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Commentary

RNA therapeutics for heart disease

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ABSTRACT

The majority of the human genome encodes non-coding RNAs (ncRNAs), species of RNA without protein-coding potential but with powerful regulatory functions in disease onset and progression. Functional studies demonstrate that both coding and ncRNAs underlie various mechanisms in heart disease and that molecules targeting RNA species show promising efficacy in preclinical development. Accompanying the exciting developments in basic RNA biology, an equally provocative field has flourished for the design of RNA-based strategies to generate innovative types of therapeutics against these new "druggable" targets, going beyond our current repertoire of small chemistry or biologics. Here, we review the (bio)chemical basis of RNA-based drug design, provide examples that show promise as translatable drug products in preclinical studies, give an insight in the current barriers that hamper straight-forward clinical translation and discuss future directions that may overcome these hurdles to expand the current pharmacotherapy for myocardial disorders.

1. RNA avenues for heart disease

Heart diseases are a major cause of morbidity and death in Western societies with little recent progress to reduce their high mortality and are now also steeply on the rise in the developing world [1]. The diseases are preceded by ventricular remodelling and changes in left ventricular mass and volume of the myocardium in response to alterations in loading conditions [2,3]. The molecular events that underlie myocardial remodelling still remain poorly understood. Another major hurdle towards improved therapeutics is that currently cardiovascular disease therapy mainly follows a generic 'one-size-fits-all' approach, ignoring the inter-individual differences caused by underlying genetic susceptibility, age, gender or stages of disease. This calls for better disease stratification and introduction of medical breakthroughs to address the key biological mechanisms that derail in subsets of patients, an approach that will likely signal the end of the big block-buster treatments for vast numbers of patients, introducing the concept of "Precision Medicine". With "Precision Medicine" is meant medical decisions, practices, and interventions tailored to the individual patient who will benefit, sparing expenses and side effects for those who will not.

Heart diseases are most commonly provoked by the acute and chronic loss of cardiomyocyte function secondary to coronary artery disease, hypertension, diabetes or combinations thereof, which places a volume load or a pressure load, respectively, on the surviving portion of the myocardium. Chronic "load" causes quite generic responses in the myocardium, such as hypertrophy of cardiac myocytes, cardiac dilatation and interstitial fibrosis. These structural cardiac changes are preceded by changes in the "genomics" of the heart: changes in transcript abundance of protein-coding or non-coding genes and the re-expression of genes that are normally only observed in the embryonic heart [4]. These complex forms of heart disease are referred to as "acquired heart diseases" [5]. Acquired forms of heart disease are contrasted by monogenetic forms of heart disease caused by mutations in single genes identified using candidate gene studies or linkage analysis, leading to hereditary, familial dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC) [5].

Since cardiovascular disease mechanisms are typically genomic in nature (hereditary cardiovascular diseases comprise a very small subset of the patient population), new developments will likely derive from a better understanding of our genome. The results of the Encyclopedia of DNA Elements (ENCODE) project demonstrate that at least 80% of our genome is functional, has a regulatory role and is transcribed into various classes of non-coding, regulatory RNAs. In short, RNA species beyond mRNAs include intronic RNAs, microRNAs (miRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs) and extracellular RNAs. Collectively, these are known as non-coding RNAs (ncRNAs)

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because they lack clear coding potential, although notable exceptions exist [6]. Here, we will focus on coding and ncRNAs as direct drug targets for heart disease using RNA to silence or mimic the expression of the target RNA molecule. Additionally, we will give examples how the versatility of RNA as drug substance can be employed to function as homing devices or delivery tools to locate other molecules in close proximity of a desired drug target, as is in the case of aptamers or short guide RNAs in CRISPR technology.

2. Modulation of RNA activity: antisense oligonucleotides

Modulation of endogenous ncRNA activity can be efficiently accomplished by the use of antisense oligonucleotide (ASO) technology or modified RNA mimics, such as plasmid or viral vectors carrying RNA sequences to cells and tissues. ASOs were first discovered decades ago and are defined as short, single-stranded chains of nucleotides that hybridize with complementary RNA sequences following Watson-Crick base pairing; they exert their function either by leading to the degradation of the endogenous ncRNA, mediated by RNase H activity, or by sterically blocking the access to its targets [7]. ASOs have already been successfully employed as tools to inhibit ncRNAs and the simple biological principles of their function makes ASO design relatively straightforward. However, since several exo- and endonucleases in organisms rapidly degrade RNA-like structures, premature degradation of ASOs severely hampers their bioavailability. In this context, chemical modifications of ASOs are often employed to alter their pharmacokinetic properties and enhance cellular uptake without concomitant loss of target binding (Fig. 1). Here, we will list the most commonly used chemical nucleotide modifications of ASOs employed for the treatment of heart diseases *in vivo* (Fig. 2).

2.1. ASO: 2′-OMe modifications

Methylation of the hydroxyl group at the 2′ position of the ribose unit is a relatively simple and attractive approach to increase resistance to nuclease attack. The 2′-O-methyl (2′-OMe) ASOs have been widely used in the past decade and have proven *in vivo* efficacy. Besides enhanced resistance to nuclease attack, 2′-OMe ASOs show improved binding affinity to their corresponding RNA sequence when compared to unmodified ASOs. To further enhance resistance to nuclease attack, phosphorothioate moieties in the linking backbone are often introduced: these sulphur analogues of phosphate can be incorporated near the 5′ or the 3′ end, showing, in combination with a 3′-cholesterol unit, enhanced ASO stability in cell cultures. Krützfeldt et al. were the first to explore the potential of 2′-OMe ASO modifications to modulate microRNA activity *in vivo*. Systemic injection of 2′-OMe-modified oligonucleotides with a partial or fully modified phosphorothioate backbone and a 3′ cholesterol conjugation through a hydroxyprolinol linkage, termed “antagomirs”, were able to completely silence endogenous miR-122 levels in the liver through microRNA degradation; moreover, high sequence specificity was demonstrated by introducing position specific mismatches that abolished ASOs activity. The authors reported that the administration of a miR-122-targeting antagomir at a dose of 80 mg/kg for 3 consecutive days effectively reduced miR-122

