Dissertation

Mechanisms of trigger induced cardiac remodelling

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

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Disclosures

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Every form of art requires an inspiration. Without naming anyone, I have found among people and circumstances the inspiration I needed.

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Abreviations

ACTA1 skeletal alpha (α)-actin

AFib atrial fibrillation

CaMKII Ca^{2+}/calmodulin-dependent protein kinase II

DCM dilated cardiomyopathy

dD diastolic dysfunction

FHL1 four and a half LIM domains 1

HFpEF heart failure with preserved ejection fraction

IPA Ingenuity Pathway Analysis

MC myocytes

miRNA micro-RNA

NCX sodium-calcium exchanger

NCXI the sodium-calcium exchanger inhibition

NF non-failing

NMC non-myocytes

NppA proatrial natriuretic peptide A

NppB proatrial natriuretic peptide B

NRVCM neonatal rat ventricular cardiomyocytes

RCAN1 regulator of calcineurin 1

SR synus rhythm when referencing human patients otherwise sarcoplasmic reticulum when referencing intracellular events

ST stretch

TC tachycardia

TRPC transient receptor potential canonical channel

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Zusamenfasung

Zelluläre Dehnung und Tachykardie können Auslöser einer transkriptionellen und strukturellen Remodellierung im Herzen sein. Diese Veränderungen sind anfangs asymptomatisch und kaum zu diagnostizieren. Das Ziel dieser Doktorarbeit ist ein verbessertes Verständnis der frühen molekularen Remodellierungsprozesse durch Dehnung und Tachykardie. Dazu wurden Untersuchungen durchgeführt in (1) neonatalen Ratten-Kardiomyozyten (in vitro) für spezifische Signalgebung und zelltyp-abhängige Prozesse und (2) Trabekeln aus menschlichen Herzen für die Antwort auf Gewebe-Ebene.

In vitro (Teil 1) fand sich sowohl bei Dehnung als auch Tachykardie in den zwei häufigsten Zelltypen, Myozyten und Fibroblasten, eine exzentrische Hypertrophie. Beide Trigger führten in Myozyten zur Hochregulation von Remodellierungs-Genen mit unterschiedlichen Expressionsmustern, in Nicht-Myozyten hatte lediglich Tachykardie einen Effekt. Nachfolgend wurde die Exzitations-Transkripitions-Kopplung (ETC) mittels Inhibitoren von Ionen-transportierenden Proteinen untersucht hinsichtlich ihrer Effekte auf einzelne und genomweite Genexpressionsänderungen. Wir konnten nachweisen, dass: (a) der Rückwärts-Modus des Natrium-Kalizium-Austauschers (NCX) maßgeblich bei sowohl Dehnung als auch Tachykardie beteiligt ist, (b) dies bei Dehnung genomweit 40% und bei Tachykardie 18% ausmacht, (c) beide Trigger den NCX aktivieren, wobei nur Dehnung zusätzlich auch die Expression des NCX steigert, (d) die Kalzium-Calmodulin-anhängige Proteinkinase II (CaMKII) nur bei Tachykardie über den NCX aktiviert wird.

In atrialen Trabekeln aus Humanherzen (Teil 2) fanden wir genomweit eine große Fraktion unabhängig regulierter Gene durch die beiden oben genannten Trigger. Das am stärksten regulierte Gen und somit potenzieller Biomarker fand sich aber in der kleineren gemeinsam regulierten Fraktion: Die micro-RNA „MIR1183“, sowie zwei ihrer Zielgene. MIR1183 erwies sich nicht nur durch akute Dehnung und Tachykardie als hochreguliert, sondern auch durch chronisches Vorhofflimmern und bei dilatativer Kardiomyopathie.

Schlussfolgernd beschreibt die vorliegende Arbeit Schlüsselmerkmale der frühen Remodellierung unter Dehnung und Tachykardie, die Großteils Trigger-spezifisch und kleineren Teils gemeinsam reguliert sind. Sie liefert außerdem einen vielversprechenden gemeinsamen Biomarker für kardiale Remodellierung mit klinischer Bedeutung.

Abstract

Stretch and tachycardia are pathological events capable of triggering the heart to enter transcriptional and structural remodelling. Initially these events remain asymptomatic and are difficult to diagnose, until more severe changes occur. We aimed to better understand the molecular basis of how stretch and tachycardia induce the early events in cardiac remodelling. In this thesis, we performed two independent study parts by using (1) neonatal rat cell culture, suitable for dissecting pathological signalling events and cell specific actions, and (2) human multicellular trabeculae, which simulate a tissue specific response.

By applying either stretch or tachycardia as a trigger *in vitro* we found an eccentric hypertrophy in the two prevalent cell types: myocytes and fibroblasts. Both triggers induced early remodelling genes in myocytes, but with different patterns. In non-myocytes only tachycardia had significant effects. Next we investigated the excitation transcription coupling (ETC) by inhibition of various ion handling proteins and characterized their effects on marker gene expression and whole genome wide microarrays. We could determine that: (a) stretch and tachycardia mediated effects mainly depend on the activation state of reverse mode of the sodium calcium exchanger (NCX), (b) inhibiting NCX prevented these effects as revealed by microarray analysis (40% of the effects in stretch and 18% in tachycardia), (c) stretch and tachycardia increased NCX activity, but stretch also increased NCX expression and (d) Calcium/Calmodulin-dependent protein kinase II (CaMKII) participated in the tachycardia mediated response downstream of NCX but not in the stretch response.

In human atrial trabeculae (part 2) we applied acute stretch or tachycardia and observed largely independent gene regulations by using microarray technology. Only a small commonly regulated fraction was present. But within this we identified a miRNA precursor gene - MIR1183 as the most promising biomarker along with two of its putative downstream targets. MIR1183 was not only upregulated in acute stretch and tachycardia, but also in a chronic state of atrial fibrillation and in dilative cardiomyopathy.

In conclusion, this thesis helps in taking a step forward in describing key features of early excitation transcription coupling, which are either stretch/tachycardia specific, or shared in their regulation including a promising biomarker for clinically relevant cardiac remodelling.

1 Introduction

1.1 Introduction to role of stretch, tachycardia and sodium-calcium exchanger in induction of early cardiac remodelling.

1.1.1 Epidemiology of Cardiovascular disease

Heart diseases resulting in broad manifestation of clinical symptoms are the leading cause of death worldwide, both among men and women affecting in approximation 20 million people. Cardiac disorders, with either structural or functional background, ultimately result in heart failure - a debilitating syndrome which is reaching epidemical proportions particularly affecting the population older than 65 with a tendency in upcoming years to further increase mortality and morbidity and expand healthcare burden (1). Great efforts had been invested in minimizing risk factors that contribute to the development of cardiovascular disorders e.g. coronary heart disease. Consequently, epidemiological studies of heart failure report that the decrease in incidence occurs as a result of improvements in prevention strategies. However, the prevalence remains, and moreover it is even increased. Advancements in therapy following cardiac events such as myocardial infarction contribute to higher survival rates but increased prevalence of heart failure. Long-term health care with frequent hospital admissions (approximation of 1 million per year) and readmissions has an impact on economy, which will continue to represent a burden with significant rise in the next decade. Other factors that influence the estimated 46% prevalence increase are prolonged life span of the aging population. Mayor efforts must be focused therefore on reducing heart failure prevalence with benefits such as less socio-economic burden and an improvement in life quality.

1.1.2 Heart failure and cardiac remodelling

What differentiates the diseased heart from the healthy is the inability to keep up with the physiological needs of the body. The main hallmark of heart failure is pump decompensation leading to its impaired hemodynamic function. Heart failure can be initiated gradually following numerous risk factors such as coronary heart disease and hypertension

or it can be a result of a sudden insult such as a myocardial infarction. Heart failure is categorized as HFrEF – heart failure with reduced ejection fraction (EF < 40%) and HFpEF – Heart failure with preserved ejection fraction (EF ≥ 50%). A new term introduced by the European Society of Cardiology categorizes patients with borderline EF between 40 – 49% as the mid-range reduced ejection fraction or HFmrEF group (2). Regardless of the form, heart failure always manifests itself on cellular level altering cardiomyocyte morphology that then translates to the organ level by decreasing its functionality and ability to provide enough blood supply to meet the metabolic demands. Before the manifestation of the first clinical symptoms the heart, as an early response to a specific triggering stress, undergoes a series of molecular events, which are known as the remodelling process. Early cardiac remodelling represents a crucial step in manifestation of structural changes on the heart. Importance of early cardiac remodelling is most notably seen when taking into consideration the asymptomatic nature of this first stage towards heart failure. Early cardiac remodelling is succeeded by later stages where patients develop first signs of functional changes. Remodelling may be in form of mechanical, structural and electrical remodelling (3) Mechanical remodelling revolves around altered contraction and relaxation properties of the heart muscle. Structural remodelling manifests itself as hypertrophy, fibrosis and inflammation both on cellular and organ level. Finally, electrical remodelling gives rise to different types of cardiac arrhythmias for e.g. atrial fibrillation and tachycardia.

1.1.3 Mechanical cardiac remodelling

Preserved contraction-relaxation coupling is an important feature in maintaining normal pump functionality. Diastolic dysfunction or inability of the heart to relax efficiently during diastolic filling is one of the hallmarks of mechanical remodelling. Diastolic dysfunction is usually present in both HFrEF and HFpEF, however the underlying mechanisms are poorly understood especially when it comes to HFpEF. Heart failure with reduced ejection fraction is exclusively linked to systolic dysfunction and it represents the inability of the heart to pump blood efficiently due to LV chamber dilation, impaired cardiomyocyte contractility and increased stiffness. Systolic dysfunction is often accompanied by diastolic dysfunction (4). Molecular mechanisms predominantly involved in HFrEF are tied to biological processes such as smooth muscle cell proliferation, nitric oxid biosynthesis, transcription regulation while HFpEF is linked more with biological processes involved in the

inflammatory response as well as ECM organization. It is challenging to clearly differentiate between HFrEF and HFpEF since they share most biomarkers, however to a different magnitude. Prevailing biomarkers of HFrEF include AMP-dependent transcription factor activating transcription factor 2 (ATF2), N-terminal pro–B-type natriuretic peptide (NT-proBNP), Growth differentiation factor 15 (GDF-15) and Interleukin 1 receptor-like 1 (IL1RL1). On the other hand HFpEF shows more often changes in levels of Integrin Subunit Beta 2 and Catenin Beta 1 markers (5).

1.1.4 Structural cardiac remodelling

Hypertrophy is the most prominent structural change which represents an increase in mass, overall heart dimensions, as well as increase in the left ventricular cavity along with thickening of the LV wall. In respect to cell size hypertrophy is defined as increase in the number of sarcomeres (cardiomyocyte contraction units) either longitudinally (eccentric hypertrophy) or in parallel (concentric hypertrophy). Hypertrophy can be divided into two categories – physiological and pathological. Both types are a form of an adaptation – a compensatory mechanism that the heart undergoes during changes in preload and afterload visible on gene expression levels and functional parameters, however, through a different mechanism of action (6). The main difference between the two categories can be attributed to the detrimental effects of pathological hypertrophy on cardiac performance. What is known is that pathological hypertrophic remodelling involves initiation of fetal gene expression making the heart more resilient under altered conditions, which require increased cardiac output (7). The genes involved are mainly responsible for increasing contraction and excitation properties of the cell. These genes consist of ANP, BNP, ß-MHC, SkA, SMA (8) and are commonly used as biomarkers of heart failure. Along with fetal gene re-expression there is an increase in transcriptional factor activity that either have a hypertrophic or anti-hypertrophic effect. Most notable pro-hypertrophic transcription factors include the families of GATA, MEF2, Csx/Nkx-2.5, SRF, myocardin and the HAND transcription factors. The anti-hypertrophic mediators include FoxO, MITF, YYI, CHF1/Hey2 (9). These transcriptional factors act directly on the target genes or can mediate the response through an interactive way. Discrimination between types of pathological hypertrophy is based on the trigger involved. Concentric hypertrophy is a result of pressure overload and in pathological conditions it manifests during events such as myocardial infarction, chronic

valve disease, hypertension, aortic stenosis while physiologically it is a result of pregnancy, exercise or growth factor stimulation. Volume overload can result from conditions such as mitral valve regurgitation or tachyarrhythmia. In physiologically normal conditions there is evidence that eccentric hypertrophy is more beneficial for enhancing muscle performance when investigating skeletal muscle during exercise (10). However, in pathological conditions it is also proposed that eccentric hypertrophy is associated with a poorer prognosis than it would be the case with concentric hypertrophy in the same conditions (11). Besides the effects on cardiomyocytes, remodelling affects the non-myocytes as well which also undergo morphological changes (12). This leads to the enlargement of the myocardial cavity as well as changes in elasticity and wall thickness. Cardiac fibrosis is the structural change specifically linked to cardiac fibroblasts and manifests itself through differentiation to myofibroblasts and excess collagen production, deposition, decreased breakdown and extracellular matrix remodelling. This process results all together in increased stiffness of the ventricular wall, consequently decreasing cardiac performance. Fibrosis can occur as a form of compensation, for the loss in the number of cardiomyocyte e.g post myocardial infarction or it can be also a result of persistent pressure and volume overload (13). There are many regulators of fibrosis, but essentially its initiation is bound to an inflammatory reaction. Autocrine and paracrine signals from mediators such as growth factor PDGF, components of the RAAS angiotensin II and aldosterone, various cytokines such as TGFß and interleukins activate myofibroblasts into producing collagen and play a crucial role in inflammation induced fibrosis (14).

