microRNAs in cardiac development and regeneration

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Abstract
Heart development involves the precise orchestration of gene expression during cardiac differentiation and morphogenesis by evolutionarily conserved regulatory networks. miRNAs (microRNAs) play important roles in the post-transcriptional regulation of gene expression, and recent studies have established critical functions for these tiny RNAs in almost every facet of cardiac development and disease. The realization that miRNAs are amenable to therapeutic manipulation has also generated considerable interest in the potential of miRNA-based drugs for the treatment of a number of human diseases, including cardiovascular disease. In the present review, I discuss well-established and emerging roles of miRNAs in cardiac development, their relevance to congenital heart disease and unresolved questions in the field for future investigation, as well as emerging therapeutic possibilities for cardiac regeneration.

Key words: cardiac regeneration, cardiovascular development, cardiovascular disease, congenital heart disease, microRNA, non-coding RNA

INTRODUCTION
The heart is the first organ to form during mammalian embryogenesis and its uninterrupted development and function are integral to organismal survival. Heart development begins when a population of mesodermal stem cells commits to the cardiogenic fate and eventually migrates and fuses to form the linear heart tube [1]. Rhythmic contractions begin shortly thereafter. The developing heart subsequently undergoes a series of transformations, including looping morphogenesis, septation, chamber specification and cardiac valve formation, to form the multi-chambered heart. A wave of cardiomyocyte proliferation subsequently ensures rapid growth of the heart prior to birth. Shortly after birth, the majority of mammalian cardiomyocytes withdraw from the cell cycle and rapid post-natal heart growth is achieved predominantly through cellular hypertrophy [2].

At the molecular level, complex gene regulatory networks involving a number of transcription factors, transcriptional co-activators and repressors, their corresponding enhancer and promoter elements and chromatin-modifying enzymes coordinate heart development. The transcriptional regulation of heart development is highly conserved across species and heterozygous mutations in a number of cardiac-expressed transcription factors underlie several human cardiac malformations [3]. The recent identification of profound and unexpected roles for a new class of genes encoding non-protein-coding RNA, known as miRNAs (microRNAs), has added a new layer of regulatory complexity to the gene networks that govern heart development and disease. miRNAs can act as context-dependent ‘rheostats’ or ‘molecular switches’ of gene expression and are often intertwined in complex cardiac signalling and transcriptional circuits, where they have been found to modulate almost every facet of cardiac biology [4]. In the present review, I will provide an overview of recent studies highlighting the important roles of miRNAs in cardiac development, as well as emerging prospects for their therapeutic application in the context of regenerative medicine.
miRNA DISCOVERY, BIOGENESIS AND FUNCTION

miRNAs are a class of evolutionarily conserved small (20–26 nucleotides in length) non-protein-coding RNAs that negatively regulate gene expression by affecting mRNA stability and/or translation [5]. miRNAs were initially discovered in Caenorhabditis elegans in the early 1990s, where the lin-4 gene was found to encode a small RNA responsible for the regulation of developmental timing in nematodes through suppression of LIN-14 protein expression [6,7]. The evolutionary conservation of miRNAs across all eukaryotes, including vertebrates, was not appreciated for almost a decade after their initial discovery in nematodes [8,9]. However, it is now recognized that the human genome contains greater than 1000 miRNAs and it is predicted that more than one third of all protein-coding genes in the mammalian genome are regulated by at least one miRNA [10]. One of the most exciting recent developments has been the realization that miRNAs can be therapeutically manipulated, and Santaris Pharma launched the first in-human clinical trial of a miRNA therapeutic for hepatitis C virus in 2010, which has since successfully progressed to Phase IIb [11]. The results of these clinical trials are eagerly awaited by the miRNA community and may usher in a new era of RNA-based therapeutics for the treatment of a range of human diseases on the backing of exciting results for many miRNA-based drugs in pre-clinical animal models.

miRNAs are transcribed by RNA polymerase II as long precursor RNAs called pri-miRNAs (primary miRNAs), which are sequentially processed by enzymes in the nucleus and cytoplasm to generate mature miRNAs of ~22 nucleotides in length. The pri-miRNAs are cleaved in the nucleus by the RNase III endonuclease Drosha, which associates with the double-stranded RNA-binding protein DGC8 (DiGeorge syndrome critical region 8) and other cofactors, to produce ~70-nucleotide-long pre-miRNAs (precursor-miRNAs) [5]. In some cases, specific miRNAs, known as mirtrons, can bypass Drosha processing and the pre-miRNA in this case is generated directly through splicing of intronic sequences [12]. The pre-miRNAs have a characteristic hairpin structure and are exported from the nucleus to the cytoplasm by exportin-5, where Dicer, another RNase III endonuclease, subsequently cleaves the hairpin to release a ~22 nucleotide miRNA duplex [5]. One strand of the miRNA duplex becomes the mature miRNA and the other strand is typically, but not always, rapidly degraded [13]. The mature single-stranded miRNA is then loaded into RISC (RNA-induced silencing complex), where a multi-protein complex consisting of Argonaute proteins facilitates interactions between the miRNA and target mRNAs [5]. The miRNA interaction with the target transcript is mediated by Watson–Crick base pairing with sequences that are canonically located in the 3′-UTR (untranslated region) of target mRNAs, although there is a growing list of examples of miRNA interactions with 5′-UTRs, protein-coding sequences and introns [14]. Association of the miRNA with its mRNA target typically results in mRNA degradation and/or translational inhibition [5], but some rare examples of translational activation have also been reported [15]. Current evidence suggests that the primary mechanism for miRNA-mediated target gene repression is via mRNA decay rather than translational repression [16].

The primary determinant of miRNA target recognition is the pairing of nucleotides 2–7 at the 5′ end of the mature miRNA (known as the ‘seed’ region) and the target mRNA [5]. miRNA ‘families’ are classified on the basis of a common ‘seed’ sequence that is shared between all family members and they are believed to have largely redundant functions. However, the other regions of the miRNA are also important for specificity and stabilization of miRNA–mRNA binding [17]. Current estimates from biochemical miRNA target identification studies [e.g. HITS-CLIP (high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation)] suggest that a single miRNA can interact with hundreds of target mRNAs in a cell, although target gene regulation is typically modest with, on average, only 20–50% changes in mRNA expression levels detected for most target transcripts [16,18]. Proteomic analyses similarly indicate that most targets are modestly repressed (<2-fold) by miRNAs [19,20]. These results indicate that miRNAs probably function through the modest repression of a number of mRNA targets, which act in concert to mediate the biological actions of miRNAs. In addition to miRNA ‘seed’ redundancy, the promiscuity of miRNA interactions with target transcripts and the targeting of individual transcripts by several different miRNAs [4], as well as the potential for generation of multiple mature miRNAs from the same miRNA precursor (isomirs) [21], allows for enormous regulatory complexity and functional redundancy. Indeed, there is immense sequence diversity among miRNAs and a large proportion of cardiac-expressed miRNAs, including miR-133, have prevalent 5′ isomirs, which can affect miRNA targeting [21]. In light of these findings, it is not entirely surprising that only 10% of genetic miRNA deletions in C. elegans have revealed grossly abnormal phenotypes in mutant animals [22]. Similarly, genetic deletions of miRNAs in mice have, in most cases, failed to reveal striking functions during development or under normal homeostatic conditions in adulthood [23]. However, major functions are often unmasked in response to specific injury or stress conditions [24]. It is unclear why compensatory mechanisms can overcome the effects of loss-of-function for most of the miRNAs tested so far during development, but not in response to specific stress signals. Nevertheless, critical roles for a number of cardiac miRNAs have emerged and their roles in heart development will be discussed in the following sections.

miRNAs AND CARDIAC DEVELOPMENT

miRNAs are essential for heart development: lessons from Dicer- and DGC8-knockout studies

