Non-coding RNA species in heart failure

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Non-coding RNA species in heart failure

Regulators of cardiac hypertrophy, fibrosis and inflammation

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Non-coding RNA species in heart failure

Regulators of cardiac hypertrophy, fibrosis and inflammation

DISSERTATION

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in accordance with the decision of the Board of Deans,
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by

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Chapter 1

General introduction and outline of this thesis

The aim of this thesis is to extend our knowledge of the influence of non-coding RNAs, such as microRNAs and lncRNAs, on the development of heart failure, with a focus on cardiac pressure overload and viral myocarditis. Heart failure (HF) is a severe disabling condition that results from the inability of the heart to sustain sufficient blood flow through the body. The prevalence of heart failure is approximately 1-2% in the western world², with 5.1 million and 15 million people affected in the US and Europe respectively^{3,4}. Importantly, the prevalence rises to 7-10% in people aged 75 years or older², making it a common disease of the elderly and thus an increasing problem in aging societies. Despite advances in medical care, the prognosis of HF is still poor, with reported 5-year mortality ranging from 40% to 65%^{2,3}. Heart failure is the common consequence of several underlying cardiovascular diseases, most importantly ischemic heart disease, hypertension, and inherited or acquired cardiomyopathies². In 75% of the HF cases in the US, patients were found to have preceding hypertension, which leads to pressure overload of the left ventricle (LV), resulting in myocardial hypertrophy and subsequent HF³. Dilated cardiomyopathy on the other hand, is very often a consequence of myocarditis, which can for example be triggered by toxins (including chemotherapeutics) or by infections with parasites, bacteria or viruses. The incidence of myocarditis is estimated to be up to 12%, and it was found in 8.6% of cases of sudden cardiac death in young adults and in 9.6% of patients with unexplained HF⁵⁻⁷. Therefore, this thesis focusses on HF due to LV pressure overload (chapters 2, 3 and 5) and viral myocarditis (chapter 6).

PRESSURE OVERLOAD-INDUCED HF

Elevated blood pressure is a highly prevalent disease not only in western societies but also in many low- and middle income countries. In fact, the global prevalence of hypertension was 20-25% in 2014⁸, thus predisposing up to ¼ of the global population to HF. Pressure overload-induced HF goes along with changes in morphology and histology of the heart, as well as cardiomyocyte metabolism, function, and gene expression: The hypertrophic LV adopts a concentric or eccentric form, apparent either as a thickened wall with unchanged or reduced LV lumen, or as dilated ventricle with thin walls in relation to the lumen, respectively. Concentric hypertrophy can restrict the filling of the LV, thus causing symptoms of heart failure despite preserved ejection fraction (HFpEF), or develop into dilated hypertrophy and heart failure with reduced ejection fraction (HFrEF). However, the factors that influence the progression of hypertensive heart disease into HFpEF or HFrEF are only incompletely understood⁹. On the histological level, HF goes along with cardiomyocyte hypertrophy, fibrosis, and immune cell infiltration. Cardiomyocyte hypertrophy presents as thickening or lengthening of individual cells and thus forms the basis for overall concentric or eccentric LV hypertrophy¹⁰. Fibrosis of the LV myocardium may be observed as diffuse interstitial deposition of collagen, “patchy” replacement fibrosis (microscopic scarring) or perivascular fibrosis. Myocardial fibrosis leads to increased LV stiffness, impaired LV contraction, arrhythmias, and reduced oxygen supply and thereby contributes to the progression of cardiac dysfunction¹¹. The importance of cardiac inflammation in LV

hypertrophy and heart failure is less well understood, although a contribution of cardiac inflammation to hypertrophy and fibrosis is acknowledged¹². For example, we have recently reported that infiltrating macrophages drive pressure overload-induced LV hypertrophy by releasing pro-hypertrophic factors¹³. On the cellular level, cardiomyocytes in a failing heart present with an altered metabolism. This goes along with reduced oxidation of fatty acids, which are the predominant energy source of the healthy heart, and (at least transiently) increased utilization of glucose as fuel¹⁴. Simultaneously, contractile performance of cardiomyocytes is impaired due to de-regulation of proteins involved in signaling pathways (e.g. β -adrenergic receptor desensitization) and calcium handling (e.g. reduced expression of SERCA)¹⁵⁻¹⁷. Simultaneously, changes in the gene expression pattern of affected cardiomyocytes occur, including re-expression of fetal genes (so called “fetal gene program”)¹⁸ and alternative mRNA splicing of several genes, such as the sarcomere protein titin^{19,20}.

In patients, LV pressure overload often results from aortic valve stenosis or from hypertension. For that reason, we made use of two mouse models in which pressure overload is inflicted on the LV (a) by surgically narrowing the diameter of the thoracic aorta (thoracic aortic constriction, TAC), thus mimicking aortic valve stenosis, or (b) by chronic subcutaneous infusion of the hormone angiotensin II (AngII), which increases the arterial blood pressure and above that has direct hormonal effects on the myocardium²¹.

VIRAL MYOCARDITIS

In addition to pressure overload, HF can be preceded by myocarditis, an inflammatory state of the myocardium and an important cause of dilated cardiomyopathy (DCM). Myocarditis can be a consequence of viral infections, as highlighted in this thesis, or be triggered by bacteria, parasites or toxins, including chemotherapeutic drugs^{22,23}. Despite improvements in cardiac imaging techniques, to date a definite diagnosis of viral myocarditis (VM) can only be based on histological analysis of endomyocardial biopsies (EMB) and PCR-based detection of viral genomes. Treatment of VM is largely limited to standard heart failure therapy, although immunosuppression, immunomodulation, or antiviral therapies are currently being developed and have shown promise²³. In about $\frac{2}{3}$ of idiopathic DCM cases, viral genomes are detectable in EMBs, with enteroviruses accounting for 10-30% of the VM cases²⁴⁻²⁶. Human coxsackievirus group B serotype 3 (CVB3) is an enterovirus frequently found in viral myocarditis, and above that CVB3 is used for modeling VM in myocarditis-prone mouse strains, such as C3H mice. In these mice CVB3 infects the heart and triggers an inflammatory response that leads to cardiac damage and eventually heart failure. Research on the course of VM in animal models has shown that destruction of cardiac tissue is not only a consequence of viral replication and subsequent cell death but to a large part results from collateral damage caused by infiltrating immune cells²⁷. In general, the course of VM can be divided into an acute, subacute, and chronic phase: Initially, viral entry and replication in cardiac cells leads to myocardial damage and initiation of the immune response. In the

subacute phase, infiltrating cells of the innate and adaptive immune system kill infected cells and eliminate the virus. Finally, inflammation is resolved or progresses into a chronic state²⁷. Progression of myocarditis, eventually leading to DCM, can either be a consequence of ineffective clearance of the virus or of an autoimmune reaction that is probably initiated by molecular mimicry in the subacute phase²³. The genetic or environmental factors that determine if VM is resolved or progresses into a chronic phase remain enigmatic.

NON-CODING RNA

Given the various pathomechanisms involved in HF, there is a clear lack of tailored treatment options. To date, HF therapy is mostly limited to reducing the strain on the heart and improving contractility, without specifically aiming to restore normal cellular function. Only over the last years, another layer for therapeutic intervention has been introduced by our expanding knowledge about regulatory RNA species. RNA molecules have long been believed to be relevant only for the production of proteins, either by encoding the amino acid sequence (messenger RNA, mRNA) or by assisting the ribosomal machinery to translate this code (ribosomal RNA, rRNA; transfer RNA, tRNA). The existence of regulatory RNA species that influence gene transcription and translation has only been acknowledged in the last 20-30 years. This thesis focusses on two major classes of regulatory RNA – microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) – and consequently consists of two parts: In chapter 2 and 3 we explore the role of different miRNAs in HF, while the chapters 4 to 6 deal with the role of lncRNAs. In the following, I will introduce the biogenesis and function of miRNAs, whereas background information about lncRNAs and their functions in the heart is given in chapter 4 of this thesis.

In 1993, the first evidence for repression of an mRNA by the small non-coding RNA lin-4 (nowadays recognized as a miRNA) was found in nematodes²⁸. However, it took 5 more years until the general concept was conceived that double-stranded RNAs may inhibit translation of mRNA into protein in a process termed RNA interference (RNAi)^{29,30}. Since then, this novel concept has had a major impact on our understanding of gene regulation and was honored with the Nobel Prize in Physiology or Medicine 2006. MicroRNAs are endogenous effectors of RNAi and have attracted much attention in fundamental research but very soon after also in translational research. MiRNAs are usually around 22-nucleotide-long RNA molecules derived from independent genes or processed from introns of mRNA or lncRNA transcripts. The canonical miRNA biogenesis pathway is outlined in Figure 1. The first processing step from the primary transcript (pri-miRNA) is the formation of a shorter hair-pin RNA of about 70 nucleotides, called pre-miRNA, by the endonuclease complex Drosha/DGCR8 or, in cases of intronic miRNAs (called mirtrons), by the splicing machinery³¹⁻³³. The pre-miRNA is exported to the cytoplasm by Exportin-5 and further processed by the nuclease Dicer, which removes the loop of the hair-pin. This liberates a duplex of two RNA strands from the 5' (5p) and the 3' (3p) arm of the pre-miRNA but in most cases a function could only be assigned to one of the two emerging strands. This

phenomenon, the so-called asymmetry rule, results from the selection of one of the two strands for further processing, based on stability parameters of the miRNA duplex^{34,35}. Usually, the binding of the two strands is less strong at one side of the duplex and the strand having its 5' end at this side is retained ("guide strand"), whereas the other strand is usually discarded ("passenger strand"). However, it should be noted that by now some instances are known in which both strands are functional³⁶⁻⁴⁰. After being produced by Dicer, the mature miRNA (miR) is incorporated in a so-called "RNA-induced silencing complex" (RISC) consisting of the miR as bait and a protein complex that can block target mRNA translation by different means⁴¹. Argonaute (AGO) proteins are at the core of RISC assembly. The AGO family members 1-4 are all able to load miRs and appear to have largely overlapping functions, but only AGO2 has an endonuclease activity and is therefore able to cleave a target mRNAs upon RISC-binding⁴². However, direct AGO-mediated mRNA degradation is only one possible way of translation repression, while in the majority of cases RISC function appears to be carried out by accessory proteins that (a) induce de-adenylation of the mRNA, leading to subsequent mRNA degradation, (b) block initiation of translation by inhibiting assembly of the ribosomal subunits at the mRNA, (c) slow down or prevent translocation of the ribosome along the mRNA, or (d) degrade the nascent protein⁴³.

Figure 1: MicroRNA biogenesis. Reproduced with permission from Winter et al.¹

Several excellent review articles have to date summarized the importance of different miRNAs in the development and progression of heart failure in general⁴⁴, as well as in different aspects of cardiac disease such as hypertension⁴⁵, myocardial hypertrophy⁴⁶⁻⁴⁸, fibrosis⁴⁹, and hypoxia/ischemia^{47,50,51}. Importantly, most investigations are to date triggered by the observation that a miRNA is differentially expressed (i.e. up- or downregulated) in a certain human disease or animal disease model. If forced overexpression or inhibition of such a candidate miRNA in animal models indeed confirms a causal relationship with the disease, the target mRNA is searched to unravel the mechanism of action. For this purpose, several algorithms have been developed to predict miRNA target genes *in silico*, based on miRNA:mRNA sequence complementarity and thermodynamic stability, and on target site conservation and accessibility. Such tools to date reach precision levels of 50-60% (predicted genes that can be experimentally validated) and thus are of great help for miRNA research⁵².

OUTLINE OF THIS THESIS

Several cell types are critically involved in HF including cardiomyocytes, fibroblasts, and immune cells. In chapter 2 we analyze the functional importance of miRNA-139 in cardiomyocytes *in vitro* and its effects on pressure overload-induced cardiac hypertrophy and failure *in vivo*. Myocardial fibrosis is a common feature of HF and in chapter 3 we report an important role for the miRNA-221/222 family in this process by affecting fibroblast function. Chapter 4 gives a general introduction to cardiac lncRNAs, while chapter 5 specifically deals with the lncRNA Malat-1 in cardiac pressure overload. A putative role for mascRNA, a processed product of Malat-1, in immune cells and consequently VM is described in chapter 6. Finally, chapter 7 provides a summary of the experimental work and discusses limitations and opportunities of non-coding RNAs as future therapeutic targets in HF.

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Chapter 2

MicroRNA-139 targets phosphodiesterases 3 and 4, and modulates calcium cycling in cardiomyocytes

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ABSTRACT

Background

Adrenergic signaling in the heart is mediated by the second messenger cyclic AMP (cAMP) and limited by the cAMP-degrading phosphodiesterases (PDEs). cAMP-dependent protein kinase (PKA) is an important effector of adrenergic signaling by modulating the function of several calcium channels and pumps involved in calcium cycling during contraction. Heart failure, a disabling disease with high morbidity and mortality worldwide, is hallmarked by abnormal cAMP/PDE signaling. However, the regulation of PDE function is only incompletely understood. While microRNAs have emerged as powerful regulators of cardiac (patho-)physiology, their potential role in regulating PDEs in heart failure has not been investigated so far.

Results

We found cardiac microRNA-139-5p (miR-139) to be down regulated in patients with aortic valve stenosis and a reporter gene assay confirmed the mRNAs of *Pde3a*, *Pde4a* and *Pde4d* as novel targets of this microRNA. Overexpression of miR-139 in rat cardiomyocytes reduced spontaneous calcium release from the sarcoplasmic reticulum under adrenergic stimulation, possibly by altering the phosphorylation patterns of proteins downstream of cAMP/PDE signaling. In a mouse model of angiotensin II-induced cardiac pressure overload we found overexpression of miR-139 to reduce adaptive concentric hypertrophy. Instead, miR-139 promoted left ventricular dilation and dysfunction, whereas inhibition of miR-139 mildly reduced AngII-induced LV dilation.

Conclusions

Taken together, miR-139 appears to regulate PDE function in cardiomyocytes and contributes to the development of pressure overload-induced cardiac hypertrophy and failure. Further studies are warranted to understand the precise link between downregulation of miR-139 in aortic valve stenosis, deregulation of PDEs, and the consequences for progression of HF.

INTRODUCTION

Cardiac hypertrophy and consequent contractile dysfunction that ultimately leads to chronic heart failure (HF) and death are a major global health burden. Pressure overload of the left ventricle (LV) by aortic valve stenosis or arterial hypertension is an important cause of cardiac hypertrophy and failure, but to date the molecular mechanisms that eventually lead to progression of cardiac hypertrophy into HF are poorly understood.

Cyclic nucleotides are fundamental second messengers of signal transduction in all three domains of terrestrial life¹. In the mammalian heart, cyclic adenosine monophosphate (cAMP) is a second messenger of the sympathetic nervous system and a key regulator of cardiac contraction and rhythm. Ligand binding to β_1 and β_2 adrenoreceptors (β -AR) leads to dissociation of the $G\alpha_s$ subunit from the receptor and subsequent activation of adenylate cyclase (AD) which converts ATP into cAMP. Consequently, cAMP can bind to effector proteins such as exchange factor directly activated by cAMP 1 (EPAC1) and cAMP-dependent proteinase kinase (PKA). EPAC1 and PKA both have important effects on hypertrophic gene regulation in cardiomyocytes²⁻⁴. Above that, activated PKA is the main mediator of adrenergic signaling on cardiomyocyte contraction. PKA-dependent phosphorylation of L-type calcium channels (LTCC), cardiac ryanodine receptors (RyR2), phospholamban (PLB, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} -ATPase, SERCA) and the sarcolemmal Na^+/Ca^{2+} exchanger (NCX) directly link cAMP to calcium entry, intracellular release, and extrusion during the cardiomyocyte excitation cycle. At the sarcomeres, PKA mediates lusitropic and inotropic effects by phosphorylation of troponin I (TnI) and myosin-binding protein c (MyBPC)⁵. Given the broad cellular effects that are mediated by cAMP, a tight spatial and temporal regulation is needed to determine the precise outcome of cAMP production. Research over the last years has highlighted the importance of phosphodiesterases (PDEs) to limit cAMP signaling to dynamic subcellular domains by degrading cAMP to adenosine monophosphate (AMP)^{6,7}. The PDE superfamily comprises eleven PDE gene families (*PDE1-PDE11*), each consisting of 1-4 family members (e.g. *PDE4A*, *B*, *C* and *D*), which can again generate different splicing isoforms (e.g. *PDE4D5*, *PDE4D8*). In total there are probably around 100 PDE isozymes that vary in substrate specificity (cAMP, cGMP or both), allosteric regulation, and sub-cellular localization⁸. In the mammalian heart, *PDE1-5* are expressed, with PDE3 and PDE4 being central in cardiomyocytes⁹. Interestingly, PDE activity appears to be unchanged or increased in hypertrophied hearts, whereas heart failure is accompanied by reduced PDE activity⁹⁻¹¹. However, to date specific therapies aiming at restoring physiological cAMP/PDE signaling during HF are lacking.

MicroRNAs (miRNAs) are small (~22 nucleotides) RNA molecules that can bind to partially complementary sequences in the 3' untranslated region (3' UTR) of target messenger RNAs and suppress their translation. They are therefore powerful modulators of protein expression and by now established regulators of cardiac hypertrophy and failure¹²⁻¹⁴. The miRNA-139 is encoded in an intron of the *PDE2A* gene and the mature miR-139-5p (miR-139) is predicted to suppress translation of several PDEs. The purpose of this study was therefore

to understand the influence of miR-139 on cAMP signaling in cardiomyocytes and its relevance in the diseased heart.

MATERIAL AND METHODS

Microarray analysis of human biopsy samples

Endomyocardial biopsies were obtained from 11 patients with cardiac hypertrophy due to aortic valve stenosis (AoS) and 6 patients undergoing coronary artery bypass grafting (CABG) for coronary artery disease but without cardiac hypertrophy. Total RNA was isolated using the mirVana miRNA isolation kit (Ambion) and hybridized to Illumina Human miRNAv2 Expression Panel arrays, containing 858 human miRNA sequences described in Sanger Institute miRBase release 12.05, plus additional novel content derived using Illumina sequencing technologies, making up a total of 1146 assays per chip. The lumi R package was used for annotation, quality control and normalization¹⁵, and a quantile normalization was applied¹⁶. Differential expression was analyzed using limma R package¹⁷. MiRNAs were considered differentially expressed with a corrected p-value <0.05. Differential expression of miR-139-5p was validated by RT-PCR in an expanded set of endomyocardial biopsy samples using the miScript RT kit (Qiagen) and a specific miR-139-5p LNA primer set (Exiqon). U6 snRNA served as internal control.

In-situ hybridization

Formalin fixed murine heart tissue was probed with digoxigenin labeled miR-139-5p probe (Exiqon) and developed using an alkaline phosphatase-conjugated antibody and NBT/BCIP as substrate (Roche). No counterstain was performed.

Validation of miR-139 targets

The genomic region surrounding the predicted target sites for miR-139 in the 3' UTR of *Pde3a*, *Pde4a* and *Pde4d* was cloned from genomic mouse DNA into the pmirGLO reporter plasmid (Promega). HEK293 cells were grown in DMEM 31966 with 10% FBS until sub-confluent. Then the medium was changed to low serum (0.1% FBS) and cells were transfected with 100 ng pmirGLO plasmid and 10 nM miR-139 mimics or control oligonucleotides (Dharmacon) using Lipofectamine 2000. After 24 hours, firefly luciferase and *Renilla* luciferase activity were measured according to the manufacturer's instruction in a Victor3 Multilabel Plate Counter (Perkin Elmer). Sequences of cloning primers are listed in Table 1.

Table 1: Primers for cloning 3' UTRs into pmirGLO. Restriction sites underlined.

Target	Forward primer (5'→3')	Reverse primer (5'→3')	Fragment size
Mouse <i>Pde3a</i>	<u>TGTTTAAAC</u>	<u>CGCTAGC</u>	~500 bp
	AGAGAAGGGGAAGCCAAGAG	GCTCAGTCTGGAGCCTGTTT	
Mouse <i>Pde4a</i>	<u>AATGTTTAAAC</u>	<u>GTTGCTAGC</u>	~1700 bp
	ATTCTGCCGTCTCTCTGCT	AACTTCCCACAAACCGACAG	
Mouse <i>Pde4d</i>	<u>ACGAGAGCTC</u>	<u>CCTATCTAGA</u>	~700 bp
	TCGTCACTCCTGTGTTCAAC	TAGGCAGAATCAACCCATGC	

Culture and stimulation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes (nRCMs) were isolated from 1-3 day old Wistar rat pups by enzymatic dissociation. After removing the atria, the ventricles were cut in 8-12 equally sized parts and digested with a mixture of 0.3 mg/ml collagenase (Sigma #C2674) and 0.3 mg/ml pancreatin (Sigma #P3292) in ADS buffer (in mM: 120 NaCl, 5 KCl, 0.8 MgSO₄, 0.5 KH₂PO₄, 0.3 Na₂HPO₄, 20 HEPES, 5.6 Glucose, pH 7.35) at 37°C. Every 20 minutes the supernatant was collected, suspended in 10% newborn calf serum (NBCS), and fresh enzyme solution was added to the residual tissue. After 5 rounds of incubation, cell solutions were pooled, centrifuged, re-suspended in plating medium (DMEM 11966, 17% M199 medium, 10% horse serum, 5% NBCS) and pre-plated onto 162cm² Corning Costar cell culture flasks (Sigma #CLS3151). After incubation in a cell culture incubator for one hour, the supernatant, containing mainly nRCMs, was collected and 10⁶ cells per well were plated on gelatin coated 6-well plates. The next day, medium was changed to experimental medium supplemented with antibiotics (DMEM 11966, 20% M199 medium, 1.6 g/L glucose, 10 mg/L bovine serum albumin, 0.25 µU insulin, 250 µM carnitine, 10 µM cytarabine) and the cells were transfected with 20 pmol miR-139 mimics or unrelated control (Dharmacon) using Lipofectamine 2000.

For expression analysis, medium was refreshed 24 hours after transfection and cells were stimulated with isoprenaline (Iso, 10 µM), phenylephrine (PE, 10 µM) or endothelin 1 (ET1, 100 nM) for 48 hours. RNA isolation and analysis are described below. To assess phosphorylation of PKA target proteins, nRCMs were transfected and after 48 hours acutely stimulated with Iso (10 nM, 5 min). Protein extraction and Western Blot assay were performed as described previously¹⁸. Briefly, cardiomyocytes were scraped in ice-cold buffer containing 120 mM NaCl, 50 mM Tris-HCl (pH 8.0) and 1% Triton X-100, supplemented with protease and phosphatase inhibitors. After 15 minutes incubation on ice, lysates were centrifuged (10,000 g, 10 min, 4°C) and supernatants were used directly for Western blotting using standard procedures. The primary antibodies used were: phospho-Phospholamban Ser-16 and total Phospholamban (Millipore), phospho-LTCC and total LTCC rabbit polyclonal antibodies (a kind gift from William Catterall, University of Washington, Seattle).

Measurement of calcium transients in adult rat cardiomyocytes (aRCMs)

Cardiomyocytes were isolated from adult Lewis rats by Langendorff perfusion according to the method of Fischer, Rose and Kammermeier¹⁹. Rats were injected with heparin and euthanized with an overdose of pentobarbital. The heart was quickly dissected, cannulated through the aorta and mounted to a Langendorff perfusion system. The coronary arteries were perfused with CO₂-bubbled modified Krebs-Henseleit solution at 37°C (MKR buffer; in mM: 117 NaCl, 2.6 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 10 NaHCO₃, 10 HEPES) to remove residual blood from the coronary arteries. To digest connective tissue, the heart was perfused with collagenase buffer at increasing pump rate for 16 minutes (MKR buffer with 250 U/mL Collagenase Type II (Worthington), 0.7% BSA (MP Biomedicals), 15 mM butanedione monoxime (Sigma) and 2 mM D-glucose). The flaccid heart was then torn in pieces, transferred to a shaking Erlenmeyer flask and further digested for 15 minutes. During the

last 5 minutes, CaCl_2 was slowly re-introduced to the solution to a final concentration of 0.56 mM. Cells were pelleted by centrifugation at 17 g, washed two times (MKR with 1 mM CaCl_2 , 2% BSA, 2 mM D-glucose), resuspended and finally sedimented by gravity. The purified cells were resuspended in adhesion medium (MKR with 0.45% BSA, 2 mM D-glucose) and rolled on a tumbler at ambient temperature for ≥ 1 hour to recover. The cells were then seeded onto glass slides coated with mouse laminin (Sigma). After 1.5-2 hours the medium was exchanged for basal medium (Medium 199 with 5 mM creatine, 3.2 mM carnitine, 3.1 M taurine, 0.427% BSA, 20 μM palmitate). At the day of isolation, cells were transfected with 10 nM miR-139 mimics or unrelated control (Dharmacon) using Lipofectamine RNAiMAX. Two days after transfection the cells were loaded with calcium dye (2 μM Fluo-4 in DMSO, 20% w/v pluronic F-127, 1 mM probenecid) and the glass slides were transferred to a Nikon Eclipse TE2000U inverted microscope, equipped with a Nikon Digital Eclipse C1 confocal scanner. The cells were superfused with normal Tyrode's solution (in mM: 136 NaCl, 5 KCl, 1 MgCl, 1.8 CaCl_2 , 10 HEPES, 10 Glucose, pH 7.4) and paced with 5 ms rectangular voltage at 1 Hz (IonOptix). The Fluo-4 signal was recorded during pacing and during rest to analyze calcium transients and spontaneous calcium release events ("sparks"), respectively. Experiments were carried out in absence or presence of 3 nM Iso. After measuring cells in presence of Iso, caffeine (10 mM) was administered to assess total SR calcium content.

