



## Review article

# Small changes can make a big difference – MicroRNA regulation of cardiac hypertrophy

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## ABSTRACT

Cardiac hypertrophy is a thickening of the heart muscle that results in enlargement of the ventricles, which is the primary response of the myocardium to stress or mechanical overload. Cardiac pathological and physiological hemodynamic overload causes enhanced protein synthesis, sarcomeric reorganization and density, and increased cardiomyocyte size, all culminating into structural remodeling of the heart. With clinical evidence demonstrating that sustained hypertrophy is a key risk factor in heart failure development, much effort is centered on the identification of signals and pathways leading to pathological hypertrophy for future rational drug design in heart failure therapy. A wide variety of studies indicate that individual microRNAs exhibit altered expression profiles under experimental and clinical conditions of cardiac hypertrophy and heart failure. Here we review the recent literature, illustrating how single microRNAs regulate cardiac hypertrophy by classifying them by their prohypertrophic or antihypertrophic properties and their specific effects on intracellular signaling cascades, ubiquitination processes, sarcomere composition and by promoting inter-cellular communication.

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## 1. Introduction

Heart failure, or the inability of the heart to meet hemodynamic demands, represents the end-stage of various forms of cardiac disease [1]. In the Western world, the prevalence and incidence of heart failure are increasing steadily and heart failure is now the leading cause of hospitalization in the elderly. The leading cause of heart failure is left ventricular hypertrophy, defined as an increase in heart size

without a change in myocyte number, because chronically hypertrophied hearts remodel and dilate [2, 3]. Cardiac hypertrophy is the primary response of the heart to stress caused by pathological and physiological hemodynamic overload and entails enhanced protein synthesis, sarcomeric reorganization and density, increased cardiomyocyte size, all culminating into structural remodeling of the heart. With clinical evidence demonstrating that sustained hypertrophy is a key risk factor in heart failure development, much effort is centered on the identification of signals and pathways leading to pathological hypertrophy for future rational drug design in heart failure therapy [4–6].

MicroRNAs (miRs) are ~22 nucleotide long, evolutionary conserved, noncoding RNA molecules that play an important role in

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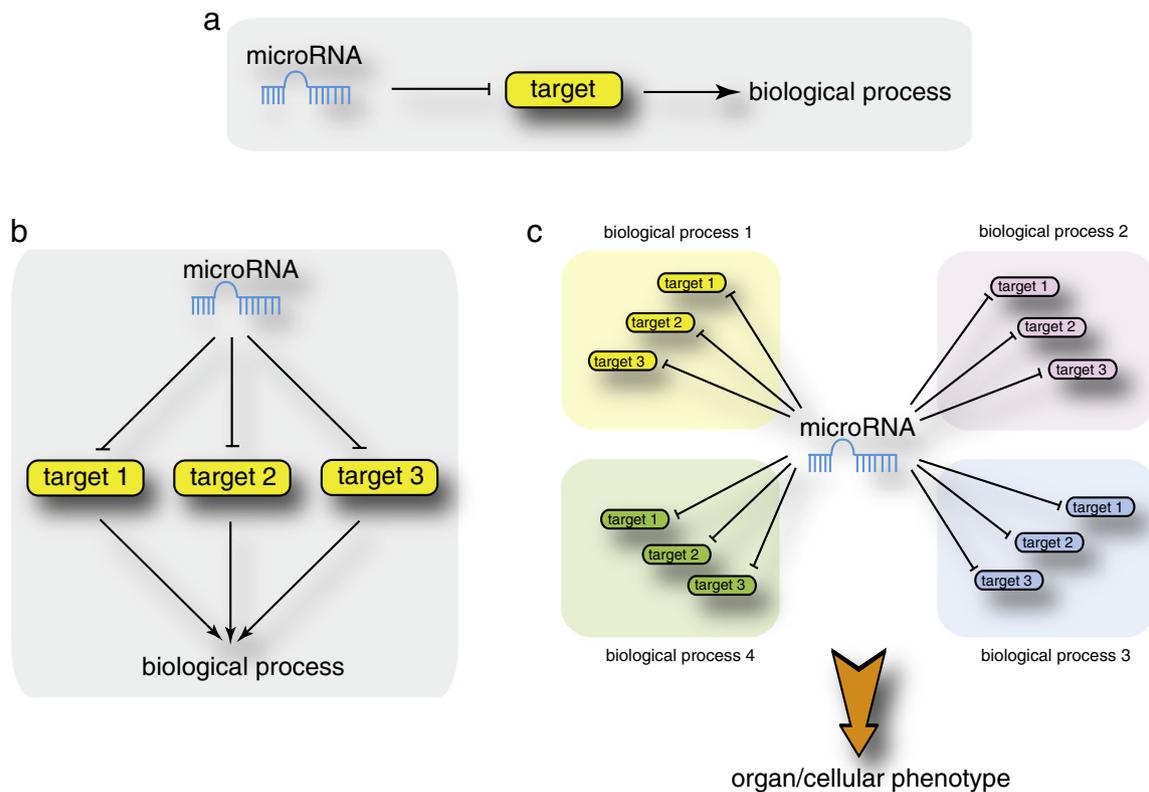
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post-transcriptional gene regulation [7–10]. By imperfect binding to the 3' untranslated region (UTR) of messenger RNAs (mRNAs), microRNAs are able to suppress translation and/or induce mRNA degradation. MicroRNAs are transcribed by RNA polymerase II as primary microRNAs (pri-miRs) that can encode one or multiple microRNAs [7]. Pri-miRs create imperfectly base-paired hairpins cleaved by the RNase III endonuclease Droscha to create multiple 60–100 basepair long hairpin-like structures called precursor microRNAs (pre-miRs) [11]. Hereafter, the pre-miR is transported from the nucleus in an exportin 5-dependent manner and cleaved again by the RNase III Dicer to yield miR-miR\* duplexes [12]. In most cases, the start sequence of the opposite strand (miR\*) will be degraded, yielding a mature microRNA. The mature strand is loaded into the RNA-induced silencing complex (RISC), which guides the mature strand to cognate target mRNAs to induce post-transcriptional gene silencing [7].

