MicroRNAs in heart failure: Small molecules with major impact

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ABSTRACT
MicroRNAs (miRNAs) have emerged as potent modulators of mammalian gene expression, thereby broadening the spectrum of molecular mechanisms orchestrating human physiological and pathological cellular functions. Growing evidence suggests that these small non-coding RNA molecules are pivotal regulators of cardiovascular development and disease. Importantly, multiple miRNAs have been specifically implicated in the onset and progression of heart failure, thus providing a new platform for battling this multi-faceted disease. This review introduces the basic concepts of miRNA biology, describes representative examples of miRNAs associated with multiple aspects of HF pathogenesis, and explores the prognostic, diagnostic and therapeutic potential of miRNAs in the cardiology clinic.

Keywords: microRNA, heart failure, biomarkers, therapeutics
INTRODUCTION
Heart failure (HF) is a complex clinical syndrome that may arise as a consequence of a spectrum of structural and functional cardiac abnormalities, as well as rhythm and conduction disorders. Symptomatic HF has an incidence of 10/1000 for individuals aged over 65, whilst at the age of 40 the lifetime risk of developing HF is approximately 1 in 5. Systolic dysfunction is considered the most common underlying pathology of HF, and it may occur as a result of ischemic cardiac disease (coronary artery disease, CAD, myocardial infarction, MI) and/or hypertension, idiopathic dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM) and valve lesions. Extensive molecular studies have revealed multiple genetic and epigenetic factors implicated in the aforementioned primary pathologies. MiRNAs are now emerging as important regulators of gene expression that act to modulate numerous biological properties, under normal and pathological conditions. As such, their role in the cardiovascular system and HF in specific is being thoroughly investigated, with direct implications in our understanding of the cardiac molecular physiology/pathophysiology and future molecular therapeutic targeting of HF.

1. MIRNAS: IMPORTANT POST-TRANSCRIPTIONAL REGULATORS OF GENE EXPRESSION
MiRNAs are a group of small (18-25 nucleotide-long), non-coding (i.e. not translated to proteins) RNA molecules that have the ability to bind mature mRNA molecules and affect their translation, thus serving as important post-transcriptional modulators of gene expression. MiRNAs are produced through an elaborate molecular mechanism. Initially, the corresponding DNA region (intergenic, intronic or polycistronic) is transcribed to produce hairpin-shaped primary transcripts called pri-miRNAs. Pri-miRNAs are appropriately processed by the microprocessor complex (Dorsh uclease and Pasha protein) inside the nucleus, to generate 70 nucleotide-long miRNAs called pre-miRNAs. Pre-miRNAs are in turn transported to the cytoplasm by exportin 5, where they are cleaved by the Dicer protein to form mature double-stranded miRNA molecules. These double-stranded molecules are then cut into two single stranded miRNAs, and one of them is selected by the argonaute protein to serve as the “active” one. The chosen single stranded miRNA is then embodied in an active RNA-induced silencing complex (RISC), containing Dicer and many associated proteins, which is also known as a microRNA ribonucleoprotein complex (miRNP). The remaining single stranded miRNA is decomposed.

Each of the miRNP complexes targets specific (one or more) mRNAs, dictated by their 3’-UTR (mRNA untranslated region) base-pair complementarity. Once an miRNA binds an mRNA molecule, it leads to suppression of its translation to protein via two distinct routes, depending on the extent of the miRNA-mRNA complementarity. In the case of perfect or near-perfect base-pairing the target mRNA is destroyed, whereas imperfect binding is more likely to result in reduced synthesis of the corresponding protein, with minimum effect on the mRNA levels. Importantly, a single miRNA may regulate the expression of hundreds of genes, and an mRNA may be targeted by multiple miRNAs. Independently of the mechanism and the extent of mRNA degradation and/or translation repression, the overall outcome is post-transcriptional gene silencing (PTGS).

The scientific evidence available to date suggest that the human genome encodes over a thousand human miRNAs, targeting over 60% of the mammalian genes and more than one third of human protein-coding genes. Thus, it comes as no surprise that miRNAs emerge as regulators of numerous physiological functions and have been also implicated in a broad spectrum of human disorders.

The key biological functions affected by miRNAs include cell growth, apoptosis, cell- and tissue-specific differentiation and development, whilst dysregulation in miRNA synthesis and function underlies pathological conditions that affect the majority of human tissues. In cardiology, the latest advances in miRNA research techniques have allowed the high-throughput, genome-wide screening of miRNA expression as well as the prediction of new miRNA-mRNA interactions, thus unveiling the multidimensional role of miRNAs in cardiac development, function and disease (reviewed in ).
Herein, the latest advances in heart failure (HF) miRNA research are reviewed, starting with the role of miRNAs in normal cardiac development, in HF pathogenesis, and proceeding with their emerging value in early and improved diagnosis and prognosis, as well as the development of new therapeutic approaches.

2. **MIRNAS PLAY A CENTRAL ROLE IN CARDIAC DEVELOPMENT**

2. a. Discoveries from animal models

*Aberrations in miRNA biosynthesis lead to abnormal cardiovascular development and embryonic death*

Several lines of evidence have indicated an essential role of miRNAs in heart development. Firstly, a series of studies investigating the impact of mutations in the miRNA processing enzyme Dicer have shown that Dicer activity is required for normal cardiovascular development of the embryo. In particular, loss of Dicer in mice resulted in embryonic lethality at embryonic day 7.5,34 whilst in zebrafish embryos developmental arrest occurred at day 10.35 In mice, deletion of the first two exons and hypomorphic expression of Dicer have been related to impaired angiogenesis,36,37 and neural crest cell-specific deletion of Dicer led to a spectrum of cardiovascular abnormalities resembling congenital heart syndromes (i.e. Type B Interrupted Aortic Arch, IAA-B, Double Outlet Right Ventricle, Double Inlet Left Ventricle, Double outlet Right Ventricle, Transposition of the Great Arteries, Interrupted Aortic Arch Types A, B, and C, Hypoplastic Left Heart Syndrome, Tetralogy of Fallot, Pulmonary Atresia with VSD, and Aortic Arch Syndrome).

Figure 1. “The biology of microRNAs. Schematic representation of microRNAs’ formation and course of action. MicroRNAs (miRNAs) are transcribed from intergenic, intronic or polycistronic DNA, in the first instance as hairpin-shaped molecules (primary transcript or pri- miRNAs), which bear a 5’ cap and a poly (A) adenosine tail on the 3’ edge. Inside the nucleus, these primary transcripts are modified by the Microprocessor complex (Dorsha nuclease and Pasha protein) to form 70 nucleotide-long pre-miRNAs. Exportin 5 facilitate premiRNAs translocation to the cytoplasm, where they are cleaved by the Dicer protein to form mature double stranded 18-25 nucleotide-long miRNA molecules. After being cut into two single stranded miRNAs by a helicase, the “active” one is selected by the Argonaut protein and embodied in a protein complex to form the active miRNP. The remaining single stranded miRNA is decomposed. The miRNP complexes are directed to the appropriate mRNA targets and bind multiple, partially complementary sites in their 3′-UTR regions. In the case of perfect base-pairing the target mRNA is destroyed, whereas partial binding multiple miRNAs to an mRNA results in reduced levels of the corresponding protein with minimum effect on the mRNA levels. 3′UTR = three prime untranslated regions; DNA = deoxyribonucleic acid; ds = double-stranded; mRNA = messenger RNA; RNA = ribonucleic acid; RISC = RNA-induced silencing complex, also known as a microRNA ribonucleoprotein complex (miRNP); ss = single-stranded.”
DORV, Ventricular Septal Defect, VSD). Zebrafish embryos devoid of Dicer function presented with a tubular heart and pericardial edema, lacking the formation of the two chambers, characteristic of the wild-type heart. Moreover, another group reported excessive endocardial cushion formation (impaired heart septation) in mutant Dicer zebrafish embryos, amongst developmental defects in other tissues.

The role of mature miRNAs in the developing heart was further elucidated through cardiac-specific deletion of Dicer in mice. In specific, conditional ablation of Dicer after the initial commitment of cardiac progenitors (from embryonic day 8.5), during heart patterning and differentiation, led to heart failure and embryonic lethality (embryonic day 12.5). The observed developmental defects included DORV with a concurrent ventricular septal defect, implying an essential role for Dicer in proper chamber septation and cardiac outflow tract alignment. A critical role for Dicer has also been proposed in murine epicardial cell development, and their consequent differentiation into coronary smooth muscle cells. Specifically, when Dicer was deleted from the epicardium of normal mice, neonates presented with severe cardiac defects including impaired coronary vessel development, and experienced early death.

