MicroRNAs in cardiac diseases: The devil is in the details
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Since their discovery in 1993, it has become clear that microRNAs (miRNAs) constitute a completely new layer of gene regulation. miRNAs are ~22 nucleotide long, non-coding RNA sequences that regulate gene expression by binding to the 3'UTR of messenger RNAs (mRNAs), resulting in repression of translation into protein. Many miRNAs show specific expression patterns and their level of expression is influenced by physiological and pathological conditions. In this thesis we investigated miRNAs in three aspects of cardiac biology. In the first part we studied the biological role of three specific miRNAs (i.e. miR-30c, miR-133 and miR-15) in cardiac hypertrophy and heart failure. In the second part of this thesis, we reveal the contribution of miRNAs to disease severity in patients with long QT syndrome, and in the third part we investigated whether miRNAs in plasma can be used as diagnostic biomarkers for heart failure.

The first part focuses on miRNAs differentially expressed in the failing myocardium. In chapter 2 we provide an overview of the literature on the role of miRNAs in non-myocyte cardiac cells in heart failure.

In our lab we performed miRNA-arrays on RNA isolated from hypertrophic and failing rat myocardium. Among the many miRNAs that were differentially expressed between the hypertrophic, failing and healthy control rat hearts, three were studied in greater detail: miR-30c, miR-133b, and miR-15b. In chapter 3 of this thesis we show that miR-30c and miR-133 are two miRNAs highly expressed in the heart, which are downregulated in cardiac hypertrophy and heart failure. CTGF is an important signaling molecule in the development of fibrosis. We provide several lines of evidence that miR-133 and miR-30c regulate the levels of CTGF. We show that overexpression of miR-133 and miR-30c in cultured cardiac myocytes and fibroblasts results in downregulation of CTGF on the mRNA and protein levels. On the other hand we show that knockdown of these miRNAs in cultured cardiac myocytes and fibroblasts results in upregulation of CTGF on the mRNA and protein level, which was accompanied by increased production of collagens. In addition, we show that miR-133 and miR-30c are able to directly target the 3'UTR of CTGF. Together, these data indicate that the decrease of these two miRNAs in cardiac remodeling allows CTGF levels to increase, which contributes to fibrosis development.

The TGFβ-pathway is known to induce cardiomyocyte hypertrophy mainly via the non-canonical TGFβ-pathway (acting via TAK1 and p38) and fibrosis through the canonical pathway (via SMAD signaling). In chapter 4 of this thesis we identified the miR-15 family to be upregulated in rodent and human hypertrophy and heart failure. In this chapter we show that this miRNA-family repressed TGFβ signaling via direct targeting of several genes in the canonical and non-canonical TGFβ-pathway. Inhibition of miR-15b by subcutaneous LNA-based antimiR injections in transverse aorta constriction (TAC) in mice aggravated cardiomyocyte hypertrophy and fibrosis. Adult (8-12 weeks) and aged (8-12 months) cardiomyocyte-specific miR-15b transgenic mice had smaller hearts. However, this cardiomyocyte-specific overexpression of miR-15b did not influence cardiomyocyte
hypertrophy or fibrosis upon pressure overload induced by TAC. In conclusion, we show that miR-15 represses the TGFβ-pathway and that loss of this repression by miR-15 inhibition results in aggravated hypertrophy and fibrosis in response to TAC.

Chapter 5 is a technical chapter in which we describe the optimization of real-time PCR for the detection of miRNAs. Because of several characteristics of miRNAs, e.g. their small size and the absence of a poly-A tail, real-time PCR methods had to be adapted for miRNAs. Several methods to overcome these problems are commercially available, however it is unclear whether they are also reliable in experiments were miRNA levels were manipulated. We show the optimization of the miScript system and compared its performance to other (golden standard) systems in in vitro and in vivo experiments where we overexpressed or inhibited miRNA levels. We show that the miScript system can be as reliable as the golden standard system but that this depends on the type of miRNA experiments performed. For example in vitro inhibition experiments require different qPCR systems depending on the chemistry of the transfected inhibitor. Inhibition after transfection of antagoniRs can be reliably measured by the miScript system, whereas inhibition after transfection of tiny LNAs requires methods with a miRNA-specific RT reaction.

