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Review

Interactions between microRNAs and long non-coding RNAs in cardiac development and repair

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ABSTRACT

Non-coding RNAs (ncRNAs) are emerging players in muscle regulation. Based on their length and differences in molecular structure, ncRNAs are subdivided into several categories including small interfering RNAs, stable non-coding RNAs, microRNAs (miRs), long non-coding RNAs (lncRNAs), and circular RNAs. miRs and lncRNAs are able to post-transcriptionally regulate many genes and bring into play several traits simultaneously due to a myriad of different targets. Recent studies have emphasized their importance in cardiac regeneration and repair. As their altered expression affects cardiac function, miRs and lncRNAs could be potential targets for therapeutic intervention. In this context, miR- and lncRNA-based gene therapies are an interesting field for harnessing the complexity of ncRNA-based therapeutic approaches in cardiac diseases. In this review we will focus on lncRNA- and miR-driven regulations of cardiac development and repair. Finally, we will summarize miRs and lncRNAs as promising candidates for the treatment of heart diseases.

Contents

Abbreviations: AAV, adeno-associated virus; AMI, acute myocardial infarction; APF, autophagy promoting factor; *Bvht*, *Braveheart*; *CARL/Carl*, cardiac apoptosis-related lncRNA; *CARMEN/Carmen*, cardiac mesoderm enhancer-associated non-coding RNA; CHAER/Chaer, cardiac-hypertrophy-associated epigenetic regulator; CHAST/Chast, cardiac hypertrophy associated transcript; *CHRF*, cardiac hypertrophy related factor; circRNA, circular RNA; elncRNA, enhancer-associated long non-coding RNA; ESC, embryonic stem cells; EZH2, enhancer of zeste homolog 2; *FENDRR/Fendrr*, Fetal-lethal or Foxf1 adjacent non-coding developmental regulatory RNA; HF, heart failure; KO, knockout; LNA, locked nucleic acid; lincRNA, long intergenic non-coding RNA; lncRNA, long non-coding RNA; Mesp1, mesoderm posterior basic helix-loop-helix transcription factor 1; *MHRT/Myheart*, myosin heavy chain associated RNA transcripts; MIAT, myocardial infarction-associated transcript; miR, microRNA; mRNA, messenger RNA; MyHC, myosin heavy chain; ncRNA, non-coding RNA; PRC2, Polycomb Repressive Complex 2; RISC, RNA-induced silencing complex; ROR, regulator of reprogramming; snoRNA, stable non-coding RNA; SUZ12/Suz12, suppressor of zeste 12 protein homolog.

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1. Introduction: miR and lncRNA biogenesis and delivery

Non-coding RNAs (ncRNAs) represent a class of transcripts with high density of stop codons and lacking extensive 'Open Reading Frame' (ORF) [1]. However, several studies using genome-wide techniques (mass spectrometry proteomics, deep RNA sequencing, ribosome profiling) are shedding light on possible cryptic ORFs inside ncRNAs that sparked controversy in the real nature of ncRNAs [2]. ncRNAs are commonly considered as RNA transcripts that, although not having a direct protein-coding potential, are biologically functional RNAs with fine-tuned mechanism of protein-coding gene regulation.

Early studies carried out in 1991 by Bartolomei et al. in mice discovered the first ncRNA (H19) spanning 2.5-kilobase. The authors demonstrated the crucial role of H19 not only in mouse development but also in imprinting, as only the maternally-inherited allele was shown to be active [3]. After this finding, several ncRNAs have emerged for their role in imprinting, such as Xist [4]. The discovery of ncRNAs changed the central dogma of molecular biology 'DNA makes RNA makes proteins' stated by Francis Crick [5]. Indeed, in the early 1960s it was believed that only a small fraction of genome (around 2%) was covered by protein-coding genes, while the remainder (up to 97% of the entire genome) was made of non-transcribed 'junk DNA' regions [6,7]. However, with the birth of the ENCODE Project in 2000 (see https://www.encodeproject.org/) that aimed to characterize all the functional elements in the human genome, huge steps forward were made in order to understand the eukaryotic genome complexity [7]. In fact, the ENCODE project helped researchers in reshaping the concept of 'junk DNA' by implementing new genome-wide techniques (tiling microarrays, RNA-seq, ChIP-seq, FAIRE-seq) with computational tools, such as protein-coding gene prediction software, to uncover the role of non-transcribed DNA regions. By means of whole transcriptome analyses, it was uncovered that up to 90% of the eukaryotic genomic DNA is transcriptionally active, including RNAs lacking their coding function, i.e. ncRNAs. A study from 2007 strengthened the idea that ncRNAs are crucial in the evolutionary scale [8], since it was demonstrated that the evolution of biological complexity in any given organism relies on the number of non-protein-coding genes rather than the number of protein-coding genes [9].

Researchers have classified ncRNAs into three different subgroups based on their length [10]:

1) Short-sized ncRNAs, including microRNAs (miRs) [18–24 nucleotides], short interfering RNAs [~21 nucleotides] and piwi-interacting RNAs [26–31 nucleotides];
2) Medium-sized ncRNAs including stable non-coding RNAs [60–300 nucleotides];
3) Long-sized ncRNAs, including long intergenic ncRNAs (lincRNAs), transcribed ultraconserved ncRNAs and other long ncRNAs (lncRNAs) [>200 nucleotides].