The role of Dicer has also been investigated during the course of postnatal heart development. In specific, conditional Dicer loss in the postnatal myocardium of 3-week-old mice led to premature death within 1 week, with the main histopathological findings including mild ventricular remodeling and dramatic atrial enlargement. The observed cardiac hypertrophy was accompanied by the reactivation of the fetal cardiac gene program. The targeted deletion of Dicer in adult mouse myocardium has also uncovered a critical role for miRNAs in maintaining adult splicing programs, via modulating the expression of alternative splicing regulators.

The combined findings from these studies on Dicer deletion suggest that mature miRNAs are essential for the successful transition from the prenatal cardiac gene program to the adult expression profile.

Multiple miRNAs implicated in different aspects of cardiac development

To date, a wide range of miRNAs has been specifically implicated in different aspects of cardiovascular development. For example, miR-1, -133a, -133b, comprise a subset of skeletal- and cardiac-muscle specific miRNAs that are induced during and regulate muscle differentiation (reviewed in ). MiR-1 and miR-133 are two highly conserved miRNAs derived from a common precursor transcript, that exhibit cardiac- and skeletal- muscle specific expression during development and adult life. According to studies, miR-1 (miR-1-1, mir-1-2) targets, amongst others, the transcription factor (TF) Hand2, a promoter of ventricular cardiomyocyte expansion, whose levels are critical for normal cardiomyocyte morphogenesis and development. Studies utilizing knockout mice of mir-1-2 have reported dysregulation of cardiac conduction, cell cycle and defective heart development in these animals, a subset of which suffered from early lethality, thereby proposing a distinct role of miR-1-1 and mir-1-2 in cardiac development. MiR133a is also critical for cardiac development. Interestingly, miR-133a-1 and miR-133a-2 present with at least partly overlapping roles, since the deletion of either one at a time results in phenotypically normal the mice. However, the double-mutant miR-133a mouse embryos and neonates present with ventricular-septal defects often leading to early lethality, whilst the surviving animals are prone to dilated cardiomyopathy and heart failure. MiR-133a gene targets include Cyclin D2 and Serum Response Factor, the upregulation of which possibly underlies the dysregulation of cell cycle control and the aberrant activation of the smooth muscle gene program, as observed in miR-133a-1/ miR-133a-2 double mutant mice. Cyclin D2 is also targeted by miR-29a, and this process has been shown to suppress cardiomyocyte proliferation during postnatal development in rats.

A recent global microRNA profiling study reported another miRNA, namely miR-27b, displaying a greatly elevated myocardial expression during heart development in mice. Interestingly, the TF Mef2c, which is involved in cardiac morphogenesis, was shown to be a target of miR-27b.

A series of studies in zebrafish has also provided valuable data for miRNAs implicated in heart development. For example, miR-23 has been shown to inhibit Hyaluronan synthase 2 (Has2) expression and extracellular hyaluronic acid production. Has2 is an extracellular remodeling enzyme which is required for endocardial cushion and valve formation, and when inhibited by miR-23 the number of endocardial cells that differentiate into endocardial cushion cells during development in zebrafish embryos was restricted. Endocardial cushions develop on the atrio-ventricular canal and play a role in proper heart septation during development. MiR-21 appears to control valve formation upon its flow-dependent expression induction in the developing zebrafish heart. Notably, miR-21 exerts
its actions by regulating the expression of the same target genes as mouse/human miR-21, namely Sprouty, Pdcd4, and Ptenb. MiR-138, which was specifically expressed in the developing ventricular chamber, was shown to be required for establishment of chamber-specific gene expression patterns. MiR-138 acts by targeting multiple members of the retinoic acid signaling pathway, to prevent ventricular expansion of gene expression normally restricted to the atrio-ventricular valve region. Last, but not least, a recent study reported a putative mutual cross-regulation mechanism between the TF Tbx5 and miR-218-1, and demonstrated its implication in heart development in zebrafish. Of note, Tbx5 gene expression levels have an overt effect on heart development, and their dysregulation has been related with the establishment of congenital heart defects. Similarly, the Tbx5 downstream targets miR-218-1 and its host gene Slit2 are known to be involved in heart development. Specifically, miR-218-1 was shown to suppress the expression of Robo receptors (Robo1,2), which interact with Slit family ligands to facilitate cell guidance during development. Evidently, the miR-218-1 and Slit/Robo form a regulatory loop required for heart tube formation in zebrafish. The exact role of miR-218-1 in Tbx-5 regulation, though, is still being explored.

Additional information on cardiac development-related miRNAs has emerged from studies in the Mexican axolotl (salamander). Interestingly, a group investigated the role of a human fetal heart microRNA which is thought to be related to the human miR-499 family, and was therefore named miR-499c, in mutant axolotl hearts in organ culture. Accordingly, the axolotl hearts with abnormal development (without tropomyosin expression, sporadically beating etc) were incubated with the miR-499c, which was able to induce expression of cardiac markers (tropomyosin, troponin, α-syntrophin) in these hearts. Evidently, miR-499c treatment promoted the formation of cardiac myofibrils in mutant axolotl hearts, thus showing the potential to restore normal embryonic heart development in this species. As presented in the following section, miR-499c facilitates the role during human cardiomyocyte (CMC) differentiation, and hence the role of the new miR-499c in cardiac development requires further investigation.

2. b. miRNA expression in embryonic stem cell-derived cardiomyocytes

However informative studies in animal models may be, they still have to be validated in humans. To this end, human embryonic stem cell-derived cardiomyocytes (hESC-derived CMCs) are now providing valuable new insights. The first miRNA profiling study of hESC-derived CMCs led to the identification of 711 unique miRNAs. Amongst them are miR-1 and miR-133 which have been previously related to heart development, miR-208 which has been shown to be implicated in the reactivation of the cardiac fetal gene program by regulating myosin heavy chain gene (α-, β-MHC) expression, as well as the novel miR-499. MiR-499 was found to share several predicted gene targets with miR-208, while its overexpression in hESCs led to elevated protein levels of the cardiac TF MEF2C, which is required for cardiac contractile gene activation and for the structural development of the heart. Moreover, miR-1 overexpression in hESCs triggered upregulation of the TF GATA4, which is essential during early heart development. Accordingly, both miRNAs promoted cardiac specification of the hESCs. A consecutive study explored the distinct roles of miR-1 and -499 in the differentiation of hESCs to CMCs, and reported that miR-499 promotes ventricular specification of hESCs, whereas miR-1 facilitates electrophysiological maturation.

3. MIRNAS IN HF PATHOGENESIS

In addition to cardiac physiology, miRNAs are increasingly associated with pathological cardiac phenotypes. In the setting of HF, despite the multitude of molecular factors already implicated, miRNAs are emerging as novel contributors to both the preceding pathologies and to HF itself.

3. a. miRNA signatures of human failing hearts

To date, miRNA profiling studies conducted in the human failing heart have identified significant miRNA alterations implicated in both pathogenesis and/or progression. Numerous miRNome studies have been conducted using microarrays, amongst other methodologies. For example, Ikeda et al measured the expression of 428 miRNAs in the failing left ventricles of patients with ICM, DCM and aortic stenosis (AS), and detected 87 miRNAs, of which 43 were differentially expressed in at least one diagnostic group. The pro-hypertrophic miR-214 appeared upregulated across all disease groups (2- to 2.8-fold), whereas the anti-hypertrophic...
miR-17
-76 was downregulated in DCM and AS. The miR-19 family was the most downregulated (miR-19a and -19b 2-2.7 fold in DCM, AS), possibly contributing to the regulation of ECM protein levels in the heart, as supported by recent studies. Another microarray study investigated the miRNA expression pattern of the end-stage failing myocardium, by measuring 467 miRNAs. Twenty-eight miRNAs were significantly upregulated and eight of these (miR-21, -23a, -24, -26b, -27, -125, -195, -199a-3p) emerged as directly associated with HF pathophysiology. In a similar fashion, Sucharov et al. assessed 470 miRNAs in idiopathic DCM and ICM hearts and found that, amongst other miRs, miR-100 was upregulated and miR-133(-a, b) was downregulated in HF. Further experiments demonstrated that miR-100 over-expression is implicated in the β-adrenergic receptor-mediated repression of "adult" cardiac genes (i.e. α-MHC, SERCA2a), whilst miR-133b overexpression acts to prevent alterations in gene expression that are due to β-adrenergic receptor stimulation. These findings imply that the HF-related shift in the expression of these two miRNAs may serve to reactivate the "fetal gene expression program" (see also). Overall, although all studies agree that significant miRNA expression changes occur in HF, the fine details thereof remain unclear and, in some cases, even contradictory. These discrepancies may reflect the existence of distinct miRNA signatures in the failing hearts of different etiologies, or to different stages of disease progression.