1.1.5 Electrical cardiac remodelling

Changes in electrophysiological properties of the heart termed electrical remodelling can affect both the atria and the ventricles. Electrical remodelling can be termed as primary and secondary remodelling. Primary remodelling is characterized as the stage before structural abnormalities in the heart and is the result of activation dependent changes. Pacing rate dependent changes, as well as a flaw in transmitting the electrical impulse from one cell to the other are the primary source of this type of remodelling. On the other hand, secondary remodelling shows signs of structural alterations and is a consequence of hypertrophic structural remodelling, heart failure and a sudden insult such as myocardial infarction. Both types of remodelling contribute to arrhythmogenesis. When electrical remodelling happens

in the atria it is very often a result of sustained atrial fibrillation (15) contrasting the electrical remodelling initiated in the ventricle (16). In advance stages of cardiac remodelling where heart failure is initiated the ventricular myocytes show prolongation of the action potential duration (APD) irrespective of the disease etiology. These changes are accompanied with either early after depolarisation (EAD) or delayed after depolarisation (DAD) (17, 18). Changes in ion channel conductance, expression and distribution are one of the mechanisms by which arrhythmogenesis is promoted. These changes are associated predominantly with potassium, sodium and calcium channel abnormalities. For EAD to occur there needs to be a reduction in outward and an increase in inward currents (19). The reduction is observed to be present in slow and rapid delayed rectifier potassium channels I_{ks} and I_{kr}, I_{CaL}, I_{NCX} during EAD (20). Calcium induced calcium release and increased late I_{Na} contribute to the propagation of EADs. The exact mechanism by which late I_{Na} is initiated remains to be fully determined (21). On the other hand, the mechanisms behind DADs are mainly directed towards increase of intracellular calcium. The calcium increase is a consequence of NCX reverse mode action, together with the unspecific ion current. Both are initiated during spontaneous calcium sarcoplasmic reticulum (SR) release (22). Cell to cell signal propagation is also responsible for arrhythmogenesis, specifically through abnormalities in expression, structure and distribution of connexin 43 (Cx43). Major studies have been directed to establish the underlying mechanisms of atrial fibrillation propagation.

1.1.6 Triggering events of cardiac remodelling

In order to undergo pathological cardiac remodelling a cell needs a specific trigger that initiates the maladaptive signaling pathway which then further leads to functional remodelling. Early on these triggers act as stressors to the cardiac wall and they can be hemodynamically categorized as pressure or volume overload. Neurohormonal stimulation is categorized as a remodelling enhancer.

1.1.7 Mechanical Stretch

As a consequence of acute mechanical load the heart undergoes an immediate adaptation known as the Frank-Starling mechanism due to increased myofilament sensitivity towards

calcium (23-25). If the stretch condition persists, the contractility increases gradually through a mechanism known as the slow force response mediated by involvement of NHE1 ion transporter (26). However, sustained pressure (27) or volume overload imposes persistent wall stress and will in turn cause left ventricular hypertrophy (LVH). There are numerous methodologies used in studying the pathology behind stretch mediated onset and progression of cardiac disease. *In vitro* cyclic stretch is applied to study the molecular changes and make correlations with physiological changes that occur in animal models during overload of the cardiac muscle (28, 29). *In vivo*, transverse aortic constriction (TAC) along with other adapted techniques that involve aortic banding (30-32) is an experimental method that induces hemodynamic overload in animal models (33) and is used to study hypertrophy (34) and load dependent heart failure (35). Although it has been reported that the hypertrophic response is strongly dependent on the synergistic interplay between stretch and neurohumoral stimulation e.g. angiotensin (36), respectively, stretch can act independently of neurohumoral stimulation to promote hypertrophy (37). Additionally, *ex vivo* methods were used to simulate increased load conditions in multicellular tissue preparations and to study functional and mechanistical properties behind the slow-force response (38, 39) connexin 43 (Cx43) (40), myofilament contractile properties (41) arrhytmogenesis (42) and many more.

1.1.8 Neurohormonal activation

Neurohormonal activation is initially evoked as a result of decrease in cardiac output and therefore is regarded as a compensatory mechanism. However, when the activation is persistent it can further advance congestive heart failure. The mechanism of action is exerted mainly through the components of the renin-angiotensin-aldosterone system (RAAS) followed by activation of arginine – vasopressin (AVP) and other vasoactive components (43). Impairment in pump function prevents the heart in maintaining normal circulation. When the fluid homeostasis is disrupted baroreceptor signals are received by the sympathetic nervous system which in turn enhances cardiomyocyte contractility, vasoconstriction and increases heart rate. In cooperation with the SNS the RAAS system works towards increase of blood pressure (44). An enzyme - renin secreted by the kidneys converts angiotensinogen to angiotensin I, which is sub sequentially, by actions of ACE, converted to angiotensin II. Angiotensin II promotes vasoconstriction of blood vessels during hypovolemic conditions.

Angiotensin II also influences secretion of aldosterone - a mineralocorticoid hormone which acts in the renal colleting tubes and promotes Na^+ absorption and K^+ and H^+ excretion increasing thus volume (45, 46). Vasopressin acts through vasopressin receptors V1, V2, V3 found in kidney, nervous and smooth muscle cells. Its main action revolves around osmoregulation (47). Other vasoactive molecules such as catecholamines also play an important role in relieving hypotension (48). However, persistent activation state of these systems results in sustained hypertension (49) which is one of the major factors that contribute to congestive heart failure. ACE inhibitors are a main strategy in reducing symptoms of congestive heart failure by decreasing levels of circulating angiotensin II and aldosterone (50). Besides the systemic effects, neurohormonal agonists can also act directly on the cells via several receptors. The angiotensin receptor 1 inhibitor losartan is widely used in patients that are not candidates for ACE inhibitors and several studies have proven its beneficial effects in restoring hemodynamic homeostasis (51). The AT1 receptor can exhibit GPCR dependent and independent mechanisms (52). Also it interacts with downstream signaling molecules NADPH oxidase and is thus involved in ROS generation (53). It activates both the WNT/ß catenin and Notch signaling pathways which are significant for cellular development and found to be active during cardiac injury and fibrosis promoting cell to cell interactions (54). Angiotensin II induces mitochondrial injury leading to generation of ROS (55). The AT2 receptor is also proposed to be necessary for the hypertension induced hypertrophic phenotype (56). Besides the RAAS system, natriuretic peptides have importance in cardiac remodelling: ANP and BNP promote antihypertrophic effects and reduce volume overload in heart failure (57). Both peptides are negatively regulated by the vasopeptidase neprilysin. Pharmacological inhibition of neprilysin in combination with an angiotensin receptor blocker has strong reverse remodelling effects and strongly improves outcome in heart failure (58).

1.1.9 Tachycardia

Tachycardia is often recurrent and can lead to sudden death or decompensated heart failure (59), which speaks of the need to characterize the very early events in remodelling. Frequently performed *in vitro* tachycardia studies involve research done on gap junction modulation as a primary source of cell to cell electrical propagation. One such study uncovered modifications of gap junction associated protein Cx43 by changes in ß-catenin

expression which is in turn ANGII/MAPK related (60). Another study confirmed the involvement of ANGII/MAPK in rapid electrical stimulation modification of Conexin 43 (Cx43) (61). Studies done in large animal models (62) (63) used chronic short-term and long-term pacing to uncover the mechanisms behind atrial fibrillation (64, 65). Field stimulation at sustained low frequencies or gradually increased frequency rates is widely used in tissue engineering to improve maturation (66) induce differentiation (67) and enhance cell contractility properties (68). By using induced pluripotent stem cells (iPSC) it has been shown that functional remodelling is achieved when combining mechanical stress and electrical stimulation and is beneficial for cardiac engineering (69). Physiological frequency rates depend on the model used to study rapid pacing. Therefore, studies done *in vitro* reveal that electrical stimulation at 2 Hz induces impairment of mitochondrial function in the P19 mouse cell line (70) and after 3 Hz in iPSC (71) and are considered pathological frequencies. However, other studies done in engineered tissue (EHM) show positive force frequency relationship even at 4 Hz stimulation rate (72) which is therefore considered as still physiological. This particular frequency is at the submaximal rate before pathological insult which is shown to be damaging on long-term in adult rat cardiomyocytes (73). Electrical remodelling on the other hand was shown to be induced at 6 Hz frequency for HL-1 cells (74). Atrial cardiomyocytes show disturbance in calcium homeostasis mediated via changes in CAMKII expression after 10 Hz frequency (75). Neonatal rat ventricular cardiomyocytes (NRVCM) show changes in contractility starting at 5 – 8 Hz (76). NRVCM cells can tolerate long term stimulation rates as high as 99 Hz as long as they are subjected to subthreshold voltage and pulse duration (77). Multicellular trabeculae were also used in conditions of increasing frequency rates to study the force frequency relationship (FFR) in normal (78) and failing ventricular myocardium (79, 80). Similar to *in vitro* findings the FFR is positive under physiological conditions for the selected tissue (81) and starts to deteriorate under higher frequencies (82) accompanied with occurrence of electrical alternans at supraphysiological frequencies (83). Therefore, tachycardic insults were generally more studied in terms of functional effects *ex vivo*.

1.1.10 Mechanism of communication between cardiomyocytes and non-myocytes

Crosstalk mechanisms between cardiomyocytes and non-myocytes are known to promote cardiac remodelling. Mediators of paracrine signaling have been identified as

exosomes that are released from one type of cell and transported to the neighboring cell where they are taken up and their content is released intracellularly. This conclusion followed the observation that cardiomyocytes begin to develop hypertrophy when grown in fibroblast conditioned media. MiRNAs such as Mir21; mir29b, mir30c were targeted as mediators in paracrine signaling between cardiomyocytes and non-myocytes during ATII stimulation (84, 85). The role of specific MiRNAs have been also investigated during stretch (86) in enriched NRVCM and in generation of tachyarrhythmias in an atrial HL1 cell (87) however the cell specific contribution to paracrine signaling is still not fully understood. Apart from exosomes, trigger affected cells secrete molecules that directly bind to receptors on the target cells thus initiating the maladaptive signaling pathway. These molecules include TGFß, IL-6, angiotensin II, endothelin 1. The mentioned mediators are produced in non-myocytes and can act in a paracrine mediated way or as another possibility in an autocrine fashion as shown in the case of TGFß (88). For example the crosstalk between atrial myocytes and non-myocytes during high rate frequency stimulation leads to myocyte mediated effects on fibroblasts through secretion of TGFß (89). Additionally, single cell RNA sequencing performed on cardiac non-myocytes showed pathways of intercommunication between different non-myocyte cell populations (90). Extracellular matrix remodelling is known to contribute to heart failure by promoting cardiac fibrosis (91). Non-myocytes predominantly secrete ECM proteins such as collagen and fibronectin which can affect the cardiomyocytes directly (92). Collagen was reported to play a significant role in the regulation of TGFß (93) and AT1 receptor 2 signalling (94) while fibronectin was found to mainly act through integrin-linked signalling pathway in cardiomyocytes (95). Matrix metalloproteinases (MMPs) and their inhibitor (TIMP) are significant modulators of cardiac fibrosis post myocardial infarction (MI) (96, 97) and their secretion from myocytes and non-myocytes is found to be bidirectional (98). Other relevant forms of communication involve direct cell to cell contact between cardiomyocytes and non-myocytes. Stretch induced mechanical coupling propagates contraction from one cell to another through existing junctions and enforces synchronized beating of the embryonic heart (99). This is opposed to the electro-mechanical coupling that regulates heart beat in adult myocardium. However, formation of mechanical junctions in the adult heart has negative effect on cardiomyocyte contractility by impairing electrical conduction (100). This effect is mediated by an adhesion receptor - N-cadherin. Another form of cell adhesion involves integrins which can act in concordance with cadherins to control cell movement and motility (101). Gap-junctions enable electrical coupling of cardiomyocytes (102) and changes in their

distribution contributes to arrhytmogenesis (103). However, it is also described that cardiomyocytes and non-myocytes may form gap junctions as well (104). Interestingly an essential component in many forms of cell communication including paracrine mechanism, nanotube related mechanical cell interaction and electrical coupling appears to be connexin-43 a protein localized in the gap junction area (105). Irrespective of the mode by which cardiomyocytes and non-myocytes develop a joint interplay it is essential to take in account both cell types while making conclusive statements that concern cardiac remodelling.

1.1.11 Sarcolemma proteins and ET coupling (ETC)

Proteins expressed on the cell membrane known as the sarcolemma are essential for the process of excitation contraction coupling (ECC) and also excitation transcription coupling (ETC). When a specific trigger promotes cardiac remodelling it starts by initiating changes in expression levels of proteins, respectively ion channels or modifying their interaction with downstream signaling mediators and indirectly or even directly with the target genes. The most important sarcolemma proteins necessary for normal contraction properties are the sodium family of channels, L type Ca channel (LTCC), potassium channels, sodium-potassium ATPase, sodium-calcium exchanger (NCX).

