Journal of Molecular and Cellular Cardiology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Review article

Non-coding RNA in control of gene regulatory programs in cardiac development and disease

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ARTICLE INFO

Article history:
Received 30 December 2014
Received in revised form 19 March 2015
Accepted 20 March 2015
Available online xxxx

Keywords:
Non-coding RNA
MicroRNA
lncRNA
Gene expression

ABSTRACT

Organogenesis of the vertebrate heart is a highly specialized process involving progressive specification and differentiation of distinct embryonic cardiac progenitor cell populations driven by specialized gene programming events. Likewise, the onset of pathologies in the adult heart, including cardiac hypertrophy, involves the reactivation of embryonic gene programs. In both cases, these intricate genomic events are temporally and spatially regulated by complex signaling networks and gene regulatory networks. Apart from well-established transcriptional mechanisms, increasing evidence indicates that gene programming in both the developing and the diseased myocardium are under epigenetic control by non-coding RNAs (ncRNAs). MicroRNAs regulate gene expression at the post-transcriptional level, and numerous studies have now established critical roles for this species of tiny RNAs in a broad range of aspects from cardiogenesis towards adult heart failure. Recent reports now also implicate the larger family of long non-coding RNAs (lncRNAs) in these processes as well. Here we discuss the involvement of these two ncRNA classes in proper cardiac development and hypertrophic disease processes of the adult myocardium. This article is part of a Special Issue entitled: Non-coding RNAs.

Contents

1. Introduction

For decades, cardiovascular scientists have been studying the molecular processes involved in pathological hypertrophy and observed how these resemble the molecular changes during fetal cardiac development [1]. During mammalian embryogenesis, the heart is the first organ to form, and it starts when a population of mesodermal stem cells commits to the cardiogenic fate. These mesodermal progenitors form the primary heart field in the anterior mesoderm and migrate ventromedially to shape a linear heart tube [2,3]. A second population of mesodermal cardiac progenitors, known as the secondary heart field, is derived from the pharyngeal mesoderm located medial and anterior to the primary heart field. These cells migrate from behind the heart tube into the anterior and posterior poles of the heart tube as it begins to undergo rightward looping as a result of uneven growth and remodeling, leading to the

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http://dx.doi.org/10.1016/j.yjmcc.2015.03.014
0022-2828/

formation of primitive ventricles and atria [2,3]. Maturation of the heart in higher organisms includes septation, leading to the formation of ventricles and atria, as well as valve formation and outflow vessel development. The cells in the primary heart field contribute to most of the left ventricle and part of the right and left atria. The secondary heart field contributes to the right ventricle, most of the outflow tract and part of the atria [2,3]. Cardiac neural crest cells migrating from the dorsal neural tube into the outflow tract participate in separation of the outflow tract [4]. Cardiogenesis in mammals requires exquisite control of gene expression as their distinct patterns define each region of the heart, including individual chambers and valves. Uninterrupted cardiac development is integral to organismal survival.

Studies of cardiogenesis in the simple model organism *Drosophila melanogaster* have defined many of the essential regulators of cardiac specification and differentiation and revealed that the cardiac gene regulatory network shows a remarkable evolutionary conservation over hundreds of millions of years. These studies, combined with many studies in vertebrate systems, have shown that the gene regulatory events and cellular movements that control cardiogenesis are temporally and spatially regulated by complex signaling networks. The formation of the vertebrate heart is controlled by intrinsic and extrinsic signals, and crosstalk occurs between myogenic transcription factors, their downstream target genes, and upstream signaling pathways that direct cardiac cell fate, myocyte differentiation, and cardiac morphogenesis in the primary heart field and secondary heart field. Inductive signals such as bone morphogenetic protein (BMP), Notch, WNT and sonic hedgehog (SHH) in vertebrates activate a set of genes encoding transcriptional activators that are expressed in the primary and secondary heart fields. Core groups of transcription factors required for cardiogenesis include NK class of homeodomain proteins, GATA zinc-finger transcription factors, the MADS domain transcription factor MEF2, T-box and Forkhead transcription factors, and the Hand class of basic helix–loop–helix (bHLH) factors [5]. Nkx2-5 plays a crucial role during embryonic heart development. Mice with a ventricular-restricted deletion of Nkx2-5 display no structural defects but have progressive complete heart block, and progressive cardiomyopathy [6]. Also, the calcineurin-regulated NFATc1 transcription factor is expressed in the murine endocardium and second heart field during cardiogenesis and is required for proper valve elongation and semilunar valve development [7].

The molecular fingerprints observed during the process of heart failure resemble those observed during cardiogenesis, and therefore adult heart failure is often described as being accompanied by the reactivation of a "fetal gene program" [8]. The results of the Encyclopedia of DNA Elements (ENCODE) project have widened our understanding of genomics at an unprecedented level and indicate that at least 80% of the genome is functional and is transcribed into the well-studied coding RNAs as well as a much larger quantity of non-coding, regulatory RNAs [9–11]. Roughly speaking, non-coding RNAs (ncRNAs) can be classified according to their transcript length into either small or long ncRNAs (lncRNAs), with lncRNAs arbitrarily defined as being larger than 200 nucleotides in length. The discovery of the epigenetic control of gene regulatory processes by non-coding RNAs has added a new layer of complexity to our understanding of heart function both during embryonic development and disease. Especially, microRNAs and lncRNAs have been shown to play an essential role in normal heart development and as regulators of the stress response in the adult heart (Fig. 1).

Here, we will provide an overview of ncRNA-mediated control of gene regulation of the cardiac fetal gene program in the developing embryo and how it is reiterated in the processes that contribute to hypertrophic heart disease of the adult myocardium (Fig. 2).

