MicroRNAs orchestra the cellular processes driving failure of the heart

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MicroRNAs Orchestrate the Cellular Processes Driving Failure of the Heart

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ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof dr. Rianne M. Letschert
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in het openbaar te verdedigen
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door

Robin Henricus Karel Oliver Verjans

Contents

Chapter 1:	General Introduction	7
Chapter 2:	MiRNA Deregulation in Cardiac Aging and Associated Disorders	19
Chapter 3:	MicroRNAs Act as Pleiotropic Regulators of Cellular Processes Underlying Heart Failure: <i>A High-Throughput Phenotypical Screen in Three Primary Cardiac Cell Types</i>	75
Chapter 4:	The MicroRNA-221/222 Family Counteracts Myocardial Fibrosis in Pressure Overload-Induced Heart Failure.	145
Chapter 5:	MiR-151 Represses Cardiomyocyte Hypertrophy and Oxidative Metabolism by Targeting PGC1 α .	169
Chapter 6:	Cardiomyocyte Cell Cycle Activity is Under the Control miR-125a	191
Chapter 7:	General Discussion	211
	Summary / Samenvatting	221
	Valorisation	229
	Dankwoord	235
	Curriculum Vitae	243

CHAPTER 1

General Introduction

1.1 Failure of the Heart

Cardiovascular disease (CVD) is the leading global cause of death, accounting for more than 17.3 million deaths per year, representing 1 of every 3 deaths [1]. In the Netherlands only, more than 100 people die from cardiovascular disease each day, an average of 1 death every ~15 minutes [2]. This life-threatening disease is accompanied with enormous global costs, reaching \$863 billion in 2010 [3]. Almost half of the patients with newly diagnosed CVD have heart failure (HF)[4], representing the only cardiovascular disease that has increased in prevalence over the last 20 years [5]. In the Netherlands, currently ~142.000 patients suffer from HF [2]. Especially the elderly population is at risk to develop HF. The incidence rates in males approximately doubles with each 10-year age increase from 65 to 85 years, while even tripling for females between ages 65 to 74 and 75 to 84 years [1]. These numbers mark that HF currently forms a major global health problem but HF will form an even greater health problem in the future, since the general population ages and affective treatment is still lacking. According to the American Heart Failure Association, the prevalence of HF will increase with 46% from 2012 to 2030, resulting in more than 8 million people with an age of 18 years or older with HF [6]. Similarly, the total cost for HF in the US was \$30.7 billion in 2012 and are expected to increase to \$69.7 billion by 2030 [3, 6]. Strikingly, the high five-year mortality of HF patients (52.6%) did not decline since 2000, highlighting the ineffectiveness of traditional therapeutics and the urgent need to develop novel treatment strategies.

1.2 MicroRNAs

MicroRNAs (miRNAs) are small endogenous regulatory RNAs belonging to a family of short single-stranded ribonucleic acids. These critical posttranscriptional regulators are tightly regulated in a temporally restrained and tissue-specific manner during development and pathological conditions. MiRNAs were first discovered in the early 1990s but only after 2001 a dedicated field focused on the study of these regulatory RNAs, following the identification of numerous endogenously expressed small RNAs [7–9]. In the ensuing decade, miRNA biology has attracted remarkable attention, resulting in rapid advances in understanding their function during development and disease. We have learned that mammalian genome encodes 300 conserved miRNA genes, and high-throughput sequencing studies have identified 1000 or more additional loci that produce small RNAs structurally resembling miRNAs [10, 11]. At least 30% of all human genes are thought to be regulated by miRNAs [12], marking the importance of miRNA-regulated networks in maintaining homeostasis.

1.3 MiRNA Biogenesis

The generation of miRNAs is a multistage process (Figure 1). Although some miRNAs are individually transcribed by RNA polymerase II/III from separate transcription units, many

more miRNAs are produced from transcription units that make more than one product. A transcript may encode clusters of distinct miRNAs, or it may encode a miRNA and a protein [13–15]. The resulting ~60–80 nucleotide long hairpin structures contain a terminal loop and flanking segments from the miRNA sequence, forming the primary miRNA (pri-miRNA) transcript [14]. Two sequential processing reactions are required to excise the transcript into the mature miRNA.

Figure 1 MicroRNA biogenesis. The miRNA biogenesis pathway includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha with the aid of DGCR8 in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5. In the cytoplasm, the RNase Dicer cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it functions as a guide to silence target mRNAs, whereas the passenger strand (black) is degraded.

The first processing step occurs in the nucleus and is performed by Drosha with the aid of DGCR8 (DiGeorge critical region 8). DGCR8 directly interacts with the pri-miRNA to serve as a molecular ruler that properly positions Drosha to form the Microprocessor complex [16, 17]. With the help of DGCR8, the catalytic site of Drosha is positioned 11 nucleotides from the base of the hairpin structure, allowing Drosha to excise the stem-loop to create a ~70 nucleotide stem-loop precursor miRNA (pre-miRNA) [15, 18]. However, microprocessor-mediated cleavage is not the only way to produce pre-miRNAs. Alternatively, splicing of pri-miRNA transcripts can take place to liberate introns that precisely mimic the structural features of pre-miRNAs [19, 20]. These mirtrons then enter the miRNA-processing pathway without the aid of the Microprocessor.

The pre-miRNA is subsequently exported to the cytoplasm via Exportin-5 to undergo the second processing step. This reaction is catalysed by Dicer, excising the terminal loop from the pre-miRNA stem to yield a mature miRNA duplex of approximately ~22 nucleotides in length [13, 15]. The processed miRNA duplex comprises characteristic 5' and 3' ends, carrying a monophosphate group and a dinucleotide overhang, respectively that ensure efficient loading onto Argonaute 2 (AGO2) to form the RISC complex (RNA-induced silencing complex) [21–23]. Subsequent to loading, the so-called guide strand is selected to form the miRNA effector as part of the RISC, while the remaining passenger strand is released and degraded [24–30].

1.4 MiRNA-induced posttranscriptional repression

The miRNA strand incorporated into RISC acts as a sequence-specific adaptor to recognize and regulate complementary target messengerRNAs (mRNAs). Typically, miRNA-binding sites in mRNAs reside in the 3' untranslated region and are usually present in multiple copies. Although the degree of miRNA-mRNA complementarity has been considered a key determinant of the regulatory mechanism, most miRNAs bind with imperfect complementarity. The “seed” sequence (nucleotides 2–8 at the 5' end) of miRNAs is most critical in determination of target recognition, and in some cases fully sufficient for target selection [31–35].

Although target recognition is greatly understood, the mechanisms of miRNA-mediated silencing have been subject to on-going debate. Different possible mechanisms of RISC-mediated repression of translation have been postulated. Normally, translation initiation begins with the recognition of the mRNA 5' terminal cap by eIF4E, one of the subunits of the eIF4F complex. Subsequently, the eIF4F complex recruits the 40S ribosomal subunit and joins with the 60S ribosomal subunit at the AUG codon to begin elongation. RISC is postulated to repress initiation of translation by competing with eIF4E to bind to the 5' terminal cap [36, 37], and by blocking the association of the 60S ribosomal subunit with the 40S pre-initiation complex [38].

The eIF4F complex additionally interacts with the poly(A)-binding protein PABP1 that decorates the 3' end of the message. The ability of the eIF4F complex to interact simultaneously with eIF4E and PAB1 effectively circularizes the mRNA molecule and greatly enhances translation efficiency [39]. However, RISC can interfere with this process by deadenylation of the mRNA, limiting the circularization of the cap and PABP1-free tail of the deadenylated mRNA [40]. Alternatively, RISC is suggested to promote premature ribosome dissociation from mRNAs. Rapid dissociation of ribosomes in a miRNA-dependent manner represses elongation and prevents mRNA translation [41]. The current paradigm prevails that miRNA-mRNA target interaction may lead to reductions in mRNA abundance due to an increase in mRNA degradation because of deadenylation, decapping, and exonucleolytic digestion of the mRNA [42, 43]. In support of this model, many repressed mRNAs are deadenylated by miRNAs *in vivo* [42] and *in vitro* [40].

1.5 MiRNAs in Cardiovascular Disease

Irrespective of the precise mechanism of MiRNA-induced repression of mRNA translation, miRNAs profoundly influence the responses of tissues to physiologic and pathophysiologic stress [44]. This suggests a central role for miRNA-regulated networks in disease states, which often represent an insufficient or aberrant response under conditions of stress or injury. MiRNAs are involved in many disease states, including the aging and failing heart [45, 46]. Numerous miRNA profiling studies in mouse models of cardiovascular disease and in human biopsies has revealed deregulated expression patterns of miRNAs in various cardiovascular disorders, including atherosclerosis [47], myocardial infarction [48], and heart failure [49]. Gain- and loss-of-function studies in rodents have also validated the importance of specific miRNAs in the pathophysiology of many of these cardiovascular disorders [46, 50]. Taken together, these studies indicate an altered miRNA profile during cardiac pathophysiology and the ability to regulate disease progression, highlighting their potential as therapeutic target.

1.6 Aims and Outline of this Thesis

The main **aims** of this thesis are: **1) to identify miRNAs that regulate cellular processes driving failure of the myocardium**, and **2) to explore if the manipulation of some promising miRNA candidates (identified under aim 1) is able to retard heart failure progression**. Thereto, we first performed a literature review and an *in vitro* high-throughput phenotypical screen to identify miRNAs that potentially play a role in the development of heart failure. Subsequently, we aimed to probe their efficacy in retarding heart failure progression in more detail through their manipulation *in vitro* and in various animal models.

In **chapter 2**, we review the cardiac molecular and cellular processes driving myocardial aging, and describe the regulating role of senescence-associated miRNAs in these processes. Given the high similarity in molecular mechanisms of cardiac aging and HF, we additionally discuss miRNAs implicated in HF, focussing on miRNAs that show differential expression in the aged heart.

Chapter 3 describes an *in vitro* high-throughput screening approach in which we selected 194 miRNAs associated with HF development, and assessed their regulatory function in different cellular processes underlying HF, namely cardiomyocyte hypertrophy, cardiac fibrosis, and inflammation, in parallel. I have done this research was performed at Cenix BioScience, Dresden, Germany, where I have worked for 2 years. Cenix Bioscience was a company that was renowned internationally for its expertise in high-throughput screening.

Chapter 4 through 6 will provide the experimental studies performed to determine the effect of individual miRNAs or miRNAs families on cellular processes underlying heart failure development. **Chapter 4** focuses on the important role of the miRNA-221/222 family in the process of myocardial fibrosis formation as an underlying factor of HF progression. **Chapter 5** elaborates on the function of miR-151a in cardiomyocyte hypertrophy and its ability to modulate cardiomyocyte metabolism by targeting PGC-1 α . **Chapter 6** addresses the effect of miR-125a on cardiomyocyte cell cycle activity and the hypertrophic response.

Finally, **chapter 7** provides a summary of the described research and will discuss the value, limitations, and opportunities of these findings in a broader perspective, as well as its clinical utility.

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CHAPTER 2

MiRNA Deregulation in Cardiac Aging and Associated Disorders

Adapted from International Review
of Cell and Molecular Biology

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Abstract

The prevalence of age-related diseases is increasing dramatically, among which cardiac disease represents the leading cause of death. Aging of the heart is characterised by various molecular and cellular hallmarks impairing both cardiomyocytes and non-cardiomyocytes, and resulting in functional deteriorations of the cardiac system. The aging process includes desensitization of β -adrenergic receptor (β AR)-signalling and decreased calcium handling, altered growth signalling and cardiac hypertrophy, mitochondrial dysfunction and impaired autophagy, increased programmed cell death, low-grade inflammation of non-canonical inflammatory cells, and increased ECM deposition.

MiRNAs play a fundamental role in regulating the processes underlying these detrimental changes in the cardiac system, indicating that miRNAs are crucially involved in aging. Among others, miR-34, miR-146a, and members of the miR-17–92 cluster, are deregulated during senescence and drive cardiac aging processes. It is therefore suggested that miRNAs form possible therapeutic targets to stabilize the aged failing myocardium.

Keywords:

Aging / Autophagy / Extracellular matrix / Fibrosis / Hypertrophy / Inflammation / Mitochondria / MicroRNA.

Introduction

The global population ages dramatically as the average life expectancy increased the past century. In the United States, currently $\pm 12\%$ of the population is over 65 years, and is estimated to further increase to $\pm 20\%$ by 2030 [1]. This increase in global age is accompanied by a decrease in quality of life in the elderly population and a dramatically growing prevalence of age-related diseases. Cardiac disease represents the leading cause of death among these age-related diseases, accounting for $\pm 40\%$ of all deaths in the elderly population [1, 2]. Heart failure (HF), the inability of the heart to pump sufficient blood into the body, is associated with frequent hospitalizations [3], resulting in high consumption of medical resources and a poor 5-year survival rate [4]. Heart failure (HF) in particular is generally accepted as a typical age-associated cardiac disease and is reaching epidemic proportions, with $\pm 88\%$ of HF deaths and $> 75\%$ of all HF hospitalizations in the United States occurring in adults aged over 65 years [5, 6]. HF is estimated to form an even greater problem in the future since, besides the fact that the increasing aged population has a high HF prevalence, effective treatment is still lacking. Besides the increasing age-associated prevalence of several cardiac risk factors including hypertension, obesity, and insulin resistance, aging itself is a major independent risk factor for cardiac disease [7, 8], stressing the importance of understanding the processes underlying intrinsic myocardial aging.

MicroRNAs (miRNAs) are a class of small ± 22 nucleotides long non-coding RNA molecules that negatively regulate target gene expression [9, 10]. MiRNAs are implicated in cardiac disease and increasing evidence links miRNAs to cardiac aging. Regulatory functions of age-associated miRNAs may potentially explain observed age-related deleterious phenotypes.

Cardiac aging

Due to the lack of proliferative capacity of cardiomyocytes, it is suggested that the heart is one of the organs most affected by detrimental age-associated changes. The senescent heart is characterized by structural remodelling at both organ, cellular, and molecular level. Remodelling of the aging cardiac system includes an increase in left ventricular wall thickness, stiffening of the heart, increased deposition of collagen, and collagen cross-linking [11]. On a cellular level, aging is associated with prolongation of cardiomyocyte action potential [12], decreased cardiomyocyte survival, as well as a decline in the number of mitochondria and cardiac stem cells [13–15]. Furthermore, endothelial dysfunction, migration/proliferation of vascular smooth muscle cells (VSMCs), increased fibroblast proliferation, and pro-inflammatory signalling of inflammatory cells are additional cellular processes underlying cardiac aging [16, 17]. These structural changes lead to an altered function of the aged myocardium, including an increase in the prevalence of atrial fibrillation [6], a lowered cardiac reserve [15], as well as declined diastolic function due to increased left ventricular diastolic stiffness, decreased filling [14, 18], and decreased contraction and relaxation velocity [12, 19–21].

The associated molecular processes include 1) desensitization of β -adrenergic receptor (β AR)-signalling and decreased calcium handling, 2) altered growth signalling, 3) mitochondrial dysfunction and impaired autophagy, 4) increased programmed cell death, 5) low-grade inflammation of non-canonical inflammatory cells, and 6) increased extracellular matrix (ECM) deposition [6, 14, 15, 22]. Thus, the intrinsic mechanisms associated with age-induced impairment of the myocardium are identified and mimic those implicated in HF. Unfortunately, precise knowledge on what initiates these cellular changes and the role of miRNAs therein is still lacking. This review provides an overview of the cardiac molecular and cellular processes driving myocardial aging listed above, and describes the regulating role of senescence-associated miRNAs in these processes. Given the similarity in molecular mechanisms of the aging and failing heart, we will as well discuss miRNAs implicated in HF of other causes than aging, focussing on miRNAs that show differential expression in the aged heart.

β AR desensitization and calcium handling

The sympathetic nervous system undergoes a series of rearrangements with aging. With normal aging, circulating norepinephrine levels increase by 10–15% per decade [23] and cardiac norepinephrine levels increase due to diminished reuptake and increased tissue spill over [24]. Chronically increased β AR occupancy by norepinephrine leads to β AR desensitization [25–27] and impairs cardiomyocyte calcium handling, eventually leading to deficits in cellular contraction and relaxation [28, 29]. Prolonged myocardial relaxation time forms a hallmark of age-related diastolic dysfunction and is caused, among others, by impaired active relaxation of cardiomyocytes due to depressed Ca^{2+} -uptake and Ca^{2+} -release activities in the aging heart [30–32]. In the normal situation, β AR activation regulates Ca^{2+} homeostasis and contraction through accumulation of the second messenger cAMP, which activates protein kinase A (PKA). In its turn, PKA phosphorylates phospholamban (PLB) and relieves its inhibitory effect on calcium-transporter SERCA2a, promoting sarcoplasmic reticulum cycling [33, 34] (Figure 1). Decreased expression [18] and activity [27] of SERCA2a are thought to be the primary mechanisms for the impaired cardiac relaxation in the aged myocardium. In addition, age-associated alterations in proteins able to regulate SERCA2a activity, including PLB, [35–38], PKA [39], and CAMKII [40] have also been documented in the aged heart. Thus, β AR signalling plays a crucial role in maintaining a balanced calcium homeostasis and regulating cardiomyocyte contraction and relaxation, and dysregulated β AR signalling forms one of the major cellular hallmarks of the senescent myocardium.