1.1.12 The sodium-calcium exchanger (NCX)

The sodium-calcium exchanger antiporters consist of 3 isoforms and 22 splice variants encoded by the SLC8A superfamily of genes. NCX1.1 splicing form is highly expressed in the heart and encoded by the SLC8A1 gene. NCX is involved in maintaining the intracellular calcium homeostasis by extruding one Ca^{2+} ion in exchange for three Na^{+} ions and this is referred to as the forward mode of action. The mechanism behind the antiporter mode of transport involves transport of each ion type in succession. The sodium influx creates a concentration gradient that facilitates and makes it possible for Ca^{2+} to move outwards - a mechanism known as the secondary active transport (106). The NCX exchanger operates predominantly in forward mode which is important as accumulation of intracellular calcium leads to pathological conditions. Calcium overload contributes to conditions of arrhytmogenesis following CPVT (107), monomorphic ventricular tachycardia as a result of

digitalis exposure (108), ischemia reperfusion injury (109) and heart failure (110). Some of these conditions are a result of alterations in LTCC function increasing extracellular Ca^{2+} entry (111), defects in SR uptake (112) and release (113) maintaining a high intracellular Ca^{2+} concentration and an improper Ca^{2+} cell removal where NCX plays a crucial role. The extent to which NCX is involved in Ca^{2+} extrusion and thus relaxation is species dependent (114) which somewhat limits the translation approach to human myocardium. NCX involvement is highly dependent on the species expression profile and electrolyte levels. The relationship between SR ATPase (SERCA) and NCX activity is in reverse correlation, where a higher involvement of NCX and reduced SERCA activity is present in humans while rodents show the opposite. Different studies showed differential relationship between NCX and SERCA during heart failure. Increase in NCX expression and therefore Ca^{2+}extrusion is seen as a consequence of reduced SERCA activity and impairment of Ca^{2+} reuptake to the SR. This increased expression can be viewed as a compensatory mechanism for contraction enhancement since reduced SERCA activity reduces intracellular Ca^{2+} (115). Additionally, during heart failure the LTCC, which are the main source of extracellular Ca^{2+}, are reported to show changes in density (116) as well as structural alterations (117) which ultimately decrease the LTCC current. Consequently, there is a higher need for an alternative Ca^{2+} influx. This is the point where NCX reverse mode activation can be beneficial and becomes the main way of Ca^{2+} entry. However, an initial compensatory mechanism can lead to Ca^{2+} overload and arrhythmogenesis. NCX reverse mode is highly dependent on Na^{+} concentration that can be increased by blocking the Na^{+}/K^{+} ATPase or prolongation of the action potential thus using the energy of the Ca^{2+} electrochemical gradient to move the Ca^{2+} intracellularly. Many pharmacological inhibitors have been designed in an attempt to specifically block NCX. Unfortunately, most of the available inhibitory compounds can block additionally other ion channels albeit in a different concentration necessary for NCX inhibition. One of the first synthesized NCX inhibitors is KB-R7934, a compound also known for its affinity to block the NCX reverse mode. KB-R7934 is used in a wide concentration range depending on the species involved. The IC50 for rat cardiomyocytes ranges from 7 to 10μM concentration (118). In order to inhibit the NCX forward mode with KB-R7934 a concentration higher than 10μM is necessary. SEA0400 (119, 120) and SN6 compounds are also widely used in NCX inhibition. SEA0400 was shown to inhibit NCX with a lower IC50 of 90nM and therefore more potency (121); however an increase in concentration prompted inhibition of other ion channels starting from 1μM SEA0400 (122). A newly developed NCX inhibitor ORM-10962 decreased occurrence of arrhythmic events

in oubain pre-treated animal models (123). Although there is clinical relevance in reducing the inward Ca^{2+} current via NCX reverse mode inhibition the pre-existing compounds are currently not applicable for trials due to selectivity and specificity challenges.

1.1.13 The sodium-proton exchanger (NHE)

The Na^+/H^+ exchanger (NHE) is encoded by the solute carrier SLC9 superfamily with three subfamilies: SLC9A, SLC9B, SLC9C (124). NHE1 along with eight more isoforms is encoded by the SLC9A subfamily and is the predominantly expressed isoform in the heart (125). The sodium proton exchanger is also part of the antiporter protein family exchanging one H^+ for one Na^+ and managing thus intracellular pH in cardiac cells among other types of cells. Other physiological roles of the Na^+/H^+ exchanger include regulation of cellular volume (126), growth and motility (127). Role of Na^+/H^+ exchanger in regulating cell growth is important not only in tumor biology but also in cardiac remodelling since inhibition of NHE was shown to attenuate hypertrophy *in vivo* (128) and *in vitro* (129). It was also shown that NHE1 participates in angiotensin II induced cardiac remodelling (130). Inhibition of NHE is therefore a promising tool in prevention of existing hypertrophy and therefore cardiac remodelling and heart failure (131, 132). Additionally, NHE plays a role in ischemia reperfusion injury (133) with an important involvement of NCX (134). There is an interaction between NHE and NCX since accumulation of Na^+ is subsequently removed by reverse mode NCX accumulating Ca^{2+} intracellularly leading to calcium overload. Functionally NHE and NCX are involved in the slow force response in the human ventricle initiated via stretch (38). Inhibitors of NHE include amiloride found already in clinical use for hypertensive patients (135). However, there is controversial data on whether amiloride would be of use for ischemic patients (136) although it is proven to be effective against cerebral ischemia (137). The more specific NHE inhibitor cariporide (HOE-694) is used in treatment of cancer patients (138). Given prior to CABG surgery it was found to be beneficial against ischemia however a higher dosage was linked to increase in stroke events (139). Results from the GUARDIAN trial done earlier reveal a beneficial effect against MI (140).

1.1.14 The L-type calcium channel (LTCC)

The L-type calcium channel is a voltage-gated calcium channel and is the main mediator of calcium influx into the cardiomyocytes during EC-coupling. The LTCC is by structure a heterotetramer constituted of a main pore forming transmembrane α1subunit encoded by *CACNA1C* along with the auxiliary extracellular α2δ subunit encoded by *CACNA2D* and the ß intracellular subunit encoded by *CACNB2* gene. Gain or loss of function mutations in the mentioned genes usually give rise to various forms of cardiac arrhythmias (141). Several intracellular molecules control calcium flux and trafficking from LTCC. First of all, calmodulin is bound to the alpha 1.2 subunit and is important in regulating the LTCC current either by facilitating or inhibiting calcium entry (142). Repression of the LTCC activity is associated with calcineurin mediated development of cardiac hypertrophy and progression to heart failure (143) while downregulation of *CACNA1C* gene via CAMKII has the same outcome (144). CAMKII is also important during excessive LTCC activity typically seen during heart failure where it phosphorylates the β2a LTCC subunit and thereby contributes to calcium overload and arrhytmogenesis (145). Mechanical stretch on the other hand was shown to preserve LTCC expression via Akt signaling (146). Channel inhibition is accomplished by a variety of available inhibitory compounds. The most commonly used include: verapamil - used in treating tachyarrhythmias (147), atrial fibrillation (148, 149) and has a long history of clinical application (150). Other drugs include nifedipine for hypertension (151, 152) , diltiazem in treatment of HCM together with verapamil (153) and is also used for research purposes *in vitro* (154-156) and *in vivo* (157, 158) :

1.1.15 Potassium channels

According to the currents that they generate there are three main groups of potassium channels present in the heart: transient outward current, delayed outward rectifying current and inward rectifying current. In the initial phase of the AP I_{to} and I_{kur} currents are responsible for rapid repolarization and highly expressed in the atria while in later stages there is a presence of I_{kr} and I_{ks} currents more expressed in the ventricle. In contrast to the mentioned currents, inward rectifying potassium channels (K_{ir}) have a possibility to conduct K^+ both outward and inward (159). Inward-rectifier potassium current (I_{K1}) is a major

determinant of the terminal AP repolarization and crucial for the setting of resting membrane potential of atrial and ventricular cardiomyocytes. On one hand, increased-I_{K1} accelerates AP repolarization and shortens AP duration, while on the other hand, the shift of I_{K1} reversal potential toward hyperpolarized membrane potentials eliminates arrhythmia (159, 160). Therefore, K_{ir} channels play a crucial role in ventricular tachycardia propagation. The difficulty in treating this condition also comes from the inability to block the channels due to their importance in regulating the membrane potential at physiological frequencies (161). Recently, K_{ir} channels have been recognized as important players in regulating calcium homeostasis in cardiac fibroblasts during atrial fibrillation (162).

1.1.16 TRP channels

The transient receptor superfamily consist of seven families of TRP channels which include: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin) and TRPN (NOMPC-like). With the exception of TRPN all TRP channels have shown a certain degree of expression in the heart (163) while in context of cardiac remodelling TRPC, TRPV and TRPM are the most studied families and have been shown to play a significant role in this process (164). The canonic transient receptor potential channels or TRPC are a family of non-selective cation ion channels most abundantly expressed in the heart where they play a significant role in processes associated to excitation-contraction coupling. A major feature is their ubiquitous expression in the heart as well as their role in cardiac remodelling specifically oriented towards hypertrophy. Certain members of the TRP families are prone to activation in presence of an external mechanical force and they are therefore termed as mechanosensitive TRP channels. To date ten TRP channels (TRPC1, 5, 6; TRPV1, 2, 4; TRPM3, 7; TRPA1; TRPP2), are characterized as mechanosensitive and play a role in cardiac diseases related to increase pressure load (165). There is evidence of synergistic effects of NCX1 with TRPC3 (166) and TRPC6 (167) channels by functional coupling. Additionally, although KB-R7943 is primarily a selective inhibitor of the reverse mode NCX, it is known to affect other signaling molecules such as TRP channels specifically TRPC3, TRPC5 and TRPC6 (168).

1.1.17 Intracellular calcium signaling pathways contributing to heart failure

The major components of the intracellular machinery that is often associated with early changes following pathological insult are linked to the calcium signaling. Intracellular mediators of calcium signaling, previously associated to pathological remodelling, include primarily the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and Calcineurin. The CaMKII mediated gene regulation involves a signaling cascade and several transcription factors. CaMKII is a serine/threonine holoenzyme activated by the Ca^{2+}/Calmodulin complex. Different isoforms of CaMKII are coded by four genes (α, β, γ, and δ) with CaMKIIδ_B and CaMKIIδ_C splicing variants being the most abundantly expressed in the heart (169). All isoforms have the following in common: the N-terminal catalytic domain, a regulatory domain that is responsible for CaM binding and auto-inhibition and the C-terminal terminal association domain (170). An important feature of CaMKII is the ability to auto-phosphorylate, meaning that once it is activated the activation state persists even in absence of calcium. CaMKIIδ_B and CaMKIIδ_C are implicated as important players in cardiac hypertrophy as they regulate expression of pro-hypertrophic genes via MEF2 transcriptional factor. MEF2 is inhibited by HDAC4, however when CaMKII is activated it phosphorylates HDAC4 and enables dislocation from the nucleus therefore promoting MEF2 initiated gene expression (171). MEF2A and MEF2D are specifically expressed in the adult human heart, MEF2D being specifically important in pathological remodelling (172). Other signaling pathways activated via CaMKII include indirect or direct phosphorylation of transcriptional factors with differential effects on cardiac remodelling. These transcription factors include CREB (173) , ATF1 (174) , NFKB (175), AP1 (176) , HSF1 (177) , SRF (178). CaMKII can also repress transcription indirectly via repressor DREAM (144). Another mechanism of action is phosphorylation of Calcineurin which disables NFAT nuclear translocation (179). Common inhibitors used to suppress the CamKII activity are KN-62 and KN-93 (180) which block the calmodulin binding sites, AIP which blocks the catalytic domain (181) and AC3-I which blocks auto-phosphorylation (182). Elevation of intracellular calcium facilitates, as mentioned previously, Calcineurin mediated NFAT translocation. The enzyme is a serine/threonine protein phosphatase which forms a heterodimer from calcineurin A and calcineurin B (183). Calcineurin A is composed of a catalytic and regulatory domain while Calcineurin B contains four calcium binding sites while NFAT on the other hand contains an N-terminal transactivation domain, regulatory domain and a DNA binding domain (184). The NFAT transcription factor family consists of NFAT1, NFAT2, NFAT3, NFAT4 and

NFAT5 (185). NFAT is found in the cytoplasm in a hyper-phosphorylated state maintained by CK1, DYRK1 and GSK-3 (186). Once de-phosphorylated by calcineurin it engages in a conformational change that reveals its nuclear localization site and enables translocation to the nucleus (187). NFAT interacts with transcriptional factors in the nucleus to co-regulate gene expression. An interesting co-regulation in terms of cardiac remodelling is that of NFAT with NFkB (188), GATA4 (189) or MEF2 (190) promoting hypertrophy. Increase in calcineurin activity is known to promote cardiac hypertrophy and may affect the transition to heart failure (191) but can also act solely on the hypertrophic response (192). Moreover, calcineurin/NFAT is found to play a role only in pathological hypertrophy, excluding therefore physiological hypertrophy (193, 194). A total of fourteen genes negatively regulate Calcineurin/NFAT signaling, most notably *FHL2* thus reducing cardiac hypertrophy (195), and *RCAN1* via a negative feedback loop (196). Most frequently used inhibitors of the Calcineurin/NFAT signaling cascade include cyclosporine A (197) and tacrolimus (FK506) (198). On the other hand, calcineurin is not exclusively tied to pathological remodelling. Namely, a CnAβ1 splicing isoform of calcineurin is reported to have a protective role by preventing atrophy (199) and reducing hypertrophy (200). The isoforms that do contribute to heart failure may exert their actions by modulating phospholamban (PLB) phosphorylation and thus SERCA activity (201) which is the essential component of the excitation contraction coupling machinery governing Ca^{2+}uptake after the myofilament contraction cycle. Its activity highly depends on the phosphorylation status of the inhibitory PLB protein. Intracellular proteins that control PLB phosphorylation involve ß agonist triggered PLB phosphorylation by PKA at Ser16 (202) and CAMKIIδ (203) phosphorylation of Thr17 (204) which is implicated in heart rate control (205). Expression of PLB is under control of several transcription factors that either reduce its expression such as ATF3 (206) TRα1 alone or in complex with RXR (207) or increase via transcription factors such as GATA4 (208, 209). Under physiological conditions SERCA is responsible for 92 % of intracellular calcium reuptake while the rest is subjected to NCX removal (210). However, during heart failure, NCX plays an important role and its actions are directed towards extruding the increased diastolic calcium caused by downregulation of SERCA and its inability to efficiently fill the SR stores (211). Additionally, NCX reverse mode compensates for contractile dysfunction inflicted by empty SR (212) .

