Review Article

The role of small and long non-coding RNAs in cardiac pathologies

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Abstract: Cardiovascular disorders (CVDs) still remain the leading cause of death worldwide. The current lack of therapeutic approaches that efficiently improve CVD quality of care and prevent the development of life-threatening complications, prompted the scientific community to continue a deeper investigation of the molecular mechanisms driving the onset and progression of these pathologies. In this context, non-coding RNAs (ncRNAs) were demonstrated to be involved in the onset of different forms of CVDs. In this review we will discuss basic aspects of some classes of ncRNAs as well as their mechanism of action and involvement in CVDs. The potential therapeutic use of ncRNAs in the clinical practice will also be addressed.

Keywords: Cardiovascular disorders (CVDs); non-coding RNAs (ncRNAs); microRNAs (miRNAs); and long non-coding RNAs (lncRNAs); nanotechnology

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Introduction

Cardiovascular disorders (CVDs) define a wide range of pathologies affecting heart muscle and its associated circulatory system (1,2). In particular, heart failure (HF) and myocardial infarction (MI) still represent the leading cause of morbidity in the industrialized world and it is also estimated to became the major source of death in the developing countries by 2020 (3,4). Additionally, the occurrence of co-morbidities such as hypertension, diabetes, and coronary artery disease threaten the efficacy of current pharmacological therapies that just alleviate the consequence of HF and fail to completely restore the functionality of injured myocardium (5). As consequence, heart-transplantation still remains the gold standard treatment for end-stage HF. However, due to the growing number of patients in combination with the lack of donors as well as the related immunological complications following organ transplantation, alternative and more efficient clinical approaches are strongly required to be discovered. In line with this, an increasing interest for deeper characterization of key regulators of molecular mechanisms underlying cardiac dysfunctions has started by the scientific community (6,7). In this context, innovative findings were indeed obtained leading to the displacement of a central dogma that has dominated the scientific landscape for half a century, the fact that only proteins represent the leading effectors of cellular functions and so responsible for the onset of diseases (8). Initiatives like encyclopaedia of DNA elements (ENCODE) (9) followed by the latest advances of deep DNA/RNA-sequencing technologies [next generation sequencing (NGS)] (10) and the large amount of public available bioinformatics tools for collecting and processing data, allowed the discover that around 98% of the entire genome is actively transcribed into RNA without coding potential, thus called non-coding RNAs (ncRNAs) (11-13). ncRNAs, initially thought to be "functionless-transcriptional noise", were on the other hand extensively demonstrated

to provide an active contribution in a plethora of biological processes. A significant correlation between their altered expression and the onset or progression of different human disorders, included CVDs, was found (11,14). Since then, the ncRNA field has increasingly received a great attention from both the research community and industry with a particular interest for their potential use as diagnostic and therapeutic tools in medicine (15).

In this review, the role of the two major groups of ncRNAs [microRNAs (miRNAs) and long ncRNAs (lncRNAs)] in cardiac pathologies will be discussed. In particular we will focus on ncRNAs participating to the pathogenesis of different CVDs and how they can be used as therapeutic tools for novel clinical strategies (16). Furthermore, there will be an overview of up-to-date challenges and possible strategies for their clinical applications.

Categories of ncRNAs and basic function

ncRNAs can be classified based on an arbitrary threshold of 200 nucleotides (nt), separating small ncRNAs (sncRNAs) and mid-size RNAs (mid-sizeRNAs) from long ncRNAs (lncRNAs) (17,18). The sncRNA family includes microRNAs (miRNAs), small interference RNAs (siRNAs), and piwi-interacting RNA (piRNA), while the mid-size category comprises transfer RNAs (tRNAs) and small nucleolar RNAs (snoRNAs). Ultimately, the long ncRNA groups mainly refers to long intergenic non-coding RNA (lincRNA) and natural antisense transcripts (NATs) (19,20). In addition, other type of ncRNAs, which fall into both sides of canonical length cut-off groups, such as enhancer-associated RNAs (eRNAs) and the more recently emerged circular RNAs (circRNAs), have been recently identified (17).

