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The therapeutic relevance of microRNA-199b in preclinical models of heart failure

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Summary

Heart failure (HF) evolves as a result of various pathological conditions including myocardial infarction (MI), hypertension and atrial fibrillation.[1] Depending on the pathological cause, the myocardium undergoes different remodeling processes leading to deteriorated heart function.[2,3] Despite advances in treatment strategies, prevalence and incidence of HF remain to be substantial since more than 23 million people worldwide have been diagnosed with HF.[4-6] Therefore, a better understanding of the underlying mechanisms of development and progression to HF is required to generate novel and more efficient therapeutic strategies (Chapter 2). In this regard, microRNAs (miRNAs) have drawn great attention for their involvement in the regulation of key signaling pathways and for being beneficial therapeutic targets in preclinical models.[7,8] In this thesis, we aimed at demonstrating the involvement of microRNA-199b (miR-199b), a previously defined pro-hypertrophic miRNA,[9] in right ventricular remodeling after pulmonary artery banding in mice (Chapter 4) and also in left ventricular remodeling post-myocardial infarction (MI) (Chapter 5). We revealed that cardiac miR-199b expression is upregulated under stress conditions in both right and left ventricular (**Figure 7.1**). In the left ventricular, overexpression of miR-199b results in exaggerated cardiac function after volume overload and moreover, inhibition of miR-199b levels leads to partial improvement of cardiac dysfunction induced by MI, indicating that miR-199b has an important function in the left ventricular under stress conditions and thus, inhibition of miR-199b provides a promising treatment strategy for left sided heart failure.

In our study (Chapter 5), we inhibited miR-199b by treating animals with an antagomir, a widely used microRNA inhibitor in preclinical research, also previously used by us.[9] Since anti-sense oligonucleotide technology is rapidly advancing with several different chemistry-based antimiRs being developed with altered properties such as nuclease resistance, binding affinity and cellular uptake, we also evaluated different chemistry-based antimiR oligonucleotides regarding their efficiency to decrease miR-199b expression levels in the heart (Chapter 6). From all tested chemistries, antagomir and LNA were shown to be the most potent inhibitors of miR-199b in the heart. This potency was further improved by changing the cholesterol moiety from the 3' to the 5'end of the molecule leading to almost 100% inhibition of miR-199b cardiac expression levels. Although LNA-RNA molecules displayed an increased inhibitory capacity, in comparison to LNA-DNA, the efficiency of antagomir (harboring the cholesterol group at the 5'end) was still much higher. Moreover, both antagomir and LNA molecules were able to inhibit miR-199b expression levels in other organs such as lung, liver and kidney, indicating the need of developing organ-specific delivery methods for anti-microRNA therapeutics to avoid unwanted side effects.