1.1.18 Transcriptomic studies involving trigger induced cardiac remodelling

Genome wide screening studies are a useful tool for identification of specific key regulating molecules that can be considered as valuable targets in prevention of cardiac disease onset. Studies focusing on neuro-hormonal induced remodelling showed specific expression profiles under angiotensin II stimulation *in vitro* (213, 214) and *in vivo* (215, 216). Another neuro-hormonal agonist used for transcriptomic studies is endothelin-1 (217, 218). Pressure overload initiated via mechanical strain was studied extensively in order to establish changes in gene expression that are time dependent. Frank et al. confirmed that biomechanical stretch induces morphological changes comparable in effect size to those of phenylephrine involving, however, a distinct gene expression program (219). Rysä et al. used neonatal rat ventricular cardiomyocytes for 48 h gene expression profiling and concluded that the majority of upregulation constitutes within the 24 h time point whereas a large portion of genes are downregulated after 48 h of stretch (220). More recently another microarray study concentrated also on time dependent expression of stretch induced gene alterations and singled out Rnd1 as a key player in mediating cell proliferation and hypertrophy (221). Cardiac non-myocytes have been also a key point of investigation when it comes to cardiac remodelling. Transcriptomic studies performed on fibroblasts confirmed the existence of a gene expression program related to inflammation and fibrosis (222) as well as development and function (223). Profiling of a hypertensive mouse model linked endothelial cells as well to a gene expression program promoting cardiac fibrosis (224). Microarrays were also used to show beneficial effects of co-culturing NRVCM with stem cells. In addition to microarrays, transcriptomic studies were also performed using single cell RNA sequencing. A study focusing on multicellular transcriptome analysis used neonatal and adult cardiomyocytes to investigate the regenerative capacity dependent on the cellular developmental stage and described the programs as active during the neonatal phase which might be limiting the adult regenerative capacity (225). Another study differentiated between phases of hypertrophy and transition from adaptive to pathological that involves pathways related to oxidative stress (226).

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1.1.19 Specific aims to role of stretch, tachycardia and sodium-calcium exchanger in induction of early cardiac remodelling

1. Examine the presence of morphological hypertrophy after subjecting the neonatal rat cardiac myocytes and non-myocytes to stretch or tachycardia.
2. Determine specific cell composition of pure non-myocytes in order to reveal the prevailing cell type.
3. Compare the pattern and time kinetics of stretch and tachycardia induced expression of hypertrophy-inducing marker genes.
4. Discriminate between myocyte specific actions and the extent of non-myocyte contribution on acute gene expression.
5. Use inhibitors of sarcolemma and intracellular signaling mediators in order to dissect mechanisms of action on gene expression level. Examine protein expression and activity of relevant mediators.
6. Perform genome wide screening and asses the possibility to prevent adverse gene expression by inhibiting the signaling mediator of interest.
7. Use bioinformatics tools to perform characterization of predicted molecular pathways and their association to functional cellular processes.

1.2 Introduction to remodelling in human atrial myocardium

1.2.1 Mechanisms and consequences of atrial remodelling

Mechanisms of atrial remodelling revolve around two different contributors – tachycardia and volume/pressure overload consistent with heart failure with distinct electrical remodelling properties (227). As a consequence of increased atrial pressure the atria become dilated. The enlarged atrium has a greater risk for arrhythmia propensity. It has been shown so far that there is an increase in the risk of atrial fibrillation occurrence (AFib) with progression from non-structural phase of cardiac ventricular remodelling to hypertrophy, as well as within different types of hypertrophy itself where eccentric hypertrophy carries a greater risk of AFib manifestation compared to concentric hypertrophy (228). Increased volume overload typically seen during valve disease such as mitral valve regurgitation and aortic valve regurgitation is a factor that contributes to the development of eccentric hypertrophy. In most cases, conditions leading to valve regurgitation are myocardial infarction (229), inflammation (230), rheumatic fever (231) and tissue degeneration. While a significant number of studies had been done taking left atrial remodelling into consideration, there is still a greater gap of knowledge when it comes to right atrial remodelling. Increased pulmonary arterial pressure (PAH) leads to right ventricular heart failure. Consequently right ventricular dysfunction influences atrial remodelling. Effects on the right atrial remodelling include before mentioned atrial dilatation through valve insufficiency subsequently leading to AFib. PAH is managed depending on the underlying cause, e.g. surgically by repair of the defective mitral valve or in case of primary PAH by a combination of drugs known as a triple therapy. Chronic and acute stretch (232) as well as supraventricular tachycardia (233) had been recognized as contributors to AFib events while AFib itself further propagates tachyarrhythmia events and is responsible for tachycardia-induced cardiomyopathy (234). Based on the duration and contributing factors AFib can be divided into paroxysmal, persistent, long-standing persistent and permanent. The sequence of atrial remodelling in most cases can be presented as electrical, mechanical and structural (235) although in specific cases electrical and structural remodelling can be absent (236). Electrical remodelling contributes to AFib by shortening the action potential duration (237) thus influencing repolarization (238) and by interfering with Ca^{2+} signaling causing Ca^{2+} overload (239). A repolarising ion current specific for the

atria is the ultra-rapid potassium outward current I_{Kur} and is found to be involved in the generation of AFib (240) .

1.2.2 Omics studies on acute and chronic atrial remodelling

The majority of omics studies on atrial remodelling focus primarily on different stages of atrial fibrillation. Therefore, transcriptomic profiling of the acute phases of atrial fibrillation managed to reveal parallel progression of electrical and mechanical remodelling tied to LTCC current deregulation in goat atria (241). Another study on induction of atrial fibrillation examined the left atria of a porcine model tachypaced to brief self-terminating episodes of AFib and concluded that paroxysmal AFib results foremost in induction of different transcription factors and microRNAs (242). Paroxysmal atrial fibrillation has also been the centre of interest of research dealing with early gene expression characterisation using right atrial appendages (243, 244) and left atrial appendages (245) of AFib versus sinus rhythm (SR) patients. Another research group managed to show through means of transcriptomics and proteomics the involvement of chloride channels in initiation of AFib using right and left atrial tissue from patients with rheumatoid heart disease (246). Even more attention has been directed in revealing mechanisms behind chronic models of atrial fibrillation. Using combined omics approaches in analysing atrial appendage samples enabled scientist to draw conclusions about changes in ketone body metabolism during persistent AFib (247) but also changes in alpha-keto acid metabolism during heart failure induced AFib (248). Therefore, heart failure can be the contributor as well as a consequences of atrial arrhythmias. The relationship between chronic AFib and heart failure was previously assessed in participants of the Framingham study (249). Different stages of heart failure coincide with the risk of AFib events marking early heart failure transcriptome changes as initiators of AFib in a canine model of chronic tachypacing (250). Comparing atrial tissue during fibrillation versus ventricular tissue has provided significant insight in similarities and differences of AFib gene expression profiles. When right atrial appendages from patients with persistent AFib were evaluated for gene expression changes versus the SR group it was concluded that downregulation of the majority of genes, including calcium signalling dependent genes is a feature of chronic atrial remodelling. Additionally, it was revealed that atrial AFib-samples and non-failing ventricular samples share a common pattern of gene regulation (251). The Framingham study had been also used to assess

differential gene expression of prevalent and incident atrial fibrillation (252). Permanent AFib was also evaluated on different levels starting from transcriptome patterns between the left and right atria (253), to factors that contribute to structural atrial remodelling (254) and epigenetics (255). Other transcriptomic studies focus on atrial and ventricular development in the fetal heart (256, 257) as well as AFib contributing factors in the fetal versus adult heart (258) but also cardiac aging and atrial and ventricular remodelling (259, 260).

1.2.3 Significance and use of biomarkers in cardiac disease

Biomarkers of different biotype origin either serve a purpose as early indicators of pathological changes leading to cardiovascular disease or show a prognostic value in therapy based and end-point outcome such as disease transition from acute to chronic phase cardiac disease. Thereby biomarkers show a promising role in determining and minimizing cardiovascular risk and are valuable in disease prevention and diagnosis but also in better cardiovascular outcome prediction. Overall classification of biomarkers as antecedent, screening, diagnostic, staging and prognostic relies on the stage when they were first detected (261). Depending on the screening method they can also be biomolecules or predefined cardiovascular parameters e.g. echocardiography parameters. As per accessibility, circulatory biomarkers as those found in plasma have an advantage over markers found in tissue however, they are of equal importance. Based on the biotype vast majorities of omics studies are carried out in an attempt to define biomarkers that are reliable, non-invasive and specific. A study based on the Framingham participants used numerous protein markers out of which 28 were able to predict cardiovascular outcome and are associated with cardiovascular death and mortality (262). The most well-known and highly reliable protein biomarkers of cardiac disease are the peptides BNP and NT-proBNP (263). Gene based studies are useful in associating different mRNAs with acute myocardial infarction (264), hypertension (265), hypertrophy (8), coronary artery disease (266) and heart failure of different etiology (267). A prime example of a promising gene marker is ST2 which was first detected in stretched cardiomyocytes with implication in myocardial infarction (268) and subsequently addressed as a biomarker for heart failure (269). Epigenetics also holds a potential to present the scientific community with a novel way of viewing biomarkers (270-272). A substantial amount of current research is directed towards investigating different types of non-coding biomolecules such as micro-RNAs (miRNAs)

(273) and circular-RNAs (circRNAs) (274-276). Circulating miRNAs have an advantage over mRNA markers when it comes down to half-life which makes them more superior for detection (277). The ones investigated so far are highly prevalent in type 2 diabetes, coronary artery disease, acute myocardial infarction and heart failure (278). Few challenges are presented regarding reliable quantification of circulating miRNAs (279). However, taking into account increased stability and tissue specificity the future perspective and application of miRNAs in cardiovascular disease is more than promising. A large number of the presented examples were studied in regards to ventricular remodelling and are very successful in tackling major cardiac outcomes. A set of well-known cardiac disease markers have been investigated in tissue (280) and circulation (281) and proposed as relevant in recognizing atrial remodelling. However, these markers lack atrial specificity. Instead echocardiographic parameters such as the right atrial diameter is to date a more relevant prognostic marker in correlating right atrial remodelling with heart failure outcome (282). What's more, early and late AFib remains the main domain of interest when dealing with biomarker studies (283, 284). Dealing with conditions that lead to initial electrical remodelling and are potential substrate for AFib propagation need to be investigated thoroughly since this would give rise in new risk-stratification methods and better prognosis for the disease outcome.

1.2.4 Specific aims to remodelling in human atrial myocardium:

1. Perform a comprehensive transcriptomic analysis of stretch and tachycardia stimulated specimens of human origin using microarray technology.
2. Screen for coding and non-coding genes with the best biomarker potential.
3. Verify or refute atrial specificity by examining the gene candidate expression in human ventricular myocardium.
4. Perform an initial screen for other key players that are potentially in relation with the proposed biomarker using bioinformatics prediction.

2 Material and Methods

2.1 Cell culture

The ethical approval for animal experiments was issued by the institutional animal care and use committee. Isolation of primary neonatal rat ventricular cardiomyocytes (MC) and cardiac non-myocytes (NCM) was conducted on 1 to 3-day old neonatal Wistar rats. The hearts were excised after decapitation and placed in ice-cold phosphate buffered saline – PBS (Sigma Aldrich, St. Louis, Missouri, USA). The ventricles were removed from the atria and minced to mechanically disrupt the tissue. The complete excision and mechanical disruption was done under the maximal recommended duration of 1h. In the next step the tissue was subjected to enzymatic digestion in a digestion solution (Table 1) with a pre-set pH of 7.3 and placed on a magnetic stirrer and placed in an incubator at 37°C with 0% CO_2. After 10 min of the first digestion step the supernatant was discarded to remove cell debris. The digestion procedure was repeated four more times for 15 min. Following each digestion step the supernatant was collected and centrifuged for 8 min at 350 g. The cell pellet was re-suspended in DMEM/F-12 GlutaMAX™cell culture medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% fetal bovine serum – FBS (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stored on ice. Single cell suspensions were sterile filtered through a 100 µm cell strainer and pulled together. The anti-proliferative agent BrdU (Sigma Aldrich, St. Louis, Missouri, USA) was added in concentration of 100 µM to prevent the proliferation of the remaining non-myocytes. A cardiomyocyte fraction was obtained after 90 min of pre-plating in non-coated cell culture flasks. The cell suspension containing cardiomyocytes was collected and the total and viable number of cells was determined using a TC20™ Automated Cell Counter (Bio-Rad, Hercules, California, US) as well as using the Neubauer Chamber (D&D Laborservice, Hennigsdorf, Germany). Cell culture dishes were previously coated with 1% gelatin (Sigma Aldrich, St. Louis, Missouri, US) and kept for drying for a minimum time of 30 min. The cell suspension was diluted to 10^6 cell/ml and $2x10^6$ cells were plated per cell culture dish and maintained at 37°C in a humidified atmosphere enriched with 5% CO2. The second day of cell culture the medium containing DMEM/F-12 GlutaMAX™ supplemented with 10% FBS, 1% Penicillin/Streptomycin (Sigma Aldrich, St. Louis, Missouri, US) and BrdU was replaced by a mixture of 80% DMEM/F-12 GlutaMAX™and 20% Medium M199 (Sigma

Aldrich, St. Louis, Missouri, US), GlutaMAX™ supplemented with 1% Penicillin/Streptomycin and serum starved for 3 days before stimulation. The stimulation was carried out on the fourth day following the isolation to reassure that the cells had properly attached and to provide sufficient time for cells to recover from the isolation procedure.