In this review we will focus on miRs and lncRNAs due to their main relevance for potential therapeutic strategies in cardiovascular diseases.

MiRs are conserved non-protein-coding RNA molecules with a typical length of ~22 nucleotides. Currently 2588 sequences of mature miRs are reported in humans, while 1915 mature miRs are described in mice (see mirbase.org). MiRs regulate gene expression by the unique mechanism of directly targeting protein-coding and non-coding genes, leading to post-translational gene silencing. Moreover, miRs have a pleiotropic effect as they can target hundreds of mRNAs [11].

The genes encoding for miRs are represented in the genome as intergenic clusters or independent transcriptional units. MiRs derive from either introns or exons of both protein-coding and non-coding genes [12,13]. During their biogenesis, miRs are synthesized by RNA polymerase II and, to a lesser extent, by RNA polymerase III [14]. RNA polymerase II transcribes miR genes into pri-miRs, accounting for long primary transcripts. pri-miRs have secondary stem-loop structure and incorporate a 5′ cap and 3′ polyadenylation tail. Subsequently, the microprocessor complex, made up by RNase-III Drosha and its co-factor Dgcr-8, cleaves the pri-miRs into pre-miRs, which are ~70 nt-long precursor molecules [15]. Pre-miRs are then shuttled into the cytosol by a nuclear export protein called exportin-5. Once inside the cytosol, the RNase-III Dicer and its RNA binding cofactor trans-activation response RNA binding protein (TRBP) complex cleaves pre-miRs to form ~22 nt-long double-stranded miR molecules [16,17]. Next, miRs go through the RNA-induced silencing complex (RISC). For this purpose, mature miR duplexes are separated into two strands, collectively known as the guide and the passenger strand. The guide strand (miR), which shows the more unstable base pair at the 5′ end, is the one that RISC complex easily incorporates, while the passenger strand (miR*) undergoes degradation. The RISC complex is formed by Argonaute proteins, such as Ago-2, and is in charge of directing the miRs to their mRNA targets. Several other enzymes take part into processes of decapping, deadenylation and subsequent degradation of mRNA target.

LncRNAs, the widest subgroup of ncRNAs, exert both beneficial and detrimental effects by acting at transcriptional, post-transcriptional or epigenetic levels. In accordance with NONCODE database (http://www.noncode.org), to date there are 144,134 and 126,415 lncRNA genes for human and mouse, respectively. Interestingly, the pool of annotated lncRNAs remarkably increased over the past few years (see http://www.lncrnadb.org/). At variance with miRs, described to have only a negative post-transcriptional regulation on protein-coding genes [18], many of the lncRNAs functions remain still unknown. It has not yet been confirmed whether all of the identified lcnRNAs are functional, since most of them are transcribed at very low levels and can be also considered merely artifacts. The critical function of lncRNAs resides in their ability to interact with specific regulatory proteins and assembling them in complexes. In this sense, lncRNAs can serve as factors recruiting proteins to specific genomic loci or, once transcribed, they can directly open the chromatin around adjacent genes [19] acting as either guide, decoy or scaffold. Concerning their guidance function, lncRNAs can modulate gene expression by means of epigenetic regulation, acting as guides recruiting proteins to target genes [20]. In principle, lncRNAs can guide chromatin rearrangement in *cis* in a co-transcriptional manner via RNA polymerase II tethering, while guidance in *trans* can occur by binding to target DNA as a RNA–DNA heteroduplex, as RNA-DNA–DNA triplex or by recognizing specific chromatin features. Conversely, by acting as decoys lncRNAs exploit their function of sequestering specific transcription factors, chromatin remodeling complexes or mainly miRs. Thus, in this review we will focus on the cross-talk miRNA-lncRNA and its potential role for designing future therapeutic strategies.

Most lncRNA genomic loci rely on intergenic regions (long intergenic non-coding RNAs or lincRNAs), although they can also be found inside introns of protein-coding genes [21], or in genomic enhancer regions (enhancer-associated lncRNAs or elncRNAs). Regarding the adjacent protein-coding gene, lncRNAs can be transcribed from the sense or antisense strand [22]. In addition, bidirectional lncRNAs are located on the antisense strand of a protein-coding gene whose transcription initiates less than 1 kb away. In order to reach mature forms the lncRNAs, as well as other immature RNA transcripts, undergo several different co-transcriptional and post-transcriptional processes including 5′-capping, splicing, polyadenylation and chemical base modification [23]. In some nuclear lncRNAs, including metastasis-associated

lung adenocarcinoma transcript 1 (*MALAT1*) and nuclear enriched abundant transcript 1 (*NEAT1*), the RNase P ribonucleoprotein mediates the cleavage of transport RNA (tRNA)-like structures from the nuclear lncRNA resulting in mature lncRNA transcripts [24,25]. Another example of lncRNA processing is the circular RNAs (circRNAs), a subpopulation of lncRNAs whose existence and function is still under debate [26]. CircRNAs are generated by means of special 5′- and 3′-processing such as back-splicing of introns (resulting in a circularized transcript with spliced 3′ sequence upstream of 5′sequence) or canonical intron splicing (giving rise to stable by-products of splicing called circular intronic lncRNAs or ciRNAs). Moreover, few lncRNAs harbor miRs and, in these lncRNAs, the transcriptional termination as well as 3′ maturation by means of canonical polyadenylation pathway is not employed. Indeed, lncpri-miRs are produced by means of polyadenylation-independent cleavage, and are eventually processed into both miRs and lncRNAs.