Evidence shows that altered β AR activity shapes cardiac miRNA profiles while specific miRNAs, including β AR-responsive ones, are able to influence β AR signalling. This implies an important role for miRNAs in age-associated desensitization of β AR signalling. Hou et al. [41] profiled rat cardiac miRNA expression under conditions of β AR activation and inhibition, identifying miRNAs controlling β AR pathway activity. Activation of β AR signal-

ling upregulated 43 while downregulating 9 miRNAs. On the other hand, β AR inhibition upregulated and downregulated 5 and 28 miRNAs, respectively [41]. Similarly, Carrillo et al. [42] found that the expression of 33 miRNAs was significantly changed in cardiac lysates from rats stimulated with the β AR agonist isoproterenol (ISO). The expression levels of miR-21, miR-132, miR-214, and miR-222 were more than 7-fold increased. MiR-21, miR-132 and miR-222 are also directly involved in controlling calcium levels via targeting, beta-2 subunit of the voltage-dependent calcium channel (CACNB2) [42]. Moreover, Ling et al. [43] have shown that miR-26b, miR-21, and miR-320 levels are affected by changes in β AR activity.

β AR signalling is shown to control one of the most abundantly cardiac expressed miRNA clusters, the miR-1/133 cluster via a cAMP response element in the promoter region of muscle specific miRNA cluster miR-1/miR-133a [44–47]. Intriguingly, miR-1 potentiates β AR signalling, causing hyperphosphorylation of ryanodine receptor 2 (RyR2) channels thereby increasing excitation contraction coupling [48]. On the contrary, miR-133 inhibits β AR-signalling at multiple levels. Transgenic cardiac specific miR-133 expression preserves cardiac performance and attenuates cardiac remodelling upon pressure overload [49]. This protective miR-133 effect is associated with decreased cAMP/PKA-dependent phosphorylation of PLB, and resulting from miR-133 directed targeting of the β 1AR (ADBR1) and several other components of the β 1AR signalling cascade [49]. Thus, while both miRNAs are under the regulation of β AR-signalling, these miRNAs have opposite roles. Exactly how their balance is regulated requires further investigation.

The cardio-protective effect of biased β -blocker carvedilol (Carv) involves enhanced β -arrestin-mediated signalling, thereby affecting pri-miRNA processing of specific miRNAs by Drosha. As a consequence, the maturation of miR-125a-5p, miR-125b-5p, miR-150, miR-199a-3p, and miR-214 is upregulated in the mouse heart [50, 51]. These Carv-responsive miRNAs may potentially explain the cardio-protective effect of biased β AR signalling [52]. In addition, their expression is altered in the aged heart [51, 53], suggesting their potential involvement in the cardiac aging process.

Collectively, the aging-myocardium is characterised by β AR desensitization and impaired calcium handling. Particularly miR-1/133a seem to function in opposite β AR feedback loops, and the exact biological reasoning remains to be clarified.

Altered growth signalling and hypertrophy

Cardiac hypertrophy is a hallmark of cardiac aging. Although this adaptive response minimizes myocardial wall stress and maintains cardiac function on the short term, persistent hypertrophic signalling on the long-term is detrimental and underlies cardiac pathology [54, 55]. Cellular changes of cardiac hypertrophy are characterized by increased cardiomyocyte volume, expression of the fetal gene program and increased protein synthesis [56–58].

Figure 1) Age-associated miRNAs in control of the cardiomyocyte β -Adrenergic signalling and the hypertrophic response. Activation of β_1 -adrenergic receptor (β_1 -AR) controls cardiomyocyte calcium homeostasis via accumulation of the second messenger cAMP, which activates the phospholamban (PLB) inhibitor, protein kinase A (PKA). Inhibition of PLB de-represses the calcium-transporter SERCA2a and promotes calcium (Ca^{2+}) entry into the sarcoplasmic reticulum. Sarcoplasmic Ca^{2+} is in its turn exported to the cytoplasm by ryanodine receptor 2 (RyR2) to regulate cardiomyocyte contraction. Stimulation of major growth signalling pathways, including the Calcineurin/NFAT, mTOR, and IGF/AKT/PI3K pathway induces a hypertrophic response characterised by transcription of hypertrophic genes: atrial natriuretic factor (ANF), brain natriuretic factor (BNP), and β -Myosin Heavy chain (β -MHC). At every level, miRNAs are implicated in regulating these processes.

Numerous miRNA profiling studies have been performed in human hypertrophic patients and rodent models of cardiac hypertrophy, identifying various miRNAs which actively participate in cardiomyocyte hypertrophic signalling, including miR-1/miR-133 [59] miR-208 [60–62], miR-18 [63], miR-98/let-7 [64], miR-9 [65], miR-214 [66, 67], miR-199a [68, 69], miR-199b [70], miR-23a [71], and more recently the miR-212/132 family [72–74].

Deregulation of major growth signalling pathways, including the mTOR/P70/S6K [75], IGF/AKT/PI3K [76, 77], and Calcineurin/NFAT pathway [78], have been implicated in hypertrophic growth of aging cardiomyocytes (Figure 1).

MiRNAs affecting mTOR/P70/S6K signalling

The mammalian target of rapamycin (mTOR) pathway plays a critical role in cardiomyocyte hypertrophy, function, and structure via integration of nutrient and hormonal signals to regulate growth and is a major modulator of aging and age-related disease [75]. Inhibition of the pro-hypertrophic mTOR/P70/S6k signalling pathway has cardioprotective effects when the heart ages or suffers from ischaemia or overload [79–82]. These findings suggest that miRNAs targeting mTOR signalling in the failing or senescent heart could protect the heart from detrimental remodelling. Indeed, several different miRNAs are shown to suppress mTOR, directly or indirectly, thereby exerting cardioprotective functions *in vitro* and *in vivo*.

For example, miR-99a was shown to negatively regulate mTOR expression and P70/S6K activation, preventing cardiomyocyte hypertrophy under stress conditions *in vitro* and *in vivo* [83]. MiR-145 also exerts its anti-hypertrophic effect by decreasing the activity of the mTOR/P70/S6K signalling cascade. MiR-145 expression is proven to be significantly up-regulated in end-stage HF patients [84]. Li et al. [85] have shown that miR-145 attenuates hypertrophy of cardiomyocytes induced by the β AR agonist ISO by suppressing the expression and nuclear translocation of GATA6. In its turn, pro-hypertrophic transcription factor GATA6 has been found to be involved in the mTOR-induced pathological hypertrophy [86, 87] activating transcription of the fetal genes ANF, BNP, and β -MHC [88] (Figure 1). These results indicate that miR-145 attenuates the cardiac hypertrophic response by decreasing mTOR activity indirectly. MiR-451 levels were significantly increased in type 2 diabetes mellitus (DM) murine hearts and in neonatal rat cardiac myocytes stimulated with palmitic acid. Loss of miR-451 function reduced palmitate-induced lipotoxicity in nRCMs. HFD-fed, cardiomyocyte-specific, miR-451 knockout mice were protected from hypertrophy and displayed lower levels of phosphorylated mTOR, implying that miR-451 is involved in diabetic cardiomyopathy, at least partly, through suppression of mTOR activity [89].

Thus, different studies suggest that miRNA-exerted regulation of mTOR plays an important role in cardiac aging and its inhibition protects the heart from detrimental hypertrophic remodelling under pathological conditions.

MiRNAs affecting IGF/AKT/PI3K signalling

Insulin/IGF signalling plays a critical role in aging. Human serum IGF-1 levels decline over time, correlating with increased risk of HF [90]. Both Akt as well as the class IA isoform of phosphoinositide 3-kinase (PI3K) are important factors downstream of insulin and IGF receptors, and regulate cell growth, survival and metabolism [91].

Muscle-specific miR-1 is one of the most cardiac abundant miRNAs, and different studies have shown an inverse correlation between miR-1 expression and cardiac hypertrophy [45, 92]. Several miR-1 cardiac targets involved in the control of hypertrophy have been identified, including HDAC4 [93], Hand2 [94], ras GTPase-activating protein (Ras-GAP), and ras homolog enriched in brain (Rheb) [92]. Besides, miR-1 additionally modulates the cardiac hypertrophic response via direct targeting of IGF-1 [95, 96] and IGFR-1 [96], influencing insulin signalling. The pro-hypertrophic IGF-1 acts as a negative regulator of cardiac enriched miRNA-378. In turn, this miRNA regulates cardiac remodelling by both direct targeting of IGF-1R and indirect inhibition of IGF/PI3K/AKT signalling through targeting of Grb-2, a known cardiac hypertrophy modulator and an essential component of the Ras signalling pathway that is downstream of IGF-1 and other growth factors [97, 98]. In the end, miR-378 blocks growth activity, leading to suppression of fetal gene expression and inhibition of hypertrophy of cardiomyocytes [97, 98].

MiRNAs affecting Calcineurin/NFAT signalling

Calcineurin-dependent NFAT (nuclear factor of activated T cells) activation is part of a central pro-hypertrophic signalling cascade. NFAT functions as a calcineurin effector, trafficking between the cytosol and the nucleus to regulate the cardiac hypertrophic response [78, 99]. Increased cardiac activation of calcineurin/NFAT signalling is observed in aging mice [18]. Two miRNAs, miR-133a and miR-9, have so far been shown to be able to decrease Calcineurin/NFAT signalling and ameliorate hypertrophic cell growth. Firstly, myocardial expression of miR-133a is decreased in human and animal hypertrophy [45, 92, 100], and is inversely related to cardiac hypertrophy [45]. MiR-133a directly targets calcineurin [101] and NFAT [102], preventing phenylephrine-induced hypertrophic cell growth and repression of the fetal gene program [103]. In line, loss of miR-133a increased angiotensinogen expression and hypertrophy [104]. The second calcineurin/NFAT associated miRNA, miR-9 indirectly suppresses NFAT through translational suppression of myocardin, which is necessary for nuclear activation of NFAT [65].

Although the majority of identified hypertrophy-regulating miRNAs has not been studied in the process of aging, these reports indicate that many miRNAs play a crucial role in controlling the hypertrophic response in cardiomyocytes. MiRNAs exert their hypertrophy-regulatory function via impacting major growth signalling pathways, which are also associated with age-related diseases. Cellular hypertrophic signalling as a result of senescence could potentially be caused by dysregulation of these hypertrophy-regulating miRNAs. Further research should be conducted to determine if the age-associated hypertrophic response is directly caused by altered expression of these miRNAs, or a secondary result.

Maintaining metabolic & mitochondrial function with age

Metabolic dysfunction is associated with aging and many age-related disease [105]. Whereas fatty acid oxidation is the primary source of energy in the adult healthy myocardium, both the senescent and failing heart display a striking impairment of mitochondrial fatty acid oxidation and oxidative phosphorylation and an increased reliance on glucose metabolism [106, 107]. Despite intense research in this area, there is little consensus on the causes of this “metabolic substrate shift” or whether the shift in substrate utilization in failing and aging myocardium is detrimental nor compensatory [108]. Also the role of miRNAs in the metabolic shift needs further clarification.

By feeding mice different diets it was demonstrated that high fat feeding induced distinct cardiac miRNA expression patterns as assessed by RNA deep sequencing [109]. This included downregulation of senescence-associated miRNAs, miR-29b and miR-15. Moreover, high fat feeding upregulated expression levels of aging miRNAs miR-21a, miR-29c, miR-22, and miR-223 [109]. Not only fatty acid levels affect miRNA profiles, recent studies have shown that high glucose significantly reduced expression levels of miR-126 and miR-375 [110–112]. These findings suggest that age-associated alterations in the metabolic environment may cause a distinct miRNA profile. More importantly, a growing body of evidence suggests that numerous miRNAs are implicated in the regulation of metabolic homeostasis by targeting genes with a prominent role in 1) fatty acid or 2) glucose metabolism, and 3) energy production via controlling mitochondrial-biogenesis, -function, and -autophagy.

MiRNAs regulating fatty acid metabolism

The healthy adult myocardium relies primarily on mitochondrial fatty acid oxidation for its energy production. Numerous mitochondrial enzymes involved in fatty acid β -oxidation have been shown to be under the control of miRNAs (Figure 2). The miR-199a/214 cluster has been shown to play a regulatory role in the metabolic switch from mitochondrial fatty acid oxidation in the healthy myocardium towards glucose utilization at the onset of HF, by actively repressing PPAR β/δ [113]. In addition, MiR-199a suppresses PPAR α indirectly, resulting in repression of different PPAR α -target genes involved within the β -oxidation [114]. Both miR-199a and miR-214 are increased in failing human and rodent hearts [100], and both decrease reliance on fatty acid oxidation through inhibition of PPAR δ [113]. Over-expression results in mitochondrial dysfunction and decreased mitochondrial fatty acid oxidation. Conversely, silencing of miR-199a/214 in mice subjected to pressure overload de-repressed cardiac PPAR δ levels, normalized mitochondrial fatty acid oxidation and ultimately lead to improved cardiac function [113].

MiR-370 regulates the rate of β -oxidation by suppressing expression of carnitine palmitoyltransferase 1a (CPT1a), an enzyme involved in the import of fatty acids into the mitochondria. In addition, other genes important for fatty acid utilization, such as Diacylglycerol O-Acyltransferase 2 (DGAT2), and fatty Acid Synthase (FAS) are also targeted by miR-370 strengthening its functional participation in fatty acid metabolism [115]. Also,

miR-132 and miR-212 decreased the transporting rate of long-chain acylcarnitines into the mitochondria for β -oxidation via translational regulation of CPT1a expression [116, 117]. MiR-33a and miR-33b inhibit the expression of various metabolic enzymes, marking them as crucial players in controlling fatty acid β -oxidation [118]. MiR-1, which we already described as central in hypertrophy and contraction, negatively regulates the expression of heart-type fatty acid binding protein 3 (FABP3), leading to decreased transport of fatty acids within the cardiomyocyte and subsequent impaired β -oxidation [119]. MiR-27b targets PPAR γ thereby regulating the cardiac metabolic shift through suppression of fatty acid fuelling of mitochondria [120]. MiR-27b transgenic mice displayed lower cardiac levels of PPAR γ while silencing of endogenous miR-27b in mice showed a higher expression of cardiac PPAR γ , which resulted in increased fatty acid oxidation and attenuation of cardiac dysfunction in a pressure overload model [120].

These results reveal the intricate regulation of fatty acid β -oxidation by miRNAs, promoting the metabolic switch towards increased reliance on glycolysis. Whether similar processes are involved in the metabolic substrate shift driving myocardial aging, remains to be elucidated.

MiRNAs regulating glucose metabolism

In addition to fatty acid oxidation (Figure 2), various miRNAs able to impact cardiac glucose metabolism have been identified. Larger reliance on glucose utilization for energy production is a consistent feature of the failing and aging heart [121, 122] which has a major impact on the available energy levels due to the aerobic nature of the heart [123]. Interestingly, the age-associated miRNA, miR-34a, directly targets several glycolytic enzymes, including hexokinase 1, hexokinase 2, glucose-6-phosphate isomerase, and pyruvate dehydrogenase kinase 1, thereby repressing cellular glycolysis, potentially attempting to attenuate the age-associated increased cardiac reliance on glucose metabolism [124].

Additional miRNAs shown to inhibit glucose metabolism include let-7, miR-208, miR-195, miR-143, miR-133, miR-93, and miR-103/107, while miR-223 stimulates glycolysis. The lin28/let 7 pathway functions as a central regulator of mammalian glucose metabolism. Expression of members of the let-7 family was enhanced in the diabetic heart and overexpression induces glucose intolerance due to diminished glucose-induced insulin secretion as a result of translational inhibition of IGF-1R, insulin receptor (INSR) and GLUT4 [125–127]. Moreover, RNA-binding proteins Lin28a and Lin28b increase insulin sensitivity and resistance to diabetes through blocking of let-7 maturation and subsequent de-repression of multiple let-7 targets [125, 128–130]. Similarly, heart specific miR-208 negatively regulates med13, a regulator of energy expenditure and numerous other genes involved in energy balance in the heart. Indeed, inhibition of miR-208 in mice conferred resistance to obesity, improved systemic insulin sensitivity, and glucose tolerance [131]. MiR-195 suppresses the expression of INSR impairing insulin signalling cascade and glycogen synthesis [132]. Silencing of miR-103/107 modulates expression of insulin receptor regulator caveolin 1

Figure 2) MiRNAs regulating fatty acid and glucose metabolism in the aging myocardium. Fatty acid (FA) metabolism is facilitated by fatty acid binding protein 3 (FABP3), which transports FAs into the cell. Here, FAs undergo β -oxidation after CPT-1 α -dependent transport into the mitochondrion. Nuclear translocation of different peroxisome proliferator-activated receptors (PPAR $\alpha/\beta/\delta$) induces transcription of various genes involved in β -oxidation, such as long chain- and very long chain - acyl-CoA dehydrogenase (ACADL and ACADVL), and Hydroxyacyl-CoA Dehydrogenase (HADH). Glucose metabolism is highly dependent on insulin-mediated activation of insulin receptor (INSR), which stimulates glucose transport by GLUT4. Central factors in these metabolic pathways are regulated by miRNAs.

