above, there have been 2 lncRNA studies performed in large animals. LncRNA *CHROME*, identified by Hennessy et al,[41] was found to be upregulated in nonhuman primates with atherosclerotic vascular disease. Further in vitro data showed that the overexpression of *CHROME* in HepG2 cells reduced miR-33 expression and de-repressed the miR-33-targeted genes, including *ABCA1*, while the inhibition of *CHROME* by shRNA or LNA GapmeR in primary human hepatocytes and HepG2 cells had opposite effects. Likewise, Li et al[42] demonstrated that the expression of lncRNA *H19* increased in 2 abdominal

aortic aneurysm mouse models as well as a low-density lipoprotein receptor (*LDLR*) knockout mini-pig aneurysm model. The in vitro knockdown of *H19* decreased the apoptotic rate of human smooth muscle cells. Overall, further and more therapeutic experiments in large animal models are needed, since no lncRNA therapeutic approach has been performed in large animals thus far.

Despite their low sequence conservation, lncRNAs have higher tissue specificity. According to a study published by Cabili et al,[75] 78% of lncRNAs may be tissue-specific, which is much higher than the percentage reported for mRNAs (19%). This conclusion is also supported by a recently published large-scale RNA-seq analysis that revealed 51% to 63% of lncRNAs to be tissue specific.[71] This specificity makes certain conserved lncRNAs promising targets for drug development, since drugs designed to act based on tissue-specific lncRNAs and their interaction may produce less remote off-target effects.

Circular RNAs (circRNAs) are another novel class of RNA molecules, have a structure featuring covalently linked 3' to 5' ends,[76] and are highly abundant in the human genome.[2,77] A review published recently summarized the role of circRNAs in cardiovascular biology. In this review, Aufiero et al[78] listed several circRNAs with functions in rodent heart disease models. For example, a circRNA termed heart-related circRNA (HRCR) was reported to have cardioprotective functions via sponging miR-223. The overexpression of *HRCR* inhibited miR-223 activity and de-repressed the downstream protein ARC and therefore attenuated hypertrophic responses.[79] Hansen et al[80] reported that a circRNA called *ciRS-7* (currently named *Cdr1as*) could serve as a miRNA sponge and be involved in heart diseases. Later, *Cdr1as* was further proven to promote myocardial infarction by sponging miR-7.[81] In addition to cardiomyocytes, Garikipati et al demonstrated that the overexpression of *circFndc3b* in endothelial cells enhanced angiogenic activity and reduced endothelial apoptosis. The cardioprotective mechanism of *circFndc3b* was to interact with the RNA-binding protein FUS to regulate the VEGF-A signaling pathway.[82] Since circRNAs stem from mRNAs, recently, several studies have also reported that lncRNA/circRNA-mRNA-miRNA networks play an important role in heart development and disease, such as AF and atherosclerosis.[83–86] For example, Zhang et al[86] found 7 circRNAs that functioned in cell adhesion, cell activation, and the immune response, which provided an overall better understanding of the pathogenesis of atherosclerosis. With the increasing importance of machine learning algorithms and artificial intelligence, we hope that the better interpretation of such network interactions can lead to an improved understanding of ncRNA networks and their effects on diseases. With the help of network prediction, *Cdr1as* was also shown to regulate neuronal activity in brains by forming a specific ncRNA regulatory network together with the lncRNA *Cyrano* and miR-7/miR-671.[87] High sequence conservation, abundant quantity, and a higher stability than mRNA are all other advantages of circRNA in terms of its potential to be studied in large animal models and its future consideration as a therapeutic target in humans.[88–90] Several studies have reported that 15% to 30% of circRNAs are conserved between 3 main species: mouse-human, mouse-pig, and pig-human.[91–93]

Human EHTs and Living Myocardial Slices

Although data from large animal models may be more predictive for human cardiovascular diseases, these studies also continue to possess certain limitations. For example, large animals require larger breeding space and higher maintenance costs, and experimental interventions may be time-consuming and do not allow for high repetition. Such circumstance makes it difficult for researchers to collect enough samples in a reasonable time to achieve statistical significance.[12,94] In addition, large animals have longer gestation times, which makes it difficult to generate gene knockout/knockin models, although the recent emergence of the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/Cas9) technique may help solve this problem.[11,95] Due to the limitations mentioned above, EHT and living myocardial slice models derived from human cells or tissues may serve as a bridge between in vitro and in vivo models (Figure 2).[96,97]