In contrast to miRs, which localize in the cytoplasm, most of lncRNAs seem to permanently reside in the nucleus. Intriguingly, a subset of lncRNAs is located in both the nucleus and the cytoplasm while others have been selectively localized only in the cytoplasm [27]. The localization of lncRNAs is critical to design an effective lncRNA-based therapy, as the expression of nuclear lncRNAs is more effectively ablated using antisense oligonucleotides while the expression of cytoplasmic lncRNAs is more effectively knocked-out using siRNA-mediated RNA interference mechanism.

As the ncRNAs have an intrinsic potential for clinical applications, the optimal delivery method needs to be exploited. To this end, several ncRNA delivery approaches are being investigated, including liposomes, polymers, cell penetrating peptides and aptamers. Liposomes for ncRNA delivery are the most promising approach due to their ease in preparation, their low cost and their relative non-toxicity. Moreover, lipids are very effective in increasing the uptake and protecting the cargo from nuclease degradation and renal clearance. Indeed, the use of liposomes in clinical settings has already undergone phase I clinical trials [28]. However, liposomes have major side effects mainly caused by their accumulation in liver, lungs and spleen. When the therapeutic approach requires lncRNA overexpression, the use of gene delivery vectors needs to be considered. Generally, viral-based systems exploit the use of retroviruses, lentiviruses, and adenoviruses or adeno-associated viruses (AAV) [29]. In addition, gene gun, electroporation, hydrodynamic, ultrasound, laser-based energy and inorganic carriers are being investigated in order to improve the efficiency of ncRNA delivery. Although these innovative approaches represent promising solutions for ncRNA delivery, an effective gene-based therapy is still far from being obtained.

2. MiRs and cardiac development

The heart is the first fully functioning and organized organ being developed during embryogenesis in humans and vertebrates. In the regulation of the complex developmental organization system, a crucial role is played by miRs [30]. An overview of miRs involved in the different stages of cardiac development is given in Fig. 1.

The miR-218 family (miR-218a-1, miR-218a-2, miR-218b) is evolutionarily preserved in mammals, with its host gene *slit2* being essential for the proper development of the heart tube as seen in a zebrafish model [31]. Additionally, miR-218 family belongs to the regulatory circuit through which tbx5 transcription factor controls cardiac morphogenesis. Both the overexpression of tbx5 [32] and the downregulation of miR-218 family caused heart-looping defects with chamber abnormalities, while the upregulation of miR-218-1 is able to rescue tbx5 overexpression-related cardiac defects [33]. Moreover, during cardiogenesis the involvement of miR-99a/let-7c in embryonic development has been described. While let-7c has been shown to induce cardiogenesis, the expression of miR-99a was shown to repress cardiogenesis by altering Nodal signaling and Smad2 phosphorylation levels, leading to reduced cardiac differentiation. Intriguingly, while these experiments were carried out in mice, the miR-99a/let-7c cluster was shown to be overexpressed in human Down syndrome fetal heart tissues, hinting at its plausible role in the congenital heart defects observed in Down syndrome patients [34].

During cardiac looping, the miR-17/92 cluster (miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, miR-92-1) influences the differentiation of cardiac progenitors helping in the elongation of the heart tube, i.e. second heart field [35]. The depletion of miR-17/92 cluster in mice has been shown to cause death after birth due to ventricular septal defects and lung hypoplasia, while its overexpression causes repressed fibronectin expression with subsequent cellular defects and overall delayed organ growth [36]. As the expression of the miR-17/92 cluster has been shown to increase dramatically with gastrulating cells differentiating into mesoderm and mesendoderm, the role of the cluster was investigated. Indeed it was shown that, by suppressing *Bmpr2* expression at primitive streak, the miR-17/92 influences gastrulation and affects the regulation of actin dynamics eventually leading to mesoderm invasion [37]. Similarly, the expression of miR-143 at the onset of cardiac looping was seen to have an essential role for the formation and function of cardiac chambers by means of myocardial cell morphology adjustment [38].

Muscle-specific miRs, or myomiRs (including miR-1, miR-133a, miR-208a, miR-208b, and miR-499) have been associated with several important steps in cardiac commitment, and the fine regulation of myomiRs is essential for the proper embryological development of the heart [39]. The overexpression of miR-1 negatively regulates ventricular cardiomyocyte proliferation in developing mouse embryo via *Hand2* mRNA targeting, causing thin-walled ventricles and heart failure eventually leading to embryonic developmental arrest [40]. Conversely, members of the miR-133a family require a stable level of expression as they are crucial for the growth of progenitor cells, with overexpression and knockdown of miR-133a reducing the proliferation of cardiomyocytes and hampering cardiomyogenesis respectively [41]. As the lack of miR-133a-1/miR-133a-2 in double knockout (KO) mice resulted in growth rate increase of cardiomyocytes [41], a crucial role for miR-133 family in maintaining the homeostasis of cardiomyocytes has been suggested.

The two isoforms of myosin heavy chain (MyHC), i.e. α-MyHC and β-MyHC, are differentially regulated during embryological development of the heart, with the latter isoform being highly expressed in fetal life. MyomiRs-208a, −208b and −499 are encoded by introns of *Myh6* (α-MyHC gene), *Myh7* (β-MyHC gene) and *Myh7b,* respectively [42]. During embryological development *Myh7* and *Myh7b* are highly expressed in the heart while *Myh6* expression is detected specifically in the adult heart. Although the concurrent high expression of miR-208b and miR-499 during embryological development has been observed, it has been suggested that myosin regulatory circuitry of miRs has no role in fetal development [42]. By creating miR-208-KO murine models, normal expression of β-MyHC and *Myh7* was observed. Hence, myomiRs seem to primarily function in adult cardiac gene expression adaptation to physiological and pathological signaling [42,43].

