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SPOTLIGHT REVIEW

MicroRNAs in control of cardiac hypertrophy

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Abstract MicroRNAs refer to a subfamily of small non-coding RNA species that are designed to influence gene expression in nearly all cell types studied to date. Studies from the past decade have demonstrated that microRNAs are atypically expressed in the cardiovascular system under specific pathological conditions. Gain- and loss-of-function studies using *in vitro* and *in vivo* models have revealed distinct roles for specific microRNAs in cardiovascular development, physiological functions, and cardiac pathological conditions. In this review, the current relevant findings on the role of microRNAs in cardiac hypertrophic growth are updated, the target genes of these microRNAs are summarized, and the future of microRNAs as potential therapeutic targets is discussed.

Keywords Cardiac hypertrophy • Post-transcriptional regulation • MicroRNAs

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1. Introduction

Cardiac hypertrophy or enlargement of the heart muscle is defined as an increase in heart size without changes in myocyte number. Cardiac hypertrophic growth occurs in response to pathological and physiological haemodynamic overload due to different forms of injury or stress, such as hypertension, valve disease, and myocardial infarction (pathological), or regular physical activity and chronic exercise training (physiological).[1,2] Both pathological and physiological cardiac hypertrophy result in increased myocardial mass, but where pathological hypertrophy is accompanied by deleterious events such as foetal gene upregulation, myocardial fibrosis, and cardiac dysfunction, physiological hypertrophy is characterized by overall normal cardiac structure and function.[3] Distinct signalling pathways mediate each type of cardiac hypertrophy, and because sustained hypertrophy is a key risk factor in the development of heart failure,[4] much effort has been dedicated to the identification of signals and pathways, inducing pathological hypertrophy for rational drug design for heart failure.

Species of non-coding RNA molecules, microRNAs (miRNAs), have been shown to regulate these complex processes.[5–7] The recognition of miRNAs as essential regulators of cardiac morphology and function in the past decade has fundamentally changed our view of the physiological and pathological aspects of the cardiovascular system. MicroRNAs not only target single genes but often functionally related gene networks, yielding complex gene regulatory networks. This review comprises the most recent progress made in demonstrating how single miRNAs can regulate different aspects of cardiac hypertrophy and explores future directions of miRNA therapeutics in heart failure.

2. MicroRNA regulation of cardiac hypertrophy: microRNA maturation

MicroRNA maturation is a complex process where any step is subject to tight molecular regulation. MicroRNAs are initially transcribed by RNA polymerase II into primary transcripts (pri-miRNAs) that can encode one or more miRNAs.[8] Hereafter, multiple 60–100 bp long hairpin-like structures are released by the action of RNase III endonuclease Drosha, followed by export of these precursor stem-loop structures (pre-miRNA) from the nucleus in a Ran/GTP/Exportin-5-dependent manner. Once in the cytoplasm, pre-miRNAs are further cleaved and processed to generate mature miRNA species.[9–11]

3. Regulation of miRNA processing: Drosha complex

Primary miRNAs are several thousand nucleotides (nt) long that undergo a nuclear cleavage step by endonuclease Drosha, generating a 60–100 nt long pre-miRNA.[12,13] The Drosha microprocessor complex is formed by several associated polypeptides, and the fact that recombinant Drosha *in vitro* is unable to generate pre-miRNAs supports the requirement of additional cofactors for proper Drosha catalytic activity.[14] DiGeorge syndrome critical region gene 8 (DGCR8), one of those cofactors, is known to stabilize Drosha levels and to promote accurate cleavage of the stem loops within the pri-miRNAs.[15] Although Drosha expression and activity is regulated in certain cancers,[16–18] limited information on its precise regulation in cardiovascular diseases is available. While a homologous

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chromosomal deletion of the region where Drosha-DGCR8 is located leads to a modest decrease in expression of ~60 miRNAs,[19] a cardiomyocyte-specific deletion of DGCR8 results in dilated cardiomyopathy and early lethality,[20] suggesting an important role of DGCR-8-dependent miRNAs in cardiac biology and function.

Deregulation of components of the microprocessor will directly affect the maturation and expression profile of specific miRNAs. The DEAD-box RNA helicases p68 and p72, two Drosha-regulating proteins,[21,22] have been shown to be involved in processing of miR-199a and miR-214, with both miRNAs being expressed at low levels in p68-p72 null fibroblasts.[23,24] This suggests that proper recruitment of the microprocessor to specific pri-miRNAs requires the functional presence of those two helicases. Curiously, miR-21 has also been shown to be dependent on the association of specific proteins with Drosha and p68 in *Arabidopsis*.[25] Whether these proteins are also required during regulation of miR-21 expression in cardiac cells remains to be investigated.

In a similar way, other miRNAs have been described to be dependent on Drosha regulation by specific auxiliary proteins, including miR-let-7a and miR-206, involved in pulmonary hypertension[26] and muscle diseases,[27] respectively. These miRNAs require the KH-type splicing regulatory protein (KSRP), a factor that targets G-rich regions of a set of pri-miRNAs during processing[28] and which is also involved in post-transcriptional regulation of miR-155,[29] an inflammation-related miRNA.

4. Regulation of miRNA processing: Dicer and related proteins

Once generated, pre-miRNAs are transported via exportin-5 into the cytoplasm where they are processed by the ribonuclease III Dicer into ~22 nt species.[30] In vertebrates, Dicer is expressed through all stages of embryonic and adult development,[31] and its proper function is critical during processing of most pre-miRNAs into their mature form.[32,33] Accordingly, a Dicer null mutation in mice leads to an embryonic lethal phenotype at E7.5,[34] and embryonic stem cells lacking Dicer display severe differentiation defects.[35] Aside from playing an important role during normal foetal development and in stem cell maintenance, a requirement for Dicer has also been described in a variety of physiological and pathological cellular processes in adult mammals.