MicroRNAs are thought to 'fine tune' gene expression under homeostatic circumstances, but under conditions of stress, the regulatory functions of microRNAs become far more pronounced and seem to play more decisive roles in disease processes. The identification of microRNA targets is still quite a challenging undertaking, since microRNA target selection is based upon partial Watson–Crick base pairing in short stretches of sequences. Accordingly, the open source predictive algorithms that were developed to predict potential microRNA targets yield extensive lists filled with many false positive targets, and insufficient information on cellular network information [13]. In addition, most studies to date mainly focused on very a simplistic role of a single microRNA robustly targeting one single mRNA and thereby having an effect on one particular pathway or biological process (Fig. 1a). It is, however, more probable that microRNAs have evolved as gene regulators that employ modest inhibitory effects on many mRNAs targets simultaneously rather than dramatic regulation of single genes (Fig. 1b). There are examples in the literature in

support of this notion, where miR-29 regulates multiple proteins involved in the fibrotic response of the heart [14] and miR-145 coordinately regulating multiple genes involved in stem-cell pluripotency [15]. On a more sophisticated level of complexity, it also becomes plausible that single microRNA gene regulatory networks cooperate to form a higher-order, microRNA-based regulatory axis. The biological effects of such a regulatory axis would only become visible when multiple microRNAs are simultaneously (mis)expressed. The combined effects on DNA, RNA and protein can be interrogated by techniques to visualize genome-wide coverage of the cellular transcriptome and proteome, allowing the creation of models where the combinatorial activity and dynamical properties of microRNA-based networks controlling cellular behavior are visualized (Fig. 1c) [16].

Because the nucleases Droscha and Dicer have a critical role during proper microRNA processing, early studies on the impact of miRs at onset and development of cardiac disease used the approach of deleting either one of these processing enzymes which impacts global microRNA maturation and expression. The first report using this strategy involved germline deletion of the *Dicer* gene with concomitant effects on microRNA production in the entire organism and throughout all developmental stages [17]. The fact that this deletion caused embryonic lethality provided strong indications that proper processing and microRNA expression is required for normal fetal development. To circumvent the observed lethality and focus further on microRNA function in the cardiovascular system, a series of mice were generated with cardiac-specific ablation of *Dicer*, either in the early embryonic heart (driven by a *nkx2.5-Cre* deleter strain) [18,19], the postnatal myocardium within a few days after birth (driven by a *myh6-Cre* deleter strain) [20], or conditionally in the juvenile or adult murine heart (driven by a *myh6*-driven, tamoxifen activated MerCreMer deleter strain) [21]. All of the above approaches disturbed microRNA processing and resulted in cardiac dysfunction: targeting



**Fig. 1.** Different models of microRNA-based regulation of gene expression. a) Simplistic model where one microRNA robustly affects the expression of one single target leading to one single biological process. b) An evolving model of microRNA function, involving modest repression of several target mRNAs in a common biological process. c) MicroRNA networks that cooperate to form a higher-order regulatory axis. The combinatorial activity and dynamical properties of microRNA-based networks integrate to control cellular behavior.

*Dicer* in early cardiac development provoked lethal intra-uterine cardiac hypoplasia, while postnatal deletion of *Dicer* generated cardiomyopathies displaying pathological cardiac gene expression, abnormal sarcomere structure and cardiomyocyte hypertrophy. Rao and colleagues interrupted microRNA processing at a more proximal step by striated muscle-specific deletion of DiGeorge syndrome critical region 8 (*Dgcr8*), which is associated to *Drosha* during intranuclear microRNA processing [22]. *Dgcr8* deletion induced left ventricular remodeling and progression to heart failure, and further provided evidence for an essential role of microRNAs during cardiac development and homeostasis [22].

Although these studies provide strong evidence for a critical combinatorial role for proper microRNA processing and maturation in cardiac development and homeostasis, they failed to clarify which signaling pathways and/or biological processes in the heart are being regulated by single microRNAs. Subsequently, a variety of studies demonstrated that individual microRNAs exhibit altered expression profiles under experimental and clinical conditions of cardiac hypertrophy and heart failure [23,24], which sparked experimental studies that revealed the precise biological function of single microRNAs in the genesis of cardiac hypertrophy and heart failure. Here we review this recent literature, illustrating how single microRNAs regulate cardiac hypertrophy (Table 1) by affecting signaling cascades and ubiquitination processes, changing sarcomere composition and by intercellular communication (Fig. 2).