MiR-15 and miR-29 families are implicated in the regulatory mechanisms promoting fetal to adulthood switch in cardiomyocytes, and the inhibition of miR-15 family (composed of miR-15a, miR-15b, miR-16-2, miR-195, and miR-497) has been associated with persistent cardiomyocyte mitosis [44]. Locked nucleic acid (LNA)-antagomiRs-mediated knockdown of miR-195 in neonatal mice resulted in higher number of proliferating cardiomyocytes, while the overexpression of miR-195 led to right ventricular

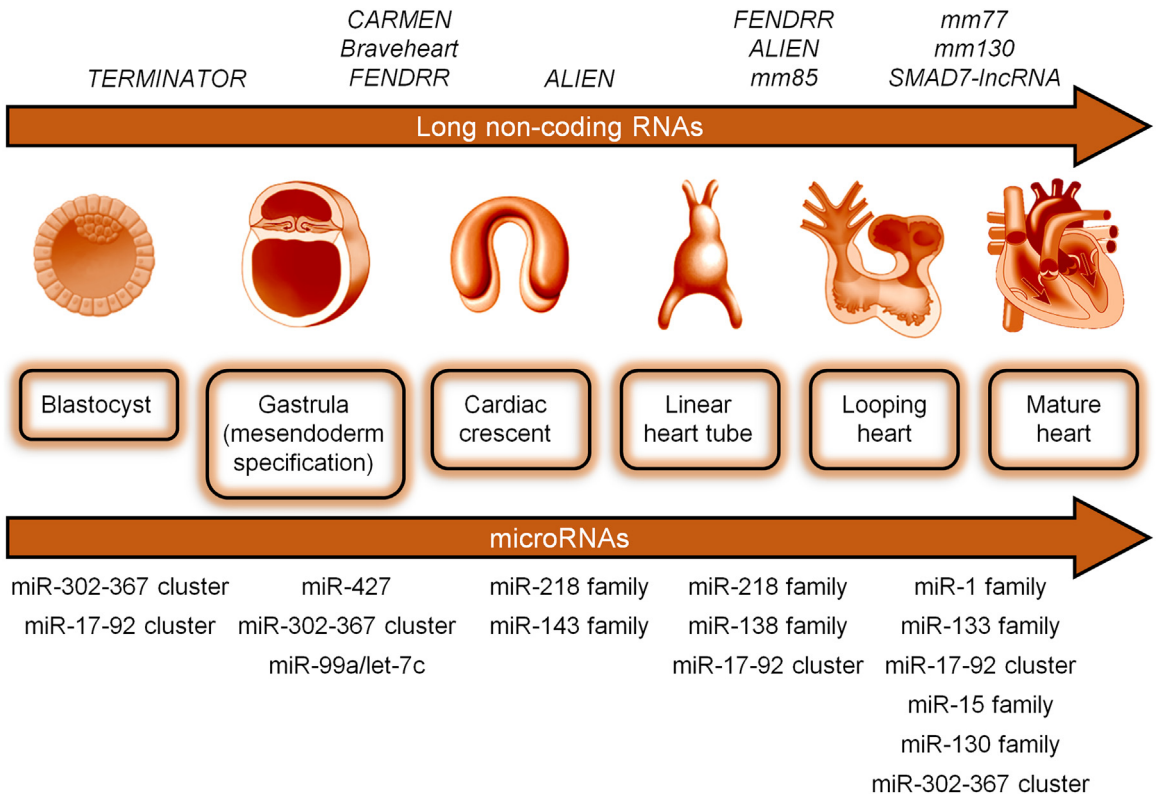

Fig. 1. Overview of miRs and lncRNAs involved in cardiac development. Summary of current miRs and lncRNAs regulating cardiac development.

hypoplasia as well as ventricular septal defects [44]. Similarly, other miRs were shown to have an important role in the final phases of cardiac maturation, both for cardiomyocyte maturation (miR-138-1 and miR-138 [45]) and ventricular wall development (miR-130a and miR-130b [46]).

Finally, miR-302-367 cluster inhibits the core components of the Hippo pathway in early mouse cardiac development eventually promoting cardiomyocyte proliferation. Conversely, the overexpression of mi*R*-302-367 causes severe cardiomegaly and excessive cardiomyocyte proliferation. Intriguingly, by re-expressing miR-302-367 postnatally the reactivation of cell cycle in cardiomyocytes was observed, resulting in reduced scar formation after experimental myocardial infarction [47]. It has also been demonstrated that miR-302-367 downregulation during the first stages of ESCs differentiation leads to the complete shift towards the mesendodermal lineage. This mechanism occurs by modulating Nodal pathway via inhibition of Lefty1 and Lefty2, i.e. Nodal inhibitors. Nodal signaling is crucial for the proper mesoendoderm development, and miR-427 controls the Nodal pathway in parallel with miR-302 [48]. Interestingly, the depletion of miR-427 in early stages of embryonic development of Xenopus leads to the reduction of brachyury levels and impairs its role in mesoderm formation and cellular differentiation.