(CAV1) and improves insulin sensitivity and glucose homeostasis, whereas forced expression of miR-103/107 impaired glucose homeostasis [133]. Moreover, miR-143 [134], miR-133 [135], and miR-93 [136] have also been shown to control glucose homeostasis, in which the latter two are shown to inhibit cardiomyocyte glucose uptake via direct targeting of glucose transporter GLUT4.

On the other hand, miR-223 overexpression increased GLUT4 expression, resulting in an increase in glucose uptake and an adaptive response to restore normal glucose uptake in the diabetic heart. Conversely, systemic inhibitor-mediated silencing of miR-223 resulted in a significant decrease in cardiac GLUT4 expression [137]. Collectively, these studies highlight the central role of miRNAs in glucose metabolism. MiRNAs exerting a glucose-stimulating function may potentially be responsible for the observed age-induced increased reliance on glucose metabolism.

Mitochondrial biogenesis and function under the control of miRNAs

Not only the “metabolic substrate shift” plays a prominent role in the aging heart, mitochondrial dysfunction and dysregulation are also associated with aging and many age-related disease [138]. Due to the constant demand for high ATP levels for maintaining contractile activity in the heart, the loss of mitochondrial number and function during cellular aging has adverse impact on cardiac muscle [139, 140]. The main causes of age-associated mitochondrial impairment are reduced mitochondrial biogenesis and increased number of dysfunctional mitochondria, the latter being partly due to decreased autophagy [141]. Mitochondrial biogenesis is regulated by several pathways, most of which rely on peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α) [142]. PGC-1 α enhances mitochondrial function but cardiac expression is repressed in the failing heart [143]. PGC-1 α is regulated by miR-696 in skeletal muscle in response to physical activity, and mitochondrial biogenesis and fatty acid oxidation were decreased following miR-696 overexpression [144]. Further research needs to be conducted using cardiomyocytes to interrogate the possible detrimental impact of miR-696 on mitochondrial biogenesis during myocardial senescence.

In addition to decreased formation of mitochondria, impaired mitochondrial functioning forms a driving factor of cellular aging in the heart. Different miRNAs have been identified to influence ATP generation by inhibiting mitochondrial function (Figure 3). Members of the aging-miRNA family, miR-15a and miR-15b, reduced ATP generation and mitochondrial integrity in rat cardiomyocytes [145, 146]. Also, miR-181 family members, affect ATP generation. MiR-181c causes electron transport chain complex IV remodelling through regulation of the mitochondrial cytochrome c oxidase subunit 1 (mt COX1) at the translational level [147], while miR-181a modulates the citric acid cycle [148]. Moreover, both miR-338 and miR-210 modulate ATP production by depressing the expression of the main components in the electron transfer chain, cytochrome c oxidase IV (COXIV) and cytochrome c oxidase assembly protein 10 (COX10)[149]. Finally, miR-484 negatively regulates mitochondrial fission in the heart via targeting mitochondrial fission protein (Fis1), thereby disrupting maintenance of normal mitochondrial function [150].

Impaired mitochondrial function does not only limit ATP production, dysfunctional mitochondria also form the most important source of ROS (Figure 3). Although generation of ROS is an inevitable by-product of aerobic metabolism and low levels of ROS are necessary for intracellular signalling [151], the aged and failing myocardium are characterised with an excess of ROS produced by various types of cardiac cells, that seems to be under the control of miRNAs as well [152–154]. MiR-210, which expression is induced in senescent fibroblasts [155], induces the generation of ROS through impairment of mitochondrial function via suppressing activity of OXPHOS complex I and II [156, 157]. Similarly, miR-494 induction in senescent fibroblasts increases ROS production [155]. ROS can also be generated by various enzymes, including the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidases [158]. MiR-448 is shown to be in control of ROS production through

repression of the NOX2 regulatory subunit Ncf1. Downregulation of cardiac miR-448 led to increased Ncf1 expression and abnormally elevated ROS production and ultimately dilated cardiomyopathy and arrhythmias [158].

The cellular defense mechanism to counter the overproduction of ROS is the scavenging of free radicals by an array of antioxidants, such as superoxide dismutase (SOD) [159, 160]. Both miR-34a and miR-335 are up-regulated in kidneys of old rodents and target ROS scavenging enzymes SOD2 and Thioredoxin Reductase 2 (Txnrd2), thereby causing oxidative stress [161]. Similarly, miR-21 increases oxidative stress via targeting of SOD2 in angiogenic progenitor cells [162]. MiR-146a downregulates the expression of NOX4, the major catalytic subunit of NADPH oxidase [163]. Aged cells display increased expression of NOX4 and decreased miR-146a levels and overexpression of miR-146a decreases the rate of HUVEC senescence [163]. Moreover, miR-1 modulates oxidative stress in cardiomyocytes via targeting multiple antioxidant enzymes, including SOD1, the catalytic subunit of the glutamate cysteine ligase (Gclc), and glucose-6-phosphate dehydrogenase (G6PD) [164]. Gclc is a positive regulator of glutathione (GSH), promoting scavenging of ROS. MiR-1 transgenic mice hearts display repressed expression of SOD1, Gclc, and G6PD, contributing to the increased ROS level and susceptibility to oxidative stress within these hearts [164]. In addition, miR-144 levels are repressed in cultured cardiomyocytes exposed to high-glucose and in hearts of streptozotocin (STZ)-induced diabetic mice. MiR-144 promotes ROS-induced oxidative damage through directly targeting of the central regulator of the response to oxidative stress, nuclear factor-erythroid 2-related factor 2 (Nrf2) [165].

Remarkably, miRNAs themselves can become oxidatively modified by ROS, which may in turn alter their stability, integrity, binding affinity, and functionality, thereby contributing to altered redox signalling and disease mechanisms [166, 167]. More specifically, miR-184 is shown to interact with the 3' UTRs of Bcl-xL and Bcl-w only after being subjected to oxidative modification, initiating apoptosis in the heart cell line H9c2 [167]. These results suggest that miRNAs have a pivotal role in maintaining cardiac mitochondrial function and energy metabolism in the presence and absence of oxidative stress, preserving myocardial homeostasis.

MiRNAs orchestrating autophagy

As described earlier, the senescent myocardium is associated with defective mitochondria [14, 168, 169] due to the infrequent replacement of cardiomyocytes and their high susceptibility to mitochondrial damage [170, 171]. Senescent cardiomyocytes counteract this detrimental process via autophagy, which is characterized by the delivery of intracellular components to the lysosome, maintaining cardiomyocyte homeostasis and mitochondrial activity through the renewal/recycling of cytoplasmic materials and organelles, removal of aggregated proteins, and the provision of energy and biomolecules to cells [172–174] (Figure 3). Although autophagy plays a relatively minor role in proliferating non-cardiomyocyte cells (such as endothelial cells and fibroblasts), it has been shown that a progressive

decline in autophagy efficiency in senescent cardiomyocytes plays an important role in age-related cardiac diseases [175]. Due to the loss of autophagy, dysfunctional mitochondria accumulate as the cells ages, and promote the formation of ROS, leading to further accumulation of damaged mitochondria and ROS creating a positive feedback loop that results in the “aging” phenotype with a decline in autophagy at its core [170, 176].

Strikingly, Gibbings and co-workers have shown that autophagic activity is crucial in regulating miRNA processing through engaging post-transcriptional regulation of DICER and establishes a checkpoint required for continued loading of miRNAs into AGO2 [177]. Autophagy-deficient cells exhibit decreased ago-loading with miRNAs, diminished miRNA levels and reduced silencing activity of exogenous siRNA duplexes [177]. Thus, autophagy is required for continued loading of miRNA duplexes into AGO and for their durable activity. This is supported by the fact that DICER protein accumulates in cells depleted of different autophagy related genes [178]. These results suggest that autophagy influences the expression of genes crucial in miRNA processing and thereby maturation of miRNAs. An age-related decline in autophagy may therefore affect miRNA processing and thus mature miRNA profiles in the heart.

The role of miRNAs in autophagy is gaining more interest and one examples of an age-associated miRNA that promotes autophagy is miR-155. MiR-155 negatively regulates the autophagy-inhibitor mTOR through targeting of multiple genes involved within this pathway, including RHEB, RPTOR Independent Companion Of MTOR Complex 2 (RICTOR), and Ribosomal Protein S6 Kinase B2 (RPS6KB2)[179]. These results suggest that miR-155 possesses the potential to exert a beneficial role in the aging myocardium via prevention of an age-induced deleterious decrease in autophagy.

On the other hand, several studies report that different forms of stress can provoke an increase in cardiomyocyte autophagic activity, and link this prolonged/excessive up-regulation of autophagy with the development of cardiac hypertrophy [180, 181]. In these situations, miRNAs functioning as negative regulators of autophagy may benefit cardiac remodelling and function. Furthermore, miR-30a decreases autophagic activity through suppression of Beclin 1 (Becn1) expression, which is able to regulate the expression of the main autophagy gene LC3B [182, 183]. Strikingly, miR-30 family members are down-regulated in human and mouse hypertrophic hearts [184, 185] and AngII-treatment is shown to result in autophagy-induced myocardial hypertrophy, at least partly, via decreasing the expression of miR-30 [182]. Initiation of autophagy by the unc-51 like autophagy activating kinase 1 (ULK1) complex is suppressed by miR-20a and miR-106b [186]. Other negative regulators of autophagy include miR-101 [187, 188], miR-199a [189], miR-221 [190, 191], miR-222 [192], and miR-374a [193]. Collectively, miRNAs able to increase autophagic activity potentially have beneficial a role during myocardial aging whereas autophagy suppressing-miRNAs may aggravate age-related myocardial phenotypes.

Figure 3) Aging miRNAs negatively regulate mitochondrial function via controlling mitochondrial ATP production, biogenesis, and autophagy. Mitochondria generate energy in the form of ATP, which is accompanied by the production of reactive oxygen species (ROS). Beclin 1 (BECN1) and unc-51 like autophagy activating kinase 1 (ULK-1) initiate the formation of autophagosomes to protect the cell against dysfunctional, ROS-producing mitochondria. Autophagosomal content undergoes lysis after fusion with a lysosome. Degraded components can be recycled during PPARγ Coactivator 1 Alpha (PGC1-α)-mediated mitochondrial biogenesis.

Programmed cell death

Cardiomyocyte renewal occurs at a low rate ($\pm 1\%$ /year) in youth and seems to slow down even further with advancing age [194]. This non-mitotic nature of cardiomyocytes marks the importance of a balanced homeostasis to prevent cell death. Aging in the mammalian heart is characterized by a gradual loss of myocytes and hypertrophy of the remaining myocytes [54]. Cardiomyocyte loss is primarily due to necrosis and apoptosis (Figure 4). Deregulation of both cell death pathways has been observed in heart disease [195] as well as during normal myocardial senescence [196, 197].

MiRNAs regulating necrotic cell death

Necrosis is morphologically characterized by an increase in cell volume, dilation of organelles, rupture of the plasma membrane and subsequent loss of intracellular contents [198,

199]. Cardiomyocytes can undergo necrosis when being exposed to excessive stress due to, for example the lack of oxygen and nutrients, as during myocardial ischemia [200]. So far, only limited evidence has been gathered on the role of miRNAs in regulation of cardiac necrosis. Age-associated miR-155 was reported to attenuate hydrogen peroxide (H₂O₂)-induced necrotic cell death in cardiomyocyte progenitor cells (CMPCs)[201]. Strikingly, transplantation of CMPCs overexpressing miR-155 in the infarcted myocardium induced an anti-necrotic response through repression of receptor interacting protein 1 (RIP1)[201]. Although certain miRNAs have been shown to influence cardiac necrotic cell death *in vitro* as well as *in vivo*, the role of miRNAs in regulation of necrotic cell death pathways is under-investigated, especially in the context of age-associated necrosis.

MiRNAs promoting apoptotic cell death

Unlike necrosis, myocardial apoptotic cell death and the role of miRNAs therein have been well documented. Apoptosis is a highly regulated process in which signalling is mediated by the mitochondria [202], regulated by a family of both pro-apoptotic and anti-apoptotic member proteins called Bcl-2 [203], and executed by a family of cysteine proteases, called caspases [204].

A large body of work describes the impact of miRNAs on cardiac apoptosis, including miRNAs implicated in myocardial aging, such as miR-34a. MiR-34a is upregulated in cardiomyocyte apoptosis induced by doxorubicin or excessive ROS accumulation [205], and its inhibition has cardioprotective effects, partially diminishing apoptosis both *in vitro* and *in vivo* [205–208]. Interestingly, miR-34a has been identified as a predictive plasma marker of future HF in patients after acute MI [205]. Moreover, miR-92a, a member of the aging miRNA cluster miR-17–92, has been proven to regulate cardiomyocyte apoptosis. Circulating expression of miR-92a is elevated in acute MI patients as well as in post-MI cardiac and blood samples of rodents [209]. Knock down of miR-92a has cardio-protective effects through reduction of apoptosis while overexpression has the opposite effect [209–212].

Two different age-associated miRNA families, the miR-15- and miR-29 family are proven to regulate apoptotic signalling through direct inhibition of anti-apoptotic gene BCL-2. Various miR-15 family members were found to be up-regulated in the overloaded heart [213] while being downregulated in both border and infarct zone of mice subjected to MI [214]. Several of these members are proven to promote apoptosis, including miR-15a, miR-15b, miR-16, miR-195, and miR-497, and worsen myocardial ischemia-induced cardiac injury [100, 215–218]. Furthermore, miR-29 family members, miR-29a/c protect cultured cardiomyocytes from I/R injury, evidenced by increased cell survival and decreased Casp3 activity after inhibition [219].

Various other miRNAs have been identified to modify cell death via targeting of additional negative regulators of the apoptotic pathway. For example, apoptosis-induced miR-140 promotes apoptosis via targeting of anti-apoptotic mitofusin 1 [220]. Silencing of

miR-140 *in vitro* and *in vivo* attenuates apoptosis and reduces infarct size in a mouse model of I/R injury [220].

MiR-155 is, in addition to prevention of necrosis, suggested to stimulate apoptosis. Inhibition of miR-155 in LPS-treated mice attenuated cardiac apoptosis and preserved cardiac function, while forced expression of miR-155 aggravated cardiac dysfunction [221]. These results are indicative for a pro-apoptotic function of miR-155 in the heart during sepsis. The discrepancy between the observed anti-necrotic and pro-apoptotic effect can possibly be explained by the fact that the role of miR-155 was investigated in different cell types. Next to that, Wang et al. studied the effect of the well-known pro-inflammatory miR-155 on cardiomyocyte apoptosis in a LPS-induced sepsis model. It is possible that the decreased apoptosis after inhibition of miR-155 is rather due to a diminished inflammatory response than a direct effect on apoptotic signalling.

Additional miRNAs enhancing apoptosis via targeting negative regulators/factors of the intrinsic apoptotic pathway include miR-1 [222], miR-100 [223], and miR-132 [224]. These results suggest that various miRNAs are able to provoke apoptosis in cardiomyocytes, in most, but not all, cases via direct or indirect suppression of activity of anti-apoptotic gene BCL-2, thereby aggravating myocardial function.

MiRNAs inhibiting apoptotic cell death

Other miRNA have been shown to inhibit apoptosis, including the senescence-associated miRNA cluster miR-17–92. Hypoxia decreases expression of various miR-17–92 cluster members, and overexpression of this cluster markedly attenuates apoptosis [225]. For example, miR-17 exert its anti-apoptotic function through suppression of Casp9 and apoptotic protease-activating factor 1 (Apaf-1)[226] while miR-19b prevents apoptosis via phosphatase and tensin homolog (PTEN) suppression [227].

One of the best-studied anti-apoptotic miRNAs is miR-21. MiR-21 displays increased expression levels in border areas in infarcted hearts while being down-regulated in infarcted areas [228, 229]. Silencing of miR-21 aggravates H₂O₂-induced apoptosis whereas overexpression of miR-21 protects against apoptosis and subsequently decreases MI size and myocardial remodelling [229, 230]. The anti-apoptotic effect of miR-21 is established via targeting of different pro-apoptotic genes, such as PTEN [231, 232], PDCD4 [233, 234], and TPM1 [235]. Interestingly, Huang and co-workers showed that miR-21/miR-146a synergistically reduced ischemia/hypoxia-induced cardiomyocyte apoptosis *in vitro* and in a murine MI-model, the combination of the miRNAs being superior to each miRNA alone [236].