RT-PCR

Total RNA was isolated from cells or snap frozen tissue using the mirVana miRNA isolation kit (Ambion). RNA was cleared from remaining DNA by treatment with DNase I (DNA-free, Ambion) and reverse transcribed using miScript RT kit (Qiagen). RT-qPCR was performed on an iQ5 system (Bio-Rad) using SybrGreen. An LNA based primer was used for detection of miR-139-5p (Exiqon), whereas mRNA was detected by custom made DNA primers (Table 2). *U6 snRNA*, *Gapdh* or *Ppia* served as internal control.

Table 2: Primer sequences for RT-PCR.

Target	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
<i>Pde2a</i>	CAGACAAAGGGCTGGAAGAC	TGAAGCTCAGGGATGTAGGC
<i>Pde3a</i>	AGGAAGAGGCTGAAACACCA	GGTGCTGAGTTATTTGGCAGT
<i>Pde4a</i>	GTGACAAGCACACAGCCTCT	TACCAATCCCGTTGTCTTC
<i>Pde4d</i>	ATCCTGGGGTGTCAAATCAA	TCTCTAGGACCGAGGAGTCG
<i>Nppb</i>	GTTTGGGCTGTAAACGACTGA	GAAAGAGACCCAGGCAGAGTCA
<i>Acta1</i>	TGAGACCACCTACAACAGCA	CCAGAGCTGTGATCTCCTTC
<i>Myh7</i>	AGAGAATGGCAAGACGGTG	GATCTTGTCGAACTTGGGAGG
<i>Gapdh</i>	GGTGGACCTCATGGCCTACA	CTCTCTTGCTCAGTGTCTTGCT
<i>U6</i>	CGCTTCGGCAGCACATATAC	TTCACGAATTTCGTGTCTAT
<i>Ppia</i>	CAATGCTGGACCAAACACAA	GCCATCCAGCCATTTCAGTCT

Production of AAV9

AAV9 vectors were produced at the AVU Facility at ICGEB, Trieste. The viral genes were removed and replaced by an expression cassette containing the mouse miRNA-139 gene under control of a CMV promoter. AAV9 stocks were produced by a dual plasmid transfection procedure, in which the vector plasmid and an adenovirus-derived helper plasmid are co-transfected into HEK293 cells. Vectors were purified from cellular lysates by centrifugation over a CsCl gradient, followed by dialysis, and vector titration was performed by RT-PCR.

Angiotensin II infusion model

Cardiac pressure overload was induced in male C57BL/6 mice by infusion of angiotensin II (Bachem, $2.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) for 28 days using osmotic minipumps (Alzet, Model 2004). Prior to AngII infusion, cardiac expression levels of miR-139 were manipulated: To overexpress miR-139 in the myocardium, 5 week old mice were infected with AAV9-miR139 or empty control vector (2.5×10^{11} viral genomes) via tail vein injection some weeks prior to minipump implantation. For knockdown studies, 9 to 10 week old mice received tail vein injections of 20 mg/kg of 2' O-methylated, phosphorothioate-modified and cholesterol-tagged antagomirs targeting murine miR-139-5p (Fidelity Systems Inc.) on three consecutive days prior to minipump implantation. Control mice received the same amount of a scrambled control antagomir. After 4 weeks of AngII infusion, mice underwent echocardiography under isoflurane anesthesia and B-mode images of the LV short axis were acquired at the height of the papillary muscles using a Vevo770 machine. Mice were then sacrificed and the hearts excised and weighed. The left ventricular apex was snap frozen for molecular-biological analysis, whereas the heart base was fixed in 4% formalin and paraffin embedded. Heart sections were stained with Picrosirius Red (Klinipath) and pictures were taken on a Leica DM2000 equipped with a Leica DFC450C at random positions covering the complete left ventricle. The area of interstitial collagen normalized to the tissue area was assessed. To analyze myocyte cross sectional area, sections were stained with FITC conjugated Wheat Germ Agglutinin (Sigma, L4895) and Hoechst 33342 (Sigma, B2261), and fluorescent images were taken on a Leica DMI3000B equipped with a Leica DFC350FX camera. The area of myocytes was measured in cells cut transversely at the height of the nucleus. All quantifications were performed in a blinded manner using ImageJ software.

Statistics

Data are presented as mean $\pm$ SEM, unless stated differently. Prism (GraphPad) and SPSS (IBM) were used for statistical analysis. Student's t-test was used for comparing two groups, with Welch's correction in case of unequal variances. For multiple comparisons versus a control group Dunnett's test was performed. Differences in phosphorylation, calcium handling, and *in vivo* parameters were compared between all groups by Tukey's multiple comparisons test. Games-Howell test was used in cases of unequal group variances, as determined by Brown-Forsythe test. Gene expression changes in stimulated nRCMs were tested by Dunnett's test (to compare the three stimulated with the control group) and by

Sidak’s test (to assess the effect of miR-139 mimics per stimulus). In all cases a (corrected) p-value <0.05 was considered statistically significant.

RESULTS

Regulation of miRNAs in human aortic valve stenosis

In a microRNA array of heart biopsies of patients with aortic valve stenosis (AoS) we found 16 and 85 miRNAs to be significantly (corrected p<0.05) up- and downregulated, respectively, versus samples of patients that underwent coronary artery bypass grafting (CABG) (Figure 1A and Supplementary Table S1). One of the downregulated miRNAs, miR-139, caught our special interest because it is encoded in an intron of phosphodiesterase 2a (*PDE2A*) with which it is co-expressed²⁰. Most importantly, the online tool TargetScan (Release 6.2) predicted miR-139-5p (miR-139 in the following) to target the mRNAs of *PDE3A*, *PDE4A* and *PDE4D*, all of which are important regulators of cAMP sub-domains in cardiomyocytes. This opened the possibility of a crosstalk between transcription of *PDE2A* and translation of other PDEs targeted by miR-139. Only one other miRNA, miR-582, is also encoded in a PDE-intron (*PDE4D*). Interestingly, miR-582-5p has a strikingly similar seed sequence to miR-139-5p (Figure 1B) and is also predicted to target other PDE family members. We decided to focus on miR-139 in our studies because miR-582-5p was expressed at a much lower level on the microarray and did not show regulation by aortic valve stenosis (Figure 1C). Differential expression of miR-139 was validated by RT-PCR in an expanded set of biopsies (Figure 1D). Patient characteristics are given in Table 3.

Table 3: Characteristics of patients enrolled in the validation study. CABG: coronary artery bypass grafting; AoS: aortic valve stenosis.

		CABG (n=6)	AoS (n=15)	t-test
		Mean ± SD	Mean ± SD	
Age	[years]	66 ± 7	70 ± 9	n.s.
Ejection fraction	[%]	59.7 ± 4.2	54.8 ± 9.2	n.s.
End diastolic LV dimension	[mm]	47.2 ± 5.0	59.6 ± 6.3	n.s.
End diastolic LV wall thickness	[mm]	9.0 ± 1.2	11.5 ± 1.7	p<0.01
LV mass index	[g/m ²]	85.00 ± 12.70	132.90 ± 26.96	p<0.05

Figure 1: MiR-139 is differentially expressed in the pressure-overloaded heart. (A) Volcano plot of differentially expressed miRNAs in endomyocardial biopsies of patients with aortic valve stenosis (AoS). (B) The miRNAs 139-5p and 582-5p are encoded in introns of PDE2A and PDE4D, respectively, and have highly similar seed sequences. (C) MiR-139-5p, but not miR-582-5p, was differentially regulated in AoS samples. (D) Validation of downregulation of miR-139 in AoS by RT-PCR.

MiR-139 is expressed by cardiomyocytes *in vivo* and regulated by hypertrophic stimulation *in vitro*

MiR-139 has previously been shown to be differentially regulated in cardiac disease in mouse and human, but the cell types expressing miR-139 in the heart have so far not been investigated. *In situ* hybridization on mouse heart sections identified expression of miR-139 in cardiomyocytes but also endothelial cells and other non-myocytes (Figure 2A). For future studies we focused on a possible function of miR-139 in cardiomyocytes. In neonatal rat cardiomyocytes (nRCMs) we found downregulation of miR-139 after 24 hours of stimulation with phenylephrine (PE) or endothelin-1 (ET1) (Figure 2B).

Figure 2: MiR-139 is expressed in cardiomyocytes *in vivo* and *in vitro*. (A) In situ hybridization detected miR-139 (purple) in cardiomyocytes ("C"), endothelial cells ("E") and interstitial cells ("I") of murine LV tissue. No counterstaining was performed. (B) MiR-139 was downregulated in nRCMs after 24 hours of stimulation with phenylephrine (10 μ M) or endothelin-1 (100 nM). * $p < 0.05$, ** $p < 0.01$ versus PBS.

***Pde3a*, *Pde4a* and *Pde4d* are bona fide targets of miR-139 in mice**

The miRNA target site prediction tool TargetScan predicted the phosphodiesterases *Pde3a*, *Pde4a* and *Pde4d* to contain binding sites for miR-139, and these sites are conserved between mouse and human (Figure 3A). A luciferase reporter plasmid containing a 3' UTR fragment of any of the three genes was sensitive to repression by miR-139 (Figure 3B). Differential regulation of miR-139 during heart disease may thus lead to changes in the relative abundance of different PDE isoforms and thereby contribute to changes in cardiomyocyte function.

A

hsa-miR-139-5p	3' GACCUCUGUGCACGUGACAUCU	Complementarity within seed
	: : : : :	: Complementarity outside seed
hsa-PDE3A	5' ...AUUUAGACACAAC-ACUGUAGA...	
mmu-Pde3a	5' ...ACUUAGACACAAC- <u>ACUGUAGA</u> ...	
hsa-miR-139-5p	3' GACCUCUGUGCACGUGACAUCU	
	: : : :	
hsa-PDE4A site 1	5' ...AAAGAAACACAGCAACUGUAGA...	
mmu-Pde4a site 1	5' ...AGUGAAACACAUCAGC <u>UGUAGA</u> ...	
hsa-miR-139-5p	3' GACCUCUGUGCACGUGACAUCU	
	: : : : :	
hsa-PDE4A site 2	5' ...UUCUGAGCACACG-GUACUGUAGC...	
mmu-Pde4a site 2	5' ...AUCUCUGUACACG-GUAC <u>UGUAGC</u> ...	
hsa-miR-139-5p	3' GACCUCUGUGCACGUGACAUCU	
	: : : : :	
hsa-PDE4D	5' ...UUUUUGACACAA--ACUGUAGA...	
mmu-Pde4d	5' ...UUUUUGACACAA-- <u>ACUGUAGA</u> ...	

B

Figure 3: *Pde3a*, *Pde4a* and *Pde4d* are bona fide targets of miR-139. (A) Alignment of miR-139 and its predicted target sites in the 3' UTRs of mouse and human *Pde3a*, *Pde4a* and *Pde4d*. (B) Transfection of miR-139 mimics repressed expression of a luciferase gene containing 3' UTR fragments from mouse *Pde3a*, *Pde4a* or *Pde4d*. In case of *Pde3a*, this could be rescued by mutating the miR-139 binding site (underlined in A). Results for mutated *Pde4a* and *Pde4d* are pending.

MiR-139 regulates mRNA levels of PDEs but not hypertrophy marker genes in nRCMs

To investigate cellular pathways that might be modulated by miR-139 we stimulated neonatal rat cardiomyocytes (nRCMs) with the pro-hypertrophic hormones isoprenaline (Iso), phenylephrine (PE) or endothelin-1 (ET1) while overexpressing miR-139. After 48 hours of stimulation with PE, miR-139 levels were still mildly reduced, whereas miR-139 expression returned to normal levels after 48 hours of ET1 stimulation (compare Figure 4A with 2B). Importantly, overexpression of miR-139 decreased *Pde4a* mRNA levels significantly and *Pde3a* mRNA levels by trend (Figure 4C-D). *Pde3a* and *Pde4a* were more sensitive to

regulation by miR-139 than *Pde4d*, as also indicated by the reporter assay (Figure 3B). It should be noted, however, that miRNA-mediated inhibition of mRNA translation does not necessarily go along with mRNA degradation²¹. Hypertrophic stimulation did not affect mRNA levels of the host gene of miR-139, *Pde2a*, nor of *Pde3a* and *Pde4a*, except for mild reduction of *Pde3a* by PE (Figure 4B-D). Interestingly, treatment with Iso increased expression of *Pde4d* (Figure 4E), possibly as a compensatory mechanism to limit excessive β -adrenergic signaling.

As expected, the hypertrophy markers *Nppb* and *Acta1* were highly upregulated by PE and ET1 (Figure 4F-G). Above that, PE significantly induced expression of the slow twitch myosin isoform *Myh7*, while Iso led to downregulation of *Myh7*, possibly as adaptation to higher contraction frequency (Figure 4H). Interestingly, miR-139 blunted the increased expression of *Myh7* and by trend reduced the upregulation of *Acta1* after stimulation with PE. This indicates an involvement of miR-139 in expression of sarcomeric genes after pro-hypertrophic stimulation. In conclusion, miR-139 regulates the mRNA levels of *Pde4a* in primary cardiomyocytes and appears to affect expression of contractility-related genes during PE stimulation.

Figure 4 (next page): Gene expression in stimulated nRCMs. (A) Endogenous miR-139 levels were mildly increased by Iso and mildly reduced by PE after 48 hours. (B) *Pde2a* expression was not affected by stimulation or miR-139 mimics. (C) *Pde3a* showed a consistent but statistically non-significant downregulation by miR-139 mimics. (D) The same effect was observed for *Pde4a* and in this case reached significance after stimulation with Iso or ET1. (E) *Pde4d* was induced by Iso but not affected by miR-139 mimics. (F) The cardiac stress marker *Nppb* was significantly increased after stimulation with PE or ET1. (G) *Acta1*, a core component of sarcomeres, was induced by PE and ET1. (H) Expression of *Myh7* was reduced by Iso and increased by PE. MiR-139 mimics blunted the upregulation induced by PE. All data normalized to *Gapdh* and *Ppia*. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 Stimulation versus PBS; +p<0.05, ++p<0.01, +++p<0.0001 Mimics versus Ctrl.

Phosphorylation of PKA target proteins may be altered by overexpression of miR-139

Inhibition of PDE3 and PDE4 isoforms increases cAMP-dependent phosphorylation of PKA target proteins involved in excitation contraction coupling and calcium cycling. We therefore assessed the phosphorylation status of PKA targets after acute stimulation with the β -AR agonist Iso in nRCMs. Western blots showed that overexpression of miR-139 mildly increased phosphorylation of LTCC, which surprisingly was not affected by acute stimulation with Iso (Figure 5A). In contrast, PLB phosphorylation was significantly increased by Iso but not influenced by overexpression of miR-139 (Figure 5B). These preliminary data indicate that the expression level of miR-139 may affect PKA target phosphorylation in specific subcellular domains.

Figure 5: Phosphorylation of PKA targets in neonatal rat cardiomyocytes. (A) Phosphorylation of LTCC was non-significantly higher in cells overexpressing miR-139. (B) Iso-stimulated phosphorylation of PLB appeared independent of miR-139. * $p < 0.05$ Iso versus PBS.

Overexpression of miR-139 reduces calcium sparks in isolated adult cardiomyocytes

We next aimed at assessing functional consequences of miR-139 overexpression in cardiomyocytes. Since nRCMs have immature sarcomere organization we used isolated adult rat cardiomyocytes to measure calcium transients and SR calcium sparks 48 hours after transfection with miR-139 mimics. Calcium transients during pacing were increased by stimulation with 3 nM Iso but not affected by overexpression of miR-139 (Figure 6A). In contrast, miR-139 mimics significantly reduced the influence of Iso on spark frequency in resting cardiomyocytes (Figure 6B). This effect appeared to be independent of SR calcium content, although there was a mild reduction in SR calcium content as assessed by administration of caffeine (Figure 6C-D). Calcium removal from the cytosol, which is dominated by SERCA activity, was more rapid after Iso stimulation but independent of miR-139 (Figure 6E). These data indicate a reduction in spontaneous calcium-induced calcium release events in the RyR2/LTCC compartment by miR-139, independent of SR calcium content and SERCA.

Figure 6: Calcium handling in adult rat cardiomyocytes after overexpression of miR-139. Stimulation with Iso led to higher calcium transients (A) and higher spark frequency (B), which was significantly reduced by miR-139 overexpression. SR calcium concentration was mildly reduced by miR-139 (C), but this did not correlate with reduced spark frequency (D). Iso also increased the decline rate in cytosolic calcium levels, and this was independent of miR-139 (E). X/Y indicates number of cells/isolations. *** $p < 0.001$, **** $p < 0.0001$ Iso versus No Iso; + $p < 0.05$ miR-139 versus Ctrl.

miR-139 aggravates pressure overload-induced cardiac dilation and dysfunction

We hypothesized that modulation of miR-139 levels may alter cardiomyocyte function *in vivo* and especially under stressed conditions. To test this hypothesis we made use of the angiotensin II infusion model (AngII) to induce arterial hypertension and cardiac hypertrophy in mice. We deployed an AAV9 vector to overexpress miR-139 in the heart, before implanting AngII-filled minipumps to induce cardiac pressure overload. After 4 weeks we found only mildly increased heart weight to body weight ratios in the mice receiving AngII compared with sham, whereas mice overexpressing miR-139 showed a significant increase in HW after AngII infusion (Figure 7A). Echocardiography revealed that AngII infusion induced concentric thickening of the LV wall, with mildly reduced LV inner diameter. In contrast, mice overexpressing miR-139 showed an enlargement of the LV inner diameter rather than an increase in wall thickness (Figure 7B-C). Consequently, cardiac function was significantly compromised by pressure overload only in mice overexpressing miR-139, whereas hypertensive control animals did not yet show significantly reduced LV fractional shortening (Figure 7D).

Figure 7: AAV9-mediated overexpression of miR-139 *in vivo* affects the outcome of cardiac pressure overload. Heart weight was significantly increased by AngII infusion when miR-139 was overexpressed but not in control mice (A). Hypertensive mice receiving AAV9-miR139 failed to increase wall thickness (B) but underwent LV dilation (C). Cardiac function was more dramatically impaired in hypertensive mice overexpressing miR-139 (D). n=5-7/group. **p<0.01, ***p<0.001 AngII versus Sham; ++p<0.01 miR-139 versus Ctrl.

On the histological level, AngII induced significant cardiomyocyte hypertrophy (Figure 8A-B) and interstitial fibrosis (Figure 8C-E). However, both observations were independent of miR-139 overexpression, suggesting a role for miR-139 mostly in contractility.

To confirm if miR-139 is able to aggravate the effect of pressure overload, we performed a rescue experiment in which we inhibited miR-139 by use of antagomirs before subjecting mice to AngII infusion. Cardiac weight and wall thickness after AngII were comparable between both groups (Figure 9A-B). Importantly, the increased LV dilation as seen with overexpression of miR-139 was largely prevented by miR-139 antagomirs (Figure 9C). In this experiment, a significant reduction in LV fractional shortening was not yet apparent in any group. In summary the *in vivo* results indicate accelerated LV dilation and dysfunction after AngII in mice overexpressing miR-139, whereas LV dilation appears reduced after inhibition of miR-139.

Figure 8: Histological analysis of pressure-overloaded hearts after overexpression of miR-139. (A) Cardiomyocyte cross sectional area was increased by AngII independently of miR-139. (B) Representative WGA stained sections. Scale bar 75 μm . (C-D) Interstitial fibrosis was increased by AngII. Because of non-normality the data were $\log_2$ -transformed for statistical analysis. (E) Representative Picrosirius Red-stained sections. Scale bar 1 mm. n=4-7/group. *p<0.05, **p<0.01, ***p<0.001 AngII versus Sham.

Figure 9: Inhibition of miR-139 reduces LV dilation after AngII infusion. The AngII-induced increase in heart weight (A) and LV wall thickness (B) was not significantly altered by knockdown of miR-139. (C) AngII induced dilation of the LV and this effect was reduced by inhibition of miR-139. (D) LV fractional shortening was mildly impaired in mice receiving AngII but not further affected by inhibition of miR-139. n=4-7/group. *p<0.05 AngII versus Sham.

DISCUSSION

The purpose of this study was to understand the influence of miRNA-139 on cAMP signaling in cardiomyocytes and its relevance in the diseased heart. We found miR-139 to be downregulated in endomyocardial biopsies of patients with aortic valve stenosis, and previous studies have reported downregulation of this miRNA in cardiomyopathies in mouse and human^{12,22,23}. However, the relation between deregulation of miR-139 and cardiac dysfunction had not been addressed so far. Here we have shown that *Pde3a*, *Pde4a* and *Pde4d* are *bona fide* targets of miR-139 and that overexpression of miR-139 in cardiomyocytes alters calcium handling, possibly by affecting PKA phosphorylation patterns. In a mouse model of cardiac pressure overload we found that AAV9-mediated overexpression of miR-139 promotes cardiac dilation and predisposes to HF, whereas knockdown of miR-139 attenuates LV dilation.

MiR-139 as regulator of PDEs and calcium handling

The PDE3 and PDE4 families, consisting of in total 6 genes (PDE3A-B and PDE4A-D), are of special importance for cardiomyocyte contraction in mammals²⁴, and PDE3A predominates over PDE3B in the mouse heart²⁵. We confirmed *Pde3a*, *Pde4a* and *Pde4d* as targets of

miR-139, therefore suggesting miR-139 as regulator of cardiac cAMP degradation and compartmentalization. In mouse cardiomyocytes, PDE3 limits PKA activity close to PLB and RyR2, while PDE4 acts close to PLB²⁶, with conflicting data on its relevance at RyR2 and LTCC²⁶⁻²⁹. Therefore, we expected miR-139 to affect phosphorylation of PLB and RyR2 but not necessarily LTCC. This should lead to increased SR calcium content and “leaky” RyR2, which are thought to increase the frequency of calcium sparks^{5,30}. Our data on phosphorylation of PKA targets after overexpression of miR-139 are still preliminary and await further validation. So far, however, they indicate that miR-139 increases phosphorylation of LTCC but not PLB in neonatal cardiomyocytes, suggesting an effect on calcium entry rather than SR function. In contrast, when studying adult rat cardiomyocytes we found that miR-139 was able to diminish Iso-induced sparks, without affecting calcium transients or SR calcium content, suggesting a reduced RyR2 leak or improved calcium buffering in the SR. These somewhat contradictory data may result from different degrees of maturation between neonatal and adult cardiomyocytes and from our miRNA-based study design, which differs considerably from the common protein-targeted approaches to study PDE function: Our current knowledge on PDE function relies on complete blockade of PDE activity by genomic deletion or pharmacological inhibition. In contrast, miRNAs are fine-tuners of gene expression and while available drugs often inhibit a complete PDE family, miR-139 targets specific family members. Above that, our overexpression studies required cultivation of cardiomyocytes, whereas experiments with PDE inhibitors or genetically modified animals can be performed with freshly isolated cells. Nevertheless, our results introduce miR-139 as regulator of the complex network of PDE isoforms in the heart.

Effect of miR-139 *in vivo*

Transgenic overexpression of $G\alpha_s$ to mimic constitutive β -AR activation in mice augments the physiological responses to adrenergic stimulation and after 15 months results in LV dilation and dysfunction^{31,32}. Interestingly, genetic deletion of *Pde4d* in mice has very similar effects: At 3 months of age, isolated working hearts of *Pde4d* knockout mice (*Pde4d* KO) exhibit increased contractility. Isolated cardiomyocytes show increased phosphorylation of phospholamban with consequently elevated calcium transients and SR calcium content²⁸. 15 months old mice, however, display arrhythmias and spontaneous dilated cardiomyopathy²⁷. We confirmed *Pde4d* as target of miR-139 and found that AAV9-mediated overexpression of miR-139 in mice aggravates pressure overload-induced LV dilation, whereas inhibition of miR-139 showed a protective effect. Aging experiments such as in $G\alpha_s$ transgenic and *Pde4d* KO mice were beyond the scope of this study but appear worthwhile. On the histological level, miR-139 overexpression did not affect myocardial fibrosis or cardiomyocyte cross-sectional area. However, lengthening of cardiomyocyte may be present in mice overexpressing miR-139, resulting in increased LV dilation. Detrimental effects of increased cAMP levels have been attributed to activation of PKA³³ and EPAC1³. So far, we have investigated the effect of miR-139 on PKA target proteins, but it remains to be seen if the effects *in vivo* are predominantly mediated by PKA or EPAC1 signaling. Above that, a better understanding of the link between cAMP/PDE deregulation, cardiomyocyte

contractility and overall cardiac morphology is needed to explain the precise role of miR-139 *in vivo*.

Interestingly, miR-139 has been reported to be co-expressed with its host gene *PDE2A*²⁰, opening the possibility of a cross-talk between expression of PDE2A and the PDEs targeted by miR-139. This would be of great interest because very recently Zoccarato et al. found opposing roles for PDE2 and PDE3/4 in cardiomyocyte hypertrophy. The authors showed that inhibition of PDE3 and PDE4 has pro-hypertrophic effects *in vitro* while inhibition of PDE2 protects from hypertrophy *in vitro* and *in vivo*³⁴. However, their finding that adenoviral expression of PDE2 induces spontaneous hypertrophy in aRCMs is in stark contrast to another publication showing that adenoviral expression of PDE2 protects aRCMs from hypertrophy after adrenergic stimulation³⁵. This striking discrepancy suggests cell culture-dependent alterations in cAMP/PDE signaling and highlights the need for a better understanding of context-specific PDE functions. So far, we conclude that downregulation of miR-139 by pressure overload has a protective effect, possibly by altering cardiomyocyte calcium handling and contractility.