The non-myocyte fraction that stayed attached to the un-coated surface was kept in DMEM/F-12 GlutaMAX™ supplemented with 10%FBS and 1% Penicillin/Streptomycin for 4 days. Afterwards the cells were detached by the following protocol: 5 min PBS incubation, 5 min 0.1 % EDTA (Sigma Aldrich, St. Louis, Missouri, US) incubation and finally 15 min of Accutase (Sigma Aldrich, St. Louis, Missouri, US) incubation all done at 37°C and re-plated on non-coated cell dishes or bioflex membranes. The cell were kept in DMEM/F-12 GlutaMAX™ supplemented with 10% FBS overnight and serum starved until stimulation. The stimulation was carried out on the third day following re-plating.

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Table 1. Components of the digestion solution

Content	Volume (ml)	Final concentration
Liberase 8 x 0,3 ml (overall 12 mg)	2,4	0,08 mg/ml
Trypsin 2,5%	6	0,1 %
CaCl (0,1M)	0,015	10 µM
10 x HBSS	15	1 x
DNase I	0,150	
Water (sterile filtered)	126,6	
Total	150	

2.2 Multicellular muscle strip preparation

Human atrial trabeculae were isolated from right atrial appendages obtained from patients undergoing cardiac surgery (coronary artery bypass graft or aortic valve replacement surgery) at the Department of Surgery, Division of Cardiac Surgery. Human ventricular muscle strips were obtained from donor non-failing hearts that failed to meet transplantation

requirements. The collected tissue was transported in 4 °C carbogen-oxygenated Tyrode solution containing 2,3-Butanedione monoxime – BDM (Sigma Aldrich, St. Louis, Missouri, USA) which enables preservation for a shorter period of time and exhibits cardioplegic properties. BDM prevents spontaneous contractions via inhibition of the sodium calcium exchanger (NCX). Trabeculae were dissected from the myocardium in maximal diameter of 1mm to avoid central necrosis. The BDM was washed out in three washing steps (placing the muscle strips in a Tyrode solution filled cups). Trabeculae were mounted on hooks, stimulated at the frequency of 1 Hz and kept in an oxygenized (95% O2; 5 % CO2) organ bath setup (Scientific Instruments, Gilching, Germany) at 37 °C. The bath was filled with a Tyrode solution with addition of Actrapid human insulin (Novo Nordisk, Bagsværd, Denmark). Calcium chloride (Merck, Kenilworth, New Jersey, US) was added in three successive steps reaching from 0.2 mM as start to 2.5 mM as the end concentration . The pH was set to 7.4 for all solutions and kept on the same value until the end of the experiment.

For mechanical stretch trabeculae were pulled on the hooks to their Lmax (length where contraction is isometric and maximal force is developed) for a period of 3h, 6h and 8h. Control trabeculae were set to slack length.

For tachycardia experiments, all trabeculae were put on slack length and stimulated either on a 1 Hz frequency (control) or 2.5 Hz (tachycardia) for 3 h and 6 h time points. Muscle strips were submerged in the organ bath setup and kept under the same conditions as for the stretch experiments.

The patient's medical history was assessed to determine a possible correlation between confounding factors such as diagnosis, EF, previous medication etc. with experimental outcome and gene expression dataset.

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2.3 Mechanical strain

MC and NCM were plated at a density of $2x10^6$ on collagen type IV pre- coated BioFlex cell culture plates (Dunn Labortechnik, Asbach, Germany). The cell culture medium was exchanged to fresh medium one hour prior to stimulation in order to remove dead and detached cells. Cyclic stretch (mechanical strain) was applied using the Flexcell FX-5000™ Tension System (Flexcell International Corp, Burlington, USA). The stimulation was applied for a period of 24 h or 72 h. Total biaxial stretch reached 120% at 0.5 Hz frequency for the selected time periods. Cell contraction and viability was microscopically evaluated. After the experiment the cells were either harvested in quizol lysis reagent for RNA isolation or fixed in formaldehyde for immunofluorescent staining.

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2.4 Electrical field stimulation

MC and NCM were plated at a density of $2x10^6$ cells on 35 mm dishes (Grainer Bio-One, Kremsmuenster, Austria) coated with 1% gelatin. Cell culture medium was exchanged to fresh medium one hour prior to stimulation in order to remove dead and detached cells. Field stimulation was applied using a C-Pace EP cell culture stimulator (IonOptix, Westwood, USA). Pacing was applied at a stimulation rate of 1 Hz, 5 Hz and 8 Hz frequency, 5-8V/cm electrode distance and 4 ms pulse duration for 3 h, 6 h, 9 h, 12 h, 2 4h and 48h time periods. Unstimulated cells were kept for the same time points. A C-dish unstimulated control cells were kept for the same time points as well. The purpose was to exclude potential stimulation related effects unrelated to the applied frequency. The stimulation voltage was defined after determining the threshold value with at least 50% capture. Cell contraction was visually verified under microscope at the starting point and followed during the whole stimulation period. Electrolysis byproducts generated on the electrodes as well as cell metabolites altered the media pH which led to toxic effects. Therefore, it was necessary to exchange the cell culture media every 4 h during stimulation in order to remove the dead and detached cells and eliminate toxic confounders. The cells were collected starting from 3 h up to 24 h time

points in a lysis reagent for RNA isolation or fixed in 4% formaldehyde after 48 h for immunofluorescent staining.

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2.5 Immunofluorescent staining for cell size

Prior to immunostaining MC and NMC cells were fixed in 4% formaldehyde for 15 minutes and kept in PBS. Cells were then permeabilized with 0.1% of Triton X-100 and 0.1% Na-citrate. A blocking solution (PBS + 5% BSA) was incubated for 30 minutes to prevent unspecific biding. Cell were incubated either over night or for 90 minutes with a mouse monoclonal anti-desmin antibody (1:150, clone D33, DAKO, #M0760, Jena, Germany) and rabbit monoclonal anti - P4HB antibody (1:500, Abcam, #ab137110, Cambridge, UK). The fluorophore-conjugated secondary antibodies (1:1000) were incubated for 45 minutes. In order to label their nuclei, cells were incubated for 10 minutes with DAPI (1:500). The cells were visualised with an inverted fluorescent microscope and photographed at 10x and 20x magnifications. Images were analysed using the ImageJ software (ImageJ 1.46r, NIH, USA). Regions of interest were taken to assess cell surface area and elongation parameters – long and short axis of each cell. The results were presented as changes in the mentioned parameters expressed in percentage increase from control conditions.

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2.6 Immunofluorescent staining for signalling pathway characterisation

MC cells were fixed in 4% PFA for 10 min at room temperature. Cells were permeabilized with 0.1% Triton-X-100 (Sigma-Aldrich, now Merck), washed with PBS, and incubated overnight at 4°C with primary antibodies: rabbit polyclonal anti-CaMKII (phospho T286) antibody (1:200, Abcam, #ab32678), mouse monoclonal anti-Na^{+}/Ca^{2+-}

exchanger antibody (1:200, SWANT, #R3F1) or rabbit polyclonal anti-ADATS13 antibody (1:200, Abcam, #ab28274). Cells were washed twice with an antibody wash solution composed of PBS and 0.01% Triton-X-100, incubated with fluorophore-conjugated secondary antibodies (1:200) for 2 h at room temperature, and visualized on Zeiss LSM 510 Meta confocal microscope equipped with a plan neofluar 40x/1.3 oil DIC objective. Images were analysed using the ImageJ software (ImageJ 1.46r, NIH, USA) by evaluating mean fluorescence arbitrary units.

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2.7 NMC Immunofluorescence Staining for characterizing cellular composition

The NCM fraction was detached from the cell culture flask using trypsin, and 0.2 x 105 cells per well were seeded on an 8-well chamber slide. Cells were allowed to attach and were then fixed with ice-cold 1% PFA for 30 min. NCM cells were permeabilized with 0.2% saponin dissolved in PBS containing 3% BSA for 60 min at room temperature, and stained overnight at 4°C with the primary antibodies: goat polyclonal anti-DDR2 antibody (1:200, Santa Cruz, #sc-7555), mouse anti-vimentin antibody (1:1000, Abcam, #ab20346) and rabbit monoclonal anti - P4HB antibody (1:500, Abcam, #ab137110). Cells were incubated with fluorophore-conjugated secondary antibodies for 2 h and a mounting media with DAPI was added for the visualization on a Nikon A1 confocal microscope (Nikon, Japan) at 10x, 20x, 40x and 60x magnification.

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2.8 Flow Cytometric Staining

The NCM fraction was detached from the cell culture flask using trypsin and 0.5 x 10^6 cells were evaluated by means of flow cytometric analysis. Cell viability was determined

using eBioscience™ Fixable Viability Dye eFluor™ 506 (1:1000, Thermo Fisher Scientific, #65-0866-14) dissolved in PBS for 30 min at room temperature. Cells were then transferred into PBS, which contained 2% FBS and 1mM EDTA, and stained for 30 min at room temperature with the following antibodies: APC/Fire 750 anti-rat CD45 antibody (1:20, BioLegend #202221) and PE anti-rat CD31 antibody (1:50, Miltenyi Biotec, #130-116-505). Cells were washed twice, resuspended in PBS enriched with 2% FBS and 1mM EDTA and analyzed on a Cytoflex S (Beckman Coulter, Brea, California, USA).

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2.9 Total mRNA isolation

Total RNA extraction was performed using the miRNeasy on column isolation kit (Qiagen, Hilden, Germany). The cell lysates were collected with a cell scraper and further homogenized by vortexing. Human muscle strips were subjected to homogenization using the MagNA Lyzer (Roche, Basel, Switzerland) for 4x17 seconds at 6500 rpm. In order to remove genomic DNA a DNase treatment was applied for 15 minutes. RNA was eluted in a total volume of 30 µl. RNA yield was measured using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). RNA quality was validated by the 2100 Bioanalyzer using the Eukaryote total RNA 6000 Pico assay (Agilent Technologies, Waldbronn, Germany). The absence of the genomic DNA content was confirmed with the QiaXpert (Qiagen, Hilden, Germany).

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2.10 Quantitative real time PCR (RT-qPCR)

cDNA was generated using the QuantiTect Reverse transcription kit (Qiagen, Hilden, Germany). The cDNA samples were diluted to 2.5 ng/µl (10 ng per reaction). Total reaction volume was 10µl (4µl of cDNA added to 6µl of Mastermix). Quantitive real time PCR for

NRVCM was performed using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, California, USA). Primers (Microsynth, Balgach, Switzerland) for the genes *GAPDH, ACTA1, FHL1, NppA, NppB, RCAN1* were used for the qPCR reaction (Table 2). mRNA levels of the selected marker genes were normalised to relative mRNA levels of the GAPDH housekeeping gene. Validation of human microarray gene candidates was performed using the TaqMan expression gene assay (Thermo Fischer Scientific) for the following genes: *CNN1* (Cat.#Hs00959434_m1), *SNRPN* (Cat.#Hs01374551_m1), *TPI1* (Cat.#Hs03806547_s1), *IL1A* (Cat.#Hs00174092_m1), *PLA2G7* (Cat.#Hs00965837_m1), *ADAM20* (Cat.#Hs01083178_s1), *MIR1183* (Cat.#Hs04273420_s1), *GAPDH* (Cat.#Hs03929097_g1) along with TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Verification of *MIR1183* gene expression was carried out using the TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA). GenEx 5.4.4. software (MultiD, Gothenburg, Sweden) had been used to correct primer efficiency followed by $2^{-\Delta\Delta ct}$ relative quantification. The results are presented as fold change in gene expression normalised to control.

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Table 2. NRVCM qPCR oligonucleotide primer sequences.

Target gene transcript	Oligonucleotide sequence	
ACTin, Alpha 1 **(skeletal muscle) ACTA1**	*Forward* *Reverse*	5‘-AGA GTC AGA GCA GCA GAA ACT AGA-3‘ 5‘-CAC GAT GGA TGG GAA CAC AGC-3‘
Regulator of Calcineurin 1 **RCAN1**	*Forward* *Reverse*	5‘-GTG GCA AAC GGT GAT GTC TTC-3‘ 5‘-CCC AGG AAC TCT GTC TTA TGC-3
Four and a Half LIM **Domains 1** **FHL1**	*Forward* *Reverse*	5‘-TAC AGG CAG GGC TGG GTTTC-3‘ 5‘-TGG AAA TGA GGT GTG GGC ATCT-3‘
Proatrial natriuretic **peptide A** **NppA**	*Forward* *Reverse*	5‘-ATC ACC AAG GGC TTC TTC CT-3‘ 5‘-TGT TGG ACA CCG CAC TGT AT-3‘
Proatrial natriuretic **peptide B** **NppB**	*Forward* *Reverse*	5‘-AGT CGC TTG GGC TGT GAC GG-3‘ 5‘-AAG AGC CGC AGG CAG AGT CA-3‘
GAPDH	*Forward* *Reverse*	5‘-GCA ACT CCC ATT CTT CCA CCT TT-3‘ 5‘-TAT CCT TGC TGG GCT GGG TG-3‘

2.11 Treatment with antagonists of membrane proteins and intracellular signaling mediators

CM grown on BioFlex membranes were treated with several sarcolemma and intracellular protein inhibitory compounds (Table 3) in different concentrations. A dose response curve marked 10 µM concentration as the maximum effect dose without effect on cell number and viability for the calcineurin and CaM-Kinase II inhibitors while concentrations of 1 µM, 2 µM, 5 µM failed to show any prominent inhibiting effect. Other inhibitory compounds were

tested as well in corresponding concentrations. The following substances: FK-506, KN-93, KB-R7934, HOE-642, SKF-96365 were dissolved in 100% DMSO. Diltiazem was dissolved in ddH_2O while Strophanthidin was dissolved in 100% ethanol. The solvent was added to the cells without the inhibitor treatment (end concentration 0.1 %) to exclude inhibitor unrelated effects. The inhibitor alone was applied to determine possible treatment independent effects. The NCX inhibitory compound (KB-R7934) had been tested on MC undergoing field stimulation as well.