Similarly, miR-24 and miR-93 are reported to target, among others, PTEN and attenuate cardiomyocyte apoptosis and myocardial injury *in vivo* [237–239]. Although miR-24 prevents apoptosis in cardiomyocytes, forced expression of miR-24 in endothelial cells promotes apoptosis through direct targeting of GATA2 and PAK4, which in their turn, impinge on BCL2-associated agonist of cell death [240] Silencing of endothelial miR-24 in mice

with MI attenuated cardiac endothelial cell apoptosis, reduced infarct size and preserved cardiac function [240].

Caspases are crucial in apoptotic signalling and miR-133 is in control of multiple of these key players, exerting an anti-apoptotic function. Hearts from cardiac-specific miR-133 transgenic mice displayed reduced Casp3 activation and apoptosis during pressure overload [49]. In addition, miR-133 protects cardiomyocytes from cell death through suppression of Casp9 explaining, at least partly, the beneficial effect of ischemic post-conditioning [241, 242].

Several members of the miR-30 family, miR-30e in particular, display reduced expression in response to doxorubicin-induced cardiotoxicity and were found to mediate apoptosis through inhibition of apoptotic signalling by mitochondria [243, 244]. In addition, miR-30e inhibits the expression of BCL2/adenovirus E1B-interacting protein 3-like (BNIP3L or NIX)[243].

Many additional miRNAs have been shown to attenuate apoptotic cell death in cardiomyocytes exposed to H₂O₂ or doxorubicin, including miR-199a [245], miR-22 [246, 247], miR-23a [248, 249], miR-125b [250], miR-132 [224], miR-150 [251], and miR-206 [252]. These reports demonstrate that many different miRNAs are able to inhibit apoptotic cell death in the myocardium, including various age-associated miRNAs. Decreased expression of these anti-apoptotic miRNAs in the aging heart may explain the observed increase in apoptotic cell death.

Figure 4) Both cell death signalling pathways, apoptosis and necrosis, are affected by aging miRNAs. Necrotic cell death can be caused by myocardial ischemia via activation of receptor interacting protein 1 (RIP1). Apoptotic cell death is regulated by B-cell lymphoma 2 (BCL-2) and executed by various caspases, including Casp3 and Casp9, and pro-apoptotic gene phosphatase and tensin homolog (PTEN).

Inflammatory endothelial activation and macrophage recruitment

Aging is associated with a systemic proinflammatory state, sometimes referred to as “inflammaging”, which can activate signalling cascades leading to structural and functional remodelling of the heart [253–255]. A growing body of evidence indicates that proinflammatory signalling is a key factor driving remodelling of the heart with advancing age (reviewed in [256]). Allostatic-associated proinflammatory signalling molecules are up-regulated whereas anti-inflammatory molecules are down-regulated in the myocardium. Importantly, the aging-associated pro-inflammatory response is not performed by “professional immune cells”; aged cardiac cells, including endothelial cells, smooth muscle cells and fibroblasts, acquire a senescence-associated secretory pro-inflammatory phenotype [17, 257]. In particular, senescence of endothelial cells (ECs) and the accompanying dysfunction promotes the pathogenesis of age-related coronary disease, including atherosclerosis [258–260]. Disturbed flow enhances cell senescence and primes endothelial cells for inflammatory gene expression, and might promote accumulation of monocyte-derived macrophages at damaged areas in the arterial intima [261–263]. Thus, miRNAs controlling endothelial activation/senescence and the potential following recruitment of macrophages can influence cellular aging and the progression of age-related cardiac diseases (Figure 5).

MiRNAs regulating endothelial activation

Interestingly, among the most abundant miRNAs in endothelial cells are age-deregulated miRNAs such as miR-146a, miR-21, miR-126a, and members of the miR-17–92 cluster [264]. Moreover, shear stress forms the main cause of accelerated cellular senescence and shapes the miRNA signature of endothelial cells [265–267], altering the expression of senescence miRNAs [268, 269]. Among these miRNAs, miR-146a represses NFκB signalling via direct inhibition of IRAK 1 and TRAF6 and, thus, cellular enrichment of miR-146a through systemic delivery of mimetics suppresses NFκB activation and reduces macrophage recruitment [270]. In addition, miR-146a^{-/-} mice are resistant to endothelial activation following treatment with IL-1β because of reduced induction of pro-inflammatory adhesion molecules [271]. Conversely, overexpression of miR-146a suppresses both chemokine production and monocyte adhesion to the endothelium, supporting the hypothesis that miR-146a ameliorates endothelial inflammation and the progression of atherosclerosis [272]. Similarly, EC-enriched miRNA miR-126a is shown to be involved in endothelial activation via targeting vascular cell adhesion molecule-1 expression (VCAM-1), preventing leukocyte adhesion to endothelial cells [264, 273, 274].

Shear stress-inducible miR-19a and miR-21 promote endothelial inflammation and disease progression. Circulating miR-21 may be an inflammatory biomarker linking the aging process to cardiac disease [275], displaying an age-related increase in the plasma miR-21 levels [276]. Here, miR-21 expression and IL-6 and IL-8 levels correlated, suggesting a potential role of miR-21 in inflammatory gene expression. On the contrary, disturbed blood

flow-induced upregulation of miR-21 in endothelial cells suppresses translation of PPAR α , inducing the expression of vascular VCAM-1 and CCL2, and promoting endothelial inflammation [277]. Moreover, miR-19a is upregulated in atherosclerotic ECs, and increases NF κ B activity by targeting several negative regulators of NF κ B, functioning as a pro-atherogenic factor [278].

Additional senescent-associated miRNAs induced by endothelial shear stress include miR-181b, miR-23b, and miR-150, whereas shear stress downregulates expression of miR-24, miR-103, miR-210, and miR-221 [269, 279–281]. MiR-181b dampens the inflammatory response in the endothelium by limiting the translocation of NF κ B from the cytoplasm to the nucleus, decreasing NF κ B activity and contributing to the anti-inflammatory phenotype of endothelial cells at athero-protected regions of (coronary) arteries [272, 282, 283]. Furthermore, members of the let-7 miRNA family are upregulated in endothelial cells at athero-protected sites, whereas miR-103 and miR-221 are upregulated at athero-prone sites [284]. More specifically, overexpression of let-7g and let-7i has been shown to suppress endothelial and cardiac inflammatory activation, whereas a pro-inflammatory phenotype was acquired in the absence of let-7g or let-7i [285, 286]. Similarly, increased miR-103 expression enhances endothelial inflammation, while blocking of miR-103 reduces macrophage accumulation and chemokine expression, suggesting a pro-inflammatory role for miR-103 [287, 288]. Finally, miR-221 is able to decrease the expression of NF κ B target genes such as VCAM-1 and CCL2 via targeting transcription factor ETS-1 [289].

MiRNAs influence macrophage polarization and function

Acquisition of a senescence-associated secretory pro-inflammatory phenotype during endothelial aging is followed by recruitment of inflammatory cells. Following their adhesion to the endothelium, inflammatory cells can migrate into the intima, differentiate into macrophages and enhance the local inflammatory response. Aging is associated with changes in cardiac tissue macrophage composition, gene expression, and function, altering the inflammatory response and increasing myocardial susceptibility [290]. More specifically, senescent mice show a suppressed post-infarct inflammatory response, evidenced by a decreased and delayed neutrophil and macrophage infiltration, markedly reduced cytokine and chemokine expression and impaired phagocytosis of death cardiomyocytes [291]. It is now recognized that miRNAs can profoundly influence the biology of inflammatory cells, in particular the differentiation of monocytes into polarized macrophages. A large number of age-associated miRNAs are differentially regulated during the differentiation of monocytes into macrophages, for example miR-24, miR-30b, miR-142–3p, and miR-199a-5p are down regulated in this process [292, 293]. In particular, miR-24, miR-30b, and miR-142–3p impair macrophage differentiation and activation, as evidenced by reduced macrophage phagocytosis and reduced expression of inflammatory cytokines upon overexpression [292, 294, 295].

Once differentiated, recruited macrophages can take on two extreme states [296, 297]. The initiation phase of an inflammatory response is characterized by pro-inflammatory

macrophages, also known as classically activated or M1 macrophages, which secrete inflammatory cytokines, such as IL-12, IL-1 β and tumor necrosis factor (TNF)- α [297, 298]. Anti-inflammatory macrophages, also known as alternatively activated or M2 macrophages, play important roles in inflammation resolution and tissue repair by secreting anti-inflammatory cytokines such as IL-10 [298]. Pro-inflammatory M1 macrophages have increased expression of age-associated miRNAs miR-29b, miR-125a, miR-26a-2, miR-147, and miR-155-5p, whereas anti-inflammatory M2 macrophages have increased expression of miR-193b or miR-27a, miR-29b-1, miR-132, miR-222 and miR-155-3p respectively [299–301] (Figure 5). Moreover, forced expression of miRNAs affects macrophage polarisation

Figure 5) Macrophage polarisation shapes their miRNA profile, subsequently affecting endothelium activation and their inflammatory response. Macrophages can polarise into either pro-inflammatory M1 macrophages, or anti-inflammatory M2 macrophages, which display distinct miRNA profiles. NFκB signalling plays a central role in endothelium activation, increasing the expression of pro-inflammatory cytokines and chemokines, including Vascular Cell Adhesion Molecule-1 (VCAM-1), and C-C Motif Chemokine Ligand 2 (CCL2). Expression of CCL2 leads to the recruitment of M1 macrophages, which in their turn secrete pro-inflammatory cytokines to promote further endothelial activation.

sation status. For example, *in vitro* overexpression of miR-155-5p and miR-29b induced differentiation towards a M1 macrophage expression pattern [299, 301].

One of the most well-investigated miRNAs in cardiac inflammatory disease, miR-155, is upregulated in the peripheral blood of aged women [302], in human B cells from elderly individuals [303] and is suggested to form a senescence associated secretory marker [304]. Moreover, Corsten et al. [305] have shown that elevated miR-155 levels were found in cardiac tissue of both human and mice with viral-induced myocarditis [305]. MiR-155 functions, among others, as a positive regulator of TLR-signalling and its silencing during viral-induced myocarditis mice models resulted in attenuation of cardiac inflammation [306]. MiR-155 is highly expressed in both mouse and human aortic atherosclerotic lesions but is reported to have a controversial role in atherosclerotic inflammation, reported to function both anti- and pro-atherogenic. Transplantation of miR-155^{-/-} bone marrow cells into LDLr^{-/-} mice was reported to amplify macrophage inflammation, increase atherosclerotic lesion size, and decrease plaque stability [307], while both whole body miR-155^{-/-}, ApoE^{-/-} mice and ApoE^{-/-} mice receiving bone marrow transplant of miR-155^{-/-} cells, showed markedly reduced macrophage inflammation and decreased atherosclerotic lesions [301, 308]. Importantly, the differences in the ability of macrophage miR-155 to reduce lesion progression may greatly depend on the stage of atherosclerotic development (i.e. early versus advanced) when the expression of key target genes is stage-specific [309].

Aging-related miRNAs miR-21 and miR-146b were found to be upregulated in myocarditis patients. These miRNAs also have a central role in immune activation and inflammation [305]. Silencing of these miRNAs by specific inhibitors showed a strong attenuation of viral-induced myocarditis mice [310]. The exact role of these miRNAs in the pathology of myocarditis is not elucidated, however it is shown that the expression of miR-21 and miR-146b is correlated with IL-17 expression, implicating an immune modulating function for both age-associated miRNAs [310].

Additionally, anti-inflammatory miRNAs, miR-106a and miR-93 were downregulated in the acute phase of human myocarditis [305], while being upregulated in the senescent heart [311]. Both are involved in anti-inflammatory responses by inhibiting the production of inflammatory cytokines [312, 313].

Strikingly, miR-181b is also increased in EC-extracellular vesicles-treated monocytic cells, while this miRNAs is shown to suppress monocyte activation upon overexpression [314]. These results suggest that crosstalk between the endothelium and monocytes/macrophages may be mediated via secreted extracellular vesicles containing anti-inflammatory miRNAs. This finding marks the important function of miRNAs in controlling endothelial activation and the inflammatory response. We can conclude from these studies that miRNA are key regulators of endothelial activation, monocyte differentiation, and macrophage activation and polarization. Dysregulation of these miRNAs affect the aging process of the myocardium, potentially aggravating the processes driving cellular senescence.

Extracellular matrix (ECM) remodelling

The extracellular matrix (ECM) is a complex collection of proteins, including fibronectin and different types of collagens, and functions as the structural backbone of the heart in addition to being a critical regulator of cellular processes like proliferation, migration, and differentiation, through the control of signalling via growth factors and bioactive molecules [315]. Importantly, excessive ECM hallmarks the senescent myocardium, increasing cardiac stiffness and mediating diastolic dysfunction [316–318]. In the heart, fibroblasts are the most abundant cell type [319, 320] and they play a crucial role in myocardial homeostasis by preserving the balance of ECM synthesis and degradation [321]. In response to various stresses, such as myocardial injury and exposure to cytokines or growth factors, fibroblasts undergo transformation to myofibroblasts [322–326], which control ECM composition by regulating the secretion and activity of proteolytic enzymes, including members of the ECM degrading superfamily of matrix metalloproteinase (MMPs) and their tissue inhibitors (TIMPs) [327, 328]. The main pro-fibrogenic growth factor identified as a central player of myofibroblasts transformation and ECM remodelling is transforming growth factor β (TGF β) [328, 329], which signals through MAPK or SMAD proteins to drive fibrotic gene expression (Figure 6).

TGF β signalling does not only induce fibroblast activation and differentiation, it also shapes the fibroblast miRNA profile. SMAD proteins, have a dual function; 1) they bind to the DNA to regulate the transcription of miRNAs and 2) they interact with the Drosha/DGCR8 complex to influence miRNA maturation [330]. For example, TGF β 1 induces expression of miR-21 [331], while reducing expression of the miR-30 family [332]. Additionally, miR-212/132 levels were increased after TGF β treatment in endothelial cells [333]. These results suggest a possible link between the fibrotic response and miRNAs. Different miRNAs have been shown to possess the ability to regulate pathological fibrosis, including miRNAs displaying an age-associated dysregulation.

MiRNAs suppressing ECM production

Members of the age-associated miR-17–92 miRNA cluster, including miR-18a and miR-19b, have been implicated as potential regulators of ECM production during aging cardiomyopathy, through their targets CTGF and TSP-1 [334]. Moreover, miR-19a/b are shown to be a negative regulator of TGF β signalling via direct targeting of TGF β R2, resulting in inhibition of interstitial fibrosis and attenuation of cardiac remodelling [335]. MiR-22, another age-associated miRNA, is one of the most abundantly expressed miRNAs in the heart [336] whose expression increases during aging [337]. This miRNA promotes senescence and activation of cultured cardiac fibroblasts via repression of its target, the small leucine rich proteoglycan osteoglycin (OGN) [337]. Although miR-22 knockout mice do not display any cardiac abnormalities under basal conditions, upon cardiac stress, they quickly develop dilated cardiomyopathy accompanied with excessive cardiac fibrosis [338, 339]. MiR-22

inhibition might therefore be a potential therapeutic target, to block fibrosis development in the elderly heart patient.

The well-documented miR-34a has been implicated in cardiac aging and fibrosis formation. It is part of a positive feedback loop, induced by TGF β and itself promoting TGF β -induced activation of cardiac fibroblasts by targeting co-smad and SMAD4 [340]. MiR-34a is upregulated in senescent murine hearts as well as in aged human myocardial biopsies [207]. Silencing of miR-34a protected the heart against excessive cardiac fibrosis and remodelling upon pressure overload or MI [207, 341–343].

The most well studied miRNA for its role in fibrosis is age-associated miR-21. This cardiac fibroblast-enriched miRNA is increased in human and murine HF [100, 344, 345], and both its myocardial and plasma levels correlated with intra-cardiac fibrosis in aortic stenosis patients [344]. MiR-21 has multiple targets implicated in matrix deposition, including sprout homologue 1 [345], PTEN [346], SMAD7 [347] and TGF β R3 [348], and their inhibition synergizes to provoke a pronounced cardiac fibrosis phenotype.

Furthermore, the aging-associated miR-29 family is downregulated in response to cardiac stress [100, 349]. Notably, circulating levels of miR-29a correlated inversely with cardiac fibrosis [350, 351]. This family protects against cardiac fibrosis via direct targeting of a number of ECM genes and subsequent inhibition of MAPK- and TGF β /SMAD3-signalling, preventing fibroblast activation and proliferation [352–355]. Strikingly, rescue of miR-29b expression 14 days after AngII treatment reduced cardiac fibrosis and restored cardiac function, indicating the therapeutic potential in established hypertensive heart disease [354].