1.1. MicroRNAs as regulators of the cardiac regulatory network

MicroRNAs are a class of evolutionary conserved small (18–24 nucleotide) single-stranded RNAs, encoded by the genome and affect gene expression by repressing mRNA translation and/or stability. In recent years, around 1000 microRNAs have been identified and functionally characterized to certain detail. The importance of microRNA gene regulation for normal heart development and function was first underscored by generating loss of function mutations of Dicer, Drosha, Ago2 and DGCR8, four enzymes essential for microRNA biogenesis [12–15]. Cardiac specific deletion of Dicer, the enzyme required to process microRNAs into their active mature forms, resulted in embryonic lethality in mice due to cardiac failure at day E12.5. Embryos lacking Dicer in the developing heart showed pericardial edema and a poorly developed ventricular myocardium, indicating an essential role for microRNA function in cardiogenesis [16]. DGCR8 inactivation in neural crest cells using a Wnt1-Cre-mediated deletion of the floxed DGCR8 allele resulted in major cardiovascular defects at E18.5, including arteriosus, persistent truncus interrupted aortic arch, cervical aortic arch and aberrant origin of the right subclavian artery [17]. Likewise, Dicer activity is also required for normal functioning of the postnatal heart, as targeted Dicer deletion in adult mice resulted in a high incidence of sudden death, pronounced cardiac hypertrophy, ventricular fibrosis and reactivation of the fetal cardiac gene program [18]. Combined, these studies demonstrate that modification of microRNA biogenesis at specific developmental windows impacts embryonic and adult myocardial morphology and function (Table 1).

1.1.1. Bicistronic miR-1/miR-133 families

Among the most abundantly expressed microRNAs in the heart is miR-1. In vertebrates, members of the miR-1 (*miR-1-1, miR1-2, miR-206*) and miR-133 (*miR-133a-1, miR-133a-2, miR-133b*) families are co-transcribed from the same bi-cistronic transcripts. MiR-1-1/miR-133a-2 are clustered in an intergenic region of human chromosome 20, whereas the miR-1-2/miR-133a-1 cluster is located in the antisense orientation within an intron of the Mind bomb1 (Mib1) gene on human chromosome 18. The expression of miR-1 and miR-133a is cardiac and skeletal-muscle specific, whereas the related miR-206 and miR-133b genes are restricted to skeletal muscle [19,20]. Transcription of the miR-1/miR-133 bi-cistronic precursors is directly regulated by serum response factor (SRF) in cardiac muscle, and by MyoD/Mef2 in skeletal muscles. In cardiomyocytes, SRF binds the enhancer regions of the miR-1/miR-133 cluster and regulates microRNA expression [20]. Gain- and loss-of function studies revealed that miR-1/miR-133 family members play crucial roles in the developing heart. Overexpression of miR-1 specifically in the developing heart resulted in decreased ventricular cardiomyocyte proliferation, ventricular wall thinning, heart failure and subsequent embryonic lethality at E13.5 [20]. In line, introduction of miR-1 into developing Xenopus embryos interfered with heart development and injection of miR-133 in embryos resulted in highly disorganized cardiac tissue, absence of cardiac looping or chamber formation, revealing that correct temporal expression and amounts of both miR-1 and miR-133 are required for proper skeletal muscle and heart development [19].

One of the validated targets of miR-1 is Hand2, a bHLH transcription factor involved in ventricular cardiomyocyte expansion [20]. Interestingly, targeted gene deletion of Hand2 in mouse embryos resulted in embryonic lethality at embryonic day 10.5 from heart failure, similarly to miR-1 transgenic mice [21]. Furthermore, targeted deletion of miR-1-2 revealed that miR-1-2 regulates cardiac morphogenesis, electrical conduction, and cardiac cell-cycle control [16]. Mice lacking either miR-133a-1 or miR-133a-2 do not display any overt developmental phenotype, whereas deletion of both microRNAs causes lethal ventricular–septal defects in approximately half of double-mutant embryos or neonates. The miR-133a double-mutant mice that survive to adulthood succumb to dilated cardiomyopathy by 5–6 months of age and heart failure. The absence of miR-133a expression is associated with aberrant cardiomyocyte proliferation and ectopic expression of smooth muscle genes in the heart. These abnormalities were proposed to be due to direct targeting of SRF and cyclin D2, both of which contain functional binding sites for miR-133a in their 3′-

