MicroRNAs in cardiac diseases: The devil is in the details
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CHAPTER 1
INTRODUCTION AND OUTLINE OF THIS THESIS
MICRORNAS

Since their discovery in 1993, it has become clear that microRNAs (miRNAs) constitute a completely new layer of gene regulation. They regulate gene expression by binding to the 3' untranslated region (3'UTR) of messenger RNAs (mRNAs), resulting in repression of translation into protein. Within the 20 years after their discovery the therapeutic potential of miRNA inhibition is already investigated in phase 2a clinical trials, where the effectiveness and safety of inhibition of miR-122 in hepatitis C patients was shown. In the heart, miRNAs are identified as important regulators of gene expression that are involved in many biological processes in cardiomyocytes and other cardiac cells. Also in the heart, the clinical potential of miRNAs is promising, mainly because of observations in small animal models of myocardial infarction and heart failure, where inhibition of specific miRNAs has proven to be beneficial.

Remarkably, miRNAs also appear to be highly stable in plasma and specific profiles of circulating miRNAs have been identified for a number of cardiac diseases. This has raised the possibility that miRNAs may be measured in the circulation and can serve as novel diagnostic biomarkers.

MiRNAs are ~22 nucleotide long, non-coding RNA sequences, which are highly conserved among species. Many miRNAs show specific expression patterns and their level of expression is influenced by physiological and pathological conditions. For instance, miR-208a is encoded by an intron of the αMHC gene and therefore this miRNA is specifically expressed in cardiomyocytes. Furthermore several miRNAs are found to be up- or downregulated in the human and rodent failing myocardium. In the human genome, the estimated number of miRNAs is as high as 1000. Together they are predicted to regulate as many as 30% of mRNA transcripts and thereby virtually every biological process. Many of these miRNAs are classified in miRNA-families based on a shared 'seed' sequence, which are nucleotide 2-7 at the 5'-end of the miRNA, and these family-members often only differ at a couple of nucleotides in the 3'-part of the miRNA.

MiRNAs are located in intronic and intergenic regions of the genome as individual miRNA genes or clusters of several miRNAs, which enter a biogenesis pathway after transcription by RNA polymerase II (Figure 1). The RNA transcripts of these miRNA genes can be several thousand nucleotides long and form the pri-miRNAs which contain hairpin-shaped structures. Next, these pri-miRNAs are cleaved by Drosha to the 70-100 nucleotide long hairpin-shaped pre-miRNAs. Exportin-5 transports these pre-miRNAs from the nucleus to the cytoplasm, where they are further processed to a miRNA-duplex by Dicer. The two strands of the miRNA-duplex are separated as the single-stranded mature miRNAs are incorporated into the RNA-induced silencing complex (RISC). Since both strands of the miRNA duplex might enter the RISC complex as mature miRNAs, they are named to the part of the hairpin they are derived from by suffixes, 5p and 3p for the mature miRNA derived from the 5'- and 3'-side of the pre-miRNA, respectively.
The mature miRNA within the RISC complex is able to bind to the 3'UTR of specific mRNA sequences and to inhibit translation or promote degradation of these transcripts. The primary determinant for binding of a miRNA to a 3'UTR is its ‘seed’ region, which requires Watson-Crick pairing between the miRNA and its mRNA target. This ‘seed’ binding can be supplemented by binding of the 3'-part of the miRNA and binding of this 3'-part can also compensate for nucleotide bulges or mismatches within the ‘seed’ binding. An important characteristic of miRNA function is that an individual miRNA is able to bind many mRNA targets and that one 3'UTR is targeted by multiple miRNAs, allowing for redundant and cooperative regulation of cellular processes.

The crucial role of miRNAs in cardiac biology was demonstrated by cardiomyocyte-specific deletion of dicer, an essential component of the maturation pathway of miRNAs. Cardiomyocyte-specific ablation of Dicer resulted in early postnatal death due to impaired cardiac function, as evidenced by dilated ventricles containing blood clots. Conditional cardiomyocyte-specific deletion of Dicer using a tamoxifen-inducible system in juvenile (3 weeks) mice resulted in increased cardiac size and sudden death within 2 weeks, probably due to fatal arrhythmias. In contrast, deletion of Dicer in these mice at an adult age (8 weeks) did not result in sudden death, but in development of severe heart failure as evidenced by a rapid dilation of the left ventricle and a strong decrease in cardiac function.