## 2. Anti hypertrophic microRNAs: the abundant microRNA-1/-133 cluster, miR-9 and miR-98

MEF2 and SRF cooperatively regulate the expression of two bicistronic microRNA clusters encoding miR-133a-1/miR-1-2 and miR-133a-2/miR-1-1 in cardiac and skeletal muscle [25,26]. A third bicistronic microRNA cluster comprised of miR-206 and miR-133b is expressed specifically in skeletal muscle but not in the heart [27]. miR-133a-1 and miR-133a-2 are identical in sequence, whereas miR-133b differs by only 2 nt at the 3' terminus [28]. Similarly, miR-1-1 and miR-1-2 are identical and differ from miR-206 by 4 nt [27]. miR-1-1/miR-1-2 are among the most abundantly expressed microRNAs in the human heart [29–31], providing a first hint that they may have a specified role in cardiomyocyte morphology and function. *Twinfilin1* (*Twf1*), a cytoskeletal regulatory protein that binds to actin monomers, preventing their assembly into filaments, was reported to be a target

gene of miR-1 [30]. The expression level of *Twf1* is low in the adult heart and inversely correlates with high expression of miR-1. There are two functional binding sites for miR-1 on the 3'UTR sequence of *Twf1* gene, unlike *Twf2*, a homolog protein, which has no miR-1 binding sites. Downregulation of miR-1 by hypertrophic stimuli, such as aortic banding or alpha-adrenergic stimulation with phenylephrine (PE), results in increased *Twf1* expression, which induces cardiac hypertrophy by regulation of cardiac cytoskeleton [30]. In fact, as *Twf1* overexpression is sufficient to induce cardiac hypertrophy in neonatal rat cardiomyocytes [30], appropriate manipulation of *Twf1* expression might be of therapeutic relevance to attenuate cardiac hypertrophy.

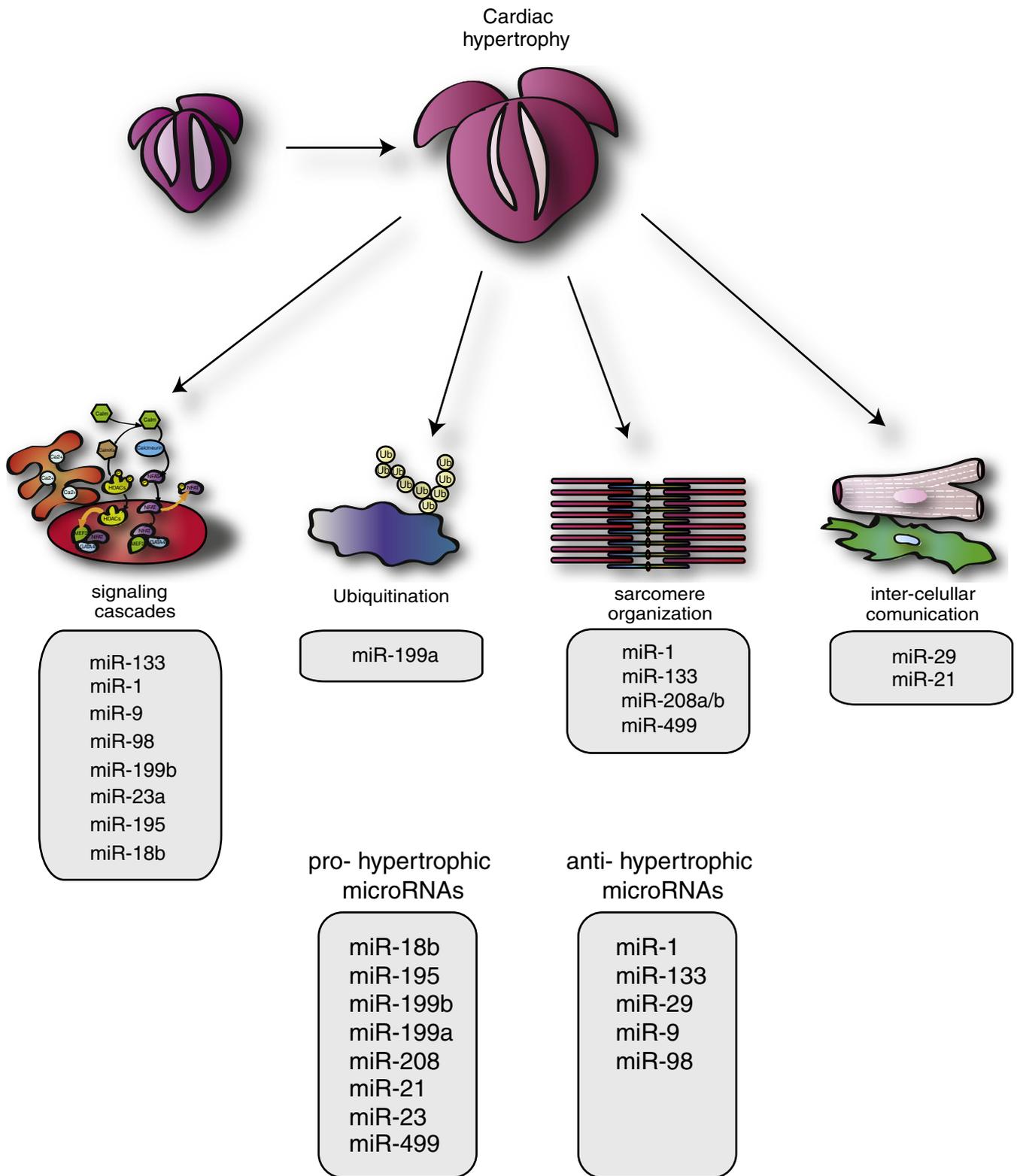
Insulin-like growth factor (IGF-1), a key regulator of growth, survival and differentiation in most cell types [13], is a validated miR-1 target gene. In models of cardiac hypertrophy and failure, there is a repression of miR-1 and concomitant upregulation of IGF-1 [32]. In line, acromegalic patients, where IGF-1 is over-expressed following aberrant synthesis of this growth hormone, display increased cardiac mass and wall thickness. IGF-1 has only one potential miR-1 binding site on its 3'UTR sequence, proven to be functional by luciferase activity assays [32]. Furthermore, IGF-1 not only is a target of miR-1, but miR-1 expression depends on the activation state of IGF-1, through activation of the PI3K/AKT signaling pathway and repression of its downstream transcription factor *Foxo3a*, providing evidence for the existence of a new feed forward paradigm to understand how IGF-1 modulates cardiac and skeletal muscle structure and function [32].