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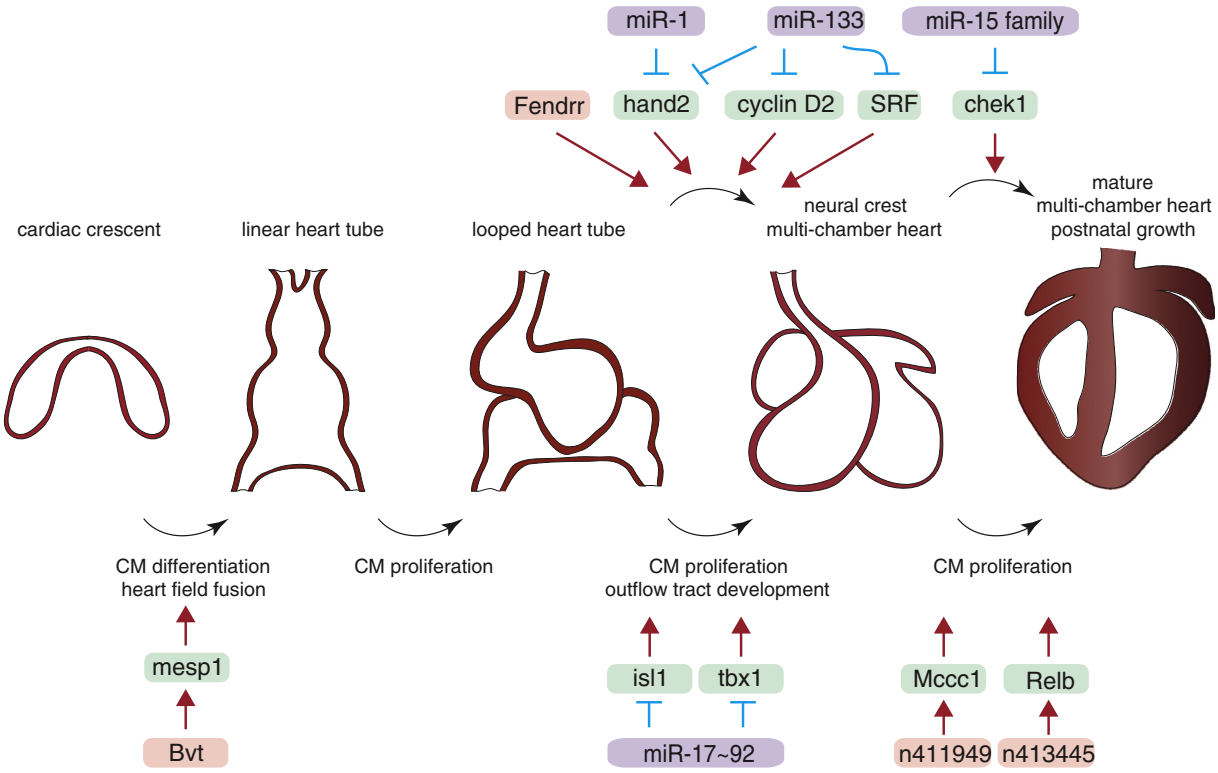

Fig. 1. Non-coding RNAs having a functional role during embryonic development. Already at early developmental stages, lncRNA Braveheart (Bvt) becomes expressed in mouse embryonic stem cells. Bvht is required for expression of core gene regulatory networks involved in defining cardiovascular cell fate and acts upstream of mesoderm posterior (MesP1), a master gene of cardiovascular lineage commitment. After cardiac looping, the miR-1-2/miR-133a-1 cluster becomes expressed, which plays a role in ventricular cardiomyocyte expansion. MiR-1 directly targets Hand2, a bHLH transcription factor involved in ventricular cardiomyocyte expansion, while miR-133 directly regulates expression of serum response factor (SRF) and cyclin D2. The miR-17~92 cluster is involved in myocardial differentiation of cardiac progenitors in the secondary heart field, which is required for normal cardiac outflow tract (OFT) formation by repressing the expression of cardiac progenitor genes Isl1 and Tbx1. Fendrr is specifically expressed in nascent lateral plate mesoderm and is required for proper development of the heart and body wall in the mouse. The lncRNA n413445 regulates Relb mRNA levels, a transcription factor that is an essential component in the NFκB pathway that is important during fetal cardiac growth. Also, increased expression of the lncRNA n411949-regulated mRNA Mccc1 is of importance during embryonic growth. After birth, the miR-15 family becomes important during the neonatal period governing cardiomyocyte cell cycle withdrawal and binucleation by directly targeting chek1.

UTRs. These findings have established critical and redundant roles for miR-133a-1 and miR-133a-2 in orchestrating cardiac development, gene expression, and function [22].

Also in the adult heart, these microRNA clusters fulfill a critical role since altered expression of miR-1 and miR-133 is associated with heart failure both in rodents and humans [23]. Sequestering endogenous miR-133 with a targeted 3′UTR decoy resulted in marked myocyte hypertrophy, significantly increased protein synthesis, increased fetal gene expression, and perinuclear expression of atrial natriuretic factor. In cultured neonatal cardiomyocytes, overexpression of miR-1 or miR-133 inhibited phenylephrine and endothelin-1-induced cardiomyocyte hypertrophy. In line, in vivo inhibition of miR-133, using an antagomir treatment, caused marked cardiac hypertrophy by means of derepressing RhoA, a GDP-GTP exchange protein regulating cardiac hypertrophy, Cdc42, a signal transduction kinase implicated in hypertrophy, and Nelf-A/WHSC2, a nuclear factor involved in cardiogenesis. This significant myocardial hypertrophy was associated with re-induction of the fetal gene program [23]. Cardiac delivery of AAV9-miR-1 in male Sprague–Dawley rats subjected to aortic stenosis, reversed hypertrophic remodeling, reduced cardiac fibrosis and apoptosis and improved calcium handling. One of the validated targets in this study was Fibullin-2, a secreted protein implicated in extracellular matrix remodeling [24]. In line, SERCA2a gene therapy, which improves cardiac function both in animals and humans with heart failure, was shown to restore cardiac miR-1 expression via an Akt/Foxo3-dependent pathway, indicating that miR-1 expression is critical for proper cardiac function [25]. Recently, miR-133 was shown to target multiple components of the β_1AR-signaling cascade, thereby protecting cardiomyocytes from β_1AR-induced apoptosis. Among the direct targets of miR-133 are the β_1-receptor itself and its downstream effectors adenylate cyclase VI (AC_{VI}) and PKA C-β, a key modulator of the β_1AR-mediated accumulation of the second messenger cAMP. Using a cardiac-specific TetON-miR-133 inducible transgenic mouse model, the authors showed that after pressure overload TetON-miR-133 inducible transgenic mice maintained cardiac performance and displayed attenuated apoptosis and decreased fibrosis compared to control mice [26]. Another receptor pathway that is targeted by members of this cluster is the insulin-like growth factor 1 (IGF-1)/insulin-like growth factor 1 receptor signal transduction pathway, a key regulator of cardiac muscle tropism and function. Both IGF-1 and IGF-1 receptor are direct targets of miR-1. Moreover, Foxo3a, a component of the IGF-1 signaling pathway, transcriptionally regulates miR-1 expression levels, providing a feedback loop between miR-1 expression and IGF-1 signaling. In line, miR-1 expression levels in myocardial biopsies from acromegalic patients, where IGF-1 is overproduced after aberrant synthesis of growth hormone, are inversely correlated with cardiac mass and wall thickness [27].