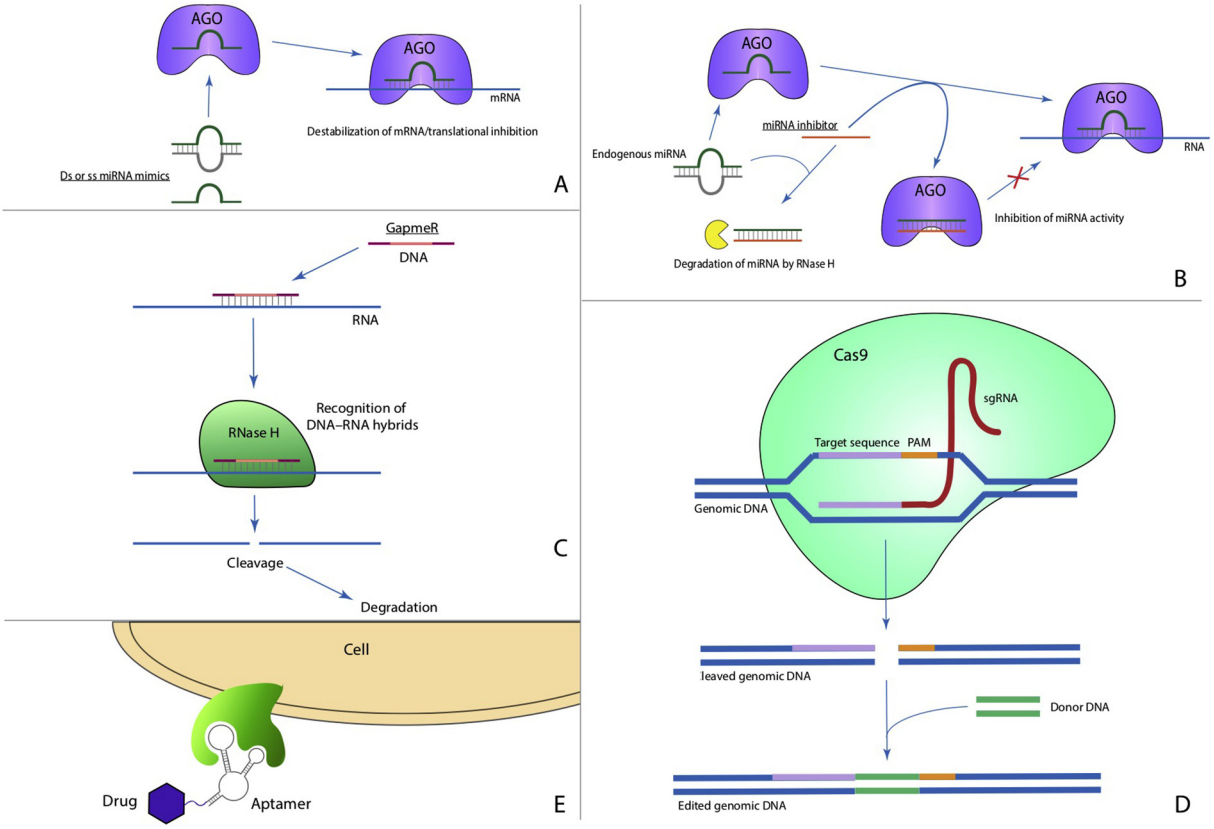

Fig. 1. Strategies for the regulation of RNA levels with antisense oligonucleotides, ncRNA mimics and CRISPR/Cas9. A) Transcription regulation by miRNA mimics. Double-stranded (ds) or single-stranded (ss) miRNA mimics reduce transcription of target mRNAs by destabilization or inhibition of the translation of the transcript they couple with. B) Regulation by miRNA inhibitors. MiRNA inhibitors can form DNA-RNA duplexes with their miRNA targets that are then cleaved by RNase H. Alternatively, miRNA inhibitors can act by binding their miRNA targets when loaded in the Argonaute protein (AGO), impairing their activity. C) Reduction of mRNAs by antisense oligonucleotides GapmeRs. RNase H recognizes the duplex formed by the GapmeR and its mRNA target and degrades it. D) Genome editing strategy using CRISPR/Cas9 tool. SgRNA molecule coupled with Cas9 enzyme binds to the target DNA region, Cas9 introduces a DSB upstream its PAM sequence and if a donor DNA molecule is present it can be used for homology-directed repair (HDR) of the DSB. E) Aptamers function as nucleotide analogues of antibodies for the tissue or cell-specific delivery of drugs.