miR-1 was also demonstrated to negatively regulate the expression of the key calcium-signaling components calmodulin (CaM) and myocyte enhancer factor-2a (*Mef2a*) [33]. In line, reduction of miR-1 expression was accompanied by elevated expression of calmodulin and *Mef2a* at the protein level. Increases in intracellular calcium and concomitant activation of the calcium binding protein CaM, activate the phosphatase calcineurin (CnA), which in turn will dephosphorylate members of the nuclear factor of activated T cells (NFAT) [34]. As a consequence, NFAT translocates to the nucleus to drive the expression of a heart failure gene program [34,35]. Two of the three CaM-encoding genes, *Calml1* and *Calml2*, contain highly conserved miR-1 seed match sequences in their 3'UTR. In a mouse model of heart failure ( $\alpha$ MHC-CnA) [34], miR-1 is downregulated while *Calml1* and *Calml2* are upregulated at the protein level [33], consistent with a direct regulatory role of miR-1 and establishing a novel mechanism for modulating calcium-activated stress signaling by post-transcriptional regulation of CaM. Overexpression of *Mef2a*, a downstream target of the CnA/NFAT

**Table 1**

Anti- and pro-hypertrophic microRNAs involved in cardiac hypertrophy and their described target genes with established function. Different expression of each microRNA is described as 'down' and 'up', representing down- or upregulation during cardiac hypertrophy, respectively. Target genes of each miRNA were selected based on in vitro or/and in vivo validation experiments.

|                             | miRNA    | Expression  | Validated target                                    | Function of target gene(s)   |
|-----------------------------|----------|-------------|---|--|
| Anti-hypertrophic microRNAs | miR-1    | Down        | <i>Twf1</i>   | Cytoskeleton regulatory protein                                    |
|                             |          |             | <i>IGF-1</i>  | Pro-hypertrophic regulator of growth, survival and differentiation |
|                             | miR-133  | Down        | <i>Cam1</i> , <i>Cam2</i> , <i>Mef2</i>             | Pro-hypertrophic signaling   |
|                             |          |             | <i>CnA<math>\beta</math></i>                        | Pro-hypertrophic protein phosphatase                               |
|                             |          |             | <i>NFATc4</i>                                       | Pro-hypertrophic transcription factor                              |
|                             |          |             | <i>Rhoa</i> , <i>Cdc42</i>                          | GTP binding proteins, cytoskeletal composition                     |
| Pro-hypertrophic microRNAs  | miR-29   | Down        | <i>NELF-A/Whsc2</i>                                 | Negative regulator of RNA polymerase                               |
|                             | miR-9    | Down        | <i>ELN</i> , <i>FBN</i> , <i>Col1</i> , <i>Col3</i> | Fibrotic genes   |
|                             | miR-98   | Down        | <i>Myocardin</i>                                    | Pro-hypertrophic transcriptional cofactor                          |
|                             | miR-18b  | Up          | <i>CyclinD2</i>                                     | Pro-apoptotic protein phosphatase                                  |
|                             | miR-195  | Up          |   |  |
|                             | miR-199b | Up          | <i>Dyrk1a</i>                                       | Anti-hypertrophic kinase   |
|                             | miR-199a | Up          | <i>Hif-1<math>\alpha</math></i>                     | Transcription factor, regulate hypoxia induced genes               |
|                             |          |             | <i>Ube2i</i> , <i>Ube2g1</i>                        | Ubiquitin-conjugating enzymes                                      |
|                             | miR-208  | Up          | <i>THRAP</i>  | Anti-hypertrophic signaling  |
|                             | miR-21   | Up          | <i>Spry1</i>  | Inhibitor of the Ras/MEK/ERK pathway                               |
| miR-23                      | Up       | <i>Murf</i> | Anti-hypertrophic protein                           |  |
| miR-499                     | Down     | <i>CnA</i>  | Pro-hypertrophic protein phosphatase                |  |



**Fig. 2.** Schematic representation of the processes responsible for the development of cardiac hypertrophy and indication of the specific involved microRNAs. MicroRNAs can regulate cardiac hypertrophy through their specific effects on signaling cascades (miR-133, miR-1, miR-9, miR-98, miR-199b, miR-23a), ubiquitination processes (miR-199a), sarcomere organization (miR-1, miR-133, miR-208a/b and miR-499) and inter-cellular communication (miR-29 and miR-21).

signaling pathway is sufficient to induce cardiac hypertrophy [36]. Ikeda and colleagues identified two conserved and functional miR-1 binding sites in the 3'UTR sequence of Mef2a [33]. Moreover, gain and loss of function studies for miR-1 showed an inverse correlation

between miR-1 expression and Mef2a protein levels in neonatal rat cardiomyocytes [33].