Figure 1. The miRNA biogenesis pathway. MiRNA genes are transcribed by RNA polymerase II and form pri-miRNAs which contain hairpin-shaped structures. These pri-miRNAs are cleaved by the Drosha-complex to hairpin-shaped pre-miRNAs of 70-100 nucleotides, which are subsequently transported to the cytoplasm by Exportin 5. In the cytoplasm pre-miRNAs are further processed by the Dicer-complex into miRNA-duplexes, of which the two strands are separated and the single-stranded mature miRNAs incorporated into the RNA-induced silencing complex (RISC).
CHAPTER 1

In this thesis we investigated three aspects of miRNAs in cardiac biology. The first part focuses on miRNAs differentially expressed in the failing myocardium and identifies the function of three miRNAs in particular (miR-15b, miR-30c and miR-133b) in cardiac hypertrophy and fibrosis. In the second part we describe a novel implication of miRNA biology on human cardiac disease by revealing that miRNAs contribute to disease severity in patients with a mutation that causes long QT syndrome. The third part of this thesis further translates miRNA biology towards the use of circulating miRNAs as biomarkers for heart failure.

PART 1: MINIRNAS IN THE PATHOGENESIS OF HEART FAILURE

Heart failure is defined as a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood.\textsuperscript{14} Heart failure is characterized by symptoms as dyspnea, fatigue and edema and commonly caused by cardiac remodeling due to coronary artery disease, hypertension, valvular heart disease, and gene mutations.\textsuperscript{14,15} Heart failure is a frequent and life-threatening disease, as evidenced by its incidence of 10 per 1000 in a population aged over 65\textsuperscript{14} and the death-rates of 30-40\% and 60-70\% of patients within respectively 1 and 5 years after diagnosis.\textsuperscript{15}

The heart responds to injury or stress, which for example results from coronary artery disease or hypertension, through a number of structural alterations commonly referred to as cardiac remodeling. One aspect of this remodeling process is cardiac hypertrophy, which is an increase in cardiomyocyte cell size that results in an increase in cardiac mass.\textsuperscript{16} Two forms of hypertrophy are distinguished, which are caused by different forms of cardiac stress. First, concentric hypertrophy is caused by pressure overload and results in an increase in wall thickness caused by an increase in cardiomyocyte width. On the other hand, eccentric hypertrophy, caused by volume overload, results from an increase in cardiomyocyte length and is characterized by dilation of the ventricle.\textsuperscript{16} Both forms of hypertrophy are accompanied by changes in expression of genes regulating energy metabolism, calcium handling and genes normally expressed in the embryonic heart.\textsuperscript{16} Another important aspect of cardiac remodeling is cardiac fibrosis, which is the excessive accumulation of extracellular matrix proteins in the interstitial and perivascular regions of the myocardium.\textsuperscript{17} Fibrosis increases mechanical stiffness of the heart, impairs myocyte contractility, disrupts electrical coupling and worsens tissue hypoxia.\textsuperscript{17} Together these structural and cellular changes result in a dysfunctional myocardium, which contributes to the development of heart failure.

The last decade it became increasingly clear that many miRNAs govern cellular processes underlying cardiac remodeling. In chapter 2 we provide an overview of the role of miRNAs in cardiac remodeling. We focus on the non-cardiomyocytes cell compartment of the heart and discuss the role of miRNAs in fibroblasts, endothelial cells and inflammatory cells.

In chapter 3 we show in an in vitro study that miR-133 and miR-30c directly target connective tissue growth factor (CTGF), which is an important signaling molecule in the development of fibrosis. Downregulation of miR-133 and miR-30c in cardiac hypertrophy and
heart failure results in relief of CTGF repression by these miRNAs, which allows for increased signaling to develop fibrosis. In chapter 4, we show that miR-15 inhibits the transforming growth factor β (TGFβ) pathway via targeting of several genes in this pathway. In vivo inhibition of miR-15 by LNA-based antimiR injections in mice resulted in an increased TGFβ-signaling leading to more severe cardiac remodeling.

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 compare its performance to other (golden standard) systems in in vitro and in vivo experiments where we overexpress or inhibit miRNA levels.