miRNAs

Among small ncRNAs, miRNAs are so far the most widely studied class (16). miRNAs are endogenous, single-stranded ncRNA molecules of 18–24 nt in length. It is estimated to be the most abundant family in the human genome, which indeed hosts more than 2000 different loci for miRNAs production (21). To date the majority of identified miRNAs are "intragenic" and processed mainly from introns rather than exons of protein coding genes, while the remaining are "intergenic", transcribed independently of a host gene and controlled by their own promoters (22-24).

miRNAs are produced via a complex sequence of events orchestrated by a multitude of enzymes and proteins (25). In brief, miRNA gene is initially transcribed by RNA polymerase II into a primary transcript (pri-miRNA), which it is then processed into a shorter precursor (pre - miRNAs) by the ribonuclease III termed DROSHA and exported outside the nucleus (26). In the cytosol, the RNAse III protein called Dicer, converts the pre - miRNA into a double - stranded mature miRNA molecule. Finally, one strand of the mature miRNA is loaded into the Argonaute (AGO) protein to form the miRNA-induced silencing complex (miRISC) complex. This complex recognizes either the 3′ or 5′ untranslated region of different mRNA genes, inducing their translational repression and/or mRNA degradation (27,28). Beyond the classical cytosolic post-transcriptional mechanism of action, numerous studies highlighted how miRNAs can regulate the expression of target gene also in different subcellular compartments such as rough endoplasmic reticulum (rER) (29,30), processing (P)-bodies (30), mitochondria (31) and the nucleus (32-34) (and Di Mauro et al, unpublished). In particular within nuclei, mature miRNAs where demonstrated to elicit novel and unconventional functions, although poorly understood, such as the induction of epigenetic changes by a direct binding to DNA (35), or even the promotion of more efficient splicing or alternative splicing profiles of nascent mRNA transcripts (32). Ultimately, when loaded into secreted vesicles, miRNAs can be released in extracellular fluids and possibly elicit their function in other cell types or tissue districts (36).

piRNAs

The piRNAs represent another large family of small ncRNAs, existing throughout the animal kingdom, particularly enriched in the germ-line tissues (37,38). piRNAs are 24–32 nt in length and are generated from the RNA transcripts of transposons, protein-coding genes, and specific intergenic loci (39). Similarly to miRNAs, piRNAs are able to form a RISC with a specific sub-group of AGO protein family, called PIWI (P-element induced wimpy testis) protein clade (40). The resulting RNA-protein complex (piRISC) is well known to negatively regulate gene transcription via endonucleolytic cleavage (slicing) of the target sequence after complementary base-pair recognition through the piRISC. Such target sequences are mainly represented by transposable elements (TEs), which are autonomous pieces of DNA that replicate and able to insert into the genome and therefore potentially prone to cause

DNA damage (38,41,42). Moreover, new functions beyond transposon silencing were uncovered, such as the regulation of proteins coding gene via DNA methylation (43) or repression by mRNA deadenylation (44,45). Surprisingly, recent papers demonstrated that piRNAs are not confined in germ line tissues but they are also present in somatic cells of brain, liver, and heart (46). In the cardiovascular area, the knowledge of piRNAs is still far from being completed defined. Nevertheless there is strong evidence demonstrating that piRNAs might be involved in the control of signaling pathways activated during stress stimuli (47,48) as well as being differentially expressed in exosomes isolated from serum of HF patients (49). Altogether, these data suggest both an active role of piRNAs in the onset of pathological cardiac remodelling and their potential use as prognostic biomarkers.

siRNAs

This class of ncRNAs and their related mechanism of action, called RNA interference (RNAi), was firstly described in 1998 by a pioneering work in *C.elegans* (50). In this paper the authors showed in worms that the injection of double-stranded RNAs coding for specific proteins were able to silence genes carrying the same sequence (51). From a structural and functional point of view, siRNAs resemble miRNAs, however, some key differences between these two classes of ncRNAs are present:

(I) siRNA molecules are shorter, with a mean length of 21nt (51);
(II) siRNA molecules require a perfect complementarity with target mRNA, thus allowing the knock down of gene with less off-target exceptions (52);
(III) siRNA-mediated gene silencing occurs trough an endonucleolytic cleavage of mRNA (52,53).

Initially defined as extragenomic RNA material, siRNAs are now accepted to be present endogenously in organisms such as plant, flies, and mice (54-56). However, the complete comprehension of endogenous siRNAs is still quite limited, therefore in order to further dissect the origin and biogenesis in mammals of this class of ncRNAs, an increase research in this field is essential.