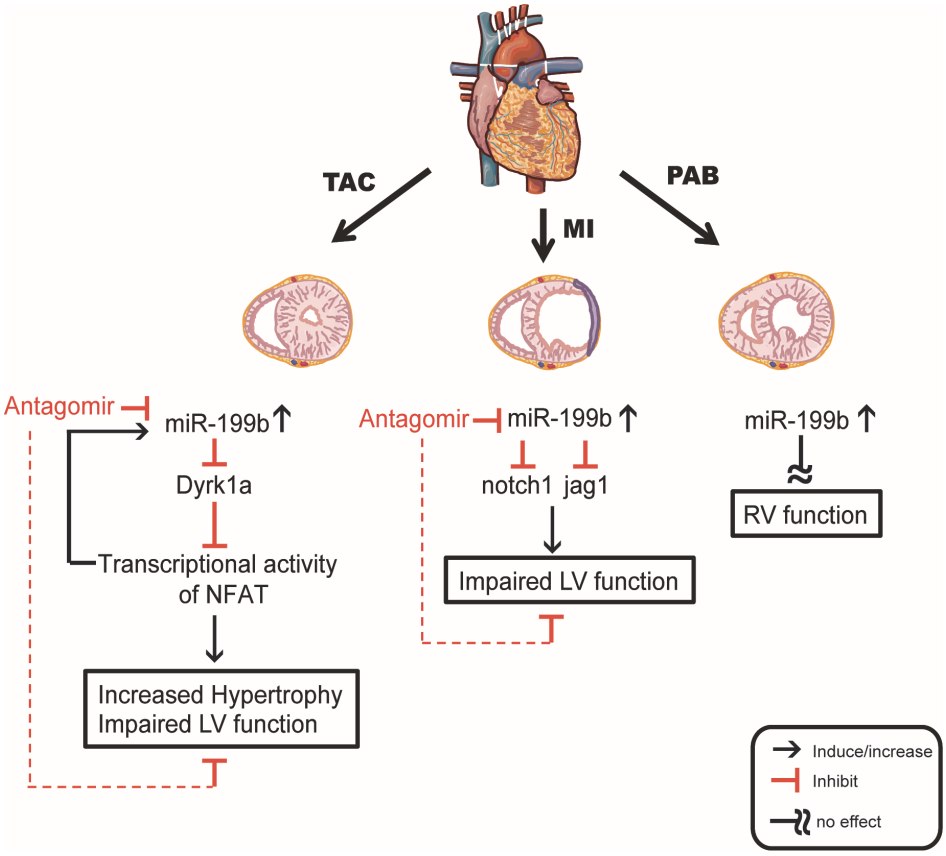

Figure 7.1 **miR-199b involvement in heart failure.** miR-199b expression is upregulated in three different preclinical models of heart failure. Upon pressure overload by trans aortic constriction (TAC), miR-199b regulates the activity of calcineurin/ nuclear factor of activated T-cell (NFAT) in an auto-amplification loop by targeting Dual specificity tyrosine-phosphorylation-regulated kinase 1A (Dyrk1a) resulting in increased hypertrophic growth and impaired LV function. Moreover, inhibition of miR-199b by an antagomir, after TAC, results in efficient recovery of pathological remodeling and cardiac dysfunction due to decreased activity of the calcineurin/NFAT pathway. Also, overexpression of miR-199b in a murine model of myocardial infarction (MI) is associated with decreased levels of notch and jagged1, two members of the Notch signaling pathway, giving rise to impaired LV function. Furthermore, antagomir treatment after MI partially rescues pathological phenotype. On contrary to the adverse effect of miR-199b overexpression in the LV, miR-199b expression failed to effect cardiac function in the RV after pulmonary artery banding (PAB). All these studies highlight the distinct involvement of miR-199b in different etiologies of heart failure.

Here, we demonstrated the therapeutic value of miR-199b in different preclinical models mimicking distinct etiologies of human heart failure. It holds great importance to comprehensively understand the underlying mechanisms of a complex disease such as heart failure in order to develop new and efficient treatment strategies. In addition, advancements in anti-sense technology enable efficient targeting of miRNAs *in vivo* and provide promising clinical applications for the treatment of heart failure in the near future.

Regulatory roles of microRNAs in heart failure

MiRNAs have been associated with a wide range of human pathologies by functioning as essential regulators of gene expression.[10] As summarized in Chapter 3, the role of specific miRNAs have been described in the different processes of pathological cardiac remodeling such as cardiac hypertrophy (miR-25, miR-378, miR499, miR-208, miR-133, miR-1 and miR-199b), fibrosis (miR-29, miR-21 and miR-101a), angiogenesis (miR-17~92, miR-24, miR-126, miR-26a and miR-146a) and inflammation (miR-155) in preclinical models of HF.[8] As HF is the end stage of various cardiac pathologies, the key to develop novel miRNA-based therapeutic strategies lies under the specification of miRNA activity for each of those pathologies.

Previously, miR-199b has been identified as an important inducer of pathological hypertrophy of the LV under chronic pressure overload conditions.[9] Considering the fact that substantial morphological, genetic and molecular differences exist between left and right ventricle,[11,12] differentially expressed miRNAs have been identified in murine models when comparing hypertrophic remodeling and/or failure between LV and RV. Four miRNAs (miR-34a, -28, -148a and -93) were found to be upregulated in the RV after pressure overload while they remained unchanged or downregulated in the LV under pressure.[13] In Chapter 4, we determined to investigate whether pro-hypertrophic function of miR-199b is also involved in the right ventricular remodeling after increased pulmonary artery pressure. Different animal models have been developed to study pulmonary artery hypertension[14] and, as expected, each model having specific limitations and advantages.[15] In our study, we employed a model of pulmonary artery banding (PAB), induced by a permanent banding around the pulmonary artery to establish increased RV afterload.[16] The main advantages of this model are the induction of pressure overload and, subsequently, RV hypertrophy without pulmonary vascular remodeling[17] as well as stable constriction of blood flow which enables evaluation of RV remodeling under pressure. In addition, the mechanistic similarities with the TAC model, where LV afterload is induced by transaortic banding,[16] enable to determine whether miR-199b executes comparable function in the LV and RV hypertrophy. In this regard, miR-199b is