Outlook

The diverse cardiac PDE isoforms appear to have selective non-redundant functions, which has important clinical implications⁸. *PDE3A* expression is significantly reduced in human failing hearts¹⁰, but additional inhibition of PDE3 with milrinone still has beneficial acute effects in HF patients. However, long term administration of milrinone increases mortality in HF patients³⁶, which is why it is approved only for acute treatment. This case highlights our lack of knowledge about the role of PDE deregulation in HF and calls for alternative treatment options. Activity and sub-cellular localization of PDE4 are similar between rodents and human. However, PDE4 activity accounts for only 10% of total PDE activity in human, whereas it makes up for 30-50% of total PDE activity in rodents³⁷. Consequently, repression of *Pde4* in rodents may have more global consequences than in human. MiR-139 is deregulated in murine and human HF and here we have shown a role for miR-139 in regulation of different PDEs exemplarily in rodent models. Studies on human cardiomyocytes would be of great interest to translate our findings on the miR-139/PDE system to humans.

Usually, studies on PDE function employ pharmacological inhibition of a complete PDE family or knockout of a specific PDE gene. MicroRNAs, however, can affect different PDE families but still be selective for certain genes within a family. Above that, there are multiple splice variants of several PDE genes and these variants not only differ in their binding partners (e.g. β_1 -AR for PDE4D8 versus β_2 -AR for PDE4D5³⁸), but also in the length of their 3' UTR. Importantly, it is known that shortening of 3' UTRs can render mRNAs resistant to regulation by miRNAs^{39,40}. Therefore, miR-139 may fine-tune PDE activity in a much more precise way than usual drugs, thus allowing for more targeted treatment options. Detailed studies are

warranted to unravel exactly which PDE isoforms and cAMP domains are affected by miR-139 and to explain our unexpected *in vitro* results.

Next to the heart, miR-139 has a high expression level in the brain and above that acts as tumor suppressor that is consistently downregulated in many malignancies (recently reviewed by Zhang et al.⁴¹). PDE3 and PDE4 not only play central roles in the heart but are also involved in many other diseases, including different mental disorders and cancer^{8,42,43}. We therefore propose a potential therapeutic use of miR-139 as fine-tuner in cardiovascular, mental and malignant diseases. Similarly, miR-582, which has a strikingly similar seed-sequence as miR-139 and is likewise predicted to repress several PDE genes, may play an important role in regulating cAMP signaling in non-cardiovascular diseases.

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SUPPLEMENTARY TABLE

Table S1: Differentially regulated miRNAs. Log normalized expression $\pm$ SD. AoS: aortic valve stenosis; CABG: coronary artery bypass grafting; P: corrected p-value; FC: fold change

	AoS			CABG			P	log FC
let-7c*	8.19	$\pm$	0.18	8.38	$\pm$	0.11	0.039	-0.188
let-7f-2*	8.03	$\pm$	0.20	8.24	$\pm$	0.07	0.032	-0.203
miR-1	14.54	$\pm$	0.08	14.64	$\pm$	0.08	0.029	-0.101
miR-1182	7.47	$\pm$	0.05	7.63	$\pm$	0.06	4.30E-05	-0.151
miR-1203	7.39	$\pm$	0.05	7.46	$\pm$	0.06	0.027	-0.066
miR-1208	8.25	$\pm$	0.19	8.53	$\pm$	0.23	0.015	-0.285
miR-1224-5p	7.39	$\pm$	0.15	7.20	$\pm$	0.03	0.009	0.184
miR-1226*	7.51	$\pm$	0.06	7.37	$\pm$	0.02	6.10E-05	0.132
miR-1238	10.92	$\pm$	2.82	7.96	$\pm$	0.56	0.024	2.955
miR-1245	7.62	$\pm$	0.16	7.78	$\pm$	0.10	0.040	-0.162
miR-1257	9.09	$\pm$	0.22	9.36	$\pm$	0.25	0.038	-0.266
miR-1258	7.90	$\pm$	0.17	8.13	$\pm$	0.08	0.008	-0.229
miR-125a-3p	8.78	$\pm$	0.25	9.04	$\pm$	0.17	0.032	-0.267
miR-126	14.48	$\pm$	0.07	14.57	$\pm$	0.02	0.010	-0.081
miR-1262	8.07	$\pm$	0.15	8.30	$\pm$	0.15	0.009	-0.228
miR-1266	7.11	$\pm$	0.05	7.18	$\pm$	0.08	0.035	-0.070
miR-1276	7.47	$\pm$	0.11	7.62	$\pm$	0.11	0.019	-0.147
miR-1278	8.07	$\pm$	0.21	8.27	$\pm$	0.11	0.043	-0.201
miR-1279	7.30	$\pm$	0.07	7.36	$\pm$	0.05	0.043	-0.069
miR-1281	8.57	$\pm$	1.06	7.60	$\pm$	0.25	0.045	0.971
miR-1283	8.10	$\pm$	0.20	8.28	$\pm$	0.06	0.041	-0.185
miR-1299	8.12	$\pm$	0.14	8.34	$\pm$	0.21	0.023	-0.217
miR-1302	7.20	$\pm$	0.07	7.29	$\pm$	0.07	0.022	-0.088
miR-1304	7.66	$\pm$	0.08	7.57	$\pm$	0.06	0.036	0.090
miR-1322	8.20	$\pm$	0.22	8.47	$\pm$	0.10	0.014	-0.268
miR-1323	9.03	$\pm$	0.24	9.29	$\pm$	0.20	0.042	-0.253
miR-135b*	7.49	$\pm$	0.12	7.62	$\pm$	0.07	0.021	-0.136
miR-139-3p	8.89	$\pm$	0.40	9.29	$\pm$	0.25	0.043	-0.401
miR-139-5p	12.78	$\pm$	0.25	13.06	$\pm$	0.05	0.019	-0.275
miR-148a	13.57	$\pm$	0.12	13.74	$\pm$	0.08	0.007	-0.171
miR-148b*	8.34	$\pm$	0.28	8.60	$\pm$	0.17	0.049	-0.266
miR-150	13.83	$\pm$	0.16	14.02	$\pm$	0.06	0.017	-0.189
miR-182*	8.60	$\pm$	0.23	8.88	$\pm$	0.21	0.028	-0.278
miR-190	9.19	$\pm$	0.43	9.83	$\pm$	0.27	0.005	-0.642
miR-194*	9.62	$\pm$	0.35	10.14	$\pm$	0.38	0.012	-0.524
miR-199a-3p	13.58	$\pm$	0.16	13.39	$\pm$	0.17	0.042	0.185
miR-200c*	7.22	$\pm$	0.04	7.33	$\pm$	0.10	0.004	-0.111
miR-205	7.91	$\pm$	0.11	8.16	$\pm$	0.26	0.012	-0.250
miR-20a	13.76	$\pm$	0.08	13.87	$\pm$	0.04	0.005	-0.118
miR-22	14.28	$\pm$	0.09	14.38	$\pm$	0.08	0.039	-0.103
miR-26a	14.44	$\pm$	0.08	14.55	$\pm$	0.02	0.005	-0.105

	AoS		CABG		P	log FC
miR-298	8.09	± 0.19	8.34	± 0.13	0.012	-0.250
miR-29c	14.42	± 0.10	14.54	± 0.05	0.022	-0.113
miR-300	8.50	± 0.20	8.77	± 0.14	0.009	-0.274
miR-302c*	7.88	± 0.15	8.19	± 0.08	2.97E-04	-0.304
miR-30b*	8.34	± 0.20	8.58	± 0.10	0.016	-0.239
miR-34c-3p	8.14	± 0.19	8.38	± 0.06	0.007	-0.245
miR-372	8.65	± 0.20	9.03	± 0.30	0.008	-0.374
miR-378*	11.61	± 0.59	12.28	± 0.28	0.021	-0.668
miR-409-3p	8.71	± 0.33	8.29	± 0.29	0.019	0.424
miR-409-5p	7.85	± 0.22	7.60	± 0.17	0.032	0.246
miR-449b	9.10	± 0.32	9.44	± 0.32	0.050	-0.344
miR-454*	8.78	± 0.30	8.47	± 0.18	0.038	0.308
miR-484	13.07	± 0.10	13.19	± 0.10	0.027	-0.119
miR-490-5p	10.64	± 0.69	11.35	± 0.47	0.042	-0.705
miR-499-5p	14.63	± 0.07	14.72	± 0.08	0.033	-0.089
miR-506	7.84	± 0.17	8.06	± 0.14	0.016	-0.220
miR-511	8.63	± 0.27	8.28	± 0.26	0.021	0.347
miR-513a-3p	7.30	± 0.08	7.43	± 0.08	0.006	-0.130
miR-516a-3p	8.54	± 0.26	8.84	± 0.28	0.041	-0.299
miR-517c	8.42	± 0.28	9.02	± 0.27	0.001	-0.598
miR-518c*	8.25	± 0.21	8.48	± 0.18	0.046	-0.222
miR-518d-3p	8.27	± 0.22	8.61	± 0.19	0.007	-0.337
miR-520b	7.44	± 0.15	7.60	± 0.05	0.025	-0.161
miR-520c-3p	7.89	± 0.23	8.12	± 0.12	0.033	-0.236
miR-525-3p	7.80	± 0.19	8.12	± 0.09	0.002	-0.315
miR-541	8.10	± 0.19	8.29	± 0.08	0.032	-0.191
miR-542-3p	10.36	± 0.55	9.73	± 0.39	0.027	0.626
miR-548b-3p	8.52	± 0.20	8.76	± 0.21	0.038	-0.236
miR-548d-5p	8.88	± 0.34	9.21	± 0.10	0.036	-0.330
miR-548e	8.19	± 0.20	8.40	± 0.09	0.027	-0.215
miR-548i	7.34	± 0.08	7.47	± 0.07	0.005	-0.129
miR-548k	7.18	± 0.05	7.25	± 0.08	0.040	-0.068
miR-551b*	7.86	± 0.22	8.09	± 0.07	0.027	-0.230
miR-563	7.85	± 0.12	8.06	± 0.08	0.002	-0.206
miR-574-5p	8.63	± 0.17	8.45	± 0.12	0.042	0.176
miR-590-5p	7.77	± 0.14	7.93	± 0.07	0.021	-0.160
miR-591	9.21	± 0.38	9.62	± 0.39	0.049	-0.416
miR-602	7.75	± 0.14	7.89	± 0.08	0.036	-0.143
miR-603	7.61	± 0.07	7.71	± 0.06	0.008	-0.105
miR-604	7.36	± 0.11	7.25	± 0.06	0.032	0.111
miR-609	8.86	± 0.26	9.20	± 0.25	0.017	-0.347
miR-613	7.77	± 0.14	7.91	± 0.10	0.045	-0.142
miR-617	7.85	± 0.22	8.09	± 0.15	0.029	-0.241
miR-620	7.39	± 0.06	7.47	± 0.04	0.013	-0.074
miR-631	7.46	± 0.07	7.61	± 0.16	0.017	-0.147

	AoS			CABG			P	log FC
miR-634	7.50	±	0.09	7.66	±	0.11	0.005	-0.156
miR-641	8.81	±	0.29	9.22	±	0.36	0.022	-0.411
miR-653	7.85	±	0.15	8.09	±	0.10	0.003	-0.235
miR-657	7.76	±	0.14	7.95	±	0.10	0.011	-0.189
miR-671-3p	8.19	±	0.13	8.05	±	0.11	0.040	0.142
miR-7-1*	11.66	±	2.34	9.36	±	0.38	0.032	2.307
miR-802	7.55	±	0.09	7.68	±	0.13	0.022	-0.131
miR-874	11.05	±	0.51	11.65	±	0.33	0.020	-0.600
miR-876-3p	7.46	±	0.09	7.60	±	0.06	0.005	-0.140
miR-888	7.68	±	0.15	7.86	±	0.11	0.022	-0.179
miR-892a	7.16	±	0.05	7.22	±	0.02	0.017	-0.060
miR-920	7.70	±	0.11	7.85	±	0.08	0.010	-0.150
miR-924	7.54	±	0.07	7.63	±	0.10	0.040	-0.095
miR-92a-1*	7.65	±	0.16	7.87	±	0.13	0.013	-0.219
miR-92b	9.88	±	0.40	9.40	±	0.18	0.015	0.485

Chapter 3

The microRNA-221/222 family counteracts myocardial fibrosis in pressure overload-induced heart failure

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ABSTRACT

Background

Pressure overload-induced pathologic remodeling of the heart includes cardiomyocyte hypertrophy and myocardial interstitial fibrosis. While initially adaptive, cardiac remodeling will eventually lead to decreased cardiac function, symptomatic heart failure, and death. A better understanding of the molecular mechanisms by which cardiac pressure overload induces cardiac remodeling will impact its treatment. The microRNA-221/222 family, consisting of miR-221-3p and miR-222-3p is differentially regulated in mouse and human cardiac pathology and associated with kidney and liver fibrosis.

Results

We investigated the role of the microRNA-221/222 family during pressure overload-induced cardiac remodeling and heart failure. In myocardial biopsies of patients with aortic valve stenosis (AoS), microRNA-221/222 expression levels correlated negatively with cardiac fibrosis and stiffness but not with hypertrophic parameters. In angiotensin II-mediated pressure overload in mice, inhibition of both microRNAs 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 microRNAs reduced the effect of TGF β on pro-fibrotic signaling.

Conclusions

In conclusion, our findings identify the microRNA-221/222 family as repressor of TGF β -induced fibroblast activation and consequent myocardial fibrosis in the pressure-overloaded heart.

INTRODUCTION

Hypertension and aortic valve stenosis (AoS) induce pressure overload of the heart, which goes along with left ventricular hypertrophic and fibrotic remodeling, culminating in left ventricular dilation and 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 the development of cardiac hypertrophy and HF. Myocardial fibrosis determines patient outcome even under optimal medical treatment by contributing to cardiac dysfunction, arrhythmias, and possibly ischemia², and therefore is a particularly interesting therapeutic target.

MicroRNAs (miRNAs) are a class of small (~22 nucleotides 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 miRNA can regulate an entire signaling cascade by repressing translation of several genes involved in that pathway³. In addition, different miRNAs can have additive or synergistic functions by repressing the same target or different targets within a single signaling cascade. Since miRNAs are such powerful regulators of gene expression, they are likely implicated in the regulation of the major fibrotic signaling pathways, including transforming growth factor beta (TGFβ)-SMAD2/3, ERK1/2-MAPK, and platelet derived growth factor (PDGF) signaling⁴. Indeed, the potential of miRNAs to modulate myocardial fibrosis has been shown exemplarily for miR-21⁵ and miR-122⁶ by stimulating fibroblast ERK-MAPK signaling and by directly targeting TGFβ₁, respectively. However, our mechanistic understanding of the role of miRNAs in pressure overload-induced cardiac fibrosis and failure 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 miRNA family. Both miRNAs are differentially regulated in human cardiomyopathies^{7,8,9} and in mouse models of cardiac diseases^{10,11,12}, and we recently showed a role for these miRNAs in viral replication and the cardiac immune response during viral myocarditis¹³. So far, there are no reports on a possible role of miR-221/222 in the development of pressure overload-induced cardiac dysfunction. The aim of this study was therefore to determine the role of the miRNA-221/222 family during pressure overload-induced cardiac remodeling. We investigated miR-221/222 levels in endomyocardial biopsies of AoS patients and found a negative correlation with cardiac interstitial fibrosis and left ventricular stiffness. Inhibition of the miRNA family in hypertensive mice resulted in increased cardiac fibrotic remodeling, dilation, and dysfunction. Finally, in isolated rat cardiac fibroblasts, miR-221/222 were found to counteract pro-fibrotic signaling and myofibroblast differentiation.

METHODS

Expanded methods can be found in the *Supplementary Methods* section.

Human samples

The study groups enrolled in this correlation analysis were explained in detail previously⁶. Briefly, septal biopsies of 28 patients with severe isolated aortic valve stenosis (AoS) were analyzed for myocardial fibrosis, cardiomyocyte size, and miRNA expression. Echocardiographic measurements were performed and left ventricular early filling deceleration time as well as chamber stiffness constant (KLV) were assessed.

Animal models

All animal experiments were carried out in accordance with Dutch law and approved by the local ethics committee. Male C57BL/6J mice (Charles River) received intravenous injections of miR-221 and miR-222 antagomirs (Fidelity Systems Inc.) at days -3, -2, -1, +13 and +14 of the study. Minipumps (Alzet) were implanted at day 0 to infuse angiotensin II ($2.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). After four weeks, echocardiography was performed and animals were sacrificed to harvest organs for histological and molecular-biological analysis.

In vitro experiments

Neonatal rat cardiac fibroblasts (nRCF) were transfected with miRIDIAN mimics (Dharmacon, 1 nM miR-221 and miR-222 each or 2 nM control) or Power Inhibitors (Exiqon, 20 nM miR-221 and miR-222 each or 40 nM control) in low serum medium (0.1% FBS) and stimulated with 10 ng/mL recombinant human TGF β_1 (Peprotech) for 24 hours.

Statistics

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

RESULTS

MiR-221/222 expression correlates with severity of fibrosis in patients with aortic valve stenosis

Aortic valve stenosis (AoS) increases the afterload on the left ventricle (LV) and induces cardiac remodeling including increased deposition of interstitial collagen. We determined myocardial expression levels of miR-221-3p and miR-222-3p in biopsies of AoS patients with either severe fibrosis (SF, collagen volume fraction CVF >15%) or non-severe fibrosis (NSF, collagen volume fraction ≤15%). Levels of both miRNAs were significantly decreased in patients with SF and consequently CVF correlated negatively with miRNA levels (Figure 1A-B). Echocardiographic analysis revealed a positive correlation of myocardial miRNA expression with early filling deceleration time (Figure 1C), indicating slower left ventricular filling in patients with low miRNA levels. In line with this, left ventricular stiffness (KLV) negatively correlated with the expression of both miRNAs (Figure 1D) although this did not reach statistical significance for miR-222 ($p=0.069$). Cardiomyocyte size did not correlate with expression of either miR-221 or miR-222 (Supplementary Figure S1A) indicating that miR-221/222 levels may modulate 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 be negatively correlated with cardiac CVF, identifying miR-221 as a potential biomarker for myocardial fibrosis (Supplementary Figure S1B).

Figure 1 (next page): Expression of the miR-221/222 family correlates negatively with human cardiac interstitial fibrosis and stiffness. Expression of both miRs is significantly lower in the myocardium of patients with severe fibrosis versus non-severe fibrosis (A) and correlates negatively with collagen volume fraction (B). A positive correlation of miR-221/222 expression with deceleration time of the early mitral filling wave (C) and a negative correlation with KLV (D) indicate the negative association of miR-221/222 levels with left ventricular stiffness. NSF: non-severe fibrosis (empty circles, $n=13$); SF: severe fibrosis (filled circles, $n=14-15$); KLV: left ventricular chamber stiffness constant. ** $p<0.01$, *** $p<0.001$.

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

To investigate if the miR-221/222 family has indeed a causative role in pressure overload-induced left ventricular fibrosis, we subjected mice to 4 weeks of infusion with angiotensin II (AngII) while inhibiting miR-221/222 using antagomirs, resulting in about 50% cardiac knockdown of both miRNAs (Supplementary 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 (Figure 2A-B). In addition, myocardial fibrosis was influenced by knockdown of miR-221/222. Histological analysis revealed significantly increased interstitial fibrosis in hypertensive mice receiving miR-221/222 antagomirs (Figure 2C-D). Perivascular fibrosis remained unchanged (Supplementary Figure S2B). The AngII-induced increase in systolic blood pressure was similar in antagomir- and control-oligonucleotide-treated mice (Supplementary Figure S2C), 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 but not in cardiac hypertrophy.

Figure 2 (next page): 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 upon pressure overload in mice receiving miR-221/222 antagomirs as compared to negative control oligonucleotides (C). (D) Representative images of Picrosirius Red-stained left ventricular sections (scale bar: 100µm). 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. (H) Representative images of laminin staining of left ventricular sections (scale bar: 100µm). 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.

The miR-221/222 family regulates pro-fibrotic signaling in neonatal rat 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 fibrosis¹⁴. In the myocardium, AngII promotes activation of resident fibroblasts via induction of TGF β signaling¹⁵. To test how miR-221/222 modulate the function of isolated primary cardiac fibroblasts, we investigated the role of the miRNA family in nRCFs. We found the expression of miR-221/222 in nRCFs to be downregulated by TGF β ₁ (Figure 3A). Rescue of this response by the artificial overexpression of both miRNAs using miR mimics reduced TGF β ₁-induced fibroblast activation. This was evidenced by decreased upregulation of connective tissue growth factor (Ctgf), fibronectin 1 (Fn1), and alpha smooth muscle actin (α SMA) (Figure 3B-D), indicating impaired trans-differentiation of fibroblasts into collagen producing myofibroblasts¹⁶.

Figure 3: Expression of miR-221/222 in cardiac fibroblasts is regulated by TGF β ₁, and overexpression of miR-221/222 impairs fibroblast activation after TGF β ₁ stimulation. MiR-221/222 are downregulated in neonatal rat cardiac fibroblasts after stimulation with TGF β ₁ (A). RT-PCR shows that trans-differentiation of fibroblasts to myofibroblasts upon TGF β ₁ stimulation is impaired by overexpression of miR-221/222 as evidenced by reduced induction of Ctgf (B), Fn1 (C), and α SMA (D) mRNA expression. n=3-6/group. *p<0.05, **p<0.01, ***p<0.001 TGF β ₁ versus Buffer; +p<0.05, ++p<0.01, +++p<0.001 miR-mimics versus control oligonucleotides.

Western blot analysis confirmed that exogenous manipulation of the miRNAs affected the activation of pro-fibrotic TGF β /SMAD-signaling and downstream gene expression of CTGF in nRCFs. Inhibition of the miRNA family by use of antimir prior to TGF β ₁ stimulation further increased the phosphorylation of SMAD2 and the subsequent induction of CTGF gene expression (Figure 4A-C), which is in line with increased myocardial fibrosis in AngII-treated

mice after inhibition of miR-221/222 (Figure 2C-D). In contrast, simultaneous overexpression of both miRNAs reduced the induction of CTGF upon TGF β_1 stimulation, with repressive though non-significant effects on SMAD2 phosphorylation (Figure 4D-F), confirming that the increased presence of miR-221/222 can restrict pro-fibrotic signaling in stimulated cardiac fibroblasts. These data identify this miRNA family as a potential therapeutic candidate in cardiac fibrosis and consequent dysfunction.

Figure 4: The protein level of CTGF in TGF β_1 -stimulated neonatal rat cardiac fibroblasts depends on miR-221/222 levels. Representative western blots of nRCFs stimulated with TGF β_1 after inhibition of miR-221/222 (A). Inhibition of both miRs increases TGF β_1 -induced SMAD2 phosphorylation (B) and CTGF induction (C). Representative western blots of nRCFs stimulated with TGF β_1 after overexpression of miR-221/222 (D). MiR-221/222 mimics do not significantly affect TGF β_1 -induced SMAD2 phosphorylation (E) but have significant effects on CTGF expression (F). n=3-6/group. *p<0.05, **p<0.01, ***p<0.001 TGF β_1 versus Buffer; +p<0.05, ++p<0.01 miR-inhibitors or miR-mimics versus respective control oligonucleotides.

DISCUSSION

Pathological stressors such as hypertension and aortic valve stenosis cause myocardial fibrosis, thereby disrupting electrical and mechanical properties and predisposing to the development of HF². In the last years, factors involved in myocardial fibrosis including miRNAs have been identified², but our mechanistic insight into the molecular processes underlying fibrosis is still limited. Downregulation of miR-122 in human AoS was found to de-repress its target TGF β ₁ and may play a role in severe cardiac fibrosis⁶. In addition, miR-21, which is consistently upregulated in HF, was found to promote cardiac fibroblast survival and growth factor secretion by affecting the ERK/MAPK and PTEN/SMAD7 signaling pathways^{5,17}. In the present study, we identify two novel miRNAs, the miRNA-221/222 family, that modulate pressure overload-induced myocardial fibrosis and consequent HF by inhibiting fibroblast activation. In patients with severe AoS, lower levels of miR-221/222 correlate 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 leads 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 fibroblasts¹⁵. 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 established¹⁸. Here we showed that TGF β also induces the downregulation of miR-221/222 in cardiac fibroblasts. TGF β /SMAD signaling directly induces expression of CTGF, a secreted growth hormone that augments induction of extracellular matrix synthesis¹⁹. Persistent fibrosis appears to be initially induced by TGF β signaling whereas CTGF acts as maintenance factor for fibrotic remodeling²⁰. Importantly, TGF β does not directly induce collagen synthesis in cardiac fibroblasts but rather induces a trans-differentiation into collagen synthesizing myofibroblasts¹⁶. This trans-differentiation is augmented by CTGF/ERK signaling²¹ and goes along with upregulation of the myofibroblast markers α SMA^{22,23} and fibronectin^{24,25}. 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 miRNA-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 development, as is indeed suggested by the negative correlation between miR-221/222 levels and interstitial fibrosis in AoS patients.