Calcineurin is negatively regulated by the other member of the miR-133/miR-1 bicistronic cluster, miR-133 [37]. Although two

potential binding sites for miR-133 on the 3'UTR sequence of CnA $\beta$  were reported, only one site displayed sensitivity to altered miR-133 levels [37]. The mechanism of interaction between miR-133 and calcineurin was established using cellular models and in vivo models of cardiac hypertrophy, where increased activity and expression of calcineurin were accompanied by a decrease in miR-133 expression levels. Treatment of neonatal rat cardiomyocytes or mice with cyclosporin A, an inhibitor of calcineurin, prevented miR-133 downregulation [37]. In line, antisense oligonucleotides designed against the catalytic subunit of CnA $\beta$  and NFAT specific decoy oligonucleotides were both capable of increasing miR-133 expression in cultured primary cardiomyocytes [37]. NFATc4 is one of the four calcineurin-activated NFAT family members and its transgenic overexpression suffices to promote cardiac hypertrophy in vivo [34], although functional redundancy exists between NFAT isoforms in their requirement to evoke a full hypertrophic response [38,39]. There are two conserved and functional binding sites for miR-133 on the 3'UTR sequence of NFATc4. Gain of function approaches demonstrated that miR-133 is able to reduce NFATc4 mRNA levels, as well as the hypertrophic response to phenylephrine mediated agonist-stimulation in primary cardiomyocytes [37]. On the other hand, miR-133 loss-of-function caused NFATc4 overexpression and a spontaneous hypertrophic response. Thus, by studying miR-133 expression and its target genes, these findings have unmasked homeostatic regulation of several players in the same signaling cascade, further lending support to the importance of calcium-activated signaling in pathological remodeling of the heart.

Bioinformatic approaches suggested that RhoA and Cdc42, members of the Rho subfamily of small GTP-binding proteins associated with cytoskeletal and myofibrillar rearrangements during cardiac hypertrophy [40] could be target genes of miR-133. Care et al. also postulated that NELFA/Whsc2, which was previously demonstrated to be involved in cardiac hypertrophy [40], could be regulated by miR-133 [40,41]. Using both neonatal and adult cardiomyocytes, suppression or overexpression of miR-133 caused an increase or a decrease in the level of those three proteins, respectively [40]. Although these three target genes were previously involved in cardiac hypertrophy, these studies uncovered the post-transcriptional mechanism driving their expression in the hypertrophied heart.

miR-9 was demonstrated to be a negative regulator of cardiac hypertrophy by targeting myocardin, a transcriptional coactivator that promotes cardiac hypertrophic responses [42,43]. While myocardin is expressed at very low levels under physiological conditions, it is increased in expression upon hypertrophic stimulation as a downstream target of NFATc3, a player in calcium-induced hypertrophic signaling. Expression of myocardin is elevated in response to hypertrophic stimulation with isoproterenol and aldosterone [43]. Under those conditions, adenoviral-mediated overexpression of miR-9 in vitro as well as the introduction of a miR-9 mimic in vivo led to inhibition of cardiac hypertrophy and decrease in myocardin expression levels, providing further evidence that myocardin is a direct target of miR-9 [43].

Thioredoxin1 (Trx1), an ubiquitously expressed antioxidant, can suppress cardiac hypertrophy through multiple mechanisms, by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), Ras and apoptosis signal-regulating kinase 1 (ASK-1) [44,45], thereby negatively regulating protein kinase cascades known to stimulate hypertrophy. miR-98/let-7 family members are upregulated in hearts of transgenic mice with cardiac overexpression of Trx1, with miR-98 exhibiting the highest level of upregulation by Trx1 in the mouse heart and indicating Trx1 as a regulator of this microRNA family [46]. In line, miR-98 was shown to be downregulated in cardiac hypertrophy and its overexpression by adenoviral delivery contributed to the suppression of angiotensin II-stimulated cardiomyocyte hypertrophy. Cyclin D2, a member of the G1-phase cyclin family and playing an essential role in mediating cardiac hypertrophy [47,48], was predicted as one of the miR-98 target genes by

virtue of the existence of three potential miR-98 binding sites in its 3'UTR sequence [46]. Additionally, luciferase activity assays demonstrated that miR-98 upregulation significantly reduces cyclin D2 expression levels. Altogether, downregulation of Trx1 leads to upregulation of miR-98 and concomitant suppression of hypertrophy, wherein cyclin D2 plays an essential role by mediating the anti-hypertrophic actions of miR-98.

### 3. MicroRNA-29/-21: where fibrosis meets hypertrophy

Cardiac hypertrophy and heart failure are accompanied by characteristic changes in the expression of collagen-related genes [14]. miR-29 is a regulator of cardiac fibrosis by targeting multiple mRNAs that encode proteins involved in fibrosis, including collagen, fibrillin and elastin [14]. Cardiac fibrotic genes such as elastin, fibrillin, collagen type I, alpha 1 and 2 and collagen type III, alpha 1 contain one or more conserved seed sequences for miR-29 and were identified as target genes of this microRNA [14]. Knockdown of miR-29 in vivo by using specific cholesterol-modified and reverse complement oligonucleotides to this microRNA stimulated collagen production in vivo. Additionally, transforming growth factor beta (TGF $\beta$ ), a known agonist in the production and deposition of collagens in the heart was identified as an upstream regulator of miR-29 [14]. Real-time PCR analysis on cardiac fibroblasts treated with TGF $\beta$  revealed a decrease in miR-29 expression indicating that downregulation of miR-29 is TGF $\beta$  dependent [14].