regulated by calcineurin (CnA)/ nuclear factor of activated T-cell (NFAT) activity,[9] an established signaling cascade responsible for the hypertrophic response in the myocardium,[18,19] and transgenic mice overexpressing an active form of CnA develop massive biventricular hypertrophy precipitating in severe cardiac dysfunction and HF as early as 18 days after birth.[20,21] Interestingly, inhibition of CnA activity results in attenuation of hypertrophy and pathological remodeling in both animals models of left-side HF[20,22-25] and hypoxia-induced pulmonary hypertension and RV hypertrophy.[26] In agreement, we observed induced activity of CnA/NFAT in the RV of wildtype mice after PAB.[27]

In order to gain more insights on the function of miR-199b in different etiologies of HF, we investigated the effect of cardiac overexpression of miR-199b in response to volume overload induced by MI. LV remodeling after MI reveals a unique pattern involving the infarcted region (fibrotic scar) and leading to hypertrophic growth of the non-infarcted myocardium and eventually to cardiac dilatation.[28] Previous findings suggest the involvement of CnA/NFAT signaling during post-MI remodeling.[19,29] Since miR-199b was identified as a pro-hypertrophic miRNA regulated by the CnA/NFAT pathway, we hypothesized that miR-199b is also involved in LV remodeling after MI. Indeed, cardiac overexpression of miR-199b sensitized the heart to volume overload as demonstrated by exaggerated cardiac dysfunction and abrupt collagen deposition in the border zone of the infarct (Chapter 5). However, in contrast to previous reports,[9] miR-199b overexpression does not further activate the CnA/NFAT signaling pathway after MI. These somewhat opposite findings suggest that different cardiac stressors may alter specific molecular mechanisms in order to obtain distinct remodeling patterns. In agreement, a recent study showed that anti-miR-208a treatment resulted in activation of different genes in MI-operated rats compared to Dahl salt sensitive rats on a high salt diet as a model for hypertension induced heart failure indicating the divergent regulatory capacity of miR-208a depending on the cause of disease.[30] Moreover, comparison of differentially expressed genes between rat hearts subjected to either pressure or volume overload revealed that besides the commonly regulated genes, several other genes are stress-specific regulated.[31,32]

Next, we investigated whether other possible targets of miR-199b could be responsible for the observed MI-induced phenotypes. In cancer, the regulatory function of miR-199b has been firmly established through the Notch pathway,[33-36] with notch1 and jagged1 being predicted target genes. A few reports indicate upregulation of notch1 and its ligand jagged1 in the adult myocardium upon cardiac stress and a subsequent protective role following cardiac injury whereas their pharmaceutical or genetic ablation results in detrimental effects.[37-41] We also observed an increase in expression of notch1

and jagged1 in the adult hearts subjected to MI. Moreover, after MI, miRNA-199b transgenic hearts revealed reduced expression of notch1 and jagged1 providing a possible explanation for the exaggerated pathological phenotype seen in these mice compared to wildtype. Induction of Notch signaling takes place in the border zone and is associated with anti-hypertrophic, anti-fibrotic and pro-angiogenic responses in the adult myocardium.[38,40,42,43] Similarly, the elevated fibrosis observed in the border zone of MI-miRNA-199b transgenic hearts could be related to blunted Notch activity by inhibition of notch1 and jagged1 expression after cardiac restricted upregulation of miR-199b.