Compelling evidence for a critical role of miRNAs during cardiac development has been garnered from loss-of-function mutations of the miRNA-processing enzymes Dicer and Drosha/DGC8. Although global deletion of miRNA-processing enzymes causes lethality during early gestation [25], conditional knockout of these enzymes in specific cell lineages has highlighted essential roles during heart development. Conditional deletion of Dicer...
using Cre recombinase under the control of the endogenous Nkx2.5 (Nk2 homeobox 5) regulatory region (Nkx2.5–Cre) abolished the processing of pre-miRNAs into their mature form in early cardiac progenitors at E8.5 (embryonic day 8.5) [26]. Early deletion of Dicer in cardiac progenitors led to embryonic lethality resulting from cardiac failure at E12.5 [26]. The ventricular myocardium of Nkx2.5–Cre-knockout mice was poorly developed and the heart exhibited signs of pericardial oedema. However, most markers of initial cardiac differentiation and patterning, such as Tbx5 (T-box 5), Hand (heart and neural crest derivatives expressed) 1, Hand2 and Mlec2v, were expressed normally [26]. Using a similar approach, but employing an alternative Nkx2.5–Cre line with slightly different spatiotemporal kinetics [27], it was demonstrated further that miRNAs are necessary for cardiac outflow tract alignment and chamber septation [28]. Interestingly, Dicer deletion was associated with a reduced number of apoptotic cells in the outflow tract at E13.5 compared with wild-type controls [28]. Although both Nkx2.5–Cre Dicer mutant strains died in utero, the extended survival period of the cardiac Dicer-deficient mice generated by Tabin’s group [28] allowed for a longer time window for the visualization of the effects of miRNA on outflow tract morphogenesis and chamber septation. The different phenotypes of these two Nkx2.5–Cre Dicer mutants are most likely due to background strain differences and disparities in the timing of transgene expression between the two Nkx2.5–Cre constructs.

The development of the outflow tract involves the co-ordinated regulation of cell proliferation, differentiation, migration and apoptosis of multiple cell types, including those derived from the neural crest. Neural crest cells are required for proper development of craniofacial structures, as well as development of the neural crest. Neural crest cells are required for proper development of craniofacial and cardiovascular structures, including VSD (ventricular septal defect), double outlet right ventricle and type B interrupted aortic arch [30–32]. Although miRNAs were shown to be dispensable for the survival of cardiac neural crest cells, they were required for migration and patterning of these cells throughout the outflow tract [30]. Although the effects of Dicer deletion in cardiac neural crest were postulated to be due to miR-21- and miR-181a-dependent regulation of ERK (extracellular-signal-regulated kinase) 1/2 signalling [30], it should be noted that miR-21-knockout mice do not display any overt developmental phenotype [33]. Further studies employing combinatorial deletion of miR-21 and miR-181a in neural crest cells will be required in order to establish further the relative importance of these individual miRNAs in developing neural crest cells.

miRNAs are also essential for the maintenance of postnatal heart function. Deletion of Dicer in cardiomyocytes using an αMHC (α-myosin heavy chain) promoter-driven Cre recombinase (αMHC–Cre) resulted in the death of mutant mice between post-natal days 0 and 4 [34]. Post-natal lethality in Dicer-mutant mice was associated with dilated cardiomyopathy and heart failure. Interestingly, αMHC–Cre Dicer-mutant hearts displayed decreased expression levels of mature miRNAs as early as E14.5, but, in contrast with Nkx2.5–Cre-mutant mice, αMHC–Cre-mutants were born at expected Mendelian frequencies [34]. αMHC–Cre-mediated Dicer deletion did not affect cell number or cardiomyocyte size at P0 (post-natal day 0), but was associated with depressed cardiac contractility, which was accompanied by misexpression of cardiac contractile proteins and profound sarcomere disarray [34]. Further support for a critical role of miRNAs during neonatal heart development comes from genetic deletion of Dgcr8 using a MCK (muscle creatine kinase)-promoter-driven Cre recombinase (MCK–Cre), which is activated around birth with cardiac transgene activity declining by P10 [35]. Similar to Dicer-mutant hearts, disruption of Dgcr8 in neonatal cardiomyocytes resulted in depressed cardiac contractility and conduction abnormalities with progression to dilated cardiomyopathy [35]. Premature death occurred within 2 months after birth [35]. Collectively, these studies have established that canonical miRNA biogenesis is essential for the maintenance of normal cardiac function and animal survival during neonatal life.

The miRNA processing machinery has also been disrupted in cardiomyocytes at several different post-natal ages using a tamoxifen-inducible αMHC–Cre line. Deletion of Dicer in juvenile (3-week-old) hearts resulted in mild ventricular remodelling, dramatic atrial enlargement, selective re-activation of fetal cardiac genes and sudden cardiac death within 1 week after the start of tamoxifen treatment [36]. In contrast, cardiac-restricted Dicer deletion in adult (8-week-old) mice was accompanied by dramatic ventricular hypertrophy, myofibre disarray, cardiac fibrosis and marked induction of fetal cardiac genes [36]. Similar results were obtained in a separate study, which also reported pathological ventricular remodelling and contractile dysfunction following tamoxifen-inducible Dicer deletion in 6-week-old adult mice [37]. Therefore miRNAs are not only required for normal embryonic and neonatal heart development, but are also crucial for cardiac growth and maintenance of cardiac function in juvenile and adult stages.

The development of the vasculature is also under miRNA control. Deletion of Dicer in vascular smooth muscle using SM22α (smooth muscle cell–specific protein of 22 kDa)–Cre resulted in embryonic lethality around E16–E17 [38]. SM22α–Cre mutants exhibited thinner blood vessel walls, and reduced smooth muscle cell proliferation and differentiation, as well as impaired vascular contractility and haemorrhaging. Deletion of Dicer in endothelial cells using Tie2 (tunica internal endothelial cell kinase 2)–Cre or inducible VE-cadherin–Cre has also established essential roles for miRNAs in post-natal angiogenesis [39]. Furthermore, Dicer deletion in the developing epicardium using Gata5 (GATA-binding protein 5)–Cre has identified a crucial role for miRNAs in coronary vessel development [40]. Gata5–Cre-mutant mice die shortly after birth and display defects in epicardial epithelial-to-mesenchymal transition, proliferation and differentiation into coronary vascular smooth muscle cells [40]. Taken together, these findings have clearly established important roles for Dicer, Drosha/Dgcr8 and, by inference, miRNAs in cardiovascular development. However, it is unclear whether the phenotypes resulting from Dicer and Dgcr8 deletion reflect the critical importance of specific miRNAs in cardiac developmental processes or whether they are the consequence of disruption of multiple miRNAs with overlapping roles in multiple essential processes. Manipulation of individual miRNAs allows for
Essential roles of the miR-1/miR-133 bicistronic cluster during heart development

miR-1 is the most abundant miRNA in the mammalian heart, accounting for almost 40% of all miRNAs in the adult murine heart [35]. miR-1 is highly conserved from fruit flies to humans and it was the first miRNA to be implicated in heart development. In vertebrates, the genes encoding miR-1 and miR-133 family members are clustered and generated from common bicistronic transcripts. The miR-1-1/miR-133a-2 cluster is located in an intergenic region on human chromosome 20, whereas the miR-1-2/miR-133a-1 cluster is positioned in the antisense orientation within an intron of the Mib1 gene on human chromosome 18. miR-1 and miR-133a are expressed in both heart and skeletal muscle, whereas the closely related miR-206/miR-133b cluster is exclusively expressed in skeletal muscle [26,43,44].