3. LncRNAs and cardiac development

The involvement of lncRNAs regulation in development has been widely investigated over the last years [49]. Hundreds of lncRNAs have been found to be cardiac-specific during development or in pathological conditions, although their function needs further studies [50,51]. Forty lncRNAs involved in the lateral plate mesoderm formation and cardiomyocyte differentiation were observed to be differentially expressed during embryonic carcinoma cell differentiation into cardiac myocytes [52]. Furthermore, during development it was found that lncRNAs function as regulators of key cardiac transcriptional pathways [53] with major differences in the profile expression of lncRNAs between embryonic and adult heart. Conversely, fewer differences were observed among healthy adult heart and a heart model with cardiomyopathy, suggesting lncRNAs primary role in cardiac development. For an overview of the most studied lncRNAs involved in the different stages of heart development, see Fig. 1.

The first lncRNA identified as a key regulator in cardiac development in the mouse embryo was *Braveheart* (*Bvht*) [54]. Its expression has been detected in cardiac mesoderm and cardiomyocytes, since *Bvht* seems to be essential for the transition from nascent to cardiac mesoderm and moreover for cardiomyocyte differentiation. Indeed, during embryonic stem cell differentiation, the depletion of *Bvht* results in loss of beating cardiomyocytes and deactivation of genes specifying key cardiac transcription factors. *Bvht* functions upstream of the critical transcriptor factor for cardiac precursors mesoderm posterior basic helix-loop-helix transcription factor 1 (*Mesp1*) by sequestering suppressor of zeste 12 protein homolog (Suz12) from the promoters of genes that determine the cardiac lineage, including *Mesp1*. Suz12 is a component of the Polycomb Repressive Complex 2 (PRC2), which trimethylates histone H3 on lysine K27 (H3K27me3), causing a mark that silences gene expression [55]. Therefore, *Bvht* functions as an epigenetic modulator decreasing the presence of Suz12/PRC2 in the promoters of cardiac-specifying genes, resulting in their expression. However, *Bvht* expression was identified in mouse ESCs and no homologs have been found in humans. In fact, the human orthologous genomic region is not actively transcribed. Nevertheless, due to the critical importance of *Braveheart* in cardiac lineage commitment, an homologous transcript could potentially be transcribed from a different genomic locus [49].

Another identified lncRNA that acts as a critical regulator in cardiac development is Fetal-lethal or Foxf1 adjacent non-

coding developmental regulatory RNA (*Fendrr*) [56]. It is exclusively expressed in the lateral plate mesoderm and regulates its differentiation into the heart and ventral body wall. Disruption of the *Fendrr* transcript results in embryonic lethality, partially due to heart failure [56,57]. Similar to *Bvht*, *Fendrr* functions as a chromatin modulator interacting with the repressing complex PRC2, in addition to the activating complex trithorax-group/mixed lineage leukaemia protein (TrxG/MLL) which trimethylates histone H3 on lysine K4 (H3K4me3). While *Bvht* decreases the presence of PRC2 in the promoters of genes that determine cardiac lineage, *Fendrr* promotes anchorage of PRC2. Therefore, *Fendrr* and *Bvht* are likely to counteract each other in order to regulate the PRC2 binding on these cardiac lineage-determining gene promoters. Unlike *Bvht*, *Fendrr* presents the human homolog transcript *FENDRR* [57].

Recently, human embryonic stem cells cardiac differentiation has provided new insights into the discovery and functional identification of novel human lncRNAs involved in cardiovascular development [49]. Kurian et al. identified two novel human lncRNAs that are also necessary in cardiac development and are partially conserved across vertebrates [58]. *TERMINATOR* (expressed in pluripotent stem cells) was found crucial for pluripotency and early mesendodermal differentiation and survival, while *ALIEN* (expressed in cardiovascular progenitors) was found crucial for the correct mesodermal specification and cardiac chamber formation.

More recently, another human lncRNA required for cardiac cell differentiation was identified and named cardiac mesoderm enhancer-associated non-coding RNA (*CARMEN*) [59]. In the same way as *Bvht*, this lncRNA functions upstream of the genes that specify cardiac mesoderm through epigenetic regulation. In addition, it also interacts with SUZ12 as well as enhancer of zeste homolog 2 (EZH2), the latter being another subunit of PRC2. In fact, *Carmen* loss-of-function experiments in mouse embryonic carcinoma cells revealed block of *Bvht* induction, suggesting that *Carmen* controls *Bvht* expression in mouse. Therefore, disruption of *Carmen* caused inhibition of cardiac cell fate determination and impairment of cardiomyocyte differentiation.

Moreover, Ounzain et al. identified seven elncRNAs (mm67, −77, −85, −104, −130, −132 and −172) expressed during cardiomyocyte differentiation of murine embryonic stem cells [50]. Four of these elncRNAs (mm67, −85, −130 and −132) appear to be evolutionary conserved in humans. In addition, the knockdown of mm85 and SMAD7-lncRNA specifically reduced the expression of their predicted target genes (Myocardin and Smad7, respectively).

4. MiR-based therapy for cardiovascular diseases

The pathological dysregulation of miRs in several cardiovascular pathologies has increasingly posed the question of whether miRs could be used both as diagnostic and therapeutic tools [60]. Table 1 summarizes a selection of therapeutic miRs in cardiovascular diseases, while an overview of possible therapeutic approaches by combining lncRNAs and miRs is depicted in Fig. 2.

