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Celik, Selvi

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SELVI CELIK
DIVISION OF CARDIOLOGY, IKVL | FACULTY OF MEDICINE | LUND UNIVERSITY

FACULTY OF
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Translational studies of cardiac non-coding RNAs

Selvi Celik

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DOCTORAL DISSERTATION

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Abstract

Cadiovascular disease (CVD) is the leading cause of death worldwide. CVDs such as ischemic heart disease and hypertension often progress into heart failure, a clinical syndrome characterized by inability of the heart to maintain sufficient output to meet the demands of the body that is associated with considerable mortality and morbidity. Additional therapeutic alternatives to improve outcomes in heart failure are needed.

Many biological pathways have been explored for a role in heart failure, but relatively few studies have examined non-coding RNAs. In humans, only 2 % of the transcribed genome is protein-coding. The non-coding transcriptome comprises hundreds of thousands of transcripts with distinct functions, including microRNAs (miRNAs), small non-coding RNAs that inhibit gene expression by posttranscriptional regulation, and natural antisense transcripts (NATs), long non-coding RNAs (lncRNAs) transcribed antisense to a protein-coding gene and with diverse regulatory functions. The regulatory capacity and tissue-specific expression profiles of many miRNAs and NATs make them promising therapeutic targets. The aim of this thesis was to investigate potential roles of cardiac non-coding RNAs in the context of ischemic heart disease and heart failure. In paper I-III, we focused on non-coding RNAs regulating the natriuretic peptide system, representing potential therapeutic targets in heart failure, while in paper IV we investigated cardioprotective mechanisms involving lncRNAs.

In paper I, we show that the antisense transcript NPPA-AS1 is a negative regulator of its sense transcript NPPA in human cardiomyocytes, achieved via recruitment of the transcription factor REST. In vivo inhibition of NPPA-AS1 resulted in increased levels of circulating ANP and lower blood pressure. In paper II, we screened for miRNAs regulating the activity of CORIN, the transmembrane protease that produces the biologically active form of ANP. miR-1-3p was identified as a regulator of ANP biogenesis through direct interaction with CORIN mRNA and additional transcriptional regulators of NPPA. In paper III, we studied miRNAs involved in ANP biogenesis and could confirm a negative effect of miR-105, miR-155 and miR-425 on NPPA gene and ANP protein levels in human cardiomyocytes. In paper IV, we show that the lncRNA Neat1 is involved in modulating the sensitivity of cardiomyocytes to hypoxia-induced injury via decreased primary miRNA processing.

In summary, this thesis contributes new knowledge about non-coding regulatory mechanisms in cardiomyocytes, which could inform new RNA-based therapeutic strategies related to CVD and heart failure.

Key words Cardiovascular disease, heart failure, non-coding RNA, miRNA, lnc-RNA, atrial natriuretic peptide

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."– Marie Curie

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II. **Celik S**, Karbalaei-Sadegh M, Rådegran G, Smith JG, Gidlöf O. Functional Screening Identifies MicroRNA Regulators of Corin Activity and Atrial Natriuretic Peptide Biogenesis. *Mol Cell Biol*. 2019 Nov 12;39(23). pii: e00271-19. doi: 10.1128/MCB.00271-19.

III. **Celik S**, Smith JG, Gidlöf O. Identification of stretch-sensitive microRNAs regulating the expression of atrial natriuretic peptide. *Manuscript.*

IV. Olof Gidlöf, Kerstin Bader, **Selvi Celik**, Shinichi Nakagawa, Tetsuro Hirose, Bernhard Metzler, Björn Olde, David Erlinge. Inhibition of the long non-coding RNA NEAT1 protects human cardiomyocytes from hypoxia via decreased pri-miRNA processing. *Manuscript in revision*.

Papers not included in the thesis

Knutsson A, Hsiung S, **Celik S**, Rattik S, Mattisson IY, Wigren M, Scher HI, Nilsson J, Hultgårdh-Nilsson A. Treatment with a GnRH receptor agonist, but not the GnRH receptor antagonist degarelix, induces atherosclerotic plaque instability in ApoE(-/-) mice. *Sci Rep.* 2016 May 18;6:26220. doi: 10.1038/srep26220. PMID: 27189011; PMCID: PMC4870555.

Gidlöf O, Johnstone AL, Bader K, Khomtchouk BB, O'Reilly JJ, **Celik S**, Van Booven DJ, Wahlestedt C, Metzler B, Erlinge D. Ischemic Preconditioning Confers Epigenetic Repression of Mtor and Induction of Autophagy Through G9a-Dependent H3K9 Dimethylation. *J Am Heart Assoc.* 2016 Dec 22;5(12):e004076. doi: 10.1161/JAHA.116.004076. PMID: 28007739; PMCID: PMC5210409.

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Egerstedt A, Berntsson J, Smith ML, Gidlöf O, Nilsson R, Benson M, Wells QS, **Celik S**, Lejonberg C, Farrell L, Sinha S, Shen D, Lundgren J, Rådegran G, Ngo D, Engström G, Yang Q, Wang TJ, Gerszten RE, Smith JG. Profiling of the plasma proteome across different stages of human heart failure. *Nat Commun.* 2019 Dec 20;10(1):5830. doi: 10.1038/s41467-019-13306-y. PMID: 31862877; PMCID: PMC6925199.

Populärvetenskaplig sammanfattning

Idag är hjärt- och kärlsjukdomar den ledande dödsorsaken i Sverige och i världen. Många av dessa sjukdomar, så som hjärtinfarkt och högt blodtryck, kan leda till hjärtsvikt. Då orkar inte hjärtat att pumpa tillräckligt med syre- och näringsrikt blod ut till kroppens alla organ. I Sverige har omkring 200 000 personer hjärtsvikt och risken för hjärtsvikt ökar med åldern. Prognosen efter en hjärtsviktsdiagnos beror bl.a. på ålder och andra underliggande sjukdomar, men sjukdomen kräver oftast livslång behandling. Hjärtsvikt innebär ofta låg livskvalitet med upprepade besök på sjukhus för symptom så som andningssvårigheter och vätskeansamling i benen, vilket i sin tur bidrar till stora samhällskostnader och hög belastning på vården.

Idag finns det inga botemedel för hjärtsvikt, bara behandlingar som lättar på hjärtats belastning, sänker blodtrycket och därmed minskar symtomen. Ibland är detta inte tillräckligt och man måste då genomgå en pacemaker-operation eller i riktigt svåra fall genomgå en hjärttransplantation, där i många fall patienter avlider i väntan på ett passande hjärta.

Hjärtat är beroende av fungerande hjärtmuskelceller, så kallade kardiomyocyter, för sin förmåga att pumpa ut blod. Kardiomyocyterna påverkas molekylärt och strukturellt som ett svar på den ökade belastning som hjärtsvikt innebär, processer som på sikt leder till ett stelare och sämre fungerande hjärta. Idag saknas behandlingsmål som kan förhindra dessa processer helt.

Större delen av vår arvsmassa kodar inte för proteiner, utan resulterar istället i s.k. icke-kodande RNA. Förhållandevis lite är känt om dessa icke-kodande RNA, men de fungerar i stor utsträckning som naturliga bromsar på uttrycket av protein-kodande gener. Att med hjälp av speciella läkemedel lätta på dessa bromsar skulle kunna användas som en ny typ av behandlingsstrategi vid vissa sjukdomstillstånd, däribland hjärtsvikt.

Syftet med denna avhandling har varit att utforska och förstå sjukdomsmekanismer som involverar icke-kodande RNA i det sviktande hjärtat för att på så sätt kunna identifiera nya och mer specifika behandlingsstrategier för hjärtsvikt.

Vi har i den här avhandlingen jobbat med humana celler i en hjärtsvikts-lik miljö, hjärtvävnad från patienter samt djurförsök. Med hjälp av dessa verktyg har vi studerat olika sjukdomsrelaterade mekanismer i kardiomyocyterna och kunnat identifiera kroppsegna icke-kodande RNA som på olika sätt kan utnyttjas för att skydda och minska belastningen på hjärtat. I de 3 första arbetena fokuserade vi på olika sätt att öka nivåerna av hjärt-hormonet atriell natriuretisk peptid (ANP) som har till uppgift att minska belastningen på hjärtat genom att minska blodtryck och blodvolym. I det första arbetet undersökte vi betydelsen av ett långt icke-kodande

RNA för produceringen av detta hjärt-hormon. Här kunde vi se att det icke-kodande RNA:t har en negativ påverkan på produktionen av hormonet. I det andra arbetet inriktade vi in oss på s.k. mikroRNA (miRNA) som kan ha viktiga egenskaper i regleringen av aktivering av hjärthormonet. miRNA är ett kort RNA-transkript som inte kodar för ett protein men spelar viktiga roller i olika biologiska mekanismer. Vi kunde se att ett hjärt-specifikt miRNA har en negativ effekt på molekylen som aktiverar hjärthormonet. I det tredje arbetet undersökte vi egenskaperna av tre andra miRNA och kunde visa att de har en negativ påverkan på produceringen av hjärthormonet och att sträckning av cellerna påverkar deras uttryck. I det sista delarbetet kunde vi identifiera ett annat lång icke-kodande RNA som är involverat i att skydda kardiomyocyterna från syrebrist.

Denna avhandling bidrar med ny kunskap kring olika sjukdomsmekanismer i hjärtats celler, som på sikt kan leda till bättre och effektivare behandlingsmetoder för hjärtsvikt och ischemisk hjärtsjukdom.

Selected Abbreviations

3' UTR	3 prim untranslated region
ANP	Atrial natriuretic peptide
ARNI	Angiotensin receptor-neprilysin inhibitor
BNP	Brain natriuretic peptide
cGMP	Cyclic guanosine monophosphate
ChIP	Chromatin immunoprecipitation
ChIRP	Chromatin isolation by RNA purificaion
CNP	C-type natriuretic peptide
CVD	Cardiovascular disease
HF	Heart failure
IPC	Ischemic precondition
iPS	Induced pluripotent stem
lncRNA	Long non-coding RNA
miRNA	micro RNA
NAT	Natural antisense transcript
NPR	Natriuretic peptide receptor
PCI	Percutaneous coronary intervention
qRT-PCR	Quantitative real time-polymerase chain reaction
ROS	Reactive oxygen species
siRNA	Small interfering RNA

Introduction

The Heart

The human heart is a muscular organ, located between the lungs in the chest, which pumps blood to the organs through the circulatory system. It consists of four chambers; left and right atria and left and right ventricles (Figure 1). The heart also contains 4 valves between the different chambers to prevent backflow of the blood; the tricuspid valve between right atrium and ventricle, the pulmonary valve between right ventricle and pulmonary vein, the mitral valve between left atrium and ventricle and the aortic valve between left ventricle and aorta. The heart is protected by the pericardium, a protective sac containing a small amount of fluid, which reduces friction between the heart and the surrounding structures. The heart muscle wall is made up of three different layers; the epicardium, an outer protective layer, the myocardium, a middle layer containing the cardiac muscle cells named cardiomyocytes, and the endocardium, the inner layer which lines the inner cavity of the heart chambers.
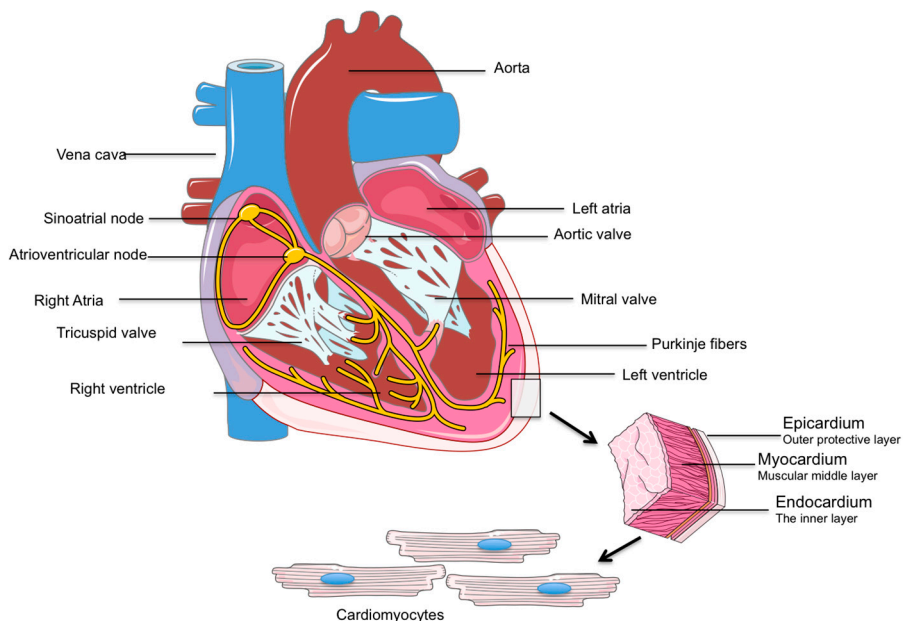

Figure 1. Anatomy of the human heart. Parts of the figure were based on images from Servier Medical Art.