Mid-size ncRNAs

tRNAs represent a type of ubiquitous RNAs, codified by tRNA genes and subsequently transcribed by RNA polymerase III into macromolecules whose function is to match an mRNA codon with the amino acid it codes for (57). As a matter of fact tRNAs transfer activated amino-acids from aminoacyl-tRNA synthetases to the ribosome, where they are used for the protein synthesis (5). However, various works pointed out additional roles for tRNAs beyond being a simple adaptor molecule as well as tRNA-associated genetic disorders (58,59). snoRNAs are a conserved class of 60–300 nt long stable RNAs, present in nucleolus of cells that play important role in ribosome biogenesis (60), as well as in the regulation of alternative splicing and posttranscriptional modification of mRNA (61).

lncRNAs

The overwhelming advance in transcriptome sequencing, epi-genomic technologies, and computational prediction techniques allowed the discovery of lncRNAs, which among all sub-categories of ncRNAs represent the most recent and thus the least characterized family (62-64). Similarly to sncRNAs, lncRNAs were found in basically all type of organisms, which include, despite a poor rate of conservation between species, virus, yeast, plants, and animals (65-68). The biogenesis of lncRNAs starts into the nucleus where RNA polymerase II generate transcripts, which bear some features resembling mRNA molecules such as the classical splice sites (GU/AG), the presence of intron and exon structures with related alternative splicing, 5'-capping and a final polyadenilation (69). However, in contrast to mRNAs, the lncRNA transcripts have little or no functional open reading frame (ORF) (62,70). So far it was estimated that 75–90% of the human genome generate a series of lncRNAs (71,72), but despite this, to date, a standard definition for this family of ncRNAs is still missing. Some empirical features can be used for a sort of classification:

(I) Length: lncRNAs are transcripts whose length span from 200 nt up to 100 kilobases (kb) (72);
(II) Genomic location and context: based on the genomic position relative to the nearest coding gene, lncRNA genes can be divided into:
  (i) Intergenic (also called lincRNAs): transcribed from introns of annotated genes (69);
  (ii) Intronic: transcribed entirely from introns of protein-coding genes (69);
  (iii) Bidirectional (divergent): generated from both sense and antisense direction of transcription start areas (69,73);
  (iv) Sense or overlapped (intronic or exonic):

produced from the sense strand of annotated transcription units (UTs). These transcripts can contain exons and derive from protein-coding genes, can overlap with part of protein-coding genes or cover the entire sequence of a protein-coding gene through an intron (74);

(v) Antisense (intronic or NAT, natural antisense transcript) produced from the antisense strand of annotated transcription units (UTs) (75);

(III) *Subcellular localization*: mounting lines of evidence demonstrated a link between subcellular distributions of lncRNAs and their function (76). So far the majority of annotated lncRNAs were found more enriched in the nucleus where they are involved in nuclear processes such as transcription and RNA processing (77). On the contrary, other lncRNAs were found more abundant in the cytosolic compartment, where their function can impact on mRNA stability and translation or even on localization of final protein products (76). Interestingly, in the last years different several works demonstrated the presence of lncRNAs in the extracellular space by a selective sorting into exosomes, thus revealing an additional role in mediating cell-cell communication (78,79).

(IV) Function: based on the evidence that many lncRNAs have a cell-type specific expression and respond to different stimuli, they are considered "signal molecules" due to their ability to integrate different hints in precise cellular and temporal contest (80). lncRNAs are also known to exert a role of "molecular decoy", whose function consists in binding and titrating away proteins to target genes (81). Additionally, lncRNAs can act as "guides" by interacting with regulatory proteins, such us chromatin-remodelling complex, and directing them to specific DNA loci in order to induce epigenetic modifications (13,62). Other lncRNAs can play a "scaffold role" acting as platforms to bring different proteins together, in the nucleus or cytosol, at the aim to activate or repress the transcription or translation of target gene (62). Moreover, lncRNAs can counteract the action of miRNAs, by "sponging" them, therefore favoring the expression of repressed target mRNAs (5).