In the setting of acute myocardial infarction (AMI), the use of miR-based therapies to prevent post-infarction cardiac remodeling, reduce the size of infarct, and stimulate cardiomyocyte proliferation is compelling [61]. In adult cardiomyocytes, the proliferative capacity aimed at heart repair following cardiomyocyte loss is restricted. In order to study miRs that could be potentially used to enhance adult cardiomyocyte proliferation, Eulalio et al. identified forty miRs that increased cardiomyocytic DNA synthesis and cytokinesis [62]. In this subset of miRs, miR-590 and miR-199a were able to increase cardiac cell proliferation in adult animals, as well as promoting *ex vivo* cell cycle re-entry of adult cardiomyocytes. Moreover, following induction of myocardial infarction in mice, miR-590 and miR-199a were able to reduce the infarct size by stimulating cardiomyocytes proliferation with recovery of cardiac functional parameters in adult mice.

The compensatory response to several stress stimuli in the heart, including AMI, leads to cardiac remodeling with impaired contraction, hypertrophy of cardiomyocytes, and decreased ejection fraction. When untreated, chronic remodeling may eventually lead to heart failure (HF) and sudden death [63]. Anatomically, cardiac remodeling consists in a thickening of the interventricular wall and/or septum, while a reduction in ventricular size. This is translated into morphological changes at extracellular matrix level with an increased deposition of fibrotic tissue (fibrillar collagen) and infiltration, in addition to the cellular component. Specifically, it has been shown that both fibroblasts and endothelial cells re-enter the cell cycle, while cardiomyocyte pool decreases in number, due to the activation of their programmed death (apoptosis), necrosis or autophagy [64].

As the pathological expression of several miRs has been observed in HF patients, it has been hypothesized that the use of miR inhibitors by blocking overexpressed miRs in HF may help in attenuating the pathological remodeling while improving cardiac function. Gupta et al. employed an aged mice model to study the effect of anti-miR22 therapy following AMI and, compared to younger mice, old mice were shown to have higher expression levels of miR-22, a key regulator for cardiac autophagy [65]. Intriguingly, by blocking miR-22 post-AMI, the activation of cardiac autophagy led to the prevention of post-infarction remodeling with improved cardiac function. In a similar study, Montgomery et al. observed how, by inhibiting miR-208a in HF murine model, the cardiac remodeling was reduced together with improved cardiac function [66]. By using a different miR inhibitor, Bernardo et al. assessed how the administration of LNA antimiR-652 in HF mice halted the hypertrophic remodeling associated with cardiomyopathy [67]. Notably, other hallmarks of heart failure including fibrosis, cardiomyocyte apoptosis and reduced angiogenesis, were reversed to physiological state. Similarly, Da Costa Martins et al. showed how the *in vivo* inhibition of miR-199b in mice model of HF ameliorated the function of the cardiac muscle, by reducing consistently fibrosis and reversing hypertrophy [68]. Moreover, Ucar et al. suggested that miRNA-212/132 family is pivotal for the cardiac hypertrophy in HF murine models [69]. Intriguingly, this study showed that, by inhibiting miR-132, the heart showed decreased cardiac fibrosis and preserved cardiac function and dilatation, eventually preventing the development of pressure overload-induced HF.

In recent years, the use of stem cell-based therapeutic strategies in cardiovascular diseases for the protection of endogenous cardiac progenitors and the direct use of differentiated cardiomyocytes has emerged. Stem cells hold the intrinsic ability of enhanced self-renewal, and can differentiate in several lineages and cell types. Different populations of stem cells have allured researchers to investigate their cardiac regeneration potential [70], with pluripotent stem cells (i.e. ESC and iPSC) coming under the spotlight as therapeutics for cardiac pathologies. ESC cells successfully differentiate into cardiac precursors and have been shown to contribute to the regeneration of post-AMI heart in large animal models [71]. However, the therapeutic use of ESCs implies several risks, including the observed teratoma formation after transplantation of unpurified ESC-derived cardiomyocyte population [72]. Therefore, therapeutic strategies exploiting ESC regenerative capacity in the heart while avoiding the issues related with cell transplantation are needed. To this end, Tian et al. analyzed miRs expression during early embryogenesis in ESCs to identify miRs that could be used for the stimulation of postnatal cardiomyocyte proliferation [47]. By demonstrating the expression of miR302-367 in early mouse cardiac development, they sought to assess whether re-expression of miR302-367 could increase cardiomyocyte proliferation in adult mice. Indeed, the increased expression of miR302-367 in cardiomy-

Table 1
List of therapeutic miRs.

Pathology	miR		Observed effect	Model	Reference
AMI	antimiR-22	inhibitor	activation of cardiac autophagy, prevention of post-infarction remodeling, improved cardiac function	mouse	[65]
	miR-590	mimic	Reduced infarct size, improved cardiac function, stimulated cardiomyocyte proliferation	mouse	[62]
	miR-199a	mimic	Reduced infarct size, improved cardiac function, stimulated cardiomyocyte proliferation	mouse	[62]
	miR302-367	mimic	increased cardiomyocyte proliferation, decreased apoptosis, reduction in fibrotic scarring, increased ejection fraction, increased *peri*-infarct angiogenesis	mouse	[47]
	miR-294	exosome	endogenous cardiac stem cell activation	mouse	[73]
HF	antimiR-208a	inhibitor	improved cardiac function, health and survival	rat	[66]
	antimiR-199b	inhibitor	ameliorated the function of the cardiac muscle, reducing consistently fibrosis and reversing hypertrophy	mouse	[68]
	antimiR-132	inhibitor	suppression of pressure-overload and attenuated development of cardiac hypertrophy	mouse	[69]
	antimiR-652	inhibitor	restored heart function, attenuation of adverse remodeling	mouse	[67]
DCM	miR-669a	mimic	long term treatment showing reduced adverse remodeling and enhanced systolic fractional shortening of left ventricle	mouse	[74]

AMI acute myocardial infarction, HF heart failure, DCM dilated cardiomyopathy.