A cardiac cycle is the time between two heartbeats, consisting of a relaxation and a contraction event. The cardiac contraction is coordinated by an electrical impulse, generated in the pacemaker cells of the sinoatrial node in the right atrium, which subsequently travels through the atrioventricular node and a specialized conduction system, along its course triggering contraction of the heart. During the relaxation period, called diastole, deoxygenated blood from the systemic circulation first enters the right atrium of the heart through the venae cavae and then passes on to the right ventricle. During contraction, or systole, the blood is pumped into the pulmonary circulation to exchange carbon dioxide for oxygen. The oxygenated blood enters into the left atrium in diastole, passes to the left ventricle and is then pumped out to the body through the aorta in systole.[1] Diastole by definition starts when the aortic valve closes and ends when the mitral valve closes. Systole begins when the mitral valve closes and ends with the closure of the aortic valve.[1, 2]

Diseases of the heart

Diseases or conditions affecting the heart and the blood vessels are collectively referred to as cardiovascular disease (CVD) and represent the leading cause of death worldwide.[3] Of particular importance in developed countries are diseases of the vasculature, specifically atherosclerotic vascular disease and hypertension, which both may result in the end-stage of all heart disease, heart failure.

Ischemic heart disease

Atherosclerosis is a disease process within the arterial wall where lipid-filled macrophages, also called foam cells, accumulate and form a plaque, resulting in narrowing of the lumen. The rupture of an atherosclerotic plaque triggers the formation of a thrombus, leading to complete or partial occlusion of the vessel. Occlusion of a coronary artery results in myocardial infarction, also often referred to as a heart attack.[4] This arterial occlusion leads to ischemia in the region of the myocardium supplied by the artery. The lack of oxygen and nutrients, as well as the accumulation of carbon dioxide, dramatically alters cardiomyocyte structure, function and metabolism.[1] During ischemia/reperfusion, the cardiomyocytes suffer from sustained shortening and stiffening that leads to cytoskeletal defects and impaired cellular integrity.[5] Cardiomyocytes also become energy-depleted and develop Ca^{2+} overload in the cytosol. Restoration of coronary blood flow is achieved primarily through percutaneous coronary intervention (PCI), an endovascular procedure where widening of the artery is performed by insertion of an angioplasty balloon through puncture of a peripheral artery, retrograde introduction through the aorta system into a coronary artery, and balloon inflation

at the occlusion site. Reperfusion can also be attained pharmacologically via administration of thrombolytic drugs. While necessary to salvage ischemic myocardium, reperfusion in itself results in cardiomyocyte dysfunction and death.[6] Reperfusion with oxygenated blood results in generation of reactive oxygen species (ROS) and calcium overload leading to dysfunctional contractile and activation of the inflammatory response. [7] Therapeutic alternatives to protect the myocardium from reperfusion injury is currently lacking. Ischemic preconditioning (IPC), i.e. short repeated cycles of ischemia and reperfusion followed by a longer period of reperfusion has been shown to produce resistance to hypoxia in cardiomyocytes.[8] Since preconditioning has to be applied prior to the ischemic event, it is less relevant in a clinical context, but is useful to identify cardioprotective mechanisms and pathways.[9]

Heart failure

Heart failure (HF) is a syndrome in which the heart is unable to meet the circulatory demands of the body at normal filling pressures [10] because of changes in the structure or function of the heart. Typical symtoms include shortness of breath, tiredness and swelling in the legs, resulting either from limitations in cardiac output or from increased intracardiac pressures being transmitted retrogradely through the venous system into the lungs and other organs. HF can rarely be cured, and current treatments are primarily aimed at decreasing cardiac workload through neurohormonal inhibition and prevention of adverse remodelling. In cases of advanced HF, a ventricular assist device or heart transplantation may be needed but are only indicated for a very small subset of cases.[11]

Heart failure most often results from left ventricular dysfunction but can involve either or both ventricles. Left ventricular dysfunction is usually divided into diastolic or systolic dysfunction. In diastolic dysfunction, left ventricular relaxation is impaired due to changes in the ventricular wall such as thickening and increased fibrosis, resulting in reduced filling and elevated filling pressures. In systolic failure, the left ventricle is unable to contract properly to eject sufficient quantities of blood, causing a decreased ejection fraction and increased intracardiac pressure often resulting in increased cardiac dilation which is able to maintain output to some extent through the Frank-Starling mechanism.[1] Patients with systolic and diastolic dysfunction largely experience the same symptoms.

Reduced cardiac output results in decreased systemic arterial pressure and tissue perfusion, which triggers the activation of various compensatory mechanisms, including neurohormonal activation and ventricular remodelling. The natriuretic peptide system, discussed in detail below, represents an important neurohormonal response to increased intracardiac pressures and exerts beneficial effects on natriuresis, diuresis and vasodilation in heart failure patients. [12] Another

compensatory mechanism is cardiac remodeling, a change in ventricular shape, structure and size in order to maintain cardiac output. Remodeling will however often eventually lead to increased tension in the ventricular wall and fibrosis, resulting in further impaired contractility.[1]

Cardiomyocytes

Structure and function

The myocardium consists of cardiac muscle cells, or cardiomyocytes, that are 100 um long with a tubular shape[13, 14]. These cells are located in atria and ventricles and have the ability to lengthen and shorten the sarcomeric cytoskeleton to be able to contract and stretch as the heart beats. Cardiomyocytes are connected to each other through cellular junctions called intercalated discs, which allow calcium, sodium and potassium to pass from cell to cell, propagating the electrochemical impulse. This allows the heart to contract in a coordinated manner.

Cardiomyocytes contain a chain of rod-like units called myofibrils. These consist of several repeating layers of sarcomeres that are essential for the contraction of the cardiomyocytes. Sarcomeres are divided into thin or thick myofilaments, which are oriented to be able to slide past each other. Thin myofilaments largely consist of the protein actin while thick filaments consist of myosin. The electrical impulse activates the release of calcium from the sarcoplasmic reticulum which initiates contraction, a process called excitation-contraction coupling. A cross-bridge is then formed between actin and myosin, in which the myosin filaments actively slide into the actin filaments, resulting in contraction of the cardiomyocyte.

Dysfunction of cardiomyocytes in heart diseases

Cardiomyocytes proliferate during fetal development and directly after birth. However, after birth cardiomyocyte proliferation is negligible, so in response to strain they only increase in size without increase in number. [15] Therefore, when there is a loss of cardiomyocytes it results in increased workload for the remaining cardiomyocytes.

Cell death of cardiomyocytes is a hallmark of myocardial ischemia and infarction. [16, 17] During an ischemic phase, cardiomyocytes are deprived of oxygen and nutrients, while reperfusion results in oxidative stress and inflammation. This leads to wide-spread cardiomyocyte death within the ischemic zone. [18] This type

of cardiomyocyte death occurs by apoptosis, necrosis or by autophagy that is usually complete within 24 hourse after an insult. [19, 20] However, death of cardiomyocytes in HF may exhibit elevated but sustained level of apoptosis. [21]

Due to cardiac injury, there is loss of cardiomyocytes and increased myocardial strain on the remaining cardiomyocytes. To compensate for this increased workload, cardiomyocytes reactivate a fetal gene expression program, including natriuretic peptide genes, transforming growth factor β (TGF-β) and proteins involved in sarcomere structure, e.g. genes coding for the fetal myosin heavy chain isoform.[12, 22, 23] Moreover, the size and structure of cardiac cells is altered in response to increased biomechanical stress, including sarcomere remodelling, cellular hypertrophy and apoptosis. [24, 25]

Isolation and cellular culturing of cardiomyocytes

Isolating and maintenance of cardiomyocytes in vitro is challenging. Cardiomyocytes do not proliferate and thus do not multiply in cellular culture. Moreover, the gap junctions which are essential for the passage of ions and small molecules in order to generate contraction are disturbed during isolation. To overcome this, the cells should be maintained in calcium free medium until membrane integrity has been restored. [26] Due to the inability of primary cardiomyocytes to proliferate, cardiomyocyte-like cell lines have been generated. H9c2 cells are derived from rat embryonic hearts and even if these cells have functioning channels, they do not exhibit organized sarcomeres, a hallmark of adult cardiomyocytes.[27] HL-1 cells are cardiomyocytes derived from mouse atrial tumour cell line transformed by cardiac-directed expression of SV40 large T antigen. [28] These cells contract spontaneously, express organized sarcomeres, exhibit genes and proteins characteristic for adult cardiomyocytes and can proliferate and be passaged in cell culture.

Induced pluripotent stem (iPS) cells are stem cells derived from somatic cells by reprograming and introduction of transcription factors. [29] iPS cells can self-renew and differentiate into all cell types. However, the efficiency of proliferation and differentiation of iPS-derived cardiomyocytes remain low. [30] Even if iPS derived cardiomyocytes remain the best model for investigating cardiac diseases, there are still conflicting studies regarding purity and maturity of these cells. [31, 32] They display properties of immature or fetal cardiomyocytes and are often phenotypically diverse, consisting of a mix of atrial, ventricular and nodal cardiomyocytes.[33, 34]

Natriuretic peptides

Natriuretic peptides are hormones mainly produced and secreted by the cardiomyocytes of the heart. In 1981, de Bold identified atrial natriuretic peptide (ANP) in rat cardiomyocytes with properties of natriuresis, diuresis and vasodilation. [12] Later, brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) were isolated from porcine brain tissue, however, BNP was later discovered to be synthesized mainly in cardiac ventricular cells, with similar biological properties to ANP. [35, 36] There are three natriuretic peptide receptors including natriuretic receptor -A, -B and -C. While ANP and BNP bind to NPR-A, CNP binds to NPR-B. NPR-C is a clearance receptor for all three natriuretic peptides and degrades them by endocytosis. [37, 38]

Biogenesis of ANP

ANP is encoded by the *NPPA* gene located on chromosome 1 which consists of three exons and two introns (Figure 2). ANP mRNA is explicitly expressed in atrial cardiomyocytes of the heart.

ANP is synthesized as a 151 amino acid long pre-proANP peptide. The first 25-amino acids signal sequence is cleaved to yield a 126-amino acid long peptide called proANP, which is stored in atrial cardiomyocytes.[39] In response to stimuli, the stored proANP is released and then cleaved by the cardiac transmembrane serine protease CORIN, resulting in a 28–amino acid long active peptide of mature ANP and a biologically inactive peptide called N-Terminal-proANP.[40] The release of proANP is stimulated in response to atrial wall stretch caused by increased intravascular blood volume and increased intracardiac pressures, but also directly by hormones such as angiotensin II, norepinephrine, endothelin-1 and cytokines.[41-43]. When cleaved, the active ANP circulates in plasma. The ANP plasma levels are low in healthy individuals but can be elevated up to 30-fold in heart failure patients.[44] Degradation of ANP is achieved through two pathways: cleavage by the endopeptidase Neprilysin or internalization by the natriuretic clearance receptor (NPR-C), resulting in a halftime of approximately 2 minutes for ANP. [45, 46]
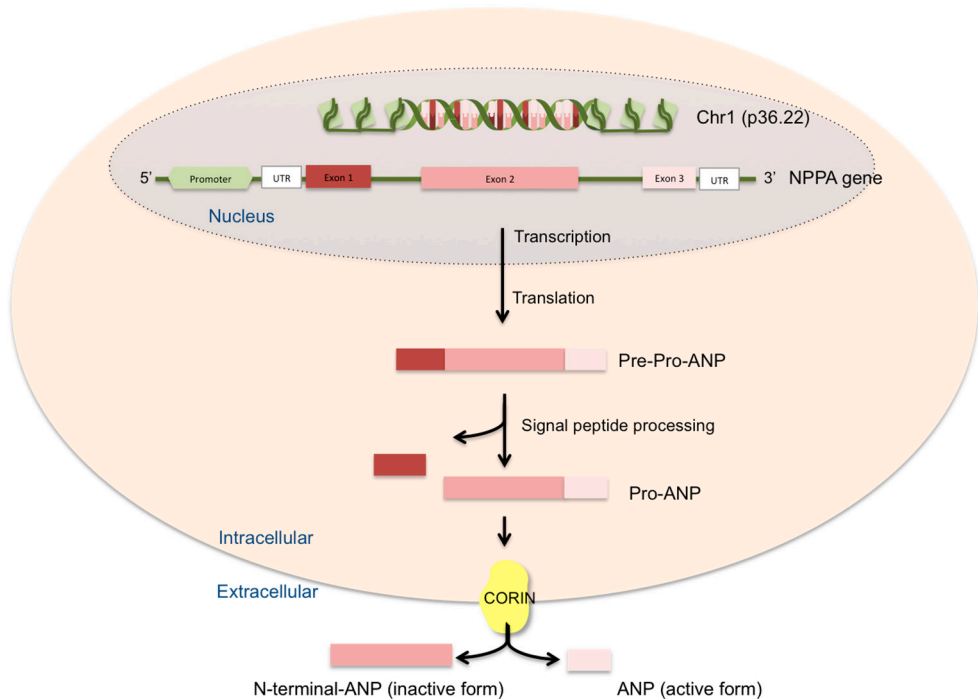
Figure 2. Schematic overview of miRNA biogenesis. Parts of the figure were based on images from Servier Medical Art.