Several studies have specifically assessed the global role of miRNAs in cardiac development and function by generating animal models with cardiac-specific ablation of Dicer. Zhao *et al*.[36] deleted a floxed Dicer allele using Cre recombinase under the control of the Nkx2.5 regulatory region and allowing for early embryonic deletion in the cardiogenic region. This deletion decreased the presence of functional miRNAs during cardiac development and resulted in chamber malformation and embryonic death at E12.5 with the hearts displaying pericardial oedema and impaired cardiac ventricular function.[36,37] Similar results were obtained in another study that investigated the effect of Dicer deletion in the postnatal myocardium immediately after birth (driven by a *myh6*-Cre deleter strain).[6] To circumvent the observed lethality and analyse the role of Dicer in the juvenile and adult heart, Cre deleter mice with conditional cardiac ablation of Dicer (*myh6-driven*, tamoxifen activated MerCreMer deleter strain) were used.[5] While Dicer loss in cardiomyocytes of young animals resulted in sudden death due to arrhythmias and a mild form of cardiac dysmorphogenesis, Dicer deletion in adult animals led to the development of considerable cardiac hypertrophy, fibrosis, and severe cardiac dysfunction.

The observation of reduced Dicer expression in failing human hearts[6] suggests the presence of functional Dicer regulatory mechanisms in cardiac disease. In fact, and similar to Drosha, Dicer also associates with several regulatory proteins, such as the TAR RNA-binding protein (TRBP) and protein kinase R-activating protein (PACT)[38] to promote stability and processing activity. Although the specific role of these proteins during cardiovascular diseases is currently unknown, the fact that TRBP phosphorylation is dependent on ERK-MAP kinase,[39] a kinase involved in cardiac hypertrophy[40] and fibrosis,[41] implies that Dicer may be subject to regulation by cellular signalling pathways in certain cardiovascular diseases.

Dicer associates with other proteins to form the RISC-loading complex, where the cleavage of the pre-miRNA by Dicer results in an unstable miR–miR* duplex composed of an active guide strand (miRNA) and the passenger opposite strand (miRNA*). Degradation of the miRNA* strand mostly yields a mature miRNA that, once loaded into the RNA-induced silencing complex (RISC), is guided to cognate target mRNAs to induce post-transcriptional gene regulation either by mRNA degradation or translational inhibition, ultimately decreasing target gene protein expression.[42] Argonaut (AGO) proteins play a crucial role in miRNA-mediated mRNA silencing,[43,44] and although there are four AGO proteins in humans, AGO2 is the only one with cleavage and stabilizing activity in mammals with its deletion resulting in reduced levels of mature miRNAs.[44,45] Because AGO stability is affected by certain growth factors,[46] AGO regulation may have clear ramifications for miRNA processing in cardiac hypertrophy.

5. Regulation of miRNA processing: modification, editing, and decay

Similar to protein-coding mRNA genes, miRNAs, more specifically pri-miRNAs, also have a 7-methy-guanylate cap at the 5′-end and a poly(A) tail at the 3′-end.[47] Modifications at the 3′-end of specific miRNAs result in altered stability. Adenosine deaminases that act on RNA (ADARs) are known to affect pri- and pre-miRNA stability by converting adenosine to ionosine in dsRNA molecules.[48] One specific splicing variant of ADAR2 is enriched in cardiac disease,[49] but its biological role and relevance remain to be explored.

The half-life of some miRNAs in cardiac cells, among others, is relatively long, and alterations in their expression and stability may be related to miRNA decay mechanisms. While some tissues, such as neural tissue, display a high turnover of certain miRNAs,[50] in cardiac cells, miR-208, involved in stress-dependent hypertrophic cardiac growth, was shown to have a half-life of 12 days.[51] Although these mechanisms have been mostly described in lower organisms such as flies and worms,[52] they may also be effective and relevant in mammals as an explanation for the spatiotemporal changes observed in the expression pattern of certain miRNAs.