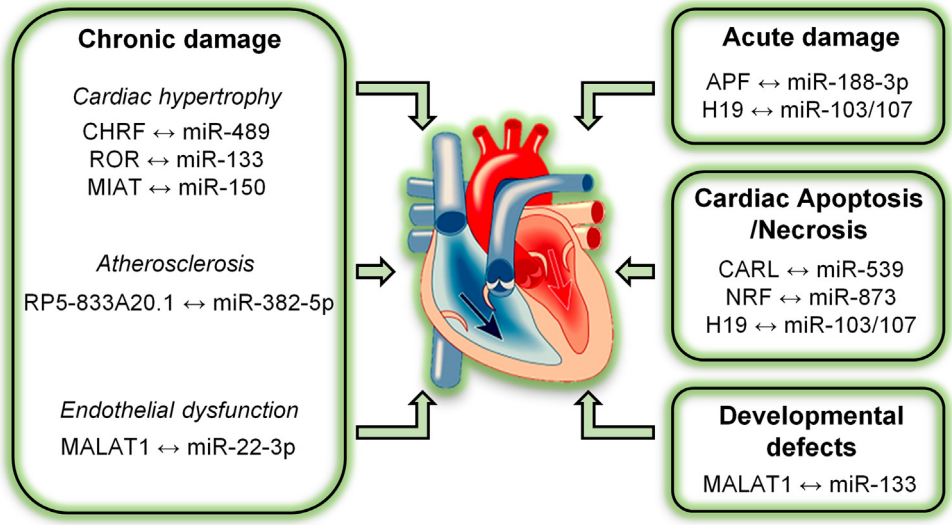

Fig. 2. Overview of miR and lncRNA interactions. Summary of interactions between miRs and lncRNAs important for cardiac development and repair.

ocytes increased the proliferation of cells, leading to eventually cardiomegaly if the miRs were expressed persistently. Conversely, by transiently treating mice with mi*R*-302-367, the correct regeneration of the murine heart was promoted. Similarly, Khan et al. sought to explore whether exosomes isolated from mouse ESCs could have the same beneficial effect of ESCs in post-AMI [73]. Exosomes from ESCs were first analyzed, showing their enrichment of miR-291, miR-294, and miR-295. By administering ESCs-derived exosomes in a post-AMI murine model, several beneficial effects including increased vascularization, improved cardiomyocyte survival, as well as protection of cardiac progenitor cells survival in the heart were observed. Similar striking results were obtained by using a miR-294 mimic; hence, the authors suggested that the aforementioned effects are dependent on ESC-exosome-mediated delivery of miR-294 [73].

Finally, Quattrocelli et al. reported that cardiac pericytes from dystrophic mice can differentiate into skeletal pericytes by means of miR-669a/q administration [74]. Notably, by transducing the cardiac muscle in neonatal mice with miR-669a-expressing AAV2/9 a partial remission from cardiomyopathies was observed.

5. LncRNA-based therapy for cardiovascular diseases

Recently, lncRNA-based therapies have emerged for the treatment of several cardiac complications, such as cardiac hypertrophy, AMI and ultimately HF. In this context, lncRNAs are described to exert their beneficial effect by acting directly on specific cardiac pathways, tackling the onset of cardiovascular diseases. Cardiomyocyte apoptosis represents one of the most useful targets to be addressed in potential therapies (see Section 4). One lncRNA observed in association with the apoptotic mechanism is cardiac apoptosis–related lncRNA (*CARL*), which has been described as an inhibitor of cardiomyocyte apoptosis targeting miR-539 [75]. An overview of possible therapeutic approaches by combining lncRNAs and miRs is depicted in Fig. 2. In mouse cardiomyocytes, *Carl* sequesters miR-539, a microRNA binding the mRNA of the PHB2 subunit of prohibitin. Prohibitin is a protein present in the inner mitochondrial membrane [76] where it has a beneficial role in mitochondrial homeostasis acting likely as chaperone for respiration chain proteins. Downregulation of PHB2 during pathological insults, such as anoxia and ischemia/reperfusion, brings to upregulation of miR-539, triggering mitochondrial remodeling and subsequent cardiomyocyte apoptosis. Therefore, *CARL* functions as

a miR-539 trap, regulating mitochondrial morphology and preventing cellular death. In conclusion, *CARL* has been demonstrated to reduce ischemia/reperfusion injury *in vivo*; hence, overexpression of *CARL* might represent a future strategy to counteract the cardiomyocyte loss, a common hallmark of myocardial infarction [75]. In contrast to the protective effect of *CARL*, cardiac hypertrophy related factor (*CHRF*) has been shown to trigger cardiomyocyte hypertrophy *in vitro* and apoptosis when injected in mice. Briefly, *CHRF* exerts its detrimental effect by targeting the antihypertrophic miR-489, which in turn downregulates *MYD88*, an important gene for the development of hypertrophy. Indeed, *myd88-KO* mice displayed resistance to hypertrophy caused by angiotensin II. *CHRF* is also well conserved in humans highlighting that CHRF-miR-489-MYD88 pathway might be modulated in order to define a tailored therapy for cardiac hypertrophy [77].