Therapeutic potential of miRNAs in heart failure

The capacity of miRNAs to regulate different molecular pathways involved in a specific disease process establishes them as suitable therapeutic targets in the treatment of various pathologies including HF.[7] As a consequence, advances in miRNA-based technologies enable us to modulate miRNA expression in an organism and currently, several preclinical studies using these technologies have generated promising outcomes for HF, as summarized in Chapter 3. Formerly, the therapeutic efficacy of targeting miR-199b in a pressure overload murine model of HF using an anti-microRNA (antimiR) conjugated to a cholesterol moiety (antagomir), has been established.[9,44] In this thesis, we provide evidence of a regulatory role of miR-199b during post-MI remodeling and of the therapeutic relevance of inhibiting miR-199b post MI as demonstrated by enhanced cardiac function after antagomir treatment.

Our main focus was to obtain efficient inhibition of miR-199b in the heart. However, further phenotyping in chapter 6 revealed that miR-199b is also expressed in other organs such as kidney, lung and liver. Furthermore, our most potent inhibitors antagomir and LNA reduced the expression of miR-199b in these organs. The physiological and functional significance of these findings is still unknown. It is actually conceivable that miR-199b may exert important reno-protective effects, especially in diabetic nephropathy, a condition in which miRNAs play a pivotal role[45] and inhibition of the NFAT/calcineurin pathway has been shown to be beneficial.[46]

Therefore, future studies should address whether antagomirs directed against miR-199b have pleiotropic beneficial effects or may cause unforeseen side effects in other organs. Reassuringly, we did not observe any mortality in our animal studies, and no apparent changes in gross anatomy of kidney, liver and lungs, albeit we cannot fully exclude that miR-199b may have some, yet unknown, protective effects. To circumvent such issues, Hinkel *et al*, showed that local delivery of LNA directed against miR-92a to the heart with a catheter

can greatly enhance the therapeutic value, while limiting side effects compared to systemic delivery.[47] A similar set-up may be devised to achieve strong and tissue specific knockdown of miR-199b in the heart. This is not an unrealistic approach, since many patients are already undergoing catheterization in both the work-up and in the acute treatment of myocardial infarction, making this procedure already suitable for local drug delivery.

In this thesis, we draw attention mainly to inhibitors of miRNAs as therapeutic tools since our miRNA of interest (miR-199b) is increased in response to cardiac stress and hence its inhibition provides therapeutic impact. However, it is also possible to restore the expression of miRNAs *in vivo* when the downregulation of a miRNA occurs in a disease state. One way is to apply synthetic RNA duplexes that are developed to mimic endogenous miRNA functions. These mimics can carry chemical modifications[48] or can be applied using cationic lipids to facilitate cellular uptake and stability.[49] Alternatively, a novel delivery system using nonviable minicells generated from bacterial cells after inactivating their cell division has been developed.[50] In addition, these carriers can be coated with anti- epithelial growth factor receptor (EGFR) antibody to achieve cancer cell specific targeted therapy.[50] Currently, in Phase I clinical trial MesomiR 1 (ClinicalTrials.gov:NCT02369198) is testing the effect of miR-15/16 mimics packed in such nanocells (EDV^{TM}) targeted with anti-EGFR antibody in malignant pleural mesothelioma (MPM) patients.[51] On the other hand, Phase I clinical studies of MRX34, miR-34 mimic, in multiple cancers was terminated due to multiple immune-related severe adverse events highlighting the significance of targeted delivery of miRNA-based therapeutics to avoid off-target affects (ClinicalTrials.gov:NCT01829971). Another way to retrieve miRNA expression is by the use of viral vectors such as adeno associated viruses (AVV).[52] The number of different serotype of AVV allow for tissue-specific targeting which can be further improved in combination with tissue specific promoters.[53]

MicroRNA expression levels have been shown to be differentially regulated in response to conventional heart failure treatments such as beta blockers in idiopathic dilated cardiomyopathy patients (iDCM).[54] Moreover, increased levels of miR-320 and reduced levels of miR-26b and miR-21 in Dahl sensitive hypertensive rat model were reversed to normal levels after treatment with a selective beta blocker, nebivolol but not treatment with atenolol.[55] Additional study revealed that both nebivolol and atenolol have an effect on miR-133 levels in high salt-treated rats.[56] These studies point out the importance of potential effects of standard heart failure treatments on miRNA biology and likelihood to intervene with novel therapeutic strategies. Further investigation of the effects combining current with new therapies is highly valuable since clinical trials can be performed in addition to standard treatments.