The miR-15 family (miR-15a, miR-15b, miR-16, miR-195, miR-497, and miR-322) is induced in response to cardiac stress [100, 216] and targets TGF β R1, SMAD3, and SMAD7 among others, presumably to counteract TGF β activity and to inhibit ECM remodelling [213]. Indeed, inhibition of miR-15b specifically aggravated fibrosis in pressure overloaded mice [213]. On the other hand, upon experimental ischemia-reperfusion, inhibition of the complete miR-15 family yielded smaller infarct sizes and less fibrosis [216]. The basis for the different outcomes of miR-15 inhibition remains unclear, but may be owed to the different nature of the insults to the heart and inhibition of the complete miR-15 family versus only one of the family members.

Cardiac fibrosis during experimental pressure overload is, associated with the down-regulation of miR-133a [356] and concomitant de-repression of its targets fibronectin, Col1a1, and Col4a1 [46, 356, 357]. Also, the senescence-associated miR-30 is a fibroblast-enriched miRNA, downregulated in the failing human and rodent heart [100], exerting its anti-fibrotic function via targeting of CTGF [185].

Many other miRNAs associated with aging have been identified as negative regulators of the cardiac fibrotic response by inhibiting crucial pro-fibrotic signalling cascades, such as the TGF β and MAPK/ERK pathways, including miR-24, miR-122, miR-101, miR-19a/b, and miR-150. MiR-24 is downregulated shortly after MI and negatively regulates cardiac fibrosis

by controlling the expression of Furin, which is necessary for TGF β excretion [358]. Overexpression of miR-24 into the mouse myocardium before inducing MI led to a reduced infarct size and improved cardiac function [358]. The same protective effect of miR-122 on myocardial fibrosis in human AOS was revealed and was dependent on the targeting of TGF β 1

Figure 6) Senescence-associated miRNAs alter cardiac fibroblast activation and proliferation via targeting of the MAPK and TGF β pathway, resulting in disbalance of the extracellular matrix. Activation of transforming growth factor beta receptors (TGF β R1/2/3) promotes MAPK/ERK and/or SMAD signalling. Both pathways stimulate the fibrotic response, which is characterised by increased transcription of connective tissue growth factor (CTGF), alpha smooth muscle actin (α SMA), osteoglycin (OGN), and different collagens (COL1 α 1 and COL3 α 1). Increased expression of these pro-fibrotic genes leads to excessive extracellular matrix (ECM) deposition through activation and proliferation of cardiac fibroblasts.

[359]. Adenovirus-mediated overexpression of miR-101 can attenuate interstitial fibrosis formation and inhibits MI-induced cardiac dysfunction in rats via silencing of TGF β R1 and the transcription factor c-Fos, suppressing proliferation of cardiac fibroblasts [360, 361].

Next to TGF β , MAPK/ERK signalling plays a critical role in activation of cardiac fibroblasts and collagen formation [362]. Interestingly, miR-7a/b inhibits both the TGF β and MAPK pathways and subsequently reduces collagen I expression and inhibits cardiac fibroblast proliferation and migration [363]. MiR-150 is decreased in murine hearts suffering from pressure overload and inhibits cardiac fibroblast activation upon overexpression *in vitro*, while miR-150 KO mice display a more severe deterioration of cardiac function due to excessive cardiac fibrosis in comparison with wild-type mice [364].

MiRNAs promoting ECM production

Age-associated and TGF β -induced miR-125b promotes fibrosis through inhibition of the antifibrotic secreted frizzled-related protein 5 (SFRP5), resulting in both proliferation and activation of cardiac fibroblasts and derepression of α SMA and collagen I and III [365].

Overall, age-associated changes in the molecular environment, such as increased TGF β signalling, are accompanied with altered miRNA expression profiles. In turn, these miRNAs are able to influence fibroblast activation, differentiation, and collagen production, marking them as potential therapeutic targets to fine-tune ECM deposition.

Conclusion & Discussion

Various cellular processes are identified to drive cardiac senescence resulting in age-associated functional deterioration. Therewith, described molecular hallmarks of cardiac aging also drive the progression of HF, implying that HF is an accelerated state of myocardial aging, involving similar processes. Still, “healthy aging” suggests the presence of a heart that is functional enough to meet the body’s demand and such heart will by definition differ from a failing heart. Therefore, using HF-miRNA studies to understand the cardiac aging process better as we did here is acceptable. Notably, recent studies suggest that age-associated functional and structural changes in the heart are, at least partly, caused by exhaustion of the stem cell pool in the myocardium, leading to impaired repair and regeneration in the aged heart (reviewed in [366]). However, the role of stem cells in cardiac aging was not discussed within this review since the abundance and biological relevance of stem cells in the heart is still under debate. Future research should be conducted to study the role of miRNAs more extensively in the context of the healthy aging heart.

As highlighted throughout this review, cardiac and circulating miRNA expression profiles change during aging. The dysregulation of these responsive miRNAs can be the cause of age-induced alterations in the microenvironment, including desensitization of β -adrenergic signalling, local pro-inflammatory stimulation, ECM-deriving growth factors, and induction of apoptosis. In their turn, miRNAs are able to orchestrate the processes

underlying senescence and potentially aggravate or counterbalance the cardiac aging phenotypes. This bidirectional connection between the cellular hallmarks of aging and miRNA function stresses the importance to gain knowledge about 1) the effect of micro-environmental aging on miRNA expression and 2) the exact regulatory function of miRNAs in cellular processes driving cardiac aging.

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CHAPTER 3

MicroRNAs Act as Pleiotropic Regulators of Cellular Processes Underlying Heart Failure:

A High-Throughput Phenotypical Screen in Three Primary Cardiac Cell Types

In preparation

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CHAPTER 4

The microRNA-221/222 Family Counteracts Myocardial Fibrosis in Pressure Overload-Induced Heart Failure

Adapted from Hypertension

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Abstract

Pressure overload-induced pathologic remodeling of the heart involves myocardial interstitial fibrosis. In response to various stresses, cardiac fibroblasts become activated and undergo transformation to myofibroblasts. These myofibroblasts can form excessive amounts of fibrosis, increasing myocardial stiffness and mediating diastolic dysfunction. A better understanding of the molecular mechanisms by which cardiac pressure overload, most often resulting from hypertension or aortic valve stenosis, induces cardiac remodeling will have important implications for future treatment strategies.

The microRNA-221/222 family, consisting of miR-221-3p and miR-222-3p, is differentially regulated in mouse and human cardiac pathology and inversely associated with kidney and liver fibrosis. Here we investigated the role of this microRNA family during pressure overload-induced cardiac remodeling and heart failure. In myocardial biopsies of patients with severe fibrosis and idiopathic dilated cardiomyopathy or aortic stenosis, we found significantly lower microRNA-221/222 levels as compared to matched patients without fibrosis. In addition, microRNA-221/222 levels in aortic stenosis patients correlated negatively with cardiac stiffness.

Inhibition of both miRNAs during angiotensin II-mediated pressure overload in mice led to increased fibrosis and aggravated left ventricular dilation and dysfunction. In isolated rat cardiac fibroblasts, inhibition of microRNA-221/222 de-repressed the activation of TGF β -mediated pro-fibrotic SMAD2 signaling and downstream gene expression, while overexpression of both miRNAs blunted TGF β -induced pro-fibrotic signaling. We found evidence that the microRNA-221/222 family may target several genes, next to validated target ETS Proto-Oncogene 1 (ETS-1), involved in TGF β signaling, including c-Jun N-terminal kinase 1 (JNK1), TGF β -receptor-1 (TGF β R1), and -2 (TGF β R2). Our findings identify downregulation of the microRNA-221/222 family to enable fibrotic signaling in the pressure-overloaded heart.

Keywords:

Aortic Valve Stenosis / Fibrosis / Heart Failure / miR-221 / miR-222 / Pressure Overload

Introduction

Hypertension and aortic valve stenosis induce pressure overload of the heart which goes along with left ventricular hypertrophic and fibrotic remodeling, culminating in heart failure (HF). In 2014, the global prevalence of elevated blood pressure was around 20–25% [1], meaning that every 4th person on earth is predisposed to HF. Myocardial fibrosis determines patient outcome even under optimal medical treatment by contributing to cardiac dysfunction, arrhythmias and possibly ischemia [2], and therefore is a particularly interesting therapeutic target.

MicroRNAs (miRNAs) are a class of small (~22 nucleotide long) non protein-coding RNAs that regulate expression of target genes via binding to (partially) complementary regions in their messenger RNAs thereby repressing translation. Importantly, a single microRNA can regulate an entire signaling cascade by repressing translation of several genes involved in that pathway [3]. As such, miRNAs have been implicated in the regulation of the major fibrotic signaling pathways, including transforming growth factor beta (TGF β)-SMAD2/3, ERK1/2-MAP kinase and platelet derived growth factor (PDGF) signaling [4]. Indeed, the potential of miRNAs to modulate myocardial fibrosis has been shown exemplarily for miR-21 [5] and miR-122 [6] by the stimulation of fibroblast ERK-MAP kinase signaling and by directly targeting of TGF β , respectively. However, our mechanistic understanding of the role of miRNAs in pressure overload-induced cardiac fibrosis and HF is still incomplete.

The miRNAs 221–3p and 222–3p (miR-221/222) are both encoded in the same genomic region on the human X chromosome and show a profound sequence similarity. Consequently, both miRNAs have a large overlap in their target genes and are considered a microRNA family. Both miRNAs are differentially regulated in human cardiomyopathies [7–9] and in mouse models of cardiac diseases [10–12] and we recently showed a role for these miRNAs in viral replication and the cardiac immune response during viral myocarditis [13]. So far, there are no reports on a physiological role of the miR-221/222 family in the development of pressure overload-induced cardiac dysfunction. Although some studies investigated the role of miR-221 or miR-222 in the heart, these made use of generated cardiomyocyte-specific overexpressing transgenic mice, not taking into account endogenous miRNA levels and cell source(s) [14–16]. In organs other than the heart, both miR-221 and miR-222 have been linked to fibrosis formation. In particular, miR-221 levels are increased in diabetic nephropathy [17] and is shown to regulate renal fibroblast activation and fibrosis by targeting ETS-1 [18]. Similarly, the miR-221/222 family is upregulated in the liver in a fibrosis progression [19], protecting the liver from excessive fibrosis formation, at least partly, via targeting of ETS-1 [20–22].

The aim of this study was therefore to investigate the physiological role of the microRNA-221/222 family during pressure overload-induced cardiac remodeling. We determined miR-221/222 levels in endomyocardial biopsies of patients with idiopathic dilated cardiomyopathy (DCM) and aortic stenosis (AOS) with either severe or non-severe fibrosis. In addition, we examined the role of these miRNAs in murine pressure overload-induced cardiac fibrosis and failure. Finally, we studied the role of miR-221/222 in pro-fibrotic signaling and myofibroblast differentiation of isolated rat cardiac fibroblasts.

Results

Decreased miR-221/222 expression is associated with severe cardiac interstitial fibrosis in patients with dilated cardiomyopathy or aortic stenosis

In myocardial biopsies from DCM patients with either non-severe (DCM-NSF, n = 41) or severe interstitial fibrosis (DCM-SF, n = 38) (Table 1), cardiac miR-221-3p and miR-222-3p levels were determined. Clinical parameters in both groups were similar, including distri-

Table 1 DCM Patient characteristics

	DCM-NSF (n = 41)	DCM-SF (n = 38)	p value
Demographics			
Male (%)	21(51,2%)	21(55,3%)	0.719
Age at onset, yrs	54.4 ± 1.7	51.8 ± 2.2	0.338
Heart rate, beats/min	73.4 ± 2.4	72.0 ± 2.3	0.659
SBP, mm Hg	133.4 ± 2.7	126.9 ± 4.1	0.182
DBP, mm Hg	80.8 ± 1.6	77.6 ± 2.6	0.297
Body height, cm	172.6 ± 1.6	173.8 ± 1.8	0.606
Body weight, kg	80.3 ± 2.6	80.9 ± 3.1	0.876
Symptoms			
NYHA class I	10(24,4%)	8(21,1%)	0,724
NYHA class II	19(46,3%)	17(44,7%)	0,886
NYHA class III	11(26,8%)	9(23,7%)	0,748
NYHA class IV	1(2,4%)	4(10,5%)	0,190
Echocardiography			
LVEF, %	29.8 ± 1.8	25.83 ± 1.8	0.131
LVEDD, mm	62.0 ± 1.3	64.69 ± 1.5	0.168
LVMI	112.6 ± 5.0	120.5 ± 5.0	0.272
Comorbidities			
Hyperlipidemia	6(14,6%)	2(5,3%)	0.266
Diabetes mellitus	7(17,1%)	2(5,3%)	0.157
Cardiac incident			
CRTD	7(17,1%)	6(15,8%)	0,878
CRT	3(7,3%)	1(2,6%)	0,616
Pacemaker	2(4,9%)	0(0,0%)	0.494
HTx	0(0,0%)	1(2,6%)	0,481
Death	5(12,2%)	3(7,9%)	0,713

Values are mean ± SEM, or n (%) BSA=body surface area; CRT=cardiac resynchronization therapy; CRTD=cardiac resynchronization therapy defibrillator; DBP=diastolic blood pressure; DCM=dilated cardiomyopathy; HTx=heart transplantation; LVEDD=left ventricular end-diastolic diameter; LVEF=left ventricular ejection fraction; LVMI; left ventricular mass index; NSF=non-severe fibrosis; NYHA=New York Heart Association; SBP=systolic blood pressure; SF=severe fibrosis.

bution of NYHA classification, left ventricular ejection fraction (LVEF), left ventricular mass index (LVMI), and occurrence of cardiac incidents. Figure 1A shows that DCM-SF patients had significantly lower myocardial levels of both miR-221 and miR-222 in comparison with the DCM-NSF group. These results indicate that decreased myocardial expression of miR-221/222 is associated with increased fibrosis formation in patients diagnosed with DCM.

Aortic stenosis (AOS) increases the afterload on the left ventricle and induces cardiac remodeling including increased deposition of interstitial fibrosis. Myocardial expression levels of both miR-221-3p and miR-222-3p were significantly decreased in AOS-SF patients as compared to AOS-NSF (Figure 1B). In addition, both miRNA levels correlated negatively with CVF (Figure 1C) and positively with early filling deceleration time (Figure 1D), indicating more fibrosis and slower left ventricular filling in patients with low microRNA levels. In line with this, left ventricular stiffness (KLV) correlated negatively with the expression

Figure 1: Myocardial miR-221 and -222 levels are decreased in patients with dilated cardiomyopathy or aortic stenosis with severe fibrosis. Myocardial biopsies from dilated cardiomyopathy (DCM) patients showed either non-severe (NSF, $n = 41$) or severe fibrosis (SF, $n = 38$). Expression of both miR-221 and miR-222 is significantly lower in the myocardium of DCM patients with SF versus NSF (A). In left ventricular biopsies of aortic stenosis patients, expression of both miRNAs is significantly lower in patients with SF (filled circles, $n = 15$) versus NSF (empty circles, $n = 13$, B) and correlates negatively with collagen volume fraction (C). A positive correlation of miR-221/222 expression with deceleration time of the early mitral filling wave (D) and a negative correlation with left ventricular chamber stiffness constant KLV (E) indicate the negative association of miR-221/222 levels with left ventricular stiffness. ** $p < 0.01$; *** $p < 0.001$.

of both miRNAs (Figure 1E) although this did not reach statistical significance for miR-222 ($p = 0.069$). On the other hand, cardiomyocyte size did not correlate with expression of either miR-221 or miR-222 (Suppl. Figure S1A) indicating that miR-221/222 levels may be involved in the fibrotic but not hypertrophic response to cardiac pressure overload. Taken together, decreased myocardial miR-221/222 levels are significantly associated with increased myocardial interstitial fibrosis and stiffness during human pressure overload. Additionally, we found circulating levels of miR-221, but not miR-222, to negatively correlate with cardiac CVF, identifying miR-221 as a potential biomarker of myocardial fibrosis (Supplemental Figure 1B).