Since the generation of human-induced pluripotent stem cells (hiPSCs) was reported,[98,99] studies on the differentiation of hiPSCs into various functional cell types, including cardiomyocytes, have rapidly increased in number.[100,101] However, hiPSC-derived cardiomyocytes (hiPSC-CMs) cultured in monolayer systems show immature and fetal phenotypes that do not reflect the adult heart and fail to recapitulate chronic heart disease phenotypes.[102] EHTs composed of hiPSC-CMs and additional supporting cells in a 3-dimensional culture system may better reflect a fully developed heart under corresponding disease models.[103,104] The EHT, sometimes mixed with fibroblast or endothelial cells, has shown improved adult phenotypes, including rod-shaped cardiomyocytes with well-organized sarcomere structures, systolic contraction, and inotropic responses to drug stimulation.[105,106] Tiburcy et al[105] further treated isoprenaline, a β1- and β2-adrenoceptor agonist to hiPSC-CM EHTs, to mimic hypertrophic responses, which demonstrated the possibility of using EHT as heart failure and cardiac repair models. HiPSCs can not only be generated from healthy individuals but also from patients who suffering from heart disease. Prondzynski et al[107] generated EHTs from hypertrophic cardiomyopathy patient-derived hiPSC-CMs, and the hypertrophic cardiomyopathy-EHTs showed phenotypes including cardiac hypertrophy, hypercontractility, and higher myofilament calcium sensitivity. The overall

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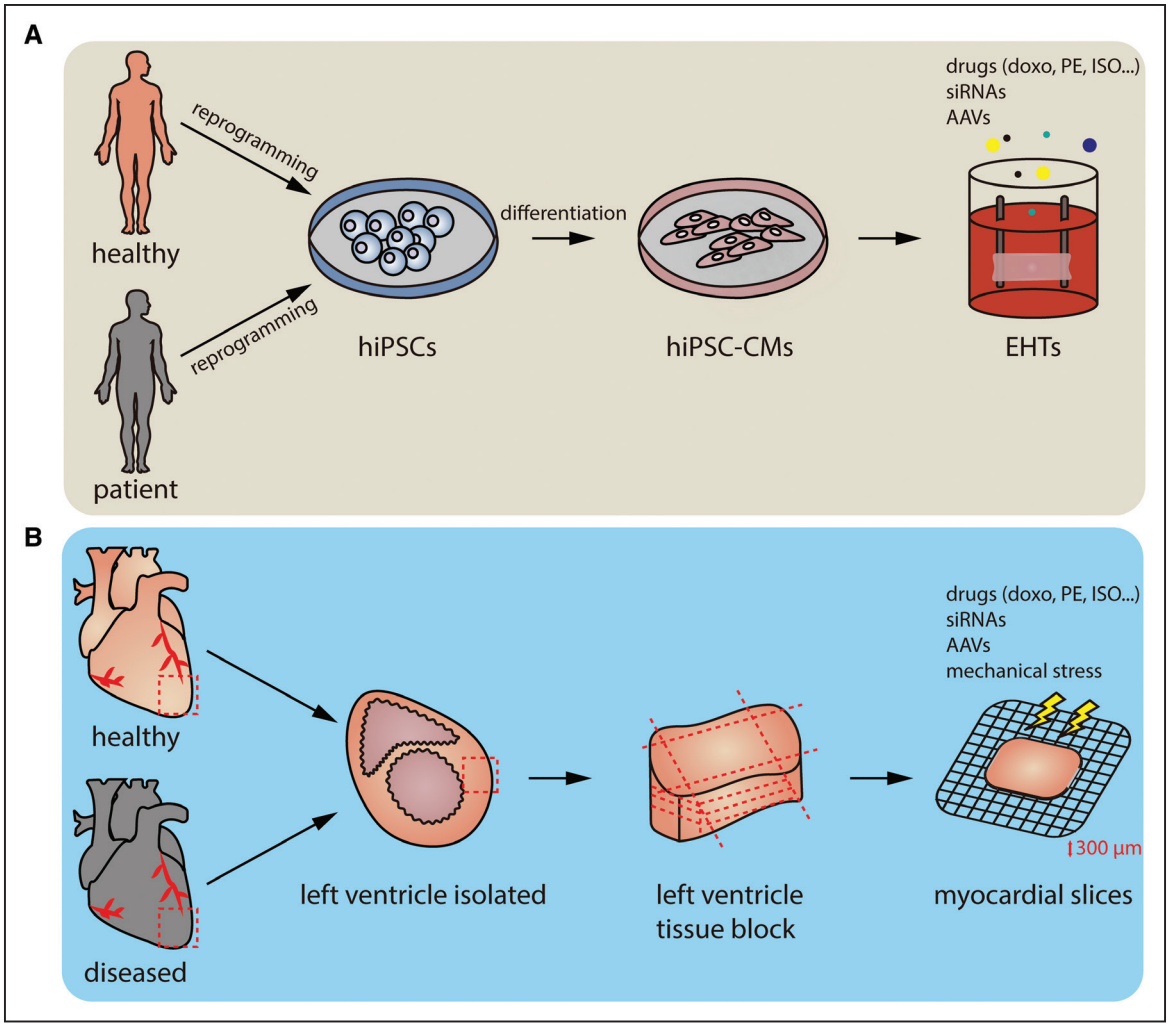