miR-21 has been found to be upregulated in many pathological conditions including cancer and after multiple types of cardiac stress [49,50]. Thum and colleagues examined the function of miR-21 in cardiac remodeling and demonstrated its actions on cardiac geometry and function by regulating the ERK–MAPK signaling pathway in cardiac fibroblasts [51]. miR-21 expression is strongly induced in the failing myocardium and most predominantly in fibroblasts. The fibroblast-restricted expression pattern of microRNA-21 was previously suggested in a model of heart muscle-restricted Dicer depletion, where miR-21 was one of the few microRNAs to be upregulated, likely in a secondary response to the cardiac remodeling processes of the Dicer-depleted myocardium [12]. miR-21 inhibition using a specific cholesterol-modified antagomir induced an ERK–MAPK signaling-sensitive apoptotic response in cardiac fibroblasts. Conversely, overexpression of this microRNA led to ERK–MAPK activation, suggesting that miR-21 is a mediator of ERK–MAPK signaling, a crucial signaling pathway for fibroblast survival and activation, also known to play important roles in many cancers [52]. Furthermore, validation experiments revealed Sprouty-1 (SPRY1), a negative regulator of ERK–MAPK signaling, as a direct target gene of miR-21. Treatment of mice subjected to pressure overload of the left ventricle by transverse aortic constriction (TAC) with an antagomir against miR-21, normalized changes in SPRY1 expression, reduced cardiac MAPK activity, inhibited interstitial fibrosis and attenuated cardiac dysfunction. These findings indicate that miR-21, like miR-29; can contribute to myocardial remodeling by primarily acting within cardiac fibroblasts.

Tatsuguchi and colleagues [50] demonstrated that miR-21 expression has a mild, but reproducible, inhibitory effect on cardiac hypertrophy, whereas LNA-based miR-21 inhibition induces hypertrophy. This was confirmed by a study by Roy et al. [53] demonstrating that phosphatase and tensin homologue (PTEN) is a direct target of miR-21 in cardiac fibroblasts in a murine model of ischemia/reperfusion (I/R). Recently, readouts from a model of ischemic preconditioning (IP) and I/R have shown that silencing miR-21 exacerbated cardiac injury, suggesting a protective function of miR-21 under these conditions [54]. Furthermore, similar data was obtained from in vitro hypoxia/reoxygenation studies using isolated primary cardiac muscle cell preparations, indicating that miR-21 is also expressed in cardiac muscle cells and has separate cellular functions in this cell type, likely unrelated to cardiomyocyte hypertrophy. Recent data from Patrick et al. [55] demonstrated that mice harboring a genetic null allele of the

complete miR-21 sequence responded normally to stress induction by TAC, calcineurin overexpression or angiotensin II treatment. Also, injection of very short (8-nucleotide long) locked nucleotide acid (LNA) modified oligonucleotides against the seed region of miR-21, which are short-acting in the myocardium and excreted fast from the animal [6], chemically different from the 22 nucleotide long, cholesterol conjugated oligonucleotides used by Thum et al. [51], failed to block the remodeling cardiac response to stress conditions, suggesting that the chemical nature of oligonucleotides used for miR-21 silencing approach *in vivo* has a significant impact on the phenotypic outcome and therapeutic benefit [56]. These data also indicate that genetic ablation strategies, where genetic deletion of the complete genomic precursor sequence and simultaneous deletion of both the mature 5' microRNA and its 3' star sequence, differs from pharmacological strategies aimed to silence only one mature microRNA. Finally, the phenotypic differences can also derive from the genetic strategy on the one hand, where miR-21 was ablated from the early embryo allowing for biological compensatory mechanisms, and the more instant, antagomir-mediated silencing strategy in the adult myocardium, on the other [57].

#### 4. Pro-hypertrophic microRNAs: myomiRs regulating contractility and hypertrophy

miR-208a, miR-208b and miR-499 belong to a family of microRNAs embedded in myosin genes, designated MyoMirs [58]. MHC proteins,  $\alpha$ -MHC (Myh6) and  $\beta$ -MHC (Myh7) are responsible for proper cardiac muscle contraction. In the embryonic rodent heart the slow ATPase  $\beta$ -MHC isoform is predominant, while the fast ATPase  $\alpha$ -MHC isoform is more highly expressed in adult rodent heart. Cardiac stress results in a switch of myosin isoform content with relative upregulation of  $\beta$ -MHC and downregulation of  $\alpha$ -MHC, resulting in a negative effect on cardiac contractility and function. miR-499 is an evolutionarily conserved muscle-specific microRNA that is encoded within an intron of the *myh7b* gene and seems to play a role in myosin gene regulation [58,59]. Although this microRNA is highly expressed in the heart under normal conditions [58,60], less miR-499 expression was observed in ischemic hearts and neonatal rat cardiomyocytes exposed to anoxia [61]. Transgenic mice overexpressing miR-499 in the heart-muscle and subjected to ischemia/reperfusion (I/R) displayed decreased levels of apoptosis, smaller infarct size and better cardiac function compared to the wild type control animals. Conversely, knockdown of miR-499 with a specific antimir resulted in increased apoptosis, increased myocardial infarct size and decreased cardiac function upon I/R, suggesting a crucial role for miR-499 in cardiac survival and function.