More recently, next generation sequencing has also been used for the analysis of human failing left ventricles of HCM or DCM etiology, and demonstrated significant changes in more than 250 of the 800 known human miRNAs, with approximately twice as many annotated miRNAs expressed in HF than unaffected cardiac tissue. Amongst the ten most abundant miRNAs in the HF samples that have been previously described in CVD studies, four have been shown to promote (miR-23a) or inhibit cardiac hypertrophy (miR-17
-76), or negatively regulate fibrosis (miR-24, -133a). Importantly, amongst the top ten overexpressed miRNAs that have not been described in previous profiling studies in HF (miR-23b, -30d, -125a, -143, -165, -193, -197, -342, -365, -455), miR-145 emerges as an important new player in cardiovascular disease, and in left ventricle pathological remodeling, in specific.

With regards to the precise miRNA mechanisms impaired in HF, Thum et al demonstrated that 87% of the over-expressed miRNAs and 84% of the under-expressed miRNAs were similar to the miRNA expression profiles of fetal cardiac tissue (e.g. miR-21, -29, -30, -129, -212), suggesting the activation of the "fetal gene expression program". The reactivation of the "fetal gene expression program" is a hallmark of the hypertrophic and failing myocardium, often accompanying pathological left ventricular remodeling. In order to prove this concept, Thum et al showed that simultaneous re-expression of three of the miRNAs overexpressed in both HF and fetal tissue (miR-21, -129, -212) resulted in activation of fetal gene program and HF-related changes, like hypertrophy, in neonatal and adult CMCs. In specific, the miRNA-regulated fetal genes included ANP, BNP, β-MHC, α-skeletal actin and MEF2a, amongst others. This study shed light to significant aspects of the reactivation of the cardiac fetal gene program during HF, and revealed possible molecular players of left ventricular pathological remodeling.

MiR-21, miR-29 and miR-30 are some of the miRNAs whose HF expression parallels this of fetal hearts, and have been studied extensively in the context of HF. miR-21 appears upregulated in cardiac fibroblasts of DCM-related HF, likely following activation of the STAT3 and NFκB transcription regulators. This is consistent with the emerging topic of miRNA participation in a feedback loop with TFS that regulate their transcription. MiR-29 targets multiple ECM proteins (collagens, fibrillins etc) and miR-30 targets the pro-fibrotic factor CTGF, with the former being downregulated in the proximal region to myocardial infarcts, and the latter down-regulated during pathological left ventricular hypertrophy caused by AS. The differential regulation of those three miRNAs during HF possibly facilitates the extensive ECM remodeling observed in the myocardium.

Other studies have pinpointed miRNAs related to HF-associated pathologies, such as hypertrophy, HCM, DCM and ICM. In specific, studies in left ventricular tissue acquired from HCM patients revealed increased expression of miR-221, which was also upregulated in the hypertrophic (2 weeks) and failing hearts (9 weeks) of TAC mice. Further studies in rat CMCs demonstrated that forced expression of miR-221 by miRNA mimics is capable of inducing hypertrophy and re-expression of fetal genes in vitro, whilst knockdown of endogenous miR-221 abolished these effects. Moreover, in silico target prediction and experimental assays indicated that miR-221 possibly acts via targeting the suppressor of cardiac hypertrophy p27. MiR-499 upregulation in human hypertrophied and failing hearts was associated with decreased expression of an array of predicted targets. Interestingly, studies in mice showed that miR-499 suffices for the induction of HF and acceleration of the pathological remodeling, upon pressure overload. AKT and MAPKs were amongst the miR-499 numerous targets.
miR-499-induced cardiomyopathy was associated with changes in protein phosphorylation (e.g. HSP90, PP1α), thus revealing a spectrum of putative mechanisms via which miR-499 may contribute to cardiac pathophysiology. Of particular interest is also the upregulation of miR-24 in cardiac tissue of ICM and DCM-related HF, which seemingly accounts for the under-expression of junctophilin 2 (JP2). JP2 is a structural protein that anchors the sarcoplasmic reticulum (SR) to the transverse tubules (TT) of the plasma membrane, which are the major sites of the excitation–contraction coupling. Importantly, transmission electron microscopic imaging revealed a significant reduction in SR-TT junctions in the ICM and DCM specimens, indicating that miR-24 and JP2 dysregulation may ultimately lead to defective excitation-contraction coupling, a characteristic of failing CMCs. Examples of miRNAs associated with age-related HF include the downregulated miR-18a, -19a and -19b leading to upregulation of the ECM proteins CTGF and TSP1, possibly in the context of ECM remodeling during HF pathogenesis.

3. b. miRNAs signatures in animal models of HF
   3. b. i. miRNAs signatures during the development of cardiac pathologies preceding HF: A close up in hypertrophy

Besides investigations in human HF, a series of animal model studies, predominantly involving transverse aortic constriction (TAC), have provided valuable insights into the miRNA expression alterations contributing to pathogenesis of hypertrophy and HF. For example, Sayed et al measured the expression of 334 miRNAs at days 1, 7 and 14 post TAC operation in mice and observed significant changed expression in 26, 22 and 51 miRNAs, respectively. Importantly, miR-1 was downregulated prior to hypertrophy development (1d) and persisted until later stages of hypertrophy (14d), and specifically up to 1 week before the presentations of HF in the TAC model. Moreover, five of the miRNAs that were upregulated during hypertrophy development (7d) (miR-199a, -199a*, -199b, -21, -214) and persisted until day 14 were the ones that exhibited the greatest change (>2 fold). These findings indicate a distinct stage-specific role of miR-1 and the latter five miRNAs in the development of hypertrophy in the TAC mouse model.

Similar miRNA expression changes were observed in another study, utilizing both the TAC mouse model and mice with cardiac-specific expression of activated calcineurin (CnA) (aimed at inducing pathological cardiac remodeling and hypertrophic growth). Accordingly, 42 miRNAs were differentially expressed in TAC hearts and 47 in CnA, with the two groups sharing 21 upregulated and 7 downregulated miRNAs. Importantly, six of these miRNAs (miR-23, -24, -125, -195, -199a, -214) were consistent with findings in idiopathic end-stage human failing heart tissue, suggesting the conservation of pathogenic processes across species and highlighting their importance in HF.

The comparative study of a preload versus afterload cardiac hypertrophy mouse model, revealed that miRNA expression changes several days post TAC or shunt, suggesting that these mechanisms are involved in the later stages of remodeling post cardiac overload. The hypertrophy related miRNA-133, -30 and -208, were regulated only in the afterload model, consistently with the direct role of miR-208 in ß-MHC upregulation. The preload hypertrophy model presented with changes in miR-140, -320 and -455. MiR-320 has been associated with apoptosis, while both miR-320 and miR-140 are upregulated in human HF.

Studies conducted in the left ventricles of a rat model of hypertrophy induced by banding of the ascending aorta, detected four upregulated miRNAs (miR-23a, -27b, -125b and -195), 14 days post operation, when the hypertrophy was already established (left ventricle weight/ body weight ratio increased by 65%). Importantly, miR-23a,-27b and -195 are known to favor CMC hypertrophic growth (see section 3.c.i). The observed changes in the expression of miRNAs in this rat model of hypertrophy are in line with previous studies in mice and human tissue, thus strengthening the notion of intra-species conservation of HF-related miRNA mechanisms.

3. b. ii. miRNA signatures change during disease progression to HF

The expression of miRNA is a highly dynamic process, with different molecules and combinations thereof being implicated in the different stages of conditions leading to HF.

The most representative example of miRNA expression pattern shift during HF development is that of miR-1 and miR-133. Both miRNAs are downregulated during the development of hypertrophy, but overexpressed in chronic HF. In specific, miR-1 and miR-133a have been found to be downregulated in mouse and rat models of hypertrophy, but upregulated in canine hearts isolated from animals with
chronic HF. Moreover, in the chronic HF animals, miR-1 and miR-133 were shown to be implicated in the development of arrhythmogenesis, a characteristic observed in approximately 50% of congestive HF cases. These findings indicate that miR-1 and miR-133 serve distinct stage-specific roles during the course of HF. Their precise mode of action is discussed in subsequent sections.