Future perspectives and concluding remarks

In this thesis, we emphasized the significance of better understanding miRNA activity in different pathologies of HF in order to develop reliable and more efficient therapeutic strategies than the current ones.

We identified new potential targets of miR-199b, jagged1 and notch1, players in the Notch pathway, supporting the possibility that miRNAs may involve in various molecular mechanisms depending on the induced cardiac stress. Upregulation of notch1 and its ligand jagged1 in the adult myocardium after stress was previously reported to provide protection from cardiac injury, whereas their pharmaceutical or genetic ablation causes exaggerated cardiac dysfunction. Consequently, a better understanding of the miRNA activity in underlying mechanisms of a disease is a prerequisite before establishing an individual miRNA as a therapeutic target in a certain disease. Hence, further identification of miR-199b targets is essential in order to profile the complex gene network wherein miR-199b is involved. Currently, various high throughput techniques enable the identification of miRNA targets in a large scale. In this regard, RNA sequencing can be applied to determine the change in transcript abundance after alterations in miRNA levels either by overexpression using mimics or downregulation by antimiRs.[57] Moreover, proteomics tools enable the detection of alterations in global protein levels after modification of miRNA expression.[58] The drawback of the mentioned approaches is the lack of information regarding indirect molecular interactions. However, strategies using co-immunoprecipitation of Argonaute -2 (AGO2), a component of RNA silencing complex (RISC), along with the mRNA:miRNA duplex and further identification by microarray or deep sequencing allow the determination of direct targets.[59] The application of these approaches in the future would greatly increase our understanding of the biological role of miR-199b.

As highlighted in Chapter 5, limitations of antimiR technology such as organ specific delivery, cellular uptake and optimal dosages to obtain efficient inhibition of target miRNA remain to be overcome prior to clinical application of antimiR drugs. One promising approach to optimize the bioavailability of antimiR oligonucleotides is to develop polymer-based nanoparticles as vehicles for *in vivo* applications. For instance, polyethylenimine (PEI), a broadly used polymer, due to its high cationic charge density potential, aids in the cellular uptake of the therapeutics.[60] Another option is to use poly(lactide-co-glycolide) (PLGA) particles with the advantage of high loading capacity and various surface modifications for beneficial pharmacodynamics.[61,62] Furthermore, studies from siRNA technology revealed the possibility of antibody conjugation in order to obtain tissue/cell type specific delivery of this anti-sense therapeutics.[63,64] In cancer biology, antibodies targeting highly expressed cancer cell surface

proteins can be utilized in order to achieve tumor-specific delivery. For instance, linking human epidermal growth factor receptor 2 (HER2) antibody to a nanocarrier of siRNA resulted in 80% inhibition of targeted mRNA and protein level in a xenograft model of ovarian cancer.[65] A relatively recent report has shown that conjugation of anti-CD71 (Tf receptor) Fab' fragment to a siRNA targeting hypoxanthine-guanine phosphoribosyltransferase (HPRT) efficiently and stably downregulated HPRT gene in calf and cardiac muscle but not in the liver or the spleen.[66] Moreover, when intramuscular injection of a conjugate generated by linking siRNA against myostatin gene and anti-CD71 Fab' fragment was applied to a mouse model of peripheral artery disease, calf muscles were hypertrophied leading to increased running performance.[66] Since miR-199b is also expressed in other tissues besides the heart, application of the above-mentioned delivery methods would corroborate the therapeutic potency of miR-199b in the future.

To generate comprehensive knowledge on the biological function of miR-199b is essential before designing and initiating any clinical studies. For this purpose, generation of a genetic cardiac-specific knockout miR-199b mouse line is a future crucial step in gaining mechanistic insight on the role of miR-199b in cardiac pathologies. This goal can be achieved by using conditional targeted gene knockout technologies[67] which enable elimination of single gene expression in a specific tissue or even in a cell type at a desired time. This approach, although not therapeutically feasible, could provide a better view over the specific effects of silencing one specific microRNA in one specific tissue since antimiRs, although therapeutically relevant, result in global inhibitory effects on different cell types and tissues which makes it difficult to determine the specific role of a miRNA in the desired cell type, tissue or organ. In addition, cardiac tissue from knockout animals can be used for RNA sequencing or proteomics to determine the changes in global gene expression after silencing miR-199b under physiological or pathological conditions.

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