1.1.2. MyomiRs: miR-208a, miR-208b and miR-499

In the rodent heart, α-MyHC (encoded by the *Myh6* gene), a fast ATPase, is highly expressed in adulthood, whereas β-MyHC (encoded by the adjacent *Myh7 gene*), a slow ATPase, is the predominant myosin isoform in cardiomyocytes in the embryonic stage [28]. The relative expression of these two myosin isoforms is correlated with the contractile velocity of cardiac muscle. Several pathologic stimuli can cause a shift in the MyHC composition of the rodent ventricle from α-MyHC to β-MyHC. The opposite holds true in humans, where β-MyHC is the predominant cardiac myosin isoform expressed [29]. Interestingly, these muscle-specific myosin genes harbor a family of microRNAs, called

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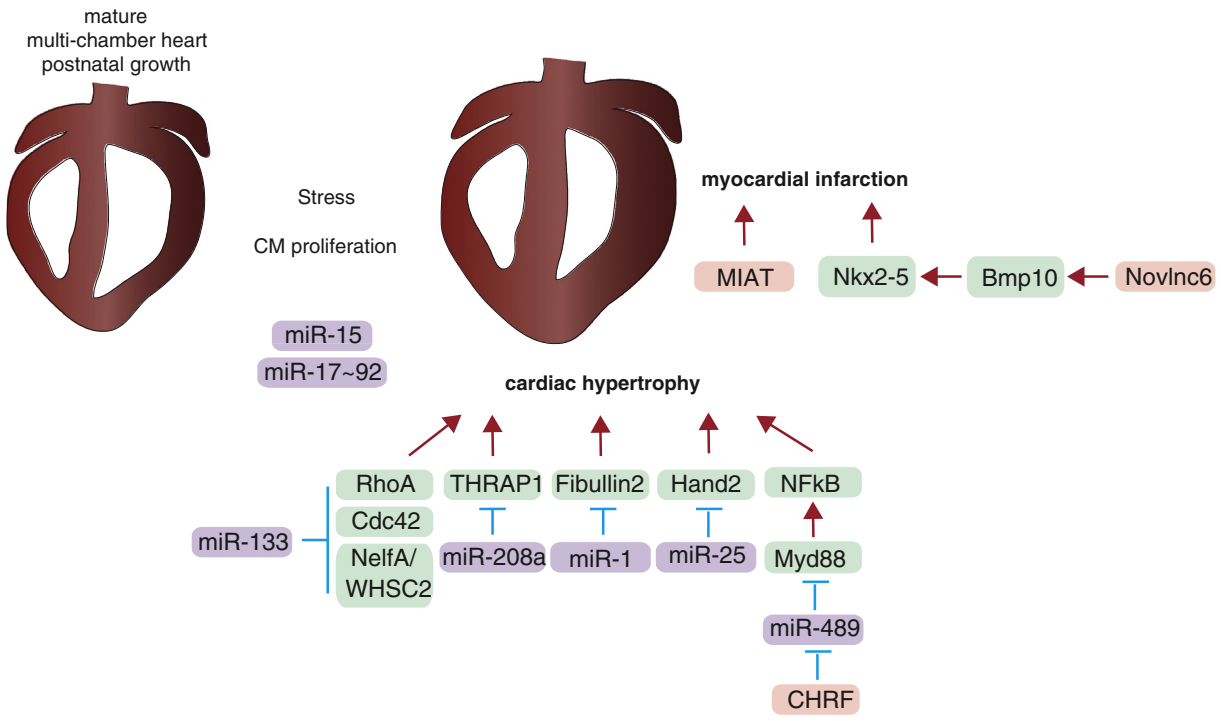

Fig. 2. The role of non-coding RNAs in adult heart disease. During processes leading to adult cardiac hypertrophy, the miR-1–133 cluster becomes downregulated. miR-1 directly targets Fibullin-2, while miR-133 repressing leads to direct derepression of RhoA, a GDP-GTP exchange protein regulating cardiac hypertrophy, Cdc42, a signal transduction kinase implicated in hypertrophy, and Nelf-A/WHSC2, a nuclear factor involved in cardiogenesis. Also, repression of miR-25 by the calcineurin/NFAT pathway leads to derepression of the transcription factor Hand2, leading to pathological cardiac remodeling. In response to cardiac stress stimuli, miR-208a expression leads to the upregulation of β-MyHC expression, thereby inducing myosin isoform switching, which is at least in part due to direct inhibition of the thyroid receptor-associated protein THRAP1, a transcriptional coregulator of the thyroid receptor, leading to increased thyroid hormone signaling. The lncRNA cardiac hypertrophy related factor (CHRF) regulates cardiac hypertrophy by directly binding to miR-489, which on its turn targets myeloid differentiation primary response gene 88 (Myd88). Overexpression of CHRF results in the upregulation of Myd88 expression and the activation of NF-κB signaling, leading to hypertrophy. Furthermore, variants in myocardial infarction associated transcript (MIAT, also known as Gomafu/RNCR2) and Novlinc6 were identified by GWAS as a risk factor for myocardial infarction and cardiac disease. Novlnc6 regulates expression of BMP10, a key signaling ligand for cardiogenesis, which maintains Nkx2.5 expression.