The time course of HCM-HF progression has also been explored in the DBL transgenic mouse model of HCM, which bears mutations in troponin I and myosin heavy chain genes (TnI-203/MHC-403) and presents with severe HCM, HF, and premature death. Measurements in 335 miRNAs showed downregulation of miR-1 and miR-133 in a pre-disease state, and this change preceded upregulation of target genes causal of cardiac hypertrophy and ECM remodeling, thus implying a role in early disease development, consistently with other studies. In end-stage HCM the miRNA signature comprised of 16 miRNAs and corresponded to those of cardiac stress and hypertrophy, including downregulation of miR-1, -133, -30 and -150, and overexpression of miR-21, -199 and -214. This group also engaged microarrays to detect differentially expressed mRNAs in end-stage HCM, and bioinformatical analysis to predict mRNA-miRNA interactions amongst the significantly changed transcripts and miRNAs. As a result, some of the altered miRNAs (miR-1, -21, -30, -31, -133, -150, -222, -486) were further associated with hypertrophy, CMC proliferation, cardiac electrophysiology, calcium signaling, fibrosis, and the TGF-β pathway, based on their predicted interaction with the dysregulated transcripts and the Gene Ontology annotations of the latter. These findings suggest that miRNAs play a critical role in the cardiac pathophysiology of the DBL mouse model during end-stage HCM.

In search of the distinct miRNAs implicated in different stages of hypertrophy-induced HF, miRNA expression alterations have also been investigated during the transition from right ventricular hypertrophy (RVH) to HF in mice that underwent pulmonary artery constriction (PAC). In addition to left ventricular pathological remodeling, which accompanies the majority of failing hearts, RVH may also lead to failure, predominantly in cases with congenital right-sided cardiac defects. Reddy et al used microarrays to measure the expression of 567 miRNAs in the right ventricle of mice at 2, 4, 10 days post-PAC or sham operation, time points which correspond to early compensated hypertrophy, early decompensated hypertrophy and overt HF, respectively. Although no significant changes were detected at 2 days, at 4 and 10 days, 32 and 49 miRNAs, respectively, were deregulated. In addition, following global mRNA expression profiling of the right ventricles at 10 days, this group associated the deregulated miRNAs with their respective altered mRNA targets. Importantly, during decompensated RVH they reported alterations in miRNA expression that can enhance CMC hypertrophic growth (miR-199a-3p, let-7c), abnormal vascular tone (miR-143/145 cluster), resistance to apoptosis (miR-181a, let 7) and increase collagen synthesis (miR-30). At the HF phase, they reported changes that coincided with reactivation of the fetal gene program in HF (miR-208a, -208b), enhanced apoptosis (miR-34b-34c, miR-144/451 cluster) and inhibition of endothelial cell proliferation and migration (miR-379, -503). Hypertrophy and HF shared 21 miRNA alterations, with some of them associated with CMC survival and adaptation to stress (miR-21, -210, -214, -199a), apoptosis (-34a), upregulation of collagens (miR-26b, -133, -149) and fibrosis (miR-21, -29c, -150, -499). These findings further support the notion that miRNA expression is a dynamic process during HF development.

The study by Reddy et al also pointed out the differences between RVH/HF in the PAC model and LVH/HF in the TAC mouse model. Specifically, they compared the miRNA profile of RVH/HF with publically available microarray data for miRNA expression in TAC mice, and found four miRNAs (miR-143/145 cluster), resistance to apoptosis (miR-181a, let 7) and increase collagen synthesis (miR-30). At the HF phase, they reported changes that coincided with reactivation of the fetal gene program in HF (miR-208a, -208b), enhanced apoptosis (miR-34b-34c, miR-144/451 cluster) and inhibition of endothelial cell proliferation and migration (miR-379, -503). Hypertrophy and HF shared 21 miRNA alterations, with some of them associated with CMC survival and adaptation to stress (miR-21, -210, -214, -199a), apoptosis (-34a), upregulation of collagens (miR-26b, -133, -149) and fibrosis (miR-21, -29c, -150, -499). These findings further support the notion that miRNA expression is a dynamic process during HF development.

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3. c. Characterization of the role of specific miRNAs in HF and associated pathologies in an experimental setting

The miRNA profiling studies in humans and in animal models of HF brought to light several miRNAs with altered expression and putative roles in HF development, many of which were subjected to further investigation. The studies presented below utilized animal model hearts and cell culture (CMCs, CFs) aiming to prove direct relations between miRNAs and HF or HF-associated pathologies.
3. c. i. Can miRNAs control cardiac hypertrophy?

Aiming to demonstrate a direct and sufficient role of selected miRNAs in the induction of cardiac hypertrophy, four teams specifically overexpressed putative pro-hypertrophic miRNAs in vitro and in vivo. Van Rooij et al overexpressed a selected group of miRs (previously found upregulated in mice undergone TAC, in mice with cardiac overexpression of activated calcineurin, and in idiopathic end-stage human failing heart tissue) in primary rat CMCs. These five microRNAs (miR-23a, -23b, -24, -195, and -214) proved to be individually capable of inducing hypertrophic growth in vitro.70 Three of those miRNAs, (mir-24, -195, and -214) were then overexpressed individually in the heart of wild type (WT) mice. The miR-195 transgenic animals developed pathological cardiac hypertrophy and HF, thus revealing a direct and sufficient role of miR-195 in the development of HF in mice. Mir-24 transgenic mice died at the embryonic stage, whilst overexpression of mir-214 had no phenotypic effect. Similarly, Ucar et al investigated the role of miR-212 and -132, also upregulated during hypertrophy in the heart of TAC mice.103 Transgenic mice with cardiac specific overexpression of miR-212 and -132 presented with hypertrophic hearts, exhibited signs of severe HF and experienced premature death. In vitro experiments from the same group showed that miR-212 and -132 target the anti-hypertrophic TF Foxo3a, whilst overexpression of miR-212 and -132 results in hyperactivation of pro-hypertrophic calcineurin/NFAT signaling.103 MiR-23a levels have also been found elevated in rodent models of hypertrophy, and specifically upon isoproterenol-induced cardiac hypertrophy in mice,60 as well as pressure overload-induced hypertrophy in both mice and rats.73 In order to elucidate its role, Wang et al produced transgenic mice with cardiac overexpression of miR-23a, which presented with exaggerated hypertrophy upon phenylephrine (PE) treatment or pressure overload induced by TAC.81 This study also reported that endogenous miR-23a was upregulated by hypertrophic stimuli (PE, endothelin, ET) in cultured CMCs, thereby indicating that miR-23a participates in the transduction of the hypertrophic signal. Moreover, they identified the anti-hypertrophic Foxo3a as a target of miR-23a, and showed that miR-23a and Foxo3a bi-transgenic mice present with an attenuated hypertrophic response, in comparison to the miR-23a transgenic mice alone.81 Interestingly, in vitro studies by other teams revealed additional molecular players in the putative miR-23a pro-hypertrophic machinery. Specifically, experiments in CMCs showed that the nuclear factor of activated T cells 3 (NFATc3) induces miR-23a, which in turn targets the negative regulator of hypertrophy muscle ring finger1 (MuRF1) thereby triggering cardiac hypertrophy (Figure 2).80,104 Mir-27b has been seen upregulated in cardiac hypertrophy, and specifically in the cardiac-specific Smad4 knockout mouse model.105 Importantly, cardiac-specific overexpression of mir-27b in transgenic mice was sufficient to induce hypertrophy and heart dysfunction,105 thereby implying a direct association. Moreover, in vitro studies showed that mir-27b targets the peroxisome proliferator-activated receptor-gamma (PPAR-gamma) and is inhibited by TGF-beta1, findings that were also validated in the TAC mouse model of HF.105 Overall, miR-195, -212, -132, -27b emerge as potent inducers of cardiac hypertrophy, while miR-23a appears to serve as a contributive factor to the establishment of this pathology.