Figure 2. Workflow of 2 3-dimensional ex vivo models, engineered heart tissues (EHTs), and living myocardial slices. **A**, Somatic cells are isolated from human blood cells or skin cells, reprogrammed into human induced pluripotent stem cells (hiPSCs), and differentiated into hiPSC-derived cardiomyocytes (hiPSC-CMs). The hiPSC-CMs are seeded onto the scaffolds to generate beating EHTs. Compared with a 2-dimensional cell culture system, EHTs exhibit a better structure and matured phenotypes that are similar to adult CMs. Through modifying the stiffness of the scaffold, different disease models, such as hypertrophic cardiomyopathy, can be established. EHTs can further be tested for drugs or as gene modulation tools. Not only stemming from healthy humans, EHTs can also be made from patients suffering from heart disease for disease modeling. **B**, To prepare living myocardial slices, small or large mammalian hearts, including human hearts, are explanted. The left ventricles or other parts of the heart are then isolated and dissected into small tissue blocks. Hundred to four hundred micrometers myocardial slices are sliced and used for further functional studies, for example, by treating with different drugs or adjusting the voltage that stimulates the contraction of the myocardial slices. Similar to EHTs, the living myocardial slices could also be prepared from diseased animal models. Doxo indicates doxorubicin; ISO, isoproterenol; and PE, phenylephrine.

results exhibited the possibility of using EHTs in personalized medicine approaches in the near future.

Recently, living myocardial slice technology has emerged as another option for further experimental evaluation before and in addition to large animal models or clinical trials. Here, cardiac tissue is cut into thin slices by a vibratome, and such slices provide a 3-dimensional structure containing various cell types and exhibit preserved electrical and mechanical connection. This technology has proven to be a platform for studying electrophysiology, drug screening, cardiac fibrosis, and heart failure in cardiac slices that are obtained from several animals, including rats, guinea pigs, rabbits, dogs and, recently, also humans.[108–113] Watson et al described a detailed protocol for the preparation of adult ventricular myocardial slices with preserved cardiomyocyte viability (97%) and functionality for up to 1 week. The thickness of each myocardial slice is 100 to 400 µm, which allowed for oxygen and small compounds to diffuse through the slice. Moreover, ultrathin slices also make it possible to produce many experiments from the same heart and therefore reduce the number of animals needed in a study.[97,114]

Clinical Experiences With Coding and Noncoding RNA Therapeutics

Since the biological relevance of ncRNAs has been recognized, the cardiovascular community has begun to

develop modulators of these targets as a new generation of cardiovascular therapeutics. In fact, RNA-based therapeutics were first developed in the 1990s, and the first Food and Drug Administration-approved RNA-based drug dates back to 1998, when a 21-mer phosphorothionate oligonucleotide (fomivirsen) targeting CMV IE-2 protein received Food and Drug Administration approval.[14] Since then, 6 more compounds have been approved by the Food and Drug Administration based on an anti-RNA mechanism targeting mRNAs relevant to age-related macular degeneration, neuromuscular disorders, familial hypercholesterolemia, and transthyretin-mediated amyloidosis, which is involved in heart failure due to the cardiac deposition of TTR amyloid fibrils.[115] Thus, almost 50% of these innovative drugs focused on indications in the cardiovascular field (Table 4). However, Mipomersen (a GapmeR targeting Apolipoprotein B-100) is no longer marketed in the United States, and 2 recently approved drugs for the treatment of transthyretin-mediated amyloidosis still need to exhibit clinical success in a competitive market, in which the high costs of treatment could be a major drawback.[116]