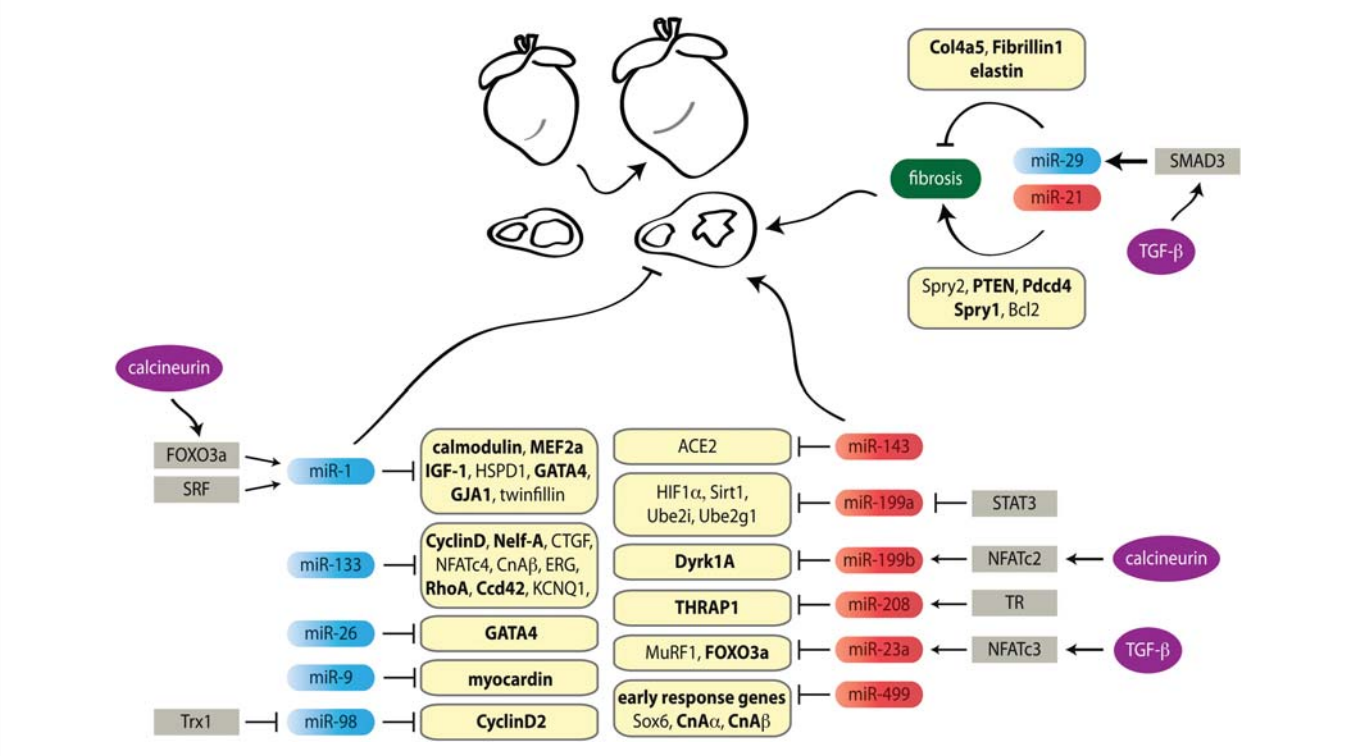

Figure 1 Regulation of pathological cardiac hypertrophy by microRNAs. Schematic representation of the microRNAs that are involved in the development of cardiac hypertrophy. MicroRNAs can have an anti-hypertrophic function (miR-1, miR-133, miR-26, miR-9, miR-98, and miR-29), while others are agonists of the hypertrophic response (miR-143, miR-199a, miR-199b, miR-208, miR-23a, miR-499, and miR-21). miR-21 and miR-29 primarily act within fibroblasts to modulate a pathological fibrotic response and thereby contributing to myocardial remodelling. A subset of stress-activated transcription factors have been revealed to regulate microRNA transcription, mechanistically linking microRNA downstream genes with molecular hypertrophic and fibrotic targets in the setting of adult hypertrophic cardiac remodelling and heart failure. Target genes of each miRNA were selected based on their identification from *in vitro* and/or *in vivo* (marked in bold) experiments.

6. miRNA regulation of pathological cardiac hypertrophy: differential expression of miRNAs

One reoccurring phenomenon that is observed in situations of pathological cardiac hypertrophy entails a switch from gene expression profiles characteristic for the adult myocardium to profiles that normally are present in the foetal heart.[53–56] One piece of evidence that miRNAs play a role in the induction of a foetal gene profile derives from the observation that the expression of miRNAs also changes during this canonical switch in gene expression.[57] A number of studies have used high-density array platforms for miRNA profiling in order to measure alterations in the expression of individual miRNAs under either experimental or clinical conditions of cardiac hypertrophy and heart failure.[58–62] Regarding the specific role of miRNAs in cardiac hypertrophy, van Rooij *et al.*[59] have reported the first microarray study using two animal models of pathological cardiac hypertrophy: the thoracic aortic-banded mouse model and calcineurin-overexpressing transgenic mice. Their results show that the miRNA expression pattern in the two animal models were similar, implying the presence of similar miRNA-controlled hypertrophic mechanisms.[59] Moreover, Thum *et al.*[63] demonstrated that miRNA expression in the failing myocardium shifts towards a foetal heart miRNA expression program. Expression of messenger RNAs and miRNAs showed an inverse correlation in failing hearts, suggesting that altered miRNA expression accounts, at least partially, for alterations in the transcriptome of the failing human heart. Collectively, these studies demonstrate that the human transcriptome is influenced by or even controlled by differing miRNA expression profiles.

7. miRNA regulation of pathological cardiac hypertrophy: antagonists

miR-1-1 and miR-1-2 are among the most abundantly expressed miRNAs in the human heart, providing a first clue that they may have a specific role in cardiomyocyte morphology and function (*Figure 1* and *Table 1*).[64–66] Whereas miR-1 has a crucial role in the developing heart, with its overexpression causing developmental arrest due to dilated ventricles and heart failure at E9.0,[67] in the adult heart, downregulation of miR-1 is one of the earliest changes observed after subjecting a mouse heart to increased pressure overload, preceding changes in the expression of other miRNAs, an increase in cardiac mass, and contractile dysfunction.[68,69] This observation, together with a study demonstrating that adenoviral miR-1 overexpression attenuates cardiomyocyte hypertrophy,[69–71] provides compelling evidence that miR-1 downregulation has a

Table 1 Anti- (blue) and pro-hypertrophic (red) microRNAs involved in cardiac hypertrophy; the animal models of cardiac disease where they have been shown to be differentially expressed and the approaches used to modulate their expression *in vivo*

miRNA	Model cardiac disease	*In vivo* modulation
miR-1	αMHC-CnA, αMHC-Akt, TAC, exercise	Genetic KO, gene transfer (AMO), Ad-miR
miR-133	αMHC-CnA, αMHC-Akt, TAC, exercise	Genetic KO, antagomir αMHC-miR, Ad-miR, Ad-Decoy
miR-26	TAC	LNA
miR-9	Iso/Aldo infusion	miR-mimic
miR-98	AngII infusion	Ad-miR Ad-anti-miR
miR-29	αMHC-CnA, TAB, MI	Antagomir
miR-143	Exercise	n.a.
miR-199a	αMHC-CnA, αMHC-β2AR TAC	n.a.
miR-199b	αMHC-CnA, TAC	Antagomir, αMHC-miR
miR-208	αMHC-CnA, TAC	Genetic KO, αMHC-miR
miR-23a	PE infusion	Antagomir, αMHC-miR
miR-499	MI, I/R	Antagomir, αMHC-miR
miR-21	αMHC-β1AR, TAC, I/R, Iso/AngII infusion	Genetic KO, antagomir, LNA