In vivo inhibition of miR-221/222 aggravates Angiotensin II-induced cardiac dysfunction and fibrosis

To investigate if the miR-221/222 family plays a causative role in pressure overload-induced left ventricular fibrotic remodeling, we subjected mice to 4 weeks of angiotensin II (AngII)

Figure 2: Inhibition of miR-221/222 during angiotensin II-induced pressure overload exaggerates cardiac dysfunction by contributing to cardiac fibrosis but not hypertrophy. Cardiac function as indicated by echocardiographically established left ventricular ejection fraction decreases after 4 weeks of angiotensin II-induced pressure overload, and further deteriorates when miR-221/222 are inhibited (A). In line, cardiac dimensions as indicated by diastolic left ventricular inner diameter (LVIDdia) are significantly increased upon pressure overload in combination with miR-221/222 inhibition (B). This adverse remodeling goes along with increased cardiac interstitial fibrosis deposition upon pressure overload in mice receiving miR-221/222 antagomirs as compared to negative control oligonucleotides (C). Representative images of Sirius red-stained left ventricular sections (D; scale bar: 100 μm). Cardiac mass corrected for tibia length (E), diastolic left ventricular wall thickness (LVWTdia) measured by echocardiography (F) and cardiomyocyte cross-sectional area (G) as measures of cardiac hypertrophy increase significantly in mice subjected to angiotensin II without being affected by inhibition of miR-221/222. Representative images of laminin staining of left ventricular sections (H; scale bar: 100 μm). AngII, angiotensin II; $n = 5-8$ for sham groups; $n = 6-11$ for AngII groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ AngII versus Sham; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ Antagomir versus Control.

infusion while inhibiting miR-221/222 using antagomirs, resulting in about 50% cardiac knockdown of both miRNAs (Suppl. Figure S2A). During AngII-induced pressure overload, inhibition of miR-221/222 significantly aggravated left ventricular dilation and dysfunction as compared to mice receiving scrambled control oligonucleotides, indicating increased susceptibility to pressure overload-induced HF upon miR-221/222 inhibition (Figure 2A-B). In addition, while AngII induced the deposition of cardiac interstitial fibrosis in both antagomir- and control oligonucleotide-treated mice, knockdown of miR-221/222 further increased AngII-induced cardiac fibrotic remodeling (Figure 2C-D). The AngII-induced increase in systolic blood pressure was similar in antagomir- and control oligonucleotide-treated mice (Suppl. Figure S2C) and also perivascular fibrosis remained unchanged (Suppl. Figure S2B), indicating that aggravated left ventricular dysfunction after inhibition of miR-221/222 was not due to differences in pressure overload. In line, cardiac hypertrophy was comparable in antagomir- and control oligonucleotide-treated mice as indicated by similar AngII-effects on heart weight (Figure 2E), left ventricular wall thickness (Figure 2F) and cardiomyocyte cross-sectional area (Figure 2G-H). In conclusion, these data implicate the miR-221/222 family in pressure overload-induced myocardial fibrosis and dysfunction, whereas endogenous miR-221/222 have no role in cardiac hypertrophy.

The miR-221/222 family regulates pro-fibrotic signaling in neonatal cardiac fibroblasts

During cardiac pressure overload, an intracardiac renin-angiotensin system is activated that increases local AngII signaling and contributes to the development of cardiac hypertrophy and fibrosis [23]. In the myocardium, AngII promotes activation of resident fibroblasts via induction of TGF β signaling [24]. To investigate if and how miR-221/222 modulate the fibrotic response of cardiac fibroblasts to TGF β , we examined the role of the miRNA family in nRCFs. We found that miR-221/222 is able to control fibroblast proliferation. Forced expression of miR-221/222 decreased BrdU incorporation in nRCFs while miRNA inhibition had opposite effects (Figure 3A-B). The expression of miR-221/222 in nRCFs was downregulated by TGF β (Figure 3C), in line with the decreased miR-221/222 levels we found in human DCM-SF and AOS-SF. Rescue of this response by the artificial overexpression of both miRNAs using miRNA mimics reduced TGF β -induced fibroblast activation. This was evidenced by miR-221/222-dependent repression of TGF β -induced upregulation of the pro-fibrotic genes Connective tissue growth factor (Ctgf), fibronectin (Fn 1) and alpha smooth muscle actin (α SMA) (Figure 3D-F), indicative of impaired trans-differentiation of fibroblasts into collagen producing myofibroblasts [25]. Western blot analysis confirmed that miR-221/222 modulate TGF β -induced pro-fibrotic SMAD-signaling and downstream CTGF expression in nRCFs.

Inhibition of the microRNA family by use of miRNA inhibitors prior to TGF β stimulation further increased the phosphorylation of SMAD2 and the subsequent induction of CTGF gene expression (Figure 4A-C), which is in line with the observed increased myocardial fibrosis in AngII-treated mice after inhibition of miR-221/222. In contrast, simultaneous

Figure 3: Expression of miR-221/222 in cardiac fibroblasts is regulated by TGFβ and miR-221/222 controls proliferation and blunts fibroblast activation after TGFβ stimulation. MiR-221/222 controls fibroblast proliferation, evidenced by decreased BrdU incorporation upon miRNA overexpression (A) while miRNA inhibition leads to increased BrdU incorporation (B). MiR-221/222 are downregulated in neonatal rat cardiac fibroblasts (nRCF) after stimulation with TGFβ (C). RT-PCR shows that trans-differentiation of fibroblasts to myofibroblasts upon TGFβ stimulation is blunted by overexpression of miR-221/222 as evidenced by reduced induction of Ctgf (D), fibronectin (Fn1, E) and alpha smooth muscle actin (αSMA, F) mRNA expression. A-B n = 18-24/group; E-F n = 3-6; *p < 0.05, **p < 0.01, ***p < 0.001: TGFβ versus Buffer; +p < 0.05, ++p < 0.01: miR-inhibitors or -mimics versus respective control oligonucleotides.

overexpression of both miRNAs reduced the induction of CTGF upon TGFβ stimulation, with repressive though non-significant effects on SMAD2 phosphorylation (Figure 4D-F), confirming that the increased presence of the microRNA-221/222 family can restrict pro-fibrotic signaling in stimulated cardiac fibroblasts. These results indicate that downregulation of miR-221/222 upon cardiac stress allows for fibroblast activation and trans-differentiation, characterised by induction of pro-fibrotic gene expression and fibroblast proliferation, and contributes to cardiac remodelling. These data identify this microRNA family as a potential therapeutic candidate in cardiac fibrosis and consequent dysfunction.

The miR-221/222 family may directly target ETS-1, JNK1, TGFβR1, and TGFβR2

The miR-221/222 family is shown to influence the fibrotic response, at least partly, through targeting of the transcription factor ETS Proto-Oncogene 1 (ETS-1) [13, 18, 26]. To determine the full underlying mechanism how miR-221/222 exerts its fibrosis-regulatory function, we identified 3 additional predicted target genes involved in TGFβ-signalling: c-Jun N-terminal kinase 1 (JNK1), TGFβ-receptor-1 (TGFβR1), and -2 (TGFβR2) (Figure 5A). Silencing of the miR-221/222 family in pressure overloaded-hearts increased the transcript expression

Figure 4: Protein levels of CTGF in TGFβ stimulated cardiac fibroblasts depend on miR-221/222 levels.

Representative western blots of neonatal rat cardiac fibroblast stimulated with TGFβ after inhibition of miR-221/222 (A). Inhibition of both miRNAs increases TGFβ-induced SMAD2 phosphorylation (B) and CTGF induction (C). Representative western blots of neonatal rat cardiac fibroblast stimulated with TGFβ after overexpression of miR-221/222 (D). TGFβ-induced SMAD2 phosphorylation is non-significantly reduced by miR-221/222 mimics (E), with significant downstream effects on CTGF expression (F). $n = 3-6/\text{group}$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: TGFβ versus Control; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$: miR-inhibitors or -mimics versus respective control oligonucleotides.

Figure 5: The miR-221/222 family targets ETS-1, JNK1, TGFβR1, and TGFβR2.

The miR-221/222 family is predicted to target ETS-1, JNK1, TGFβR1, and TGFβR2 (A). Silencing of of miR-221/222 during angiotensin II-induced pressure overload increased murine cardiac transcript expression of ETS-1, JNK1, TGFβR1, and TGFβR2 (B). Overexpression of miR-221/222 in nRCFs decreases transcript expression levels of predicted target genes with a crucial role in TGFβ-signalling, including ETS-1, JNK1, TGFβR1, and TGFβR2 (C). For nRCFs: $n = 6/\text{group}$; ** $p < 0.01$, *** $p < 0.001$: miR-mimics versus control oligonucleotides. For murine hearts: AngII, angiotensin II; $n = 5-8$ for sham groups; $n = 6-9$ for AngII groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Antagomir versus Control.

of ETS-1, JNK1, TGF β R1, and TGF β R2 (Figure 5B), while transcript levels of these 4 genes decreased upon overexpression of miR-221/222 in cultured cardiac fibroblasts (Figure 5C). Taken together, these results indicate that miR-221/222 may regulate the fibrotic response through targeting of ETS-1, JNK1, TGF β R1, and TGF β R2.

Conclusion & Discussion

Pathological stressors such as hypertension and aortic stenosis cause myocardial interstitial fibrosis, disrupting electrical and mechanical properties and predisposing to the development of HF [2]. In the last years, factors involved in myocardial fibrosis including miRNAs have been identified [2], but our mechanistic insight into the molecular processes underlying fibrosis is still limited. Downregulation of miR-122 in human aortic stenosis was found to de-repress its target TGF β and may play a role in severe cardiac fibrosis [6]. In addition, miR-21, which is consistently up-regulated in HF, was found to promote cardiac fibroblast survival and growth factor secretion by targeting the ERK/MAPK and PTEN/SMAD7 signaling pathways [5, 27]. In the present study, we identify two novel miRNAs, the microRNA-221/222 family, which downregulation upon pressure overload contributes to myocardial fibrosis and consequent HF by allowing fibroblast activation and differentiation. Dilated cardiomyopathy patients with severe fibrosis formation displayed lower myocardial levels of miR-221/222. Similarly, in patients with severe aortic valve stenosis, lower levels of miR-221/222 correlated significantly with more severe myocardial fibrosis and left ventricular stiffness. In line with these findings, inhibition of miR-221/222 in a mouse model of angiotensin II-induced pressure overload lead to increased myocardial interstitial fibrosis, left ventricular dilation and cardiac dysfunction.

Angiotensin II can exert its pro-fibrotic effects via induction of TGF β in cardiac fibroblasts [24]. TGF β expression is increased in the myocardium of both patients and experimental models with pressure overload and HF. It is one of the most potent inducers of extracellular matrix proteins, and its role in the pathogenesis of fibrosis is well established [28]. In cardiac fibroblasts, TGF β induced the downregulation of miR-221/222, pointing to TGF β as a potential regulator of these miRNAs and identifying a feedback mechanism to potentiate TGF β signaling. TGF β /SMAD signaling directly induces expression of CTGF, a secreted growth hormone that augments induction of extracellular matrix synthesis [29]. Persistent fibrosis appears to be initially induced by TGF β signaling whereas CTGF acts as maintenance factor for fibrotic remodelling [30]. Importantly, TGF β does not directly induce collagen synthesis in cardiac fibroblasts, but rather induces fibroblast activation which is accompanied by increased proliferation and trans-differentiation into collagen synthesizing myofibroblasts [25]. Here, we found that this proliferation is under the control of miR-221/222, evidenced by decreased BrdU incorporation upon miRNA overexpression while silencing of the miRNA-family increased BrdU incorporation in cardiac fibroblasts. Fibroblast trans-differentiation is augmented by CTGF signaling [31] and goes along with

upregulation of the myofibroblast markers α SMA [32, 33] and fibronectin [34, 35]. Indeed, we found that overexpression of miR-221/222 significantly blunted the induction of these myofibroblast markers after stimulation with TGF β , indicating that downregulation of these miRNAs upon pressure overload contributes to myofibroblast differentiation and collagen production. On the protein level, we confirmed that overexpression of miR-221/222 impedes the TGF β -induced expression of CTGF, whereas inhibition of both miRNAs significantly augmented SMAD2 phosphorylation and subsequent CTGF induction. These results provide evidence that the microRNA-221/222 family inhibits fibroblast to myofibroblast trans-differentiation after TGF β stimulation. Downregulation of these miRNAs in cardiac fibroblasts upon pressure overload will therefore facilitate interstitial fibrosis deposition, as is indeed suggested by the negative correlation between miR-221/222 levels and interstitial fibrosis in aortic stenosis patients. Interestingly, we found a non-significant increase of miR-221/222 levels by AngII in whole heart extracts, apparently contradicting their down-regulation in fibroblasts. However, AngII has been shown to increase expression of both miRNAs in vascular smooth muscle cells [36] and miR-222 was found to be increased in cardiomyocytes of mice after wheel running, whereas its expression was slightly reduced in non-cardiomyocytes [14]. This indicates that AngII may induce expression of miR-221/222 in VSMCs and cardiomyocytes, while subsequent activation of TGF β signaling leads to repression of the miR-221/222 family in fibroblasts.

Recently, miR-222 has been suggested to drive liver fibrosis by exerting pro-proliferative effects on stellate cells [20, 37], apparently contradicting our findings. However, both studies relate to biliary atresia, in which occlusion of the bile duct leads to cholestasis with secondary fibrosis of the liver [38]. It is therefore conceivable, that the high miR-222 levels found in these studies do not directly correlate with pro-fibrotic signaling, but rather relate to increased stellate cell proliferation with secondary effects on the degree of cholestasis and fibrosis.

We found evidence that the microRNA-221/222 family may target several genes involved in TGF β signaling, including ETS-1, JNK1, TGF β R1, and TGF β R2. The transcription factor ETS-1 synergizes with TGF β to induce CTGF expression [39] and was found to mediate the pro-fibrotic effects of angiotensin II on renal fibroblast [40]. ETS-1 expression is repressed by both miR-221 and miR-222 [13, 18, 26]. In particular, overexpression of miR-221 in normal rat kidney fibroblasts (NRK-49F cells) is able to prevent the angiotensin II-induced expression of fibronectin and alpha smooth muscle actin (α SMA) through targeting of ETS-1 [18]. Various reports have shown that the stress-activated protein kinase JNK1 is required for the development of fibrosis in multiple organs, including the liver [41, 42], lung [43, 44], kidney [45], and heart [46]. In particular, JNK1 is necessary for expression of profibrotic genes through stimulation of fibroblast activation, proliferation and transdifferentiation into myofibroblasts [45, 47–49]. JNK1-deficient mice are protected against the development of hepatic [41, 49] and pulmonary fibrosis [44]. Furthermore, enhanced expression of JNK1 is associated with an accelerated progression of diabetic myocardial interstitial fibrosis [46]. Similarly, both TGF β R1 and TGF β R2 play a crucial role in heart failure development since

TGF β signals through binding a heterotetrameric receptor complex of these 2 receptors [50]. Upon TGF β binding, TGF β R2 phosphorylates and thereby activates TGF β R1 serine/threonine kinases that in their turn activate SMAD2/3 transcription factors. In addition to this canonical TGF β pathway, TGF β R2-mediated activation of TGF β -activated kinase 1 (TAK1) results in downstream stimulation of JNK1 and p38 [51, 52]. Thus, TGF β R1 and -R2 mediate activation of both the canonical and non-canonical TGF β signaling pathways, playing a key role in the cardiac fibrotic response. Therapeutical inhibition of TGF β R1 after TAC prevented cardiac fibrosis and attenuated cardiac dysfunction [53]. Similarly, blocking of TGF β via soluble TGF β R2 treatment upon myocardial infarction prevented cardiac dilatation and dysfunction through diminishing cardiomyocyte hypertrophy and interstitial fibrosis [54]. Taken together, these results suggest that restoration of miR-221/222-induced inhibition of TGF β signalling upon cardiac stress, through their targeting of ETS-1, JNK1, TGF β R1, and TGF β R2, may provide a suitable therapy to prevent excessive fibrosis formation and heart failure development.

Recently, two groups have investigated the cardiac effects of miR-221 and miR-222 in cardiomyocyte restricted overexpression models. Generated transgenic mice overexpressing miR-221 or miR-222 in the cardiomyocytes develop HF spontaneously, accompanied by impaired autophagy, cardiomyocyte dysfunction and death, and increased fibrosis [15, 16]. On the contrary, mice with inducible cardiomyocyte-specific miR-222 expression were protected against adverse cardiac remodeling and dysfunction after ischemia/reperfusion injury, as evidenced by reduced cardiomyocyte death and reduced myocardial fibrosis [14]. In these models of cardiomyocyte-restricted miR-221/222 overexpression, myocardial fibrosis most probably occurs secondary to an influence of these miRNAs on cardiomyocyte function and survival. In contrast to these models, we manipulated miR-221/222 in the adult whole heart including cardiac fibroblasts and found a direct effect on the pro-fibrotic phenotype of these cells.

Altogether, we show that lower levels of microRNA-221 and -222 correlate with higher levels of fibrosis and left ventricular stiffness in patients with HF, and provide evidence that decreased levels of miR-221/222 contribute to pressure overload-induced myocardial fibrosis and dysfunction in mice. Additionally, we found that in cardiac fibroblasts, TGF β downregulates the expression of miR-221/222, alleviating their interference with its own pro-fibrotic signaling. Therefore, we propose that myocardial TGF β signaling leads to downregulation of the miR-221/222 family to facilitate trans-differentiation of cardiac fibroblasts into collagen producing myofibroblasts, contributing to the development of myocardial fibrosis and HF.