Despite the fast increase of data regarding the lncRNAs, this field is still far from being completely understood, especially for what concerning the function of this class of ncRNAs. Indeed, it is plausible that in the near future, more lncRNA-mediated functions will emerge and with them also many questions and challenges associated with them.

eRNAs and circRNAs

Although enhancers have been identified more than 30 years ago, only it was only in 2010 when the existence of enhancer-related RNAs transcripts was demonstrated and subsequently termed eRNAs (82,83). Despite eRNA field is still in its infancy, here we will try to briefly report the current knowledge concerning their molecular features as well as some information about their mechanism of actions.

eRNAs can be distinguishable from lncRNAs by some specific features:

(I) Epigenetic signature: eRNAs are marked by histone modifications such as H3K4me1/2 and H3K27Ac unlike from promoters of lncRNAs which are specifically enriched in H3K3me3 (84);

(II) Origin: eRNAs are generated from bidirectional transcription of both strands of DNA from a central non-transcribed region (82,85,86);

(III) Length: eRNAs are relatively shorter than lncRNAs, with a range between 500-2000 nt (87);

(IV) Post-transcriptional modifications: generally after transcription, eRNAs are not subjected to full maturation processes, indeed they possess a 5′ cap but they are unspliced and rarely polyadenylated (88-90).

(V) Half life: eRNAs are mainly retained into nuclei therefore they show a very short half-life with a degradation process within minutes (91).

Since eRNAs were initially considered as merely transcriptional noise, also their function was consequently underestimated. Nevertheless, in the last years different functions were demonstrated to be associated to eRNAs. As a matter of fact, eRNAs can regulate activities of target or neighbor genes in different ways, such as affecting their transcriptional elongation by releasing the negative elongation factor (NELF) from paused RNA polymerase II at specific gene promoters (92,93). In addition, eRNAs can initiate the transcription of target genes by directly opening the chromatin or trough recruitment of chromatin remodelers (94). Moreover, eRNAs can also act as bridge to facilitate a physical interaction between enhancers and promoters (E:P Loop) independently from distance and orientation (95,96).

circRNAs represent a subset of ncRNAs initially

considered as byproducts of defective splicing due to their very low expression and uncommon features, therefore did not receiving much attention (97). However, the high-throughput sequencing technology coupled with development of high specific computational algorithms [e.g., circRNAs_finder (98,99), find_circ (99), CIRI (100) and CIRCexplorer (101)] overturned this vision by the discover of thousands of endogenous circRNAs (102). CircRNAs present a unique structure resulting from a 3′ to 5′ end-joining event also called backsplicing (103). CircRNAs are generated from both exons and introns of protein coding genes, although the latter are less frequent (102). Moreover, there are pieces of evidence showing that they can also took origin from intergenic regions, UTRs, and ncRNA loci (99). Once transcribed, circRNAs can be retained into nuclear compartment or exported in the cytoplasm. For what concerning their biological function, five main mechanisms have been so far proposed:

(I) miRNA-sponge: by harboring binding sites for miRNAs, circRNAs can sequester these ncRNAs and therefore indirectly regulate the activity of miRNA-target genes (99,104). However, this function is controversial and nowadays considered not a general phenomenon of circRNAs (105). In fact, to feature proper miRNA-sponge activities, circRNAs must fulfill some principal criterions:
  (i) To harbor a large number of binding sites for the putative miRNA targets;
  (ii) To target few number of miRNAs;
  (iii) To show higher circRNA-miRNA affinity compared to miRNA-mRNA ones;
  (iv) To induce degradation of miRNA targets.

(II) Post-transcriptional control: circRNAs enriched into cell nuclei can interact with elongating RNA Pol II and promote transcription of related genes (106);

(III) Rolling circle translation: circRNAs containing open reading frame (ORF) or internal ribosome entry site (IRES) can be translated into proteins via a rolling circle amplification mechanism. However, this mechanism is only demonstrated *in vitro*, and so far there are no evidence showing that natural eukaryotic endogenous circRNAs can be effectively translated into peptides (107,108);

(IV) Production of circRNA-derived pseudogenes: some circRNAs can be reversely transcribed to cDNA and even integrated into the genome (109);

(V) Control of alternative splicing: this feature is handled by circRNAs derived from exons of protein coding genes. Indeed, biogenesis of such circRNAs can hinder the pre-mRNA splicing, resulting in lower levels of linear mRNAs or even changing the composition of processed mRNA by selective exons exclusion (110).