The miR-1/miR-133a clusters are embedded within conserved gene regulatory networks involving interactions between a number of transcription factors, as well as upstream and intragenic enhancers. The miR-1/miR-133a clusters are regulated by several important myogenic transcription factors including SRF (serum response factor), Mef2 (myocyte-enhancer factor 2), MyoD (myogenic differentiation factor D) and Nkx2.5 [26,45,46]. Deletion analysis of miR-1 enhancers in mice revealed that SRF is required for cardiac expression of miR-1 [26], whereas Mef2 is necessary for expression of miR-1 and miR-133a in skeletal muscle [26,45]. SRF also regulates cardiac expression of miR-1 in Drosophila [47] and Mef2 co-operates with Twist in flies to control the expression of miR-1 in skeletal muscle [48]. Studies in fruit flies have also uncovered a regulatory loop involving cooperation between miR-1, the Rho-GTPase Cdc42 (cell division...
cycle 42) and the Nkx2.5 orthologue tinman [46]. In the fly heart, tinman negatively regulates miR-1 expression and miR-1 itself directly targets Cdc42 for translational repression. The genetic interaction between Cdc42 and Nkx2.5, as well as the negative regulation of miR-1 by Nkx2.5, is also conserved in the mouse heart [46]. The highly conserved transcriptional regulation of miR-1 and miR-133 by myogenic transcription factors suggests that there has been strong evolutionary pressure to maintain expression of these miRNAs during heart development.

Gain- and loss-of-function experiments in the mouse, zebrafish and fruit fly have established critical roles for the miR-1 and miR-133 families during heart development. Genetic loss of miR-1 in Drosophila (dmiR-1) causes embryonic lethality in the larval stages after hatching and is associated with a spectrum of muscle defects [47,48]. Nearly half of all dmiR-1-mutant flies displayed severe defects in sarcomeric gene expression, indicating a late requirement for miR-1 in muscle differentiation [47]. Furthermore, the most severely affected embryos displayed abnormal cardiac muscle patterning, and cardiac progenitors were often arrested in a proliferative state and failed to terminally differentiate [47]. The Notch ligand Delta, a known regulator of asymmetric cell division of muscle progenitors, was shown to be a direct target of miR-1 in vivo, providing a plausible mechanism by which miR-1 influences cardiac progenitor differentiation [47]. A more recent genome-wide forward genetic screen in Drosophila has revealed novel signalling pathways and functions for dmiR-1, including a previously unrecognized function for this miRNA in regulating cardiac progenitor cell polarity [49]. The transcription factor kayak, the Drosophila orthologue of mammalian c-Fos, was identified as a direct target of dmiR-1, and c-Fos was similarly dysregulated upon miR-1 gain- and loss-of-function in the mouse heart following TAB (thoracic aortic banding) [49]. Although the significance of miR-1-dependent alterations in cardiac progenitor cell polarity for mammalian heart development is currently unclear, these findings may shed light on the mechanistic basis for the spectrum of cardiac developmental phenotypes observed in miR-1-knockout mice.

Overexpression of miR-1 under the control of the βMHC (β myosin heavy chain) promoter disrupted embryonic mouse heart development and was associated with thin-walled ventricles, heart failure and lethality at E13.5 [26], miR-1 overexpression caused a reduction in the number of proliferating cardiomyocytes without affecting the number of apoptotic cells. Inhibition of cardiogenesis by miR-1 overexpression was proposed to be mediated, at least in part, through translational inhibition of the basic helix–loop–helix transcription factor Hand2 [26], which is required for ventricular cardiomyocyte proliferation during the early stages of cardiac development [50,51]. Interestingly, despite strong evolutionary conservation of both miR-1 and Hand from fruit flies to humans, the Drosophila orthologue of mammalian Hand does not contain any miR-1-binding sites, suggesting that the miR-1/Hand2 regulatory axis appeared later in evolution.

Genetic deletion of miR-1-2 provided the first demonstration of an essential role for an individual miRNA during cardiogenesis. Homozygous deletion of miR-1-2 in mice causes lethality, with incomplete penetrance, between E15.5 and birth due to VSDs [52]. Consistent with previous gain-of-function experiments, which proposed a role for miR-1 as a negative regulator of cardiomyocyte proliferation via translational repression of Hand2, miR-1-2-knockout mice have an increased number of mitotic cardiomyocytes and higher expression levels of Hand2 protein [52]. The miR-1-1-2-homozygous mice that survive to adulthood display a spectrum of cardiac phenotypes including conduction abnormalities and ~15% of miR-1-1-2-knockout mice develop dilated cardiomyopathy and die by 2–3 months of age. Cardiac electrophysiological defects in miR-1-2 mutants appear to be due to de-repression of the miR-1 target Irx5 (iroquois homeobox 5), a homeodomain transcription factor that regulates Kcnd2 (encoding potassium voltage-gated channel, Shal-related subfamily, member 2), which is involved in cardiac repolarization [52]. Although the underlying basis for the incomplete penetrance of miR-1-2 mutant phenotypes is currently unclear, this could reflect a potential role for additional epigenetic modifiers, which may be able to compensate for miR-1-1-2 loss-of-function during heart development. Alternatively, the phenotypic heterogeneity of miR-1-2-mutant mice could reflect intrinsic differences in genetic background, as it is unclear whether these mice were inbred to homozygosity on a pure genetic background. Nevertheless, the profound cardiac abnormalities observed in a subset of miR-1-2 mutants, in which miR-1-1 was unaffected, suggests that heart development is particularly sensitive to the dosage of this miRNA. Future studies employing compound deletions of miR-1-1 and miR-1-2 will allow for a more comprehensive understanding of the functions of miR-1 during cardiogenesis.

In contrast with the sensitivity of miR-1-knockout mice to haploinsufficiency, mice lacking either miR-133a-1 or miR-133a-2 do not display any overt developmental phenotype [53]. However, deletion of both miR-133a genes causes lethal VSDs in approximately half of double-mutant embryos or neonates [53]. The miR-133a double mutants that survive to adulthood eventually succumb to dilated cardiomyopathy by 5–6 months of age and at least half of these mice suffer from sudden cardiac death. As with miR-1 mutants, the precise basis for the incomplete penetrance of miR-133a-double-mutant phenotypes is unclear, but may be related to underlying genetic or epigenetic modifiers that influence stochastic variations in gene expression during critical windows of cardiac development.

miR-133a-double-mutant hearts are characterized by increased cardiomyocyte proliferation and apoptosis, profound sarcomere disarray and cardiac fibrosis [53]. Consistent with increased cardiomyocyte proliferation and sarcomere disarray, absence of miR-133a expression is associated with aberrant expression of muscle contractile genes, ectopic expression of smooth muscle genes in the heart and increased expression of cell-cycle-related genes. These effects 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’-UTRs [53]. The potent ability of miR-133a to regulate cardiomyocyte proliferation was established further by gain-of-function studies where overexpression of miR-133a in the embryonic heart under the control of the βMHC promoter led to VSDs, ventricular thinning and a diminution of cardiomyocyte proliferation [53]. Taken together, these findings have established critical roles for miR-133a in the regulation of proliferative and sarcomeric gene expression.
during cardiac development and suggest that miR-133a plays an important role in guiding cardiomyocyte maturation and proliferative arrest during late embryonic stages.

Interestingly, there appears to be significant overlap between the functions of miR-1 and miR-133a during cardiac development, despite the fact that these miRNAs have different `seed' regions and presumably regulate a distinct repertoire of target genes. A previous affinity-purification study suggests that some miR-1/miR-133 target genes may contain binding sites for both miRNAs and Hand2, for example, is regulated in vivo by both miR-1 and miR-133 in the mouse heart [54]. Similarly, it was demonstrated previously that ~54% of up-regulated transcripts in zebrafish muscle Dicer mutants contain 7–8mer binding sites for miR-1 and miR-133 [55]. Furthermore, loss of both miR-1 and miR-133 by morpholino knockdown in zebrafish results in a more severe sarcomere disorganization phenotype compared with loss of either miRNA alone [55]. It therefore appears that miR-1 and miR-133 have been co-opted into bicistronic clusters during evolution where they act in concert to shape cardiac contractile and proliferative gene expression during development. However, the functions of miR-1 and miR-133 may not be entirely overlapping. Although expression of miR-1 and miR-133 in embryonic stem cells suppresses their differentiation into endodermal or ectodermal lineages and promotes myogenic differentiation in vitro, miR-1 and miR-133 play opposing roles in this process [56]. Further studies employing compound loss-of-function of both miR-1 and miR-133 in the mouse heart will be required to further establish the overlapping functions of this bicistronic miRNA cluster during mammalian cardiacogenesis and whether or not they have any role during gastrulation and early cardiac differentiation in vivo.