In the second part of this thesis we investigated the contribution of miRNAs to disease severity in patients with long QT syndrome type I (LQT1). LQT1 is the most common cardiac arrhythmia characterized by prolongation of the QT-interval on the surface ECG and is caused by loss-of-function mutations in KCNQ1. Since these mutations are usually dominant-negative, one would expect that each mutation carrier has a similar degree of LQT1. However, arrhythmias in family members with an identical mutation vary greatly in severity. We hypothesized that this variation in severity is caused by imbalance in expression between the healthy and mutated allele, which predicts that QT prolongation is less severe when the mutated allele is expressed less and vice versa. In chapter 6 we identified three single nucleotide polymorphisms (SNPs) in the 3'UTR of KCNQ1 (rs2519184, rs8234, rs10798) of which the derived variant represses translation of the allele it is residing on, inducing imbalance in expression between the healthy and mutant allele. This novel mechanism partially explains the high variability in LQT1 disease severity. We show that when these suppressive variants reside on the patient's healthy KCNQ1 allele, they suppress expression of this healthy allele, which results in a shift towards more mutated channels. This was seen as a significant increase in QT-interval and occurrence of symptoms compared to patients lacking these variants. The opposite was also true: when the suppressive variants resided on the mutant KCNQ1 allele, QT-interval was shorter with less symptoms. We confirmed the translational repression by the variants in in vitro luciferase assays, where the variants suppressed translation of the luciferase reporter in cultured cardiomyocytes.

Together, these results suggested that the suppressive variants created stronger miRNA binding sites. Indeed, as discussed in chapter 7, bioinformatic analysis revealed 28 miRNAs predicted to bind stronger to the 3'UTR containing the suppressive variants. We investigated
whether these miRNAs were able to repress expression of a reporter plasmid containing the suppressive variants in a luciferase screen. Among others, we identified miR-378 to be able to repress expression of the luciferase reporter containing the suppressive variants. Strikingly, two of the three variants (rs2519184 and rs8234) create stronger binding sites for miR-378. Furthermore, this miRNA is abundantly expressed in cardiomyocytes. Together this allows miR-378 to repress the expression of only the allele containing the suppressive variants. In addition, we show the crucial role of miR-378 in the suppressive effects of the variants by inhibition of this miRNA in cultured cardiomyocytes.

Together, the results of chapter 6 and 7 suggest that binding of miR-378 to the suppressive variants may largely explain the variety in disease severity in LQT1 patients. This further illuminates the therapeutic potential of miR-378 inhibition specifically in patients with the suppressive variants on their normal allele, which are the most severely affected patients, to restore the balance between their normal and mutant alleles.

Remarkably, miRNAs also appeared to be highly stable in plasma and specific profiles of circulating miRNAs have been identified for a number of diseases. This has raised the possibility that miRNAs may be measured in the circulation and can serve as novel diagnostic biomarkers. The third part of this thesis focuses on circulating miRNAs as putative biomarkers for cardiac disease. In chapter 8 we discuss the specific circulating miRNA profiles in coronary artery disease, myocardial infarction, hypertension, heart failure, viral myocarditis and type 2 diabetes mellitus described in literature.

In chapter 9 we describe the identification of miR-423-5p and 6 other miRNAs (miR-18b*, miR-129-5p, HS_202.1, miR-622, miR-654-3p, and miR-1254) increased in plasma of heart failure patients. We performed a miRNA array on RNA isolated from plasma of 12 heart failure patients and 12 healthy controls. We selected 16 miRNAs for further validation in a cohort of 39 healthy controls and 50 dyspneic patients, of whom 30 were diagnosed to have dyspnea due to heart failure and 20 due to other causes. We were able to validate the increase of the 7 miRNAs mentioned above, of which miR-423-5p was most strongly related to the diagnosis of heart failure. This miRNA distinguished heart failure patients from healthy controls with an area under the ROC curve (AUC) of 0.91. In addition, miR-423-5p was correlated to disease severity and the currently used biomarker N-terminal pro brain natriuretic peptide (NT-proBNP). We suggest these 7 miRNAs as attractive candidates as putative biomarkers for heart failure.

In chapter 10 we investigated whether the increase in these circulating miRNAs might be derived from release by the hypertrophic or failing myocardium. Therefore we made use of a porcine hypertrophy model subjected to 1, 3 or 8 weeks of ascending aortic banding (AoB). In these pigs we determined the expression of these miRNAs in myocardial samples from the epi- and endocardial site of the left ventricular free wall. In addition, we were able to determine circulating miRNA levels in plasma samples derived from either arterial or selective coronary venous sampling. We compared circulating miRNA levels in AoB pigs to miRNA levels in sham-
operated pigs. Moreover, we calculated transmyocardial concentration gradients by subtracting circulating miRNA levels in the arterial blood from the levels in coronary venous blood, where a positive gradient indicates release by the myocardium and a negative gradient indicates uptake. We show that these pigs develop left ventricular hypertrophy but do not develop heart failure and that none of the measured miRNAs is regulated in the hypertrophic porcine myocardium. Furthermore, we were not able to detect any significant release or uptake of these miRNAs by the myocardium.

Within the 20 years after their discovery in 1993, the therapeutic potential of miRNA inhibition is already investigated in phase 2a clinical trials. In chapter 11 we discuss the clinical potential of miRNAs as biomarkers and as therapeutic target and focus on the clinical potential of the miRNAs described in this thesis.