Materials & Methods

Patient samples

The study groups enrolled in this correlation analysis were explained in detail previously [6, 55]. In brief, DCM patients with a left ventricular ejection fraction (LVEF) < 50% and indexed left ventricular end-diastolic diameter (LVEDDI) > 33 mm/m² (men) or > 32 mm/m² (women) in the absence of active viral myocarditis or a clinical history of myocardial infarction and/or atrial fibrillation were included (n = 79). Right ventricular septal biopsies of 41 DCM patients showed an interstitial collagen volume fraction (CVF) of below 5%, called non-severe fibrosis (DCM-NSF), and 38 had an interstitial CVF of above 5%, called severe fibrosis (DCM-SF)[55].

Similarly, endomyocardial biopsies of 28 patients with severe isolated aortic valve stenosis were collected and analysed for myocardial fibrosis and microRNA expression. M-mode and pulsed Doppler echocardiographic measurements were performed in all patients. Left ventricular chamber stiffness constant (KLV) was calculated as the ratio squared of the deceleration time of the early mitral filling wave according to the following equation [56]: $KLV = (0.07 : DT)^2$.

The fraction of myocardium occupied by collagen or collagen volume fraction (CVF) was determined by quantitative morphometry with an automated image analysis system in sections stained with Picrosirius Red as previously described⁶. A cluster analysis was performed according to CVF values, and AS patients were divided in two groups: non-severe fibrosis (AOS-NSF) group (CVF ≤ 15%, n = 13) and severe fibrosis (AOS-SF) group (CVF > 15%, n = 15). Cardiomyocyte area was analysed in Masson's trichrome stained sections. The cross-sectional area of approximately 100 cardiomyocytes (with the nucleus in central position) was measured in each sample using the AnalySIS 3.1 software (Soft Imaging System GmbH).

Myocardial and serum RNA was isolated with TRIzol or TRIzol LS reagent (Invitrogen) according to manufacturer's recommendations. Synthetic *C. elegans* microRNA 39 (cel-miR-39) was spiked into serum after the addition of the Trizol LS reagent to the samples to be used as control. Reverse transcription (RT) of microRNAs was performed using specific primers for miR-221 and miR-222, cel-miR-39 and snU6 as an endogenous control for myocardium. The cDNA obtained was pre-amplified and amplified by PCR using the specific TaqMan microRNA assays (Life Technologies) for these microRNAs and for snU6 RNA. The fluorescent signal was detected with a 7900HT Fast Real-Time PCR System (Applied Biosystems) and analysed with the SDS 2.2.2 software. MicroRNA expression was normalised by snU6 RNA in the myocardium and by cel-miR-39 in serum and expressed as arbitrary units (A.U.).

Animal models

Eight week old C57Bl/6J mice (Charles River) received subcutaneous infusion of angiotensin II (Bachem, 2.5 mg·kg⁻¹·d⁻¹) by use of osmotic minipumps (Alzet) for four weeks. To

inhibit miR-221 and miR-222 simultaneously, mice received intravenous injections of 40 mg/kg of each antagomir (Fidelity Systems) at three consecutive days prior to minipump implantation, as well as at days 13 and 14 of AngII infusion. Injections of scrambled control oligonucleotides (80 mg/kg) served as control. After four weeks, echocardiography was performed on a Vevo2100 system (Visualsonics) to acquire M-mode images at the height of the papillary muscles. Afterwards, animals were sacrificed to harvest organs for histological and molecular biological analysis. Mice were housed under social conditions with *ad libitum* access to water and standard chow. All animal experiments were carried out in accordance with Dutch law and approved by the local ethics committee.

Histology

After sacrifice, organs were rinsed in PBS and fixed in 4% neutral buffered formalin for 4 days before embedding in paraffin blocks. Sections were stained with Picrosirius Red to quantify fibrosis. Approximately 10 randomly chosen microphotographs across the left ventricle and septum were taken. Interstitial fibrosis was normalized to tissue area and perivascular fibrosis was normalized to the area of the respective vessel. Laminin staining was performed using rabbit anti-mouse laminin (Sigma, L9393) and Vectastain Elite ABC kit (Vector laboratories). Images were taken at the septum and left ventricular myocardium. Epicardial cells, as well as longitudinal cells and cells without visible nucleus were excluded from analysis. All images were taken and analysed in a blinded manner using a Leica DM2000 equipped with a Leica DFC450C camera and custom designed macros in ImageJ software.

In vitro experiments

Neonatal rat cardiac fibroblasts (nRCF) were isolated from 1–2 day old Wistar rat pups by digestion with Collagenase I (Gibco) and Pancreatin (Sigma) in spinner flasks. Adherent cells were collected and maintained in DMEM 22320 supplemented with 10% heat inactivated fetal bovine serum (FBS) and penicillin/streptomycin. After 1–2 passages cells were seeded in 6-well plates. Sub-confluent cells were transfected with miRIDIAN miR-221 and miR-222 mimics (Dharmacon, 1 nM each vs 2 nM control) or Power Inhibitors (Exiqon, 20 nM each vs 40 nM control) in low serum medium (0.1% FBS) using Lipofectamine2000 (Invitrogen). After 24 hours cells were stimulated with 10 ng/mL recombinant human TGF β_1 (Peprotech). After 15 minutes or 24 hours of stimulation cells were harvested in protein sample buffer (5mM TRIS buffer, 20% glycerol, 4% SDS, 10% β -mercaptoethanol) for western blot analysis or in lysis buffer for RNA isolation using mirVana miRNA isolation kit (Ambion).

Western Blot

Western blot of nRCF protein was performed using standard procedures. Primary antibodies used were as follows: rabbit anti-connective tissue growth factor (CTGF) (Gene-Tex #GTX26992), rabbit anti-SMAD2/3 (CellSignaling #8685P), and rabbit anti-pSMAD2/3

(CellSignaling #8828S). Mouse anti-Gapdh (Millipore MAB374) served as internal control. Secondary antibodies were HRP conjugated goat anti-rabbit (CellSignaling #7074) or horse anti-mouse (CellSignaling #7076). Enhanced chemiluminescence was measured in a custom made imaging system equipped with a cooled Artemis FS28 16-bit camera and analysed using ImageJ and Image Studio Lite software.

RNA isolation and RT-qPCR

RNA was isolated from animal tissue or cells using mirVana microRNA isolation kit (Ambion) according to manufacturer's instructions. Snap frozen heart tissue was homogenized in mirVana lysis buffer using ceramic beads (Lysing Matrix D, MP Biomedicals). RNA was reverse transcribed using miScript RT II kit (Qiagen) and RT-qPCR was performed using SYBR Green (Biorad). The expression of miR-221 and miR-222 was detected using microRNA LNA primer sets (Exiqon). Primer sequences to determine transcript levels are described in table 2.

Table 2 RT-PCR primer sequences

Gene	Forward sequence	Reverse sequence
CTGF	CACAGAGTGGAGCGCCTGTTC	GATGCACTTTTTGCCCTTCTTAATG
Fn1	AGTCCCGATGGTACCACTGGCC	CCTGCACGTCCAACGGCATG
α SMA	GTCCCAGACACCAGGGAGTGA	TCGGATACTTCAGGGTCAGGA
ETS-1	GCTGTGGCAGTTTCTTCTGG	TTCTCTTTCCCCATCTCCT
Rno-JNK1	GCGTCATAAGAGGGCAGCCG	TGCTGTCTGTATCCGAGGCCA
Mmu-JNK1	CCGAGGCCGGACGTTGC	CAAATATTCACCAAGGCCGGCAG
Rno-TGFBR1	GCGGGGAGAAGAAGTTGCCG	ACCATTGTCTTTGTTGTCTGCTGCT
Mmu-TGFBR1	GGGCCGGGCCACAAACA	ACTGTAATGCCTTCGCCCCC
Rno-TGFBR2	CCCCGTTTGGTTCCAGAGTG	CACGATCTGGATGCCCTGGTG
Mmu-TGFBR2	CAGGGGCCGGTCTATGACGA	ACATCACTGTAAACCGACTTGGA
RPL13a	CAGAAAGTTTGCTTACCTGGGG	TTCTGCCTGTTTCCTTAGCCTC
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCTAT

Statistics

Unless stated otherwise, data are presented as mean $\pm$ SEM. Statistical analysis was performed with GraphPad Prism using Student's t-test or one-way ANOVA followed by Tukey post-hoc test to compare individual groups. Measurements on human dilated cardiomyopathy patients were analysed using Student's t-test. Measurements on human samples were analysed using Mann-Whitney test or Spearman's correlation. In all cases a p -value < 0.05 was considered statistically significant.

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Supplemental Figures & Methods

Supplemental Figure 1: Myocardial expression of miR-221 and miR-222 does not correlate with myocardial TGF β levels (A) or cardiomyocyte size (B) in patients with aortic stenosis. Plasma levels of miR-221, but not miR-222 significantly correlate with myocardial fibrosis (C). NSF: non-severe fibrosis (empty circles, $n = 13$); SF: severe fibrosis (filled circles, $n = 12-13$).

Supplemental Figure 2: Systemic administration of antagomirs resulted in a significant cardiac knockdown of miR-221 and miR-222 compared to injection of scrambled control oligonucleotides (A and B). Perivascular fibrosis was not affected by angiotensin II infusion or inhibition of miR-221/222 (C). The blood pressure increase by angiotensin II infusion was similar between mice receiving antagomirs or scrambled control oligonucleotides indicating that differences in fibrosis and LV function are not due to differences in the level of pressure overload (D). AngII, angiotensin II; n = 7-8 for sham groups and n = 8-11 for AngII groups; For SBP measurements n = 3-5/group; *p < 0.05, **p < 0.01, AngII versus Sham; [†]p < 0.05, ^{††}p < 0.01, ^{†††}p < 0.001 Antagomir versus Control.

CHAPTER 5

MiR-151 Represses Cardiomyocyte Hypertrophy and Oxidative Metabolism by Targeting PGC-1 α

In preparation

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CHAPTER 6

Cardiomyocyte Cell Cycle Activity is Under The Control of miR-125a

In preparation

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CHAPTER 7
General Discussion

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General discussion

Heart failure is an age-related, multifaceted disease, driven by different cellular processes and involving various cell types. These include hypertrophic growth of cardiomyocytes, excessive extracellular matrix deposition by fibroblasts, and an exaggerated inflammatory response by immune cells. MiRNAs have a pivotal regulatory role in all those processes [1, 2]. Therefore, the **first aim** of this thesis was to **identify miRNAs that regulate cellular processes driving failure and aging of the myocardium**. To address this aim, we reviewed the current literature describing the regulatory role of miRNAs in various processes mediating cardiac senescence and failure (Chapter 2). Furthermore, we selected 194 heart failure-associated miRNAs, which were probed for their effect on cardiomyocyte size, cardiac fibroblasts collagen content, and macrophage polarization in an extensive *in vitro* screen. As a result, we identified previously undescribed roles of miRNAs in hypertrophy, fibrosis, and inflammation, and attribute new cellular effects to various well-known miRNAs (Chapter 3).

The **second aim** was to **interrogate the efficacy of miRNA-manipulation in retarding heart failure progression**. To this aim, we selected specific miRNAs and studied their ability to influence cellular processes underlying cardiac disease progression. First, the role of the miR-221/222 family was investigated during pressure overload-induced cardiac remodelling. Antagomir-mediated inhibition of miR-221/222 expression enabled exaggerated cardiac fibrotic signalling and deteriorated cardiac function (**Chapter 4**). Second, miR-151a was shown to influence cardiomyocyte hypertrophy and, at the same time regulates mitochondrial oxidative capacity of cardiomyocytes, probably via regulation of PGC-1 α levels (**Chapter 5**). Finally, cardiomyocyte cell cycle activity was found to be under the control of miR-125a, inducing, at least *in vitro*, non-pathological cell growth while increasing contractility. MiR-125a is able to induce CDKi expression in the murine heart and in neonatal cardiomyocytes, inducing cellular growth associated with G1-cell cycle arrest, and increased cardiac contractility and cardiomyocyte contraction frequency and amplitude (**Chapter 6**). The main conclusion from these studies is that miRNAs are important directors of molecular and multi-cellular processes underlying cardiac disease progression. Their regulatory function may be manipulated therapeutically in rodents – and in the future in humans- to impact disease progression.

7.1 The bigger picture: numerous miRNAs are involved in cardiac failure

Despite the existing therapies, heart failure remains a major health problem with a high global prevalence and poor clinical outcome, especially threatening the lives of the elderly population [3–6]. MiRNA-targeted therapy may provide a novel approach to treat this age-related disease. MiRNA-profiling studies show deregulated myocardial expression patterns during aging [7] and in different disease states, including heart failure (HF) [8], viral myocarditis [9], or myocardial infarction [10]. These studies associate various miRNAs with the

cellular changes related to cardiac aging and disease progression. However, the question remains if identified deregulated miRNAs are part of a disease-mediating network, or that their differential expression merely represents a secondary effect. We therefore reviewed in chapter 2 the current knowledge concerning the molecular and cellular processes driving myocardial aging, and described the regulating role of known senescence-associated miRNAs in these processes. Altered miRNA expression profile seems not to be an “innocent bystander-effect”. On the contrary, specific identified deregulated miRNAs influence healthy cardiac aging and disease.

The concept of the multi-cellular function of most of the HF-related miRNAs was confirmed in our screen of 194 miRNAs, differentially regulated in rodent and human inflammatory cardiac disease. Their effect on cardiomyocyte size, cardiac fibroblasts collagen content, and macrophage polarization was studied *in vitro* (Chapter 3). The simultaneous testing in different cell types broadened our understanding of the cellular involvement underlying the multifaceted pathogenesis of heart failure, identifying previously undescribed roles of miRNAs in hypertrophy, fibrosis, and inflammation, and attribute new cellular effects to various well-known miRNAs. Several high-throughput screens, aimed to identify on a large scale miRNAs as regulators of molecular processes crucially involved in cardiac (dys)function on a large scale, were performed by others. Cardiac processes which have been screened for the involvement of miRNAs include cardiomyocyte proliferation [11], intracellular calcium handling [12], and hypertrophic growth [13]. The uniqueness of our screen lies in its multi-cellular nature, whereas other screens only studied one specific cell type. Each screen has its own advantages and disadvantages. In general, high-throughput screening is accompanied with false-positive and false-negative rates. This necessitates further in-depth validation of the regulatory function of “top hit” miRNAs to support the process of selecting a miRNA that may impact cardiac disease outcome. Despite the shortcomings of high-throughput screening, large-scale assessment of miRNA function in one or more cellular processes forms a rich source of information and helps to identify promising miRNA candidates for therapeutic targeting.

7.2 A closer look: specific miRNAs with specialised functions

This thesis studied specific miRNAs as therapeutic candidate for their ability to regulate disease outcome in different animal models. In chapter 4, we silenced miR-221/222 via antagomir injections in a mouse model of AngII-induced pressure overload. MiR-221/222 inhibition results in an exaggerated cardiac fibrotic response along with a deterioration in cardiac function. This study indicates the pivotal role of the miR-221/222 family in controlling the fibrotic response during pressure-overload. However, the efficacy of miR-221/222 overexpression to prevent excessive fibrosis formation in the myocardium to attenuate detrimental cardiac remodelling still needs to be determined. In contrast, recent reports suggest that cardiomyocyte specific overexpression of either miR-221 or miR-222 impairs

autophagy, leading to the spontaneous development of HF [14, 15]. However, the function of miR-221/222 in the cardiomyocyte was assessed using a transgenic overexpression model, reaching artificial expression levels that by far exceeded the pathophysiological levels observed in the heart *in situ*.

Other miRNA-based therapeutics have already made the transition from rodents to primate studies [16–18], confirming their specialised function and demonstration that modulation of their activity can impact disease progression in rodents and primates. This holds great promise regarding their translation into the clinic. Indeed, the first clinical trials modulating miRNA activity in humans have been performed. The safety and efficacy of anti-miR-122 treatment (*Miravirsen*) has been evaluated in 36 patients with chronic hepatitis C virus (HCV) genotype 1 infection [19]. The use of *Miravirsen* in patients with chronic HCV genotype 1 infection resulted in a dose-dependent reduction in HCV RNA levels that endured beyond the end of active therapy, while observing no dose-limiting adverse events and no escape mutations in the miR-122 binding site of the HCV genome [19]. In addition, both senescence-associated miR-16 and miR-34 (as discussed in chapter 2) are well known for their inhibitory effect on tumor growth and metastasis [20–23] and entered phase I clinical trials to investigate the safety, pharmacokinetics and pharmacodynamics of miRNA overexpression in cancer patients. The clinical trial for miR-16 has been completed, although no study results have been reported yet (Clinical trials.gov identifier NCT02369198). For miR-34, clinical testing in cancer patients was halted in September 2016, following multiple severe immune-related adverse events (Clinical trials.gov identifier NCT01829971). These clinical trials on the safety and efficacy of modulation of miRNAs indicate that we are on the edge of implementing miRNA-based therapeutics into the clinics.