Current clinical antisense-based drug developments are numerous (recently reviewed by Bennet et al[13]) and include CVD targets such as PCSK9 in LDL-C-hypercholesterolemia (NCT01350960, NCT02597127), Apolipoprotein CIII in familial chylomicronemia syndrome (Volanesorsen received conditional marketing approval in the EU; available via the Early Access Program in the US, NCT 03544060), or Lipoprotein A (Novartis/Akcea Therapeutics, NCT04023552; Amgen, NCT03626662). Moreover, further drugs are currently in a developmental pipeline for the treatment of CVD, targeting mRNAs for angiopoietin 3, factor XI, or apolipoprotein(a), among others.[117–119] All these aforementioned therapeutic entities use an RNAseH-dependent mechanism or siRNA/RNAi to repress the expression levels of the target transcript. Other drugs targeting mRNAs make use of splicing modulation to improve the expression of a beneficial functional transcript over an altered or missing splicing product in the relevant disease, such as dystrophin in Duchenne muscular dystrophy or the SMN protein in spinal muscular atrophy (see Eteplirsen and Nusinersen in Table 4). Splicing modulation will, in principle, be a relevant mechanism for lncRNAs and circRNAs upon their being targeted as pharmaceutical agents in the future. As described above, these mechanisms are currently subjects of intensive research.

MiRNAs have so far reached the clinical stage, although clinical studies using or targeting miRNAs are still more scarce than antisense strategies for mRNAs. The majority of results from the US database of clinical trials (www.clinicaltrials.gov) refer to the evaluation of miRNA as biomarkers or prognostic factors. Still, a number of miRNAs are currently under clinical development and are summarized below (Table 3).

Organ Fibrosis

A compound mimicking miRNA-29a in clinical development aims to increase the functional levels of miRNA-29a to combat fibrosis. MiRNA-29a has been shown to reduce collagen expression and is downregulated in multiple fibrotic conditions, including, but not limited to, fibrosis of the heart, lungs, liver, and kidneys and systemic sclerosis.[120] One early comprehensive study revealed that miR-29a plays an important role in the pathological remodeling of the heart after myocardial infarction.[121] Recently, and in contrast to the proposed beneficial effects of miR-29a overexpression, it has been demonstrated that cardiomyocyte-expressed miR-29 promotes pathological remodeling of the heart by activating Wnt signaling.[122] MiRNA-29a mimic, called Remlarsen (MRG-201),[123] was successfully tested in a phase I study with drug administration to 54 healthy volunteers (NCT02603224); currently, a phase II clinical trial targeting cutaneous fibrosis is being conducted to determine if the substance can limit the formation of fibrous scar tissue in certain skin diseases (NCT03601052). These studies could pave the way toward the investigation of this drug in idiopathic pulmonary fibrosis and other conditions of pathological fibrosis.

MiR-21 is a profibrotic molecule discovered in 2008 that is currently being targeted in a clinical phase II trial. AntimiR-21 has been described as strongly antifibrotic[7] and is currently clinically developed for the treatment of Alport syndrome, a collagen IV defect

Table 4. FDA-Approved Antisense Drugs

Proprietary Name	Active Ingredient	Target/Indication	Route of Administration	FDA Approval Year
VITRAVENE (Novartis)	Fomivirsen sodium	CMV IE-2/CMV retinitis	Intravitreal	1998
MACUGEN (Valeant)	Pegaptanib sodium	VEGF165/AMD	intravitreal	2004
KYNAMRO (Kastle)	Mipomersen sodium	Apolipoprotein B-100/hoFH	Subcutaneous	2013
EXONDYS 51 (Sarepta)	Eteplirsen	Dystrophin/DMD	Intravenous	2016
SPINRAZA (Biogen)	Nusinersen sodium	SMN/SMA in infants	Intrathecal	2016
ONPATTRO (Alnylam)	Patisiran sodium	Transthyretin/hATTR in adults	Intravenous	2018
TEGSEDI (Akcea)	Inotersen sodium	Transthyretin/hATTR in adults	Subcutaneous	2018

AMD indicates age-related macular degeneration; CMV, cytomegalovirus; DMD, Duchenne muscular dystrophy; hATTR, hereditary transthyretin-mediated amyloidosis; hoFH, homozygous familial hypercholesterolemia; and SMA, spinal muscular atrophy.

causing fibrotic kidney disease, hearing loss, and eyesight problems.[124–126] A natural history study and a first-in-man trial have both been successfully completed (NCT03373786). A phase II trial for the assessment of safety, tolerability, and efficacy in reducing the decline in renal function has been initiated in a randomized, double-blind, placebo-controlled design, with weekly subcutaneous injections of either the test substance or a placebo over 48 weeks (NCT02855268).