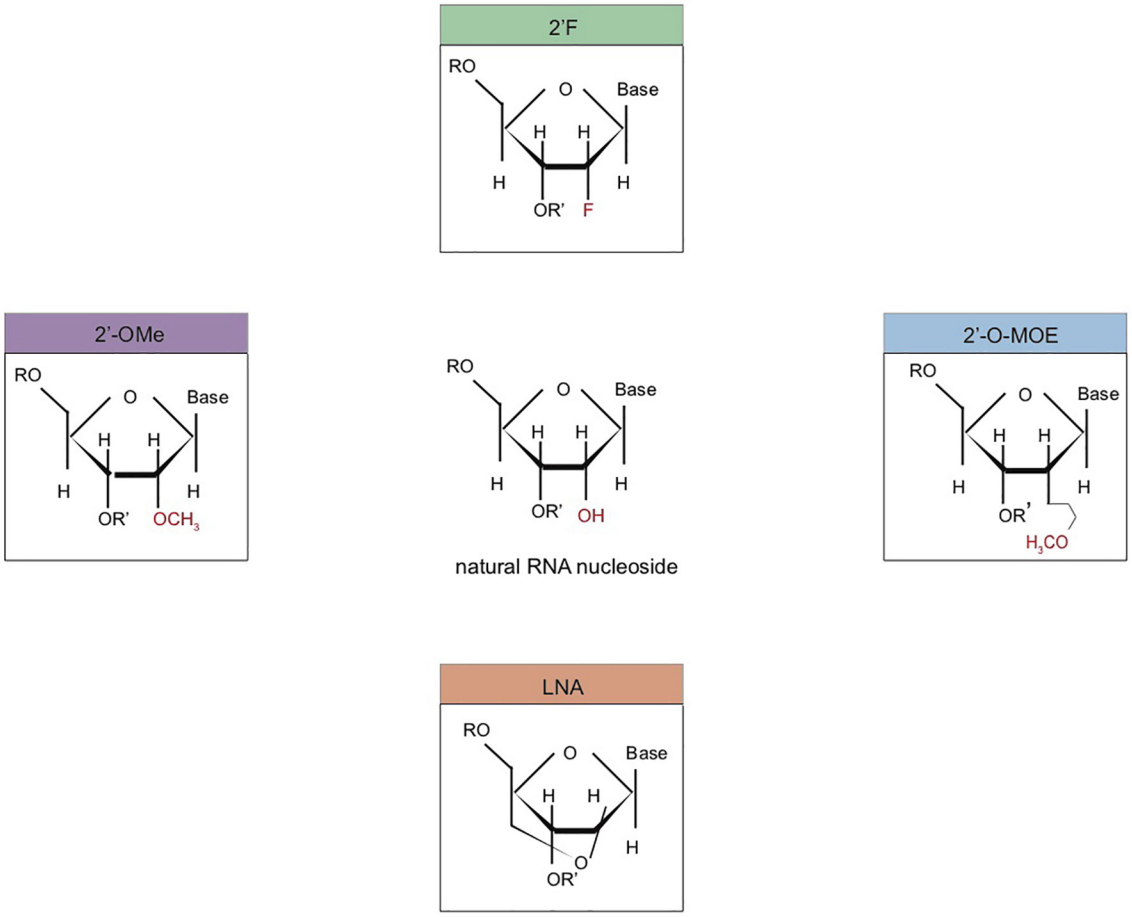

Fig. 2. Chemical modifications in RNA-based drugs. A natural RNA nucleoside is depicted for reference in the middle. Each box surrounding the natural nucleoside is colour-coded and represents a specific chemical modification.

expression levels in mice for a period of at least 23 days. By the use of fluorescently labelled antagomirs they showed that the interaction between antagomirs and microRNAs takes place in the cytoplasmic compartment, upstream of the processing bodies (P-bodies), sites that act as scaffolding centres for microRNA function. Antagomirs display a broad biodistribution and can efficiently silence their target microRNAs in all tissues tested. Although they cannot cross the placental and blood-brain barriers, injection of antagomirs directly into the cortex can efficiently produce a reduction of the targeted microRNAs levels in the brain.

The use of 2′-OMe modified antagomirs has been extensively tested in the context of heart diseases: the study of Carè et al. was one of the first demonstrating how the use of an antagomir efficiently silences endogenous miR-133, a particularly abundant miRNA species in the heart, leading to significant cardiac hypertrophy in mouse [8]. Various studies afterwards have made use of 2′-OMe antagomirs to revert pathological cardiac conditions induced in small mammals. For instance, mice subjected to pressure overload of the left ventricle by transverse aortic constriction (TAC) have been injected with antagomirs specific for miR-21, with subsequent attenuation of the impairment of left ventricular function as well as interstitial fibrosis and cardiac hypertrophy expected after TAC surgery [9]. The injection of antagomirs against miR-132 has also shown the potential to prevent the pathological cardiac remodelling induced in mice subjected to TAC [10]. Boon et al. showed that miR-34a level increases after acute myocardial infarction and that its inhibition enhances cardiac recovery; interestingly, they also demonstrated the involvement of this microRNA in cardiac ageing. MiR-34a is increased in the hearts of Ku80-/- mice with accelerated ageing and its pharmacological inhibition significantly abolishes the deterioration of cardiac function accompanied by the premature ageing phenotype in this model [11]. Further examples of pre-clinical studies against heart diseases using oligos with 2′-OMe-based chemistry are quite common [4,12–27] (Table 1). Taken together, 2′-OMe-based antagomirs follow a proven chemical design that relies more on endogenous miRNA degradation rather than steric inhibition and uses chemical modifications that were proven as a relatively safe and tolerable option in future clinical studies.

2.2. ASO: locked nucleotide acid (LNA) modifications

The pharmacokinetics of ASOs can alternatively be modified by incorporating nucleotides with a so-called “locked nucleic acid” (LNA) modification. Here, the 2′-hydroxyl group is linked to the 4′ carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene $[CH2]_n$ group bridging the 2′ oxygen atom with the 4′ carbon atom wherein n is 1 or 2. LNA-protected antisense RNA oligonucleotides exhibit extreme high thermal stability when hybridized with their corresponding RNA target molecules. In addition, LNA oligonucleotides display relatively low toxicity in rodents at very low doses, are water-soluble and show excellent target specificity even when the ASO sequence is relatively short, typically consisting of 16-mer oligonucleotides. In clinical settings, however, LNA oligos have on occasion shown a rather severe acute kidney toxicity [28], possibly hampering straightforward clinical application.

Among the LNAs tested in the context of heart dysfunction, delivery of an LNA oligo that targets miR-92a in a porcine model of myocardial infarction resulted in reduced cell death in the infarcted area, increased capillary density, reduced inflammation and overall improved cardiac

Table 1
Preclinical studies applying nucleotide-based compounds targeting miRNAs in relevant models of heart failure.