The myomiR (muscle-specific miRNA) regulatory network: an intronic miRNA signalling system for the regulation of myosin expression

The expression of muscle contractile proteins is tightly regulated during heart development and dysregulation of muscle myosin genes is a hallmark feature of pathological cardiac remodelling. In rodent, αMHC/Myh6, a fast ATPase, is highly expressed in the adult heart, whereas βMHC/Myh7, a slow ATPase, is the predominant myosin isoform in cardiomyocytes prior to birth [57]. In contrast, βMHC is the predominant cardiac myosin isoform expressed in large mammals including humans [58], indicating that there are important species differences in the regulatory mechanisms governing myosin isoform expression. In response to cardiac stress and hypothyroidism in both humans and rodents, the heart modulates MHC isoform content, resulting in an up-regulation of βMHC and a down-regulation of αMHC, which subsequently compromises cardiac contractility and function [59]. One of the most interesting and surprising discoveries in recent years has been the finding that these muscle-specific myosin genes are under the control of a family of intronic miRNAs, known as myomiRs.

The myomiRs consist of miR-208a, miR-208b and miR-499, which are embedded within the introns of Myh6, Myh7 and Myh7b respectively. The expression of the myomiRs parallels the expression of their respective host genes during development, with miR-208a/Myh7 expression levels declining rapidly after birth, whereas miR-499/Myh6 and miR-208b/Myh7b expression levels are relatively low during embryogenesis and increase postnatally [60–62]. The myomiRs do not exist in invertebrates but are highly conserved in terms of both sequence identity and genomic organization from fish to humans. miR-208a and miR-208b have identical ‘seed’ sequences and miR-499 is highly similar to miR-208 family members, with six overlapping nucleotides in the ‘seed’ region, suggesting that the myomiRs probably share common target genes and functions. These observations also imply that there has been strong selective pressure to maintain these miRNAs within myosin gene regulatory networks throughout vertebrate evolution.

Genetic loss-of-function studies in mice have revealed that miR-208a is not essential for cardiac development. miR-208a mutants are viable, fertile, have normal cardiac contractile function under baseline conditions and do not display any overt signs of cardiac pathology up to 20 weeks of age [62]. However, cardiac function in miR-208a mutants progressively declines with age (>6 months) owing to abnormalities in sarcomere structure [62]. Although these results suggest that miR-208a is dispensable for embryonic cardiac development, another study has revealed striking cardiac conduction abnormalities in 4-month-old miR-208a mutant mice, which completely lacked P waves preceding QRS complexes, indicative of atrial fibrillation [61]. Consistent with the effects of miR-208a loss-of-function, transgenic overexpression of miR-208a in the mouse heart caused a prolongation of the PR interval, indicative of arrhythmias associated with first-degree AV block [61]. The effects of miR-208a on cardiac conduction were proposed to occur through interactions with a number of target genes including the transcription factors, Hop (homeodomain-only protein) and GATA4, as well as the gap junction protein connexin-40 [61]. In contrast, acute administration of miR-208a anti-miRs in adult mice was not sufficient to induce any obvious electrophysiological abnormalities [63], suggesting that, although miR-208a may play an important role in the development of the cardiac conduction system, its function is not required for maintenance of electrical conduction in adulthood.

In contrast with the relatively subtle effects of miR-208a loss-of-function on cardiac development, miR-208a is indispensable for mediating some aspects of the cardiac stress response during pressure overload or hypothyroidism. In response to TAB or transgenic expression of activated calcineurin, which both induce cardiac hypertrophy in wild-type mice, miR-208a-mutant animals showed signs of cardiomyocyte hypertrophy or cardiac fibrosis [62]. The blunted hypertrophic response of miR-208a mutants was associated with a reduction in cardiac contractility and a profound inability to up-regulate βMHC/Myh7 expression, whereas expression levels of other cardiac stress markers, such as ANF (atrial natriuretic factor) and BNP (brain natriuretic peptide), were unaffected. Similarly, mice lacking miR-208a are incapable of up-regulating βMHC/Myh7 in response to inhibition of T3 (3,3',5-tri-iodothyronine) signalling (i.e. hypothyroidism) in the adult heart [62]. Consistent with these findings, miR-208a overexpression in transgenic mice is sufficient to induce βMHC/Myh7 up-regulation prior to the onset of any overt signs of cardiac hypertrophy and/or pathology [62]. Conversely,
developmental loss of miR-208a does not appear to have any effect on βMHC protein levels in the embryonic and neonatal heart [60–62], suggesting that distinct regulatory hierarchies control the expression of βMHC/Myh7 during embryogenesis and under conditions of cardiac stress in adulthood. Complete loss-of-function of miR-208a, miR-208b and miR-499 will be required to overcome potential issues of functional redundancy and to fully assess the functions of the myomiR network during embryonic heart development.

The molecular mechanisms by which miR-208a regulates βMHC expression in the adult heart involve a fascinating interplay between multiple members of the myomiR regulatory network and their downstream molecular targets. In the adult heart, miR-208a is essential for expression of Myh7b/miR-499 expression, as well as Myh7/miR-208b [60]. Indeed, adult miR-208a-knockout mice can essentially be considered null mutants for all of the myomiR members. Through an elegant series of genetic deletions and rescue experiments, van Rooij et al. [60] demonstrated that re-expression of miR-499 in the adult heart is sufficient to re-activate Myh7, Myh7b and miR-208b expression, as well as prevent aberrant up-regulation of fast skeletal muscle troponin isoforms, in hypothyroid miR-208a-mutant mice [60]. These results indicate that miR-499 is a major downstream effector of the actions of miR-208a in the heart and define a positive-feedback loop whereby miR-208a regulates the expression of Myh7b and its intronic miRNA miR-499 which, in turn, regulates βMHC.

The biological actions of miR-208 and miR-499 on myosin expression have been reported to occur through a suite of downstream target genes including the TRH (thyroid hormone receptor) co-regulator Thrap1/Med13 (TRH-associated protein 1/mediator complex subunit 13) [61,62], as well as several transcriptional repressors of slow myosin gene expression, including Sox [SRY (sex determining region Y)-box] 6, Purβ (purine-rich element-binding protein β), Sp3 (specificity protein 3) and HP-1β (heterochromatin protein 1β) [60]. However, βMHC expression levels are similar in wild-type and Thrap1/Med13-cardiac-transgenic mice following inhibition of T₃ signalling [64], indicating that Thrap1/Med13 may not be a major mediator of myosin switching in vivo. In contrast, recent in vivo studies of Thrap1/Med13-transgenic and -knockout mice have revealed an unexpected role for the miR-208/Thrap1 cardiac signalling circuit in the regulation of systemic insulin sensitivity and glucose tolerance following high-fat-diet-induced obesity, suggesting important roles for the myomiR regulatory network beyond control of myosin expression [64]. With regards to myosin switching, a recent in vitro study by Yeung et al. [65] supports the notion that miR-499 represses βMHC expression through repression of Sox6 and suggests that the lymphoid transcription factor Ikaros 4 (Eos) may be an important upstream activator of Myh7b/miR-499 transcription. However, another study demonstrated that, although miR-499 overexpression in the heart was associated with increased βMHC expression, Sox6 expression was unaffected [66]. Srivastava and co-workers [66] concluded that Sox6 may not be a direct target of miR-499 in the heart in vivo and an additional role for miR-499 in the regulation of immediate early response genes, which are involved in the early cardiac transcriptional response to cardiac stress, was identified. Furthermore, miR-499 also appears to be important for modulating mitochondrial dynamics, through regulation of the Drp1 (dynamin-related protein 1), and overexpression of miR-499 inhibits apoptosis and is sufficient to blunt the cardiac stress response following I/R (ischaemia/reperfusion) injury [67]. Interestingly, the expression of miR-499 in the ischemic heart appears to be under the influence of p53, which down-regulates miR-499 independently of Myh7b expression [67]. Thus miR-499 processing is sometimes uncoupled from Myh7b transcription and the regulatory actions of the myomiR network are complex, highly context-dependent and remain incompletely understood.