myomiRs, within their introns. MyomiRs consist of miR-208a, miR-208b, and miR-499, encoded within the introns of Myh6, Myh7 and Myh7b genes, respectively. The myomiRs are highly conserved and share similar sequence identity, and the expression of the myomiRs parallels the expression of their respective host genes during development [30]. Targeted deletion of miR-208a resulted in ectopic activation of the fast skeletal muscle gene expression within the heart, and mice were protected against cardiac hypertrophy and myocardial fibrosis. Mice lacking miR-208a fail to upregulate β-MyHC expression in response to cardiac stress stimuli, at least in part due to direct inhibition of the thyroid receptor–associated protein THRAP1, a transcriptional coregulator of the thyroid receptor, leading to increased thyroid hormone signaling, thereby regulating myosin isoform switching. Given that miR-208a null mice are viable, it indicates that miR-208a is not required for development of the heart and embryogenesis [31]. Inhibition of miR-208a in adult mouse hearts by systemic administration of an antimir against miR-208a prevented pathological myosin switches and cardiac remodeling upon stress, resulting in improved cardiac function and survival [32]. Furthermore, miR-208a regulates the expression of the two slow myosins and their intronic microRNAs, Myh7/miR-208b and Myh7b/miR-499, as deletion of miR-208a abolishes the expression of miR-208b and miR-499. MiR-208b and miR-499 display functional

Table 1
MicroRNAs regulating cardiac genes.

microRNA	Target	Function	Reference
miR-1	Hand2	Ventricular cardiomyocyte expansion	[20]
miR-1	Fibullin-2	Extracellular matrix remodeling	[24]
miR-1	IGF-1,	IGF-1 signal transduction, cardiac muscle tropism and function	[27]
miR-133a	IGF-1 receptor	Cardiomyocyte proliferation, ectopic expression of smooth muscle genes in the heart	[22]
miR-133	SRF,	Cardiac hypertrophy	[23]
miR-133	Cyclin D2	Cardiac hypertrophy	[26]
	RhoA	Cardiogenesis	
	Cdc42	β_1AR-signaling, β_1AR-induced	
	Nelf-A/WHSC2	apoptosis	
	β_1-receptor,		
	AC_{VI},		
	PKA C-β		
miR-208a	THRAP1	Thyroid hormone signaling	[31]
miR-17, miR-20a	Isl1, Tbx1	Cardiac progenitors	[34]
miR-25	Hand2	Cardiogenesis (second heart field formation)	[36]
miR-25	SERCA2	Sarcoplasmic reticulum calcium uptake	[37]
miR-195	Check1	Cell cycle checkpoint	[38]

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redundancy, and have crucial roles in specifying muscle fiber identity by activating slow and repressing fast myofiber gene programs [30].

1.1.3. The miR-17~92 cluster

The miR-17~92 cluster consists of six microRNAs: *miR-17*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b*, and *miR-92a*. These six microRNAs belong to four microRNA families and are generated from a common primary transcript. The miR-17~92 cluster was initially reported as a human oncogene, named Oncomir-1 and is located on human chromosome 13. This microRNA cluster has two paralogs, the miR-106b~25 cluster and the miR-106a~363 cluster. The two paralogs are located at different genetic loci, which provide an extra layer of redundancy among the four microRNA families. The miR-106b~25 cluster consists of miR-106b, miR-93 and miR-25 and is located in the 13th intron of the human *MCM7* gene. On the other hand, the miR-106a~363 cluster maps to chromosome X in both humans and mice. The miR-106a~363 and miR-106b~25 clusters contain microRNAs that are highly similar, and in some cases even identical, to those encoded by the miR-17~92 cluster. This indicates that they probably have overlapping functions by regulating a similar set of direct downstream targets [33] and most likely originated from a series of ancient evolutionary genetic duplication events. The broad conservation of sequence across species implies the importance of those microRNAs during vertebrate development [33].

Loss of function studies revealed a critical role for the miR-17~92 cluster in heart development. MiR-17~92 mutants die shortly after birth and display ventricular septal defects and lung hypoplasia. In contrast, loss of the paralogous miR-106a~93 and miR-106b~25 clusters does not affect organismal viability and these mutants do not display an obvious phenotype. This is most likely due to functional redundancy with the miR-17~92 cluster. However, miR-17~92/miR-106b~25 double null embryos and miR-17~92/miR-106b~25/miR-106a~363 triple null embryos die at mid-gestation, before E15, and exhibit a much more severe phenotype compared to embryos lacking miR-17~92 alone. At E13.5 and E14.5 in the developing mouse embryo, miR-17~92/miR-106b~25 double null embryos showed edema and vascular congestion associated with severe cardiac developmental abnormalities including defective ventricular and atrial septation and thinning of the ventricles [33].

Furthermore, the miR-17~92 cluster is involved in myocardial differentiation of cardiac progenitors in the secondary heart field, which is required for normal cardiac outflow tract (OFT) formation. The cardiac OFT is a developmentally complex structure that is often defective in patients with congenital heart disease. The bone morphogenetic proteins Bmp2 and Bmp4, direct OFT myocardial differentiation via regulation of the miR-17~92 cluster. The miR-17~92 cluster in its turn represses the expression of cardiac progenitor genes Isl1 and Tbx1 [34].

Gain-of-function studies have revealed a role for the miR-17~92 cluster in organ size regulation. Global miR-17 overexpression leads to organ growth retardation, including the heart. In contrast, overexpression of the entire miR-17~92 cluster provokes cardiomyocyte hypertrophy and hyperplasia. Recently, transgenic overexpression of the miR-17~92 cluster in cardiomyocytes was shown to be sufficient to induce cardiomyocyte proliferation in embryonic, postnatal, and adult hearts. Moreover, miR-17~92 cluster overexpression in adult cardiomyocytes protects the heart from myocardial infarction-induced injury [35].