Chemistry	RNA therapeutic	Dose/Delivery	Model	Target	Outcome	Ref
2′-OMe	21	80 mg/kg/bw three weeks post TAC; jugular vein catheter	Mouse, TAC	SPRY1	Attenuation of the impairment of cardiac function and regression of cardiac hypertrophy and fibrosis after TAC	[9]
	23a	25 mg/kg/bw for one week in combination with isoproterenol; osmotic minipumps	Mouse, Isoproterenol infusion	MuRF1	Inhibited Iso-induced hypertrophy	[12]
	24	80 mg/kg/bw for three consecutive days starting from two weeks post TAC. Three-days treatment repeated over 6–8 weeks; intravenous injection	Mouse, TAC	N/A	Prevented transition toward decompensated hypertrophy and protected the integrity of E–C coupling in hypertrophied cardiomyocytes	[13]
	24	5 and 80 mg/kg/bw, day 0 and day 2 post LAD; retroorbital injection	Mouse, LAD	GATA2, PAK4	Reduced endothelial apoptosis, enhanced vascularization, decreased infarct size and improved cardiac function	[14]
	25	80 mg/kg/bw for three consecutive days in weeks 1 and 4 post TAC; intraperitoneal injection	Mouse, TAC	Hand2	Exacerbated cardiac remodelling	[4]
	25	300 mg, three daily injection per week for 3 weeks; intravenous injection	Mouse, TAC	Serca2a	Improved cardiac function, decreased hypertrophy and fibrosis	[15]
	29b	80 mg/kg/bw for two consecutive days; intravenous injection	Mouse	COL1A1, COL1A2, COL3A1, ELN1, FBN1	Increased collagen expression as it happens at the site of tissue damage after MI	[16]
	34a	8 mg/kg/bw; intravenous injection	Mouse, MI	PNUTS	Reduced cell death and fibrosis, improved cardiac function, increased capillary density in the border zone	[11]
	21-3p	80 mg/kg/bw for 3 consecutive days before LPS treatment; intravenous	Mouse, sepsis associated cardiac dysfunction	SORBS2	Attenuated LPS-induced cardiac dysfunction and promoted survival	[17]
	19b-16	20 mg/kg/bw every two weeks for 8 weeks; subcutaneous injection	Mouse, HHD	ANDRA1A	Reduced hypertension; reduced fibrosis and apoptosis in the myocardial tissue	[18]
	1	200 pmol/kg/bw for three consecutive days; intravenous injection	Rat, I/R-IPC	Cx43	Increased expression of Cx43 and attenuated ischemia–reperfusion injury	[19]
	503	30 mg/kg/bw on days 1, 2, 8, 9, 15 and 16 post-TAC; intravenous injection	Mouse, TAC	Apelin-13	Reduced TAC-induced cardiac fibrosis	[20]
	144	20 mg/kg twice a week for seven weeks; intravenous injection	Mouse, diabetic cardiomyopathy	Nrf2	Decreased oxidative stress and reduced cardiomyocyte apoptosis	[21]
	195	5 mg/kg after LAD; intravenous injection	Rat, LAD	Bcl-2	Attenuated cardiac injury induced by MI via reduction of apoptosis	[22]
	128	80 mg/kg three days prior to LAD; intravenous injection	Rabbit, I/R	PPARG	Reduced myocardial I/R-induced cardiomyocyte apoptosis	[23]
	92a	8 mg/kg/bw days 1, 3 and 5 post MI; intravenous injection	Mouse, MI	ITGA5	Enhanced blood vessel growth, functional recovery of damaged tissue and decreased apoptosis	[24]
	132	80 mg/kg/bw on days 0 and 1 post TAC; retroorbital injection	Mouse, TAC	FoxO3	Decreased hypertrophy and fibrosis, preserved cardiac function	[10]
	199a-214	20 mg/kg/bw three daily injections; intraperitoneal injection	Mouse, TAC	PPARd	Decreased hypertrophy and fibrosis, preserved cardiac function	[25]
	199b	80 mg/kg/bw three daily injections in weeks 1 and 4; intraperitoneal injection	Mouse, TAC	Dyrk1A	Reduced nuclear NFAT activity, inhibition and reversal of hypertrophy and fibrosis	[26]
	133	80 mg/kg/bw three daily injections; osmotic minipumps	Mouse	RhoA, Cdc42 Nelf-A/ WHSC2	Marked and sustained hypertrophy	[8]
	146a	8 mg/kg/bw four injections in 1 week; intravenous injection	Mouse, Stat3 Knock-out	NRAS	Improved cardiac function, reduced fibrosis and enhanced capillary density	[27]
LNA	92a	0.03 mg/kg/bw; intravenous or catheter local delivery to the heart	Pig, I/R	N/A	Reduced infarct size, increased capillary density and decreased inflammation	[26]
	34 family	25 mg/kg/bw three daily injection; subcutaneous injection	Mouse, TAC	SIRT1, HNF4α, SOX2, CTCF, N-Myc	Improved cardiac function, reduced inflammation and fibrosis, attenuated hypertrophy	[30]
	208a	25 mg/kg/bw; intravenous injection	Mouse; Dahl salt-sensitive rats/high-salt diet	N/A	Increased survival, increased body weight, reduced fibrosis and attenuated hypertrophy	[31]
	155	10 mg/kg/bw three daily injections; intravenous injection	Mouse, Angiotensin II treatment	Socs1	Improved cardiac function, decreased heart weight and reduced inflammation	[32]
	21	10–80 mg/kg/bw on days 1 and 2 post TAC; intravenous injection	Mouse, TAC	N/A	Attenuated hypertrophy and improved cardiac function	[33]
	26a	24 mg/kg/bw; intravenous injection	Mouse, MI	Smad1	Induced angiogenesis, improved cardiac function, reduced infarct size and decreased apoptosis	[34]

(*continued on next page*)

Table 1 (*continued*)