AMO, 2′-O-methyl-modified antisense oligonucleotides; LNA, locked nucleic acid.

causative role in the pathogenesis of cardiac hypertrophy. A wealth of literature now provides evidence that calcium-mediated signalling serves as an upstream mediator of cardiomyocyte hypertrophy.[72] Interestingly, miR-1 was demonstrated to regulate two separate signalling mediators of calcium signalling, calmodulin, and Mef2a, where cardiac calmodulin transcripts of the genes *Calm1* and *Calm2* are both repressed by miR-1 in animal models of heart failure.[69,72] In addition, downstream transcriptional effectors, MEF2A and GATA4, are regulated by the same miRNA, suggesting that miR-1 plays a centralized role by regulating the expression of calcium signalling modalities simultaneously.

miR-1 was reported to target a cytoskeletal regulatory protein, twinfilin 1 (Twf1), that binds to actin monomers, preventing their assembly into filaments.[65] The expression level of Twf1 is low in the adult heart and inversely correlates with high expression of miR-1. Downregulation of miR-1 induced by hypertrophic stimuli, such as aortic banding or α-adrenergic stimulation with phenylephrine (PE), results in increased Twf1 expression. The fact that Twf1 overexpression is sufficient to induce cardiac hypertrophy in neonatal rat cardiomyocytes suggests therapeutic relevance of appropriate manipulation of Twf1 expression in attenuating cardiac hypertrophy.

Another validated target of miR-1 is insulin-like growth factor (IGF-1), a modulator of growth, survival, and differentiation in most cell types.[73] In models of cardiac hypertrophy and failure, there is a repression of miR-1 expression and concomitant upregulation of IGF-1.[68] Accordingly, acromegalic patients, in whom IGF-1 is induced following atypical synthesis of this growth hormone, display increased cardiac mass and wall thickness. Besides being a target of miR-1, miR-1 expression is dependent on the activation state of IGF-1, through activation of the PI3K/AKT signalling pathway and repression of its downstream target, Foxo3a, providing evidence for the existence of a new feed-forward model to understand how IGF-1 modulates cardiac and skeletal muscle structure and function.[68]

There are two bicistronic clusters encoding miR-1, miR-133a-1/miR-1-2, and miR-133a-2/miR-1-1 in the heart.[67] A third bicistronic miRNA cluster composed of miR-206 and miR-133b is expressed in skeletal muscle and is, therefore, not present in the heart.[67,74] There exists strong evidence that miR-133 regulates proper cardiac development and cardiac function. First, overexpression of this miRNA attenuates agonist-induced hypertrophy,[71,75] while conversely, silencing of miR-133 by using chemically modified antisense oligonucleotides (so-called antagomirs[76]) or by expression of tandem repeat antisense sequences for miR-133 (so-called miRNA sponges[77]) sensitizes the myocardium to excessive cardiac growth. Mechanistically, miR-133 represses family members of the Rho kinase family, *RhoA* and *Cdc42*, as well as *NelfA*, a negative regulator of RNA polymerase II. The derepression of these miRNA targets was demonstrated to play a role in the cardiac growth response downstream of miR-133.[68]

Genetic deficiency for both miR-133a-1 and miR-133a-2 resulted in extensive fibrosis and impaired cardiac function, but there was no significant difference in left ventricular posterior wall thickness in diastole between wild-type and double-deficient mice, confirming that those hearts were not hypertrophic.[78] Of importance are the differences in phenotypes between the miRNA knockout studies vs. antagomir silencing, and these differing approaches used to study miRNAs could underlie fundamental biological differences and have ramifications for our understanding of how to investigate miRNA cellular function. First, the likelihood that antagomirs have off-target effects is small, as a single nucleotide mismatch already leads to a dramatic loss of specificity,[76] although this possibility cannot be excluded. Likewise, miRNA silencing by antimiRs could theoretically lead to escape mechanisms by RNA editing, most commonly by ADARs[48,49] and affecting ~16% of all pri-miRNAs in the cell,[79,80] rendering the silencing approach less efficient. Finally, a genetic loss-of-function miRNA strategy, unlike those for protein coding genes, results in simultaneous loss of two mature miRNA species, the 5p sequence and 3p sequence (also referred to as the 'star' sequence and shown to be functional in several studies),[81–83] each with their individual target genes, which would not truly reflect a loss-of-function strategy for one single mature miRNA species.