Despite the number of annotated circRNAs is in rapid expansion, so far only a small group of them have been clearly correlated with CVDs (111). Indeed, compared to other RNAs, the current knowledge of circRNAs still needs to face some concrete obstacles. For example, the relatively low abundance of circRNAs as well as their unique circular structure makes current techniques for detection, manipulation, and other functional studies sill poor efficient (105). As a consequence, for an effective application of circRNAs in clinical management of cardiac diseases, the scientific community must accomplish significant efforts in the discovery and characterization of this class of ncRNAs.

The role of miRNA and lncRNAs in cardiovascular disorders

Starting from the embryonic stage to fully developed adult heart, ncRNAs were detected in many cell lineages forming the cardiac tissues where they contribute to processes such as differentiation, proliferation, contractility, and electric conduction system (112). Therefore, any alterations in ncRNA expression can determine the onset or progression of cardiac pathologies. Here we will report few examples of miRNAs and lncRNAs affecting the pathophysiology of heart.

Cardiac fibrosis

Cardiac fibrosis is a pathological consequence related to heart damages, characterized by the adverse accumulation of collagens and other extracellular matrix (ECM) proteins (113). Nowadays a number of investigations demonstrated that expression of distinct miRNAs and lncRNAs, strongly correlate with the genesis, progression, and treatment of cardiac fibrosis (114). One of the first study in this context was made by the Olson's group that found a negative correlation between the expression of miR-29 family members (miR-29a, miR-29b and miR-29c) and genes involved in ECM production and fibrosis after experimental MI (115). Another fibrotic-related miRNA was described in a work by Thum *et al*, where the authors demonstrated that miR-21 promotes cardiac fibrosis by

targeting extracellular regulated kinase inhibitor sprouty homolog 1 (Spry1) augmenting ERK-MAP kinase signaling in cardiac fibroblasts (116). Several other miRNAs have been identified as potent anti-fibrotic molecules, such us Let-7i and miR-26a which were found to attenuate collagen deposition by targeting Col1α2 and Col1α1, respectively (117,118). Although the current knowledge regarding fibrotic-related lncRNAs remains poor, there are available scientific lines of evidence underlining their involvement in controlling cardiac fibrosis. This is the case of the conserved Wisp2 super-enhancer-associated RNA (Wisper), which was described to control the progression of this disease by up-regulating pro-fibrotic genes (119). In another work, *in vivo* inhibition of lncRNA Meg3 during the early phase of cardiac remodelling was able to prevent the induction of matrix metalloproteinase-2 (MMP-2), therefore to decrease cardiac fibrosis and improved diastolic function (120).

Cardiac hypertrophy

Biomechanical stress and other pathological stimuli can trigger the initiation of a phenotypic remodelling, i.e., cardiac hypertrophy, which results in an increase in myocyte size and myofibrillar volume (121). Although beneficial for a short period, prolonged hypertrophic state can lead to HF and death (122). Among the first identified hypertrophic-related miRNAs, there are miR-1 and miR-133. The inverse correlation between their expression and progression of cardiac hypertrophy in a rodent model of cardiac hypertrophy led to the identification of a relevant role in this disorder (122). Indeed, *in vitro* studies using neonatal rodent myocytes demonstrated that miR-1 was able to target several hypertrophic genes such as Ras GTPase-activating protein (RasGAP), cyclin-dependent kinase 9 (Cdk9), Ras homolog enriched in brain (Rheb), and fibronectin (123). In addition, the work of Carè *et al*, showed for the first time that an *in vivo* administration of a synthetic molecule (i.e., antagomir, discussed in next paragraphs) able to down-regulate the level of cardiac miR-133a, led to an hypertrophic response of the myocardial tissues (124). Other miRNAs on the contrary were demonstrated to have pro-hypertrophic effects. This is the case of miR-19a/b family, whose members downregulate the anti-hypertrophic genes atrogin 1 and muscle ring finger protein 1 (Murf1) with the consequent activation of calcineurin/nuclear factor of activated T cells (NFAT) signaling (125).