Chemistry	RNA therapeutic	Dose/Delivery	Model	Target	Outcome	Ref
	652	25 mg/kg/bw three daily injections; subcutaneous injection	Mouse, TAC	Jagged1	Preserved capillary density, improved cardiac function, attenuated hypertrophy and reduced fibrosis	[35]
	154	25 mg/kg/bw over three consecutive days; subcutaneous injection	Mouse, TAC	TGFβ, Wnt pathway	Attenuated pathological cardiac remodelling and lung congestion	[36]
	208b	Intraperitoneal injection at days 0 and 7 after AngII infusion	Mouse, *Tnt* Knock-in for DCM model	MAPK8, HDAC2, ROCK1, RHOBTB3	Prevention of DCM development	[37]
	29	20 mg/kg/bw for three consecutive times; intravenous injection	Mouse, TAC	GSK3B, ICAT/CTNNBIP1, HBP1 and GLIS2 (Wnt pathway)	Protection from cardiac dysfunction, from cardiac hypertrophy at the tissue and cell level and from fibrosis	[38]
GapmeR	Chast-GapmeR	20 mg/kg weekly injections starting from four weeks post TAC; intraperitoneal injection	Mouse, TAC	Chast	Reduced fibrosis and hypertrophy and rescued EF levels after pressure overload	[39]
	Meg3-GapmeR	20 mg/kg every ten days starting from one week post TAC; intraperitoneal injection	Mouse, TAC	Meg3	Reduced fibrosis and hypertrophy and rescued diastolic functioning	[40]
	MALAT1-GapmeR	10 mg/kg every four days starting from three weeks after hypoxic exposure; intraperitoneal injection	Mouse, Pulmonary Hypertension (PH)	MALAT1	Reduced heart hypertrophy after hypoxia	[41]
2′(F)-MOE	21	10–80 mg/kg/bw; intravenous injection	Mouse, TAC	N/A	Reduced fibrosis and cardiac hypertrophy and prevented decline of cardiac functions post-TAC	[33]
AgomiR	302b/c	10 μg daily for 7 days after MI; intravenous injection	Mouse, MI	Mst1, Mob1b (Hippo pathway)	Improved cardiac repair and regeneration after MI	[42]
	145	2.5 nmol/kg/bw of miRNA encapsulated by liposomes immediately after the start of reperfusion; intravenous injection	Rabbit, I/R	FRS2, Akt pathway	Reduced MI size, improved LV function and remodelling and accelerated cardiomyocyte autophagy	[43]
	378	80 mg/kg/bw from the first day post TAC for three days; intravenous injection	Mouse, TAC	Col1a1, Col3a1, Acta2	Inhibited hypertrophy and fibrosis during pressure overload-induced cardiac remodelling	[44]
	199a-3p/590-3p	200 pMoles post-LAD; intracardiac injection	Mouse, LAD	Homer1, Clic5	Reduced hypertrophic response of cardiomyocytes after MI, rescue of cardiac functioning and presence of proliferating cardiomyocytes	[45]
AAVs delivering anti-miR	99/100	Intracardiac injection in the periphery of the infarcted area of 10^{11} viral genome particles per animal	Mouse, MI	FNTβ, SMARCA5	Regenerative response upon injury with reduction in fibrotic scarring and in infarct size and ameliorated functional heart parameters	[51]
Lentivirus delivering anti-miR	Let-7	5.25×10^7 TU four days before myocardial ischemia; injection into the free anterior wall myocardium of the left ventricle	Rat, diabetic cardiomyopathy	IGF-1R, InsR, GLUT4, P-Akt, and P-mTOR	Cardioprotective effect against I/R Injury	[49]

recovery. An important aspect addressed in this study regards the delivery method used and how drastically it can change the outcome: local administration of the compound rather than its systemic delivery proved to be key for the aforementioned positive effects on the phenotype of the ischemic animals, pointing out a central critical aspect in the use of RNA therapeutics, that is their efficient delivery to the target organ [29]. Additional examples of LNA based ASO studies in the context of heart disease have been reported [30–38] (Table 1).

GapmeRs are a relatively new class of LNA ASOs used to target lncRNAs, but still only a few examples of these compounds to treat myocardial diseases are available. Typically, GapmeRs are designed as a 20-base pair ASO with a phosphorothioate backbone along the entire length of the oligo to provide nuclease resistance, while the 2′-sugar modification is used exclusively on the first and last 5 nucleotides, leaving the middle 10 nucleotides unmodified at the 2′-sugar positions. This design provides increased target RNA binding affinity on the outer portions of the ASO allowing RNAse H cleavage at the central portion of the oligo. One example of a GapmeR tested to treat heart dysfunctions consists of the GapmeR against *Chast* (Cardiac hypertrophy–associated transcript), a lncRNA increased in pressure overloaded mouse hearts, whose administration ameliorates adverse cardiac remodelling and reduces hypertrophy [39]. Other studies addressing the use of GapmeRs to treat heart diseases have also been reported [40,41] (Table 1).

2.3. ASO: 2′(F)-MOE modifications

Another tested approach to increase the stability of ASOs is the combination of 2′-O-methoxyethyl (2′-MOE) with phosphorothioate modifications in the linking backbone. This method is similar to 2′-OMe modifications albeit using $CH_2CH_2OCH_3$ protective groups rather than a simple CH_3. Apart from 2′-MOE variations of RNA analogues, other RNA analogues contain a 2′-fluoro modification, which has proven to be a potent inhibitor of both miR-122 and miR-21 in mice. However, ASOs containing 2′-fluoro modifications require further modifications to make them resistant to nuclease attack by making single strand antagonists with a full phosphorothioate backbone or additional 2′ protective groups like 2′-MOE. By using 22-mer oligonucleotides with either the 2′-OMe or the 2′(F)-MOE modifications, successful silencing of miR-21 was reached in a study which established a central role for this miRNA in pressure overload-induced cardiac dysfunction in mice [33] (Table 1).

3. Modulation of RNA activity: ncRNA mimics

In case a ncRNA is decreased in expression in a disease setting and restoration of its expression is warranted or could have therapeutic potential, it may be desirable to reintroduce the activity of the ncRNA. Most experimental evidences to date show the restoration of miRNA activity with synthetic RNA duplexes that harbour chemical modifications to improve their stability and cellular uptake. In such double-stranded miRNA mimics, the strand identical to the miRNA of interest is named guide strand (or antisense strand), while the opposite one is called passenger strand (or sense strand). The latter strand is less stable and can be linked to a molecule, such as cholesterol, to enhance cellular uptake. In addition, the passenger strand may contain chemical modifications to prevent RISC loading while carrying minimal nucleotide modifications that might prevent it from degradation, typically consisting of 2′-fluoro modifications. Apart from chemically modified miRNA mimics, the use of lenti-, adeno-(Ad) or adeno-associated viruses (AAV) to drive expression of a given ncRNA for restoring its activity has been successfully reported by several studies in the cardiac field. Examples of either strategy are reported below.

3.1. RNA mimics: agomirs

The “agomir” miRNA mimics technology is available at Creative Biogene Biotechnology Inc. (USA) and essentially consists of chemically-modified double-strand miRNA mimics that resemble endogenous mature miRNAs and rely on the use of the natural miRNA machinery after transfection into cells or when delivered to organs to be functional. The antisense strand of the agomir has two phosphorothioates at the 5′ end, four phosphorothioate backbone modifications plus a cholesterol group at the 3′ end and a full-length nucleotide 2′-methoxy modification and is reported to exhibit enhanced cellular uptake, stability and regulatory activity *in vivo*. These compounds have been tested for therapeutic purposes, as for instance to transiently increase the miR-302-367 cluster to promote cardiac regeneration after myocardial infarction in the mouse [42]. This miRNA cluster is involved in the repression of the Hippo pathway and when temporarily overexpressed can increase cardiomyocyte proliferation and decrease apoptosis without adverse effects on cardiac function [42]. Additional examples using agomirs to treat diseased hearts are reported in the following reports [43–45] (Table 1).