Cellular and *in vivo* models of cardiac hypertrophy with increased activity and expression of calcineurin (CnA) display decreased

miR-133 expression levels, suggesting that calcineurin is a direct target of miR-133.[84] Although two potential binding sites for miR-133 on the 3′UTR of CnAβ were reported, only one site is sensitive to altered miR-133 levels.[84] Treatment of neonatal rat cardiomyocytes or mice with cyclosporin A, an inhibitor of calcineurin, prevented miR-133 downregulation.[84] In line with this, antisense oligonucleotides designed against the catalytic subunit of CnAβ and NFAT-specific decoy oligonucleotides were both capable of increasing miR-133 expression in cultured primary cardiomyocytes. The 3′UTR sequence of NFATc4, one of the four calcineurin-activated NFAT family members, has two functional binding sites for miR-133. There is functional redundancy between NFAT isoforms in their involvement to evoke a full hypertrophic response,[85–88] and NFATc4 overexpression is sufficient to promote cardiac hypertrophy *in vivo*,[86] although NFATc4-targeted ablation is not sufficient to impair the cardiac hypertrophic response *in vivo*.[86] Gain-of-function approaches showed that miR-133 is able to reduce NFATc4 mRNA levels as well as the hypertrophic response to PE-mediated stimulation in primary cardiomyocytes.[89] Accordingly, miR-133 loss-of-function caused increased NFATc4 expression and a spontaneous hypertrophic response. Jointly, these findings unveil a homeostatic regulation of several players in the same signalling cascade, further lending support to the importance of calcium-activated signalling in pathological remodelling of the heart.

The fibrotic response of the heart contributes to diastolic stiffening and may serve as a precipitate for lethal arrhythmias. One interesting finding in miR-133 knockout mice was the excessive myocardial fibrotic response accompanied by increased cardiomyocyte apoptosis,[78] which could be explained by the fact that miR-133 directly represses connective tissue growth factor,[90] although it could also represent a secondary effect by the loss of cardiomyocytes.[75]

miR-26 was recently shown to regulate myocyte survival and hypertrophy by targeting Gata4.[91] This miRNA is significantly downregulated in the heart after 1 week of pressure overload, allowing for upregulation of Gata4 expression during hypertrophy. Modulation of miR-26 expression levels by adenoviral delivery revealed that this miRNA could inhibit endothelin-1-mediated upregulation of Gata4 in a dose-dependent fashion. In contrast, knockdown of miR-26 using an adenovirus expressing a tandem repeat of antisense miR-26 induced an increase in cell size.[91]

miR-9 was demonstrated to be a negative regulator of cardiac hypertrophy by targeting myocardin, a transcriptional coactivator that promotes cardiac hypertrophic responses.[92,93] Myocardin is expressed at low levels under physiological conditions, but its expression is increased upon hypertrophic stimulation as a downstream target of NFATc3, a player in calcium-induced hypertrophic signalling. Under hypertrophic stimulation with isoproteronol and aldosterone,[92] *in vitro* adenoviral-mediated overexpression of miR-9 as well as the introduction of a miR-9 mimic *in vivo* led to inhibition of cardiac hypertrophy and decreased myocardin expression levels, providing further evidence that myocardin is a direct target gene of miR-9.[93]

Thioredoxin (Trx1) is a ubiquitously expressed antioxidant that suppresses cardiac hypertrophy by inhibiting nuclear factor kappa-light-chain enhancer of activated B cells (NF-kB), Ras, and apoptosis signal-regulating kinase 1 (ASK1),[94,95] thereby negatively regulating protein kinase cascades known to stimulate hypertrophy. miR-98/let-7b family members are upregulated in hearts of transgenic mice overexpressing cardiac Trx1, with miR-98 showing the strongest level of upregulation, indicating Trx1 is a regulator of this miRNA family. miR-98 is downregulated in cardiac hypertrophy and its overexpression contributes to suppression of angiotensin II-stimulated cardiomyocyte hypertrophy.[96] A member of the G1-phase cyclin family, cyclin D2, was identified as a direct target gene of miR-98 with luciferase activity assays showing that miR-98 upregulation significantly reduces cyclin D2 expression levels.[97,98] Altogether, downregulation of Trx1 leads to upregulation of miR-98 and simultaneous inhibition of hypertrophy, wherein cyclin D2 plays an essential role by mediating the anti-hypertrophic actions of miR-98.

8. miRNA regulation of pathological cardiac hypertrophy: agonists

miR-195 was the first-characterized miRNA involved in inducing hypertrophic growth in the adult heart (*Figure 1* and *Table 1*).[59] Array data show that this miRNA is upregulated both in human and mouse hypertrophied hearts. Adenoviral-mediated overexpression of miR-195 is sufficient to induce hypertrophy in cultured neonatal rat cardiomyocytes, and miR-195 overexpression leads to dilated cardiomyopathy and heart dysfunction *in vivo*.[59,99] Although these results suggest that miR-195 is a pro-hypertrophic factor that actively participates in the hypertrophic process, so far no direct targets of this miRNA have been reported in the context of hypertrophic heart disease.

Myosin heavy chains of the cardiac sarcomere are primarily composed of gene products from three myosin heavy chain genes: *Myh6*, which encodes the fast-twitch α-myosin heavy chain and displays higher actomyosin ATPase activity; *Myh7*, which encodes the slow-twitch β-isoform; and low amounts of *Myh7b*, another fast-twitch isoform.[100] Importantly, different species harbour different relative amounts of the α-isoform vs. β-isoforms, so that the adult heart of small rodent species primarily contains the α-isoform, while larger species such as humans are composed of more than 95% of the β-isoform. The difference between the relative compositions of the sarcomere for these proteins determines the contractile properties and energy consumption of the heart.[101] Interestingly, a family of so-called myomiRs was discovered that are encoded by introns of the separate myosin heavy chain genes, where miR-208a, miR-208b, and miR-499 are located within the *Myh6*, *Myh7*, and *Myh7b* genes, respectively. Given that the sarcomere composition in small rodent species displays strong fluctuations of myosin heavy chain isoform expression by thyroid hormone levels and in disease situations, these myomiRs are thought to play a crucial role in the regulation of myosin gene expression and the cardiac stress response in rodent species.[100] *In vivo* deletion of miR-208a results in viable animals with normal cardiac size and function at baseline, but those animals start exhibiting a mild decline in cardiac function up to 5 months of age.[51] In response to cardiac stress, the knockout hearts developed a greater cardiac dysfunction than the control animals, without evident signs of cell hypertrophic growth or fibrosis. miR-208a is controlled by the thyroid hormone receptor (TR)[102] and was demonstrated to be necessary for β-MHC upregulation in response to stress and hypothyroidism.[51] In contrast, overexpression of miR-208a was sufficient to upregulate Myh7 and to elicit cardiac hypertrophy, resulting in cardiac systolic dysfunction.[103]