Among the first lncRNAs interrogated for their involvement in hypertrophic remodeling, there is the myosin heavy chain associated RNA transcripts (*MHRT*), which antagonizes the brahma-related gene 1 (*Brg1*) by blocking its recognition sites on cardiac stress genes, thus protecting the heart from pathological HF (126). More recently, the lncRNA cardiac-hypertrophy-associated epigenetic regulator (*Chaer*) was described as an important checkpoint for the progression of cardiac hypertrophy. Indeed, *Chaer* promotes an activation of stress-related genes, by tethering away from them the polycomb repressor complex 2 (PRC2) (127). Recently, another lncRNA called *TINCR* was demonstrated to attenuate cardiac hypertrophy by the epigenetic silencing of CaMKII (128).

MI

MI is a common cardiovascular event characterized by cardiomyocytes loss via programmed cell death (129). In this contest miRNA can either promote or impair cardiomyocyte survival, thus acting as angel or devil in the regulatory network of cardiac cell death (113). An example of negative regulators of cell death is represented by miR-15 family, whose chemical inhibition was demonstrated to reduce myocardial damaged area after ischaemia–reperfusion injury (130). On the contrary miR-24, when overexpressed, was reported to exert a positive effect on cardiomyocytes survival by targeting the proapoptotic Bcl-2-like protein 11 (131). Analogously to miRNAs, there are also examples of cell death-related lncRNAs. The long noncoding RNA, named autophagy promoting factor (APF), can regulate autophagic cell death by targeting miR-188-3p and ATG7 (132). More recently, the GATA1 activated lncRNA *Galont*, was demonstrated to interact with miR-338 and to promote ATG5-mediated autophagic cell death in murine cardiomyocytes (133) (*Tables 1,2*).

miRNA and lncRNA therapeutics: on the road for promises and challenges of RNA-based therapies

Tools for modulation of ncRNAs

As consequence of the latest advances in next-generation sequencing technology and the huge availability of generated genomic data, the scientific community managed to identify a plethora of key regulators of various pathologies, many of which successfully translated into therapeutic compounds for the treatment of CVDs (10). So far the two main classes of Food and Drug Administration (FDA) approved drugs

Table 1 miRNAs involved in cardiac disorders

miRNA/miRNA family	Target	Associated disease	Reference
miR-29	Elastin (Eln); Fibrillin 1 (Fbn1); collagen type I, alpha 1 and 2 (Col1α1, Col1α2) and collagen type III, alpha 1 (Col3α1)	Cardiac fibrosis	117
miR-21	Kinase inhibitor sprouty homolog 1 (Spry1)	Cardiac fibrosis	118
Let-7i	Col1α2	Cardiac fibrosis	119
Mir-26	Col1α1	Cardiac fibrosis	120
miR-133a	Ras homolog gene family, member ARhoA; Cell division control protein 42 homolog (Cdc42); Wolf-Hirschhorn syndrome candidate 2Nelf-A/WHSC2	Cardiac hypertrophy	126
miR-1	Ras GTPase-activating protein (RasGAP), cyclin-dependent kinase 9 (Cdk9), Ras homolog enriched in brain (Rheb), and fibronectin (FN1)	Cardiac hypertrophy	125
miR-19a/b	atrogin 1 (Fbx32) and muscle ring finger protein 1 (Murf1)	Cardiac hypertrophy	127
miR-15	BCL2 Apoptosis Regulator (Bcl2)	MI	132
mir-34	Bcl-2-like protein 11 (Bcl2l11)	MI	133

MI, myocardial infarction.

Table 2 lncRNAs involved in cardiac pathologies

lncRNA	Target	Associated disease	Reference
Wisper	Col3α2, Fn1, Tgfb2	Cardiac fibrosis	121
Meg3	matrix metalloproteinase-2 (MMP-2)	Cardiac fibrosis	122
MHRT	Brahma-related gene 1 (Brg1)	Cardiac hypertrophy	128
Chaer	Polycomb repressor complex 2 (PRC2	Cardiac hypertrophy	129
TINCR	Calcium/calmodulin dependent protein kinase ii (CaMKII)	Cardiac hypertrophy	130
APF	miR-188-3p, Autophagy Related 7 (ATG7)	MI	134
Galont	miR-338	MI	135

MI, myocardial infarction.