3.2. RNA mimics: viral delivery of ncRNA

Viruses are naturally very efficient at transducing their own genetic information into host cells for their own replication. By replacing non-essential viral genes with foreign genes of therapeutic interest, recombinant viral vectors can be used to transduce the cell type that they would normally infect. Although viruses may trigger a host immune response, they also have evolved and developed efficient countermeasures, thus enabling them to reach and replicate in their target cells. Among the viral vectors most employed in preclinical cardiac disease settings there are Adenovirus, Lentivirus, Moloney Murine Leukemia Virus (MMLV) and Adeno-Associated Virus (AAV). In depth reviews on these vector types are described elsewhere [46,47]. The overall design of viral vectors for the delivery of ncRNAs includes the endogenous genomic DNA sequence encoding the ncRNA coupled to a strong promoter driving the transcription of the encoded RNA transcript (e.g. the cytomegalovirus promoter or even a cardiac cell type-selective promoter sequence). In such manner, transcription, subsequent processing and maturation of the delivered ncRNA uses the machinery of the cell. A lentivirus has been used for the delivery of lncRNA H19 in the heart of diabetic rats: as a result, H19, normally down-regulated in diabetic conditions, was found increased in the animals injected with this lentivirus, resulting in a lower cardiomyocyte apoptotic index, better left ventricular function, less inflammation and lower oxidative stress [48]. Viral vectors have also been successfully used to deliver antisense oligonucleotides as well as evidenced by the local transfection of lentiviral vectors encoding a Let-7 ASO to silence the targeted miRNA in diabetic rats subjected to temporal coronary artery occlusion. As a result, cardioprotection against ischemia-reperfusion injury was observed [49]. Apart from lentiviruses, the use of AAV vectors in cardiovascular disease settings are now increasingly reported [50]. AAV serotype 2/9 (AAV2/9) presenting cardiac tropism has been the tool for the delivery of anti-miR-99/100 to mice subjected to left anterior descending artery ligation, with subsequent improvement of contractile cardiac parameters and a significant reduction in fibrotic scarring and infarct size [51] (Table 1).

4. Aptamer delivery of RNA

Aptamers are small (ranging from 20 to 60 nucleotides) single-stranded RNA or DNA oligonucleotides able to bind target molecules with high affinity and specificity. In fact, aptamers are nucleotide analogues of antibodies, but aptamer-generation is significantly easier and cheaper than the production of antibodies. Moreover, aptamers are neither immunogenic nor toxic. All these features make aptamers ideal candidates for diagnostic and therapeutic applications, purification of target molecules from complex mixtures, or biosensor design. Aptamers are usually selected from the oligonucleotide collection that is known as

the initial oligonucleotide pool (IOP), often called “combinatorial library”, which includes > 1000 different single-chained DNA or RNA oligonucleotides conditioned to bind the target molecule. The conventional method for aptamer engineering is known as SELEX (systematic evolution of ligands by exponential enrichment): the sequences in the library are exposed to the target ligand and those that do not bind the target are removed. The bound sequences are then eluted and amplified by PCR to prepare the library for the following rounds of selection, which will be performed increasing the stringency of the elution. Maximum enrichment of the pool of aptamers with the strongest affinity for the target molecules is usually achieved after 5–15 rounds. Theoretically, aptamer-based approaches may provide efficient tools for delivery of RNA species to specific cell types or organs, increasing their intracellular uptake, processing and function [52].

The direct conjugation of (nc)RNAs to aptamers against cell-type specific receptors represents an innovative experimental approach for their selective delivery to cardiac cells. An interesting example of the use of aptamers with potential therapeutic purposes comes from the work by Li et al., where the authors succeeded in efficiently targeting the osteopontin transcript in pressure overloaded mouse hearts, resulting in the prevention of pathological cardiac remodelling and fibrosis [53]. Aptamers have been successfully used in the context of heart diseases in additional studies [54,55] (Table 2).

5. RNA to guide precision genome editing

The identification of the clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas)9 system has revolutionized the field of genetics, has greatly facilitated the generation of genetically modified animals and even opened the possibility to correct human germline monogenic mutations [56–58]. In particular, CRISPR–Cas9 is a versatile tool for recognizing specific genomic sequences and inducing double-strand breaks (DSBs). To do so, the CRISPR-associated endonuclease Cas9 is directed to specific locations in the genome via an RNA-guided system involving single-guide (sg)RNAs. DSBs induced by Cas9 are resolved by endogenous DNA repair mechanisms, preferentially using a non-homologous end-joining (NHEJ) pathway [56]. Obviously, NHEJ is inappropriate for gene correction applications because it introduces additional mutations in the form of insertions or deletions at the DSB site. Consequently, frameshift mutations might occur that can impair the expression of the gene, for instance by degradation of the transcript due to nonsense-mediated decay, leading in the end to protein loss [56]. As a result, CRISPR/Cas9 has been increasingly used to generate loss-of-function mutations in genes of interest in a variety of model organisms, including zebrafish, mice, and nonhuman primates [59]. In some cases, however, targeted cells activate an alternative DNA repair pathway called homology-directed repair (HDR) that rebuilds the DSB site using the non-mutant homologous chromosome or a supplied exogenous DNA molecule as a template, leading to correction of the mutant allele without accumulating additional indels [56]. Because HDR efficiency is relatively low, examples of genome editing for gene therapy have been limited, but notable examples of correcting human mutations leading to inherited cardiomyopathies have been reported.