Although miR-208a is required for cardiac hypertrophic growth and fibrosis in the mouse,[51,103] the role of the co-transcribed miR-208b in these pathological processes remains to be clarified. Furthermore, other, not yet identified miR-208 target genes must also contribute to cardiac hypertrophy since accumulation of isolated b-MHC isoform in the heart is not sufficient for induction of a hypertrophic response.[104] More importantly, in adult rodent myocardium, Myh6 is the major myosin heavy chain, while in humans Myh7 predominates.[102,105] Consequently, miR-208b is likely to be the major myomiR in the adult heart, as suggested by assessment of cardiac miRNA expression.[106] The mechanism by which myomiRs regulate myosin heavy chain expression in the adult heart is an important but unresolved question. In human heart disease, Myh6 is downregulated while Myh7 is upregulated;[101] however, expression of miR-208 family members has not been reported to change in human heart disease.

miR-499 is an evolutionarily conserved muscle-specific miRNA that is encoded in an intron of the *myh7* gene and likely to play a role in myosin gene regulation.[100,107] This miRNA has been implicated in suppression of apoptosis in myocardial infarction and under ischaemia/reperfusion (I/R) conditions.[108] More recently, Shieh *et al.*[109] generated transgenic mice with increased expression levels of miR-499 in the heart by expressing miR-499 under control of the cardiac Myh6 promoter. Mice displaying low levels of miR-499 expression did not develop a cardiac phenotype at baseline. In contrast, animals with elevated levels of miR-499 developed enlarged hearts and increased heart-to-body weight ratios compared with littermate controls. These animals also demonstrated reduced fractional shortening that is indicative of contractile dysfunction. Comparison of global gene expression between miR-499 transgenic mice and littermate controls revealed strong downregulation of several immediate early response genes known to be relevant for the cardiac stress response.[110] Predisposition of miR-499 transgenic animals to cardiac dysfunction may reflect a key role of immediate early response genes in the cardiac transcriptional response to stress.

Recently, miR-199b was depicted as a direct downstream target of calcineurin-NFAT signalling,[111] with increased expression in mouse and human heart failure. NFAT transcription factors,[112] and most prominently NFATc2,[85] have been established as crucial activators of the pathological cardiac remodelling gene response. This miRNA targets the dual-specificity tyrosine (Y) phosphorylation-regulated kinase 1a (Dyrk1a), a NFAT-kinase that was previously linked to dysregulation of NFAT in Down's syndrome.[113,114] miR-199b regulates Dyrk1a in a process that constitutes a pathogenic feed-forward mechanism affecting calcineurin-responsive gene expression.[111] *In vivo* inhibition of miR-199b by a specific antagomir normalized Dyrk1a expression, reduced nuclear NFAT activity, and caused marked inhibition and even reversal of hypertrophy and fibrosis in mouse models of heart failure. These findings indicate that a calcineurin/NFAT-responsive miRNA, miR-199b, destabilizes a regulatory signalling circuit encompassing calcineurin/NFAT and Dyrk1A, leading to acceleration of heart failure.

Rane *et al.*[115] demonstrated upregulation of miR-199a-5p in hypertrophic hearts from mice subjected to pressure overload. In a later stage of heart failure, there is a strong decline in the expression levels of this miRNA. miR-199a-5p was also found to be upregulated in mice overexpressing β1-adrenergic receptor (AR) or β2-AR, two transgenic models of heart failure. *In vitro* studies also confirmed upregulation of miR-199a in neonatal rat cardiomyocytes after isoproterenol stimulation. In contrast, insulin receptor-induced activation of the Akt pathway resulted in downregulation of miR-199a followed by upregulation of the transcription factor hypoxia-inducible factor 1-alpha (HIF-1α), and sirtuin (Sirt1), a histone deacetylase that induces modest hypertrophy and protects cardiomyocytes from apoptosis.[115,116] Both genes have been previously identified as direct target genes of miR-199a in studies where cardiomyocytes were subjected to hypoxia or ischaemia.[115] Under these conditions, miR-199a was found to be downregulated, resulting in HIF-1α-mediated inhibition of hypoxia-induced proapoptotic pathways.[115] Downregulation of HIF-1α has been reported to cause the transition from hypertrophy to heart failure 2 weeks after pressure overload in mice.[117] However, no changes in Sirt1 expression were reported in the same studies.[117] Overexpression of miR-199a *in vitro* with an adenoviral vector was sufficient to increase cell hypertrophy, whereas antisense oligonucleotide-mediated knockdown of miR-199a decreased cardiomyocyte size and attenuated hypertrophy induced by PE.[115] Altogether, these results suggest that alteration of miR-199a expression is a cause of heart failure rather than a consequence.

The signal transducer and activator of transcription 3 (STAT3) is expressed in postnatal hearts and is required for maintenance of cardiac integrity and function.[118] STAT3 knockout mice display elevated levels of miR-199a compared with control animals. Overexpression of miR-199a in cardiomyocytes results in disruption of sarcomere structure and, conversely, suppression of miR-199a abolishes this phenotype. In turn, miR-199a regulates ubiquitin-conjugated enzymes (Ube2i and Ube2g1), and *in vitro* knockdown of these enzymes results in cardiomyocyte dysfunction due to the loss of sarcomeric organization and reduction in α- and β-MHC expression levels.[118] In conclusion, miR-199a is negatively regulated by STAT3, a factor that controls postnatal cardiac integrity, by repressing ubiquitin-conjugating enzymes via post-transcriptional regulation.