Table 3. Inhibitory compounds for specific intracellular and sarcolemma signaling mediators.

Target signaling mediator	Compound generic name	Concentration (μM)	Manufacturer
Calcineurin	FK506 monohydrate	10	Sigma Aldrich
CaM-Kinase II (CaMKII)	KN93	10	Sigma Aldrich
Sodium channel (Nav1.5)	Flecainide acetate	10	Meda Pharma
Sodium-Calcium exchanger (NCX)	KB-R7943	10	Sigma Aldrich
Sodium proton exchanger (NHE)	HOE642	10	Tocris
L-type Calcium channel (LTCC)	Diltiazem hydrochloride	10	Sigma Aldrich
Na^+/K^+-ATPase	Strophanthidin	100	Sigma Aldrich
Transient receptor potential cation channel (TRPC)	SKF96365	10	Sigma Aldrich

2.12 Microarray hybridisation

Due to a lower RNA yield than required for microarray hybridization it was first necessary to perform a single primer isothermal amplification (SPIA) using an Ovation Pico WTA System v2 kit (NuGen Technologies, Chesapeake Dr, Redwood City, US). The samples were subsequently purified with Agencourt Beads. A total amount of 20 ng RNA used for amplification yielded 10 µg of SPIA cDNA in 20 µl volume. Amplification quality was verified on qPCR for 27 samples pre-selected for array hybridisation (human atrial tissue). The SPIA cDNA was further diluted to 5 ng, 10 ng and 20 ng in a 25 µl SYBR green reaction volume (10 µl cDNA, 15 µl Mastermix). Labelling of cRNA was done with Encore Biotin Module (NuGen Technologies, Chesapeake Dr, Redwood City, US). After the validation five human samples per group (20 samples in total) or three NRVCM samples per group (18 samples in total) were selected for hybridisation that was carried out using GeneChip™ Human Gene 2.0 ST Array, Rat Gene 2.0 ST Array, Affymetrix GeneChip® HT hybridization, Wash and Stain Kit and Affymetrix Genechip® fluidics station 450: protocol FS450_0002 for Cartridge Arrays. Visualization was performed using an Affymetrix Scanner: GCS3000; AGCC (Command Console Software AGCC 4.0.0) and subsequence data analysis was done with Affymetrix Genexpression Console (1.3.1). Data pre-processing and filtering was carried out with the Partek® Genomics Suite® software (v.6.6) software for robust multi-array average (RMA) with the purpose to subtract background signal (un-specific binding), perform quantile normalization through media polish summarization across all the chips in the array and log2 transformation. Prior to RMA, quality check was performed to assure reliable visualization on the array. Probe level intensities were checked as well as background intensities to assess sample quality. The presence of bacterial spikes (Bio-B, Bio-C, Bio-D, and Cre) with pre-labeled bacterial RNA controls was checked to ensure consistent array hybridization among all samples. PolyA spikes (Lys, Phe, Thr, and Dap) were assessed to ensure proper amplification. To show similarities between arrays (samples) a Pearson/Ward´s cluster analysis was performed. A principal component plot has been generated to show if the group dissemination was present. Finally, a potential association between the regulated genes and known canonical pathways was investigated using the Ingenuity® Pathway Analysis software – IPA (Qiagen, Hilden, Germany). IPA software calculations were based on the Fisher's exact test – right tailed. The p value reflected the probability that the genes in the dataset were involved in a particular function.

A $p < 0.05$ was considered to be significant. An IPA calculated z score predicts the activation or inhibition state of a particular pathway. Values that were ≥ 2 were considered to reflect an increase in function, while values of ≤ -2 to represent a decrease in a function. Lists obtained from IPA consisted of predicted molecular pathways, diseases and functions, molecules, networks and upstream regulators. Specific molecules and their functions were associated to known diseases. The molecules list gave a descriptive overview of the molecules in the dataset. Additionally, networks were created to assess diseases and metabolic functions in which molecules found in the dataset were participating. Analysis of the upstream regulators helped to define which downstream molecules from the dataset were potential targets of a specific regulatory molecule or a group of molecules.

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2.13 Statistical analyses

Results are represented as mean ± SEM. Results are expressed as bar charts with SEM or line plots with SEM. Statistical significance was determined by a T test, One-Sample T test for samples with normal distribution or by an equivalent non-parametric test (Mann-Whitney test) for a non-normal distribution. Groups with a small sample size were analysed using Unpaired T test with Welch's correction assuming unequal variance. For more than two groups one-way ANOVA was performed or ANOVA based on ranks for a non-normal distribution. Statistical analysis of the microarray dataset was performed using one-way and two-way ANOVA. A $p < 0,05$ was considered to be statistically significant.

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3 Results part I (NRVCM)

3.1 To aim 1: Effects of Stretch and Tachycardia on morphological changes in NRVCM culture

In order to establish a pathological phenotype cardiomyocytes (MC) were either subjected to 72 h of mechanical strain (stretch) or 48 h of field stimulation (tachycardia). The stretch group (ST) showed a slightly but significantly pronounced hypertrophy by giving an increase in MC cell surface area (Figure 3.1A) of 10% (3138 μm^2 ± 74.69 versus 2860 μm^2 ± 60.29 in control) as well as increase in cell elongation (Figure 3.1B) of 8% (3.44 ± 0.05 versus 3.16 ± 0.06 in control) implying an eccentric type of hypertrophy. Tachycardia (TC) induced frequency dependent hypertrophy of 22% (2366 μm^2 ± 32.22) after 48 h of 5 Hz field stimulation (Figure 3.1C) when compared to unstimulated cells (1911 μm^2 ± 29.13) and 10 % when compared to cells at normal rate frequency of 1 Hz (2160 μm^2 ± 27.79). Cell elongation parameters are affected by stimulation (Figure 3.1D). This effect is however independent from the applied frequency, therefore, all stimulated groups show patterns of eccentric hypertrophy ranging from 15% at 1 Hz (2.97 ± 0.05) to 18% at 5 Hz frequency (3.06 ± 0.05). Cell viability at 8 Hz showed less survival of technical replicates (data not shown) in comparison with other groups where cell survival was not affected (Figure 3.1E). Therefore, 8 Hz stimulation rate can be applied acutely but is not as preferable for longer time points.

The cardiac non-myocytes (Figure 3.1F-J) showed increase in cell surface area of 8% (3170 μm^2 ± 51.13 vs 2934 μm^2 ± 59.14 in control) during stretch (Figure 3.1F). Regarding the elongation parameters there was no difference present between the long and short axis ratio during stretch (Figure 3.1G) suggesting, therefore, a concentric type of hypertrophy. During tachycardia stimulation at 5 Hz frequency there is a significant change in cell surface area (Figure 3.1H) when comparing the trigger condition both to an unstimulated control 5% (1911 μm^2 ± 46.68 versus 1841 μm^2 ± 69.59) as well as to a 1 Hz normal rate control 12% (1911 μm^2 versus 1703 μm^2 ± 37.41). Additionally, during tachycardia stimulation there is a significant ratio difference when comparing the trigger to unstimulated control. After 5 Hz stimulation rate there is a 10% increase in cell elongation (2.08 ± 0.04) compared to 1 Hz frequency (1.90 ± 0.03) and 11% compared to unstimulated control (1.87 ± 0.03) (Figure 3.1I). Tachycardia therefore shows clearly a rate dependent eccentric pattern of hypertrophy

compared to stretch. Cell survival and density were not affected by the applied stimuli (Figure 3.1J).

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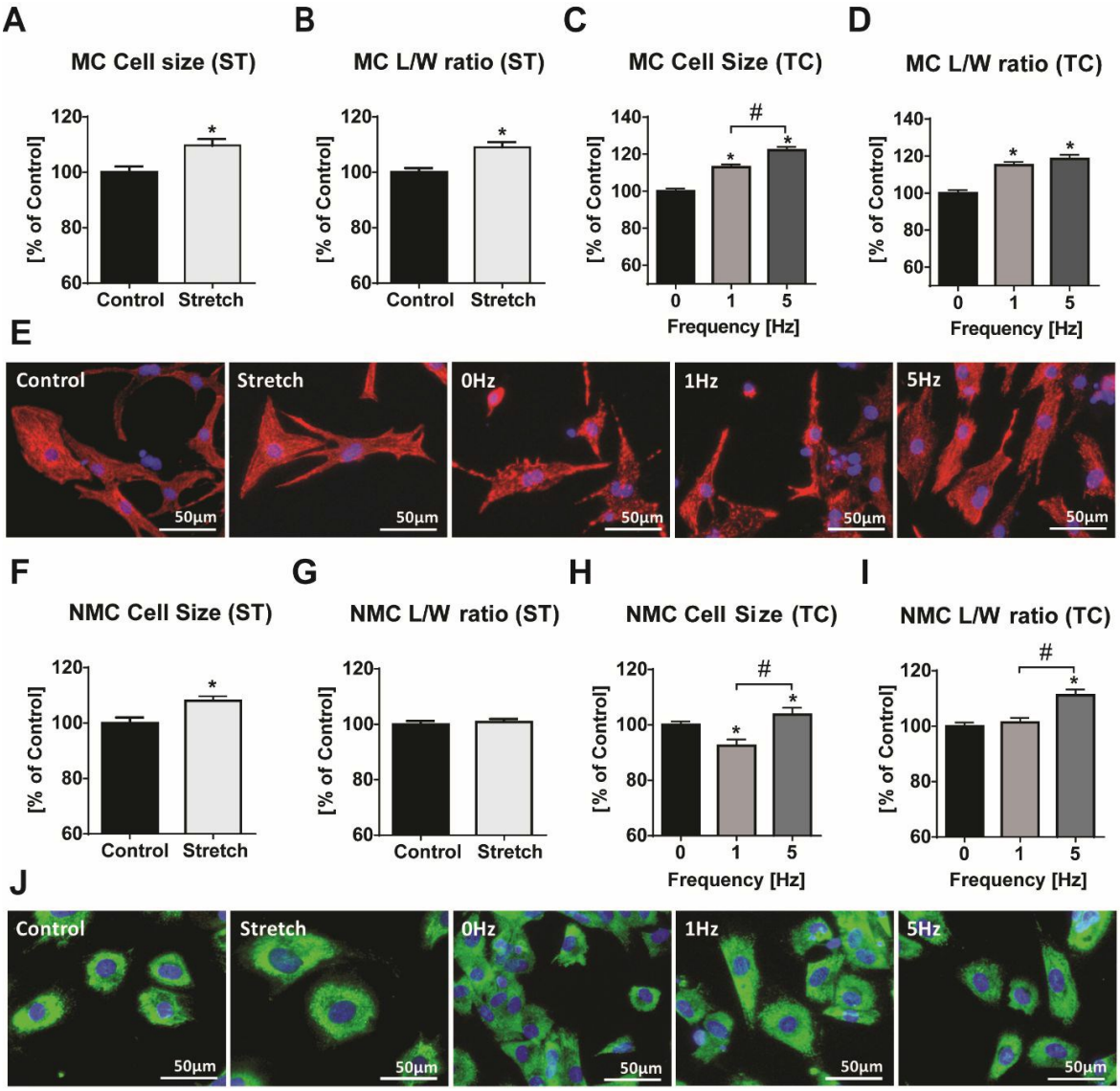

Figure 3.1 Morphological characteristics of neonatal rat ventricular cardiomyocytes (MC) and non-myocytes (NMC) under mechanical stretch (ST) and tachycardia (TC).

(A) *Induction of hypertrophy of MC after 72 h of ST.* ***(B)*** *Extended long to short axis ratio after ST* ***(C)*** *Frequency dependent hypertrophy of MC upon 48 h of TC. (**D**) Extended long*

to short axis upon 1 Hz stimulation and TC. ***(E)*** *Immunofluorescent staining of MC for desmin protein (red), nuclei stained with DAPI (blue).* ***(F-G)*** *ST induces hypertrophy of NMC after 72h without cell elongation.* ***(H-I)*** *TC results in hypertrophy of NMC with elongation* ***(J)*** *NMC stained for P4Hß (green), nuclei stained with DAPI (blue). Data are represented as mean ± SEM.* * *p <0.05.* $^{\#}$*p <0.05 for pairwise comparison. Average cell count: n=700 - 1000 per group. (This Figure was adapted from Djalinac et al, JCMM, 2020 Ref (295) with permission of the publisher (Wiley).*

3.2 To aim 2: Fibroblast as the main cellular type among cardiac non-myocytes

The composition of the available cell culture consists of approximately 70 % cardiomyocytes or MC and 30 % of other cell types referred as non-myocytes or NCM. To identify the specific composition of the NMC fraction, we first applied multicolor flow cytometry and identified a relative contribution of approximately 5% endothelial cells ($CD31^{+}CD45^{-}$) and 4% inflammatory cells ($CD31^{-}CD45^{+}$) of all viable cells (for gating strategy, please refer to Figure 3.2 A-D, and for the quantification, please see Figure 3.2 E). The majority of cells (90%) were negative for CD31 and CD45 suggesting that the remaining population represents resident mesenchymal fibroblasts. Since fibroblasts are heterogeneous cells, for which flow cytometric markers are as-of-yet not readily commercially available, we performed a triple color immunofluorescent staining of known fibroblast markers, namely P4HB, DDR2 and vimentin. All fibroblast markers stained the majority of cells, confirming our results obtained from the flow cytometric staining (Figure 3.2 F-K). The smallest $CD31^{+}CD45^{+}$ fraction likely represents progenitor cells.