7.3 Beyond the horizon: possibilities & limitations of future miRNA-based therapeutics

Based on the impact of miRNA modulation on cardiac disease progression observed in rodent and primate studies and the initiated testing of miRNA-based therapeutics in clinical trials, we can conclude that miRNAs hold great promise as potential targets for therapy in the nearby future. However, clinical trials need to determine if miRNA-based therapeutics truly comprise the ability to prevent and/or cure cardiovascular disease in the human population. In order to progress the application of miRNA-based therapeutics in the clinics, improvement of current miRNA tools to impact miRNA function *in vivo* will be necessary.

Currently used techniques to inhibit specific miRNAs include the use of antagomirs, while miRNA overexpression is achieved via the use of miRNA mimics or adeno-associated virus (AAV)-mediated gene transfer. These miRNA mimics and antagomirs can be delivered systemically by intravenous, intraperitoneal, or subcutaneous injection with little or no

toxicity [24, 25]. However, since the vast majority of oligonucleotides is cleared through the liver and kidney [26], relatively high doses are needed to achieve therapeutic efficacy in the heart [27]. These high doses are paralleled by deregulation of the same miRNA in other unintended cell types, mainly being effective in circulating inflammatory cells and endothelial cells. Thus, using systemic administration of antagomirs or mimics limits the target options, necessitating the selection of a miRNA specifically expressed and/or functional in the cell type of interest to minimize off-target effects.

Local, instead of systemic, administration of miRNA-modulating agents would circumvent this problem. A single intra-cardiac injection of miRNA mimics has benefitted the heart after myocardial infarction, inducing a myocardial overexpression that lasted for at least 12 days [28]. Furthermore, AAV9-vectors have a tropism towards to heart and have been used to induce miRNA overexpression in the cardiomyocyte with minimal off target effects [29, 30]. However, the observed overexpression levels in the heart are rather mild, forming in some cases a suboptimal model to determine the phenotypical and functional consequences of miRNA modulation. Thus, current approaches to modulate miRNA expression force the researcher to choose between a potent but aspecific, or a mild but specific therapeutic strategy.

In spite of the current challenges, our knowledge about the role of miRNAs in cardiovascular disease accumulates rapidly and their translation into clinically applicable therapeutic tools will be established in the nearby future as specific delivery of miRNA-based therapeutics improves.

7.4 Conclusions and Future Perspectives

Heart failure is a major health problem. The lives of millions of people globally are threatened due to an increasing age of the general population and, more importantly, due to the lack of effective treatment. This thesis interrogated the role of miRNAs as regulators of cellular processes driving failure and aging of the myocardium, and elaborated on the efficacy of their manipulation in retarding heart failure progression. We identify multiple miRNAs that possess the ability to regulate cellular processes central to cardiac (dys)function, and provided proof of principle that their manipulation impacts cardiac disease progression. The obtained knowledge broadens our understanding of the mechanisms underlying the multifaceted pathogenesis of heart failure and the role of miRNAs therein.

While many different miRNAs are currently being studied to alter disease progression in rodent and primate studies, few promising miRNAs have already made the translation into clinical trials, indicating that we are on the edge of implementation of miRNA-based therapeutics. Development of improved therapeutic strategies to target miRNA activity will accelerate the utility of miRNA-based therapeutics in the clinics, holding great promise for the nearby future to treat cardiac disease and ensure healthy cardiac aging.

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Summary / Samenvatting

S

Summary

Heart failure (HF) currently forms a major global health problem and its prevalence is predicted to increase in the future, since effective treatment is still lacking. This highlights the urgent need to develop novel treatment strategies to save the lives of millions of people globally. Specific miRNAs are differentially expressed during cardiac pathophysiology and possess the ability to impact cardiac disease progression, highlighting their potential as therapeutic target. Therefore, the main **aims** of this thesis are: **1) to identify miRNAs that regulate cellular processes driving failure of the myocardium**, and **2) to explore if the manipulation of some promising miRNA candidates (identified under aim 1) are able to retard heart failure progression**.

To address the first aim, we review in **chapter 2** the fundamental role of miRNAs in regulating molecular and cellular processes driving detrimental changes in the myocardium associated with senescence and heart failure. **Chapter 3** describes an *in vitro* high-throughput screening approach to determine the regulatory function of miRNAs in cardiomyocyte hypertrophy, cardiac fibrosis, and inflammation in a large scale. As a result, we identified miRNAs as pleiotropic regulators, describing previously unknown roles of miRNAs in cardiomyocyte hypertrophy, fibrosis, and inflammation, and attribute new cellular functions to various well-known miRNAs.

Addressing the second aim, in chapter 4 through 6 we explored if the manipulation of selected promising miRNA candidates is able to retard heart failure progression. **Chapter 4** addresses the pivotal role of the miR-221/222 family in the process of myocardial fibrosis formation as an underlying factor of HF progression. Cardiac miR-221/222 levels negatively correlate with myocardial fibrosis in different patient populations with heart failure. Inhibition of this miRNA family in mice subjected to pressure-overload increased cardiac fibrosis and aggravated left ventricular dilation and dysfunction. In isolated rat cardiac fibroblasts, inhibition of miR-221/222 de-repressed TGF β -signaling, while overexpression of both miRNAs blunted TGF β -induced pro-fibrotic signaling. MiR-221/222 is thus a key regulator of fibrotic signalling in the heart.

Chapter 5 focuses on the negative-regulatory function of miR-151 in cardiomyocyte hypertrophy and its ability to modulate mitochondrial oxidative capacity of cardiomyocytes, probably via regulation of PGC-1 α . Although additional research needs to be conducted to validate and clarify the underlying mechanism, these findings mark miR-151 as an important regulator of the energetic profile of the heart.

Chapter 6 addresses the effect of miR-125a –identified as the strongest inducer of cell growth in neonatal cardiomyocytes (Chapter 3)– on cardiomyocyte cell cycle activity and the hypertrophic response. MiR-125a induces cardiomyocyte CDKi expression *in vivo* and *in vitro*, enhances cellular growth, and increases cardiac contractility and cardiomyocyte contraction frequency and amplitude. MiR-125a therefore is a modulator of cardiomyocyte differentiation and cell cycle activity. Whether miR-125a and its control of cell cycle re-entry have a role in the failing heart will be subject of further investigations.

Finally, in **chapter 7** we recapitulate the findings of our studies and discuss this in a broader perspective. Furthermore, we address possibilities and limitations of miRNA-based therapeutic strategies and its potential utility in the clinic.

Samenvatting

Hartfalen (HF) vormt momenteel een wereldwijd gezondheidsprobleem en de prevalentie hiervan is voorspeld in de toekomst toe te nemen aangezien effectieve behandeling nog steeds ontbreekt. Dit geeft de dringende behoefte aan om nieuwe behandelmethoden te ontwikkelen om zodoende de levens van miljoenen mensen wereldwijd te redden. Specifieke miRNAs komen differentieel tot expressie gedurende pathofysiologie van het hart en bezitten het vermogen om de progressie van hartziekten te beïnvloeden, wat hun potentie als therapeutisch doel duidt. Om die reden zijn de hoofddoelen van deze thesis: **1) het identificeren van miRNAs die in staat zijn om cellulaire processen die hartfalen bewerkstelligen, te reguleren, en 2) het bestuderen of het manipuleren van veelbelovende miRNA kandidaten (geïdentificeerd onder doel 1) in staat is om de progressie van hartfalen te remmen.**

Om het eerste doel te realiseren, bespreken we in **hoofdstuk 2** de fundamentele rol van miRNAs in het reguleren van moleculaire en cellulaire processen die nadelige veranderingen in het hart bewerkstelligen en geassocieerd worden met ouderdom en hartfalen. **Hoofdstuk 3** beschrijft een *in vitro* high-throughput screening methode voor het op grote schaal bepalen van de regulerende functie van miRNAs in hartspier hypertrofie, cardiale fibrose en inflammatie. Als resultaat hebben we miRNAs geïdentificeerd als pleiotrope regulatoren, beschreven we voorheen onbekende functies voor miRNAs in hypertrofie, fibrose en inflammatie, en schreven we nieuwe cellulaire effecten toe aan meerdere bekende miRNAs.

Bij het adresseren van de tweede doelstelling, hebben we in hoofdstuk 4 tot en met 6 onderzocht of de manipulatie van geselecteerde veelbelovende miRNA kandidaten in staat is om de progressie van hartfalen te remmen. **Hoofdstuk 4** beschrijft de centrale rol van de miR-221/222 familie in het proces van fibrose vorming in het hart als een onderliggende factor voor de progressie van hartfalen. Cardiale miR-221/222 levels correleren negatief met fibrose in het myocard bij verschillende patiënt populaties met hartfalen. Het remmen van deze miRNA familie in muizen blootgesteld aan druk-overbelasting, vermeerderd cardiale fibrose en verergerd linker ventrikel dilatatie en dysfunctie. In geïsoleerde rat cardiale fibroblasten, remming van miR-221/222 vermindert de onderdrukking van TGFβ-signalering. MiR-221/222 is dus een belangrijke regulator van fibrose signalering in het hart.

Hoofdstuk 5 spitst zich toe op de regulerende functie van miR-151 in hartspier hypertrofie en zijn vermogen om de mitochondriale oxidatieve capaciteit van hartspieren te moduleren, waarschijnlijk via regulatie van PGC-1α. Hoewel additioneel onderzoek uitgevoerd moet worden om dit te valideren en het onderliggende mechanisme te verhelderen, markeren deze bevindingen miR-151 als een belangrijke regulator van het energie profiel van het hart.

Hoofdstuk 6 richt zich op het effect van miR-125a –geïdentificeerd als sterkste veroorzaker van celgroei in neonatale hartspieren (hoofdstuk 3)- op hartspier celcyclus activiteit en de hypertrofe response. MiR-125a induceert hartspier CDKi expressie *in vivo* en *in vitro*,

versterkt celgroei, en vermeedert contractiliteit van het hart en de contractie frequentie en amplitude van hartspieren. MiR-125a is daarom een bemiddelaar van cardiomyocyte differentiatie en celcyclus activiteit. Of miR-125a en zijn controle over celcyclus-herintrede een rol spelen in falen van het hart is onderwerp van toekomstig onderzoek.

Tenslotte, in **hoofdstuk 7** vatten we de bevindingen van onze studies samen en stellen we dit ter discussie in een breder perspectief. Bovendien bespreken we de mogelijkheden en beperkingen van miRNA-gebaseerde therapeutische strategieën en de mogelijke toepasbaarheid hiervan in de kliniek.

Valorisation

Valorisation

Societal relevance

Cardiovascular disease (CVD) is the leading global cause of death, accounting for more than 17.3 million deaths per year, representing 1 of every 3 deaths [1]. This life-threatening disease is accompanied with enormous annual costs, reaching \$863 billion in 2010 globally [2]. Almost half of the patients with newly diagnosed CVD have heart failure (HF). Importantly, HF is the only cardiovascular disease that has increased in prevalence over the last 20 years [3]. According to the American Heart Failure Association, the prevalence of HF will continue to increase with 46% from 2012 to 2030, resulting in more than 8 million people with an age of 18 years or older with HF [4]. In the Netherlands, currently ~142.000 patients suffer from HF, resulting in 940 million euros of annual costs for HF in 2011 in the Netherlands only [5]. In addition to the rising prevalence, the high five-year mortality of HF patients (52.6%) did not decline since 2000, highlighting the ineffectiveness of current therapeutics and the urgent need to develop novel treatment strategies. Thus, improving HF therapy is necessary to prevent this disease to reach global epidemic proportions and to save the lives of millions of people around the world. However, to achieve this goal, we should expand the current knowledge concerning cellular processes driving HF development, and identify molecular key players to test as potential therapeutic targets.

In this thesis, we identified multiple specific microRNA (miRNA) candidates with a pivotal regulatory function in cellular processes driving HF development. Identification of these miRNAs and interrogation of the mechanism underlying their function increases the understanding of HF development. Increased understanding of the processes driving HF progression expands the body of knowledge, benefitting the scientific community. More importantly, identified miRNA candidates can be probed for their safety and efficacy to impact cellular processes underlying HF development and to retard disease progression. In this thesis we identified several promising miRNA candidates that in a next stage should be tested for their safety and ability to intervene with HF progression.

Target groups

The content of this thesis is relevant for the scientific community, for future drug development, and on the longer term for patients. Within the scientific community, increased understanding of the function of miRNAs during HF progression will benefit cardiac researchers. In addition to the cardiac research field, basic research studying other disease etiologies may be aided by the content of this thesis. The cellular processes studied within this thesis are not restricted to be involved in cardiac disease progression only. Therefore, miRNAs described in this thesis to regulate these processes are most likely also involved in other disease settings. For example, manipulation of the miR-221/222 family may also impact hepatic or pulmonary fibrosis formation. Furthermore, inducing cell cycle arrest through induction of miR-125a may potentially affect tumor growth.

Almost half of the HF patients will die within 5-years after diagnosis. This marks the ineffectiveness of current therapeutic strategies to treat HF patients. Therefore, new drugs should be developed to save the lives of millions of people globally. Although the end result by means of a successful clinically utilised therapy lies beyond the horizon, we as basic scientists should strive for expansion of the scientific body of knowledge to increase the chances of developing life-saving therapeutic approaches. Although this thesis highlights multiple miRNAs crucially involved in processes underlying failure of the heart, different challenges need to be overcome before miRNA-based strategies can undergo widespread implementation, as discussed in the discussion section.

Described novel knowledge on how miRNAs control the development of heart failure is only of indirect value to the current patient population. Further rigorous investigation of these miRNA candidates is necessary to determine safety and efficacy in patients. However, the findings in this thesis will advance our understanding of the involvement of miRNAs in cardiac disease. Ultimately, patients will benefit from developed miRNA-based therapies, alleviating the heavy burden of HF on society.

Innovation, products, and implementation

Current ineffective therapeutic strategies to treat HF patients are not aimed at targeting miRNAs. This thesis proves an altered expression profile and functional involvement of specific miRNAs in the failing heart. These findings mark an innovative treatment approach in which therapies are designed to modulate miRNA function to impact HF progression. MiRNA identified by us using in vitro high-throughput screening, or specific miRNAs tested in animal models may form the basis for drug development. However, the process of developing a clinically applicable therapeutic strategy based on basic research is a long and costly process, making it difficult to predict the long-term value of this thesis.

Independent of the future development of miRNA-based therapeutic approaches, communication of the content of the described research is crucial for future implementation. Findings of this thesis are communicated through multiple international scientific conferences in which we were invited to present our work. Moreover, we have submitted and published our scientific results to international peer-reviewed journals, allowing the scientific community and general public to get access to it.

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Dankwoord

D

Dankwoord

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Curriculum Vitae

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Robin Verjans was born on the 5th of March 1990 in Heerlen. In 2008, he graduated from his secondary education at the Graaf Huyn College in Geleen, the Netherlands. He started his B.Sc. study Health Sciences at Maastricht University with a major in Bioregulation and a minor in Movement Sciences. During his B.Sc. studies, Robin completed a research internship at Maastricht University, department of Human Biology. The topic of this internship was the influence of C/EBP β isoforms LIP and LAP on ApoA-1 production in HepG2 cells. Element of this internship was writing the literature review: HDL Functionality as Protective Factor for Future Cardiac Diseases.

In 2011, he started the M.Sc. study Biomedical Sciences. During his first year, he completed his junior internship at the department of Pathology at the Academic Hospital Maastricht, under the supervision of Dr. Judith Sluimer. During his junior internship, the effect of reoxygenating carbogen treatment on efferocytosis efficiency and hypoxia in murine atherosclerotic plaques was studied. During the final year, he completed his senior thesis entitled “A Crucial Role for microRNAs in Heart Failure: Identifying the molecular mechanism” at the department of Cardiology at Maastricht University. He received his master’s degree with a specialisation in Cardiovascular Biology and Medicine in 2013.

Subsequently, he commenced his PhD trajectory at the department of Cardiology at Maastricht University under the supervision of promotor Prof. Dr. Stephane Heymans and co-promoters Dr. Blanche Schroen and Dr. Marc van Bilsen. The main focus of this trajectory was to study the regulatory role of microRNAs in cellular processes that drive the development of heart failure. In the context of the European CardiomiR project, Robin was seconded from October 2013 to January 2015 to RNA-interference company: *Cenix BioScience GmbH*, Dresden Germany. During this secondment he designed and performed a high-throughput screening to determine the function of a large subset of heart failure-associated microRNAs.

In August 2017, Robin was granted the CARIM Postdoctoral Talent Fellowship for the project: “Prevention of Cardiomyocyte Cell Cycle Re-entry via miR-125a Protects the Heart from Detrimental Remodeling”. This fellowship enables him to perform his postdoctoral research at the International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste Italy, as well as at Cardiovascular Research Institute Maastricht (CARIM), Maastricht the Netherlands.