Recently, miR-222 has been suggested to drive liver fibrosis by exerting pro-proliferative effects on stellate cells^{26,27}, 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 liver²⁸. 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 the miRNA-221/222 family to interfere with TGF β signaling in cardiac fibroblasts, but the direct mRNA targets involved in this regulation remain elusive. It was recently shown that overexpression of miR-221 in normal rat kidney fibroblasts (NRK-49F cells) is able to prevent pro-fibrotic signaling by targeting the transcription factor Ets-1 and knockdown of Ets-1 prevents the angiotensin II-induced expression of fibronectin and alpha smooth muscle actin in these cells²⁹. The transcription factor Ets-1 synergizes with TGF β to induce CTGF expression³⁰ and was found to mediate the pro-fibrotic effects of angiotensin II on renal fibroblast³¹. Ets-1 expression is repressed by both miR-221 and miR-222³², and downregulation of Ets-1 by miR-221/222 may therefore contribute to the anti-fibrotic effects of the miRNA-221/222 family. Interestingly, expression of miR-222 has also been shown to be repressed by Ets-1, suggesting an amplifying feedback loop of miR-222 downregulation and Ets-1 induction during fibroblast activation³³. However, while it appears likely that the anti-fibrotic effects of miR-221/222 are mediated by Ets-1, other mRNA targets may be involved in this process and further studies are needed to define the signaling cascades that are modulated by the miR-221/222 family in fibroblasts.

Very recently, two groups have investigated the cardiac effects of miR-221 and miR-222 in cardiomyocyte restricted overexpression models. Interestingly, induction of cardiomyocyte-specific miR-222 overexpression in adult mice protects against ischemia/reperfusion injury, as evidenced by reduced cardiomyocyte death and reduced myocardial fibrosis³⁴. In contrast, cardiomyocyte-specific overexpression of miR-221 seems to have the opposite effect and causes spontaneous HF accompanied by impaired autophagy, cardiomyocyte dysfunction and death, and increased fibrosis³⁵. In these two models of cardiomyocyte-restricted miR-221/222 overexpression, myocardial fibrosis most probably occurs secondary to an influence of the 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 showed that lower levels of miR-221 and miR-222 correlate with higher levels of fibrosis and left ventricular stiffness in patients with severe aortic valve stenosis and provided 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 miR-221/222 interfere with TGF β -mediated pro-fibrotic changes and that TGF β is able to downregulate the expression of both microRNAs. 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.

PERSPECTIVE

Cardiac fibrosis stiffens the heart and contributes to contractile dysfunction but is also indispensable for maintaining tissue integrity after myocardial damage. Therefore, a more detailed understanding of the molecular mechanisms involved in the development of fibrosis in different pathophysiological conditions is highly needed. The current work identifies the miRNA-221/222 family as an inhibitor of fibroblast activation during cardiac pressure overload and TGF β signaling. The therapeutic implications of the unraveled mechanism depend on the feasibility of miRNA mimic delivery *in vivo*, which is currently being developed by different research groups and companies. Next to therapeutic options, miR-221/222 may be useful as biomarkers of myocardial remodeling. Fibrosis of the cardiac atrium is a substrate for atrial fibrillation and plasma levels of miR-222 were recently found reduced in patients with atrial fibrillation³⁶. This, together with our observation that circulating miR-221 negatively correlates with myocardial collagen volume fraction in AoS patients, may bring forth miR-221 and miR-222 as biomarkers for fibrotic cardiac remodeling.

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SUPPLEMENTARY METHODS

Human samples

The study groups enrolled in this correlation analysis were explained in detail previously¹. Briefly, two septal biopsies of 28 patients with severe isolated aortic valve stenosis were collected and analyzed for myocardial fibrosis and miRNA 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 (DT) of the early mitral filling wave according to the following equation²: $KLV = (0.07/DT)^2$.

The fraction of myocardium occupied by collagen (collagen volume fraction, CVF) was determined by quantitative morphometry with an automated image analysis system in sections stained with Picrosirius Red as previously described¹. A cluster analysis was performed according to CVF values, and AoS patients were divided in two groups: non-severe fibrosis (non-SF) group (CVF $\leq 15\%$, n=13) and severe fibrosis (SF) group (CVF $>15\%$, n=15). Cardiomyocyte area was analyzed 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* miRNA-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 miRNAs 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 miRNA assays (Life Technologies) for these miRNAs and for snU6 RNA. The fluorescent signal was detected with a 7900HT Fast Real-Time PCR System (Applied Biosystems) and analyzed with the SDS 2.2.2 software. MiRNA expression was normalized to snU6 RNA in the myocardium and to 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, $2.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) 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 Inc.) 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. Conscious blood pressure was measured three weeks after minipump implantation using the CODA tail-cuff system. Care was taken that the animals entered the restrainer voluntarily, to avoid stress. After four weeks of pressure overload, 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 (Klinipath) 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 cardiomyocytes, cells cut longitudinally, and cells without visible nucleus were excluded from analysis. All images were taken and analyzed 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 versus 2 nM control) or Power Inhibitors (Exiqon, 20 nM each versus 40 nM control) in low serum medium (0.1% FBS) using Lipofectamine 2000 (Invitrogen). After 24 hours, cells were stimulated with 10 ng/mL recombinant human TGF β ₁ (Peprotech). After 24 hours of stimulation, cells were harvested in protein sample buffer (5 mM 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. The following primary antibodies were used: rabbit anti-CTGF (GeneTex #GTX26992), rabbit anti-SMAD2/3 (CellSignaling #8685P), and rabbit anti-phospho-SMAD2/3 (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 analyzed using ImageJ and Image Studio Lite software.

RNA isolation and RT-qPCR

RNA was isolated from animal tissue or cells using mirVana miRNA 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 (Bio-Rad). The expression of miR-221 and miR-222 was detected using specific miRNA LNA primer sets (Exiqon). Primer sequences for the myofibroblast markers *Ctgf*, *Fn1*, and α SMA were as follows: *Ctgf_Fw* CACAGAGTGGAGCGCCTGTTC, *Ctgf_Rev* GATGCACTTTTGCCCTTCTTAATG; *Fn1_Fw* AGTCCCGATGGTACCACTGGCC, *Fn1_Rev* CCTG CACGTCCAACGGCATG; *Acta2_Fw* GTCCCAGACACCAGGGAGTGA, *Acta2_Rev* TCGGATACTTCAGG GTCAGGA. U6 RNA served as internal control for miRNA levels in mouse heart samples, and the ribosomal *Rpl13a* was used as internal control for samples of TGF β -stimulated rat cardiac fibroblasts. Primer sequences of housekeeping genes were as follows: *U6_Fw* CGCTTCGGCAGCACATATAC, *U6_Rev* TTCACGAATTTGCGTGTCTAT; *Rpl13a_Fw* CAGAAAGTTTGCTTACCTGGGG, *Rpl13a_Rev* TTCTGC CTGTTTCCTTAGCCTC.

Statistics

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

SUPPLEMENTARY FIGURES

Figure S1: Myocardial expression of miR-221 and miR-222 does not correlate with cardiomyocyte size in patients with aortic valve stenosis, indicating no role of the miRNA family in hypertrophy (A). Plasma levels of miR-221 but not miR-222 significantly correlate with myocardial fibrosis (B). NSF: non-severe fibrosis (empty circles, n=13); SF: severe fibrosis (filled circles, n=12-13).

Figure S2: 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 increase in systolic blood pressure (SBP) 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.

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Chapter 4

Missing links in cardiology: Long non-coding RNAs enter the arena

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ABSTRACT

Heart failure as a consequence of ischemic, hypertensive, infectious, or hereditary heart disease is a major challenge in cardiology and topic of intense research. Recently, new players appeared in this field and promise deeper insights into cardiac development, function, and disease. Long non-coding RNAs are a novel class of transcripts that can regulate gene expression and may have many more functions inside the cell. Here, we present examples of lncRNA function in cardiac development and give suggestions on how lncRNAs may be involved in cardiomyocyte dysfunction, myocardial fibrosis, and inflammation, three hallmarks of the failing heart. Above that, we point out opportunities as well as challenges that should be considered in the endeavor to investigate cardiac lncRNAs.

INTRODUCTION

It has long been believed that a large fraction of the human genome is untranscribed “junk” DNA, consisting of “remains of extinct genes”¹, featuring at most structural functions. Also the decipherment of the human genome in 2001 confirmed that only about 1.1-1.5% of the human genome actually code for proteins^{2,3}. However, especially in the first decade of the 21st century research on mammalian RNA was boosted by large screenings of the genome, such as ENCODE (Encyclopedia of DNA Elements) or the RIKEN Mouse Gene Encyclopaedia Project, which is now being followed up by the FANTOM project (Functional Annotation of the Mammalian Genome)⁴. The ENCODE project was launched in 2003 to search for functional elements in the genome and came to unanticipated results. In September 2012 a coordinated publication of 30 articles by the ENCODE project triggered high interest in RNA research as it was found that up to 80% of the genome may execute biochemical functions⁵, and that about two-thirds of the DNA is transcribed⁶. At that time, some functional non-protein-coding RNA species had been found and investigated, but the vast number of putative non-coding RNA (ncRNA) genes came as a surprise and raised the question how untranslated RNA could – in contrast to the central dogma of molecular biology – steer behavior of cells, organs, and organisms.

Regulatory RNA species

Housekeeping ncRNAs, such as transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), have been known for decades and are constitutively expressed, whereas the concept of regulatory RNA species featuring differential expression patterns is quite new. Table 1 outlines the different currently known RNA species. Only recently, non-coding yet functional regulatory RNA species were discovered bit by bit, and meanwhile several classes of regulatory ncRNAs are distinguished according to size, genomic positioning, and (proposed) mechanism of action. To date, an unanticipated plethora of non-coding RNA classes and sub-classes has been identified next to tRNA and rRNA, including small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), microRNA (miRNA), Piwi-interacting RNA (piRNA) and long non-coding RNA (lncRNA). A comprehensive overview of different classes of non-coding RNAs and their potential involvement in human disease was published recently⁷.

Table 1: Classes of RNA species.

RNA species	Established function(s)
mRNA	Messenger for protein production
tRNA	Translation of RNA codon to amino acid
rRNA	Enzymatic and structural part of ribosomes
snRNA	Pre-mRNA processing
snoRNA	Modification of rRNA
miRNA	Repression of translation
piRNA	Silencing of transposons
lncRNA	Regulation of transcription, pre-mRNA processing, miRNA abundance and protein function

PiRNAs are important for silencing transposons in germ cells, whereas *snoRNAs* play a role in rRNA editing. Both classes have so far not been associated with cardiovascular development or disease and will not be discussed here. *SnRNAs* exert their function in pre-mRNA splicing and ectopic expression of modified snRNA has been shown to change splicing patterns of targeted genes⁸. In 2013, two studies were published in which this technique was employed in the heart to therapeutically restore correct splicing of dystrophin (Dmd) and cardiac myosin binding protein-C (Mybpc3), respectively, in animal models of cardiomyopathy^{9,10}. Barbash et al. used a dog model of muscular dystrophy which shows skipping of exon 7 of Dmd mRNA, destroying its open reading frame. Virus mediated ectopic expression of a modified snRNA targeting the Dmd pre-mRNA gave rise to a truncated splice variant that was in-frame again and could therefore be translated into quasi-functional Dmd protein⁹. Similarly, Gedicke-Hornung et al. could change splicing patterns of Mybpc3 in a mouse model of hypertrophic cardiomyopathy and thus rescue cardiac dimensions and function¹⁰. Despite these promising results and great therapeutic potential, snRNAs appear to attract little attention in current cardiology.

The most extensively studied although not largest class of ncRNAs is that of *miRNAs*, ~22-nucleotide-long single-stranded RNA molecules that modulate translation of target mRNAs by binding to complementary regions in their 3' UTR. It is noteworthy that a given miRNA can target dozens if not hundreds of different mRNAs and inversely one mRNA can be targeted by several different miRNAs, thus creating a complex network of post-transcriptional regulation. Many extensive reviews have been published on the involvement of miRNAs in cardiovascular development¹¹⁻¹³, and (patho)physiology¹⁴⁻¹⁷, which is why they are not covered in this review.

Figure 1: Pubmed results on "RNA, Long Noncoding"[MeSH]

Long non-coding RNAs entered the stage in 1992 with two independent articles on the lncRNA XIST published in the same issue of *Cell*^{18,19}. Since then, lncRNAs have been of rising interest, starting off with 7 publications in 1992 and reaching more than 200 in 2012 (Figure 1). The rapid increase in lncRNA research from 2012 on may be in part explained by the results of the ENCODE consortium as outlined above, and based on such groundbreaking publications a boost in lncRNA research can be anticipated. Thus, lncRNAs constitute an emerging field in biomedical research and will be of growing importance also in cardiovascular research. The present review therefore aims at illustrating functions of long non-coding RNAs, linking them to challenges in the field of cardiology, and giving directions for future research on the role of lncRNAs in cardiac (patho-)physiology. Before going into (potential) functions of lncRNAs in the heart, we will briefly recapitulate the hallmarks of cardiac disease that serve as a leitmotif for this review.

Hallmarks of cardiac disease

Cardiovascular diseases (CVD) are the number one cause of death worldwide, with nearly 17 million people dying of a CVD-related complication in 2011²⁰, and represent a major socioeconomic burden on western societies. There are several cardiovascular diseases that ultimately lead to heart failure (HF), a condition in which the heart is not able to maintain sufficient blood flow to meet the demands of the body. Important underlying causes of HF are ischemic heart disease and arterial hypertension, but also infectious or hereditary diseases can lead to pathological remodeling and failure of the heart. In the development of HF, several histological and molecular-biological changes are apparent in the heart. Cardiomyocytes respond to increased work load by becoming hypertrophic, which leads to thickening of the ventricular wall and thus reduction in wall tension. However, sustained overload of the heart eventually leads to deterioration of cardiac function. On the cellular level, this is accompanied by specific changes in transcription, especially a recurrence of a so-called “fetal gene program”²¹, and occurrence of alternative splicing²²⁻²⁴. The switch in gene expression includes metabolic as well as structural sarcomeric proteins and is thought to be an adaptation of the cardiomyocytes to increased biochemical and mechanical stress. The importance of correct splicing for integrity of the heart was nicely illustrated by Xu et al., who demonstrated that knockout of splicing factor ASF/SF2 in mice leads to development of dilated cardiomyopathy due to alternative splicing of calcium/calmodulin-dependent protein kinase II delta pre-mRNA.²⁵ Next to changes in cardiomyocyte function, failing hearts show structural remodeling that manifests histologically as myocardial fibrosis (*reviewed in this issue of Pflugers Arch.*) and myocardial inflammation²⁶. Both processes are pronounced in conditions that go along with massive cardiomyocyte death, such as myocardial infarction (MI), but are also observed in hypertension-induced heart failure.

Non-coding RNAs have been shown to play central roles in the process of cardiomyocyte hypertrophy and dysfunction, myocardial fibrosis, and inflammation and thus direct the development of HF. The involvement of miRNAs in cardiac hypertrophy, fibrosis, and inflammation has been summarized in a review series in *Cardiovascular Research*

in 2012²⁷⁻²⁹. In contrast, literature on the role of long non-coding RNAs is scarce. In the following, we will introduce general features of lncRNAs before giving examples of lncRNAs involved in cardiac development and disease.

Long non-coding RNA

Definition

Long non-coding RNAs (lncRNAs, in some publications also referred to as *large non-coding RNAs*) were discovered in the early 1990's and are nowadays defined as RNA molecules of >200 nucleotides in length, lacking a significant open reading frame (ORF). Usually, an ORF cut-off of 100 amino acid is used because >95% of coding genes have ORFs >100 amino acids³⁰ and the FANTOM consortium used an ORF length of 100 amino acids to help identify coding mRNAs³¹. lncRNAs are grouped according to their genomic positioning as sense, antisense, intronic, divergent, or intergenic. *Natural antisense transcripts* (NATs) are most likely to regulate expression of their complementary counterpart, which in most cases lies directly on the opposite strand in the same locus but can also be encoded in another genomic region. The other classes, however, are likely to have diverse functions. *Intergenic lncRNAs* are also referred to as lincRNAs, which initially was used as abbreviation for large *intervening* non-coding RNA³². More than 95% of lincRNAs appear to be evolutionary conserved³², whereas lncRNAs on average are far less conserved, which has led to the assumption that most of them are non-functional³³. Pang et al. estimate the conservation of functional lncRNA to be in general <70% between mouse and human, which is comparable to intron conservation. However, many ncRNAs appear to harbor conserved patches, which may indicate domains that are important for their function³⁴.

The somewhat vague definition of lncRNA is owed to their heterogeneous length, ranging from some 100 nucleotides up to several kilobases (kb) and to the incomplete knowledge on their involvement in physiological processes. In fact, it has recently been proposed to define a new group of *very long intergenic non-coding RNAs* (vlincRNAs) comprising intergenic lncRNA genes of at least 50 kb in length³⁵, and it seems likely that with growing knowledge about expression and function of the various lncRNAs further sub-classes will be defined. In addition, also the ORF cut-off of 100 amino acids has been challenged recently. Using ribosome profiling in zebrafish, Giraldez et al. found that short ORFs of some putative lncRNAs can actually be bound by ribosomes, indicating active translation. Above that, they could demonstrate the existence of a functional micropeptide of less than 100 amino acids that was derived from a processed RNA with so far unknown coding potential, indicating that experimental identification of the coding potential can uncover novel coding genes in the genome (personal communication, publication under review). It is thus possible that some lncRNAs can give rise to small proteins while the remaining sequence is either degraded or exerts an additional function on its own.

Number and processing

In the last decade, a large number of lncRNAs was found by screening of cDNA libraries³¹ or searching for chromatin modifications indicative for transcription of non-coding genes³². To date, the total number of lncRNAs in mammals is estimated to be several ten thousands, with the publicly available database *Lncipedia.org* covering about 32.000 human annotated lncRNA transcripts from more than 17.000 genes³⁶. Interestingly, the number of proposed intergenic lncRNAs rose from about 1.600 in 2009³² to possibly more than 50.000 in 2012³⁷. At the same time, the identification of non-intergenic lncRNAs is methodologically even more challenging, and the total number of lncRNAs may thus be much higher than currently anticipated. The exact proportions of intergenic and overlapping lncRNAs therefore remain to be established.

The majority of lincRNAs appears to be transcribed by RNA polymerase II, spliced, 5'-capped and poly-A tailed³². However, while many lncRNAs show similarities in processing, their expression patterns can be highly specific in certain tissues and several lncRNAs are differentially regulated in various diseases³⁸, which makes them attractive for biomedical research. Above that, multi-exonic lncRNAs can undergo tissue specific splicing³⁹, indicating a large complexity in lncRNA regulation and function. How lncRNA expression and splicing are regulated under physiologic and pathologic conditions remains to be investigated.

Xist, the first discovered lncRNA, regulates gene transcription

The discovery of the first lncRNA XIST (X-inactive specific transcript) was published in 1992 by two different groups in the same issue of *Cell*^{18,19}. These groups found that the product of the Xist gene in human and mouse, respectively, is a non-coding yet functional RNA. This lncRNA is exclusively expressed from the inactivated X chromosome of female mammals and plays an important role in this silencing process. After being transcribed, Xist covers the complete chromosome and recruits the polycomb repressor complex 2 (PCR2) to deposit inactivating histone marks. Interestingly, PCR2 is first recruited to shut down active loci, before attending to the remaining chromosomal regions in a two-step process⁴⁰. Today, many lncRNAs have been found to similarly regulate gene expression at specific loci by inducing modifications of the chromatin landscape⁴¹. The ability of RNA to form sequence specific triple-helices with genomic DNA proves advantageous for this. Interestingly, there are cases in which the actual lncRNA transcript is dispensable while the act of lncRNA transcription at a given locus regulates expression of neighboring genes⁴².

LncRNA functional classes

Currently, research is often focusing on transcriptional regulation by lncRNAs, but given the huge number of lncRNA genes, many more functions can be anticipated. In fact, there are already reports on post-transcriptional regulation by lncRNAs, for example regulation of splicing⁴³, and some lncRNAs have established functions in the cytoplasm or shuttle between nucleus and cytoplasm^{44,45}. The diversity in lncRNA behavior is a challenge for researchers as it calls for conceptually distinct experiments to define functions of individual lncRNAs.

To summarize the scope of their action, Wang and Chang have proposed four functional lncRNA categories: signals, decoys, guides and scaffolds⁴⁶. Firstly, due to their temporally and spatially flexible expression patterns, lncRNAs can react on stimulation and transduce *signals* either by exerting regulatory functions themselves or with the act of lncRNA transcription being the regulatory event. *Decoy* lncRNAs can sequester RNA-binding proteins or other RNA species, such as miRNAs, and thus interfere with their function. In contrast to sequestration, lncRNAs can *guide* proteins to specific genomic regions in *cis* and *trans* to modulate gene expression. Their capability to form heteroduplexes or -triplexes with DNA qualifies lncRNAs for this function. Lastly, instead of acting as decoy for proteins, lncRNAs can also form a *scaffold* for their binding partners to coordinate their individual or combined function. Although Wang and Chang focus on gene regulatory functions of lncRNAs in the proposed classes, the same concepts can be expanded to lncRNAs that steer cell behavior at the post-transcriptional level.

LNCRNAS AS PLAYERS IN THE HEART

The fact that some lncRNAs have been found to be differentially regulated in the developing or diseased heart^{47,48} provides a strong indication for their involvement in cardiac (patho-)physiology. Indeed, specific lncRNAs have already been shown to play a central role in heart development and function as evidenced by loss-of-function studies. In the following, we will emphasize the importance of lncRNAs in cardiac development before illustrating how lncRNAs may contribute to hallmarks of cardiac disease.

lncRNAs in the developing heart

The heart is the first organ in the developing fetus to take up its work. As soon as diffusion processes are insufficient to supply the growing body, blood flow is needed to distribute nutrients. The development of the heart is an intricate process that requires the cooperation of several signaling molecules and transcription factors to coordinate cell migration, replication, and differentiation. Transcription factors are master regulators of these processes as they translate extra- and intracellular signals into expression of proteins in a time- and site-specific manner.

Linc-MD1

The transcription factors Myocyte-specific enhancer factor 2C (Mef2c) and Mastermind-like protein 1 (Maml1) induce the expression of muscle-specific genes and thus play an important role in muscle development. Interestingly, the expression of both transcription factors is indirectly regulated by a single lncRNA. According to the functional classification of Wang et al. given above, this lncRNA, linc-MD1, falls in the second category as it was found to sequester the miRNAs miR-135 and miR-133 from their target mRNAs Mef2c and Maml1, respectively. The de-repression of the messenger RNAs results in increased expression of the transcription factors and thus translates into promotion of muscle differentiation⁴⁹. Importantly, Mef2c is an indispensable regulator of cardiac development in mice⁵⁰, suggesting that linc-MD1 may also be important in cardiomyocyte differentiation.

SRA1

Another transcription factor that is important for myocyte differentiation is Myogenic differentiation 1 (MyoD) which is regulated by the lncRNA Steroid receptor RNA activator 1 (SRA1, also termed SRA)⁵¹. Friedrichs et al. found that the genomic region that harbors SRA1 is in linkage disequilibrium with development of cardiomyopathy and showed that knockdown of SRA1 resulted in impaired cardiac function in zebrafish⁵². SRA1 is an excellent example for the regulatory complexity that lncRNAs can add to the central dogma, as it functions both as a coding and non-coding gene. SRA1 had initially been identified as a lncRNA⁵³ acting as transcriptional co-activator of MyoD. Besides its function on the RNA level, alternative promoter usage and splicing can also give rise to a coding SRA transcript that is translated into the SRA1 protein, SRAP^{54,55}. Only recently it has been found that the coding and non-coding transcript stand in a well-balanced interplay, in which SRAP physically interacts with SRA1 ncRNA thus preventing the activating effect of SRA ncRNA on MyoD⁵⁶ (Figure 2). Such intricate regulation illustrates the wealth of mechanisms by which lncRNAs could possibly regulate genes involved in cardiac development and function.

Figure 2: Proposed function of the coding and non-coding transcripts from the SRA1 locus. SRA ncRNA activates MyoD to promote transcription of genes that steer muscle differentiation. The protein encoded from the same SRA1 locus (SRAP) binds SRA ncRNA thereby counteracting MyoD-dependent transcriptional activation.

Braveheart

Early in 2013, a novel lncRNA, aptly named Braveheart (Bvht), was shown to be required for cardiomyocyte lineage commitment in mice. Braveheart promotes expression of Mesoderm posterior 1 (MesP1), a marker of cardiovascular progenitor cells, and other cardiac-specific genes, probably by sequestering the PCR2 from their promoters⁵⁷. However, there is no homologue of Braveheart in rat or human, which leaves the question, if there is an analogous human lncRNA with a similar function as Braveheart. The relatively low level of lncRNA conservation even between mammals as exemplified here may eventually help to understand how organisms with largely homologous coding sequences can be so different in their physical and mental appearance.