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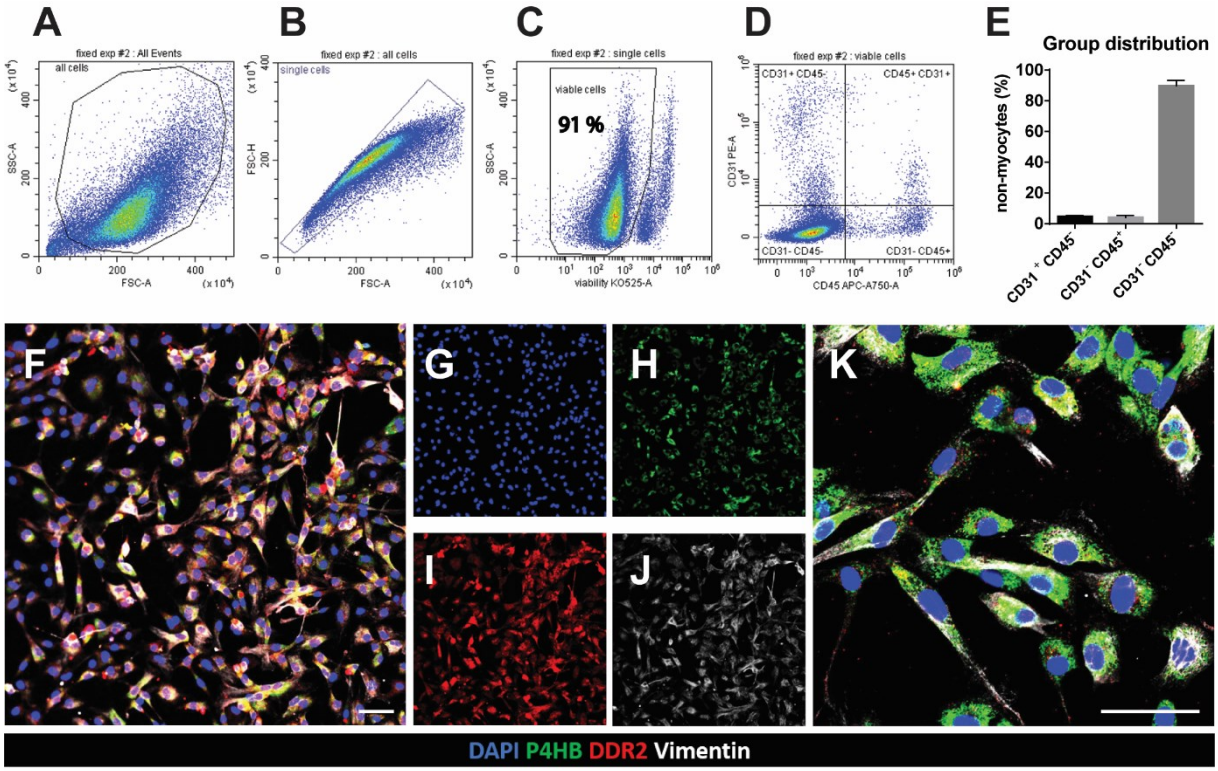

Figure 3.2 Cellular composition of non- myocytes (NMC).

*(**A-D**). Representative Flow Cytometry plots showing gating strategy for (**A**) all events, (**B**) forward scatter for single cells, and (**C**) viable cells. (**D**) Cell clustering based on CD45 and CD31 surface markers. (**E**) Distribution of $CD31^{+}CD45^{-}$, $CD31^{-}CD45^{+}$ and $CD31^{-}CD45^{-}$ cells. (**F**) Immunofluorescent co-staining of NMC with (**G**) DAPI (nuclei), (**H-J**) P4HB, DDR2 and vimentin (fibroblast markers). (**K**) Merged image of stained NMC. Scale bar = 50µm, n = 3 cell cultures. (These measurements were done by PhD Katharina Jandl, Medical University of Graz). (This Figure was adapted from Djalinac et al, JCMM, 2020 Ref (295) with permission of the publisher (Wiley).*

3.3 To aim 3: Stretch and tachycardia show differences in marker gene expression

Marker gene candidates for hypertrophy, fibrosis, inflammation and calcium signalling (see methods, Table 2) were evaluated for changes in mRNA expression levels during stretch and tachycardia conditions. Time dependent gene expression profiling identified a maximum effect gene expression time point for stretch at 24 h after the

stimulation beginning (Figure 3.3.1). A rather early 3h time point showed a slight but however, significant increase in gene expression levels of *FHL1* (1.36-fold ± 0.01, p =0.002, n=3), *NppA* (1.77-fold ± 0.07, p =0.007, n=3), *NppB* (1.53-fold ± 0.07, p =0.02, n=3) and *RCAN1* (1.23-fold ± 0.05, p =0.04, n=3) while *ACTA1* expression was still at baseline level. The 3 h time point was followed by the 24h expression peak of almost all genes: *ACTA1* (2.35-fold ± 0.07, p=0.005, n=6), *FHL1* (1.51-fold ± 0.17, p=0.03, n=6), *NppA* (2.28-fold ± 0.38, p=0.02, n=6), *NppB* (2.61-fold ± 0.37, p =0.007, n=6) except for *RCAN1* (1.67-fold ± 0.25, p=0.05, n=6) which showed a non-significant trend towards up-regulation. This allows to conclude that the stretch effect is transient with gene expression levels returning to baseline after 48 h of stimulation.

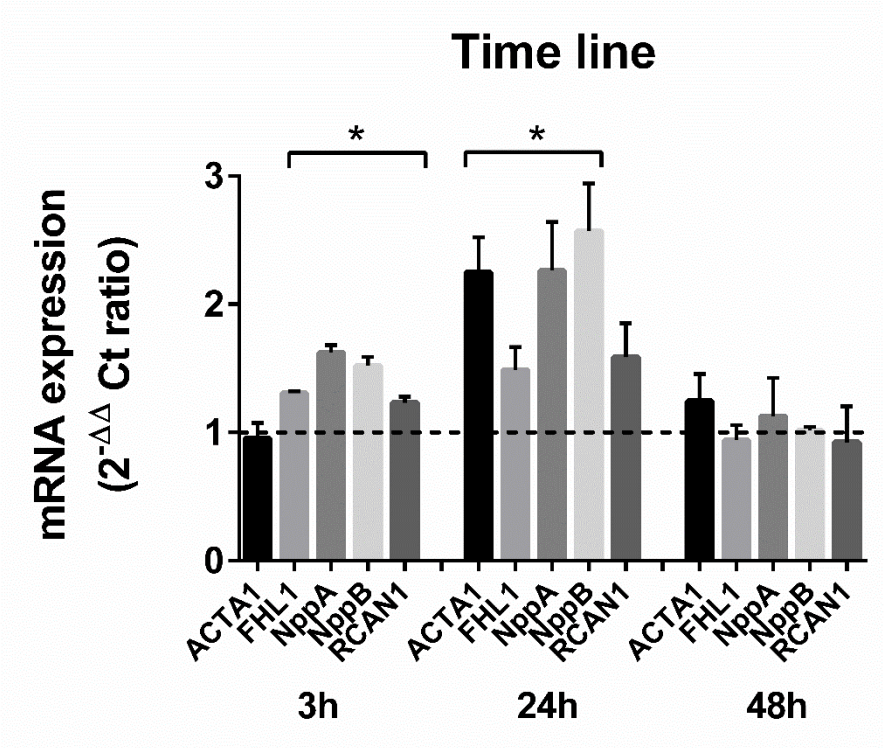

Figure 3.3.1 Stretch triggered time dependent gene expression timeline.

*The 24h time point is identified as the maximum effect time point. The results are presented as mean ± SEM. The line intersecting the y axis represents the baseline gene expression. Statistical difference was determined by One-Sample T test. n = 2 isolations (3 – 6 samples) * p <0.05 vs control, ns = not significant. (This Figure was adapted from Djalinac et al, JCMM, 2020 Ref (295) with permission of the publisher (Wiley).*

Next, we aimed to determine the magnitude of the tachycardia stimulation over a 24 h period. The 24 h screening time line had been chosen based on the observed stretch mediated effect during this period. Unstimulated cells were evaluated for basal expression of pro-hypertrophic genes over a selected period of time (Figure 3.3.2 A) and showed stable expression of *FHL1* and downregulation of the remaining genes. A gene expression up-regulation is already present at a 1 Hz normal rate frequency (Figure 3.3.2 B) as early as after 3h of stimulation when compared to unstimulated control. The initial up-regulation persists during the whole stimulation time of 24 h. A similar degree and pattern of up-regulation is present when stimulating at a higher pacing rate of 5 Hz (Figure 3.3.2 C). Since the 3 h time point already showed a convincing degree of up-regulation for most of the genes it was decided to proceed with the given time point for further gene expression evaluation.

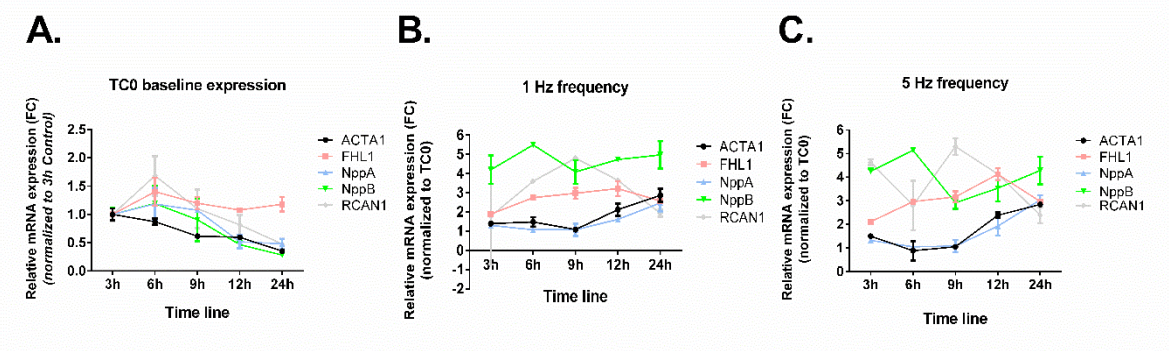

Figure 3.3.2 Time dependent gene expression in the absence or presence of electrical field stimulation during a 24 h time frame

(A). *Gene expression changes in unstimulated cells during 24h.* ***(B).*** *Cardiomyocytes stimulated at 1 Hz frequency over 24 h stimulation period show stable gene expression levels when compared to unstimulated cells.* ***(C).*** *Fluctuations in time dependent gene expression during 24 h of 5 Hz tachycardia stimulation when compared to unstimulated control. n = 1 isolation (2 samples per group). The results are presented as line graphs ± SEM.*

Next, we also looked into the difference between normal frequency stimulation and tachycardia. It was noted that at early time-points genes show a moderate trend towards up-

regulation and this trend for most of the genes stays unchanged during 24 h. Therefore, we can conclude that first of all there is a stimulation dependent effect as seen previously with the unstimulated control comparison. This effect is also frequency dependent since the initial changes during 1 Hz are slightly more pronounced by increasing the frequency to 5 Hz (Figure 5). Finally, the lack of any major gene expression changes over the time course reinforced the conclusion that the gene expression of pro-hypertrophic markers is initiated early on and maintains a stabile expression profile through 24 h when comparing stimulated groups.

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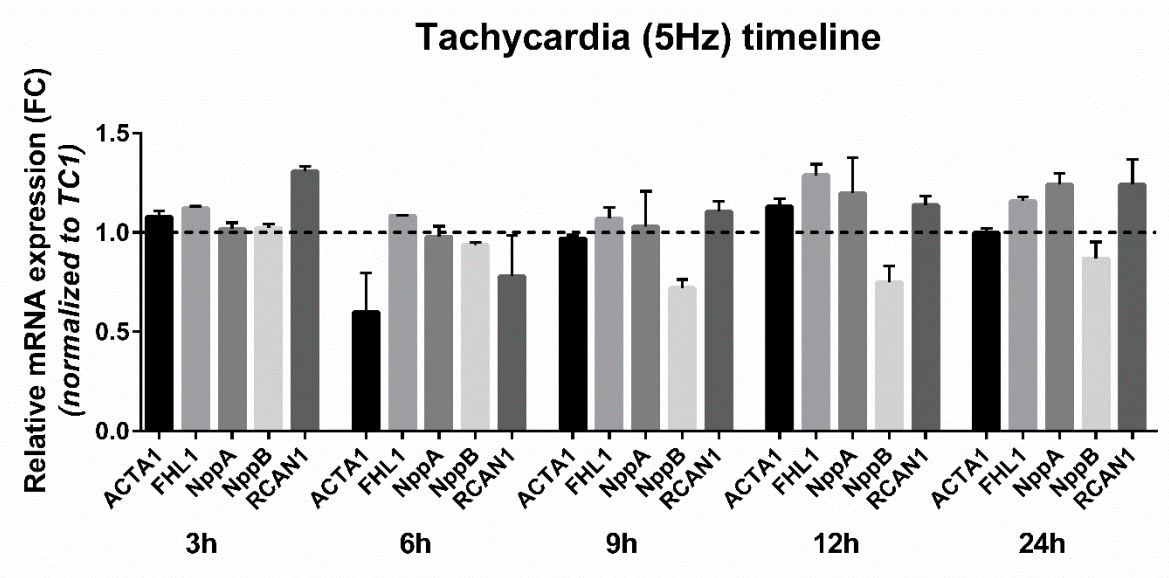

Figure 3.3.3 Time dependent gene expression during 24 h of 5Hz tachycardia stimulation.

n = 1 isolation (2 samples per group). The dashed line represents the baseline gene expression. The results are presented as mean ± SEM. (This Figure was adapted from Djalinac et al, JCMM, 2020 Ref (295) with permission of the publisher (Wiley).