Inhibition of miR-25 in vivo by 4 weeks of antagomir treatment in adult mice induced cardiac dilatation accompanied by decreased cardiac function [36]. This phenotype was even more severe in a pressure overload-induced heart failure model, due to the miR-25 mediated derepression of the transcription factor, Hand2. Hand2 is involved in the formation of the second heart field of the developing heart, and becomes re-activated in the adult failing heart by this microRNA mechanism. In line, Hand2 overexpression in mice leads to decreased cardiac function and dilatation, while conditional Hand2 gene deletion proved protective against pressure overload-induced heart failure [36]. A contrasting publication found that inhibition of miR-25 by antagomir treatment in a five-month long pressure overload study in mice increased cardiac function by derepression of SERCA2a. Further research is needed to clarify this contradiction and obtain a more integrative understanding of the functional role of the miR-106b~25 and miR-106a~363 clusters in the adult heart [37].

1.1.4. The miR-15 family

By comparing microRNA expression profiles in mouse cardiac ventricles at P1 and P10, multiple miR-15 family members were identified as regulators of postnatal cardiomyocyte mitotic arrest. The miR-15 family consists of miR-15a, miR-15b, miR-16, miR-195, and miR-497. Family member miR-195 displayed a 6-fold increase in expression at P10 compared to P1. Overexpression of miR-195 in the developing heart using a transgenic mouse model caused congenital heart abnormalities associated with premature cell cycle arrest. The miR-15 family member miR-195 directly targets cell cycle gene checkpoint kinase 1 (Chek1). Inhibition of the miR-15 family in neonatal mice with locked nucleic acid-modified anti-microRNAs was associated with an increased number of mitotic cardiomyocytes and derepression of Chek1. These results suggest that the miR-15 family is important during the neonatal period governing cardiomyocyte cell cycle withdrawal and binucleation [38]. The neonatal mammalian heart still has regenerative capacity after myocardial infarction through proliferation of preexisting cardiomyocytes. The miR-15 family modulates neonatal heart regeneration by inhibiting postnatal cardiomyocyte proliferation. Inhibition of the miR-15 family from an early postnatal age until adulthood increased myocyte proliferation in the adult heart and improved left ventricular systolic function after adult myocardial infarction in mice [39].

1.2. LncRNAs: adding layers of complexity to cardiac gene regulation

Most lncRNAs are RNA polymerase II transcribed, 5′-capped, alternatively spliced and polyadenylated. They are less conserved than microRNAs and only a small subset show evolutionary conservation of the primary sequence. Yet, lncRNAs do show tissue and cell type-specific expression, indicating that their expression must be tightly controlled. LncRNAs are distinguished by a diversity of molecular functions derived from their ability to act as scaffolds for protein–protein interactions and/or chaperones that direct protein complexes to specific RNA or DNA sequences. One of the first identified mechanisms for lncRNA action is imprinting. For imprinted genes, the expression occurs from only one allele, instead of both parental alleles being expressed equally. Secondly, LncRNAs can act both as transcriptional activators and repressors by interacting with epigenetic modifying protein complexes, regulate expression of genes located in close proximity (cis-acting) or target distant transcriptional activators or repressors (trans-acting). LncRNAs can also act as molecular decoys. In this case, the lncRNA can 'sponge' protein-based factors such as transcription factors away from chromatin or sponge tiny RNA species [40]. Finally, lncRNAs can act as molecular guides by directing ribonucleoprotein complexes to specific chromatin targets [41]. LncRNAs may serve as central platforms for assembly of complex protein components. Some lncRNAs possess distinct domains that bind different protein factors, bringing transcriptional activators or repressors together in both time and space (Table 2).

Since many cardiac diseases are heritable, series of genome-wide association studies (GWAS) have been performed to identify new disease loci for cardiovascular diseases. Remarkably, 93% of the identified disease loci fall outside protein-coding regions and could influence the expression or function of non-coding RNAs. In line, single nucleotide polymorphisms (SNPs) associated with cardiac disease have been identified to locate within lncRNA encoding genes [42], suggesting that lncRNAs may have more causal roles in disease, which justifies an in-depth analysis of individual lncRNA function.

The more detailed characterization of individual lncRNAs in development and disease of the cardiovascular system is only starting to emerge. In 2013, a hallmark publication demonstrated that lncRNA Braveheart (Bvht, AK143260) is required for cardiovascular lineage

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Table 2
LncRNAs in cardiac gene regulatory networks.

LncRNA	Target	Function	Reference
Braveheart	Mesp1	Cardiovascular lineage commitment	[42]
Fendrr	PRC2 and TrxG/MLL complexes	Histone modification	[43]
SRA	MyoD	Skeletal myogenesis	[45]
Myheart	Brg1	Chromatin remodeling, α/β-MHC ratio control	[47]
Novlnc6	Bmp10	Cardiogenesis, regulating Nkx2.5 expression	[50]
MIAT	Unknown	Myocardial infarction	[51]
n413445	Relb	Cardiogenesis (transcription factor in NFκB pathway)	[52]
n411949	Mccc1	Sensor of free leucine levels	
CHRF	miR-489	Cardiac hypertrophy (via regulation of Myd88 and NFκB)	[56]

commitment. Bvht is expressed at early developmental stages in mouse embryonic stem cells (mESCs) and is also highly expressed in the adult mouse heart. Depletion of Bvht in mESCs impaired formation of cardiomyocytes in multiple in vitro differentiation assays. Bvht depletion in primary murine neonatal ventricular cardiomyocyte cultures resulted in unevenly distributed and irregularly bundled myofibrils after 5 days in culture compared to control cells and significantly smaller overall cardiomyocyte cell surface area, indicating that neonatal cardiomyocyte structure is disrupted in Bvht-depleted cells. Bvht is required for expression of core gene regulatory networks involved in defining cardiovascular cell fate and acts upstream of mesoderm posterior (MesP1), a master gene of cardiovascular lineage commitment [43]. Furthermore, the authors show that also the downstream targets of MesP1 require upstream Bvht activation, including the core cardiac transcription factors Gata4, Gata6, Hand1, Hand2, Tbx2, and Nkx2.5, among others.