Mechanism of ANP

The main function of ANP is to regulate blood volume and pressure by stimulating natriuresis, diuresis and vasodilation. There are three known natriuretic peptide binding receptors with 450-amino acid long extracellular ligand binding domain and a single membrane-spanning region. However, only Natriuretic peptide receptor A and B (NPR-A and NPR-B) have an intracellular domain, consisting of a kinase homology domain, dimerization domain and carboxyl-terminal guanylyl cyclase domain, activating synthesis of the second messenger cyclic guanosine monophosphate (cGMP). ANP binds to NPR-A and mediate its action by the cGMP-dependent pathway, acting on cGMP-dependent protein kinases (PKG), cGMP-regulated phosphodiesterases (PDE) and cGMP-gated ion channels[47]. Nakayama et al. performed a study, where they show that lack of NPR-A in Japanese individuals resulted in hypertension and ventricular hypertrophy.[48] Mice lacking NPR-A exhibit salt-resistant hypertension and cardiac hypertrophy and fibrosis.[49] It has also been shown that NPR-A knockout mice have cardiac hypertrophy and fibrosis, and pressure overload in these mice results in reduced

left ventricle systolic function. [50, 51] However, under normal conditions, ANP knockout mice do not develop cardiac hypertrophy.[52] These studies indicate that ANP, in addition to its effect on blood pressure and volume, regulates cardiac fibrosis and remodelling.

The Natriuretic peptide receptor C (NPR-C) lacks the intracellular domain and is considered a clearance receptor. It binds to all the natriuretic peptides and degrades them through receptor-mediated internalization.[53, 54] Mice lacking NPR-C showed increased ANP half-life, mild diuresis and blood volume reduction. [55]

Importance of ANP in cardiovascular diseases

ANP has long been considered a promising therapeutic drug for cardiovascular diseases due to its multiple functions in stimulating natriuresis, diuresis and vasodilation. Although administration of synthetic ANP has been shown to confer therapeutic benefits in patients with myocardial infarction and carperitide, i.e. recombinant ANP, is approved to treat patients with heart failure in Japan,[56] its short half-life means that targeting the degradation of ANP may be more favourable. Another strategy to increase ANP levels is based on inhibition of the ANP degrading enzyme Neprilysin. Inhibiton of Neprilysin alone has variable effects on blood pressure due to its many other substrates in the circulation. The combination of Angiotensin receptor blockade and neprilysin inhibition (ARNI) with sacubitril-valsartan, has been shown to reduce mortality and hospitalization in patients with heart failure [57] and it is currently used extensively in heart failure clinics. These observations underline the therapeutic benefit of ANP-augmentation in heart failure patients.

Non-coding RNA

Even though 90% of the genome is transcribed, only 2% of the transcribed genome is protein coding. [58] The remaining transcripts are classified based on function and size, and includes long non-coding RNAs (lncRNAs) or small non-coding RNAs.

Long non-coding transcripts

Genomic location and classification
LncRNAs are defined as non-coding RNA transcripts larger than 200 nucleotides and are classified according to their genomic location compared to the protein-coding gene.[59, 60] LncRNAs transcribed from a region between two protein-

coding genes are called intergenic lncRNAs and those located in intronic regions of protein-coding genes are named intronic lncRNA. Transcripts transcribed from the sense strand and in the same direction as that of protein-coding gene is named sense lncRNAs. These transcripts can overlap partly or cover the whole sequence of the protein-coding gene. Antisense transcripts are transcribed from the opposite strand of the protein-coding gene. Bidirectional lncRNAs are located within the promoter region of protein-coding gene but are transcribed from the opposite strand.[60]

A large proportion of lncRNAs are found in the nucleus and are involved in transcriptional regulation. lncRNAs regulating expression of genes closely located loci are classified as cis-acting lncRNAs (cis-lncRNAs). On the contrary, trans-acting lncRNAs (trans-lncRNAs) regulate the expression of distant genes. Cis-lncRNAs transcribed from the promoter region can influence the transcription of the protein-coding gene by promoter binding or through interacting with transcription factors, inducing chromatin remodelling and activating downstream protein-coding transcription.[61, 62] cis-lncRNAs that are involved in chromatin modifications often recruit chromatin modification complexes leading to transcriptional silencing of target genes.[63] Trans-lncRNAs regulate gene loci located on a different chromosome from which they are transcribed.[64] Like cis-lncRNAs, trans-RNAs are also binding to chromatin modification complexes[65], in addition to transcription elongation factors[66] and RNA polymerases[67] to regulate the transcription of specific genes.

Only a minor fraction of lncRNAs have been studied in detail regarding their function. Imprinting is an epigenetic mechanism where only 1 allele of a gene is expressed. X inactive specific transcript (XIST) is a lncRNA shown to be involved in this mechanism. It regulates X chromosome inactivation, resulting in silencing of one of the X chromosomes of female mammalian cells.[68] LncRNAs can also act as scaffolds for transcription factors. The HOX antisense intergenic RNA (HOTAIR) acts as a scaffold, guiding histone modifying enzymes to its target site and resulting in silencing of the *HOXD* locus, which is involved in fetal development.[65]

Natural antisense transcripts

Definition

Natural antisense transcripts (NATs) are defined according to their genomic position compared to the protein-coding gene. cis-NATs are transcribed from the opposite strand of protein-coding gene and are complementary to the sense transcript. On the contrary, trans-NATs are transcribed from different loci and are partly complementary to the sense transcript.[69, 70]

Structure and localization

NATs, similar to mRNAs, are also capped and poly-adenylated. Like sense transcripts, the transcription of NATs are controlled by promoters and enhancers that are recognized by transcription factors to form a transcription initiation complex.[71, 72] Unlike the sense transcript, which accumulates in the cytoplasm, NATs can be localized both in the nucleus and the cytoplasm.[71]

Function and mechanism of action

The significance of NATs is still not fully understood. Up to 50 % of the protein-coding genes harbour NATs and the transcription levels of the non–coding and protein-coding genes are often correlated. NATs are involved in both transcriptional and post-transcriptional regulation of other genes.[73, 74]

Cis-NATs can be classified in three categories, including head-to-head, tail-to-tail and full overlap. In head-to-head alignment, the sense and the antisense transcripts overlap on their 5' ends, while in tail-to-tail they overlap on their 3' ends. In full overlap, one of the entire transcripts overlap with the other transcript.[75]

NATs can regulate the expression of its sense gene by transcriptional interference. This can occur both during initiation or elongation phases of transcription.[76] Promoters of head-to-head NATs are competing for the use of RNA polymerase II and common regulatory elements. This occurs in the initiation phase, while in elongation phase, interference can occur after a collision between RNA polymerase II complexes, a promoter occlusion by RNA polymerase II during elongation of antisense transcript or RNA polymerase II dislodgement by the RNA polymerase II on the opposite strand. [76] However, the mechanistic details of transcriptional interference by NATs is still under investigation.[77]

The expression of protein-coding genes can also be regulated by chromatin modifications such as DNA methylation of cytosine in CpG islands and histone modifications of lysine residues. NATs regulate the expression by interacting with proteins involved in histone modification or chromatin remodelling.[78] Another protein decoy mechanism is when NATs bind to protein complexes and prevents them from binding to the gene of target in a competitive manner, resulting in interruption of transcription and silence of the sense gene.[79]

NATs can regulate the expression of the protein-coding gene by forming a complex with the mRNA of the protein-coding gene. This RNA masking will then interfere with the splicing or translation system and also prevent binding of other molecules such as miRNAs to the mRNA.[80]

MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNAs, with a length of approximately 23 nucleotides. miRNAs are key regulators of gene expression and act on several cellular processes, including cell differentiation, cell cycle progression and apoptosis.[81, 82] miRNA are grouped into families based on shared seed-sequences, i.e. the nucleotides in position 2-8 of the microRNA which interacts with target mRNA. [83]

Biogenesis

Primary miRNA (pri-miRNA) is transcribed by RNA Polymerase II (POL II) in the nucleus as a long transcript with a hairpin structure. Pri-miRNA is then processed into precursor miRNA (pre-miRNA) by the microprocessor complex, consisting of the RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and the ribonuclease III enzyme Drosha.[84] DGCR8 recognizes and binds to motifs within pri-miRNA, while Drosha cleaves the pri-miRNA at the lower stem of the hairpin structure, resulting in pre-miRNA. The pre-miRNA is then exported to the cytoplasm by Exportin 5 (EXP5) and the Ran-GTP complex.[85] In the cytoplasm, Dicer, a RNase III endonuclease, binds to the pre-miRNA and removes the terminal loop of the hairpin structure, resulting in a mature miRNA.[86]

Mechanism

The silencing of mRNAs by miRNA is mediated by the RNA-induced silencing complex (RISC, Figure 3). The mature miRNA acts as a guide for RISC through recognition and binding of the target mRNA by Watson-Crick base pairing. This results in Argonaute 2-mediated degradation or repression of the translational machinery. [87]
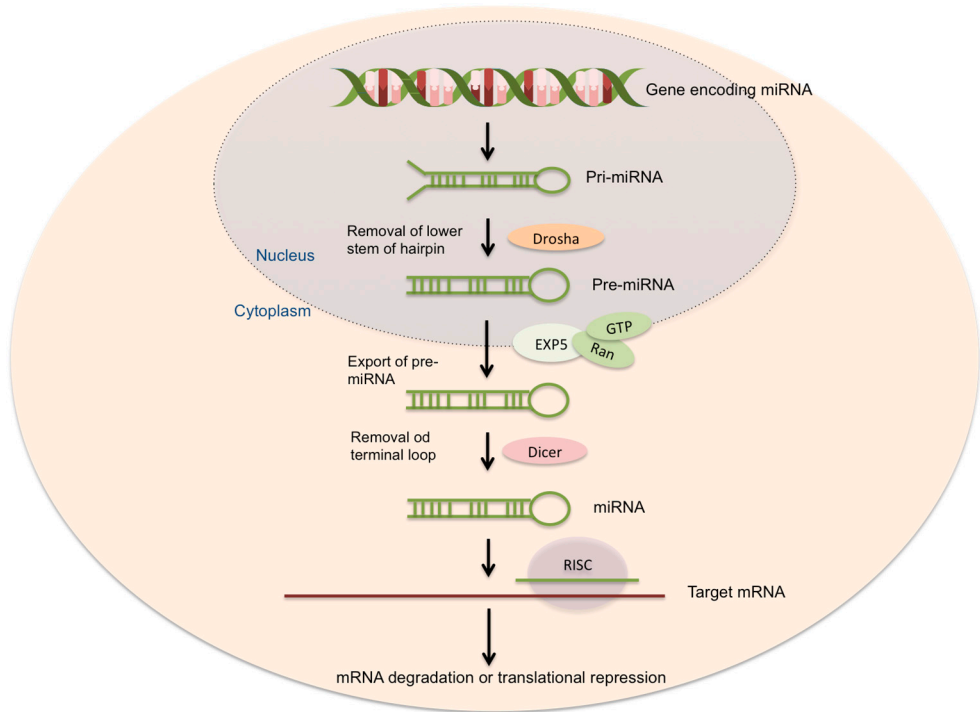
Figure 3. Schematic depiction of miRNA biogenesis.