The miR-17–92 cluster is required for heart development

The miR-17–92 cluster, also known as Oncomir-1, consists of six miRNAs, belonging to four miRNA families, that are generated from a common pri-miRNA transcript. Additionally, two paralogous clusters, miR-106a∼93 and miR-106b∼25, are transcribed from genetic loci on separate chromosomes and provide a further layer of redundancy among the four miRNA families that comprise the miR-17–92 cluster. The miR-17–92, miR-106a∼93 and miR-106b∼25 clusters probably arose through a series of gene duplication events during vertebrate evolution and the broad conservation of sequence and genomic organization across species implies important functions during vertebrate development. Loss-of-function studies in the mouse have revealed critical roles for the miR-17–92 cluster during heart development [68]. miR-17–92 mutants die shortly after birth with VSDs and lung hypoplasia. 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. However, compound mutant embryos lacking both miR-17–92 and miR-106b∼25 die at mid-gestation with an increased severity of cardiac developmental defects, including VSDs, ASDs (atrial septal defects) and ventricular wall thinning [68]. miRNAs within the miR-17–92 cluster were proposed to exert their biological effects via repression of a number of pro-apoptotic proteins, including Bim, which contains well-conserved 3′-UTR-binding sites for miR-17, miR-25/92/363 and miR-17/20/93/106 [68]. These findings highlight the essential and overlapping roles of miR-17∼92 and related clusters during heart development.

The miR-17–92 cluster also appears to influence myocardial differentiation of cardiac progenitors in the secondary heart field, which is required for normal outflow tract development. BMP (bone morphogenetic protein) signalling activates transcription of multiple members of the miR-17∼92 cluster, which in turn repress the expression of the cardiac progenitor genes Isl1 (ISL LIM homeobox 1) and Tbx1 (T-box 1) [69]. Studies in the zebrafish have also shown that miR-92 is critical for endoderm formation and overexpression of miR-92 reduces endoderm formation during gastrulation and causes cardiomyopathy [70]. The effects of miR-92 on developing endoderm were found to be due to modulation of Gata5 levels by this miRNA. Gain-of-function studies in the mouse have revealed further a function for the miR-17∼92 cluster in the regulation of organ size, as transgenic mice globally overexpressing miR-17 display growth retardation in multiple
Regulation of cardiac morphogenesis and patterning by miR-138 and miR-218

Cardiac morphogenesis and patterning require exquisite spatiotemporal control of gene expression and function. Recent studies in zebrafish suggest that miRNAs are integrated into the genetic circuitry that distinguishes chamber-specific gene expression patterns and guide early heart morphogenesis. During vertebrate development, the atria and ventricles are separated by a domain known as the AVC (atrioventricular canal), which eventually gives rise to the valves that ensure proper unidirectional flow of blood through the multi-chambered heart. In zebrafish, the AVC is characterized by expression of cspg2 (versican) and notch1b, which distinguish the AVC from adjacent cardiac chambers [73]. A role for miRNAs in the regulation of AVC-specific gene expression has emerged from loss-of-function studies. Knockdown of miR-138 by antagonomiRs in one-to-two cell zebrafish embryos results in pericardial oedema, indicative of cardiac dysfunction, impaired cardiomyocyte maturation and cardiac looping defects in 60–80% of antagonomiR-treated embryos [74]. Interestingly, cardiac patterning was also affected, with genes normally restricted to the AVC region becoming ectopically expressed in the ventricular chamber following miR-138 knockdown. miR-138 restricts AVC gene expression in the cardiac ventricle through direct repression of the gene encoding versican (cspg2), as well as through direct targeting of the retinoic acid synthesis enzyme aldehyde dehydrogenase 1a2 (aldh1a2), which itself is a positive regulator of versican expression [74]. Although these findings have identified an important role for miR-138 in establishing the distinct identity of cardiac structures in the developing heart, additional studies will be required in order to determine the relevance of this regulatory circuitry during mammalian heart development.

Further evidence for a role of miRNAs during vertebrate cardiac morphogenesis and patterning comes from recent gain- and loss-of-function studies of miR-218 in zebrafish. The miR-218 family consists of three highly conserved members including miR-218a-1, miR-218a-2 and miR-218b. In vertebrates, miR-218a and miR-218a-2 are intronically encoded in the slit2 and slit3 genes, whereas miR-218b is intergenic. The Slit ligands and their Robo (Roundabout) receptors are best known for their roles in axon guidance in the nervous system [75], but they also provide guidance cues for development of several other organ systems, including the heart and vasculature [76,77]. Fish et al. [76] recently reported that knockdown of miR-218 using two different morpholinos in one- to two-cell zebrafish embryos results in cardiac morphological defects, impaired migration of heart field progenitors to the midline during heart tube formation and pericardial oedema at 48 hpf (hours post fertilization), but does not cause any severe vascular defects [76]. Furthermore, a sub-phenotypic dose of robol morpholino significantly rescued the miR-218 morphant phenotype, suggesting that miR-218 regulates heart field migration and heart tube formation through modulation of Robo1 dosage [76]. However, Chiavacci et al. [78] could not reproduce these findings and, in contrast, reported that miR-218 knockdown, even following microinjection of very high doses of one of the identical morpholinos used in the study by Fish et al. [76], did not cause any defects in cardiac development. Chiavacci et al. [78] argued that a role for miR-218 in the migration of endocardial and myocardial progenitors is unlikely because miR-218 is not substantially expressed before 24 hpf when the fusion of migrating cardiac cells is almost complete [78]. In support of this model, Chiavacci et al. [78] reported that overexpression of miR-218 during early stages of zebrafish embryogenesis caused cardiac defects including incomplete looping, as well as impaired ventricular and atrial morphogenesis. In addition, overexpression of miR-218a disrupts cardiac patterning and is associated with ectopic expression of the normally endothelial-restricted gene tie2 in the atria and ventricles. It was also shown that expression of miR-218 correlates with Tbx5 during zebrafish heart development and down-regulation of miR-218 is sufficient to rescue most of the developmental heart defects associated with Tbx5 overexpression [78]. Given that mutations in the Tbx5 gene are a common cause of Holt–Oram syndrome, a condition associated with a wide spectrum of congenital heart abnormalities including VSDs and ASDs, the functions of miR-218 during cardiac morphogenesis in mammals warrants future investigation.