In addition to upregulated pro-hypertrophic miRNAs, disruption of anti-hypertrophic miRNAs expression has also been reported in the hypertrophied and failing myocardium. A representative example is miR-1, which was downregulated in a series of studies in rodent models of hypertrophy, HCM and HF (TAC, AKT overexpression, MHCα-CN mice, cardiac specific Dicer deletion, and DBL transgenic mice). Ikeda et al demonstrated that the size of miR-1 deficient neonatal rat CMCs was significantly increased at baseline and after treatment with pro-hypertrophic stimulus (ET), indicating that miR-1 downregulation promotes hypertrophic growth. According to further studies in CMCs, miR-1 inhibits cell growth-related targets (RasGAP, Cdk9, fibronectin, Rheb), reduces protein synthesis and cell size, and its downregulation promotes hypertrophy.74 In addition, in vitro experiments in a series of studies revealed multiple putative mechanisms of action for miR-1-mediated hypertrophy suppression,76,71–75 including targeting of Igf-1 and Igf1-r,71 calmodulin, Mef2a and Gata4.72 These data indicate that miR-1 targets key regulators of hypertrophic growth, and may thus act as a central suppressor of hypertrophy via a range of downstream effectors in the failing myocardium. Similarly, the newly described miR-378 has been shown to be down-regulated during hypertrophic growth and HF. Studies in rat CMCs have shown that deficiency of this miRNA is sufficient to induce fetal gene expression, thereby suggesting an anti-hypertrophic role in HF. MiR-378 seemingly acts by negatively regulating the MAPKs pathway. In specific, multiple components of this pathway have been identified as miR-378 targets (Mapk1, Igif1, Grb2, Ksr1) by Ganesan et al.108 In addition, recent experiments in rat CMCs showed that miR-378 directly targets Grb2 and blocks Ras activation, resulting in negative
regulation of fetal gene expression and cardiac hypertrophy. 106,107 MiR-9 is also downregulated following hypertrophic treatments, and confers anti-hypertrophic effects in the murine heart. Wang et al utilized the isoproterenol and aldosterone-induced mouse models of hypertrophy to demonstrate that NFATc3 can promote hypertrophy via induction of myocardin expression, while miR-9 targets and suppresses myocardin.109 Whether miR-9 is also underexpressed in human HF and may thus provide a target towards pathological hypertrophy HF inhibition, is yet to be determined.

3. c. ii. miRNAs impact on ECM remodeling and fibrosis

Besides the establishment of hypertrophy and/or dilatation, the failing myocardium is often accompanied by structural remodeling. However, the remodeling processes that take place during HF development are not restricted to the contractile cell component of the heart (CMCs). The non-myocyte cells of the healthy heart account for more than 60% of the cardiac cells, include cardiac fibroblasts (CFs) and endothelial cells (ECs), and are actively involved in the remodeling process.110,111 Fibroblasts, which are responsible for the synthesis of ECM components, account for approximately 90% of the non-myocyte cell mass.110,111 In the stressed myocardium, fibroblasts differentiate into active myofibroblasts upon a wide range of stimuli (e.g. TGF-β).89,110 These activated cells can regulate the secretion of ECM components and ECM degrading-enzymes (matrix metalloproteinases, MMPs) and tend to proliferate and migrate, acting to remodel the cardiac interstitium.89 This process may result in cardiac fibrosis, a hallmark of pathological hypertrophy and HF, which presents with aberrant proliferation of CFs and excessive deposition of ECM proteins in the interstitium and perivascular regions of the myocardium, ultimately impairing cardiac function.89

Several lines of evidence indicate that dysregulation of miRNAs during HF occurs in CFs, besides CMCs, thereby contributing to the development of cardiac fibrosis. In particular, the increased miR-21 expression observed in human HF,70 has been attributed mainly to fibroblasts using the TAC mouse model of HF.84 Specifically, miR-21 is selectively upregulated in the fibroblasts of the failing heart and has been shown to target Sprys, a negative regulator of ERK-MAPK pathway, which functions to enhance growth factor secretion and fibroblast survival, thus promoting interstitial fibrosis.84 MiR-21 was also

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**Figure 2.** The role of miR-132, -212 and -23a in molecular pathways of cardiac hypertrophy. Activation of calcineurin/NFAT pro-hypertrophic pathway leads to increased expression of miR-23a. MiR-23a targets the negative regulator of hypertrophy muscle ring fingers (Murf1), thereby triggering hypertrophic response via the transcriptional activity of serum response factor (SRF). Overexpression of miR-132 and -212 also promotes hypertrophy via over-activating the calcineurin/NFAT pro-hypertrophic pathway. In addition, miR-132, -212 and - may also trigger cardiac hypertrophy via targeting the anti-hypertrophic transcription factor Foxo3a.
found upregulated in CFs of the infarct zone after ischemia-reperfusion in mice, where it was shown to induce MMP2 (an ECM degrading enzyme) via direct targeting of PTEN, but its role in fibrosis was not further investigated in this model. A more recent study by Liang et al revealed additional evidence supporting a role for miR-21 in fibrosis: miR-21 was upregulated in the border zone of murine hearts after MI, whereas the negative regulator of TGFβ, TGFβRIII, was underexpressed. Further experiments in CFs showed that miR-21 overexpression can enhance collagen production, in part through TGFβRIII suppression, and conversely TGFβRIII overexpression can inhibit miR-21 and reduce collagen production in CFs. Taken together, these studies imply that miR-21 upregulation under pathologic conditions in the myocardium may impair cardiac function by contributing to cardiac fibrosis.

The miR-29 family has also been found deregulated in the failing heart and associated with the pathological mediator of fibrosis TGFβ. The members of the miR-29 family (miR-29a, b, c) are mainly expressed in the CFs of the murine heart and have been found downregulated in response to a variety of remodeling-inducing stresses (TAC, chronic calcineurin signaling, MI). In vitro experiments in cultured CFs showed that this reduction in miR-29 levels may be triggered upon TGFβ stimulus. Moreover, the miR-29 family was shown to directly target multiple genes encoding ECM components (collagens, elastin, fibrillins), whilst in vivo inhibition of miR-29 in healthy murine hearts and in vitro in CFs led to increased collagen expression, thus indicating a role of miR-29 in ECM remodeling.

MiR-24 was found to be correlated with ECM remodeling and TGFβ, in a mouse model of MI. In particular, miR-24 was reported downregulated in the infarct zone after MI, and miR-24 treatment led to fibrosis attenuation and improved cardiac function. In vitro experiments conducted in CFs showed that miR-24 upregulation could specifically decrease the differentiation and migration of CFs, and reduce fibrosis. The same team also demonstrated that miR-24 may act via suppressing its target furin, which is essential for TGF-β secretion, whose secretion is reduced upon miR-24 overexpression in CFs. In conclusion, miR-24 downregulation in response to MI possibly serves to promote cardiac fibrosis after MI, which has been identified as a contributive factor to the development of HF.

MiR-133a is observed deregulated in HF and may have a role in ECM remodeling during HF. In specific, miR-133a and miR-30 has been found downregulated in the homozygous Ren2 rat model of hypertension-induced HF, and in rats having undergone TAC. The downregulation of these miRNAs in pathological LVH paralleled the increased expression of the profibrotic protein CTGF. In vitro experiments in CMCs and CFs showed that both miRNAs target CTGF, the expression of which was associated with increased collagen synthesis. Moreover, a recent study in the DBL transgenic mouse model of HCM (described earlier in this review) reported the downregulation of mir-1 and -133 before ECM remodeling and mir-1, -133 and -30 in end-stage HCM, overall suggesting a distinct role for these miRNAs in pathological ECM remodeling throughout the course of LVH development in HF.

In addition to the pool of residing interstitial CFs, recent studies suggest that epicardial mesothelial cells (EMCs) lining the heart and microvascular endothelial cells may also contribute to the injury-induced fibrotic process in the myocardium. In adults, EMCs can undergo epithelial-to-mesenchymal transition (EMT) due to reactivation of the developmental program or during cardiac injury (e.g. MI). Several research groups have provided in vitro and in vivo evidence that EMT of EMCs occurring in the injured adult myocardium can give rise to fibroblast-like cells, which contribute to the default repair-driven fibrotic response. Interestingly, Bronnum and partners showed in 2013 that miRNAs are capable of regulating fibrogenic EMT of the EMCs in the adult heart. In specific, they found that pro-fibrotic TGF-beta treatment promoted EMT progression in EMC cultures, resulting in expression changes of numerous miRs, and especially miR-21. Evidently, the ectopic expression of miR-21 promoted the fibroblast–like phenotype resulting from fibrogenic EMT, whereas a miR-21 antagonist abolished this effect. Moreover, this study determined that several miR-21 mRNA targets were differentially expressed during fibrogenic EMT of EMCs, such as PDCD4 and SPRY1, of which miR-21-dependent suppression contributed to the development of the fibroblast-like phenotype of EMCs. Another study of the same group utilized TGF beta-induced EMT in rat-derived adult EMC cultures to investigate the role of Islet-1 (Isl1), a known marker of progenitor cells such as EMCs. They reported that Isl1 promoted the mesenchymal phenotype in untreated EMCs, whilst during TGF beta-induced EMT Isl1 was underexpressed, exerting a negative effect on EMT progression. The observed underexpression of Isl1 was in part attributable to miR-31, which was shown to act as a negative modulator of cardiac fibrogenic EMT, primarily via targeting Isl1. Overall, these studies shed light to molecular mechanisms implicated in the contribution of EMCs to cardiac fibrosis, whilst suggesting a regulatory role for miR-21 and miR-31 in the fibrogenic EMT of EMCs.
According to recent studies, endothelial cells can also provide fibroblast-like cells through endothelial-to–mesenchymal transition (EndMT), but the presence of cells of this origin in the adult myocardium occurs only under pathological conditions and is associated with fibrosis.\textsuperscript{121} Zeisberg and partners suggested that endothelial cells may undergo (EndMT) and generate CFs, and they showed that EndMT contributes to cardiac fibrosis progression in mouse models of pressure overload and chronic allograft rejection.\textsuperscript{122} More recently, Ghosh et al reported differential expression of several miRs during cardiac EndMT.\textsuperscript{123} Specifically, they treated cultured mouse cardiac endothelial cells (MCECs) with TGFbeta2 to trigger EndMT, and performed microRNA microarrays to measure total microRNA expression in fibroblast-like cells vs MCECs. They reported significant expression changes in a range of miRs in fibroblast-like cells, and amongst them were many previously associated with CVD (\textsuperscript{miR-125b, -21, -30b, -195, -Let-7c, -7g; \textsuperscript{miR-122a, -127, -196, -375}). The expression of \textsuperscript{miR-125b} was further validated by qPCR, whilst the protein levels of its target p53 were found downregulated in the EndMT-derived fibroblast-like cells. Interestingly, p53 is known to antagonize TGFbeta-induced profibrotic responses,\textsuperscript{124} therefore \textsuperscript{miR-125b} overexpression may lead to profibrotic signaling upregulation via suppressing its target p53 in these fibroblast-like cells. In conclusion, EndMT-derived fibroblast-like cells emerge as a novel cardiac fibrosis mediators, whilst their disease-specific existence in the adult myocardium may facilitate the development of miRNA based tools to target fibrosis.