Importance in cardiac diseases

In addition to the importance in different biological processes, miRNAs are also very important in the progression of different diseases, thus making them potential therapeutic targets. miR-208a is a cardiac miRNA shown to be important in the progression of cardiac hypertrophy and stress-induced fibrosis.[88] miR-24, an inhibitor of angiogenesis, is expressed in cardiac endothelial cells, and inhibition of miR-24 in mice resulted in restricted infarct size and preserved cardiac function.[89] Another miRNA important in cardiac disease is miR-499, encoded within the intron of myosin heavy chain 7B.[90] Increased levels of miR-499 results in impaired contractility during cardiac stress.[91]

Therapies with non-coding RNAs

Therapeutic olignoucleotides have become an increasingly attractive alternative to traditional small molecule or antibody-based drugs in recent years. Ease of design and production and the possibility to target virtually any gene in the genome are obvious advantages with RNA-based therapies, although challenges remain with regards to delivery and potential immune-related adverse effects.

RNA-based therapies

There are numerous studies investigating RNA-based therapies for various diseases. Antisense oligonucleotides or small interfering RNA molecules can be designed to alter the expression, translation or splicing of a specific disease-associated transcript. There are already some RNA-based therapies for cardiovascular disease in clinical use. Since 2013, Mipomersen is used as therapy for homozygous familial hypercholesterolemia. It is a single-stranded oligonucleotide that binds to the RNA encoding apolipoprotein B and marks it for RNase H-mediated cleavage. This results in reduced plasma levels of Apo-B-containing particles and low-density lipoprotein. [92] Another therapeutic antisense oligonucleotide in clinical trials is Volanesorsen. It targets Apolipoprotein CIII, which is a key component in hypertriglyceridemia and chylomicronemia. Results from clinical trials show that patients receiving Volanesorsen have lower plasma levels of apolipoprotein CIII and triglycerides. [93] Proprotein convertase subtilisin–kexin type 9 (PCSK9) is an enzyme that promotes degradation of the LDL receptor, which results in high levels of LDL cholesterol levels. [94] Inclisiran, a double stranded small interfering RNA, targets PCSK9 synthesis by binding and degrading PCSK9 mRNA via incorporation in the RNA-induced silencing complex. This results in elevated levels of the LDL receptor and subsequent increase in LDL clearance.[95] Taken together, these results underline the advantages of using oligonucleotide-based treatments as a therapeutic approach in cases where a target RNA has been identified for a disease. However, there are still limitations with this strategy that should be in consideration when designing and evaluating oligonucleotide-based treatments. There could be side effects such as activation of the immune response when administering oligonucleotides.[96, 97] Oligonucleotides could also potentially bind to transcripts other than the intended target, which could modulate the expression of other genes and cause adverse effects. Another limitation of oligonucleotide-based therapy is the delivery of the oligonucleotide to the target tissue without affecting other tissues.

miRNA-based therapies

The expression of many miRNAs is increased or decreased in different diseases, underscoring the need to investigate and understand the actions of miRNAs and to assess the benefit in using them as possible therapeutic targets. miRNA expression can be altered by using miRNA inhibitors (antagomiRs) or miRNA mimics, also called agomiRs.[98, 99] AntagomiRs are locked nucleic acid molecules that bind to target miRNA with high affinity and inhibits the miRNA from binding its targets.[100] There are also studies on using miRNA sponges and masking which downregulates the miRNA of interest.[101] However, in some diseases, upregulation of a specific miRNA is desired. miRNA expression could be increased by either using a vector overexpressing the target miRNA, or using double-stranded miRNA mimicking the target miRNA. There are already some

drugs in development targeting miRs. Miravirsen is a drug in development for the treatment of hepatitis C.[102] It targets miR-122, which has an essential role in the progression of hepatitis C virus infection. Miravirsen targets miR-122 at the pri- and pre-miRNA levels, which leads to suppressed levels of the miRNA. Cobomarsen, a drug inhibiting miR-155 is in clinical trials for T-cell lymphoma. Cobomarsen-induced inhibition of miR-155 leads to reduced proliferation and survival of T-cell lymphomas. [103] CDR132L is a drug targeting miR-132 which recently entered phase I clinical trials for the treatment of heart failure. Increased miR-132 expression has been associated with pathological cardiac remodelling, which suggests that it can constitute a potential therapeutic target in heart failure.[104] Another drug in phase I clinical trials for the treatment of heart failure is MRG-110, which targets miR-92a. It has been shown that inhibition of miR-92a results in increased angiogenesis, improving cardiac function following myocardial infarction or ischemia and in several heart diseases causing fibrosis.[105]

Material and methods

Cell culture

In all the papers, we used human cardiomyocytes derived from induced pluripotent stem cells (iPS-cells). The cells were cultured in iCell Cardiomyocyte Plating or Maintenance Medium. In addition to iPS-cells, we also used primary human cardiac fibroblasts in paper I, grown in Fibroblast Growth Medium and HL-1 cells in paper IV, grown in Dulbecco's Modified Eagle Medium (DMEM).

Animal model

In paper I, we used mice to investigate the physiological effects of *NPPA-AS1*-inhibition. C57BL/6JBomTac male mice were injected with different doses of the inhibiting compound GapmeR or the negative control GapmeR. The blood pressure was measured before and after the injection by using a non-invasive tail cuff system. After 48h post injection, the mice were sacrificed followed by collection of blood and organs. This study was approved by Lund Ethical Committee for Animal Research.

In paper II, we used a mouse model of ischemic pre-conditioning. C57BL/6J-strain mice were anesthetized and an incision was made in the left midclavicular line of the chest. A silk suture was placed around the left descending coronary artery and 4 cycles of 5 min tightening (ischemia) and 5 min loosening (reperfusion) followed by 30 min reperfusion was performed. The animals were sacrificed and the hearts were perfused with PBS before collecting biopsies from the ischemic area (area at risk) and non-ischemic area (remote myocardium). The biopsies were stored at -80°C. This study was approved by the Institutional Committee for Use and Care of Laboratory Animals, University of Innsbruck.

Human heart tissue

Human atrial and ventricular cardiac tissue were obtained from explanted hearts. Patients provided written consent before participation and the study was approved by the local ethics committee of Skåne University Hospital. The collected heart tissues were dissected and stored in RNAlater at -80°C.

Strain of cells

To mimic the conditions of heart failure in cell culture, we subjected the cells to mechanical strain. Cells were seeded on silicon chambers with flexible membranes, allowing the cells to be stretched. Cells were then strained in a uniaxial manner in one direction for 48h with 10% elongation, which is 2.0 mm in distance with a frequency of 60 cycles per minutes (Figure 4). Cells were then harvested for either protein or RNA extraction.
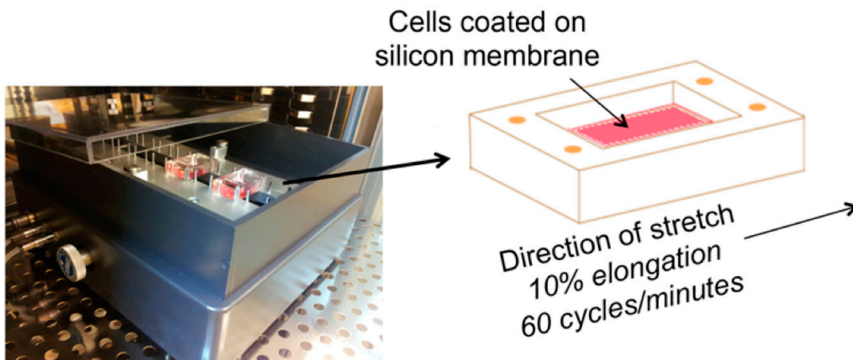
Figure 4. Overview of the strech unit.

Transfection of pre/anti-mir and sirna

Transfection with pre- or anti-miRNA is used to investigate the specific effect of the miRNA of interest. By this, the miRNA of interest can be increased or decreased in a controlled manner in the desired environment. In paper II and III, the cells were grown in medium until 80% confluence and pre- or anti- miRNA was delivered to the cells by lipid-mediated transfection. After 48h, cells were harvested for RNA extraction.

RNA extraction and Quantitative real-time PCR

Cells and heart tissues were first washed with PBS and then lysed in QIAzol or TRIzol. RNA was extracted by a RNA-preparation kit, miRNeasy, according to manufacturer's instructions. RNA concentration and validation was measured using NANODROP 2000C Spectrophotometer.

cDNA was prepared from the RNA samples by using RevertAid H Minus First Strand cDNA Synthesis Kit. Gene expression was then evaluated by quantitative Real time PCR using Taqman assays specific for genes of interest. In paper II and III, cDNA for miRNA analysis was prepared by using miRCURY LNA Universal RT microRNA cDNA kit and then expression levels of miRNA were analysed by SYBR Green and primers specific for the miRNA of interest.

One of the methods used for analysis of gene expression in this thesis is the TaqMan assay. It is based on dye-labelled probes that anneals with one of the template strands. The probe sequence, which binds to the DNA target of interest, is labelled with two fluorescent dyes. The 5'end of the probe sequence is labelled with a reporter dye and the 3'end is labelled with a quencher dye. In its unbound state, there is no fluorescence due to the inhibition of reporter dye by the quencher. However, during polymerization, the DNA polymerase with 5'exonuclease activity will degrade the 5'end of the probe sequence, releasing the reporter dye from the quencher dye, resulting in fluorescence. The other method used for gene expression analysis was SYBR Green/end-point PCR. The SYBR Green molecule emits green fluorescence when bound to double stranded DNA. Hence, the amount of fluorescence is related to the amount of PCR product. The gene expression is calculated according to the Pfaffl's method 2-$\Delta\Delta$Ct.[106] The expression of target of interest is normalized to expression of a reference gene, which must remain constant for comparative measurements.

Protein extraction and Western Blot

Western blot is a technique used to detect and quantify a specific protein in cells or tissues. Proteins are extracted from lysed cells and separated by their molecular weight using gel electrophoresis. Then, the proteins are transferred from the gel into a membrane by electro blotting to make them accessible to antibodies. To prevent non-specific binding of antibody, the membrane is blocked with non-fat dry milk or bovine serum albumin. After blocking, the membrane is incubated with primary antibody, specific for the protein of interest, followed by incubation with secondary antibody, linked with horseradish peroxide (HRP), which binds to the primary antibody. The proteins are then detected by chemiluminescent and evaluated through densitometry.

ChiRP

To discover regions of the genome that are binding to a transcript, Chromatin isolation by RNA purification (ChIRP) was performed. Nuclei from cross-linked cells are isolated and lysed to release the chromatin (Figure 5). The chromatin is further sheared into smaller fragments and then hybridized with biotin labelled oligonucleotides that have been designed to be complementary to the transcript of interest. After the hybridization, the biotin-probe:DNA fragments are captured by magnetic beads and DNA is extracted from the complex for analysis with SYBR green qPCR.
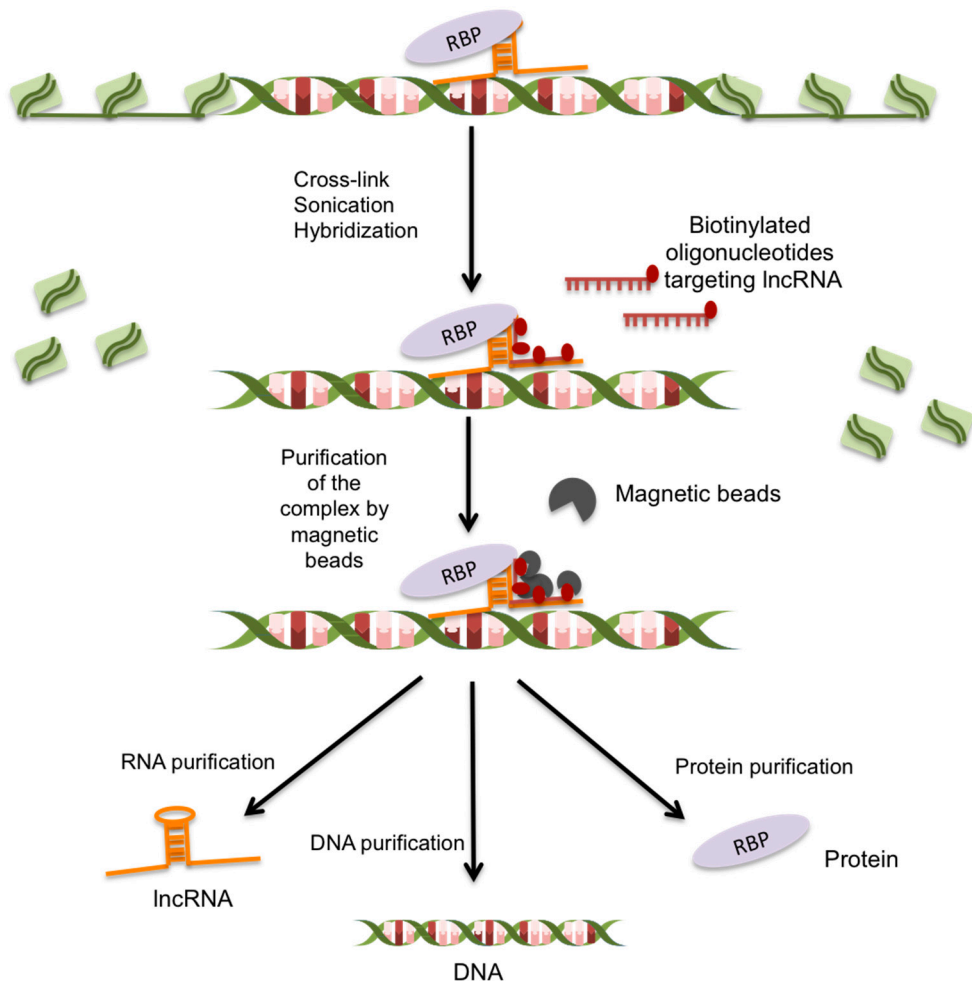

Figure 5. Overview of workflow for ChIRP. Adapted from Chu et al .[107] RBP, RNA binding protein.