One of the most striking examples involved sperm donated by an adult male patient with well-documented familial HCM caused by a heterozygous dominant 4-bp GAGT deletion in exon 16 of *MYBPC3*. It was used to fertilize healthy donor oocytes to obtain heterozygous $MYBPC3^{\Delta GAGT}$ zygotes. In these zygotes the capacity of CRISPR-Cas9 system to correct the pathological deletion was then tested by microinjecting a mixture containing sgRNAs and Cas9 protein in the cytoplasm of pronuclear stage zygotes. The overall targeting efficiency in human embryos was 72%; the majority of targeted blastomeres (63%) resolved their DSBs by HDR using the wild-type allele as a template. Moreover, the treated embryos carried normal diploid karyotypes with no evidence of detectable numerical or structural chromosomal

Table 2
Overview of alternative strategies for the targeting of ncRNAs in relevant models of heart failure.

Chemistry	RNA therapeutic	Dose/Delivery	System	Target	Outcome	Indication of HFpEF/ HFrEF	Ref
Aptamer	RNA-Apt30	RNA-Apt30 conjugated with the modified mTAT peptide (mTAT) for the delivery in the cells; effect observed at concentrations of ≥ 2.5 μM and at incubation times of 2–16 h	Isolated adult rat ventricular cardiomyocytes	PLN	Relief of PLN-mediated SERCA2a inhibition with consequent enhancement of Ca2 + transients and of contractility	HFpEF/HFrEF	[54]
	C13	GRK2 kinase activity assays using bovine rhodopsin as GRK2 substrate; the IC_{50} determined using C13 is 4.1 nM ± 1.2 nM	GRK2 kinase activity assays using bovine rhodopsin as GRK2 substrate	GRK2	Dose-dependent, strong inhibition of GRK2 kinase activity	HFrEF	[55]
	OPN aptamer	56 nmol/kg once every other day, starting at two months post-TAC; intravenous injection	Mouse, TAC with/without Osteopontin knock-out	OPN	Prevented cardiac myocyte hypertrophy and fibrosis after pressure overload; no rescue of the phenotype in $OPN^{-/-}$ animals, demonstrating that the aptamer is working through the OPN protein	HFpEF	[53]
CRSPR/Cas9	sgRNA targeting H530R mutation	AAV9-Cas9/sgRNA intraventricular injection at P4 or intravenous injection at P42	Mouse, knock-in +/H530R (PRKAG2 cardiac syndrome)	PRKAG2	Reduced hypertrophy, decreased thickness of the left ventricular wall, decreased myocardial glycogen content, regularly arranged myofibrils and fewer vacuoles and glycogen particles	HFpEF	[61]
	sgRNAs targeting PLN locus	Cas9 and sgRNAs microinjected into the pronuclei of zygotes	Mouse, CSQ-Tg (heart failure model)	PLN	Reduced incidence of arrhythmias, improved diastolic function and overall significantly improved mortality rates, especially early deaths	HFpEF	[62]
	sgRNAs targeting $MYBPC3^{\Delta GAGT}$ deletion	Cas9 and sgRNAs microinjected into the cytoplasm of pronuclear stage zygotes 18 h after fertilization	Human heterozygous $MYBPC3^{\Delta GAGT}$ zygotes (mutation causing hypertrophic cardiomyopathy)	MYBPC3	Normal development towards the blastocyst stage; ES cell lines from CRISPR–Cas9-injected blastocysts don't display cytogenetic abnormalities	N/A	[60]

(continued on next page)

Table 2 (*continued*)

Chemistry	RNA therapeutic	Dose/Delivery	System	Target	Outcome	Indication of HFpEF/ HFrEF	Ref
Lentivirus delivering pcDNA	H19/miR-675	Intracoronary injection of lentivirus pcDNA-H19	Rat, diabetic cardiomyopathy	VDAC1	Reduction of the hyperglycemia-induced left ventricular dysfunction and oxidative stress	HFpEF	[48]

rearrangements and the results of Digenome-seq, Whole-Genome Sequencing and Whole-Exome Sequencing demonstrated high on-targeting specificity of CRISPR–Cas9 without off-target effects [60]. Likewise, additional examples where this technology was successfully applied in myocardial diseases are reported [61,62] (Table 2).

6. RNA therapeutics: hurdles towards clinical application

RNA-based drugs have conceptual and practical advantages compared to conventional small chemistry-based drugs or therapeutic antibodies directed against a target protein product. For example, small chemistry-based medicinal products lack absolute target specificity and require expensive drug screening procedures before lead compounds can be refined towards an acceptable drug substance with sufficient specificity [63]. Antibodies can only recognize targets that are both druggable and secreted or extracellular, because there is no good strategy to deliver them inside cells [64]. By contrast, siRNAs and ASOs can in principle suppress any gene, even if it is highly expressed, including non-coding genes, which conventional chemical therapeutics or antibodies cannot [7]. Unlike antibodies, RNAs can be chemically synthesized, thus leading to cheaper and more easily manufactured drugs than biologics, which can suffer from batch-to-batch variability. Antibodies need to be administered every few weeks, and patients often develop immunological responses, which can limit the effectiveness of antibody therapy with continued use. Thus far, there is no evidence of adaptive immune responses against RNA therapeutics [65]. A final potential advantage of RNA drugs that utilize a Watson-Crick target-binding motif, compared to antibodies, is that an antisense oligo is straightforward to design using the complementary sequence to specifically bind and inactivate the target sequence, being either a mRNA, a ncRNA or a ssDNA [7,65].

Although a considerable number of preclinical studies involving ncRNA therapeutics for heart diseases have now been conducted in the past decade, only a remarkably small number of RNA-based therapies so far managed to move into clinical development [66]. In fact, for the cardiovascular field, only one single study using an ASO targeting miR-92 is recruiting patients for a Phase I safety/dosing study (press release Miragen Therapeutics Inc., 14-03-2018). Whereas clinical examples for RNA-based drugs for other disease areas are now increasingly progressing through all clinical phases including FDA approval for market entry [67], clinical stage RNA therapeutics for cardiovascular disorders remain conspicuously rare. This fact could well be related to the shared hurdles faced by any RNA-based drug in any disease context [7,65], but could equally well reflect the fact that genomic studies in cardiac disease were initiated later than is the case for other non-communicable disease fields, despite the staggering numbers of heart disease patients worldwide.