3. c. iii. miRNAs impact on calcium cycling

The dysregulation of \textsuperscript{miR-1} and \textsuperscript{miR-133a} appears to serve multiple and distinct roles during HF development and progression. In contrast to their downregulation in certain rodent models of HF (see sections 3.c.i, ii), \textsuperscript{miR-1} and \textsuperscript{miR-133a} were found to be upregulated in canine hearts isolated from animals with chronic HF accompanied by increased left ventricular dimensions and impaired contractility of the left ventricle.\textsuperscript{94} These miRNAs were shown to target the mRNA of the PP2A catalytic subunit of RyR2, which led to increased RyR2 phosphorylation and abnormal spontaneous sarcoplasmic reticulum Ca\textsuperscript{2+} release, thus contributing to arrhythmogenesis.\textsuperscript{94} These in vivo findings confirm previous studies in rat CMCs, where \textsuperscript{miR-1} over-expression was shown to decrease the protein phosphatase PP2A regulatory subunit B56alpha, which in turn resulted in increased phosphorylation of the L-type and RyR2 calcium channels, and ultimately enhanced cardiac excitation-contraction.\textsuperscript{125} These observations point to \textsuperscript{miR-1} and \textsuperscript{miR-133a} as regulators of CMC contractility via modulation of calcium signaling, suggesting their implication in arrhythmia manifestation during HF. The role of \textsuperscript{miR-1} in calcium signaling has been further investigated in additional rodent models of HF. Studies in the cardiomyocyte-specific SRF knock-out mouse model of HF revealed that sodium-calcium exchanger 1 (NCX1) and AnxA5 mRNAs are targets of \textsuperscript{miR-1}.\textsuperscript{126} This is consistent with previous studies in chronic post-myocardial infarction rat model of HF, where \textsuperscript{miR-1} expression was restored by SERCA2a gene therapy (AAV9.SERCA2a) in the failing heart and led to normalization of NCX1 expression.\textsuperscript{127} Of note, \textsuperscript{miR-1} expression restoration also resulted in improved cardiac function in this model.\textsuperscript{127} Moreover, studies in the mouse model of hypertrophy derived from cardiac-specific Dicer deletion, showed that \textsuperscript{miR-1} also targets sorcin, which functions as a regulator of calcium signaling and excitation-contraction coupling.\textsuperscript{76}

With regards to the role of SERCA2a in the failing myocardium, functional screening of 875 miRNAs, identified \textsuperscript{miR-25} as a suppressor of SERCA2a expression and consequently a potent regulator of intracellular calcium handling. \textsuperscript{miR-25} has also been found overexpressed in human and experimental HF. Moreover, experiments in cardiomyocyte-like HL-1 cells demonstrated that \textsuperscript{miR-25} delayed calcium uptake kinetics, whilst AAV9-mediated overexpression in a mouse model of HF led to loss of contractile function. Importantly, inhibition of \textsuperscript{miR-25} expression via antagomiRs in a mouse model of HF halted the established HF, and improved cardiac function and survival, thereby suggesting that \textsuperscript{miR-25} may be a novel therapeutic target for HF.\textsuperscript{180} These findings suggest that miRNAs, among their many mechanisms of contributing to HF, may also impair different aspects of calcium homeostasis in the cardiomyocytes.

3. c. iv. miRNAs impact on mitochondrial dysfunction underlying HF

Interestingly, recent studies suggest that miRNAs may be implicated in HF development via impairing mitochondrial function. In specific, in vivo systemic administration of cloned \textsuperscript{miR-181c} packaged in lipid-based nanoparticles in rats led to reduced exercise capacity and development of
HF symptoms. Mir-181c acts via targeting mitochondrial COX1 (cytochrome c oxidase subunit 1), and miR-181c treatment selectively affected the expression of mitochondrial complex IV genes in the heart. Importantly, following miR-181c administration several mitochondrial functions were impaired, such as O2 consumption, ROS production, matrix calcium levels as well as mitochondrial membrane potential.183 Another group sought to investigate the role of miR-30c in the heart, due to the implication of miR-30 family in several aspects of CMC function and heart pathophysiology. To this aim they generated transgenic mice specifically overexpressing miR-30c in CMCs. They observed that these animals develop severe DCM after 6 weeks of age, and expression analysis of the transgenic hearts prior to phenotype onset revealed changes indicating mitochondrial function impairment. In addition to these findings, mitochondrial oxidative phosphorylation (OXPHOS) complexes III and IV were downregulated at the protein level. These observations indicate that miR-30c triggered mitochondrial dysfunction may account for the DCM phenotype of the miR-30c transgenic mice, thus uncovering a specific role of miR-30c in cardiac physiology.182 Overall, these findings indicate a role of miRNAs in mitochondrial expression modifications that may underlie cardiac dysfunction.

4. MIRNAS IN THE DIAGNOSIS AND PROGNOSIS OF HF

Since HF is a highly heterogeneous disease, both in terms of etiology, clinical manifestation and outcome, early diagnostic and/or prognostic markers could considerably contribute towards the timely detection and more effective management of the disease.128 Towards this direction, significant ongoing efforts are aiming to depict miRNAs that could fulfill this role. A plethora of studies refer to observed changes in miRNA expression as potentially relevant in the diagnostic or prognostic setting. However, a very limited number of studies have been designed to address the per se diagnostic classification and/or prognostic value of miRNA markers. These studies have assessed cardiac tissue biopsies derived during surgery and peripheral blood.

4. a. Diagnosis

Cardiac tissue miRNAs

A recent study by Leptidis et al performed next generation sequencing in human failing left ventricles of end-stage HF patients of HCM and DCM etiology and reported over 250 significantly changed miRNAs in HF.33 Interestingly, the miRNA signatures differed significantly based on the pathology preceding HF (DCM or HCM),33 a finding consistent with other studies of distinct miRNA profiles in different HF diagnostic groups (e.g. DCM, ICM, AS). Importantly, the differences reported by the latter study appear to suffice for accurate patient classification.69 In specific, Ikeda et al used 67 microRNA signatures of control, ICM, DCM and AS heart tissue in order to develop a microRNA-based classifier. A diagnostic group label was assigned to each of the samples, which interestingly, corresponded to the clinical diagnosis 69% of the time (P < 0.001).69 Nevertheless, cardiac tissue miRNA signatures would have a limited diagnostic value, due to the requirement of a cardiac biopsy. However, if cardiac miRNA signatures prove to correlate with circulating miRNA signatures, they could be easily translated to clinical practice, facilitating patient classification, and potentially prognosis and treatment.

Circulating blood miRNAs

A number of studies have focused on the miRNA expression in HF patient peripheral blood. Among them, several have pointed to an increase in miR-423-5p, often combined with a number of other miRNAs. For example, it has been proposed that increased serum levels of miR-423-5p, along with miR-320a, -22, and miR-92 can be used to identify patients with systolic HF and correlate with clinical prognostic parameters such as elevated serum natriuretic peptide levels, a wide QRS (Q, R, S waves of an electrocardiogram) and dilatation of the left ventricle and left atrium.129 Similarly, another group suggested that increased plasma levels of miR-423-5p can be a diagnostic biomarker of HF caused by DCM, while they correlated positively with N-terminal pro-brain natriuretic peptide (NT-proBNP) levels.130 However, it should be noted, that miR-423-5p has been investigated extensively in the context of multiple cardiac pathologies, with contradictory findings to date. Additional research is therefore needed, before final conclusions can be reached and findings are translated to the clinic.