Another miRNA upregulated during pressure overload-induced and isoproterenol-induced hypertrophy is miR-23a.[59,66,70,119,120] NFATc3 is known to directly regulate this miRNA, and miR-23 expression is sufficient and required to mediate hypertrophic cardiac growth in response to activation of the calcineurin/NFAT pathway. An antagomir approach to silence miR-23a revealed negative regulator muscle ring finger 1 (MuRF1) as a direct target gene of miR-23a. Absence or low expression of miR-23a *in vitro* and *in vivo* resulted in increased levels of MuRF1 followed by strong inhibition in isoproterenol-induced cardiac hypertrophic growth and improved cardiac function, respectively. Accordingly, mice lacking MuRF1 develop a more pronounced pressure overload-induced cardiac hypertrophic phenotype.[121] miR-23a, similar to miR-199b,[111] is regulated by the calcineurin/NFAT signalling pathway and it will be interesting to know how both miRNA functions integrate to mediate calcineurin/NFAT signalling during cardiac hypertrophy and heart failure.

miR-100 and miR-92 are also involved in the hypertrophic process. miR-100 is upregulated in the failing heart, whereas miR-92 is downregulated.[62] Although studies using mimics of miR-100 in cardiomyocytes show that increased expression of miR-100 represses the expression of the adult genes α-MHC and sarco(endo)plasmic reticulum ATPases (SERCA), also increasing isoproterenol-mediated upregulation of the foetal genes ANF and β-MHC, the direct target genes of miR-100 have not yet been identified. The role of miR-92 in cardiac hypertrophic growth remains to be clarified.

In vitro and *in vivo* studies have revealed that miR-18b is increased in cardiac hypertrophy.[119] Even though miR-18b expression levels are very low in cardiomyocytes, inhibition of endogenous miR-18b is able to induce hypertrophic growth. Moreover, overexpression of

this miRNA in cardiomyocytes decreased myocyte hypertrophy and expression of hypertrophic markers but was not able to inhibit PE-induced cardiomyocyte hypertrophy. These data suggest a causative role for miR-18b in cardiomyocyte hypertrophy, but the functional mechanisms and direct targets remain to be elucidated.

9. miRNA regulation of pathological cardiac hypertrophy: fibrosis

Cardiac hypertrophy and heart failure are accompanied by adverse accumulation of collagens and other extracellular matrix proteins.[122] A variety of miRNAs display dysregulated expression in situations with excessive fibrosis, including miR-29.[122] An interesting feature of this miRNA is its demonstrated ability to simultaneously target multiple genes that target proteins closely associated with the fibrotic response, including elastin, collagens, and fibrillins.[122] Additionally, transforming growth factor beta (TGFβ), a known agonist in the production and deposition of collagens in the heart, was identified as an upstream regulator of miR-29. Real-time PCR analysis of cardiac fibroblasts treated with TGFβ revealed a decrease in miR-29 expression, indicating that downregulation of miR-29 is TGFβ dependent.[122] Downregulation of miR-149 and upregulation of miR-21, -214, and -223 were shown to accompany downregulation of miR-29, but the functional consequence of these changes is unknown.

miR-21 is upregulated in many pathological conditions including cancer and in multiple types of stress.[41,119,122] Thum *et al.*[41] demonstrated that miR-21 contributes to myocardial remodelling through regulation of the ERK-MAP kinase-signalling pathway, a crucial signalling pathway in fibroblast survival and activation.[123] miR-21 expression is strongly induced in the failing myocardium and mostly predominant in fibroblasts. This fibroblast-restricted expression pattern was previously suggested in a model of heart muscle-restricted Dicer depletion where miR-21 was one of the few miRNAs to be upregulated.[5] *In vivo* silencing of miR-21 by a specific antagomir suppressed pathological ERK-MAP kinase signalling and was able to prevent cardiac dysfunction in a mouse model of cardiac pressure overload.[41] Conversely, overexpression of miR-21 led to ERK-MAP kinase activation. Furthermore, validation experiments revealed that Sprouty-1 (SPRY1), a negative regulator of ERK-MAP kinase signalling, is a direct target gene of miR-21.[41] miR-21 regulates fibroblast survival and growth factor secretion that eventually control the extent of interstitial fibrosis and cardiac hypertrophy. Overall, these findings indicate that miR-21, similar to miR-29, can contribute to myocardial remodelling by primarily acting within cardiac fibroblasts. Downregulation of miR-21 could therefore be a beneficial approach to inhibit fibroblast proliferation in heart disease and thereby inhibit secondary cardiac remodelling.