The miR-15 family and neonatal heart development

In mammals, a number of important physiological transitions take place during the immediate post-natal period, which prepares the heart for life outside the womb. In rodents, the first 2 weeks of post-natal life are characterized by rapid cardiac growth coincident with cardiomyocyte cell-cycle withdrawal, cardiomyocyte binucleation, cellular hypertrophy, a switch from glycolytic to fatty acid substrate utilization, increased mitochondrial biogenesis and maturation, and an increase in myofibril density, as well as alterations in the composition of contractile proteins including myosin isofrom switching [79]. Genetic deletion of Dicer and DGCR8 in mice has identified essential roles for miRNAs during neonatal heart development [34,35]. Furthermore, recent expression profiling studies have revealed dynamic alterations in cardiac miRNA expression during neonatal life, including up-regulation of multiple members of the miR-15 family between P1 and P10 [80]. The miR-15 family consists of six members, which are clustered on three separate chromosomes. miR-15a and miR-16-1 are intronically encoded within the Dleu2 IncRNA (long non-coding RNA), which is frequently deleted in patients with chronic lymphocytic leukaemia. miR-15b and miR-16-2 are clustered within an intron of a putative IncRNA, termed the mir-497-195 cluster host gene (MIR497HG). Recent gain- and loss-of-function
The timing of miR-15 family up-regulation in the neonatal mouse heart coincides with the onset of cardiomyocyte cell-cycle arrest and binucleation [80]. Overexpression of miR-15 in the embryonic heart under the control of the βMHC promoter inhibits cardiomyocyte proliferation and is associated with VSDs and ventricular hypoplasia in ~30% of transgenic mice at birth [80]. The remaining mice, which do not have VSDs, develop a slow-onset dilated cardiomyopathy and die between 5 and 18 months of age. Overexpression of miR-195 in vivo is associated with a reduction in the number of mitotic cardiomyocytes, an increased number of multinucleated cardiomyocytes and repression of mitotic genes [80]. Similarly, overexpression of miR-15 family members in vitro inhibits cardiomyocyte proliferation [80,81]. Consistent with these findings, inhibition of the miR-15 family in neonatal mice by systemic administration of antimirs delays cardiomyocyte mitotic arrest [80], suggesting that post-natal up-regulation of the miR-15 family provides an important regulatory brake on the cardiac cell-cycle machinery.

At the molecular level, the miR-15 family directly targets a number of cell-cycle genes. In the heart, biochemical analysis of neonatal miR-195 transgenic mice using next-generation sequencing of RISC-associated transcripts (RISC-seq) led to the identification of a number of miR-15 family target genes involved in cell proliferation, including Chek1 (checkpoint kinase 1) [80]. Chek1 has many important functions during DNA repair and mitosis, including prevention of genomic instability, co-ordinating progression through G2/M and spindle checkpoints, chromosome segregation and cytokinesis [82]. Although recent analyses of miR-15a/-16-1-knockout mice [83], as well as human cisplatin-resistant cancer cells [84] and follicular lymphomas [85], are consistent with the notion that Chek1 is an important downstream mediator of miR-15 family functions, the role of Chek1 during cardiac development remains unknown and awaits further investigation.

The functions of the miR-15 family in cardiomyocytes extend beyond cell-cycle control and other studies indicate that the miR-15 family also regulates a number of proteins with important roles in the maintenance of mitochondrial function and cell survival. Inhibition of the miR-15 family in vitro protects cardiomyocytes from cell death, and these effects are proposed to occur via regulation of Bel-2 (B-cell CLL/lymphoma 2) [86,87] and Sirt1 (sirtuin 1) [87]. Inhibition of the miR-15 family also protects against I/R injury in vivo in adult mice, and this effect is associated with de-repression of the putative miR-15 target genes Pdk4 (pyruvate dehydrogenase kinase 4) and Sgk1 (serum-glucocorticoid-regulated kinase 1), which regulate mitochondrial function and apoptosis respectively [86]. Additionally, miR-15 family members have been found to regulate cellular ATP levels in cultured neonatal rat cardiomyocytes, and this involves repression of Arl2 (ADP-ribosylation factor-like 2) [88]. Overexpression of miR-15b also causes changes in mitochondrial morphology, including a reduction in mitochondrial size and mitochondrial degeneration, suggesting that the miR-15 family regulates mitochondrial integrity [88]. Interestingly, in addition to its metabolic function, Arl2 also acts as a regulator of microtubule biogenesis and cytokinesis [89]. Indeed, inhibition of Arl2 partially rescues the cell-cycle-inhibitory actions of miR-16 overexpression in A549 human adenocarcinoma cells, suggesting that Arl2, at least in part, mediates the effects of miR-15 family members on cell proliferation [90]. The functions of Arl2 during cardiac development remain to be elucidated, but it is tempting to speculate that the miR-15/Arl2 axis may serve a dual role in cardiomyocyte proliferative arrest and mitochondrial maturation during the neonatal period. However, it should be noted that all of the in vivo loss-of-function studies of the miR-15 family to date have utilized antisense oligonucleotides, which inhibit miR-15 family members in multiple tissues and cell types. Additionally, the miR-15 family probably recognizes a distinct repertoire of target genes in the neonatal and adult heart. Therefore delineation of miR-15 targets with specific relevance to developmental compared with disease contexts requires further attention. Future studies employing more sophisticated cell-specific loss-of-function strategies will be required in order to more fully elucidate the functions of the miR-15 family during cardiac development and disease.

**miRNAs AND CHD (CONGENITAL HEART DISEASE) IN HUMANS**

CHD accounts for approximately one third of all major congenital anomalies and is the leading cause of childhood morbidity and mortality [91]. Genetic mutations in a number of critical genes for cardiovascular development, such as NKX2.5 and GATA4, have been found to underlie a number of cardiac malformations [91]. Most of the known human heart malformations result from haploinsufficiency or heterozygous point mutations in transcription factors, suggesting that the heart is particularly sensitive to the dosage of critical developmental pathways [91]. The causes of most congenital heart defects remain undefined and, given the emerging role of miRNAs as ‘fine-tuners’ of gene expression during cardiac development, miRNAs may serve as useful diagnostic, prognostic and therapeutic targets for CHD. However, despite dozens of miRNA expression profiling studies linking specific miRNA expression signatures with cardiovascular diseases in adults, there is little information available regarding miRNAs and cardiovascular disease during childhood in humans. Further studies will be required to assess the utility of miRNAs as potential biomarkers for CHD. Furthermore, given the stability of miRNAs in the circulation, miRNA profiling in maternal, fetal or neonatal serum could also be particularly useful for assisting CHD diagnosis and prognosis in the future [92].

SNPs (single nucleotide polymorphisms) or mutations can alter miRNA expression, processing, targeting and function. For example, SNPs located in the 3′-UTR of the AGTR1 (angiotensin II type 1 receptor) gene affect miRNA-dependent regulation of AGTR1 by miR-155 and are associated with hypertension [93]. SNPs have also been identified in mature miRNA sequences. Of particular relevance to cardiac biology, systematic screening for sequence variants in the myomiR network identified a naturally occurring, rare, mutation in miR-499 [94]. Interestingly,
cardiac-specific overexpression of wild-type and mutant miR-499 revealed misdirected recruitment of a number of miR-499 target genes in mutant mice, despite the fact that this mutation is located in the 3' end of miR-499, outside of the canonical 'seed' region thought to determine miRNA target recognition [94]. Although the pathophysiological relevance of this naturally occurring miRNA mutation is currently unclear, that study provided an elegant proof-of-principle for the existence of functionally relevant mutations within miRNAs, even in regions outside of the canonical sequences required for target recognition. In addition, a functionally relevant variant of miR-196a-2 has been associated with CHD susceptibility in a Chinese population [95]. Genotype–phenotype correlation analyses revealed that the miR-196a-2 mutation was associated with increased cardiac expression levels of mature miR-196a and aberrant targeting of HOXB8 (homeobox B8), a natural target for miR-196a [95]. However, the mechanistic basis for regulation of cardiac development by miR-196a is not clear. Knockdown studies in the developing chick embryo do not indicate a central, evolutionarily conserved, role for miR-196 in vertebrate heart development, but this miRNA does appear to be particularly important for patterning of the axial skeleton [96]. Thus additional studies are required to validate the broader significance of miR-196a-2 mutations for vertebrate heart development in animal models, and these findings will also need to be replicated in different human populations.

miRNAs AND CARDIAC REGENERATION

miRNAs and cardiomyocyte proliferation

The classic dogma that the heart is terminally differentiated has been overturned in recent years by the identification of low rates of cardiomyocyte turnover during normal aging [97,98] and following myocardial infarction [98]. However, the adult mammalian heart cannot undergo appreciable regeneration following injury and, as a consequence, ischaemic heart disease remains the leading cause of death in adults in the developed world. In contrast, recent studies have identified a robust, yet transient, regenerative capacity of the neonatal mammalian heart [99,100], which displays similarities with regenerative mechanisms in lower vertebrates [101]. Cardiomyocyte proliferation appears to be the dominant cellular mechanism driving cardiomyocyte replenishment in the regenerating neonatal mouse heart following apical resection injury [99] and myocardial infarction [100].