Voellenkle et al investigated the miRNA expression pattern of peripheral blood mononuclear cells (PBMCs) in chronic HF patients suffering from ICM and nonischemic DCM.134 This group reported that three miRNAs (miR-107, -139, -142-5p) were decreased in both patient groups, while each group also
featured additional altered miRNAs, and specifically decreased miR-125b, -497 in ICM, and increased miR-142-3p,-29b in nonischemic DCM. These findings suggest that chronic HF has a distinct miRNA expression profile in PBMCs, along with etiology-dependent changes that may allow patient classification, upon further validation of these results.

4. b. Prognosis

Circulating miRNAs as prognostic markers

In the context of identifying predictors of the development of ischemic HF in post AMI patients, the analysis of 377 miRNAs pointed to three p53-responsive microRNAs, namely miR-192, -194, and -34a, that were increased in the serum of patients who developed HF within one year of AMI onset.131 Moreover, a significant correlation was observed between miR-194, -34a expression levels and left ventricular end-diastolic dimension.131 These overall findings indicate that HF-related circulating miRNAs may serve as accurate biomarkers only for specific patient groups, and require further investigation in order to specify the conditions under which they could facilitate HF diagnosis and prognosis in the clinical setting.

He et al assessed the diagnostic and prognostic value of circulating miR-328 and -134 in AMI patients. To this aim, this group performed qPCR in plasma samples of 359 AMI patients and 30 healthy volunteers, and in parallel measured high-sensitivity cardiac troponin T (hs-cTnT) levels. Whilst miR-328 and -134 plasma levels were found to be significantly higher in patients in comparison to healthy controls, the diagnostic value of these miRs as determined by ROC curve analysis was significant, but inferior to (hs-cTnT) levels for AMI diagnosis. Interestingly though, the levels of these two circulating miRNAs were found to be associated with the risk of mortality and development of HF within 6 months after infarction (miR-328: OR 7.35, 95 % confidence interval 1.07-17.83, P < 0.001, miR-134: OR 2.28, 95 % confidence interval 1.03-11.32 P < 0.001).183 As such, miR-328 and 134 could be utilized as prognostic markers of post AMI clinical outcome.

Qiang et al investigated the miRNA expression profiles of endothelial progenitor cells (EPCs) isolated from venous blood of chronic HF patients with ICM or non-ischemic CM. This study identified sixteen miRNAs as differentially expressed between the two patient groups (miR-126, -508-5p, -34a, -210, -490-3p, -513-5p, -517c, -518e, -589, -220c, -200a*, -186*, -7i*, -200b*, -595, -662) and conducted a survival analysis using the patients' two-year follow up data. As a result, the levels of two of the differentially expressed miRNAs, miR-126 and -508-5p, were identified as independent prognostic factors of survival in both patient groups (P = 0.003; HR (hazard ratio): 0.19; 95% CI (confidence intervals): 0.06-0.58, and P = 0.002; HR: 2.292; 95% CI: 1.37-3.84 respectively).132 This study brought to light two miRNAs that could be possibly used as prognostic markers of the clinical outcome of CHF.

In another study, the plasma concentrations of miR-126, -122 and -499 were measured in 33 congestive HF patients with ischemic heart disease and 17 asymptomatic controls. MiR-126 plasma levels were found to be decreased in HF patients, and negatively correlated with age, logBNP (B-type natriuretic peptide) and NYHA (New York Heart Association) class.135 Interestingly, miR-126 levels increased with improvement of the NYHA class from IV to III, in ten of the HF patients investigated. This finding is in line with a putative correlation of miR-126 with HF clinical outcome suggested by Qiang et al in 2013. However, miR-126 downregulation has also been related to coronary artery disease.136 Further investigation is required in order to assess if miR-126 downregulation is etiology-dependent or pertinent to HF development.

Goren et al aimed to identify circulating miRNAs that can be used as markers for atrial fibrillation (AF), given that AF is associated with poor prognosis in HF patients. They performed a genome wide screen for miRNAs in platelet and serum samples from 41 patients with systolic HF and 35 controls without cardiac disease. They observed that miR-150 levels were 3.2 fold lower in platelets of AF patients when compared to non AF HF patients, and 1.5 fold lower in the respective AF serum samples in comparison to non AF HF. Moreover, the serum levels of (cell-free) miR-150 in AF patients were found to be correlated with platelet levels of miR-150.184 Further investigation is required in order to assess if the markedly reduced miR-150 level in platelets and serum can be utilized as a prognostic marker for HF patients.

In agreement with HF patient studies, a recent analysis of hypertension-induced HF in rats detected significantly increased plasma levels of miR-423-5p, -16, -20b, -93, -106b, and -223.133 The levels of these miRNAs were also measured during disease progression, at 2, 4, 6, 8 weeks after the high-salt diet onset in the salt-sensitive rats. Interestingly, some of the observed changes in miRNA expression
paralleled disease progression. Specifically the levels of miR-106b and 93b showed significant upregulation at week 2 after diet onset, miR-20b at week 4, miR-19b at weeks 2 and 8, miR-423-5p at week 8 and miR-223 at weeks 6 and 8. The same study also assessed the levels of miR-16, -20b, -93, -106b, -223, and miR-423-5p after treatment with ACE inhibitors or mir-208 inhibition, and reported attenuation of their increase. These data suggest that the pattern of circulating miRNAs expression may be representative of distinct time points during HF progression, and as such they may be utilized in the prognostic setting. Additionally, early evidence indicates that circulating miRNAs could also be used to monitor response to HF treatment.

5. MIRNA AND NOVEL THERAPEUTIC APPROACHES FOR HF
5. a. RNA interference as a therapeutic approach
RNA interference (RNAi) technology has emerged as an effective method to manipulate gene expression. Importantly, RNAi has been recently proposed as a novel therapeutic strategy for manipulating dysregulated gene expression in human disease, and the first clinical trials using RNAi therapeutics are highly promising. The basic principle of RNAi is triggering gene expression silencing by an 18–27 nucleotide long small RNA that identifies the target mRNA(s) via base pairing, with the most important classes of small RNAs utilized being miRNA and small interfering RNA (siRNA). These two types of small RNAs have a similar course of action, but different biogenesis. MiRNAs originate from hairpin molecules containing ssRNAs (described in Section 1), whereas siRNAs originate from dsRNA which is in turn processed by Dicer and then directed to the target mRNA in the same manner as miRNA. In mammalian cells, two approaches are followed in order to achieve RNAi mediated gene silencing: the RNA- and the DNA-based approach. In the first approach, 21 base siRNA duplexes are chemically synthesized and delivered directly to the target cells, resulting in a transient gene silencing effect due to the short half-life of siRNA. In the DNA-based approach, short hairpin RNA (shRNA) are delivered into the cell via viral vectors, and consequently shRNAs are synthesized in the nucleus and exported to the cytoplasm through the miRNA machinery, to be processed by Dicer and become siRNA effectors, thus achieving long term gene suppression.

Being an effective tool for gene silencing, siRNA emerges as a potential therapeutic agent for CVD and HF, according to in vitro and in vivo studies. A representative example of the therapeutic applications of siRNA in HF is the knock down of phospholamban (PLN) via RNAi in the TAC rat model of HF. PLN is a muscle-specific protein acting as an inhibitor of SERCA2A, but upon its phosphorylation triggered by β-adrenergic stimulation, it fails to inhibit SERCA2A, thus leading to increased heart contractility. Notably, suppression of PLN gene underlie an inherited form of DCM that presents with severe CHF in humans, whilst suppression of Pln has been engaged aiming to preserve Serca2 activity and prevent HF in animal models of HF. Suckau et al developed a dimeric cardiotropic adeno-associated virus vector (rAAV9-shPLB), which was administered intravenously to TAC rats, in order to suppress Pln expression in the heart via RNAi. Interestingly, cardiac Pln protein levels were reduced to 25% and the observed suppression of Serca2 was restored in TAC rats, ultimately resulting in the attenuation of TAC-induced cardiac dilation, hypertrophy and fibrosis. These findings have been confirmed and expanded by other groups. Overall, it emerges that suppression of PLN or PP1 by RNAi could provide novel therapeutic strategies to fight HF.