ChIP

In paper I, we used chromatin immunoprecipitation to investigate protein:DNA interactions and to identify proteins associated with a specific region of the genome. Cells are fixed and cross-linked and chromatin is isolated and fragmented by enzymatic digestion. Then, the fragmented chromatin is subjected to immunoprecipitation by using antibodies specific to a protein of interest. The protein:DNA:antibody complex is then captured by magnetic beads and chromatin is eluted from the complex followed by reversal of cross-link to purify the DNA. The DNA can then be quantified by qPCR.

RIP

RNA immunoprecipitation (RIP) is a method used to investigate interactions between proteins and their RNA targets. In paper I, we performed this to study the interaction between *NPPA-AS1* and different transcription factors. Cells are lysed, followed by immunoprecipitation of the protein of interest and its associated RNA transcripts. This protein:RNA-complex is pulled down with the help of magnetic beads, which binds to the antibody. Afterwards, the bound protein:RNA-comples is washed and RNA attached to the protein is purified. The RNA can then be analysed by e.g. qPCR.

Serine protease activity assay

In Paper II, we adapted an assay from Chen et al. for the validation of CORIN activity in iPS-CM.[108] CORIN belongs to the transmembrane serine protease family and cleaves its target at serine protease cleavage sites. A fluorogenic bisamide substrate rhodamine 110 with two serine protease cleavage sites was added to iPS-CM plated in 96-well plates and fluorescence was monitored over time (5-60min).

Reporter assay

To determine if a protein or transcript interacts with or affects a specific target sequence, a reporter assay can be performed. The DNA sequence of interest is cloned in conjunction with a reporter gene into a plasmid, which is then transfected into cells. After transfection, the medium is collected and the activity of the reporter

gene is measured. In this thesis, a reporter plasmid containing Gaussia luciferase (GLuc) and secreted alkaline phosphatase (SEAP) was used (Figure 6). GLuc signals were normalized to the SEAP signal. In paper I, we incorporated the *NPPA* promoter region into the plasmid to investigate the interaction of this region with REST and *NPPA*-AS1. In paper II, we designed a reporter assay to confirm the interaction between the CORIN 3'UTR and miR-1-3p.
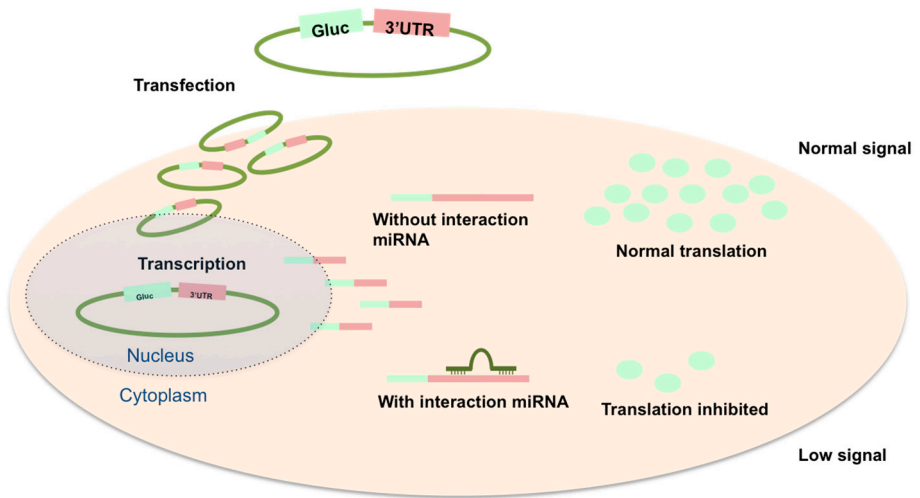

Figure 6. Schematic overview of reporter assay.

Aims and Results

Paper I

Aim

The aim of this paper was to characterize the role of the *NPPA* antisense transcript *NPPA-AS1* in regulating *NPPA* expression in human cardiomyocytes and to assess the physiological consequences of pharmacological inhibition of *NPPA-AS1* in vivo.

Results

The NPPA antisense transcript is located in atrial cardiomyocyte nuclei

First, we wanted to investigate the expression profile of the antisense transcript *NPPA-AS1*. We analyzed the expression of *NPPA* and *NPPA-AS1* by qRT-PCR in three main cell types of the heart, including cardiomyocytes, fibroblasts and endothelial cells. As expected, *NPPA* was mainly expressed in cardiomyocytes while *NPPA-AS1* was found in all the cell types (Figure 7a). To further investigate the distribution of *NPPA-AS1*, we performed FISH using a probe specific for *NPPA-AS1* and found decreased nuclear FISH staining when *NPPA-AS1* was inhibited by siRNA compared to control cells (Figure 7b).
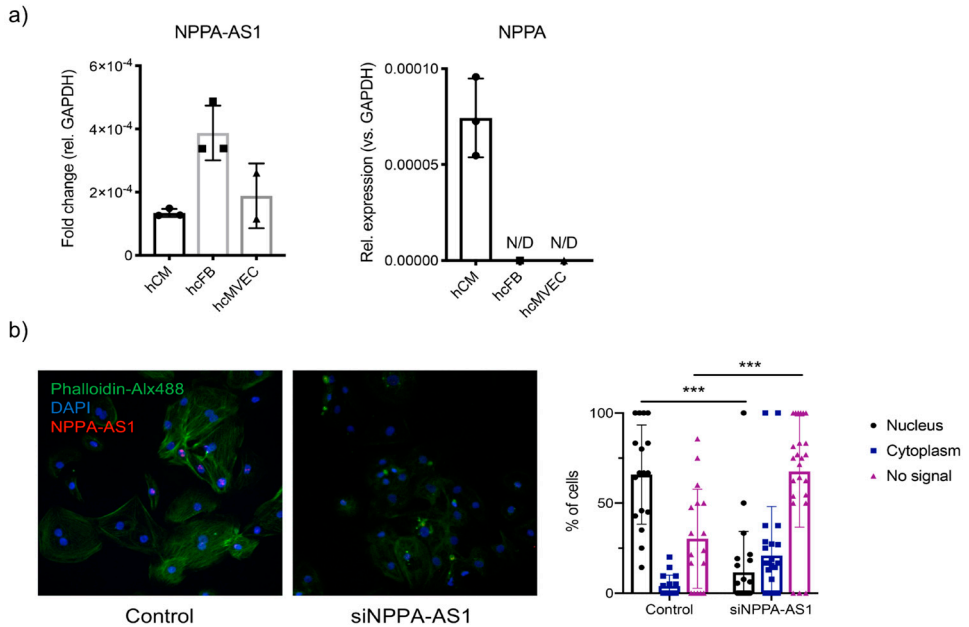
Figure 7. The antisense transcript *NPPA-AS1* is found in the nucleus of cardiomyocytes. a) *NPPA-AS1* and *NPPA* expression in cardiac cells investigated with qRT-PCR. b) Localization of *NPPA-AS1* (red) assessed with fluorescence in situ hybridization in iPS-CMs. Cells were stained with Alexa Fluor 488–conjugated phalloidin (green) and nuclei were counterstained with DAPI (blue). ***P < 0.01

NPPA-AS1 has a negative effect on NPPA expression on chromatin level

We wanted to assess the effects of *NPPA-AS1* on *NPPA* expression by using siRNA-mediated knock down of *NPPA-AS1* in iPS-CM. The results showed an increase of *NPPA* when *NPPA-AS1* was down regulated, indicating that *NPPA* is negatively regulated by *NPPA-AS1* (Figure 8a). To investigate this mechanism we considered the possibility of an interaction between *NPPA*-AS and *NPPA* mRNA and therefore performed ChIRP, where no RNA duplex formation between the transcripts could be confirmed (Figure 8b).
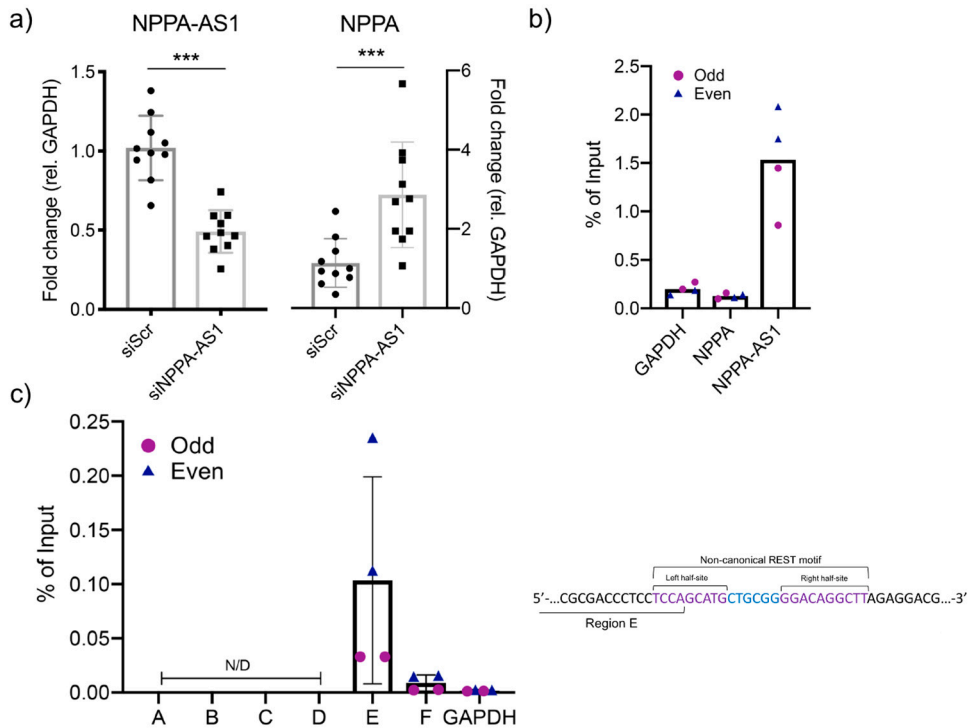
Figure 8. NPPA-AS1 is binding to the promotor of the *NPPA* gene. a) iPS-CM transfected with siRNA targeting *NPPA-AS1* display an increase in *NPPA* expression compared with cells transfected with scrambled negative control siRNA. b) No duplex formation between *NPPA-AS1* and *NPPA* mRNA was found when Chromatin Isolation by RNA Purification (ChIRP) was performed in human cardiac tissue. c) qRT-PCR analysis of human atrial DNA coprecipitated with ChIRP probe sets specific for NPPA-AS1. N/D, not detected. Illustration of the noncanonical REST motif in the *NPPA* promoter and its overlap with region E. ***P < 0.01

Due to the nuclear localization of *NPPA*-AS1, we hypothesized that *NPPA-AS1* might have an effect on the *NPPA* promoter. As seen in figure 8c, results from ChIRP showed an interaction between *NPPA-AS1* and a specific region in the *NPPA* promoter. Analyses of this region revealed this region as a binding site for RE1-silecing transcription factor (REST) and further experiments showed that *NPPA-AS1* indeed facilitates the binding of REST to the *NPPA* promoter.