are divided into (I) "small molecules", largely characterized by organic hydrophobic compounds and (II) "proteins" mainly represented by antibodies. The small molecules, thanks to their reduced size are able to cross cell membrane by rapid diffusion and directly inhibiting or activating target proteins, such as receptors or enzymes (134,135). However, the prerequisite of small molecules-druggable targets is the presence of active-site pockets, and because of only the 2–5% of proteins in the human genome possess such binding pockets, the applicability of such compounds is quite limited (135,136). In this context, antibody-based drugs can be used to overcome this limitation by directing replacing mutated or missing proteins, on the other hand a larger size does not allow the cross of cellular barrier by diffusion, thus restricting their use to extracellular targets (135). The need to overcome such limitations, combined with the discovery of altered ncRNAs in different pathologies as well as their active role in controlling disorders, fuelled the academia and industry to consider RNA - based therapeutics suitable tools for treatment and prevention of different diseases that lead up this year to the first ever FDA-approved ncRNA based drug, Onpattro (patisiran), for the treatment of polyneuropathy caused by hereditary transthyretin-mediated amyloidosis (hATTR) (137,138).

In this paragraph we will briefly discuss the current

available strategies to modulate miRNA and lncRNAs functions.

So far two approaches are commonly used to modulate miRNA activity: 1) to inhibit the miRNA function by using chemically modified anti-miR oligonucleotides; 2) to restore miRNA function trough synthetic double-stranded-based overexpression system (139). Inhibition of miRNA-targeting can be achieved by the use of antisense oligonucleotide, the so called anti-miR, and in this group are comprised the miRNA sponges, the locked nucleic acid (LNA) anti-miR constructs and the antagomir (140). The miRNA sponges approach relies on the use of overexpression vectors harbouring complementary binding sites for a miRNA (or a family) whose function is blocking it by competing for the binding with bona fide target genes (141). The last two technologies are so far the most used, and they are synthetic oligonucleotides either fully or partially complementary to a specific miRNAs, which contain chemical modifications aimed to increase their binding affinity, the biostability and pharmacokinetic properties (142). The LNA modification is a conformational restriction based on the use of a methylene bridge between the 2' oxygen with the 4'carbon of the ribose ring, demonstrated to generate a thermodynamically strong duplex formation towards complementary single-stranded RNA molecules both *in vitro* and *in vivo* (143). The antagomir molecules are characterized by the insertion of the 2′-O-methoxyethyl modification, a partial phospho-rothioate backbone (PS) and a covalent addition of a cholesterol molecule. In particular these last two modifications were demonstrated to improve the nuclease resistance and the cellular up-take respectively (144,145).

For what concerning pathological disorders related to a low level of miRNAs, so far, the most used strategy to restore miRNA level is represented by mimic molecules. miRNA-mimic molecules are double or triple stranded synthetic miRNA oligonucleotides, which inside cells are processed into a single strand form and regulate protein coding genes via miRNA-like function (146). Similarly to the antisense oligonucleotide, also miRNA-mimics possess different chemical modifications in order to ameliorate their efficiency and decreases off target effects (147). However, the available engineered modifications for miRNA-mimic are very limited because demonstrated to interfere with the loading into RISC complex, and therefore leading to a loss of effective silencing function (5). Given the relatively recent discovery and the diversity of currently known lncRNA mechanisms (some of which still yet not characterized), the available tools for their modulation are quite limited and also have to be carefully considered in the context of how the lncRNA may function (148). For what concerning the down-regulation of specific lncRNAs the RNA interference is still widely used (149). Moreover, this technology may also be applied for the lncRNA enriched in the nuclear compartment, based on the recent discovery of key components of RNAi to be present and active in cell nuclei (34,150). An alternative to RNAi for the degradation of lncRNAs is represented by gapmer molecules, which are 15–20 nt single-stranded DNA oligomers that hybridize with target lncRNA transcripts through complementarity and induce RNaseH-mediated degradation of the target transcripts (151). However, both RNAi and gapmer are reduced in efficiency because of the formation of secondary structures typical of lncRNAs. To overcome this limitation, recently the Cas13 family of CRISPR ribonucleases technology was clearly demonstrated to efficiently lower the level of specific lncRNA molecules (152). In contrast, the up regulation of lncRNAs is more complicated and to date the available system are quite limited. A potential strategy for up regulation of lncRNAs relies on the use of recombinant adeno or lentivirus. In particular cardio-trophic adeno-associated (AAV) vector were already demonstrated to be efficient for the delivery of both protein coding genes and miRNAs, therefore may also represent a promising novel approach also for lncRNAs although so far remains to be determined (13,153-155).