Ischemic Conditions and Heart Failure

Another compound intended to promote the growth of new blood vessels by inhibiting miR-92a (MRG-110) is currently under clinical development. The beneficial effects of miR-92a silencing in ischemic heart conditions and for the promotion of angiogenesis, as observed in mice and pigs, have been described above. A phase I trial for the investigation of an intradermal injection of miR-92 antimiR in wound healing and incisional complications recently completed recruitment (NCT03603431). The safety of antimiR-92 administration via intravenous injection has been assessed in healthy volunteers, but the results of the study are not yet publicly available (NCT03494712).

Recently, a clinical dose ascending and dose repetition phase 1b study was initiated to assess the safety, pharmacokinetics, and pharmacodynamic parameters of an antimiR-132 inhibitor in stable heart failure patients (NCT04045405). Preclinical data suggested this miRNA plays a key role in the pathological cardiac remodeling process.[23,40]

Other Clinical Developments

Another inhibitor against a miRNA, miR-155, previously also described in cardiovascular disease,[127] is being developed for the treatment of various blood cancers, including, but not limited to, T-cell lymphoma and chronic lymphocytic leukemia. This LNA antimiR, called Cobomarsen (MRG-106), has reached clinical phase II with 2 currently active trials, PRISM and SOLAR (NCT03713320, NCT03837457). Two other developments rely on increasing the function of miR-16 (NCT02369198) and miR-34a (NCT01829971) in patients with various advanced malignancies. A phase I trial using TargomiRs (minicells targeted to EGFR) loaded with miR-16-based mimic microRNA was completed with encouraging results.[128] However, the effects of TargomiRs in patients with malignant pleural mesothelioma require further investigation. One phase I trial with a miR-34a mimic (MRX34) enrolling 155 subjects was withdrawn by the sponsor after 5 serious immune-related adverse events (NCT01829971). This illustrates the potential immunogenicity and off-target effects induced by some RNA drugs.[129]

An antimiR-122 has been evaluated for the treatment of hepatitis C in patients who did not respond to pegylated-interferon alpha and ribavirin; however, its clinical development has so far not proceeded beyond phase II (NCT02508090, NCT02452814).

Drug Formulations and Different Routes of Administration

As mentioned above, ncRNA-based therapies have recently attracted increasing attention. Compared with other drug formulations, like small molecules or antibodies, RNA therapies have several advantages. Previous studies have shown that many protein targets (80%–85% of the protein-coding genes) are still "undruggable", mostly scaffold proteins or transcription factors.[130,131] In contrast, 98% of the human transcriptome consist of noncoding RNAs; therefore, RNA therapy provides treatment options to those diseases with "undruggable" protein targets. Drug resistance from an ABC transporter or from epigenetic modifications is a serious issue in treating cancer or infectious disease,[132,133] whereas ncRNA therapy has no such issues reported so far. Another advantage of ncRNA is the paracrine effect. Previous studies have shown that multiple cell types in the cardiovascular system generate different kinds of vesicles, such as microvesicles and exosomes, that are able to transport the ncRNAs to other organs or cell types. The paracrine effect provides ncRNA-based drugs with broader targets to the whole signaling pathway compared with antibodies or small molecules.[134,135] Additionally, with different chemical modifications, the half-life of ncRNA drugs can be long (weeks to months), and, thus, patient dosing frequency can be decreased compared with small molecules or antibodies.[136,137] A further advantage is that one or more complete disease pathway can be modulated by noncoding RNA-based therapeutic approaches.[138]

With respect to chemical modifications, the miRNA agonists and antagonists mentioned in the previous sections are all synthetic oligonucleotides but belong to different chemical classes. These range from small double-stranded RNAs (siRNA—eg, Patisiran and miRNA mimic—eg, MesomiR) over antisense DNA/RNA oligonucleotides with backbone modifications (eg, Fomivirsen, Eteplirsen). Furthermore, the second generation of antisense oligonucleotides (ASOs), which contain sugar modifications such as 2'-O-methoxyethyl (2'-O-MOE, eg, Mipomersen) or an 2'-4' ether bridge leading to a bicyclic sugar moiety, usually referred to as LNA, have also been well-developed. To maintain RNAse H cleavage, these therapeutic ASOs need to possess a complementary sequence target made up of DNA flanked by the modified residues; these entities are called GapmeR or chimeric LNA (eg, Miravirsen, Cobomarsen; Figure 1).