Given the importance of the miR-15 family and miR-133 for cardiomyocyte proliferation during development, the functions of these miRNAs in the context of neonatal mice and adult zebrafish heart regeneration have been recently assessed. Transgenic mice overexpressing the miR-15 family member miR-195 in the neonatal heart fail to regenerate following myocardial infarction and display reduced rates of cardiomyocyte proliferation, increased cardiomyocyte size, fibrosis and cardiac dysfunction compared with wild-type littermates [100]. Consistent with previously identified roles for the miR-15 family in cardiac development and disease, miR-195 overexpression was associated with repression of a large number of cell-cycle and mitochondrial genes [100]. Similar studies in the zebrafish revealed a critical role for miR-133 in the regulation of cardiomyocyte proliferation and regeneration following apical resection injury [102]. These findings suggest that miR-15 and miR-133 family members impair the cardiac regenerative response of neonatal mice and adult zebrafish respectively, through inhibition of cardiomyocyte proliferation.

Loss-of-function experiments in the mouse support a role for the miR-15 family in post-natal regulation of mammalian cardiac regenerative capacity. Pharmacological inhibition of the miR-15 family by systemic administration of antimiRs from an early post-natal age until adulthood improves cardiac function following I/R injury and is associated with induction of cardiomyocyte proliferation in adult mice [100]. Importantly, acute administration of miR-15 inhibitors following I/R injury in adult mice also improves cardiac function [86], implying that the miR-15 family may be an attractive therapeutic target for ischaemic heart disease. However, acute inhibition of the miR-15 family following I/R inhibits cell death and reduces infarct size following infarction [86], whereas chronic administration of antimiRs during neonatal development does not affect cell survival following I/R [100]. These differences probably reflect distinct subsets of target genes that are modulated by the miR-15 family in immature and mature cardiomyocytes, as well as potential differences in the subset of genes regulated during normal development compared with injury. Moreover, owing to the non-tissue-specific nature of systemic anti-miR administration and the potential risk for cancer following miR-15 loss-of-function [83,103], future studies should assess the therapeutic potential of cardiomyocyte-specific inhibition of the miR-15 family in animal models before moving to any potential clinical trials.

Further support for therapeutic strategies based on miRNA-dependent re-activation of cardiac regenerative mechanisms comes from a recent functional screen for miRNAs that induce cardiomyocyte proliferation. Eulalio et al. [81] performed a high-content high-throughput screen representing 988 mature miRNAs for identification of miRNAs that induce cardiomyocyte proliferation [81]. In addition to validating previously identified repressors of cardiomyocyte proliferation, such as the miR-15 family, 40 additional miRNAs were found to induce DNA synthesis in neonatal rat and mouse cardiomyocytes. Two of these miRNAs, miR-199a and miR-590, were shown to induce cell-cycle re-entry and cytokinesis of cultured adult cardiomyocytes in vitro. In vivo delivery of miR-199a or miR-590 to neonatal mice using the cardiotropic adenovirus-associated virus, AAV9 (adenovirus-associated virus serotype 9), resulted in widespread activation of cardiomyocyte proliferation and increased heart size, without inducing cardiomyocyte hypertrophy. Moreover, AAV9-mediated induction of miR-199a or miR-590 following myocardial infarction in adult mice induced marked cardiac regeneration, associated with augmented cardiomyocyte DNA synthesis rates and reduced fibrosis. miR-199a and miR-590 did not affect cardiomyocyte apoptosis following myocardial infarction. However, myocardial viability, using the standard redox indicator tetrazolium chloride, was not assessed in this study and a potential protective effect of these miRNAs on cell viability (possibly through protection against necrotic cell death) cannot be excluded. Analysis of potential downstream mRNA targets indicates that Homer1
Figure 3 Regulation of cardiomyocyte proliferation by miRNAs

A summary of the molecular mechanisms by which miRNAs regulate cardiomyocyte proliferation. Ccnd2, cyclin D2; CM, cardiomyocyte.

[Figure 3]

[Holter homologue 1 (Drosophila) and Hopx (HOP homeobox), previously implicated in the regulation of cardiomyocyte calcium signalling and embryonic cardiomyocyte proliferation respectively, are targeted by both miR-199a and miR-590 [81]. Further studies will be required to assess the endogenous physiological functions of miR-199a and miR-590 during cardiac development and any potential side effects associated with ectopic expression of these miRNAs in non-cardiomyocytes. Nevertheless, these studies provide a powerful demonstration of the potential utility of miRNA-based therapeutics for induction of cardiomyocyte proliferation and cardiac regeneration following myocardial infarction. A summary of miRNAs implicated in the regulation of cardiomyocyte proliferation is provided in Figure 3.

miRNAs and stem/progenitor cells

Stem cells represent a potentially attractive source of cardiac cells for the treatment of cardiovascular diseases. Over the last decade, several subsets of exogenous and endogenous cardiac stem/progenitor cells have demonstrated therapeutic potential in animal models and clinical trials. However, most of the functional benefits reported for stem cell clinical trials of ischaemic heart disease have been modest, short-lived, associated with low engraftment rates and appear to be due to paracrine effects rather than differentiation of the applied cells into cardiomyocytes [104]. Several barriers will need to be overcome in order to improve the safety, efficiency and efficacy of stem cell therapies for heart disease, including selection of an appropriate cell source, improvement of stem cell homing and survival in the infarct zone, reducing the tumorigenic potential of pluripotent stem cells and enhancing the efficiency of stem cell differentiation towards specific cardiovascular lineages. One avenue towards achieving at least some of these goals may involve the use of miRNAs to control the gene expression networks underlying self-renewal, pluripotency and differentiation decisions in stem cells.

miRNA expression profiling of human ESC (embryonic stem cell)-derived cardiomyocytes revealed that miR-1, miR-133 and the myomiRs (miR-208 and miR-499) are significantly induced during cardiomyocyte differentiation [56,105,106]. Overexpression of miR-1 induces the expression of mesodermal and cardiac marker genes in mouse and human ESCs [56], as well as human ESC-derived embryoid bodies [106] and adult cardiac progenitor cells [105]. As well as controlling the expression of sarcomeric genes in differentiating ESCs, miR-1 has also been proposed to regulate the electrophysiological maturation of ESC-derived cardiomyocytes [105]. In contrast, the bicistronically clustered miR-133, which shares many functions with miR-1 during cardiomyocyte differentiation in vivo, opposes cardiomyocyte differentiation and represses cardiac gene markers in ESCs in vitro [56]. Furthermore, Takaya et al. [107] reported that both miR-1 and miR-133 inhibit cardiomyocyte differentiation in a two-dimensional mouse ESC culture model, suggesting context-specific functions of miR-1 and miR-133 during cardiac differentiation. Consistent with a crucial role for the myomiR network in cardiac differentiation, overexpression of miR-499 in human cardiac progenitor cells and ESCs induces expression of cardiac gene markers and hastens the formation of beating embryoid bodies, whereas inhibition of miR-499 blocks cardiac differentiation in vitro [105,106,107]. The effects of miR-499 on cardiac differentiation were proposed to occur through repression of the transcription factor Sox6 [105]. However, in contrast with miR-1, overexpression of miR-499 does not induce the calcium amplitude and kinetics of calcium handling that are characteristic of more mature ventricular cardiomyocytes [108]. Therefore miR-1 and miR-499 both appear to be potent inducers of cardiomyogenic differentiation of stem cells, but miR-1 induces a more mature ventricular cardiomyocyte phenotype than miR-499.