Fendrr

Yet another lncRNA that appears to be important for cardiac development, termed Fetal-lethal noncoding developmental regulatory RNA (Fendrr), was found in 2012 by searching early embryonic mouse tissues for differentially expressed lncRNAs⁵⁸. The group found Fendrr to be expressed in lateral plate mesoderm and loss-of-function experiments showed that genetic deletion of Fendrr leads to embryonic lethality after day E12.5. According to this study, Fendrr is essential for proper development of heart wall and body wall. Importantly, the Fendrr gene lies only 1.2 kb upstream of the Foxf1 gene on the opposite strand and may overlap with sequences that regulate Foxf1 expression. To circumvent possible effects of the Fendrr knockout on Foxf1 expression, the authors replaced the first exon of Fendrr with transcription stop signals instead of removing the whole locus. Still, the authors detected increased Foxf1 expression in the caudal ends of Fendrr knockout embryos, which they explain by a *cis*-acting repressive function of Fendrr on the Foxf1 promoter. In addition, a rescue experiment performed by ectopical expression of Fendrr showed normalization of Foxf1 levels along with dramatically reduced pre-natal mortality⁵⁸ indicating that indeed a *cis*-acting function of the Fendrr transcript instead of impaired genomic integrity were causative for changes in Foxf1 expression. On the other hand, Sauvageau et al. designed a Fendrr knockout mouse in which the promoter and first exon and intron remained unchanged, whereas the complete downstream region of Fendrr was replaced with a lacZ reporter gene. Interestingly, this group detected high Fendrr promoter activity in tissues derived from lateral plate mesoderm with the highest Fendrr expression in the lung. In contrast to the study of Grote et al., these mice were born in normal Mendelian ratios, but all homozygous Fendrr knockout mice died within 24 hours after birth due to malformation of the lungs⁵⁹. The different phenotypes of Fendrr knockout observed by these groups nicely illustrate the influence that the knockout strategy can have on the outcome and consequently raises the question regarding the “true” function of Fendrr. Both constructs have pros and cons which should not be used to play one model off against the other but should be seen as complementary information. The approach of Grote et al., which blocks transcription already from the first exon onwards, cannot rule out the possibility that the passive act of transcription at the Fendrr locus has a regulatory function. This possibility is addressed by Sauvageau et al. because in their model

transcription of the knocked-in gene is taking place at the *Fendrr* locus. On the other hand, Sauvageau et al. cannot rule out the possibility that the *Fendrr* locus acts as an enhancer but this issue is addressed in the construct of Grote et al. Thus, both constructs and the combined observations of the two knockout models may be helpful in getting a complete picture of the function of *Fendrr* in mammalian development.

The examples of *linc-MD1*, *SRA1*, *Bvht* and *Fendrr* illustrate the general importance of lncRNAs in cardiac development and more indispensable lncRNAs will be discovered as the number of lncRNA knockout models increases.

LncRNAs in the diseased heart

Besides congenital heart defects that may be attributable to lncRNA function, diseases of the adult heart may be determined by lncRNAs, even though literature on lncRNAs involved in cardiac disease is scarce to date. In the following we will try and translate discoveries from other research fields to illustrate how lncRNAs could be involved in pathological processes in the heart, focusing on cardiomyocyte dysfunction, myocardial fibrosis, and myocardial inflammation, the hallmarks of cardiac dysfunction. With this we want to provide a reference point for this emerging topic and encourage research in this promising new field.

LncRNAs as potential regulators of cardiomyocyte function

A common feature of cardiac disease and indicator of increased mechanical stress on the myocardium is cardiac hypertrophy. Thickening of the cardiac muscle can temporarily reduce cardiac pressure overload by reducing wall stress. However, if the stressor persists the heart will ultimately start to decompensate and fail. A well-known although only incompletely understood feature of heart failure is a broad change of the cardiac transcriptome. A so called “fetal gene program” is reactivated that affects cellular metabolism (including a shift from fatty acid to glucose metabolism), signaling (e.g. upregulation of the natriuretic peptides A and B) and contraction (e.g. a shift from the adult isoform of myosin heavy chain to the fetal isoform). As introduced above, *XIST* as a prototypical lncRNA has a central function in shutting off genes and several other lncRNAs have been implicated in gene silencing or activation by guiding chromatin modifying enzymes to target genes. It is obvious to presume an involvement of lncRNAs in the extensive transcriptional changes in the diseased heart.

Next to up- or downregulation, different coding genes show changes in the abundance of specific isoforms²². Recently, it was found that the locus of the Dystrophin gene (*DMD*) harbors several lncRNAs which regulate the transcription of certain *DMD* messenger RNA isoforms by interacting with different *DMD* promoters⁶⁰. Interestingly, several of these lncRNAs repress expression of the full-length Dp427 isoform, without affecting the short Dp71 isoform and these two isoforms have distinct functions during myogenesis⁶¹. Differential regulation of the lncRNAs at the *Dmd* locus could therefore define which *Dmd* isoform is predominant in the healthy or diseased heart.

As stated in the introduction, some lncRNAs, termed Natural Antisense Transcripts (NATs), have a reverse-complementary sequence to already known genes and likely regulate their function. In the heart, expression of Atrial myosin light chain (Alc1) was found to be controlled by this mechanism. The expression level of Alc1 antisense RNA is increased in the heart of patients with Tetralogy of Fallot and a higher level of Alc1 mRNA is needed in these patients to achieve normal levels of Alc1 translation⁴⁷. In this way, Alc1 and other NATs may post-transcriptionally regulate gene expression in the heart.

A prominent example of a lncRNA involved in cardiac disease is Myocardial infarction associated transcript (MIAT), which was discovered in 2006⁴⁸. A large-scale association study revealed that a SNP within exon 5 of MIAT correlates with susceptibility to myocardial infarction. A mechanistic explanation, however, remains elusive. MIAT is also known as Gomafu, which is Japanese for “spotted pattern”, because it was found to form a unique granular pattern in the nucleus of neurons⁶². Recently, it was proposed that MIAT/Gomafu can sequester Splicing factor-1 and thus modulate the activity of the nuclear splicing machinery⁶³, hinting at a mechanistic explanation for the observed correlation between MIAT and MI.

Interestingly, there are two more lncRNAs, Nuclear-enriched abundant transcript 1 and 2 (Neat1 and Neat2, respectively), which show a “spotted pattern” in the nucleus and are therefore proposed to form a lncRNA family together with Gomafu⁶⁴. Neat1, also known as Multiple endocrine neoplasia ϵ/β (MEN ϵ/β) or Virus-induced non-coding transcript (VINC), is an integral part of nuclear para-speckles, which are proposed to regulate gene expression by retaining A-to-I edited mRNAs in the nucleus and possibly release them as an immediate stress-response⁶⁵. Neat2, also known as Malat-1 (Metastasis associated lung adenocarcinoma transcript 1), co-localizes with nuclear speckles and associates with splicing factors of the serine/arginine-rich family of nuclear phosphoproteins. A possible role for Malat-1 in splicing can be inferred from the finding that knockdown of Malat-1 provokes alternative splicing of several pre-mRNAs⁴³. Both MIAT and Malat-1 are therefore possibly involved in alternative splicing in the diseased heart, while Neat1 may play an important role in regulation of mRNA translation under physiological and pathological conditions. Contrary to intuition, both Neat1 and Malat-1 are dispensable for normal development in knockout mice^{66,67}. It is therefore likely that the regulatory functions of Gomafu, Neat1 and Malat-1 in the heart become apparent especially under pathological conditions when cells need to adapt to stressors, such as hypertension or ischemia.

lncRNAs and the extra-cellular matrix

Literature on the involvement of lncRNAs in extra-cellular matrix remodeling and fibrosis is scarce. However, very recently a promising study was published by Zhou et al.⁶⁸ in which the authors performed high-throughput RNA sequencing in two mouse models of fibrotic and inflammatory kidney disease, respectively, and found thousands of lncRNAs to be differentially expressed. The expression of more than 100 lncRNAs was regulated by Smad3,

a pro-inflammatory and pro-fibrotic transcription factor, as determined by RNA sequencing in Smad3 knockout mice. Interestingly, the fibrotic and inflammatory disease models showed an overlap of 21 Smad3-dependent lncRNAs, which are therefore promising candidates to play a role in both fibrosis and inflammation also in other organs⁶⁸. In another study, 210 up- and 358 downregulated lncRNAs were detected in a rat model of bleomycin-induced lung fibrosis using microarrays⁶⁹. The authors suggest that targeting some of these lncRNAs may be helpful to reduce fibrotic remodeling of the lung. Together, these studies provide first indications that lncRNAs are differentially expressed in fibrotic remodeling and can inspire scientists involved in cardiovascular research to investigate the effect of lncRNAs on extra-cellular matrix biogenesis and myocardial fibrosis.

LncRNAs in inflammation

Infiltration of immune cells in the myocardium is a feature that is frequently observed in stressed hearts and may be a consequence of myocyte death and/or release of “danger” signals by the myocardium. How this recruitment of leukocytes to the myocardium is regulated is still incompletely understood. The involvement of several miRNAs has been shown for cardiac inflammation²⁹ and there are also some candidate lncRNAs that may play a role in cardiac inflammation.

Infiltration of immune cells to the heart is largely dependent on pro-inflammatory signaling of the vasculature. By using RNA sequencing, Leung et al. found that treatment of rat vascular smooth muscle cells with angiotensin II changes the expression pattern of several coding and non-coding RNAs. Strikingly, one of the differentially expressed transcripts, lnc-Ang362, harbors the genes of the miRNA-221/222 family and knockdown of lnc-Ang362 resulted in reduced levels of miR-221 and miR-222⁷⁰. This miRNA family is downregulated in human ischemic and dilated cardiomyopathy^{71,72}, and both miRNAs can regulate macrophage adherence to endothelium by repressing translation of ICAM-1⁷³ and are implicated in leukocyte proliferation⁷⁴. Above that, miR-221 appears to be required for B cells to reside in bone marrow⁷⁵, whereas miR-222 may be involved in macrophage phenotype polarization⁷⁶. Differential regulation of lnc-Ang362 may therefore play a role in pro-inflammatory signaling of the cardiac vasculature.

The influence of the pro-inflammatory cytokine tumor necrosis factor (TNF) on the progression of heart failure is well established²⁶, as is the fact that TNF can induce transcription of target genes via activation of the NFκB pathway. Very recently, it was found that TNF stimulation of mouse embryonic fibroblasts induces the expression of several lncRNAs, some of which are novel or had previously been annotated as pseudogenes. One of the pseudogene lncRNAs, now termed Lethe, is not only transcribed in an NFκB-dependent manner but is also able to bind to RelA and repress NFκB-signaling. Moreover, Lethe is induced by TNF and Il1b but not by toll-like receptor (TLR) agonists. The authors therefore conclude that Lethe is part of a negative feedback loop that controls NFκB signaling in inflammation but is not involved in innate immunity⁷⁷.

Complementary to Lethe, other lncRNAs may very well play a role in innate immunity as shown by Carpenter et al. who found that activation of Tlr2 on bone marrow derived macrophages induces transcription of 62 lncRNAs. The authors were able to establish lincRNA-Cox2 as a positive and negative regulator of several genes involved in inflammation, such as the chemokine Ccl5, chemokine receptors (Ccr1) and interleukin 6 (IL-6)⁷⁸. The importance of cardiac IL-6/STAT3 signaling under pathologic conditions is well established⁷⁹ and suggests an involvement of lincRNA-Cox2 in cardiac disease. In summary, lncRNAs can function as positive and negative modulators in inflammation as well as innate immunity and are thus putative regulators of inflammatory processes in the diseased heart.

CONCLUSIONS

The examples presented here illustrate the challenges and opportunities in lncRNA research. Several individual lncRNAs have already been identified as important players in cardiac development and disease and yet some thousand more await investigation, thus opening a huge promising research field. Bioinformaticians are currently working on indexing the unanticipated wealth of lncRNAs in databases such as lncrnadb⁸⁰, LNCipedia³⁶, or lncRNome⁸¹. Another important task for bioinformatics is the development of potent tools to predict RNA secondary structure and – given that most lncRNAs appear to team up with RNA-binding proteins – possible protein binding domains of lncRNAs.

Several single-nucleotide polymorphisms (SNPs) are known to correlate with cardiac diseases, but so far most research has focused on protein-coding genes in their genomic neighborhood. The discovery of lncRNAs offers the intriguing possibility that disease-association of some haplotypes which were thought to relate to proteins or could not be explained so far are actually linked to non-coding genes. Next to the example of MIAT as laid out above, a SNP in the lncRNA ANRIL (Antisense non-coding RNA in the INK4 locus) has been found to confer increased risk for cardiovascular disease⁸². Given the possibility that lncRNAs guide proteins to specific genomic loci by forming sequence specific triple-helices with DNA, it is plausible that nucleotide substitutions in lncRNAs could abort this guidance or re-direct the lncRNA to new loci. It appears therefore worthwhile to screen SNPs associated with cardiac diseases for possible effects on lncRNAs. Indeed, in a set of newly identified lncRNAs, Hangauer et al. found a 5-fold enrichment for trait-associated SNPs in comparison to nonexpressed intergenic regions³⁷. We foretell that future research will identify several lncRNAs as missing links in unexplained correlations between SNPs and cardiovascular diseases. Another promising approach would be a genome wide screen for differential expression of lncRNAs in human cardiac pathologies that would likely identify several new disease-relevant candidates and thus boost lncRNA research in cardiology.

lncRNA research has proven cumbersome in some ways. The example of Fendrr highlights the influence that the technical approach can have on the phenotype. However, this has also been experienced with manipulation of coding genes over the last decades and should be seen as an opportunity. The combination of such findings can be informative on how

disturbing physical integrity or transcriptional capacity of a lncRNA locus can have different outcomes. All methods to interfere with transcription also affect physical properties of the genome, be it on the level of DNA sequence or even larger deletions/insertions that could reorganize enhancer or insulator sequences. In that regard, no construct is going to be perfect on its own and the combination of different approaches is the most powerful option to investigate different regulatory aspects of lncRNAs.

The discovery of lncRNAs has filled many gaps in the genomic landscape, making it more crowded. The effort that Grote et al. took to exclude direct physical effects of their *Fendrr* knockout construct on the nearby *Foxf1* gene nicely illustrates the challenge of discerning functions of lncRNAs from those of other genes in close genomic proximity. Vice versa, there is the worrying possibility that genomic manipulations that have been carried out for decades to interfere with coding genes also affected lncRNAs which were unknown at that time. It appears therefore likely that some published knockout phenotypes relate (partially) to off-target effects on nearby lncRNA genes. The generation of knockouts gets even more complicated if not impossible for lncRNA genes that overlap with other transcripts on the same or opposite strand like many NATs do. We expect therefore that in the near future, research on lncRNAs will first focus on the sub-group of lncRNAs in larger distance to (currently known) genes which are easier to manipulate and above that far better conserved than lncRNAs on average. However, partial knockout strategies or insertion of strong transcription stop signals or RNA destabilizing sequences into lncRNA genes remain valid tools when choosing an appropriate strategy to abort lncRNA expression.

Next to genetic knockout strategies to achieve loss-of-function phenotypes, post-transcriptional knockdown can be achieved using antisense oligonucleotides that induce degradation of targeted lncRNAs. The recent development of antisense oligonucleotides that recruit RNase H to cleave target RNA but at the same time carry modifications to render themselves nuclease resistant offers a potent tool for *in vitro* and *in vivo* knockdown of nuclear and cytosolic lncRNAs (see e.g. Wheeler et al.⁸³). However, some lncRNAs exert their function simply by the process of being transcribed, for example by modulating the accessibility of nearby genomic sequences for transcription factors. In such cases, in which the actual RNA transcript is dispensable, knockdown experiments are doomed to fail. Therefore, new experimental designs and tools will be needed to understand lncRNA function in depth.

Another challenge in lncRNA research is the poor conservation of many lncRNAs compared to coding sequences. While poor sequence conservation does not argue against functionality in general⁸⁴, it may still impede the development of animal models to study human diseases. The lncRNA *Braveheart* is fundamental for proper heart development in mice but a human homolog has not been identified⁵⁷. Also protein-centered research, which is based on much better conserved genes, has experienced problems in translating findings from animal models to humans and at the same time proven insufficient to explain differences between

species. While the nematode *Caenorhabditis elegans* has approximately the same number of coding genes as humans, the number of lncRNA genes appears to correlate much better with what we call “higher development”⁸⁵. The discovery of lncRNAs has provided us with promising candidates to finally explain the huge range of physical and mental abilities between species. Investigating long non-coding RNAs in the heart is challenging, however understanding their involvement in cardiac development, function, and disease will be fundamental for further advances in diagnosis, prognosis, and treatment of human cardiac disorders.

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Chapter 5

Long non-coding RNA Malat-1 is dispensable during pressure overload-induced cardiac remodeling and failure in mice

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ABSTRACT

Background

Long non-coding RNAs (lncRNAs) are a class of RNA molecules with diverse regulatory functions during embryonic development, normal life, and disease in higher organisms. However, research on the role of lncRNAs in cardiovascular diseases and in particular heart failure is still in its infancy. The exceptionally well-conserved nuclear lncRNA Metastasis associated lung adenocarcinoma transcript 1 (Malat-1) is a regulator of mRNA splicing and highly expressed in the heart. Malat-1 modulates hypoxia-induced vessel growth, activates ERK/MAPK signaling, and scavenges the anti-hypertrophic microRNA-133. We therefore hypothesized that Malat-1 may act as regulator of cardiac hypertrophy and failure during cardiac pressure overload induced by thoracic aortic constriction (TAC) in mice.

Results

Absence of Malat-1 did not affect cardiac hypertrophy upon pressure overload: Heart weight to tibia length ratio significantly increased in WT mice (sham: 5.78 ± 0.55 , TAC: 9.79 ± 1.82 g/mm; $p < 0.001$) but to a similar extent also in Malat-1 knockout (KO) mice (sham: 6.21 ± 1.12 , TAC: 8.91 ± 1.74 g/mm; $p < 0.01$) with no significant difference between genotypes. As expected, TAC significantly reduced left ventricular fractional shortening in WT (sham: $38.81 \pm 6.53\%$, TAC: $23.14 \pm 11.99\%$; $p < 0.01$) but to a comparable degree also in KO mice (sham: $37.01 \pm 4.19\%$, TAC: $25.98 \pm 9.75\%$; $p < 0.05$). Histological hallmarks of myocardial remodeling, such as cardiomyocyte hypertrophy, increased interstitial fibrosis, reduced capillary density, and immune cell infiltration, did not differ significantly between WT and KO mice after TAC. In line, the absence of Malat-1 did not significantly affect angiotensin II-induced cardiac hypertrophy, dysfunction, and overall remodeling. Above that, pressure overload by TAC significantly induced mRNA levels of the hypertrophy marker genes *Nppa*, *Nppb* and *Acta1*, to a similar extent in both genotypes. Alternative splicing of *Ndr2* after TAC was apparent in WT (isoform ratio; sham: 2.97 ± 0.26 , TAC 1.57 ± 0.40 ; $p < 0.0001$) and KO mice (sham: 3.64 ± 0.37 ; TAC: 2.24 ± 0.76 ; $p < 0.0001$) and interestingly differed between genotypes both at baseline and after pressure overload ($p < 0.05$ each).

Conclusions

These findings confirm a role for the lncRNA Malat-1 in mRNA splicing. However, no critical role for Malat-1 was found in pressure overload-induced heart failure in mice despite its reported role in vascularization, ERK/MAPK signaling, and regulation of miR-133.

INTRODUCTION

The complexity of an organism is not related to the size of its genome nor to the number of proteins encoded therein but rather correlates with the number of genes that produce non-coding RNA^{1,2}. Long non-coding RNAs (LncRNAs) were discovered in the early 1990's^{3,4} and are nowadays defined as RNA molecules of >200 nucleotides in length, lacking a significant open reading frame. They are able to bind other RNA or DNA species as well as proteins and may thereby regulate processes at all stages from gene transcription and translation to protein function. The functions of many LncRNAs in transcriptional regulation have recently attracted much attention in the field of developmental biology (reviewed in 5) and cancer research (reviewed in 6). However, also cardiovascular research is beginning to recognize the importance of LncRNAs for heart development (e.g. SRA1) and pathology (e.g. MIAT) as recently reviewed^{7,8}. The LncRNA Metastasis associated lung adenocarcinoma transcript 1 (Malat-1; also known as Nuclear Enriched Abundant Transcript 2, Neat2) was discovered in metastasizing non-small cell lung cancer⁹ and is highly expressed in most cell types and organs, including the heart. Increased expression of Malat-1 is by now recognized as an established feature of many tumors and indicates poor prognosis^{10,11}. Malat-1 can bind to active chromatin sites¹² and co-localizes with nuclear speckles, where it regulates pre-mRNA splicing^{13,14}. Above that, a *cis*-regulatory role has been assigned to the Malat-1 locus¹⁵. Given the high conservation and expression level of Malat-1, several research groups simultaneously undertook the effort to generate Malat-1 knockout mice. Surprisingly but consistently, none of the strains lacking Malat-1 showed any obvious abnormalities during embryonic or post-natal development¹⁵⁻¹⁷, indicating that Malat-1 is either completely dispensable or becomes important only under pathological conditions.

Heart failure (HF) is a condition in which the heart is unable to sustain sufficient blood flow through the body and is the fatal end-stage of many heart diseases. About 23 million people worldwide suffer from HF, with the highest prevalence in the growing elderly population¹⁸. A common cause for HF is cardiac pressure overload, which can be a consequence of hypertension or aortic valve stenosis. Pressure overload of the left ventricle (LV) causes a transient increase in vascularization that is necessary for adaptive cardiomyocyte growth, and myocardial vessel density correlates with cardiac function¹⁹⁻²¹. Importantly, genetic ablation of Malat-1 has recently been shown to reduce revascularization capacity after hind limb ischemia²². Above that, two groups have shown impaired myogenic differentiation after silencing of Malat-1 *in vitro*^{23,24}, possibly via regulation of microRNA-133. This microRNA has central roles in cardiac contractility and hypertrophy by repressing β_1 -adrenergic receptor and serum response factor (SRF), respectively^{25,26}. Scavenging of miR-133 by Malat-1 may therefore increase levels of SRF, an important mediator of cardiac hypertrophy²⁷. Similarly, ERK/MAPK signaling propagates pro-hypertrophic signaling in the heart²⁸, and Malat-1 was found to activate this pathway²⁹. These reports strongly suggest a role for Malat-1 in the development of cardiac hypertrophy and failure. Therefore, we subjected Malat-1 knockout mice to either thoracic aortic constriction (TAC) or chronic infusion of angiotensin II (AngII)

to induce pressure overload of the LV, mimicking aortic valve stenosis or systemic hypertension, respectively. Surprisingly, detailed analysis of cardiac morphology, function, and histology did not reveal an implication of Malat-1 in myocardial hypertrophy, angiogenesis, inflammation, fibrosis, or dysfunction upon chronic cardiac pressure overload.

METHODS

Mouse models

Heterozygous Malat-1^{+/-} mice derived from CBA x C57BL/6 chimeric animals were provided by Shinichi Nakagawa after 6 backcrosses into C57BL/6N mice¹⁷. The offspring was genotyped before the start of the studies to match group sizes and only homozygous Malat-1^{+/+} and Malat-1^{-/-} mice were used. DNA was isolated from toes of new born mice and genotyping PCR was performed using a standard 3-step protocol with 30 cycles and 62-66°C annealing temperature. Primer sequences were: WT-Fw AGAGCAGAGCAGCGTAGAGC, WT-Rev GCTCTGGTCAGCCTCCATTA, KO-Fw TTGAAGTGGCGAGCGATAC, and KO-Rev AGATCC CAGCGGTCAAAAC.

Mice were operated at 8-12 weeks of age to induce cardiac hypertrophy by either continuous infusion of angiotensin II (AngII, 2.5 mg·kg⁻¹·d⁻¹) using osmotic minipumps (Alzet) or thoracic aortic constriction (TAC) between the brachiocephalic artery and the left common carotid artery. Aortic diameter was reduced to 0.41 mm (27G needle) for mice weighing up to 25.5 g or to 0.46 mm (26G needle) for larger animals. TAC mimics the situation in patients with aortic valve stenosis with pure pressure overload but without direct hormonal effects. On the other hand, AngII infusion causes systemic hypertension and additionally has direct cellular effects in heart tissue which further promote heart failure³⁰. In both studies the duration of the experiment was 4 weeks and sham operated animals served as controls. Conscious heart rate and blood pressure of mice enrolled in the AngII study were measured by CODA tail cuff (Kent Scientific) three weeks after surgery. After four weeks of pressure overload, 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 analysis and RNA isolation. All animal experiments were carried out in accordance with Dutch law and approved by the animal experimental committee at Maastricht University.

Histology

After sacrifice, organs were rinsed in PBS and Zinc fixed for 48 hours (BD Pharmingen, #552658). 4 µm paraffin sections were cut to analyze histological changes of the left ventricle and septum. Collagen was stained with Picrosirius Red F3B (Klinipath, #80115) and interstitial collagen area was quantified after exclusion of vessels and endo- and epicardial connective tissue. Laminin staining was performed using rabbit anti-mouse laminin (Sigma, L9393) and Vectastain Elite ABC kit (Vector laboratories) to assess cardiomyocyte size. Epicardial cardiomyocytes, cells cut longitudinally, and cells without visible nucleus were

excluded from cell size analysis. CD45 positive cells were stained with rat anti-mouse CD45 antibody (BD Pharmingen, #553076) and Vectastain ABC-AP kit (Vector laboratories) and counted in the whole LV and septum. Capillaries were stained with biotinylated Griffonia (Bandeiraea) Simplicifolia Lectin I (Vector Laboratories, B-1105) and Vectastain ABC-AP kit and cross-sectioned capillaries near the endocardium were counted. All analysis was performed in a blinded manner using a Leica DM2000 equipped with a Leica DFC450C camera and ImageJ software.