The first steps in clinical development mainly used local administration, for example, intravitreal for eye diseases, intradermal for wound healing, and intrathecal for neurological disorders. Meanwhile, systemic administrations,

such as intravenous or subcutaneous injection, are preferred for most clinical applications but raise the question of tissue-specific drug delivery versus off-target effects.[139] Current nonclinical studies in heart disease models may make use of local administration, including intracoronary or intramyocardial injection, but their translation into clinical reality remains questionable and difficult (Figure 1). Therefore, different strategies have been exploited to direct therapeutic ASO to target tissue and cell types. In CVD, it may be important, depending on the pathomechanism of the clinical entity, to deliver the drug to the cardiomyocytes, endothelial cells, cardiac fibroblasts, or the immune cells in the heart. Viral and nonviral approaches are the subjects of ongoing investigations in RNA-based diagnostic and therapeutic strategies for CVD, as recently reviewed by Lu and Thum.[6] While directing ASO drugs toward the liver via GalNAc conjugation or liposomal formulation has already reached a clinical stage, including Food and Drug Administration approval (Patisiran), microRNA mimics have been shown to specifically reach cancer cells via liposomal (MRX34) or TargomiR formulations (MesoMiR). However, the delivery of cardiac-specific ASO drug remains a challenge to be solved in the future (Figure 1).

In a systematic study involving 135 large animal pigs, potential differences between intravenous and intracoronary applications of antimiR-132 were tested.[23] Based on detailed plasma PK and tissue uptake measurements of the antimiR-132, it could be shown that there were no significant PK differences between these 2 routes of administration. However, whether this could be translated to other antimiR molecules and/or chemistries remains to be tested.

Virus-based approaches, especially via adenoassociated virus, are currently powerful tools for human gene therapy but challenging due to high antibody titers found in many patients, which limits the number of eligible patients entering clinical trials.[140–143] A possible solution could be new capsid-modified adenoassociated viruses with improved specificity for delivery to the cardiovascular system and/or a decreased ability to raise an immune response.[144–146] Cardiac specific delivery strategies are also currently being developed in the ongoing EU project CardioReGenix.[147]

Finally, most studies have usually been performed in single-model systems in small rodents, hampering clinical translation. Cardiovascular studies in large-animal, nonrodent studies or human ex vivo studies may be more predictive of future clinical success in next generation ncRNA-based therapeutics.[97]

CONCLUSIONS

Here, we described the state-of-the-art ncRNA-based therapies targeting the heart, ranging from large-animal heart disease models to clinical studies (Figure 3).