PART 2: MIRNAS IN DISEASE-SEVERITY OF LONG QT SYNDROME TYPE I

The rhythmic contractions of the heart rely on the sequential electronic activation of cardiomyocytes in specific regions of the heart. First the specialized pacemaker cells in the sinoatrial node are activated, which is followed by propagation of the electrical activity through the atria to the atrioventricular node. From here the activity is conducted through the Purkinje fibers to the apex to spread through the ventricular myocardium. This specific activity pattern can be detected on the body surface and measurement of this activity can be depicted in an electrocardiogram (ECG, Figure 2A).

The action potential in individual cardiomyocytes is the basis of the electrical activity in the heart. This action potential proceeds through 5 phases (Figure 2B), of which the first one (phase 0) is the initial upstroke that results from activation of voltage-gated Na+ channels. This is followed by a transient repolarization (phase 1), which reflects the inactivation of the Na+ channels and the activation of rapid transient outward K+ currents. Phase 2 is the plateau phase of the action potential, which is constituted of the outward K+ current through two delayed rectifier K+ channels (I_{Ks} and I_{Kr}) and the influx of Ca2+ through the L-type Ca2+ channels. This inward Ca2+ is the basis for excitation-contraction coupling in the myocardium. As these Ca2+ channels are inactivated, the outward K+ currents stay activated, leading to repolarization of cardiomyocytes (phase 3) until the resting membrane potential (phase 4) is reached.

Long QT syndrome type I (LQT1) is a common hereditary cardiac arrhythmia with a prevalence of 1 in 3000 individuals, caused by heterozygous mutations in KCNQ1. These mutations are loss-of-function mutations that reduce the I_{Ks} current and thereby delay repolarization of cardiomyocytes, which results in prolongation of the ventricular action potential (Figure 2D). This prolongation of the action potential is visible as QT prolongation on the ECG (Figure 2C) and may result in life-threatening arrhythmias that lead to syncope and even sudden death.
Since KCNQ1 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 is caused by imbalance in expression between the healthy and mutated allele, which predicts that QT prolongation is more severe when the mutated allele is expressed more, and vice versa. In chapter 6 we identified three SNPs in the 3’UTR of KCNQ1 of which the derived variant represses translation of the allele it is residing on, causing the hypothesized imbalance in expression between the healthy and mutated allele and partially explaining the high variability in LQT1 disease severity. In chapter 7 we show that these variants induce repression of the allele they are residing on by creating stronger binding sites for miR-378.

Figure 2. Prolongation of QT interval and action potential in Long QT syndrome type 1 (LQT1) patients. A) Measurement of electrical activity of the healthy heart depicted in an ECG. B) The action potential in individual cardiomyocytes underlies the electrical activity of the heart and consists of 5 phases, which are characterized by opening and closing of specific ion channels. In LQT1 patients repolarization of cardiomyocytes is impaired due to mutations in the KCNQ1-encoded K^+ channel responsible for the I\_Ks current. This results in prolongation of the QT interval on the ECG (C) due to a prolonged action potential (AP) duration (D).
PART 3: CIRCULATING MiRNAs

In 2008, it was discovered that miRNAs are also present in the circulation. In plasma they are remarkably stable even under harsh conditions as boiling, low or high pH, long-term storage at room temperature and in multiple freeze-thaw cycles. Due to this stability and easy accessibility in the circulation, their tissue- and pathology-specific regulation, and the possibility to detect them in a sensitive and specific manner by sequence-specific amplification, miRNAs are currently explored for their potential as biomarkers in a wide range of diseases. In chapter 8 we provide an overview of the studies exploring miRNAs as biomarker for cardiovascular diseases and discuss the miRNAs proposed as potential biomarkers. In chapter 9 we identified miR-423-5p and 5 other miRNAs that are elevated in patients with heart failure and provide attractive candidates as putative biomarkers for HF. In chapter 10 we investigated whether the detected elevation of these miRNAs in plasma might result from release by the myocardium. Therefore we used a porcine hypertrophy model, where we determined levels of these miRNAs in the myocardium. We also determined circulating miRNA levels in plasma samples derived from either mixed arterial or selective great cardiac vein sampling.

Finally, in chapter 11 we discuss the major findings of the experimental chapters and outline future perspectives.
REFERENCES