Delivery approaches to improve efficiency and selectivity of ncRNA therapeutics

Despite the well-accepted possibility to be promising tools for the treatment of cardiovascular disorders, so far the number of available ncRNA-based drugs is very limited. This is due to the deficiency of reliable delivery tools aimed at increasing the drug local concentration in heart muscle reducing the side effect at systemic level (156). So far the available delivery approaches used for ncRNAs local release in cardiovascular system are: (I) assisted-device delivery system, (II) viral vectors, and (III) non-viral vectors.

For what concerning the assisted-device delivery system, Hinkel *et al.* provided in 2012 the first evidence that local catheter-based delivery more efficiently inhibits miR-92a expression in the heart compared with intravenous infusion (157). However, this strategy didn't completely prevent the systemic inhibitory effect of the anti-miR since the expression of miR-92a resulted affected also in other organs such as kidney and liver (158). Another example

is represented by the work of Wang *et al.*, in which they demonstrated an efficient local silencing of miR-21, with reduced systemic side effects, using a drug eluting stent (DES) in a rat model of myointimal hyperplasia (MH) (159).

The use of viral vectors has significantly improved tissue-specific enrichment of ncRNAs. One of the first research describing the use of viral vector was represented by the work of Mauro Giacca's lab in which AAV serotype 9 was used to selectively overexpress miR-199a and miR-590 into cardiomyocytes (160). Moreover, further studies from the same research team and concerning the use of AAV vectors for specific miRNA delivery recently resulted with the publication of a proof of concept for the use of AAV coding miRNA in large animals. Indeed in this work of Gabisonia *et al.*, the serotype 6 (AAV6) was demonstrated to be a valid vector for transducing pig cardiomyocytes after intramyocardial injection (161). Together with a re-increased interest in the field of gene therapy, as represented also by the recent in human successes from the leading company Audentes (https://www.audentestx.com/), the clinical use of viral vector for a target delivery of ncRNAs is promising. Nevertheless the viral approach is still not free from negative aspects such as related-carcinogenesis (162), immunogenicity (163), broad tropism (164), and chronic transgene expression (165,166). To cope with such limitations, the evaluation and development of alternative vectors based on non-viral systems is on going (167). An example is provided by the latest advances in material sciences, where a plethora of biosafe and biodegradable non-viral vectors with different size, shape, surface charge and drug cargo, has been recently explored in order to achieve an improved beneficial action of delivery but with reduced side effects (168). In the work of Bellera *et al.*, the authors demonstrated in adult pigs that a single intracoronary administration of encapsulated antagomir - 92a into microspheres prevents left ventricular remodelling with no local or distant adverse effects (169). At the clinical level, a significant effort has been done by the Nano-Athero consortium, which is currently developing efficient nanocarriers for both the imaging and the treatment of thrombus and plaque (http://www.nanoathero.eu/). A similar activity is pursued by our group, who demonstrated the potential application of nanosystems for the specific targeting and release of therapeutic compounds to the heart. In particular, we demonstrated in both mice and pigs how the inhalation of biocompatible and biodegradable calcium phosphate nanoparticles (CaPs) can represent an important tool for the release of therapeutic drugs directly into the myocardium (170,171).

Based on the concept that no one-size-fits-all solution to gene delivery, new nanoformulation are constantly being proposed and investigated, therefore opening new horizon in the clinical approach for the treatment of cardiovascular disorders (172).

Conclusions

Although ncRNAs can represent promising tools for the prevention or treatment of CVDs, the general number of approved (e.g., Mipomersen, for treatment of homozygous familial hypercholesterolemia) or still in clinical trials (e.g., Inclisiran in Phase II; IONIS ANGPTL3-LRxin Phase II) (173) therapeutic RNA-based compounds remain limited. The effort from joint ventures between scientific community and pharmaceutical industries, such as Cardior (https://www.cardior.de/) or within the H2020 Cupido project (http://www.cupidoproject.eu/), are strongly required for the establishment of new strategies to overcome obstacles related to elevated toxicity, poor cellular uptake and tissue specificity that hinder the use of RNA molecules in the modern medicine.

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Footnote

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/ncri.2019.05.03). The authors have no conflicts of interest to declare.

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