The calcineurin  $\alpha$  and  $\beta$  subunits were predicted to be target genes of miR-499 [61]. There are functional binding sites for miR-499 in the 3'UTR sequence of both subunits of calcineurin, and hearts of miR-499 transgenic mice displayed reduced levels of the calcineurin  $\alpha$  and  $\beta$  subunits after I/R, while knockdown of miR-499 elevated their expression levels. Short hairpin-mediated knockdown of both calcineurin subunits resulted in inhibition of anoxia-induced cell death. Anoxia can induce apoptosis by promoting mitochondrial death pathways. Dynamin-related protein1 (Drp1), which is required for mitochondrial fission, demonstrated to be the link between calcineurin and initiation of cell death program in cardiomyocytes with calcineurin-mediated dephosphorylation of Drp1 causing its translocation to mitochondria and promoting mitochondrial fission, with miR-499 involved in regulating the mitochondrial fission machinery.

More recently, Shieh and colleagues [62] generated transgenic mice with increased expression levels of miR-499 in the heart by expressing miR-499 under control of the cardiac Myh6 promoter. While mice expressing lower levels of miR-499 did not show a cardiac phenotype under basal conditions, the higher expressing miR-499 animals developed enlarged hearts and increased heart-to-body

weight ratios compared to littermate controls. These animals also demonstrated decreased fractional shortening indicating contractile dysfunction. Comparison of global gene expression between miR-499 transgenic mice and littermate controls revealed strong downregulation of the immediate early response genes, early growth response 1 (Egr1), FBJ osteosarcoma oncogene (*fos*) and early growth response 2 (Egr2), known to be important in the cardiac stress response [63–65]. The indication that miR-499 transgenic mice develop or are predisposed to cardiac dysfunction may reflect a key position of immediate early response genes in the hierarchy of the cardiac transcriptional response to stress.

Other microRNAs encoded by myosin genes are miR-208a, encoded by *myh6*, and miR-208b, encoded by *myh7*. miR-208a expression is clearly detectable in the adult mouse heart but is very low during heart development. The exact opposite is observed for miR-208b, which is in line with the fact that these microRNAs are co-transcribed with their host genes and follow the MHC isoform switches shortly after birth in the rodent heart [62]. Both microRNAs have similar seed regions, indicating the potential regulation of common target genes but at distinct developmental stages. miR-208a, encoded by an intronic region of the  $\alpha$ -MHC gene, which in turn is controlled by the thyroid hormone receptor (TR) [63], was demonstrated to be necessary for  $\beta$ -MHC upregulation in response to stress and hypothyroidism [64]. In fact, during pressure overload in mice, miR-208a functions in a negative feedback loop repressing its own expression and that of  $\alpha$ -MHC, while facilitating the upregulation of  $\beta$ -MHC [5]. Postnatal, heart-muscle restricted transgenic overexpression of miR-208a in the mouse also resulted in a clear hypertrophic response, but not in changes of the sarcomeric structure integrity or ANF transcript levels [62]. Manipulation of miR-208a expression in neonatal rat cardiomyocytes influenced cell growth but not other hypertrophy-related processes such as re-activation of the fetal gene program. Although miR-208a is required for cardiomyocyte hypertrophy and fibrosis in the mouse, the role of the co-transcribed miR-208b in these pathological processes remains to be clarified. Moreover, other, yet to be determined mir-208 target genes besides the slow  $\beta$ -MHC isoform, must play a role in cardiac hypertrophy, since isolated slow  $\beta$ -MHC isoform accumulation in the heart is not sufficient for the induction of a hypertrophic response [65]. More importantly, the relevance of these miR-208-dependent mechanisms in human cardiac hypertrophy and failure is less straightforward, since the MHC-isoform switches in the adult human heart, where the slow  $\beta$ -MHC isoform is already the primary isoform, are much less pronounced compared to the rodent heart [63,66].

#### 5. Pro-hypertrophic microRNAs: miR-23/-199a/-199b influence signaling and ubiquitination

Recently, miR-199b was reported as a direct downstream target of calcineurin/NFAT signaling [67]. NFAT transcription factors [68], and in particular NFATc2 [35], have been established as crucial activators of the pathological remodeling gene response. miR-199b is upregulated upon activation of calcineurin and in hearts of a murine model of cardiac pressure overload (TAC), two well established animal models of pathological cardiac remodeling. miR-199b targets the dual specificity tyrosine-phosphorylation-regulated kinase 1a (Dyrk1a), an NFAT-kinase that was previously linked to dysregulation of NFAT in Down's syndrome [69,70]. In line, reduced Dyrk1a protein expression levels in cardiac biopsies of patients with heart failure inversely correlated with increased expression of miR-199b. Mice treated with an antagomir specific to silence miR-199b and subjected to pressure overload demonstrated efficient repression of miR-199b expression, normalized levels of Dyrk1A expression, prevention of fetal gene genes, attenuated levels of interstitial fibrosis and reduced cardiomyocyte size. Analysis of cardiac function by echocardiography revealed that miR-199b silencing normalized fractional shortening

and systolic/diastolic contractile defects even in the setting of continuous pressure overload. In conclusion, these findings indicate that a calcineurin/NFAT responsive microRNA, miR-199b, destabilizes a regulatory signaling circuit encompassing calcineurin/NFAT and Dyrk1a, leading to acceleration of heart failure.