Although the mechanism of RNAi and its therapeutic efficacy are not yet fully elucidated, RNAi emerges as a promising therapeutic strategy. It has been demonstrated that RNAi techniques have great sensitivity and specificity for the target gene, whilst its cooperation with the cell’s own miRNA machinery may allow the transcriptional suppression of virtually any gene of interest. However, the therapeutic use of RNAi in humans has yet to overcome a number of obstacles, such as effective in vivo delivery method to specific tissue or cells, specific siRNAs designed for each mRNA target with diminished off-target effects, and avoidance of innate immunity activation by siRNAs.

Interestingly, these concerns may soon subside as recent studies showed that intravenous administration of nanoparticle-enclosed siRNAs is safe, and capable of triggering target-specific suppression of gene expression via an RNAi mechanism of action in cancer patients. Importantly, in a phase I trial, researchers showed that intravenous administration of the siRNA ALN-PCS -targeting the circulating protein PSCK9, in order to lower LDL plasma levels- resulted in significant plasma level reduction of PSCK9 (70%), and led to reduction of LDL (40%). To date, the latter study is the first to show that a therapeutic agent based on RNAi technology can be safely administered and affect a
clinically validated endpoint, thus indicating that novel and effective RNAi-based drugs may soon be an alternative option to existing therapeutics.

5. b. MiRNAs as therapeutic targets: AntagomiRs and miRNA mimics

As aforementioned, several dysregulated miRNAs have been associated with HF pathogenesis and HF related pathologies, thus the targeted modulation of miRNA expression and activity may be a promising therapeutic approach to improve HF clinical management.

The targeted regulation of miRNA pathways could be facilitated by a variety of molecular tools, divided into two main categories: anti-miRNAs (antagomiRs) and miRNA mimics. AntagomiRs are modified antisense nucleotides that can trigger downregulation of the intracellular levels of selected miRNAs. AntagomiRs may intervene at multiple levels on the cellular miRNA machinery, including i) binding to mature miRNA within RISC and serving as a competitive inhibitor, ii) binding to pre-miRNA and inhibiting their processing and incorporation to the RISC complex, and iii) inhibiting the processing or the exit of pre-miRNA or pri-miRNA from the nucleus. Importantly, in all cases, antagomiRs activity ultimately results in increased intracellular levels of the corresponding mRNA targets. Conversely, miRNA mimics are synthetic RNA duplexes that mimic the function of endogenous mature miRNAs, and aim to decrease the levels of selected mRNA targets.

Interestingly, several investigating groups have engaged antagomiRs in an attempt to inverse the pathological phenotype that was seemingly triggered by specific miRNAs in HF. For example, Montgomery et al administered antagomiR-208a to Dahl rats with hypertension-induced HF, and prevented the pathological “myosin switch” and cardiac remodeling, whereas cardiac function, overall health and survival were markedly improved. Ucar et al used antagomiRs to target the pro-hypertrophic miR-132 and miR-212, in mice with heart specific overexpression of these miRNAs presenting with cardiac hypertrophy and HF. Accordingly, injection of antagomiR-132 rescued cardiac hypertrophy and HF in vivo, whereas transgenic mice lacking both miR-212 and -132 were protected from TAC-induced hypertension, and were partially protected from TAC-induced cardiac fibrosis, dilatation and impaired left ventricular function. These data indicate a causal role of miR-132 and a contributing role of miR-212 in the development of hypertrophy and HF in vivo, whilst suppression of miR-132 via antagomiRs emerges as a possible therapeutic approach for HF.

In contrast, inhibition of endogenous miR-21 or miR-18b was shown to increase hypertrophic growth in cultured CMCs. However, the latter study is in contrast with the findings of other groups regarding the role of miR-21 in hypertrophy. Cheng et al performed antisense-mediated depletion of miR-21 in hypertrophic CMCs which overexpressed miR-21, and reported significantly decreased CMC hypertrophy. Moreover, Thum et al used antagomiRs to inhibit mir-21 in TAC mice, and as a result the TAC-induced cardiac hypertrophy was attenuated. Interestingly though, Patrick et al claimed that genetic deletion or inhibition of miR-21 in mice did not alter the hypertrophy they displayed in response to cardiac stressing stimuli (TAC, MI, chronic calcineurin activation, infusion of Ang II), implying that mir-21 is not essential for the development of pathological cardiac hypertrophy.

This discrepancy was subject to further discussion, and the different length of the anti-miR-21 oligonucleotides used by the groups of Thum and Patrick (22-mer vs 8-mer) were suggested as the cause of this inconsistence, whilst the difference in the phenotype of the miR-21 deficient mice has yet to be explained. MiR-21, being mainly expresses by CFs, has also emerged as a regulator of cardiac fibrosis, and as such Thun and Patrick also investigated the effect of miR-21 inhibition in this subpathology. According to Thun et al, miR-21 inhibition protected TAC mice against cardiac fibrosis, but Patrick et al has called into question the role of miR-21 in cardiac fibrosis, as well as hypertrophy. The latter reported that genetic deletion or inhibition of miR-21 in mice did not have an effect on the fibrosis the developed in response to a variety of cardiac stressing stimuli (TAC, MI, chronic calcineurin activation, infusion of Ang II). This inconsistence may be due to a technicality (antagomir length), but leaves open the possibility of a contributing role of miR-21 in the development of cardiac fibrosis.

MiRNA mimics have been utilized in the experimental setting in order to normalize the expression of miR-9 which is observed downregulated during cardiac hypertrophy. Wang et al administered a double-stranded RNA miRNA mimic for miR-9 in rats with isoproterenol-induced cardiac hypertrophy, and successfully reduced the levels of miR-9 target myocardin, ultimately leading to attenuation of cardiac hypertrophy and improvement of cardiac function. Although the efficiency of miRNA mimics is subject to a number of limitations, regarding in vivo delivery methods, cellular uptake and off-target
effects, this study provides a paradigm of a possible therapeutic approach, where exogenous supplementation of miRNA mimics could be used to replenish endogenous miRNAs that are reduced during cardiac disease. Overall, it is important to note that mimics (other than viral delivery) have thus far not proven to be a viable option in vivo. In fact, it is thought that in vivo methods, other than viral delivery of mimics, actually result in an miR inhibitory effect, rather than a mimic effect.

According to the aforementioned studies, miRNA modulation appears to be a promising tool for the development of novel therapeutic strategies against cardiac disease and HF. However, further investigation is still required in order to fully reveal the functions of antagomiRs and miRNA mimics, as well as their exact pharmacodynamic and pharmacokinetic characteristics, and the potential adverse reactions or side effects. In addition, comparisons of the specificity and efficacy of the different miRNA antagonism and mimicking chemistries will need to be made, and the most effective and safe modes of in vivo deliver are yet to be determined.

5. c. miRNAs in cardiac regeneration: a novel therapeutic approach

In the failing myocardium, systolic dysfunction may occur as a result of many subpathologies, with MI-induced cardiac injury being one of the most common. The loss of functional CMCs due to MI or HF may deteriorate cardiac function, and the adult heart has a very limited ability to repair damaged tissue through myocardial regeneration. CMCs have a very low proliferative rate during postnatal development, but recent evidence supports the increased CMC proliferation at the border zone of the heart post-MI in adult mice. Interestingly, several lines of evidence indicate that miRNAs are potent regulators of CMC cell cycle (see Section “miRNAs play a central role in cardiac development”), and could be manipulated to trigger CMC proliferation in order to achieve myocardial regeneration. For example, knock down of miR-195 increases mitotic CMCs, and inhibition of miR-29a induces cell proliferation, accelerates G1/S and G2/M transition, and enhances cell cycle gene expression, acting at least in part through cyclin D2.

Furthermore, exogenous administration of hsa-miR-590 and -199a, stimulates cardiac regeneration and reduces infarct size in animal models of MI. More importantly, miRNA-induced cardiac regeneration was accompanied by almost complete recovery of cardiac functional parameters (e.g. left ventricular ejection fraction LVEF, left ventricular fractional shortening LVFS). Similarly, the miR-17-92 cluster appears to be a potent stimulator of CMC proliferation in embryonic, postnatal and...
adult murine hearts. Overall, these findings point to miRNAs as a novel, promising approach for treating HF related CMC loss.

CONCLUSION

The continuously expanding field of miRNA research has revealed the significant contribution of these small molecules in a broad range of physiological and pathological mechanisms (Figure 3). In the context of heart biology, miRNAs prove to be potent regulators of gene expression during cardiac development and are directly implicated in the onset and progression of heart failure, amongst other pathological conditions. These valuable new insights change our perception of disease pathogenesis, and unveil promising new diagnostic and prognostic markers. Importantly, miRNAs open the way to a new line of therapeutic approaches that could play a significant role in the future of the cardiology clinics.

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