NPPA and NPPA-AS1 are increased in human heart failure patients and in strained cardiomyocytes

The next step was to determine if the expression of *NPPA-AS1* was changed during pathophysiological conditions. Human cardiomyocytes were subjected to 10% cyclical strain for 48h mimicking the pathophysiological conditions of heart failure. The expression of both *NPPA* and *NPPA-AS1* mRNA was increased after 48h of strain (Figure 9a). We further assessed whether the expression of *NPPA*

would change if *NPPA-AS1* was knocked down prior to strain. The results showed an increase in stretch-induced *NPPA* expression when *NPPA-AS1* was inhibited by siRNA (Figure 9b). We then investigated the expression of the transcripts in a cohort of heart failure and non-failure controls. Both *NPPA* and *NPPA-AS1* mRNA expression was increased in ventricular tissue of heart failure patients compared to the control group. There was also a positive correlation between these transcripts in atrial and ventricular tissue (Figure 9c).
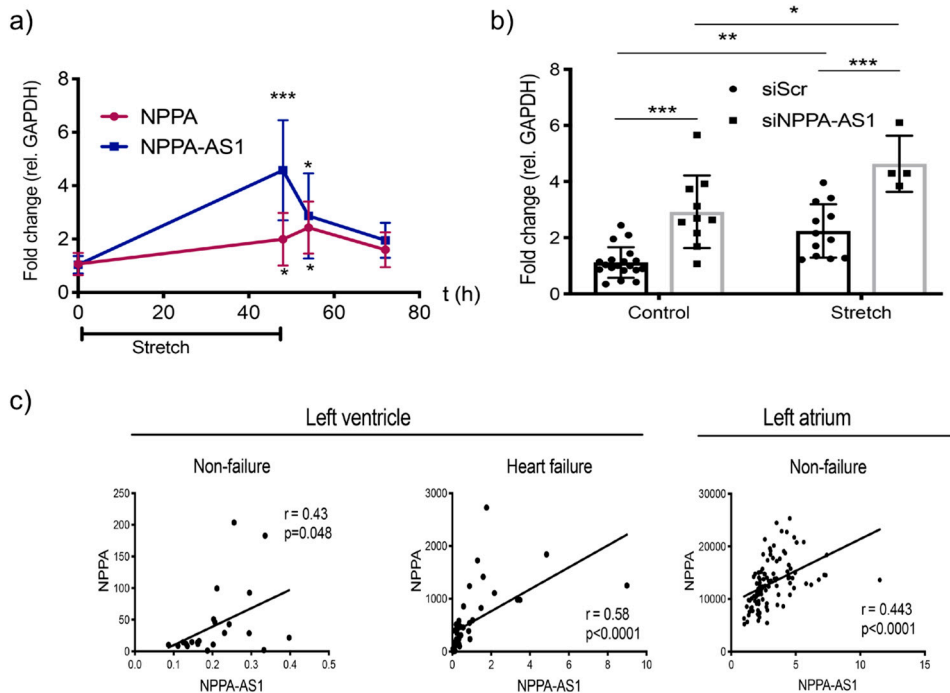

Figure 9. Increase of *NPPA* and *NPPA-AS1* in heart failure patients and cardiomyocytes subjected to strech.
a) Expression of *NPPA-AS1* and *NPPA* levels during the time course of the experiment analysed by qRT-PCR. b) *NPPA* levels in iPS-CMs transfected with siRNA targeting *NPPA-AS1* followed by 48 hours of stretch. c) Correlation between *NPPA* and *NPPA-AS1* in left ventricle and left atrium from heart failure and nonfailure donors. *P < 0.05, **P < 0.01, ***P < 0.001

Inhibition of Nppa-as by gapmeRs increased Nppa levels in mice

Due to the negative effects of *NPPA-AS1* on *NPPA* expression, inhibition of *NPPA-AS1* could be a potential therapeutic strategy to increase ANP levels. We assessed this in mice by knocking down *Nppa-as* using LNA-based antisense oligonucleotides (GapmeRs). As seen in figure 10, we could see an increase of *Nppa* and Anp expression in mice treated with gapmeR compared to the control group. We could also see a decrease in blood pressure and an increase in the second messenger cGMP in the kidney of mice treated with gapmeR.
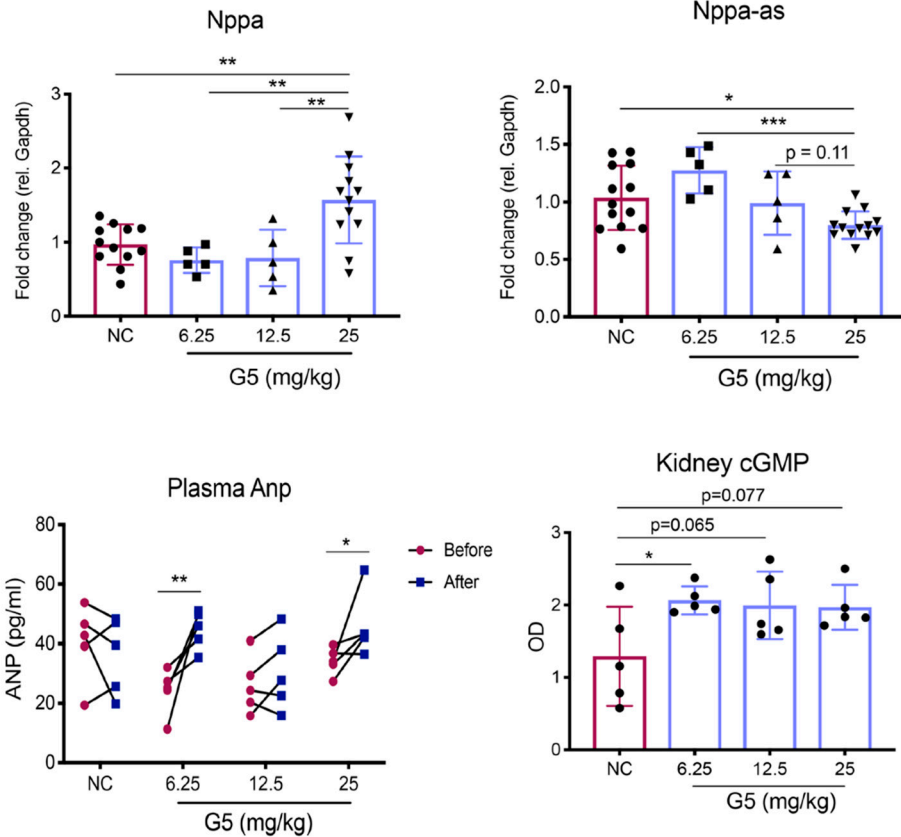
Figure 10. Increased *Nppa* expression in mice when injected with gapmeR against Nppa-as. The expression of *Nppa* and *Nppa-as* in atrial tissue, ANP levels from blood and cGMP levels from kidney of mice injected subcutaneously with 6.25, 12.5, or 25 mg/kg of GapmeR5 or 25 mg/kg of negative control GapmeR for 48 hours (n = 5–13 per group). *P < 0.05, **P < 0.01, ***P < 0.001

Paper II

Aim

In this paper we performed a screening to identify miRNAs affecting CORIN activity and ANP biogenesis in human cardiomyocytes.

Results

Validation of an assay for measuring CORIN activity in human cardiomyocytes

To be able to investigate CORIN activity in human iPS-CM, we used an adapted version of a cardiac serine protease activity assay performed by Chen et al.[108] We measured CORIN activity by adding a fluorogenic serine protease substrate to iPS-CM. We confirmed that that the fluorescent signal was linear and proportional to substrate concentration (Figure11a). To validate the assay, we treated the cells with a serine protease inhibitor (benzamidine) or with *CORIN* small interfering RNA (siCORIN). Here we could see a 50% reduction in enzymatic activity in cells treated with benzamidine compared to untreated control cells (Figure 11b). Importantly, cells treated with siCORIN displayed a 25% decrease in assay signal compared to untreated control cells, indicating that the assay was both sensitive and specific to CORIN activity.
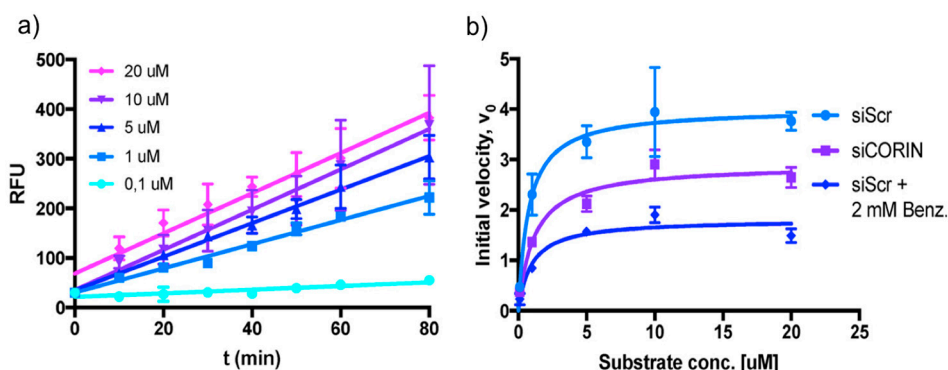

Figure 11. Validation of CORIN activity. a) The increases in fluorescence were linear and proportional to substrate concentrations in iPS-CM. b) CORIN activity measured in cells transfected with scrambled control siRNA or CORIN siRNA and cells treated with 2 mM benzamidine and presented in Michaelis-Menten kinetics.

Identification of miRNAs targeting CORIN

We used a library of miRNA family inhibitors to identify miRNAs that could affect CORIN activity. iPS-CM were transfected with miRNA family inhibitors for 72h before measuring CORIN activity. The results showed that the CORIN activity was increased when miR-1 family was inhibited compared to control (Figure 12a).

The miR-1 family consists of two miRNAs: miR-1-3p and miR-206. However, previous studies have shown that miR-1-3p is a cardiac-specific miRNA while miR-206 is restricted to skeletal muscle. Therefore, miR-1-3p was chosen for further studies. We could show that by inhibiting miR-1-3p using the antisense oligonucleotide anti-miR-1, CORIN activity was significantly increased (Figure 12b). In addition, we could see an increase in extracellular ANP levels when miR-1-3p was inhibited, indicating that miR-1-3p indeed has an effect on CORIN activity (Figure 12c).
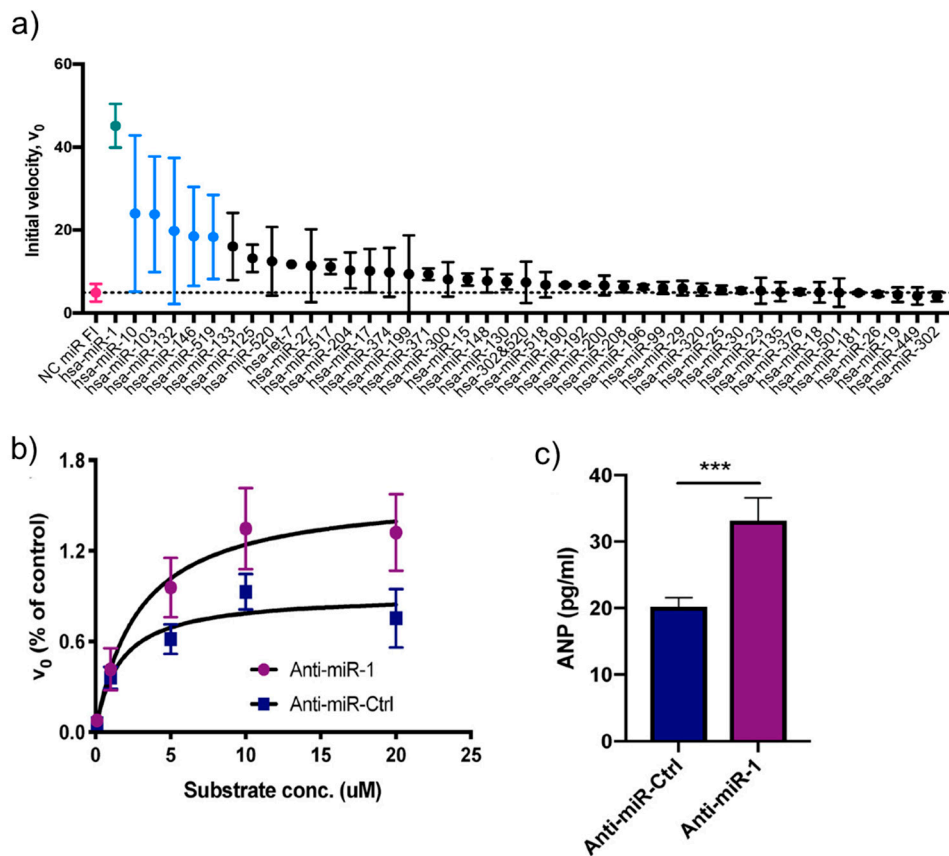
Figure 12. miR-1-3p has a negative effect on CORIN activity. a) Initial reaction velocity (V0) for each miRNA family inhibitor ranked according to effect. b) Corin activity in iPS-CM transfected with anti-miR-1 or scrambled control anti-miRNA presented in Michaelis-Menten saturation curve. c) ANP protein levels in the supernatants of iPS-CM transfected with anti-miR-1 or scrambled control anti-miRNA assessed by enzyme-linked immunosorbent assay (ELISA). *** P < 0.001.

miR-1-3p is binding to the 3'UTR of CORIN *mRNA*

To investigate whether there is a direct interaction between miR-1-3p and *CORIN* mRNA, we performed a luciferase reporter assay. We constructed a reporter vector containing the CORIN 3'UTR and could see a significant increase in the luciferase signal when cells were transfected with anti-miR-1 compared to untreated cells. We also confirmed the interaction between miR-1-3p and a predicted target site in the *CORIN* 3'UTR through site-directed mutagenesis of the reporter vector.