RNA-based therapeutics as siRNAs and ASOs still face several threats compared to more traditional drugs. First, siRNAs can show off-target effects due to partial homology of the intended target with other genes, thus leading to unwanted effects even respecting the strict complementarity between the target and the RNA-based compound [68]. Off-target activities can also arise because of additional roles of the target rather than the one of interest which would be possibly affected by the drug [68]. Secondly, the toxicity of RNA therapeutics remains to be fully explored. So far, LNA or 2′OMe-based oligonucleotides display relatively low toxicity in rodents at very low doses. In clinical settings, however, LNA oligos on occasion have shown a rather severe acute kidney toxicity [28], possibly hampering straightforward clinical application. Indeed, ASOs, in particularly those with phosphorothioate chemistry, have also been reported to activate the complement cascade by the C4d-specific classical pathway with subsequent inhibition of the intrinsic pathway of blood coagulation [68]. In addition, and even if unlikely, ASOs can show toxic effects when they trigger immune responses either directly because of their sequence or indirectly by their delivery vehicle [68].To explore these potential risks

during the development of RNA-based drugs towards clinical application, preclinical safety and toxicity studies in mice and non-human primates and in early Phase I clinical trials are recommended to be performed as have been reported for other disciplines employing RNA therapeutics [69–71].

A main challenge all forms of RNA-based drugs face is the delivery of the drug product to the site of action, in such a way that allows the accumulation of the drug candidate in high enough concentrations in the organ or cell-type of preference to have the desired effect but without potential toxicities and off-target effects [7,65]. Above, we elaborated on the chemical modifications providing resistance to degradation to the RNA drugs when exposed to the systemic environment. Today, the RNA-based therapies approved for commercial use are against spinal muscular atrophy (Spinraza, Nusinersen), homozygous familial hypercholesterolemia (Kynamro, Mipomersen), cytomegalovirus retinitis (Vitravene, Fomivirsen), and age-related macular retinal degeneration (Macugen, Pegaptanib). Part of their success may be related to the delivery routes of these compounds (intravitreal, intrathecal) to the site of action, which ensures a higher and localized drug substance accumulation without excess diffusion to other organs where toxicity and off-target effects may ensue [67]. Even in the case of the subcutaneously administered Mipomersen, the characteristic of ASOs to distribute mostly into liver and kidney following systemic delivery indicates that drug targets in the liver have a competitive drug delivery advantage. Indeed, several studies demonstrate that only trace amounts of systemically delivered RNA-based drugs will end up in the myocardium. Obviously, for treatment of heart disease patients, who may need life-long delivery of RNA therapeutics, repeated systemic delivery carries the realistic risk for hepatotoxicity and renal toxicity.

The importance of a tissue- or even a cell-specific delivery of RNA-based drugs emerges from two well-performed studies where either miR-29 or miR-21 were placed centrally as drug targets in pathological cardiac remodelling [9,16,33,38]. In one study, it was demonstrated that infusion of an ASO targeting miR-29 protects from cardiac hypertrophy, fibrosis and dysfunction in a mouse model of heart failure [38]. Although miR-29 is expressed in several cardiac cell types, the beneficial effects derive from its specific silencing in cardiomyocytes, thus because of a dominance of miR-29 activity in cardiac muscle cells compared to cardiac fibroblasts with respect of cardiac remodelling [38]. Indeed, a previous study observed a decrease of miR-29 in cardiac fibroblasts under pathological conditions, suggesting that miR-29 inhibition would facilitate adverse fibrosis and remodelling from the perspective of miR-29 function in fibroblasts [16]. Likewise, early studies demonstrated that systemic inhibition of miR-21 was beneficial against myocardial fibrosis and dysfunction, with later reports indicating a protective function of miR-21 in cardiac myocytes [9,33]. Using elaborate combinations of mice harbouring a floxed allele for miR-21 and diverse viral vectors driving Cre recombinase in distinct myocardial cell types, a therapeutic benefit was found in mice with miR-21 deficiency in nonmyocyte cardiac cells, but not in mice with global or cardiac muscle-specific ablation, indicating that miR-21 exerts its pathologic activity directly in cardiac nonmyocytes [33].

One possibility to reach the myocardium for local delivery could encompass the technology of percutaneous coronary intervention (PCI), a non-surgical procedure where the blood stream is accessed through the femoral or radial artery with small catheters, routinely used in interventional cardiology to visualize the blood vessels on X-ray imaging and treat narrowing of the coronary arteries of the heart found in coronary artery diseases. This procedure is clinically already in use for local delivery of therapeutic agents as adjuncts during percutaneous interventions and was successfully demonstrated to deliver an ASO for miR-92 by intracoronary infusion in a porcine model [29]. An innovative approach was recently published where inhalation of small biocompatible and biodegradable calcium phosphate nanoparticles were shown to rapidly translocate from the pulmonary tree to the bloodstream and myocardium. Inhalation of calcium phosphate nanoparticles carrying a therapeutic peptide resulted in improved cardiac function in a mouse model of diabetic cardiomyopathy as well as in a porcine large animal model, effectively providing a proof-of-principle that inhalation of nanoparticles could offer an effective method of targeted delivery to the heart [72]. Finally, ligand conjugation strategies could also be an avenue to increase potency and more efficient uptake by the cardiac cell type. The examples of ligand conjugation of triantennary GalNAc to siRNA or single-stranded ASOs with greatly enhanced targeted distribution of these molecules to hepatocytes (approximately 10-fold with concomitant reductions in distribution to other organs and cell types) [7], demonstrates the theoretical technical possibility to conjugate ligands or antibodies for myocardial cell surface receptors to RNA-based drugs as a way to optimize organ or cell-type specific delivery through endocytic pathways.

Taken together, several preclinical formulations have shown to be promising, thanks to their low toxicity profile and their effective delivery to the target site. The flexibility of their design leaves a lot of room for the further improvement of their drug-like properties; it would possibly allow also the production of multifunctional drugs with more than one way of action for the substitution of drug cocktails. In our opinion, RNA-based drugs have the potential to become a new tool for the treatment of heart diseases, revolutionizing the current panorama of the available “traditional” therapeutics. The current surge in genomic and proteomic data in human biology will aid in the identification of key RNA targets for drug development and pharmacotherapy in heart disease; this increased body of knowledge, coupled with a comprehensive preclinical analysis possible through novel delivery platforms, should enable RNA therapeutics in cardiovascular medicine to become a long-term clinical reality.

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