There is apparent disagreement in the literature on the existence and functional role of miR-21 in cardiomyocytes. Thum *et al.*[41] provided compelling evidence that loss- or gain-of-function approaches did not provoke morphological effects in cultures cardiomyocytes. Others observed that miR-21 overexpression induced direct downregulation of Spry2 accompanied by a peculiar phenotype characterized by long, slim cellular outgrowths.[124] In addition, there are other reports demonstrating that miR-21 has a cardioprotective role by targeting *Pdcd4*,[125] and Tatsuguchi *et al.*[119] demonstrated that miR-21 has a mild, but reproducible, inhibitory effect on cardiac hypertrophy, whereas LNA-based miR-21 inhibition induces hypertrophy. This was confirmed by a study showing that phosphatase and tensin homologue (PTEN) is a direct target of miR-21 in cardiac fibroblasts in a murine model of I/R.[126] Moreover, models of ischaemic preconditioning (IP) and I/R showed that silencing of miR-21 exacerbated cardiac injury, indicative of a protective function of miR-21 under these conditions.[125] Recent data from Patrick *et al.*[127] confirmed that mice harbouring a genetic null allele for miR-21 responded normally to cardiac stress induction (aortic constriction, calcineurin overexpression, or angiotensin II treatment). Also, injection of very short (8 nt long) LNA-modified oligonucleotides against the seed region of miR-21 that act briefly in the myocardium, are rapidly excreted from the animal,[127] and are chemically different from the antagomir used by Thum *et al.*[41] failed to suppress the cardiac remodelling response to stress conditions. This implies that the chemical nature of oligonucleotides used for *in vivo* silencing of specific miRNAs has a considerable impact on the phenotypic outcome and therapeutic benefit.[128] These data also indicate that genetic deletion of the complete genomic precursor sequence and simultaneous deletion of both the mature 5′ miRNA and its 3′ star sequence differ from pharmacological strategies aimed to silence only a single mature miRNA.[129]

10. miRNA regulation of physiological cardiac hypertrophy

Left ventricular hypertrophy (LVH) induced by aerobic exercise training is an important physiological compensatory mechanism in response to chronic increase in haemodynamic overload. This phenotype is associated with the addition of sarcomeres in series and in parallel to lengthen the cardiac cell. The increased cross-sectional area contributes to increased ventricular stroke volume and cardiac output, which improves aerobic capacity.[130] Although most of the studies relating miRNA regulation to cardiac hypertrophy focus on the pathological aspects of cardiac growth, recently these non-coding RNA molecules have shown to be also involved in physiological cardiac hypertrophy. A recent study has shown that swimming exercise training induced physiological LVH and that this was associated with altered expression of miRNAs that target renin–angiotensin system (RAS) genes.[131] Although the implication of specific miRNAs regulating RAS genes in cardiac hypertrophy induced by exercise training has not been previously described, reports confirm that miR-27a and -27b[132] directly regulate angiotensin-converting enzyme (ACE) and that ACE2 is a direct target gene of miR-143.[133] In fact, expression of these miRNAs in exercised hearts inversely correlates with the mRNA levels of ACE genes and strongly suggests that a decrease in miR-143 could upregulate cardioprotective genes in the heart, while an increase in miR-27 expression would inhibit ACE levels. In another recent study, where female rats were subjected to intensive swimming training,[134] miR-1, miR-133a, and -133b were downregulated as observed in pathological cardiac hypertrophy but without changes in pathological markers. Surprisingly, miR-29c expression was strongly increased and correlated with a decrease in collagen I and collagen III, indicating that increased miR-29c expression and decreased collagen gene expression in the heart are associated with aerobic high endurance training.

Recently, plasma-circulating miRNAs were shown to control cellular processes, but the contribution of these circulating miRNAs to human exercise remains poorly described. To determine whether circulating miRNAs are dynamically regulated in response to acute exhaustive

exercise and sustained exercise training, miRNA expression levels were determined in competitive male rowers before and after a 90-day period of rowing training.[135] Distinct patterns of circulating miRNA response to exercise were observed: miRNAs up-regulated by acute exercise before and after sustained training (miR-146a and miR-222), miRNAs responsive to acute exercise before but not after sustained training (miR-21 and miR-221), miRNAs responsive only to sustained training (miR-20a), and non-responsive miRNAs (miR-133a, miR-210, miR-328). Although these results suggest a potential value of miRNAs as exercise biomarkers, more functional studies are necessary to establish these specific miRNAs as physiological players of training-induced cardiac remodelling.

11. Therapeutic potential of miRNAs: future perspective

Recent developments in the field of cardiovascular biology have attributed a crucial role to miRNAs in the onset and development of cardiac disease. In fact, such studies, aside from emphasizing the great potential of miRNAs as therapeutic targets in cardiac disease, also bring new possibilities for the development of innovative therapeutic approaches. It was recently demonstrated (and discussed throughout this review) that a number of drugs of clinical relevance could modulate miRNA expression both *in vitro* and *in vivo*, in cellular and animal models of pathological hypertrophy, respectively. Progression of miRNA-based therapeutics is, however, confronted with several dilemmas mostly concerning (specific) delivery to target cells or organs, as in the heart. Although specific chemical modifications have been shown to prevent degradation of synthetic nucleotides in systemic circulation, much remains to be explored regarding the pharmacokinetics and pharmacodynamics of such synthetic RNA molecules in order to improve delivery efficacy. In addition to the obvious need for further mechanistic insights, the advances made thus far in the cardiovascular field regarding the biology and function of miRNAs will eventually allow for an accurate transition of knowledge from bench to bedside and transformation of miRNA-based therapies into an expected reality.

12. Summary

miRNAs have reshaped our understanding of the pathogenesis of many diseases. In the past decade, studies on miRNAs and cardiac hypertrophy have resulted in significant achievements in unveiling the signalling pathways in cardiac hypertrophy. Although there is growing evidence that miRNAs play a pivotal role in many cellular processes that contribute to cardiac hypertrophy, most of the miRNAs differentially expressed in humans have not yet been analysed and their functions need to be revealed. By studying these miRNAs and their targets and showing their essential roles during cardiac hypertrophy, we will most likely uncover novel cardiac hypertrophic mechanisms and create innovative therapeutic approaches. It is to be expected that by preventing and reversing the consequences of cardiac hypertrophy, miRNA-based diagnostic and therapeutics will greatly improve patients' quality of life and extend their longevity. With the strategies for combating cardiac hypertrophy facing a rapid development, a miRNA-based novel treatment for heart failure is an anticipated reality.

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