Another lncRNA, Fendrr (Foxf1 adjacent non-coding developmental regulatory RNA; ENSMUSG00000097336), is specifically expressed in nascent lateral plate mesoderm and is required for proper development of the heart and body wall in the mouse. Fendrr-deficient mice die around E13.5 due to abnormal functioning of the heart. Fendrr acts by modifying the chromatin signatures of genes involved in the formation and differentiation of the lateral mesoderm lineage through binding to both the PRC2 and TrxG/MLL complexes [44]. Expression of the transcription factors Nkx2.5 and Gata6 was increased upon cardiac Fendrr deletion, accompanied by corresponding H3K4me3 changes in their promoter regions, while expression of other core transcription factors, such as Gata4 or Tbx5 was not affected.

A third example of lncRNAs involved in cardiac development is the lncRNA SRA. The steroid receptor RNA activator 1 (SRA1) gene generates both steroid receptor RNA activator protein (SRAP) as well as several non-coding SRA transcripts, depending on alternative transcription start site usage and alternative splicing [45]. The lncRNA SRA binds to MyoD, which regulates skeletal myogenesis. In vitro and in vivo experiments showed that SRA is a coactivator of MyoD [46]. SRA is present in a 600-kb region of linkage disequilibrium (LD) associated with human dilated cardiomyopathy on 5q31.2-3, harboring multiple genes, in three independent Caucasian populations. Knockdown of SRA1 by Morpholino antisense in zebrafish resulted in impaired cardiac function phenotypes, with impaired contractility predominantly in ventricular heart chambers at 72 h post fertilization [47].

Recently, a cluster of cardiac-specific lncRNA transcripts from Myh7 loci was identified and named myosin heavy-chain-associated RNA transcripts (Myheart, or Mhrt). Mhrt antagonizes the function of Brg1, a chromatin-remodeling factor that is activated by stress to trigger aberrant gene expression and cardiac myopathy [48]. The ATP-dependent Brg1–HDAC–PARP complex cooperatively controls the change in the α/β-MHC ratio in failing hearts [49]. These lncRNAs, which are splice isoforms of Mhrt, have a tight correlation with the Myh6/Myh7 ratio during cardiac development and in hypertrophic hearts. Cardiac stress in mice induced by pressure overload lead to a progressive loss of Mhrt expression in cardiomyocytes along with the development of pathological hypertrophy. Restoring Mhrt expression to the prestress level attenuated pathological hypertrophic responses and restored cardiac function, demonstrating the cardioprotective role of Mhrt in vivo. Mhrt directly binds to the helicase domain of Brg1, a domain that is crucial for tethering Brg1 to chromatinized DNA targets, enabling a competitive inhibition mechanism by which Mhrt sequesters Brg1 from its genomic DNA targets to prevent chromatin remodeling and gene regulation by Brg1. This negative feedback circuit between a lncRNA and the Brg1 chromatin remodeling complex is thus crucial for heart function [48].

Likewise, lncRNA profiling of the cardiac transcriptome after myocardial infarction was performed to identify novel heart-specific lncRNAs with potential roles in both cardiac development and pathological cardiac remodeling. These novel lncRNAs also have human orthologues, which are dysregulated during disease. The novel lncRNA Novlnc6 was significantly downregulated in human dilated cardiomyopathy. Novlnc6 is associated with a bonafide cardiac developmental enhancer and modulates the expression of Nkx2.5, a master cardiac transcription factor, critical for the modulation of gene programs involved in cardiogenic differentiation, maturation, and homeostasis. Furthermore, knockdown of Novlnc6 by GapmeRs in cardiomyocytes resulted in concomitant downregulation of BMP10, a key signaling ligand for cardiogenesis, which maintains Nkx2.5 expression [50].

Additionally, variants in myocardial infarction associated transcript (MIAT, also known as Gomafu/RNCR2) were identified by GWAS as a risk factor for myocardial infarction. Six single nucleotide polymorphisms (SNPs) in MIAT showed markedly significant association with myocardial infarction [51]. To date, the molecular mechanism by which MIAT causes cardiac disease is still unknown.

By RNA sequencing of embryonic (E14), normal adult and hypertrophied adult hearts, Matkovich et al. identified 157 lncRNAs that were differentially expressed in embryonic hearts compared with adults, but relatively few fetal lncRNAs that showed altered expression in adult-onset cardiac hypertrophy. Only 17 lncRNAs were differentially expressed in hypertrophied hearts, 13 of which observed in embryonic hearts. By analyzing neighboring mRNAs within 10 kb of dynamically expressed lncRNAs, the authors revealed that 22 mRNAs were concordantly and 11 were reciprocally regulated. Using lncRNA knockdown in C2C12 myoblasts, the functional reciprocal interactions between mRNAs Mccc1 and Relb, and lncRNAs n411949 and n413445, respectively, were validated. LncRNA n413445 and n411949 are highly expressed in the embryonic heart but show quite low expression in adult hearts, with no apparent change in the pressure overloaded heart. The lncRNA n413445 regulates Relb mRNA levels, a transcription factor that is an essential component in the NFκB pathway that is important during fetal cardiac growth. An increase of the lncRNA n411949-regulated mRNA Mccc1 during embryonic growth may be of key importance for sensing free leucine levels and thus the availability of branched-chain amino acids for anabolic signaling in muscle [52].