From the same family of microRNAs, miR-199a is upregulated by 10-fold in hypertrophic hearts after abdominal aorta constriction for 12 weeks [71]. Gain and loss of function of endogenous miR-199a in cardiomyocytes causes increased or decreased cell size, respectively. Bioinformatic approaches predicted hypoxia-inducible factor 1 alpha (Hif1 $\alpha$ ) to be a potential miR-199a target gene. Hif1 $\alpha$  has previously been reported to be involved in cardiac hypertrophy [72], and recently, Rane and colleagues [73] have showed that rapid upregulation of Hif-1 $\alpha$  upon decline in oxygen tension was facilitated by downregulation of miR-199a under these conditions. In fact, restoring miR-199a levels during hypoxia reduces apoptosis by inhibition of Hif-1 $\alpha$  expression and subsequent stabilization of p53. Furthermore, miR-199a also targets Sirtuin1 (Sirt1), which is required for stabilization of Hif1 $\alpha$  by downregulation of prolyl hydroxylase 2. miR-199a acts as a crucial regulator of a hypoxia-triggered pathway and can be exploited for preconditioning cells against hypoxic damage, demonstrating a functional link between two key molecules that regulate hypoxia preconditioning and longevity. The signal transducer and activator of transcription 3 (STAT3) is expressed in postnatal hearts and is required for maintenance of cardiac integrity and function [74]. STAT3 knockout mice displayed elevated levels of miR-199a compared to wild type animals. Overexpression of miR-199a in cardiomyocytes results in disruption in sarcomere structure and, conversely, suppression of miR-199a abolishes this phenotype [74]. Ubiquitin-conjugating enzymes Ube2i and Ube2g1 have been predicted and confirmed to be target genes of miR-199a. Short hairpin-mediated knockdown of Ube2i and Ube2g1 caused major dysfunction in cultured cardiomyocytes such as loss of sarcomere organization, reduction in mRNA expression of  $\alpha$ - and  $\beta$ -MHC as well as proteins levels of sarcomeric MHC, TnT and Tm. Altogether, miR-199a is negatively regulated by STAT3, a factor which controls postnatal cardiac integrity, by repressing ubiquitin conjugating enzymes via post-transcriptional regulation [74].

Another microRNA reported to be upregulated during cardiac hypertrophy is miR-23a. This microRNA was found to be the most induced in the rat heart after two weeks of pressure overload [75]. miR-23a, a member of the microRNA-23a~27a~24-2 cluster, was upregulated in neonatal rat cardiomyocytes after exposure to isoproterenol or aldosterone, both hypertrophic agonists. However, hypertrophy could be abrogated both in vitro and in vivo by knockdown of miR-23a [58]. Accordingly, transduction of cardiomyocytes with an adenovirus harboring an active form of calcineurin induced myocyte growth and upregulation of miR-23a. Isoproterenol-induced myocyte growth and miR-23a expression could be blunted by cyclosporin A, an inhibitor of calcineurin. In fact, the calcineurin downstream target NFATc3 was found to transcriptionally activate miR-23a expression by binding to the promoter region of the microRNA-23a~27a~24-2 cluster gene locus. Bioinformatics and luciferase activity assays identified the muscle-specific ring finger protein1 (Murf1), an antihypertrophic protein, as a downstream target gene of miR-23a and its overexpression in cardiomyocytes was able to inhibit cardiac hypertrophy mediated by miR-23a.

## 6. Pro-hypertrophic microRNAs: microRNA-195/-18b: orphan microRNAs

Other microRNAs have documented function in cardiac hypertrophy, but intracellular targets remain to be defined. miR-195 is upregulated in cardiac tissue of calcineurin transgenic and aortic banded mouse models and in human failing hearts [59]. In vitro overexpression of miR-195 in primary cardiomyocytes provoked hypertrophic

growth and sarcomere assembly and cardiac-specific overexpression of miR-195 was sufficient to drive cardiac hypertrophy and progression to heart failure. While in this study no direct target genes of microRNA-195 were identified, a recent study by Zhu et al. shows that miR-195 induction also contributes to apoptosis in cardiomyocytes [76], with Sirt1 as a direct target of this microRNA, implicating miR-195 in the development of lipotoxic cardiomyopathy. miR-18b has also been reported to be upregulated in cardiac hypertrophy, both in neonatal rat cardiomyocytes, as well as in cardiac tissue of mice subjected to aortic banding [60]. Even though miR-18b expression is very low in cardiomyocytes, inhibition of endogenous miR-18b is able to induce hypertrophic growth. Moreover, overexpression of miR-18b in cardiomyocytes decreased myocyte hypertrophy and expression of hypertrophic markers but was not able to inhibit PE-induced cardiomyocyte hypertrophy. Although these data may suggest a causative role for miR-18b in cardiomyocyte hypertrophy, the functional mechanisms and direct targets remain to be clarified.

## 7. Conclusions and perspectives

To conclude, microRNAs have changed our understanding about regulation of cardiac hypertrophy. The heart, likely more than most other organs, is exquisitely sensitive to relatively subtle changes in gene dosage and to gene modifier effects. The ability of microRNAs to fine-tune gene expression programs and act as powerful stress regulators suggests their central role in many facets of cardiac biology. Given the vast number of miRNAs, we can expect to witness the discovery of additional levels of complexity for miR-dependent regulation of gene expression that contributes to the modulation of different biological programs. Our collective understanding of how these post-transcriptional gene regulators function in cellular networks may provide new molecular horizons for cures or therapies to a variety of human diseases.

## Disclosures

None.

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