39

Paper III

Aim

The aim of this study was to investigate miRNAs that regulate *NPPA gene expression* in an in vitro model of human cardiomyocyte mechanical stress.

Results

Validation of human iPS-CM

Human iPS-CM is an essential tool for investigating different molecular pathways related to heart disease. *NPPA* and ANP expression is mainly restricted to atrial cardiomyocytes and therefore, we wanted to validate these cells as a model system for our investigations. Results from whole-transcriptome microarray showed marker genes of atrial and ventricular cardiomyocytes (Figure 13a). A characteristic feature of atrial cardiomyocytes is increase of *NPPA* levels when applied to mechanical stretch. Results showed a significant increase in *NPPA* mRNA (Figure 13b) and ANP protein (Figure 11c and d) levels when applied to 10% mechanical stretch for 48h, indicating atrial-like properties in terms of *NPPA* expression.
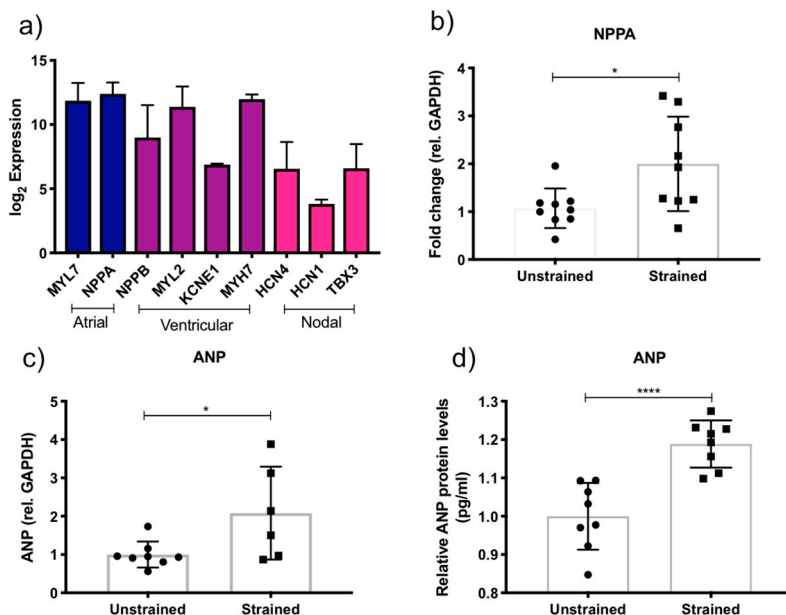
Figure 13. Validation of human iPS-CM show properties of artial cardiomyocytes. a) Expression of marker genes characteristic of atrial, ventricular and nodal cardiomyocytes in the iPS-derived cardiomyocytes used in the study. Shown are log2-transformed, normalized expression data from the Clariom D whole-transcriptome array on total RNA from iCell iPS-derived cardiomyocytes. b) Expression of b) NPPA, c) ANP and d) secreted ANP in iPS-CM subjected to 10 % elongation at 60 cycles/min. *P<0.05 ****<0.0001

NPPA expression is negatively affected by miR-105, miR-155 and miR-425

The expression of miRNAs was modified by using pre- or anti-miRs and the results were assessed by qRT-PCR. We observed a significant decrease of *NPPA* mRNA expression in cells with overexpression of each miRNA (Figure 14a). However, we could only see a significant increase of *NPPA* expression in cells transfected with anti-miR-155 (Figure 14B), which could also be confirmed in ANP protein levels (Figure 14C-D).
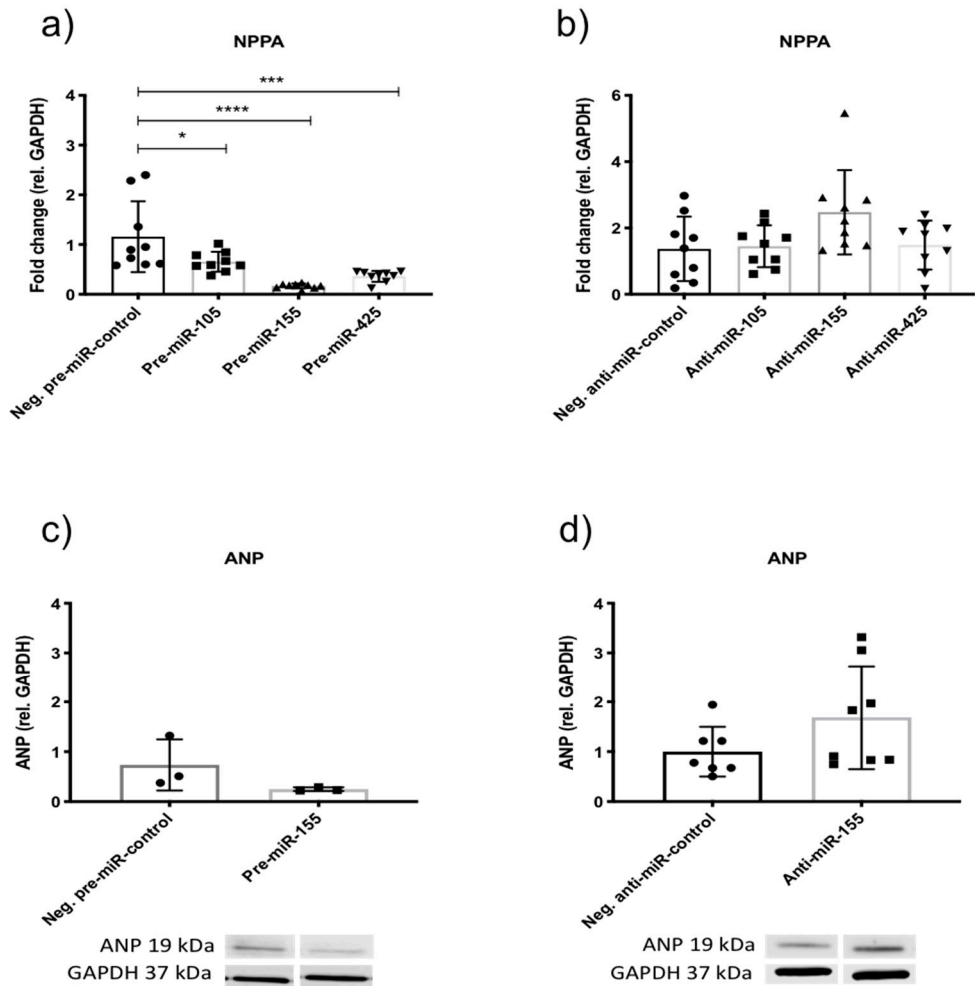
Figure 14. miR-155 has a negative effect on *NPPA* and ANP levels in iPS-CM. *NPPA* mRNA levels in cells transfected with (a) pre-miRNA or (b) anti-miRNA. ANP protein levels in cells transfected with (c) pre-miR-155 and (d) anti-miR-155 asessed by western blot. *P<0.05 ***P<0.001 ****P<0.0001

Negative effect of strain on miR-105, miR-155 and miR-425

Next we wanted to investigate the effects of strain on miR-105, miR-155 and miR-425. 10% mechanical stretch for 48h resulted in a decrease of miR-105, miR-155 and miR-425 expression compared to unstretched iPS-CM. We further wanted to investigate if stretch-induced *NPPA* mRNA expression could be enhanced by pre-treating cells with anti-miR-155. This was confirmed by qRT-PCR.
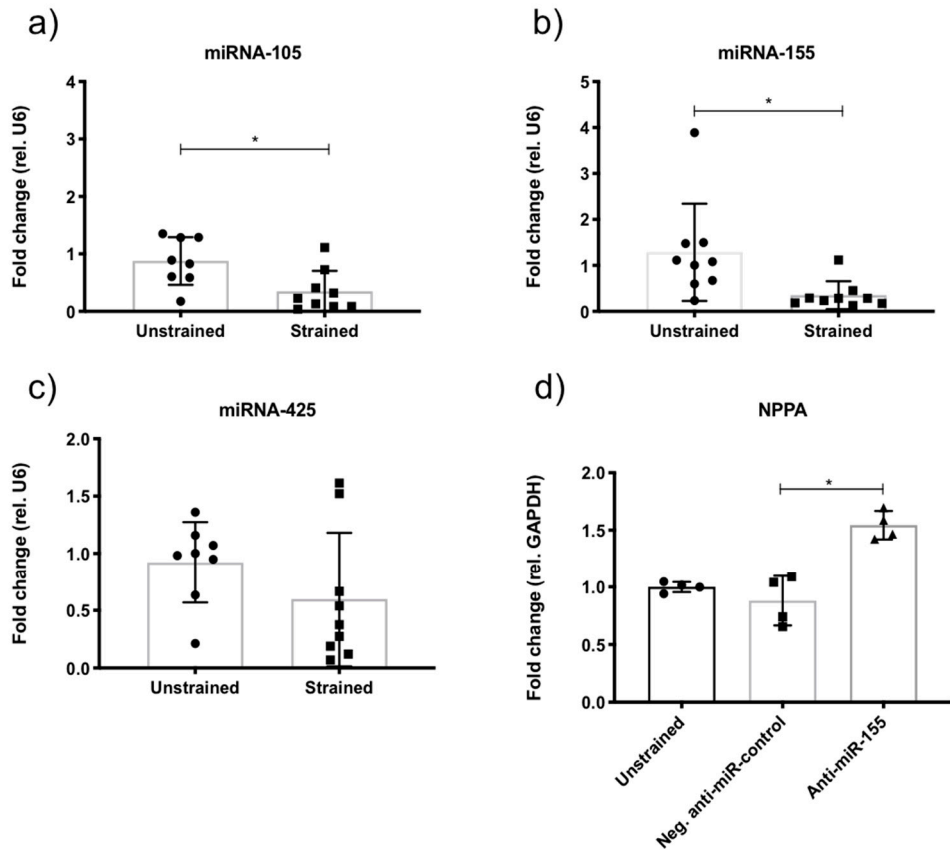
Figure 15 . Decrease of miR-105 and miR-155 in iPS-CM when subjected to mechanical strain.
Cardiomyocytes derived from human induced pluripotent stem cells were subjected to 10% elongation at 60 cycles/min for 48h. (A) miRNA-105, (B) miRNA-155 and (C) miRNA-425 expression leves in unstretched and stretched cells. (D) NPPA levels in cells transfected with anti-miRNA-155. Error bars represent mean of standard deviation. *P<0.05

Paper IV

Aim

Here, we characterize the cardiac lncRNA transcriptome in mice in response to ischemic preconditioning (IPC) in order to identify novel cardioprotective pathways.