Recent studies also support the translational potential of miRNA-mediated manipulation of stem cells for heart disease. Transplantation of ESCs overexpressing miR-1 into the border zone of infarcted mouse hearts protects the myocardium from ischaemic injury in vivo [109,110]. miR-1 inhibits expression of PTEN (phosphatase and tensin homologue deleted on chromosome 10) in ESCs, which augments PI3K (phosphoinositide 3-kinase)/Akt signalling, improves cardiac function, enhances cardiomyocyte differentiation and attenuates apoptotic cell death [109,110]. Similarily, overexpression of miR-499 in c-kit+ cardiac progenitor cells improves myocardial function, enhances cardiomyocyte differentiation and potentiates restoration of myocardial mass following injection into the border zone of infarcted rat hearts [111]. Thus ectopic expression of miRNAs in cardiac stem and progenitor cells can alter the differentiation potential of engrafted cells in vivo and could be used to improve the efficacy of stem cell therapies for heart disease.

An alternative strategy to exogenous cell replacement therapies for heart disease involves harnessing the innate regenerative potential of the mammalian heart. In addition to induction of cardiomyocyte proliferation (discussed above), several cardiac progenitor populations have been identified in the adult heart and their regenerative potential could potentially be harnessed for therapeutic purposes. Little is known with regards to the functions of miRNAs in the control of self-renewal and differentiation decisions of endogenous cardiac progenitor cells. Interestingly, c-kit progenitors undergo a maturation process during early postnatal life, which coincides with a significant loss of cardiomyogenic potential during the neonatal period [112]. Although the molecular mechanisms regulating developmental maturation of
c-kit progenitors are not understood, a recent expression profiling study has identified a number of miRNAs that are developmentally regulated in neonatal compared with adult c-kit progenitors. Among the differentially regulated miRNAs, mir-17, a member of the mir-17~92 cluster, was found to be reduced in adult compared with neonatal progenitors [113]. Overexpression of the mir-17~92 cluster increased the proliferation of adult c-kit progenitors in vivo, suggesting that developmental down-regulation of this miRNA cluster may be an important mechanism regulating the proliferative potential of cardiac progenitor cells. Further studies of the roles of mir-17~92, as well as other miRNAs, using gain- and loss-of-function strategies in vivo will be required in order to more fully elucidate the importance of miRNAs in cardiac progenitor cell populations during development and following cardiac injury.

miRNAs and direct lineage reprogramming

As an alternative to cell replacement strategies, some investigators have recently turned their attention to lineage reprogramming, which involves transforming one somatic cell type into another. The most stunning example of lineage reprogramming involves the re-induction of pluripotency in somatic cells by introduction of the so-called Yamanaka factors: Oct4/POU5F1 (POU class 5 homeobox 1), Sox2, Klf4 (Krüppel-like factor 4) and c-Myc [v-myc myelocytomatosis viral oncogene homologue (avian)] [114]. Intriguingly, overexpression of the miR-302 cluster members and miR-367 can completely substitute for the Yamanaka factors and induces more efficient reprogramming of mouse and human somatic cells than the four Yamanaka factors [115]. The subsequent generation of iPSCs (induced pluripotent stem cells) provides an ethically unencumbered source of stem cells for disease modelling, drug discovery and regenerative medicine. However, barriers to the use of iPSC technology for cardiac regeneration include very low reprogramming efficiencies, incomplete or partial cell reprogramming, functional heterogeneity and immaturity of iPSC-derived cardiomyocytes, low survival and retention of stem cells in the infarct zone, as well as a number of safety concerns. In an attempt to overcome some of these barriers, researchers have recently turned their attention to direct reprogramming of fibroblasts into cardiomyocytes, with the added bonus that the human heart provides a rich endogenous supply of fibroblasts for reprogramming following myocardial infarction.

By systematically screening a number of core components of the cardiogenic transcriptional machinery, Ieda et al. [116] provided the first demonstration that the combination of three transcription factors, Gata4, Mef2c and Tbx5, could reprogramme mouse fibroblasts directly into cardiomyocytes in vitro. Although this study provided an important proof-of-principle for direct cardiac reprogramming, the efficiency of reprogramming was very low and very few cardiomyocytes showed evidence of spontaneous beating, a hallmark feature of a fully mature ventricular cardiomyocyte. Nevertheless, two recent landmark papers by Qian et al. [117] and Song et al. [118] provide evidence that in vivo reprogramming of fibroblasts into cardiomyocytes is possible and, moreover, direct reprogramming is associated with long-lasting improvements in cardiac function following myocardial infarction.

As an alternative to transcription-factor-mediated direct reprogramming, miRNAs can also induce reprogramming of fibroblasts to cardiomyocytes in vitro and in vivo. Lentiviral-mediated delivery of miR-1, miR-133, miR-208 and miR-499 into the border zone of the mouse myocardium following myocardial infarction induces direct conversion of fibroblasts into cardiomyocytes in situ [119]. One potential advantage of miRNA-based approaches for direct reprogramming is the potential to circumvent the use of viral vectors for cardiac transduction by using chemically synthesized miRNA mimics. However, Jayawardena et al. [119] did not assess the impact of miRNA-based reprogramming on cardiac function, so the therapeutic benefits of this approach await further investigation.

FUTURE DIRECTIONS

Since the discovery that miRNAs are involved in cardiac development in 2005 [26], rapid progress has been made in deciphering the myriad roles for miRNAs in cardiovascular biology. miRNAs now constitute an important component of the regulatory circuits that govern heart development (Figure 1) and this knowledge has revolutionized our understanding of the diverse roles of non-protein-coding RNA in cardiac biology. However, with the human genome encoding over 1000 miRNAs, only a handful of which have been studied in the heart, much remains to be learned about this new class of regulatory RNA. Indeed, most of the largest and most abundant cardiac miRNA families, such as let-7 and miR-30, have not yet been extensively characterized, owing to technical difficulties associated with the requirement for compound genetic deletions of multiple miRNA family members located on multiple different chromosomes. Indeed, miRNA redundancy can also exist across different miRNA families, with multiple miRNAs recognizing the same target miRNAs and/or impinging on multiple different targets within a common biological pathway. In light of these multiple layers of functional redundancy, the often-modest effects of miRNAs on individual target genes and the accompanying subtle phenotypes associated with genetic deletion of individual miRNAs are not entirely surprising. Understanding the potential for redundancy in the regulatory hierarchy of miRNA interactions will form a critical component of future investigations in the field. Furthermore, current models of miRNA target recognition are inadequate and an enhanced understanding of the mechanisms of miRNA action is required. In particular, the importance of non-seed regions in the mature miRNA may shed light on functional redundancy outside of miRNA families. The continued development of biochemical approaches for miRNA target identification will aid our understanding of miRNA mechanisms of action, which are often clouded by biased bioinformatic approaches that are highly susceptible to false discovery.

One of the most promising avenues of miRNA research continues to be the development of diagnostic and therapeutic platforms for detection and manipulation of miRNAs. The clinical utility and safety of these approaches will be closely monitored in the coming years. With a growing list of miRNAs being implicated in cardiac developmental processes, it will be interesting to see whether this work in animal models will translate
into any meaningful clinical benefits for patients with congenital heart disease. miRNA-based therapeutics could also be harnessed in the context of regenerative medicine (Figure 2), which would usher in a new era of miRNA-based drugs for ischaemic heart disease. All of these possibilities will require ongoing exploration of the roles of miRNAs in cardiac developmental and regenerative processes, as well as the continued refinement of experimental and therapeutic approaches. Given the rapid progress that has been made in miRNA biology over the last decade, there is good reason to be optimistic about the future.

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microRNAs in cardiac development and regeneration