As described, apoptosis represents an interesting pathway to be targeted, but also strategies aiming to reverse cardiomyocyte autophagy might be encouraging. In this regard, Viereck et al. showed that a lncRNA known as cardiac hypertrophy associated transcript (*CHAST*), acts by blocking the autophagy of cardiomyocytes while promoting cardiac hypertrophy [78]. Specifically, overexpression of *Chast* using AAV-mediated gene delivery induced cardiomyocyte hypertrophy in wild-type mice, whereas gapmeR-mediated inhibition of *Chast* showed the opposite effect by preventing HF. *Chast* acts by modulating autophagy regulatory factor *Plekhm1* levels, and the use of *CHAST* as a novel target in treating cardiac hypertrophy is promising. Moreover, a recent study reported that autophagy is also modulated by the lncRNA named autophagy promoting factor (*APF*), which targets miR-188-3p and ATG7 [79]. MiR-188-3p inhibits autophagy and myocardial infarction by targeting ATG7, while APF displays negative effects, triggering autophagy [79].

Recently, the lncRNA cardiac-hypertrophy-associated epigenetic regulator (*CHAER*) has been found to be important for the development of cardiac hypertrophy. *Chaer-KO* mice show a reduced hypertrophy of the heart after transverse aortic constriction operation. In addition, *Chaer-KO mice* show an attenuated fibrosis and an amelioration of heart function. Mechanistically, specific region of *CHAER* can bind EZH2 subunit of the repressor complex PRC2. This will bring to the inhibition of histone H3 lysine 27 methylation in the promoter region of cardiac hypertrophy-associated genes [80]. Hence, the inhibition of *CHAER* expression could reduce cardiac hypertrophy and other types of heart dysfunctions. Moreover, another hypertrophic inducer is *ROR* (regulator of reprogramming) and functions as miR-133 sponge, since overexpression of miR-133 can reverse the pro-hypertrophic effect of *ROR* [81]. Conversely, a recent study discovered a class of antisense lncRNAs with cardio-protective effect, known as myosin heavy-chain associated RNA transcripts (*MHRT* or *Myheart*) [82]. This lncRNA is thought to function as a chromatin modulator, by decoying the BRG1 chromatin repressor complex and preventing it to recognize its targets in the genomic DNA. BRG1 is activated by pathological stress and results in abnormal expression of genes that ultimate cause cardiac myopathy and hypertrophy, as well as inhibition of *MHRT* expression. Therefore, in physiological condition, *MHRT* transcription can inhibit this anomalous gene expression and its pathological outcome [82]. Indeed, BRG1 could be used as a target to reduce pathological cardiac hypertrophy. Moreover, another lcnRNA has been found associated with cardiomyocytes in subjects affected by dilated cardiomyopathy, named Novlnc6. Although its role is not yet elucidated, inhibition experiments using Gapmers display that down-regulation of Novlnc6, a reduction in the expression of Bmp10 and Nkx2.5 at mRNA level is observed [50].

Finally, although so far there are no studies aiming to modulate the dysregulated immune response present in cardiac hypertrophy, emerging evidences suggest that the reduction of fibrotic tissue accumulated in the hypertrophic heart is also promising in slowing down the disease. In this regard, a novel lncRNA, known as *MIAT*, has been demonstrated to be the first pro-fibrotic lncRNA present in heart. The mechanism by which *MIAT* exerts its effects is by sequestering miR-24. Therefore, inhibition of *MIAT* paves the way for development of new anti-fibrotic strategies in the near future [83].

6. Conclusion and remarks

Among non-coding RNAs, miRs and lncRNAs were extensively studied in the last decade and some important interactions between them have been identified. Understanding this novel RNA crosstalk will shed light into gene regulatory networks and will have new implications in human development and disease. The majority of miRs are found during cardiac development, and their expression and distribution is reorganized during acute and chronic damage. LncRNAs are not only expressed during cardiac development, but they are also involved in adult cardiac homeostasis. In this context, both miRs and lncRNAs are dysregulated in pathological conditions and they were successfully therapeutically targeted. Recently, the academic world has established partnerships with biotech companies in order to develop new powerful tools, including nanotechnology and microvesicles as RNA delivery carriers, which are paving the way to establish reliable protocols for the treatment of cardiac diseases [84]. Several preclinical studies denoted that extracellular vesicles isolated from adult stem cells retain cardioprotective and proangiogenic properties when transplanted in MI animal models. For instance intra-cardiac injections of cardiac stem cell-derived extracellular vesicles reduced scar tissue and improved cardiac function in MI rat model [85]. Moreover, exosomes containing high amounts of microRNA-146a produced by cardiac stem cells were proved to ameliorate cardiac function and selective delivery of miR-146a mimics reproduced some of the beneficial effects of exosomes [86]. Nevertheless, exosomes hamper the rapid translation in clinical practice due to their complex structures and their potential immunogenic properties. Thus using cell factory approaches in autologous setting to engineer exosome mimetics with specific miRs and lncRNAs could be an alternative strategy to overcome these issues.

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