Results

Neat1 *is downregulated after IPC*

To investigate lncRNAs involved in cardioprotection induced by IPC, we compared lncRNAs between area at risk with remote myocardium from mice with microarray (Figure 16a). We found *Neat1*, a lncRNA important in formation of nuclear paraspeckles, to be significantly down regulated in the area at risk compared to remote myocardium (Figure 16b). *Neat1* has been shown to be involved in the worsening of myocardial injury, therefore the downregulation of *Neat1* might be part of the cardioprotection induced by IPC.
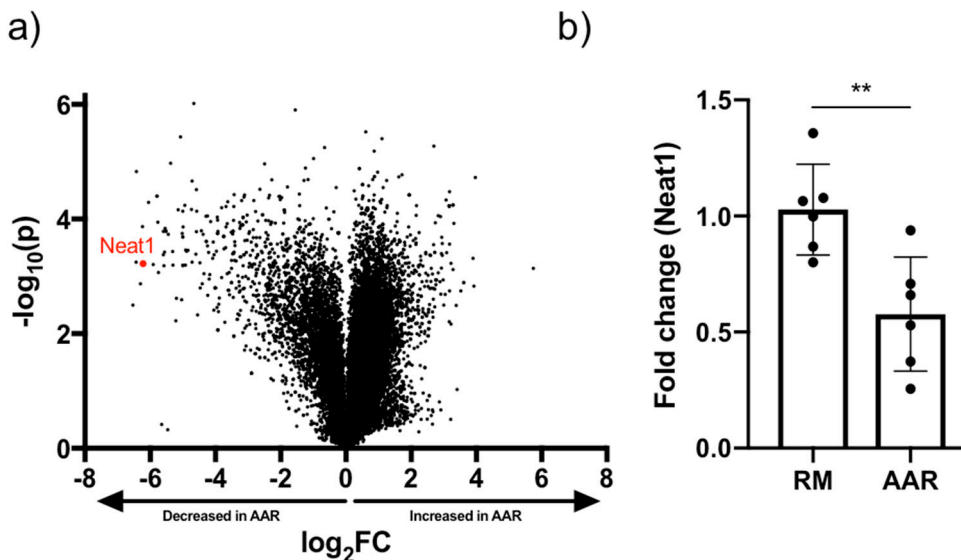

Figure 16. Identification of *Neat1* in cardiac tissue from ischemic preconditioning mouse model. a) Assessment of lncRNA expression in remote myocardium and area at risk from mice subjected to IPC. *Neat1* is shown in red. b) Expression of *Neat1* in cardiac tissue from remote myocardium and area at risk of IPC mice. Results are analyzed by qRT-PCR and normalized to *Gapdh*. **p<0.01.

Downregulation of Neat1 *protects cells from hypoxia*

We further wanted to investigate whether downregulation of Neat1 could be part of a mechanism protecting cardiomyocytes from hypoxia. HL-1 and iPS-CM cells were transfected with siRNA targeting Neat1, followed by hypoxia. Results showed Neat1 knock down led to a decrease in apoptotic cells (Figure 17a), assessed by Annexin V-staining and a Caspase 3/7 activity assay (Figure 17b).
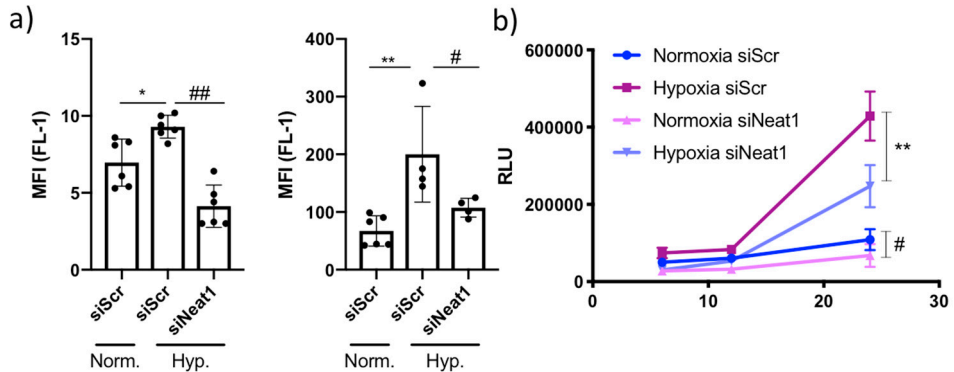

Figure 17. Protection from hypoxia by downregulation of *Neat1* in cardiomyocytes. a) Decrease of apoptotic cells in HL-1 (left) and iPS-CM (right) when pre-treated with siRNA targeting *Neat1* (siNeat1) compared to control cells treated with scrambled negative control (siScr), followed by hypoxia or normoxia and stained with Annexin V-FITC. Mean fluorescence intensity (MFI). b) Assessment of caspase 3/7 activity in iPS-CM pre-treated with siScr or siNeat1 followed by hypoxia or normoxia. *p<0.05,**p<0.01, #<p0.05, ##p<0.01

Interaction between NEAT1 *and miR-22*

Neat1 has been shown to have a role in the processing of primary miRNAs in the nucleus, and we wanted to explore this mechanism in cardiomyocytes. A screening of 96 miRNA/pri-miRNA pairs with abundant cardiac expression identified miR-22 as one of the pri-miRNAs processed by *NEAT1*. miR-22 has been shown by others to be involved in the response of cardiomyocytes to ischemia/reperfusion-induced injury. Therefore we wanted to see if the interaction between *NEAT1* and miR-22 could be involved in a cardioprotective mechanism. To confirm the pro-apoptotic role of miR-22, iPS-CM were transfected with pre-miR-22 for 24h, resulting in increased Caspase 3/7 activity compared to untreated control cells (Figure 18a). Thereafter, we assessed the role of miR-22 in hypoxia and found that cells treated with inhibitor against miR-22 reversed the hypoxia-induced apoptosis. We then wanted to assess whether there is a direct interaction between Neat1 and miR-22 by performing RNA immunoprecipitation (Figure 18b). When the miR-22 target site of Neat1 was mutated in mouse cardiomyocytes, decreased levels of pri-miR-22 were precipitated compared to control cells, indicating a direct binding between Neat1 and pri-miR-22.
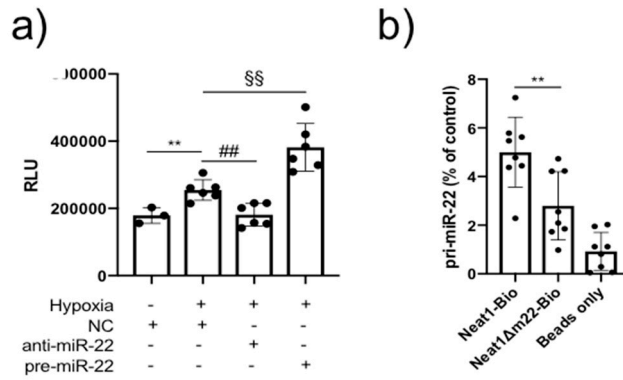
Figure 18. *NEAT1* is interacting with miR-22. Assessment of caspase 3/7 activity in iPS-CM transfected with negative control miRNA (NC), anti- or pre-miR-22 followed by normoxia or hypoxia. b) Co-precipitation of pri-miR-22 in HL-1 cells using an intact Neat1 probe, the mutated Neat1Δm22 probe or streptavidin beads only assessed with qRT-PCR.

Discussion and Future perspectives

In this thesis, we were interested in mechanisms involving non-coding RNAs and their potential roles as new therapeutic targets for the treatment of cardiac diseases. To investigate this, we used in vitro and in vivo models, as well as human heart tissue.

Paper I

In this paper, we investigated the importance of the lncRNA *NPPA-AS1* in regulation of the *NPPA* gene expression and ANP protein levels.

We confirmed *NPPA-AS1* expression not only in cardiomyocytes, but also in cardiac fibroblasts and endothelial cells. Considering that *NPPA* expression is restricted to cardiomyocytes, it is conceivable that *NPPA-AS1* might have other targets or functions in the heart. Moreover, we cannot rule out trans-acting effects of *NPPA-AS1*. Transcriptome profiling of organs from mice treated with *Nppa-as* GapmeR will elucidate the full regulatory capacity and tissue-specificity of this NAT. Further, we could see a nuclear distribution of *NPPA-AS1* and confirmed that *NPPA-AS1* might bind to *NPPA* promoter specifically at the binding site of the transcription factor REST. It has previously been shown that REST has a negative effect on *NPPA* expression in rat ventricular cardiomyocytes.[109] We propose a mechanistic model in which *NPPA-AS1* acts as a scaffold for REST and facilitates its interaction with the *NPPA* promoter, resulting in decreased *NPPA* levels.

We could see a positive correlation between *NPPA* and *NPPA-AS1* levels in human heart tissue from heart failure patients and cells subjected to mechanical strain. This does in our view not contradict a mechanism whereby NPPA-AS1 acts as a negative feedback loop on NPPA, but rather indicates common upstream regulators. These matters will be the subject of future studies.

Using a GapmeR to inhibit *Nppa-as* in mice, we observed an increase in *Nppa* expression and Anp levels, suggesting that *NPPA-AS1* could be a potential target for atrial natriuretic peptide augmentation in heart failure.

However, additional work using a mouse model of heart failure is ongoing in order to investigate the therapeutic benefit of *Nppa-as* inhibition.

Paper II

Augmentation of the natriuretic peptide system by inhibiting neprilysin has been shown to have therapeutic benefits for certain types of heart failure patients. However, more specific strategies to achieve natriuretic peptide augmentation, in order to increase therapeutic efficacy and reduce adverse effects, are needed. In this study, we focused on miRNAs that might have an effect on the biogenesis of ANP, and more specifically the enzyme CORIN, which produces biologically active ANP.

Using a functional screening, we identified the miR-1 family as potential regulators of CORIN activity and could show that miR-1-3p has a negative effect on CORIN activity by directly binding to 3'UTR of *CORIN* mRNA. In addition, we identified several other miR-1-3p targets involved in transcriptional regulation of the *NPPA* gene. The fact that miR-1-3p expression is restricted to cardiomyocytes and affects ANP biogenesis through multiple targets makes it a specific and potent target for natriuretic peptide augmentation. However, the potential of miR-1-3p as a therapeutic target should be followed up in vivo. In other studies, overexpression of miR-1 in mice resulted in prevention of cardiac remodelling[110] but also promoted arrhythmia,[111] showing the importance of further investigation.

Taken together, the results in this study suggest that miR-1-3p constitutes a potential candidate for augmentation of ANP levels in heart failure.

Paper III

miRNAs are possible targets for altering expression of specific genes or pathways that can be used for therapeutic purposes. Others have shown a negative effect of miR-105, miR-155 and miR-425 on ANP levels and in this paper, we wanted to validate this in human cardiomyocytes and further see the effects of mechanical stretch on the miRNAs.

Here, we used human iPS-CM for the investigations. However it is important to observe that these cells are not fully representative of adult cardiomyocytes. iPS-CM have structural and functional properties of immature or fetal cardiomyocytes.[33, 34] It should also be recognized that iPS-CM often consists of a mixture of atrial, ventricular and nodal cardiomyocytes. Based on transcriptional and functional analyses, we could show that the iPS-CM used in this study had properties of atrial cardiomyocytes and constitutes a relevant cellular model for the study of ANP biology.

Previous studies have shown strain to influence the expression of miRNAs.[112, 113] Here, we saw a negative effect of mechanical stretch on miR-105, miR-155 and miR-425. This indicates that the stretch-induced *NPPA* and ANP levels are due to a combination of mechanical stress and inhibition of miRNAs involved in the biogenesis of *NPPA*. Most importantly, we could see an increase of ANP expression in cells transfected with anti-miR-155 prior to strain, suggesting miR-155 as a potential therapeutic target. However more studies have to be done to evaluate the importance of miR-155 in the biogenesis of ANP. miRNAs have a pleiotropic function, which has to be carefully considered before pursuing miRNAs as therapeutic targets due to the potential off targets that could emerge.

Paper IV

lncRNAs are now recognized as key regulators of different biological processes, including the cellular response to hypoxia. In this paper, we wanted to identify lncRNAs involved in IPC-induced cardioprotection.

We found over 2000 altered lncRNA transcripts in cardiac tissue from IPC-mice, suggesting that IPC induces profound alterations of the non-coding transcriptome and is potentially linked to cardioprotection through a multitude of pathways.

The lncRNA Neat1, a key component of nuclear paraspeckles, was one of the lncRNAs decreased after IPC in mouse heart tissue. This could also be seen in human cardiomyocytes exposed to short-term hypoxia, indicating a conserved mechanism. We further showed that decreased *NEAT1* levels resulted in impaired pri-miR-22 processing and further protecting cells from hypoxia-induced injury. miR-22 have been shown to both inhibit and trigger apoptosis depending on different cell types and mouse models and here, we could see a positive correlation between hypoxia-induced injury and miR-22 levels in human cardiomyocytes. This suggest that miR-22 might have different function depending on the species and environment. However, additional work has to be done to evaluate the importance of miR-22 in hypoxia-induced injuries in human heart diseases.

Further in vivo work should be performed to assess the importance of Neat1 and miR-22 in cardioprotective mechanisms, where knock down of cardiac Neat1 is investigated in an ischemia/reperfusion mouse model.

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