RT-PCR and analysis of alternative splicing

Myocardial RNA was isolated using mirVana miRNA isolation kit (Ambion) according to manufacturer's instructions. RNA was reverse transcribed using iScript RT kit (Bio-Rad) and RT-qPCR was performed using SYBR Green (Bio-Rad). Primer sequences for Malat-1 and for cardiac hypertrophy markers Atrial natriuretic peptide (*Nppa*), Brain natriuretic peptide (*Nppb*) and Skeletal alpha actin (*Acta1*) were: *Malat-1_Fw* CTTTTCCTCCACATTCCAA, *Malat-1_Rev* CTCGTGGCTCAAGTGAGGTG; *Nppa_Fw* ATTGACAGGATTGGAGCCAGAGT, *Nppa_Rev* TGACACACCACAAGGGCTTAGGAT; *Nppb_Fw* GTTTGGGCTGTAAACGCACTGA, *Nppb_Rev* GAAAGAGACCCAGGCAGAGTCA; *Acta1_Fw* TGAGACCACCTACAACAGCA, *Acta1_Rev* CCAGAGCTGTGATCTCCTTC. Gene expression was normalized to Cyclophilin-A (*Ppia*) as internal control: *Ppia_Fw* CAAATGCTGGACCAACACAA, *Ppia_Rev* GCCATCCAGCC ATTCAGTCT.

Alternative mRNA splicing of N-myc downstream-regulated gene 2 (*Ndr2*) and Eukaryotic translation initiation factor 4H (*Eif4h*) has been reported in hypertrophic and failing mouse hearts, respectively^{31,32}. 500 ng of RNA was reverse-transcribed using Oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). Primers based on mouse *Ndr2* (exon 1 Fw: TCAAAGGCAAGTGAAGGTGG, exon 4 Rev: CGAGCCATAAGGTGTCTCCA) and *Eif4h* (exon 3 Fw: GTGGATTCCCTGAAGGAGGC, exon 6 Rev: GAAAGCGACTCCCCATTGGA) were used to detect splicing changes. PCR amplification was performed at 58°C for 30 and 35 cycles, respectively. Electrophoretically separated PCR products were quantified by densitometric analysis using ImageJ software, and the ratio of the mRNA isoforms (long/short) was calculated.

Statistics

Data are presented as median and range with individual data points depicted. Statistical analysis was performed using Prism (GraphPad) and SPSS (IBM). Equality of variances was tested by Levene's test. One-way ANOVA (with Welch's correction if appropriate) followed by Tukey or Games-Howell post-hoc test was deployed to compare groups with equal or unequal variances, respectively. In all cases a p-value <0.05 was considered statistically significant.

RESULTS

Pressure overload-induced heart failure develops independently of Malat-1

Four weeks after TAC surgery, the effect of LV pressure overload on cardiac dimensions and function were assessed by echocardiography. Both Malat-1 WT and KO mice showed a significant increase in cardiac mass and wall thickness (Figure 1A-C). Impaired heart function was evidenced by reduced fractional shortening of the left ventricle and in some cases by backward failure leading to lung edema (Figure 1D-E). Importantly, the degree of hypertrophy and dysfunction did not significantly differ between WT and Malat-1 KO mice. Similarly, chronic infusion of angiotensin II for four weeks induced comparable degrees of hypertrophy and left ventricular dysfunction in Malat-1 WT and KO mice (Supplementary Figure S1A-F). A summary of animal characteristics, including organ weights can be found in Supplementary Table S1. In conclusion, absence of Malat-1 in mice does not affect the development of heart failure upon pressure overload.

Figure 1: Gross morphological and functional analysis of Malat-1 WT and KO hearts after TAC. Increased heart weight/tibia length (A) and diastolic LV wall thickness (B) confirm concentric hypertrophy in both Malat-1 WT and KO mice, without effects on diastolic LV inner diameter (C). Decreased fractional shortening (D) and lung congestion with increased lung weight/tibia length in some animals (E) indicate heart failure independent of Malat-1 deficiency. (F) Representative photographs of mouse hearts. Scale bar: 1 cm. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ TAC versus Sham.

Myocardial remodeling during pressure overload is independent of Malat-1

Hallmarks of maladaptive cardiac remodeling, including cardiomyocyte hypertrophy, interstitial fibrosis, capillary density, and immune cell infiltration was assessed in heart tissue slides four weeks after induction of pressure overload. Hypertrophy of cardiomyocytes was

apparent upon TAC and was unaffected by knockout of Malat-1 (Figure 2A). Similarly, AngII caused a comparable increase in cardiomyocyte area in both Malat-1 WT and KO mice (Supplementary Figure S2A). The fraction of interstitial collagen area as assessed by staining with Picrosirius Red was increased significantly after both TAC and AngII, and was independent of Malat-1 expression (Figure 2B and Supplementary Figure S2B). Capillary density was significantly reduced by TAC in WT mice and showed a similar trend in Malat-1 KO mice, signifying the transition towards heart failure (Figure 2C). However, no significant difference could be found between the two genotypes, suggesting no effect of Malat-1 deficiency on myocardial microvascular perfusion during pressure overload. Finally, while TAC increased the influx of CD45⁺ immune cells into the heart, Malat-1 deficiency did not affect the number of cardiac CD45⁺ cells (Figure 2D). In this genetic background, AngII had milder effects on capillary density and immune cell infiltration than TAC, but again no effects of Malat-1 were identified (Supplementary Figure S2C-D). Taken together, these findings show that Malat-1 is dispensable for cardiac remodeling upon pressure overload.

Afterload-induced expression of fetal genes is independent of Malat-1

Cardiac hypertrophy goes along with the upregulation of mRNA levels of the natriuretic peptides A (*Nppa*) and B (*Nppb*), and the cytoskeletal protein skeletal alpha actin (*Acta1*) in the myocardium. We found mRNA levels of all three hypertrophy markers to be significantly increased after TAC, whereas ablation of Malat-1 did not interfere with upregulation of these genes (Figure 3A-C). Next to transcriptional changes, cardiac hypertrophy and failure also induce alternative splicing of certain mRNAs. We measured the fraction of alternatively spliced mRNA of *Ndr2* and *Eif4h* and found that TAC as expected induced skipping of exon 3 of *Ndr2*, as well as inclusion of exon 5 of *Eif4h*^{31,32}. Interestingly, *Ndr2* showed a higher splice ratio in Malat-1 KO mice both with and without pressure overload (Figure 4A-C) confirming a role of Malat-1 in splicing, whereas the splicing pattern of *Eif4h* was not different between genotypes.

Figure 2: Histological analysis of LV and septal myocardium after TAC. Significant cardiomyocyte hypertrophy (A) and interstitial fibrosis (B) were induced by TAC, whereas the density of endomyocardial capillaries was mildly decreased (C). Infiltration of CD45 positive leukocytes was apparent after TAC, although this did not reach statistical significance in Malat-1 KO mice (D). Importantly, no significant differences could be detected between Malat-1 WT and KO mice for any of the histological parameters. * $p < 0.05$, ** $p < 0.01$ TAC versus Sham. Scale bars: Picrosirius Red: 1 mm; all other stains: 100 μm .

Figure 3: mRNA levels of hypertrophy markers. The hypertrophy marker genes *Nppa* (A), *Nppb* (B) and *Acta1* (C) were upregulated by TAC and not different between Malat-1 WT and KO mice. (D) Malat-1 itself was not significantly deregulated after TAC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ TAC versus Sham; \$\$\$\$ $p < 0.0001$ KO versus WT.

Figure 4: Alternative splicing of *Ndrp2* and *Eif4h* is evident after TAC. (A) Enhanced skipping of *Ndrp2* exon 3 is apparent after TAC in Malat-1 WT and KO mice, but absence of Malat-1 reduces exon skipping both at baseline and after pressure overload. (B) Exon 5 inclusion of *Eif4h* is induced by TAC but not affected by absence of Malat-1. (C) Representative images of PCR products of *Ndrp2* and *Eif4h*. * $p < 0.05$, **** $p < 0.0001$ TAC versus Sham; \$ $p < 0.05$ KO versus WT.

DISCUSSION

Long non-coding RNAs are emerging as important players in several pathologic conditions, such as cancer and cardiovascular disorders. The nuclear lncRNA Malat-1 is exceptionally well conserved among vertebrates and abundantly expressed in most organs and cell types investigated so far, implying an important role throughout evolution. However, different approaches to knock out Malat-1 in mice have shown no effect on development and normal life¹⁵⁻¹⁷, leading researchers to assume that Malat-1 becomes relevant only under stressed conditions. Heart failure is the final end-stage of many cardiac diseases and a major cause of death worldwide. We investigated the effect of genetic ablation of Malat-1 on molecular, histological, morphological, and functional changes during development of heart failure in two different mouse models. Although we confirm a role for Malat-1 in mRNA splicing, we found that this lncRNA has no crucial role in pressure overload-induced cardiac hypertrophy and failure.

In vivo, Malat-1 abrogation was recently found to cause aberrant vessel growth in a model of hind limb ischemia²². However, the functional relevance of this finding was not assessed in the study. In the pressure-overloaded heart, a transient increase in capillary density and concomitantly improved perfusion is indispensable for development of compensated hypertrophy and maintained cardiac function¹⁹. Therefore, we expected Malat-1 to accelerate heart failure development by impeding the compensatory response of capillary growth upon pressure overload. However, while four weeks of TAC resulted in heart failure with decreased capillary density, we found no role for Malat-1 in regulating cardiac capillary density both under normal physiological and under pressure-overloaded conditions. Next to vessel growth, Malat-1 is essential for proliferation and migration of several cancer cell lines and was reported to activate the ERK/MAPK growth signaling pathway²⁹, which is well-known for its central role in cardiomyocyte hypertrophy. Additionally, Malat-1 has been proposed to act as a competing endogenous RNA for microRNA-133, thereby attenuating miR-133 mediated repression of serum response factor (SRF)²⁴. The muscle-specific miR-133 has critical functions in the heart and is a powerful inhibitor of cardiac hypertrophy^{25,26,33}, and the transcription factor SRF is an important regulator of several hypertrophy associated genes, such as *Nppa*, *Nppb* and *Acta1*³⁴. However, our findings indicate no role of Malat-1 on transcriptional changes or on overall cardiac hypertrophy during pressure overload. These findings argue against a relevant influence of Malat-1 on ERK/MAPK signaling or miR-133/SRF regulation in the heart.

Cardiac pro-hypertrophic signaling together with cardiomyocyte damage leads to the activation of resident fibroblasts and deposition of interstitial connective tissue. Indeed, both AngII and TAC induced myocardial collagen deposition and ablation of Malat-1 did not affect this process, ruling out an important role of this lncRNA in fibroblast activation. Additionally, the number of CD45⁺ cells in the pressure-overloaded heart was not affected by presence or absence of Malat-1, although a role for Malat-1 in pro-inflammatory cytokine production by HUVECS has recently been suggested³⁵. On the cellular level, knockdown of

Malat-1 results in alternative splicing of several genes¹⁴, and in line with this it was found that Malat-1 co-localizes with the splicing factors ASF/SF2 in nuclear speckles of mouse embryonic fibroblasts and cultured neurons³⁶. Interestingly, a deficiency of ASF/SF2 has been shown to alter cardiomyocyte function by affecting splicing of calcium/calmodulin-dependent protein kinase II delta³⁷ and perturbation of mRNA splicing is a feature of heart failure³⁸. Interestingly, we found alternative splicing of *Ndrp2*, which shows skipping of exon 3 in hypertrophic mouse hearts³¹, to be less common in Malat-1 KO mice both at baseline and after pressure overload. In contrast, alternative splicing of *Eif4h* was apparent after pressure overload as previously reported³² but not affected by ablation of Malat-1. These data confirm that Malat-1 can influence splicing of individual mRNAs but do not indicate an important role of this effect in cardiac pressure overload. The named molecular and histological changes entail effects on cardiac morphology and function both in Malat-1 WT and KO mice. Our echocardiographic analysis is limited to systolic function, but in view of the comparable cardiomyocyte hypertrophy and myocardial fibrosis it appears unlikely that diastolic function is affected by absence of Malat-1. In conclusion, despite extensive phenotyping of cardiac function, morphology, histological appearance, and gene expression no important differences could be found in the hearts of Malat-1 WT and KO mice after TAC- or AngII-induced cardiac pressure overload.

While this manuscript was in preparation, another group reported that inhibition of Malat-1 may have protective effects on LV dilation and dysfunction in a model of streptozotocin-induced diabetic cardiomyopathy. This was associated with dampening of cytokine expression and cardiomyocyte apoptosis^{39,40}. However, no information was given on possible cell types and signaling pathways responsible for this effect, which impedes a proper comparison with other findings. Above that, the power and relevance of these two short reports are difficult to assess due to the lack of information on data presentation and statistical analysis. Therefore, more detailed knowledge is needed to reconcile a possible role of Malat-1 in diabetic cardiomyopathy with its insignificance during pressure overload-induced cardiac hypertrophy and failure.

Interestingly, only an effect of Malat-1 on vascularization of the retina was shown in Malat-1 KO mice²², whereas its functions in hind limb ischemia, ERK/MAPK signaling, miR-133 scavenging, and possibly diabetic cardiomyopathy were exclusively shown by post-transcriptional knockdown of Malat-1^{22-24,29,39,40}. It is therefore conceivable that compensatory pathways are activated in Malat-1 KO mice during embryonic development that allow for normal cardiac function and adaptation in our study. Inducible knockout strategies or deep RNA sequencing may help to circumvent and to identify possible compensatory mechanisms, respectively. Importantly, the nature of such compensatory changes would directly help to deduce the regular function of Malat-1. Above that, phenotypical changes upon deletion of a lncRNA can depend on the knockout strategy employed, which constitutes a major difficulty for investigating lncRNA functions *in vivo*⁴¹. Subtle phenotypical differences have been observed between the three different Malat-1

knockout lines generated so far⁴², and it is therefore conceivable that the promoter or other parts of the Malat-1 locus may have a function independent of the actual Malat-1 transcript. However, our results clearly show that the Malat-1 lncRNA transcript is dispensable during pressure overload induced cardiac hypertrophy and dysfunction. While we cannot exclude transcript-independent functions of the Malat-1 locus or the existence of compensatory mechanisms, our findings suggest no important role for Malat-1 in heart failure.

CONCLUSIONS

We deployed two mouse models of pressure overload-induced heart failure to investigate the function of the lncRNA Malat-1 in a highly relevant human disease. Despite its reported function as regulator of vascularization, activator of ERK/MAPK signaling, and scavenger for the muscle-specific miR-133, we conclude that Malat-1 has no important role for cardiac hypertrophy and failure *in vivo*. Our findings therefore stress the importance of validating proposed lncRNA functions in clinically relevant disease models.

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SUPPLEMENTARY FIGURES AND TABLE

Figure S1: Gross morphological and functional analysis of Malat-1 WT and KO hearts after AngII infusion. Increased heart weight/body weight ratio (A) and diastolic left ventricular wall thickness (B) without changes in diastolic left ventricular inner diameter (C) indicate concentric hypertrophy in both Malat-1 WT and KO mice. Decreased fractional shortening (D) without changes in lung weight/body weight ratio (E) indicates beginning of heart failure independent of Malat-1 deficiency. (F) Representative photographs of mouse hearts. Scale bar: 1 cm. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ AngII versus Sham.

Table S1: Characteristics of Malat-1 WT and KO mice after sham surgery or pressure overload.
 BW: body weight; TL: tibia length; SAP: systolic arterial pressure.

TAC Study						
		WT/Sham Mean ± SD	WT/TAC Mean ± SD	KO/Sham Mean ± SD	KO/TAC Mean ± SD	ANOVA
Sex	[m/f]	5/5	6/5	5/4	6/6	
Age at surgery	[weeks]	9.0 ± 0.4	9.1 ± 0.4	8.4 ± 0.4	8.6 ± 0.6	
Bodyweight at surgery	[g]	22.53 ± 2.28	22.49 ± 2.46	21.10 ± 3.39	21.63 ± 3.22	p>0.05
Tibia lenght (day 28)	[mm]	17.82 ± 0.23	17.83 ± 0.20	17.60 ± 0.49	17.66 ± 0.38	p>0.05
Heart weight	[mg]	103.10 ± 10.37	174.55 ± 31.97 ***	109.67 ± 22.02	157.58 ± 32.47 **	p<0.001
HW/BW	[mg/g]	4.58 ± 0.23	7.83 ± 1.58 ***	5.19 ± 0.41 \$	7.28 ± 1.01 ***	p<0.001
HW/TL	[mg/mm]	5.78 ± 0.55	9.79 ± 1.82 ***	6.21 ± 1.12	8.91 ± 1.74 **	p<0.001
Lung weight	[mg]	142.40 ± 4.58	231.91 ± 122.69	143.33 ± 18.46	233.50 ± 122.36	p<0.05
LW/BW	[mg/g]	6.36 ± 0.51	10.49 ± 5.87	6.85 ± 0.60	10.65 ± 5.13	p<0.05
LW/TL	[mg/mm]	7.99 ± 0.19	12.99 ± 6.82	8.12 ± 0.85	13.18 ± 6.81	p<0.05
Liver weight	[mg]	1133.30 ± 142.04	1023.73 ± 195.41	1030.00 ± 154.85	1080.00 ± 220.36	p>0.05
LiW/BW	[mg/g]	50.28 ± 3.17	45.44 ± 6.43	49.33 ± 6.66	49.92 ± 6.59	p>0.05
LiW/TL	[mg/mm]	63.57 ± 7.63	57.41 ± 10.88	58.44 ± 7.98	61.07 ± 11.83	p>0.05
Kidney weight	[mg]	144.15 ± 17.11	146.27 ± 20.07	158.00 ± 37.97	144.96 ± 22.88	p>0.05
KW/BW	[mg/g]	6.39 ± 0.22	6.49 ± 0.31	7.42 ± 0.70 \$\$	6.70 ± 0.46	p<0.01
KW/TL	[mg/mm]	8.08 ± 0.91	8.20 ± 1.11	8.94 ± 1.96	8.19 ± 1.18	p>0.05
Spleen weight	[mg]	79.70 ± 6.41	86.82 ± 19.04	83.13 ± 20.78	98.25 ± 29.44	p>0.05
SW/BW	[mg/g]	3.57 ± 0.49	3.91 ± 1.03	3.79 ± 0.82	4.63 ± 1.51	p>0.05
SW/TL	[mg/mm]	4.47 ± 0.36	4.86 ± 1.04	4.68 ± 1.11	5.56 ± 1.64	p>0.05
Heart rate during echo	[bpm]	521 ± 41	521 ± 49	506 ± 69	535 ± 33	p>0.05
Fractional shortening	[%]	38.81 ± 6.53	23.14 ± 11.99 **	37.01 ± 4.19	25.98 ± 9.75 *	p<0.001
Diastolic LV wall thickness	[mm]	0.89 ± 0.24	1.16 ± 0.18 **	0.89 ± 0.09	1.10 ± 0.15 **	p<0.001
Diastolic LV inner diameter	[mm]	3.72 ± 0.12	4.14 ± 0.64	3.85 ± 0.51	4.10 ± 0.34	p<0.05

Angiotensin II Study						
		WT/Sham Mean ± SD	WT/AngII Mean ± SD	KO/Sham Mean ± SD	KO/AngII Mean ± SD	ANOVA
Sex	[m/f]	5/4	5/6	0/8	5/5	
Age at surgery	[weeks]	11.9 ± 0.2	11.6 ± 0.5	12.0 ± 0.1	12.0 ± 0.1	
Bodyweight (day 0)	[g]	25.40 ± 4.40	24.19 ± 2.95	22.07 ± 1.15	23.02 ± 2.66	p=0.084
Tibia lenght (day 28)	[mm]	18.17 ± 0.30	18.18 ± 0.25	17.96 ± 0.29	17.93 ± 0.24	p=0.089
Heart weight	[mg]	119.89 ± 21.77	128.36 ± 16.11	100.00 ± 10.92	131.70 ± 17.99 **	p<0.01
HW/BW	[mg/g]	4.73 ± 0.36	5.33 ± 0.53 *	4.43 ± 0.38	5.73 ± 0.58 ***	p<0.001
HW/TL	[mg/mm]	6.59 ± 1.13	7.05 ± 0.82	5.56 ± 0.56	7.34 ± 0.96 **	p<0.01
Lung weight	[mg]	159.89 ± 9.23	165.82 ± 10.29	149.63 ± 8.33	174.20 ± 22.17 *	p<0.01
LW/BW	[mg/g]	6.44 ± 0.99	6.92 ± 0.68	6.65 ± 0.50	7.61 ± 1.00	p=0.075
LW/TL	[mg/mm]	8.80 ± 0.44	9.12 ± 0.49	8.33 ± 0.44	9.72 ± 1.25 **	p<0.01
Liver weight	[mg]	1198.78 ± 100.17	1174.82 ± 187.09	920.13 ± 157.94 \$	1043.70 ± 206.96	p<0.01
LiW/BW	[mg/g]	48.36 ± 8.38	48.79 ± 6.82	41.04 ± 7.49	45.96 ± 10.91	p=0.233
LiW/TL	[mg/mm]	65.97 ± 5.13	64.64 ± 10.35	51.24 ± 8.65 \$	58.21 ± 11.51	p<0.01
Kidney weight	[mg]	169.78 ± 38.19	153.00 ± 20.45	132.86 ± 15.91	151.10 ± 20.23	p>0.05
KW/BW	[mg/g]	6.64 ± 0.69	6.34 ± 0.69	5.99 ± 0.58	6.59 ± 0.77	p>0.05
KW/TL	[mg/mm]	9.32 ± 2.04	8.39 ± 1.06	7.40 ± 0.86	8.41 ± 1.07	p>0.05
Spleen weight	[mg]	86.67 ± 12.33	78.00 ± 13.99	97.88 ± 9.69	84.50 ± 13.71	p<0.05
SW/BW	[mg/g]	3.48 ± 0.64	3.26 ± 0.74	4.34 ± 0.46	3.74 ± 0.81	p<0.05
SW/TL	[mg/mm]	4.77 ± 0.64	4.29 ± 0.73	5.45 ± 0.52	4.71 ± 0.77	p<0.01
Heart rate during echo	[bpm]	501 ± 43	564 ± 32 *	474 ± 58	526 ± 47	p<0.001
Fractional shortening	[%]	39.48 ± 6.07	26.71 ± 6.36 **	39.54 ± 6.13	27.62 ± 9.67 **	p<0.001
Diastolic LV wall thickness	[mm]	0.84 ± 0.07	0.98 ± 0.12	0.86 ± 0.15	1.07 ± 0.10 **	p<0.001
Diastolic LV inner diameter	[mm]	3.98 ± 0.45	3.93 ± 0.45	3.57 ± 0.54	3.95 ± 0.38	p>0.05
Conscious heart rate	[bpm]	568 ± 82	638 ± 72	625 ± 57	610 ± 78	p>0.05
Conscious SAP	[mmHg]	160.90 ± 14.81	188.94 ± 22.13 **	153.73 ± 20.81	182.34 ± 13.49 *	p<0.001

*p<0.05 vs Sham

\$ p<0.05 vs WT

*p<0.05 vs Sham

**p<0.01 vs Sham

\$\$ p<0.01 vs WT

**p<0.01 vs Sham

***p<0.001 vs Sham

Chapter 6

Antiviral properties of mascRNA, a Malat1-derived non-coding RNA

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ABSTRACT

Background

Inflammation of the cardiac muscle due to viral infection (viral myocarditis, VM) predisposes to heart failure and death, but to date diagnosis and treatment of VM lack specificity. It is well established that not only viral replication itself but also the massive infiltration of activated immune cells is responsible for the damage inflicted to the heart during VM. Over the last years, long non-coding RNAs (lncRNAs) have emerged as regulators of cellular processes in health and disease and may provide a novel class of therapeutic biomarkers or targets during VM. The lncRNA Malat-1 has a known function in cancer progression but apart from that also gives rise to a small processed product, called mascRNA, to which no function has been assigned yet.

Results

We found mascRNA to be increased in VM in mice and to be deregulated by CVB3 infection in cardiomyocytes and immune cells *in vitro*. Ectopic expression of mascRNA prevented replication of CVB3 in isolated rat cardiomyocytes *in vitro*, probably by upregulation of *Ifit* and *Ifitm* genes as cellular defense line against viruses. In a mouse model of VM, overexpression of mascRNA led to an increased fraction of circulating lymphocytes and a reduced fraction of circulating neutrophils without major consequence on the heart. In contrast, injection of antisense oligonucleotides against mascRNA increased the fraction of circulating neutrophils and led to increased infiltration of leukocytes in the heart without affecting cardiac CVB3 levels.

Conclusions

Our data provide indications that mascRNA plays a role in the innate immune response after virus infections by affecting cellular defense mechanisms and/or regulating the recruitment of immune cells during VM. Further studies are warranted to investigate potential diagnostic or therapeutic value of mascRNA in viral myocarditis.