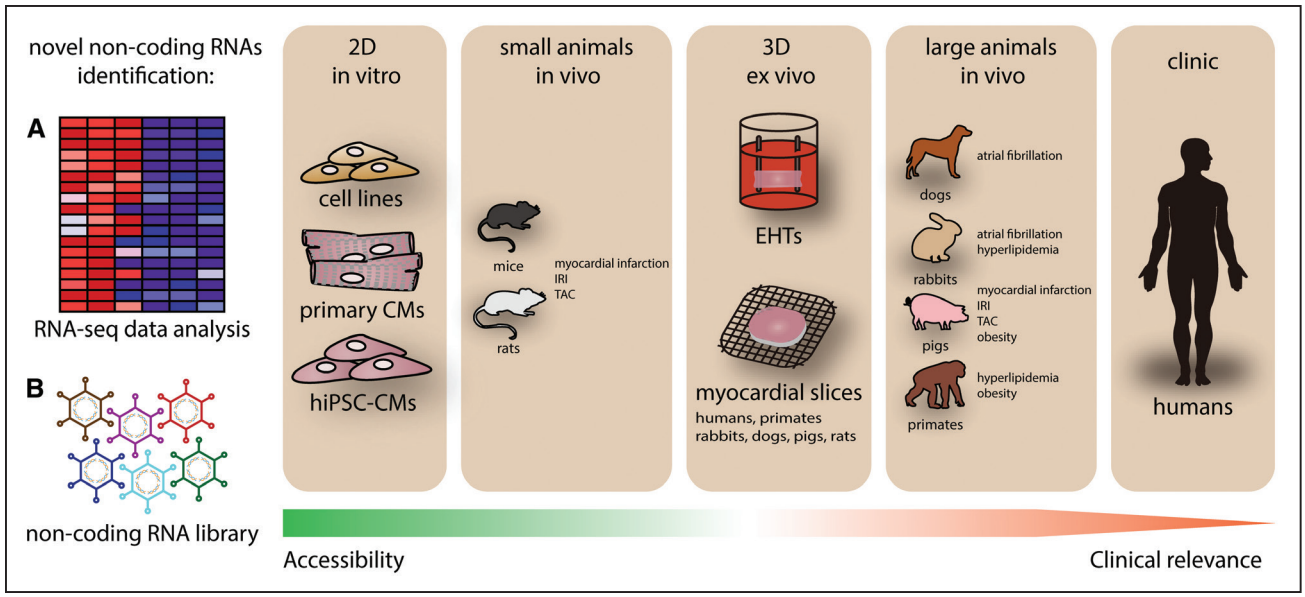

Figure 3. Processes of noncoding RNA (ncRNA)-based drug development.
Novel ncRNA candidates are selected from a RNA-seq profile or other ncRNA approaches[40,148] and then validated in cardiovascular cells (in vitro). After basic characterization, the ncRNA candidates are further investigated in animal models (in vivo). Several pathophysiological animal models of cardiovascular complication are available in different species, ranging from small to large animals, via the application of surgical techniques, genetic engineering, or diet changes. Some effective yet nontoxic ncRNA candidates are selected for further clinical development. However, successful transitions from preclinical to clinical studies are generally small in number. Often, in vitro models are easy to apply but have limited clinical relevance; while in vivo models have higher clinical relevance, they are challenging to conduct and expensive. To increase the translational efficiency of ncRNA-based therapeutic human induced pluripotent stem cells and/or other human cardiovascular cell types and living myocardial slices, could be powerful tools to bridge the gap between in vivo and clinical development. IRI indicates ischemia-reperfusion injury; and TAC, transverse aortic constriction.

The improvement of bioinformatic tools enhances our understanding of the underlying mechanisms of the lncRNA/circRNA-mRNA-miRNA network in CVDs. This then supports the discovery of novel RNA molecules, which can prove to be therapeutic targets and provide more and hopefully improved treatment options to CVD patients in the future. Furthermore, large animal models have recently been gaining increasing attention for their high clinical relevance; however, while being closer to humans than rodents, there remain limitations on the level of metabolism or immune system, which render animal studies on a whole not to be fully predictive for safety and efficacy in humans. Despite great efforts in the last few decades, promising clinical candidates continue to be eliminated from the developmental pipeline for safety reasons and/or a lack of efficacy in all clinical stages. Therefore, the rapid development of human ex vivo systems, such as EHTs and living myocardial slices, constitute new valuable tools to improve insight into the translatability of preclinical studies. Such ex vivo models will also ultimately contribute to a reduction of the number of animals used in animal studies in pharmaceutical drug development. New delivery techniques with the aim of increasing tissue and/or cell type specificity and thereby lowering off-target effects will improve the safety of new developmental drugs. Moreover, an increased understanding of certain interindividual and sex differences is a requirement before progress in personalized medicine.

In addition to developing techniques in the laboratory, it is also important to validate and qualify these new tools and methods to achieve standardized assays that can be acceptable to authorities within the regulatory procedure.

In summary, based on (1) in vitro models, (2) rodent models, (3) large animal models, (4) ex vivo studies with human cells and tissues, and (5) new delivery systems, ncRNA therapies have the potential to enable significant progress in the development of next-generation therapeutics for cardiovascular disease (Figure 3).

ARTICLE INFORMATION

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Disclosures

T. Thum has filed and licensed patents regarding the diagnostic and therapeutic use of several cardiovascular noncoding RNAs. He is also the founder of and holds shares in Cardior Pharmaceuticals GmbH, a clinical-stage biotech company. The other authors report no conflicts.

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