In order to determine the expression profile of myocardial lncRNAs and their potential role in early stage reperfusion, Liu and colleagues performed microarray analysis and validated the results using polymerase chain reaction (PCR). They identified 64 lncRNAs that were up-regulated and 87 that were down-regulated, while 50 mRNAs were up-regulated and 60 down-regulated in infarct region at all reperfusion

Please cite this article as: Philippen LE, et al, Non-coding RNA in control of gene regulatory programs in cardiac development and disease, J Mol Cell Cardiol (2015), http://dx.doi.org/10.1016/j.yjmcc.2015.03.014

sampled. Target gene-related pathway analysis showed significant changes in cytokine–cytokine receptor interaction, the chemokine signaling pathway and nucleotide oligomerization domain (NOD)-like receptor signaling pathway which have a close relationship with myocardial ischemia/reperfusion injury (MI/RI). The co-expressed network of 10 highly-dysregulated lncRNAs showed that ENSMUST00000166777 and AK156124 are in closest relation with coding RNAs (mRNAs). However, ENSMUST00000170410 and uc007prv.1 have the slightest relevance to coding RNAs (mRNAs). Six mRNAs (CXCL1, CCL9, CXCL12, EDA, TNFAIP3 and BIRC3) were targeted by corresponding lncRNAs, all of which have been linked to ischemia/reperfusion injury [53].

Deep sequencing of RNA isolated from paired nonischemic (NICM; $n = 8$) and ischemic (ICM; $n = 8$) human failing LV samples collected before and after LVAD and from nonfailing human LV ($n = 8$) revealed high abundance of mRNA (37%) and lncRNA (71%) of mitochondrial origin. The analysis identified 18,480 lncRNAs in human LV. Among the 679 (ICM) and 570 (NICM) lncRNAs differentially expressed with heart failure, ≈10% are improved or normalized with LVAD. Furthermore, the expression signature of lncRNAs, but not microRNAs or mRNAs, distinguished ICM from NICM in this study, suggesting a predominant role for lncRNAs in the pathogenesis of heart failure and in reverse remodeling by mechanical support [54].

Recently, it was shown that the lncRNA cardiac hypertrophy related factor (CHRF) regulates cardiac hypertrophy by directly binding to miR-489, acting as a functional sponge to regulate anti-hypertrophic miR-489 expression and activity. The responsible downstream target of miR-489 appeared to be myeloid differentiation primary response gene 88 (Myd88). The overexpression of CHRF resulted in the upregulation of Myd88 expression and the activation of NF-κB signaling, leading to hypertrophy [55].

2. Conclusions and perspectives

Studies on two major species of ncRNAs, microRNAs and lncRNAs, have changed our understanding about the epigenetic control of gene regulatory programs and reveal a vast overlap between the regulation of gene programs in the developing heart and adult-onset heart disease. As such, the emerging picture also demonstrates that the heart seems sensitive to relatively subtle changes in gene modifier effects. The ability of microRNAs to fine-tune gene expression programs and act as powerful stress regulators suggests their central role in many facets of cardiac biology. Since one single microRNA could possibly modulate dozens of target genes at once, and one gene could be targeted by multiple microRNAs, methods aimed to better understand the integration of microRNA control within gene regulatory networks are clearly necessary for the field. Our collective understanding of how these tiny post-transcriptional gene regulators function in cellular networks provides new molecular horizons for cures or therapies to a variety of human heart diseases, and the first examples of microRNA-based proof-of-concept therapies in small and larger animal models are well underway. Manipulation of individual microRNAs by chemically modified oligonucleotides or adeno-associated viral vectors have therapeutic potential in animal models for cardiovascular diseases. A major translational challenge will remain in understanding the pharmacology/toxicology aspects, correct dosing and correct delivery to the site of action of these fundamentally new therapeutic entities. As for lncRNAs, literally thousands of putative lncRNAs have been identified in mammalian species including humans, but only dozens have been experimentally studied in sufficient detail so far. The relatively poor evolutionary conservation of lncRNAs still poses a barrier to translate their function from animal models to humans. Likely, the identification of evolutionary conserved functional domains in lncRNAs will provide relief to latter obstacle and possibly allow therapeutic manipulation by antisense oligonucleotide-based cardiac therapy. Eventually, the detailed characterization of ncRNAs with essential roles in the developing or the diseased heart will undoubtedly lead to a richer understanding of the complex gene regulatory networks that drive proper cardiac development and maintain healthy myocardial function.

Conflict of interest disclosures

L.E.P.: none. E.D.: none. P.D.C.M.: none. L.J.D.W.: none.

Acknowledgments

E.D. was supported by a Long Term Fellowship of the EMBO Organization (ALTF 848-2013), Marie Curie actions Intra-European fellowship (PIEF-GA-2013-627539) and VENI fellowship (016.156.016) from the Netherlands Organization for Health Research and Development (ZonMW). P.D.C.M. was supported by a Leducq Career Development Award, the Dutch Heart Foundation grant NHS2010B261 and a NWO MEERVOUD grant. L.D.W. acknowledges support from the *Netherlands CardioVascular Research Initiative*: the Dutch Heart Foundation, Dutch Federation of University Medical Centers, the Netherlands Organization for Health Research and Development (ZonMW) and the Royal Netherlands Academy of Sciences. L.D.W. was further supported by grant 311549 from the European Research Council (ERC).

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