INTRODUCTION

Inflammation of the heart muscle (myocarditis) is an important cause of dilated cardiomyopathy, leading to heart failure and death^{1,2}. Viral myocarditis (VM) can be triggered by common viruses, such as parvovirus B 19 (PVB 19) and coxsackievirus group B serotype 3 (CVB3). To date a definite diagnosis of myocarditis can only be based on histological and molecular-biological analysis of endomyocardial biopsies (EMB), and in most cases VM is only recognized when first cardiac symptoms appear. Treatment of VM is still largely limited to standard heart failure therapy, although immunosuppression, immunomodulation, or antiviral therapies have shown promise². A key challenge is to develop treatment options that restrict viral replication without eliciting excessive immune response, because research has shown that destruction of cardiac tissue is not only a consequence of viral replication but to a large part results from collateral damage caused by infiltrating immune cells³. Considering the lack of specific and sensitive diagnostic biomarkers and the paucity of targeted therapy options, a better understanding of the molecular and cellular pathomechanisms of VM are much-needed.

Over the last few years, our knowledge about the mammalian transcriptome has changed dramatically. By now, the concept of pervasive transcription of the genome outside of protein-coding genes is widely accepted⁴, and especially the class of long non-coding RNAs (lncRNAs) has attracted much attention. lncRNAs are molecules of >200 nucleotides length that lack a significant open reading frame but still can be functional. lncRNAs have been shown to regulate translation, act as microRNA sponges, or serve as precursor for smaller ncRNAs. The long non-coding RNA Metastasis associated lung adenocarcinoma transcript 1 (Malat-1) is highly abundant in the nucleus of basically all cell types investigated so far and localizes to sites of active transcription⁵ as well as to nuclear speckles, where it is involved in mRNA splicing^{6,7}. Interestingly, Malat-1 also gives rise to Malat1-associated small cytoplasmic RNA (mascRNA), which is extremely well conserved between rodents and human. MascRNA is a 58-nucleotide-long ncRNA that is cleaved from the 3' end of Malat-1 by RNaseP and RNaseZ and subsequently CCA-tailed. It is hence processed in a similar way to tRNAs. However, mascRNA is smaller than regular tRNAs, has a relatively poorly conserved anti-codon loop, and is not aminoacylated in HeLa cells⁸, making it highly unlikely to act as tRNA. To date, no function could be assigned to this untypical small ncRNA. The aim of this study was to investigate a possible involvement of mascRNA in the pathophysiology of VM on the cellular and systemic level. We present evidence that mascRNA is upregulated by VM in mice and has a role in the cellular and systemic defense against VM by regulating the expression of antiviral genes in cardiomyocytes and regulating the recruitment of immune cells.

METHODS

Expression construct and viruses

Naturally occurring mascRNA is processed from its precursor Malat-1 by cleavage at the 5' and 3' site and subsequent addition of a 3' CCA-tail. For recombinant overexpression of mature mascRNA, the full mascRNA sequence including a genomically encoded CCA-tail was cloned into pAd5-TetO7 containing a U6 promoter. This construct was transfected into HEK293 cell, while the same vector containing an shRNA sequence targeting GFP served as control. Since primary cardiomyocyte are not easily transfected with plasmids, an adenovirus was generated from the plasmid, while empty vector DNA was used to produce a control adenovirus. For cardiac mascRNA overexpression *in vivo*, murine mascRNA-CCA was packed into adeno-associated virus serotype 9 (AAV9), which has a tropism for the heart and to some extent liver, skeletal muscle, and pancreas⁹. The CVB3 used for the experiments was derived from the Nancy strain (ATCC® VR-30) and produced at our own facility.

Microarray analysis after overexpression of mascRNA in HEK293 cells

HEK293 cells were cultured in high glucose DMEM (Gibco) supplemented with 10 % fetal calf serum (C.C. Pro) and penicillin-streptomycin (Sigma-Aldrich). $6 \cdot 10^5$ HEK293 cells were seeded on 6-well plates and after 24 hours transfected with 2 μ g plasmid DNA using Polyethylenimine (PEI). Changes in mRNA expression were analyzed after 72 hours using an Affymetrix Human Genome U133 Plus 2.0 Microarray.

Cell culture experiments

Neonatal rat cardiac myocytes (nRCMs) were isolated from 1-3 day old Lewis rat pups by enzymatic dissociation. After removing the atria, the ventricles were cut in 8-12 equally sized parts and digested with a mixture of 0.3 mg/ml collagenase (Sigma #C2674) and 0.3 mg/ml pancreatin (Sigma #P3292) in ADS buffer (in mM: 120 NaCl, 5 KCl, 0.8 MgSO₄, 0.5 KH₂PO₄, 0.3 Na₂HPO₄, 20 HEPES, 5.6 Glucose, pH 7.35) at 37°C. Every 20 minutes the supernatant was collected, suspended in 10% newborn calf serum (NBCS), and fresh enzyme solution was added to the residual tissue. After 5 rounds of incubation, cell solutions were pooled, centrifuged, re-suspended in plating medium (DMEM 11966, 17% M199 medium, 10% horse serum, 5% NBCS) and pre-plated onto 162cm² Corning Costar cell culture flasks (Sigma #CLS3151). After incubation in a cell culture incubator for one hour, the supernatant, containing mainly nRCMs, was collected and 10^6 cells per well were plated on gelatin coated 6-well plates. The next day, medium was changed to experimental medium supplemented with antibiotics (DMEM 11966, 20% M199 medium, 1.6 g/L glucose, 10 mg/L bovine serum albumin, 0.25 μ U insulin, 250 μ M carnitine, 10 μ M cytarabine) and the cells were incubated with adenovirus containing either an empty cloning site (Ad-ctrl) or the mascRNA-CCA sequence (Ad-mascRNA) for 2.5 hours. Unbound virions were washed away and cells were incubated in experimental medium overnight to allow transgene expression. The next day, the cells were infected with CVB3 at a multiplicity of infection of 1 and after eight hours the

medium containing unbound virions was replaced. For RNA isolation, cells were collected in mirVana lysis buffer (Ambion) at 24, 48, or 72 hours after CVB3 infection.

Knockdown of Malat-1 in nRCMs was achieved by transfection with a cocktail of three gapmers (Exiqon, 10 nM each) 24 hours prior to infection with CVB3. Unbound virions were removed after eight hours and after additional 16 hours cells were collected in mirVana lysis buffer for RNA isolation.

For expression analysis, RAW264 cells were stimulated with 10 ng/mL lipopolysaccharide (LPS) from *E. coli* for the indicated time. Bone marrow derived macrophages (BMDMs) were obtained by culturing mouse bone marrow for one week in the presence of L-cell-conditioned medium. BMDMs were then stimulated with 100 ng/mL LPS for 24 hours.

***In vivo* studies**

One week old C3H mouse pups received i.p. injections of AAV9-mascRNA or empty AAV9 control and three weeks later were infected with CVB3 to induce viral myocarditis (VM). To inhibit mascRNA *in vivo*, three week old male C3H mice received i.v. injections of 35 mg/kg LNA-modified mascRNA antisense oligonucleotides (ASO) or scrambled control (Exiqon) at days -1, +1 and +4 before and after infection with CVB3, respectively. One week after induction of VM, mice were anaesthetized and blood was collected from the vena cava and analyzed in a CELL-DYN 3700 (Abbot Diagnostics). Organs were collected, rinsed in PBS and fixed in zinc fixative (BD Pharmingen) or snap frozen for later RNA isolation. Mice were housed under social conditions with *ad libitum* access to water and standard chow. All animal experiments were carried out in accordance with Belgian law.

RT-PCR

Tissue samples were homogenized in lysis buffer using ceramic beads. RNA was isolated from cells and homogenized tissue using the mirVana miRNA isolation kit (Ambion) followed by treatment with DNase I (Ambion). An equal amount of RNA per sample was reverse transcribed using the miScript kit (Qiagen) and RT-PCR was performed using SYBR Green (Bio-Rad). Primer sequences are given in Table 1.

Statistics

Unless stated otherwise, data are presented as mean $\pm$ SEM. Statistical analysis was performed using Prism (GraphPad) and SPSS (IBM). Student's t-test was used to compare individual groups. For multiple comparisons, one-way ANOVA with Tukey's or Games-Howell post-hoc test was used to compare individual groups with equal or unequal variances, respectively. Comparison of several groups versus one control was performed using Dunnett's test.

Table 1: Primer sequences for RT-PCR.

Target	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
Rat mascRNA	CTGGTGGTTGGCACTCCT	AGACGCCGCAGGGACTTG
Rat Malat-1	ATGAGTTGGAAACGGGGAAT	CAGCTTTTCTTCACCTTCCTG
Rat Ifit1	CCGGAAAGGTGACATAAACG	CCTGCCTGTTCCAGATGTC
Rat Ifit2	GTCCCTTCAGCATCTTTGGA	GCTTGGTCCATGGAGTTGTT
Rat Ifit3	GCAGTTTCCCAGACCTGAAA	GCCAACAATCCAGCAAAAAT
Rat Ifitm1	CATGGCTATCCTCACGATCA	TAGAGTTTGGGGCAGACTGG
Rat Ifitm2	CCACCTCTGCGGTAGTCTTT	GTAAAGGGTCAAGCCCTCAG
Rat Ifitm3	TGCTCCTCGTCTCCAGACTT	AGTGTTACACCTGCGTGTCG
Rat Gapdh	GGTGGACCTCATGGCCTACA	CTCTCTTGCTCTCAGTATCCTTGCT
Mouse mascRNA	CTGGTGGCTGGCACTCCT	AGACACCGCAGGGACTTG
Mouse Ifit2	GTACCTTCAGCATCTTTGGA	TCTTGATCCAGGAAGTGGTT
Mouse Ifit3	GCAATTTCCCAGAAATGAAA	GCCAAGAATCCACCACAATT
Mouse Ifitm3	CGCTCAAAACCTTCACACTT	AGTGTTACACCTGCGTGTAG
CVB3	ACGAATCCCAGTGTTTGG	TGCTCAAAAACGGTATGGACAT

RESULTS

MascRNA is differentially regulated in viral myocarditis

In a screen for differentially regulated ncRNAs in CVB3-induced VM in mice we found mascRNA to be significantly upregulated in the heart (Figure 1A), while it was non-significantly increased in isolated cardiomyocytes after VM (Figure 1B). In addition, CVB3 infection significantly upregulated mascRNA in primary neonatal rat cardiomyocytes (Figure 1C). Besides cardiomyocytes, immune cells are a major cell type in hearts during VM. Therefore, we assessed mascRNA transcript levels in macrophages and found mascRNA to be reduced by LPS-stimulation in RAW cells, whereas no significant downregulation was observed in bone marrow derived macrophages (Figure 1D-E). Based on the expression data, we hypothesized that mascRNA may have an immune modulatory function in cardiomyocytes and/or immune cells during viral infection.

Figure 1: MascRNA is differentially regulated by pathogens and danger signals. MascRNA levels increased in whole mouse hearts after VM (A), with mild induction in the isolated cardiomyocyte fraction (B). CVB3 induced mascRNA 48 hours after infection of neonatal rat cardiomyocytes (C). LPS downregulated mascRNA in RAW cells (D) but had less pronounced effects in bone marrow-derived macrophages (E). Expression calculated as 2^{-CT} . *p < 0.05, ***p < 0.001 versus control.

Overexpression of mascRNA induces expression of antiviral genes and prevents amplification of CVB3

To test the effect of increased cellular mascRNA levels we transfected HEK293 cells with a mascRNA expression plasmid and assessed gene expression changes using an Affymetrix microarray. Among the transcripts most highly upregulated by mascRNA we found a striking predominance of antiviral genes, most of which are inducible by interferon (IFN) (Table 2). Importantly, IFN itself was not upregulated by mascRNA overexpression. Of note, the host transcript of mascRNA, Malat-1, was significantly downregulated by mascRNA overexpression, suggesting a negative feedback between mascRNA levels and host gene transcription.

Table 2: Microarray results after overexpression of mascRNA in HEK293 cells. MascRNA induces interferon-regulated genes and reduces expression of Malat-1.

Gene symbol	Gene name	Fold change
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	5.35
IFI44L	Interferon-induced protein 44-like	4.85
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	4.15
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	3.75
IFI6	Interferon-alpha-inducible protein 6	2.86
IFITM1	Interferon-induced transmembrane protein 1	2.56
IRF9	Interferon regulatory factor 9	2.50
MALAT-1	Metastasis associated lung adenocarcinoma transcript 1	-4.45

We next tested if mascRNA also regulates antiviral genes in cardiomyocytes and hence could protect them from CVB3 infection. To this end, we transduced neonatal rat cardiomyocytes with an adenovirus expressing mascRNA and subsequently infected the cells with CVB3. MascRNA levels were approximately 10-fold increased after adenoviral transduction, and in cells transduced with empty control adenovirus we again found a significant increase in mascRNA levels after 48 hours of infection with CVB3 (Figure 2A, compare to Figure 1C). Importantly, infection with CVB3 led to high levels of viral RNA in nRCMs receiving control adenovirus, whereas CVB3 levels remained strikingly low in mascRNA-overexpressing cells (Figure 2B). Induction of Ifit and Ifitm genes by overexpression of mascRNA largely mirrored the results found in HEK293 cells with Ifit2, Ifit3, Ifitm2, and Ifitm3 being significantly induced by mascRNA (Figure 2C-H). Interestingly, mascRNA seemed to prepare nRCMs to defend against a viral infection because further induction of Ifit or Ifitm genes, which was apparent in control transduced cells, was largely prevented by mascRNA.

Figure 2: Overexpression of mascRNA in nRCMs induces interferon-regulated genes and prevents viral replication. (A) MascRNA overexpression using an adenovirus was approximately 10-fold. When looking at control transduced cells, mascRNA was significantly induced by infection with CVB3. (B) The increase in viral RNA was drastically reduced in cells overexpressing mascRNA. (C-H) mascRNA led to baseline induction of Ifit2 & 3, and Ifit2 & 3, without affecting Ifit1 and Ifit1. Expression normalized to Gapdh. $^{\$}p < 0.05$, $^{SS}p < 0.01$, $^{SSS}p < 0.001$ CVB3 versus 24h no CVB3 (Dunnett's test); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ AdV-mascRNA versus AdV-Ctrl (t-test). Expression of CVB3 is relative to AdV-Ctrl condition after 24h of CVB3. Expression of all other genes is relative to AdV-Ctrl condition without CVB3.

Since overexpression of mascRNA can induce compensatory downregulation of its host transcript Malat-1, we tested if Malat-1 inhibition may have confounding effects on CVB3 replication. Preliminary data show that inhibition of Malat-1 in nRCMs using gapmers does not affect CVB3 levels 24 hours after infection (Figure 3). These data suggest that mascRNA, but not Malat-1, is involved in the cellular defense against viral infection.

Figure 3: Inhibition of Malat-1 in nRCMs using gapmers does not affect viral load after CVB3 infection. (A) Malat-1 was successfully knocked down by gapmers. (B) Viral RNA was detectable 24 hours after infection with CVB3 but was not affected by knockdown of Malat-1. Expression normalized to Gapdh. ** $p < 0.01$, *** $p < 0.001$.

AAV9-mediated overexpression of mascRNA does not reduce CVB3-induced myocarditis
We next tested if cardiac overexpression of mascRNA *in vivo* using an AAV9 vector would protect from CVB3-induced myocarditis. Surprisingly, histological analysis did not show differences in the area of leukocyte infiltrates in the heart after 7 days of VM (Figure 4A-B).

Figure 4: Cardiac transduction with AAV9-mascRNA does not influence myocardial immune cell infiltration in VM. (A) Representative H&E stained heart sections. (B) Quantification shows no effect of overexpression of mascRNA on infiltrated area.

AAV9 predominantly infects the heart and liver but not spleen or lymph nodes. However, we unexpectedly observed a slightly higher count of leukocytes, predominantly lymphocytes and monocytes, in the blood of mice receiving AAV9-mascRNA (Figure 5A-D). This led to a switch in the relative frequency of neutrophils and lymphocytes (Figure 5E-G). These data suggest that cardiac mascRNA levels play a role in “cardiosplenic” or “cardiomedullary” communication during VM that influences recruitment of immune cells from spleen or bone marrow.

Figure 5: Blood cell count of mice with VM after AAV9-mediated overexpression of mascRNA. (A) Count of white blood cells in CVB3-infected mice was mildly increased by AAV9-mascRNA. (B) Neutrophil count was unchanged by mascRNA overexpression, whereas lymphocyte (C) and monocyte count (D) were mildly increased. This led to a shift in relative abundance of neutrophils and lymphocytes (E-F), with negligible effect on monocyte relative frequency (G).

Systemic inhibition of mascRNA *in vivo* increases myocardial inflammation

Given the effect of mascRNA deregulation on immune cell subpopulations, we wondered if systemic manipulation of mascRNA would affect the outcome of VM. To this end, we injected mice with LNA-modified antisense oligonucleotides (ASO) against mascRNA (anti-mascRNA) or scrambled control ASO (Ctrl) on three days before and during infection with CVB3. Interestingly, inhibition of mascRNA led to more severe leukocyte infiltration of the myocardium after 7 days of VM as evidenced by a higher proportion of hematoxylin-stained cell nuclei in the tissue (Figure 6A-B). However, mascRNA inhibition had no direct effect on CVB3 levels, neither in heart or spleen (Figure 6C-D). In the blood, we found a non-significant increase in the number of circulating neutrophils after mascRNA knockdown, whereas

lymphocyte and monocyte count were unchanged (Figure 7A-D). This resulted in a switch in the relative frequency of neutrophils and leukocytes (Figure 7E-G), which was reciprocal to that observed after inhibition of mascRNA (Figure 5E-G) and suggests that mascRNA balances innate and adaptive immune system activation during VM. Surprisingly, we did not detect significant differences in the expression of *Ifit* or *Ifitm* genes in the heart or spleen of mice receiving mascRNA inhibitors (Figure 8A-F). Above that, we were not able to confirm knockdown of mascRNA in heart or spleen by RT-PCR (Figure 8G-H).

Figure 6: Myocardial leukocyte infiltration is increased after inhibition of mascRNA, but virus levels are not significantly altered. (A) Representative H&E stained heart sections. (B) Quantification shows significantly more infiltrated area after inhibition of mascRNA. CVB3 levels in heart (C) and spleen (D) were not significantly increased by mascRNA inhibition.

Figure 7 (next page): Blood cell count of mice with VM after inhibition of mascRNA. (A) Count of white blood cells in CVB3-infected mice was not influenced by inhibition of mascRNA. (B) Neutrophil count was non-significantly increased by mascRNA inhibition, whereas lymphocyte (C) and monocyte count (D) were unchanged. This led to a shift in relative abundance of neutrophils and lymphocytes (E-F), with no effect on monocyte fraction (G).

Figure 8: RT-PCR analysis of heart (top) and spleen (bottom) in mice with VM after inhibition of mascRNA. Expression of Ifit2 (A-B), Ifit3 (C-D), and Ifitm3 (E-F) was not significantly changed by mascRNA inhibitors in heart or spleen, although a mild increase in Ifitm3 levels in the heart was observed. Knockdown of mascRNA could not be proven by RT-PCR in heart or spleen (G-H). Expression calculated as 2^{-CT} .

DISCUSSION

In this study we have shown a putative role for mascRNA, a tRNA-like molecule derived from the lncRNA Malat-1, in the cellular and systemic defense against viral infection. We found that mascRNA is differentially expressed after CVB3 infection and regulates the expression of several genes involved in intracellular antiviral defense mechanisms, most importantly *Ifit* and *Ifitm* genes. *In vitro* studies revealed that overexpression of mascRNA can protect isolated cardiomyocytes from infection with CVB3. Furthermore, we provide first indications that modulation of mascRNA *in vivo* affects the relative abundance of circulating immune cells and may affect the severity of cardiac inflammation during VM. These findings are highly relevant for the emerging field of lncRNA research because to date hardly anything is known about the expression and function of mascRNA and mammalian tRNA-like RNAs in general.

Differential regulation of mascRNA in viral myocarditis

We found mascRNA to be differentially expressed under certain conditions of immune system activation, suggesting a role of this ncRNA in pathogen control. In whole mouse hearts, we found mascRNA to be upregulated after 7 days of VM, with a non-significant increase in the isolated cardiomyocyte fraction. This indicates that mascRNA is highly expressed by infiltrating immune cells or upregulated in resident non-cardiomyocytes. Infection of isolated nRCMs with CVB3 led to a significant increase of mascRNA levels after 48 hours. The variation in the response of cardiomyocytes *in vivo* and *in vitro* may be explained by differences in the duration of the infection or by different multiplicities of infection. Interestingly, we observed downregulation of mascRNA in LPS-stimulated macrophages, contrasting the upregulation in CVB3-infected nRCMs. This may relate to the pathogens used as stressor (bacterial LPS versus virus) or to the difference between immune cells and non-immune cells. These preliminary data warrant a detailed analysis of mascRNA expression in different cell populations of immune system and heart during VM.

MascRNA protects against CVB3 infection *in vitro*

Overexpression of mascRNA in HEK293 cells induced the expression of several IFN-regulated genes of the *Ifit*- and the *Ifitm*-family, without upregulation of IFN itself. This suggests antiviral properties of mascRNA because *Ifitm* and *Ifit* genes prevent viral entry and replication, respectively. However, IFITM proteins have only been reported to inhibit entry of enveloped viruses¹⁰ and further studies need to clarify how exactly mascRNA prevents entry and/or replication of the non-enveloped CVB3. Also the mechanism of gene regulation remains elusive, as mascRNA has been reported to exclusively localize to the cytoplasm⁸. In contrast, its endogenous precursor Malat-1 resides in the nucleus and inhibition of Malat-1 alters gene expression¹¹⁻¹³. Malat-1 expression was reduced in cells overexpressing mascRNA, suggesting a negative feedback mechanism, and it appears possible that downregulation of nuclear Malat-1 was involved in the observed transcriptional changes. However, adenoviral overexpression of mascRNA in nRCMs led to a striking reduction of CVB3 replication *in vitro*, whereas inhibition of Malat-1 prior to infection with CVB3 did not

affect viral RNA levels after 24 hours. Although we did not assess possible long-term effects of Malat-1 inhibition on CVB3 replication, these findings argue against an involvement of Malat-1 in the antiviral properties of mascRNA. Importantly, we used an adenoviral vector to transduce nRCMs, and it is possible that the expression of mascRNA from a virus genome in itself triggered an intracellular immune response. As control, we used an adenovirus lacking a transgene, instead of the more common GFP or LacZ control. The reasoning behind this choice is a matter of debate and is discussed in more detail below. However, since also plasmid-mediated overexpression of mascRNA induced expression of *Ifit* and *Ifitm* genes in HEK293 cells, we are confident that the preconditioning effect of mascRNA in nRCMs was not due to the adenoviral vector.

MascRNA affects the immune response towards CVB3 infection *in vivo*

Based on our findings *in vitro*, we reasoned that overexpression of mascRNA in cardiomyocytes would protect against VM *in vivo*. Surprisingly, infection of mice with AAV9-mascRNA prior to infection with CVB3 did not reduce migration of leukocytes into the heart nor did it influence cardiac viral levels. In contrast, administration of mascRNA antisense oligonucleotides (ASOs) led to increased immune cell infiltration of the myocardium, indicating a more severe course of disease after inhibition of mascRNA. Interestingly, the fraction of circulating neutrophils was reduced and increased in mice receiving AAV9-mascRNA and mascRNA ASOs, respectively. It is therefore tempting to postulate mascRNA as a modulator of the innate immune system. A possible mechanism, however, remains elusive because AAV9 has been reported to infect predominantly the heart, liver, skeletal muscle, and pancreas, with only negligible transfection of the spleen⁹, and we are not aware of any report on AAV9-mediated transduction of bone marrow. This suggests that humoral signals from the mascRNA-overexpressing heart (or possibly liver) can induce changes in the differentiation or mobilization of neutrophils.

Surprisingly, the viral load of heart or spleen was not significantly affected by mascRNA inhibition despite increased cardiac immune cell infiltration. This indicates a role for mascRNA in immune cell activation and recruitment rather than viral replication or clearance *in vivo*. If mascRNA indeed exerts its main function in immune cells this would also explain why cardiac-restricted modulation of mascRNA elicited weaker phenotypic changes than systemic modulation. Above that, cardiac mascRNA levels are high at baseline, and while adenoviral overexpression *in vitro* was strong enough to induce striking effects, AAV9-mediated overexpression *in vivo* may have been insufficient. In summary, based on the observed phenotypic changes we propose mascRNA as regulator of circulating immune cell populations *in vivo*.

Limitations and technical challenges of the study

When engaging in the novel field of lncRNA research we experienced some technical challenges and difficulties. First of all, we used to rely on RT-PCR based quantification of mascRNA but later found that this may be inaccurate. We were surprised to see that

myocardial or splenic knockdown of mascRNA could not be confirmed by RT-PCR, although the phenotypic effects of mascRNA ASOs *in vivo* suggested at least functional mascRNA inhibition, if not degradation. However, the almost completely closed tRNA-like structure of mascRNA may hamper binding of ASOs and above that impede reverse transcription. It is therefore impossible to tell apart if knockdown of mascRNA was inefficient or if our RT-PCR work-flow did not allow for correct quantification of mascRNA. Meanwhile, northern blot appears as a reliable technique to quantify mascRNA levels. However, inhibition of mascRNA without degradation cannot be accurately measured by northern blot either. Even visualization of mascRNA:ASO duplexes does not prove if this interaction happened intracellularly or only during the process of cell lysis and RNA isolation. Thus, proving inhibition of mascRNA is virtually impossible on the RNA level. We conclude that the observation of a phenotypic change upon manipulation of mascRNA is the most reliable indicator for effective treatment. However, the uncertainty about the tools to manipulate mascRNA comes at the cost that absence of a phenotype cannot be interpreted as absence of a function of mascRNA. Given the difficulty of establishing post-transcriptional inhibition, genomic deletion of mascRNA seems preferable. However, mascRNA is not expressed from an own promoter but processed from the 3' end of the lncRNA Malat-1⁸, which has a role in gene regulation and mRNA processing^{7,11-13}. Novel methods of genome engineering, such as the CRISPR/Cas-system, may be suitable to remove or replace the genomic mascRNA sequence in a targeted manner, without interfering with generation of a functional Malat-1 transcript. To date, however, the precise interplay of Malat-1 and mascRNA regarding biogenesis, turn-over, and cooperation are unknown, thus constituting another challenge of research on mascRNA function.

Off-target effects of virus-mediated protein overexpression are usually controlled for by expression of an unrelated protein, such as GFP or LacZ. However, effects of mascRNA cannot be compared to those of protein-coding transgenes, because the processing of the latter by mRNA processing machinery and ribosomes is very different from that of a non-coding RNA. One other short non-coding RNA, menRNA, is quite similar to mascRNA with regard to size, processing, and secondary structure^{14,15}, which at first glance makes it a suitable control for mascRNA studies. MenRNA is processed from the 3' end of the lncRNA Menβ, encoded at the Multiple endocrine neoplasia type 1 (MEN) locus, which is a paralog of Malat-1. However, this similarity also hampers its suitability as a control sequence for mascRNA studies. Often, ncRNAs are poorly conserved on the sequence level but better conserved in terms of spatial structure, and it is believed that ncRNA folding can determine its function^{16,17}. MenRNA and mascRNA differ in primary sequence but given their similar folding it cannot be excluded that both ncRNAs serve similar functions. Therefore, using menRNA as a control sequence for studying mascRNA functions may preclude finding of functions that are shared between menRNA and mascRNA. This calls for a control sequence that is very similar to mascRNA but folds differently. However, since the primary sequence determines the secondary structure of RNA molecules, such a control is difficult to design. Above that, mascRNA is highly conserved between mammals also regarding primary

sequence and we cannot exclude that the primary sequence of mascRNA is important for its function. Ideally, experiments to study the effect of mascRNA overexpression therefore need several control conditions including ncRNA molecules with (a) similar structure but not sequence, (b) different structure but similar sequence, and (c) completely unrelated sequence and structure. Above that, experimental designs that use different delivery methods for overexpression of mascRNA (viral transduction, plasmid transfection, genomic integration) can give complementary information about mascRNA function independent of its origin, therefore reducing the influence of off-target effects. These considerations illustrate the complexity of lncRNA research and the importance of proper study design for assigning functions to ncRNAs.

CONCLUSIONS

Here we report a possible role for the non-coding mascRNA in the immune response during viral myocarditis. Notwithstanding some limitations, we provide first evidence that overexpression of mascRNA may be protective during viral infection *in vitro* by inducing genes of the intracellular antiviral defense, such as Ifit and Ifitm genes. Above that, we found indications that mascRNA is involved in balancing the innate and adaptive immune response during viral myocarditis *in vivo*. Further studies are warranted to investigate (a) the binding partners through which mascRNA exerts its effects, (b) the regulation of mascRNA expression, and (c) the functional relationship between mascRNA and its host transcript Malat-1. Above that, the development of more effective tools to manipulate mascRNA levels *in vivo* in a targeted manner is desirable.

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Chapter 7

Summary and general discussion

The aim of this thesis was to extend our knowledge on the role of different non-coding RNA species in the development of heart failure (HF). Over the last years it has been acknowledged that multiple cell types are critically involved in HF, including cardiomyocytes, cardiac fibroblasts, and resident or infiltrating immune cells. This thesis comprises research on the involvement of these three cell types in HF with a focus on microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) as regulators of cell function. Only very recently, the cell type-specific effects of miRNAs in HF and their role in paracrine signaling have come to the center of attention¹⁻⁵. Here we extend this knowledge by identifying miR-139 as regulator of cardiomyocyte function and the miR-221/222 family as repressor of fibroblast activation. In comparison, the role of lncRNAs in the different cardiac cell types is to date essentially unknown. We performed an in-depth analysis of the development of HF in mice lacking the lncRNA Malat-1 but surprisingly did not find a relevant function in any of the mentioned cell types. In contrast, we propose mascRNA, a processed product of Malat-1, to act as regulator of the immune system during VM. The overall conclusion is that ncRNAs play distinct roles in different cell types that could be exploited for targeted therapy strategies in cardiac disease.

MIRNAS AS MODULATORS OF CARDIOMYOCYTE FUNCTION AND MYOCARDIAL FIBROSIS

In chapter 2 of this thesis we have shown that miR-139 is differentially expressed in human aortic valve stenosis and affects calcium handling in cardiomyocytes, probably by interfering with cAMP/PDE signaling. Stimulation of β -adrenergic receptors (β -AR) is a central physiological response to increased cardiac demands that evokes positive inotropic and lusitropic effects in cardiomyocytes⁶. The intracellular effect of β -AR activation is initiated by production of cAMP by adenylate cyclase, which leads to activation of cAMP effector proteins such as PKA and EPAC1, whereas phosphodiesterases (PDEs) terminate β -AR signaling by degrading cAMP. In HF, the adrenergic system is activated but the downstream effects are altered due to reduced number and function of the β -AR⁷ and deregulation of the intracellular signaling cascade, including PDEs⁸⁻¹¹. We validated the phosphodiesterases 3a, 4a and 4d as targets of miR-139 and found indications for increased phosphorylation of the L-type calcium channel in rat cardiomyocytes overexpressing miR-139. At the same time, miR-139 reduced spontaneous calcium release from the SR. These findings indicate that miR-139 is involved in calcium entry and intracellular cycling, although more detailed studies are needed to unravel the precise mechanism involved. *In vivo* we found that overexpression of miR-139 aggravated LV dilation after pressure overload. Defining the link between PDE repression and long-term effects after pressure overload *in vivo* was beyond the scope of this project because of the complexity of cAMP regulation and the multitude of possible miR-139 targets involved. Of note, it is conceivable that apart from affecting PKA signaling as assessed *in vitro*, miR-139 also influences transcriptional programs involved in cardiac hypertrophy, such as cAMP/EPAC1 signaling¹². Therefore, further studies are warranted to delineate the effect of miR-139 on cAMP/PDE signaling in the diseased heart.

Besides cardiomyocyte dysfunction, myocardial fibrosis is a hallmark of HF and negatively affects cardiac contraction, electrical conduction, and oxygen supply¹³. In chapter 3 we have reported that the miRNA-221/222 family correlates negatively with the level of cardiac fibrosis in patients with aortic valve stenosis. In a mouse model of cardiac pressure overload we showed that inhibition of miR-221/222 aggravates the development of cardiac dysfunction and fibrosis. These data indicate that high myocardial levels of miR-221/222 may protect against excessive fibrosis in cardiac disease. To unravel the mechanism behind this, we modulated expression of miR-221/222 in rat cardiac fibroblasts and found a repressive effect on TGF β -induced activation of fibroblasts into collagen-producing myofibroblasts. This effect is possibly mediated by the miR-221/222 target Ets1, a pro-fibrotic transcription factor that synergizes with TGF β ^{14,15}. While we show a detrimental role of miR-221/222 in HF by acting on fibroblasts, other groups have investigated cardiomyocyte-specific effects of miR-221/222: Liu et al. found that miR-222 is necessary for physiological cardiac hypertrophy and that its overexpression in cardiomyocytes attenuates ischemic injury and reduces fibrosis¹⁶. In contrast, Su et al. reported that cardiomyocyte-restricted overexpression of miR-221 leads to spontaneous development of heart failure due to cardiomyocyte dysfunction and death¹⁷. Taken together, the outcome of systemic miRNA inhibition as performed in our study differs from that of cell type-restricted intervention. The prevailing phenotype of systemic inhibition probably depends on the cell type with highest expression of the targeted miRNA, which calls for the development of more targeted delivery strategies as discussed further below.

LNCRNAS AS NOVEL PLAYERS IN HEART DISEASE

Several studies describe an important role for lncRNAs especially in cardiac development but also in heart disease as summarized in chapter 4. However, it remains challenging to identify lncRNAs with relevant functions in the heart and especially to define the precise pathway and binding partners mediating this function. Several lncRNAs have functions in the immune system¹⁸ but their role in cardiac inflammation had not been investigated so far. In chapter 6 we have provided first evidence for a protective role of mascRNA, a lncRNA-derived small ncRNA, in the regulation of the immune response during VM. We found mascRNA to induce cellular defense mechanisms against viral entry and replication by upregulating IFITM and IFIT genes. Above that, systemic manipulation of mascRNA affected circulating immune cell populations. MascRNA is therefore a novel player in the pathophysiology of VM and an interesting study object in immunology and infection research in general.

Surprisingly, we found the host transcript of mascRNA, the lncRNA Malat-1, to be dispensable for the development of pressure overload-induced HF. Malat-1 is a highly abundant nuclear lncRNA with remarkable evolutionary conservation. It has been reported to affect vascularization¹⁹, to regulate the abundance of the muscle-specific miR-133^{20,21}, and to activate the pro-hypertrophic ERK/MAPK pathway²². These reports notwithstanding, cardiac pressure overload in Malat-1 knockout mice had largely the same effect as in wild

type mice (chapter 5). We performed in-depth phenotyping including analysis of LV morphology, function, histology, and gene expression, all of which are known to be perturbed in HF. However, we only found an effect of Malat-1 ablation on splicing of *Ndr2*, which was apparently irrelevant for the clinical outcome. These unexpected observations suggest that the reported functions of Malat-1 as regulator of vascularization, scavenger of miR-133, and activator of ERK/MAPK signaling may be context-dependent and do not sum up to an important role of Malat-1 in cardiac hypertrophy and failure. Our results therefore stress that individually reported lncRNA functions need to be validated in complex disease models and highlight that sequence conservation and high expression level of a lncRNA do not necessarily indicate important (patho-)physiological functions.

NOVEL TOOLS FOR NOVEL RNA

MiRNAs are known to function primarily via target gene repression (except for anecdotal reports about enhanced target gene translation²³⁻²⁵), and there are established tools to predict and validate miRNA targets and to modulate miRNA levels *in vitro* and *in vivo* by use of miRNA mimics, inhibitors, viral vectors or antagomirs²⁶⁻²⁹. LncRNAs, in strong contrast to miRNAs, are a very heterogeneous class of RNA molecules with variable length, post-transcriptional processing, three-dimensional structure, intracellular localization, and mode of action as outlined in chapter 4. A first bioinformatic method to predict the function of lncRNAs has very recently been developed but is limited to effects on gene regulation³⁰. Therefore, research into lncRNA function so far relies on loss-of-function experiments by post-transcriptional knockdown or genomic deletion. Both approaches have their values but also some limitations, and novel approaches are needed to investigate this novel class of RNA.

LncRNAs may act in the cytoplasm or nucleus, and it is important to know the predominant location to design appropriate knockdown strategies. For example, siRNA-mediated knockdown is very efficient in the cytoplasm but its efficacy in the nucleus is controversial³¹⁻³³ and may relate to repression of transcription rather than post-transcriptional target degradation^{34,35}. Therefore, we made use of gapmers to knock down nuclear Malat-1 (chapter 6). Gapmers are antisense oligonucleotides with an LNA-DNA-LNA backbone that potently induces cleavage of nuclear lncRNAs by RNaseH^{36,37}. However, even with the correct approach, knockdown efficiency can be impaired by lncRNA secondary structure or binding partners that block the target site. For example, in chapter 6 we discussed difficulties to knock down the cytoplasmic mascRNA, probably due to its secondary structure. Nuclear lncRNAs can have functions in organizing nuclear structures (e.g. *Neat1* in paraspeckles³⁸), affect mRNA processing (e.g. Malat-1 in splicing^{39,40}), or regulate gene expression in *cis* or in *trans* (e.g. *XIST* and *HOTAIR*⁴¹). Importantly, gene regulatory effects may even be independent of the lncRNA transcript and emerge from the mere transcriptional activity at the lncRNA gene locus⁴²⁻⁴⁵. In that case, post-transcriptional knockdown of the lncRNA will either way fail to identify the function of the gene locus.

Next to antisense-mediated knockdown strategies, genomic deletion of lncRNAs is widely employed. However, interfering with genomic integrity at a lncRNA locus may remove or reorganize binding sites for regulatory factors, leading to off-target effects⁴⁶. For example, it has been shown that blocking transcription of the lncRNA *Fendrr* has different consequences than replacing the gene while keeping the locus transcriptionally active^{47,48}. Therefore, a careful study design including appropriate controls is mandatory in lncRNA research and elaborate considerations about the interpretation of *in vivo* studies have been summarized recently⁴⁶. Of note, positional off-target effects may also occur after genomic manipulation of coding genes, which has been carried out for several years. The lessons learned from lncRNA research may thus also have important implications for established methods in research on coding RNA.

Another layer of complexity is added by lncRNA processing and Malat-1 is a good example for this: The primary Malat-1 transcript is processed into a long nuclear-retained fragment with reported functions in gene transcription and mRNA splicing^{39,49-51}, but its 3' terminus also gives rise to mascRNA, a small cytoplasmic ncRNA for which no function has been published previously⁵². In chapter 6 we have described a role for mascRNA in VM, but disentangling the functions of Malat-1 and mascRNA remains challenging. Complete removal of the Malat-1 locus abolishes expression of mascRNA, whereas transcriptional blockade of Malat-1 may still allow for residual expression of downstream regions from an unknown promoter. Also post-transcriptional knockdown of a lncRNA may or may not lead to simultaneous depletion of its processed products, depending on the time frame between transcription and processing. Novel methods of genome engineering, such as the CRISPR/Cas-system, may be suitable for targeted removal of a lncRNA and/or its processed product from the genome.

In contrast to loss-of-function studies, overexpression of lncRNAs is rarely employed to date and is much more challenging than overexpression or mimicking miRNAs. The use of adeno-associated virus (AAV) vectors *in vivo* is well-established and different serotypes allow for some organ specificity in transgene delivery. In chapter 2 and 6 we employed AAV9 vectors for cardiomyocyte-specific overexpression, but these vectors are limited to transgenes smaller than 4.5 kb²⁹ and some lncRNAs including Malat-1 are simply too large to fit into viral vectors. Above that, transgene delivery is futile for lncRNAs with a *cis*-regulatory function that depends on correct chromosomal location. Interestingly, methods to enhance expression of endogenous genes by modified zinc finger proteins^{53,54} and TALEs^{55,56} have been developed in the last years. Especially the recently described CRIPR/Cas-mediated gene activation system may provide an easily applicable tool to overexpress lncRNAs from the endogenous locus⁵⁷.

THE BROAD FIELD OF NCRNA RESEARCH: WHICH WAY TO GO?

Our growing knowledge about miRNA function and the impressive potency of miRNA modulation in animal models has quickly elicited interest of pharmaceutical companies, and first miRNA-based therapeutics are already being developed and tested⁵⁸⁻⁶⁰. The transition from discovery to clinical testing has happened with remarkable speed: The first miRNA, lin-4, was discovered in 1993⁶¹ and 5 years later the concept of RNAi was introduced⁶², although the term “microRNA” was only coined in 2001⁶³⁻⁶⁵. Already in 2008, the applicability of miRNA inhibitors in primates was tested⁶⁶ and soon followed by a clinical phase 2a trial in humans, which showed promising results for the treatment of hepatitis⁶⁷. To date, we are clearly able to effectively inhibit miRNAs *in vivo*, and by now also the first miRNA mimic has entered a phase 1 clinical trial⁶⁸. However, this haste in exploiting the therapeutic potential of miRNAs also calls for a sober view on the potential limitations and dangers. Possible adverse effects include activation of the immune system by exogenous RNA and off-target effects by unintentional modulation of unrelated miRNAs⁶⁰. Above that, our results and the growing knowledge about cell type- and context-specific miRNA functions highlight the possibility of side effects by manipulating the intended miRNA in unintended cell types or organs. In chapter 3 we described anti-fibrotic effects of the miR-221/222 family in cardiac pressure overload, putting miRNA mimics forward as therapeutic option to dampen fibroblast activation. However, another group has shown that overexpression of miR-221 in cardiomyocytes causes cell death and leads to HF¹⁷. Above that, the miR-221/222 family can suppress or promote malignant diseases, depending on the type of tumor⁶⁹. In view of the Janus-faced function of miRNAs, possible off-target effects need to be carefully assessed and advances in targeted delivery methods are much-needed before the full potential of miRNA-based medicine can safely be harnessed^{60,70}.

While the first miRNAs enter the clinic, the involvement of lncRNAs in (patho-)physiological processes in the heart has only been investigated in the last few years. In chapter 4 we have summarized the progress that has been made in assigning functions to individual lncRNAs and their involvement in cardiac development, function, and disease. We showed that research on lncRNAs in cardiac disease is still in its infancy but offers a new opportunity to develop biomarkers and treatment options in heart failure. In the years while I was preparing this thesis, the number of publications on lncRNAs has steeply increased from about 100 publications indexed in PubMed in 2011 to more than 700 in 2014. lncRNAs are clearly a “hot topic”, and interest in this area was boosted in 2012 by multiple publications from the ENCODE consortium (Encyclopedia of DNA Elements), stating that the vast majority of the genome is not silent “junk” as previously considered⁷¹ but transcribed and functional^{72,73}. However, it did not take long until a debate about the interpretation of ENCODE arose^{74,75}, although part of the controversy may relate to definitions and semantics. The current interest in lncRNAs exemplifies the problem of “hypes” in scientific research, leading to high expectations and possibly a one-sided and misleading interpretation of novel observations. The lncRNA Malat-1 is remarkably well conserved, highly expressed in most

tissues, and has a function in synaptogenesis⁴⁰. Therefore, expectations were high that genomic deletion of Malat-1 would have severe consequences. Three independent groups made an effort to generate Malat-1 knockout mice, but surprisingly all three strains develop and breed normally^{51,76,77}. Somewhat later, several reports indicated a role for Malat-1 in cardiac hypertrophy but we found Malat-1 to be dispensable for the development of pressure overload-induced cardiac hypertrophy and failure (chapter 5).

Obviously, several highly important lncRNAs have been identified to date, but it remains questionable if they constitute the “tip of the iceberg” of functional lncRNAs, or if most lncRNAs are in fact redundant or even non-functional. A publication from Sauvageau et al. exemplifies this conundrum and illustrates the problem of “misleading interpretation” as mentioned above. In their methodologically remarkable and insightful study “Multiple knockout mouse models reveal lincRNAs are required for life and brain development”, the authors generated 18 lncRNA knockout mice and found clear abnormalities in 5 of them, indeed suggesting that some lncRNAs are crucial for normal development⁴⁸. However, in a detailed comment, Claudiu Bandea criticizes the misleading title and emphasizes that actually “most lncRNAs (i.e. 13 out of 18) do not appear to play critical roles *in vivo*”⁷⁸. In conclusion, research of the last years has identified several highly interesting lncRNAs but the true potential of lncRNAs to revolutionize biomedical research is still difficult to assess.

CONCLUDING REMARKS

This thesis provides a comprehensive picture of the cell types involved in the development of HF and highlights how ncRNAs can affect cell type-specific pathological mechanisms. It is evident from our data and from the large amount of work done by other groups that ncRNAs are crucial regulators of cell function and consequently important players in heart failure. The levels of knowledge, however, differ greatly when it comes to compare miRNAs and lncRNAs. Several miRNAs are known to play important roles in the development of HF, and we have contributed some more puzzle pieces to this knowledge. The utilization of miRNA-based therapeutics for HF appears to be a matter of time despite some hurdles that still need to be taken. In contrast, research on lncRNAs in heart failure is still in its infancy and is hampered by methodological challenges and the heterogeneity of lncRNA function. However, progress in science depends on sailing unknown seas and with the discovery of lncRNAs there is now a New World to explore.

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Valorization

SOCIETAL RELEVANCE

Cardiovascular diseases (CVD) are today the most common cause of death worldwide¹ and almost everyone has experienced neighbors, friends, or relatives dying from heart attack, stroke, or heart failure. The burden of CVD lies especially in the suffering of the patients and the grief of the relatives, but objectively speaking also in major health care costs, amounting to more than 100 billion euros in the EU in 2009². Several cardiovascular diseases, most importantly ischemic heart disease, hypertension, and inherited or acquired cardiomyopathies ultimately result in heart failure (HF), a disabling condition in which the heart cannot maintain sufficient blood flow through the circulation. The consequences for the patient begin with shortness of breath during physical activity and progress into complaints at rest, the inability to walk, and eventually death. Standard treatment of heart failure currently relies on reducing the hemodynamic strain on the heart and to improve muscle contraction. However, the prognosis for HF patients is very poor with reported 5-year mortality ranging from 40% to 65%^{3,4}. A better option than treating HF would be to prevent the progression of the underlying disease but our knowledge about the determining molecular mechanisms are limited. The aim of this thesis was to contribute to a better understanding of the molecular features of HF and its development due to cardiac pressure overload or viral myocarditis.

TARGET GROUPS

The focus of this thesis is on HF as consequence of pressure overload or viral infection. In 2014, the global prevalence of hypertension was 20-25%⁵, and the incidence of myocarditis is estimated to be up to 12%⁶. These numbers indicate that a large fraction of the general population may at some point suffer from HF and underlines the importance of basic research in this field. The ultimate target groups of this thesis are therefore patients with hypertensive heart disease or myocarditis that are at risk of developing HF. Above that, the findings may be transferrable to HF of different origin in which similar mechanisms are activated in the same cell types.

INNOVATION, PRODUCTS, AND IMPLEMENTATION

This thesis presents data from basic research, which only in rare cases results in directly applicable findings. However, the acquired knowledge will directly contribute to the expertise of the researchers involved, allowing them to further advance our understanding of the molecular mechanisms of HF. In addition, the resulting publications will enable scientists world-wide to build on the research presented in this thesis. The long-term societal value of basic research is often difficult to predict but in the following I will try a brief prognosis on how our finding may be helpful in the future.

The improvement of diagnostic tools and treatment options for HF will depend on a comprehensive knowledge of the cellular and molecular disease mechanisms. This thesis contributes to a better understanding by describing the role of three different non-coding RNAs in HF:

1) Cyclic adenosine monophosphate (cAMP)-signaling is deregulated in HF and inhibition of cAMP-degrading phosphodiesterases (PDEs) was thought to strengthen the failing heart. On the long run, however, the side-effects of PDE inhibition overshadowed the benefits⁷. MiR-139 may constitute an innovative target to improve cardiac contractility by specifically regulating certain PDE isoforms and thereby fine-tuning subcellular cAMP compartmentalization with reduced side-effects (chapter 2).

2) The miR-221/222 family is a fine-tuner of fibroblast function and may allow for adjusting the level of fibrosis in the myocardium (chapter 3). This is important because connective tissue is critical to stabilize wounds, for example after myocardial infarction, but is also involved in arrhythmogenesis and myocardial stiffening in HF⁸. Inhibition or activation of the miR-221/222 family as fine-tuner of fibroblast function could therefore be beneficial for stabilizing infarcted myocardium or preventing excessive scarring of hypertrophic hearts, respectively.

3) Infection with enteroviruses, such as CVB3, only induces mild symptoms in the vast majority of people but in some cases leads to massive inflammation of the heart that can progress to dilated cardiomyopathy and heart failure. A better knowledge of the molecular determinants of VM could help to explain this discrepancy and to develop better diagnostic tools and treatments. Malat1-associated small cytoplasmic RNA (mascRNA) is the first reported lncRNA-derived regulator of VM and therefore an innovative target with possibly broad applicability in viral diseases (chapter 6).

Future steps will have to confirm the findings from this thesis in larger animal models and human tissue samples. After successful translation, the named non-coding RNAs can be defined as possible targets for pharmaceutical research companies. Above that, further research into molecular pathways related to these candidates may uncover additional pharmaceutical target RNAs and proteins. Currently, there are several companies working on RNA-based therapeutics and the first drugs targeting microRNAs are in clinical trials^{9,10}. It remains to be seen how the findings from this thesis can be utilized for the development of applicable drugs.

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Curriculum vitae

Tim Peters was born on 09 May 1986 in Hannover, Germany. In 2005, he received his university-entrance diploma from the secondary school Sophienschule in Hannover. After his civilian service at Hannover Medical School he enrolled in the study program Molecular Medicine at the Georg-August-University Göttingen (Germany) in 2006. He received his B.Sc. degree in 2009 as best of class and completed the consecutive M.Sc. studies “with honors” in 2011.

During his B.Sc. studies, Tim Peters completed research internships at the University of Seville (Spain) and St Thomas' Hospital London (England), where he worked on brain function and skeletal muscle fatigue, respectively. He completed his bachelor's thesis in the group of Prof. Dr. Lars Maier at the University Medical Center Göttingen on the role of CaMKII in cardiomyocytes. His interest in cardiovascular research also led him to the Queen's Medical Research Institute Edinburgh (Scotland) during his M.Sc. studies, where he completed an internship about the hypertensive Cyp1a1-Ren2 transgenic rat. In 2010/11, he prepared his master's thesis at Bayer HealthCare (Wuppertal, Germany) on the topic of stent thrombosis. Later in 2011, Tim Peters started his PhD training in the group of Prof. Dr. Stephane Heymans at CARIM School for Cardiovascular Diseases (Maastricht, The Netherlands) under the mentoring of Dr. Blanche Schroen. During his PhD training he focused on the role of different short and long non-coding RNAs in the development of cardiac hypertrophy and failure.