3.4 To aim 4: Cardiomyocytes and Non-myocytes contribute to stretch and tachycardia differential gene expression changes in NRVCM.

Stretch and Tachycardia were applied to both myocytes and non-myocytes to study cell specific contribution in the overall regulation of pro-hypertrophic genes. The previously established 24 h during time dependent screening shows a strong stretch induced increase of expression levels for at least 4 genes (Figure 3.4 A) in cardiomyocytes: *ACTA1* (2.86-fold ± 0.28, $p < 0.0001$, n = 13), *FHL1* (1.96-fold ± 0.11, $p < 0.0001$, n =9), *NppA* (2.43-fold ± 0.20, $p < 0.0001$, n = 13) and *NppB* (2.40-fold ± 0.27, $p < 0.0001$, n = 13). In non-myocytes the trend towards up-regulation concerning *NppA* (1.60-fold ± 0.19, p = 0.01, n = 3) and *NppB* (1.35-fold ± 0.07, $p <0.001$, n = 4) genes is also present as in cardiomyocytes, however, in a lower magnitude (Figure 3.4 B) while *ACTA1*, *FHL1* and *RCAN1* are at baseline level of expression. Therefore, stretch dependent effects can be largely attributed to cardiomyocytes. Increased expression levels of *FHL1* (1.14-fold ± 0.03, $p <0.001$, n = 6) and *RCAN1* (1.29 ± 0.06, $p <0.05$, n = 5) could be observed already at a borderline supra-physiological 5 Hz frequency. An increase in the stimulation frequency from 5 Hz to 8 Hz measured after 3 h (Figure 3.4. C) prompted a greater increase in mRNA expression levels of the following pro-hypertrophic genes in cardiomyocytes: *FHL1* (1.30-fold ± 0.12, p = 0.04, n=2), *NppA* (1.17-fold ± 0.07, p=0.03, n=4), *NppB* (1.29-fold ± 0.07, $p <0.001$, n=4) and *RCAN1* (1.81-fold ± 0.14, $p <0.001$, n=4). Therefore, priority for further assessment was given to the higher rate frequency at the earlier time point. A similar pattern of gene regulation arose when stimulating NMC at the given frequencies and time point (figure 3.4 D). Only *RCAN1* showed a rate dependent effect when stimulated at both 5 Hz (1.26 ± 0.11, $p < 0.05$, n = 14) and 8 Hz (1.69 ± 0.26, $p < 0.05$, n = 14). Other genes: *FHL1* (1.21 ± 0.08, $p < 0.05$, n = 14) and *NppB* (1.45 ± 0.17, $p < 0.05$, n = 14) showed significant upregulation (versus control and 5 Hz) at the higher 8 Hz frequency. In conclusion, the tachycardia dependent effects in the enriched cell culture are derived from both MC and NMC but given the proportion of MC distribution it is safe to assume that MC as the prevailing cell type have a significantly greater impact on gene regulation.

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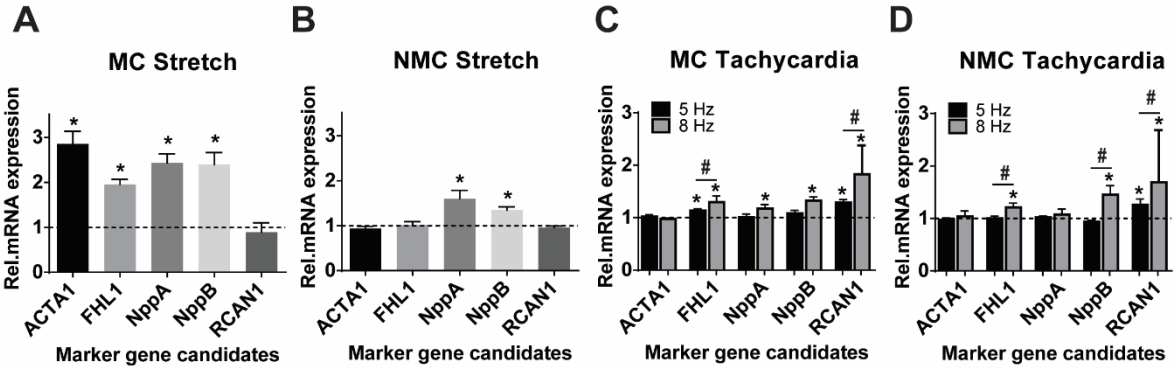

Figure 3.4 Gene expression characterization of stretch and tachycardia induced changes in neonatal rat ventricular cardiomyocytes (MC) and non-myocytes (NMC).

A. *Up-regulation of marker gene candidates associated with cardiac remodelling after 24h of stretch.* ***B.*** *Up-regulation of selected marker gene candidates upon 24 h of stretch stimulation. (**C, D**) Rate-dependent gene expression changes after 3 h of TC in MC (**C**), and NMC (**D**). The Dashed line represents the baseline expression of the corresponding control. *$p<0.05$ vs. control, #$p<0.05$ for pairwise comparison. Statistical difference was determined by One-Sample T-Test. n=4–13 cell cultures. (This Figure was adapted from Djalinac et al, JCMM, 2020 Ref (295) with permission of the publisher (Wiley).*

3.5 To aim 5: Involvement of sarcolemma and intracellular signalling mediators in stretch and tachycardia induced hypertrophy.

Following the gene expression characterization further experiments had been conducted with the aim of an in-depth dissection of signalling pathways that contribute to the pathological expression. The peak effect time points were chosen for screening purposes using a variety of available target specific inhibitory compounds (see methods, Table 3). Both inhibitors of sarcolemma ion channels as well as inhibitors of intracellular signalling mediators were used in attempt to alter the pro-hypertrophic response triggered by stretch and thus identify the upstream signalling participants (Figure 3.5.1 A). First, the screening procedure identified the sodium-calcium exchanger (NCX) as one the key mediators in the trigger induced signalling cascade. The stretch induced up-regulation of *ACTA1, FHL1* and *NppA* mRNA levels shows significant inhibition following incubation with the KB-R7934 inhibitory compound (Figure 3.5.1 A). KB-R7934 is primarily an inhibitor of the reverse-

mode NCX (285) but is shown to exhibit other off target effects mainly on TRPC, NaV1.5 and L-type Ca channels. Out of the mentioned, we confirmed the involvement of TRPC in the stretch mediated response using an additional pan-specific TRPC inhibitor. However, an overall greater involvement of NCX is apparent. Additionally, treatment with the NCX inhibitor showed that up-regulation of *RCAN1* (1.68 ± 0.15, p <0.001, n = 3) after 3 h of tachycardia stimulation (Figure 3.5.1 B) significantly reversed baseline expression (1.12-fold ± 0.04, p <0.001, n=3). The contribution of CaMKII and Calcineurin in the intracellular signalling cascade following stretch is implicated after treatments with KN93 and FK506. The rest of the inhibitory compounds failed to show a meaningful trend in expression in the preliminary screening (Figures 3.5.2 A-E). The attempt to block the L-type Ca^{2+} channel, NHE, NaV1.5 and Na^{+}/K^{+}- ATPase resulted in inconsistent findings with few genes displaying trends towards up-regulation while in contrast the rest were more prone to down-regulation. Therefore, without an obvious unidirectional pattern of expression the inhibitors were not evaluated in future cell cultures.

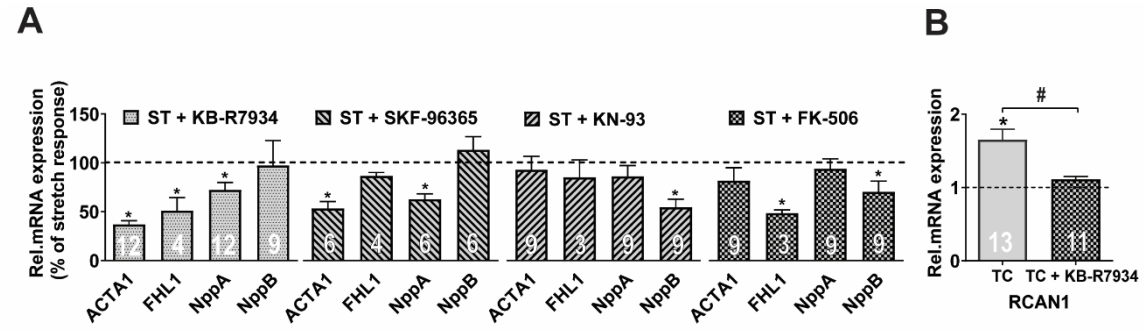

Figure 3.5.1 Gene expression characterization of stretch and tachycardia induced changes in NVRCM.

***(A)**. Marker gene expression under inhibition of sarcolemma and intracellular signaling targets showing significant contribution of the Sodium-Calcium exchanger (NCX), TRPC channel and Cam kinase II (CamKII). The dashed line represents the maximum gene up-regulation under stretch conditions. Stretched and inhibitior treated stretched cells were normalized to their own controls. **(B)** Confirmation of NCX involvement after 3 h of tachycardia stimulation. Results are represented as mean ± SEM. The dashed line represents the baseline expression, * p <0.05, # p <0.05 (stretch + inhibitor vs control + inhibitor). Statistical difference had been determined by One Sample T-Test. Numbers in bars represent*

the number of technical replicates. (This Figure was adapted from Djalinac et al, JCMM, 2020 Ref (295) with permission of the publisher (Wiley).

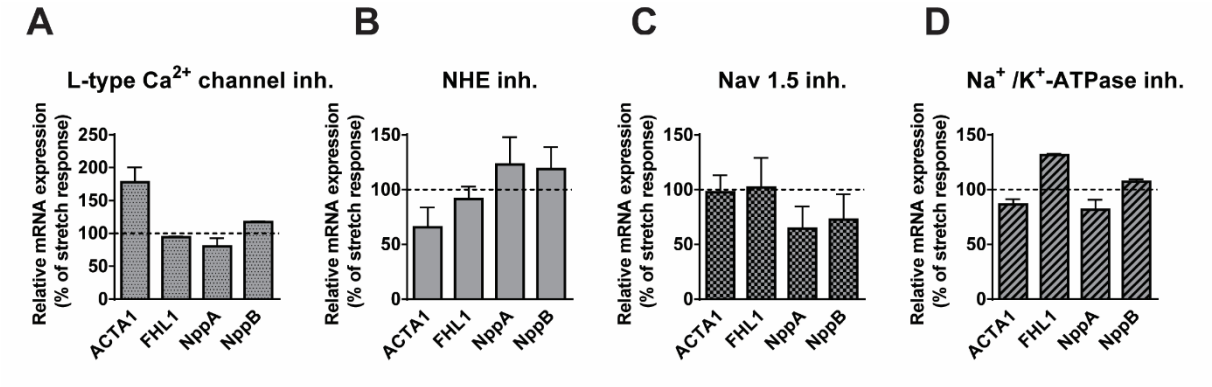

Figure 3.5.2 Gene expression screening for inhibition of stretch induced changes in NRVCM.

Gene expression after the inhibition of stretch (ST)-induced changes in myocytes. There is no obvious trend present in an attempt to inhibit ***(A)*** *diltiazem (L-type Ca^{2+} channel inhibitor),* ***(B)*** *HOE642 (the Na^+/H^+ exchanger inhibitor),* ***(C)*** *strophanthidine (Na^+ /K^+ ATPase inhibitor) and* ***(D)*** *flecainide (NaV 1.5 channel blocker). Dashed line represents the maximum gene up-regulation under stretch conditions. Gene expression levels were normalized to respective controls. n=1-2 cell cultures. (This Figure was adapted from Djalinac et al, JCMM, 2020 Ref (295) with permission of the publisher (Wiley).*

NCX activity and expression are known to go hand in hand in advanced phases of cardiac disease. Therefore, the involvement of NCX reverse mode activity as early as 24 h led us to investigate possible alterations of NCX protein levels. Indeed the NCX expression levels measured by immunofluorescence (Figure 3.5.3 A) increased upon 24 h of stretch (Figure 3.5.3 B). Additionally, application of KB-R9743, KN93 and KN92 hindered significantly the observed stretch effects. The fact that KN92 is an inactive homolog of KN93 prevented us from drawing a clear and final conclusion about CaMKII dependency. Therefore, only the effect mediated by KB-R9743 can be considered specific. Thus, we have shown that stretch leads foremost to changes in NCX reverse mode activity followed by changes in protein expression. There was no change in NCX protein levels when applying

different frequency rates for 3 h, suggesting a difference in mechanism of action between ST and TC (Figure 3.5.3 C).

We were interested to elucidate how differences in NCX activity and/or protein levels can be associated to calcium handling effectors downstream of NCX. Therefore, we evaluated the levels of CaMKII phosphorylation at the T286 autophosphorylation residue since CaMKII activity is considered to be a significant indicator of dysregulated calcium homeostasis at least in later stages of cardiovascular disease (Figure 3.5.4 A). There was a complete absence of CaMKII activation post stretch excluding it thereby from the signalling cascade downstream of NCX (Figure 3.5.4 B). However, we have seen a convincing increase of 32% in CaMKII activation post 8 Hz stimulation and partial inhibition of the TC mediated activation following KB-R7943 application (Figure 3.5.4 C). This confirms the involvement of CaMKII in TC triggered -NCX mediated excitation transcription coupling. ADAMTS13 - a novel key player in the CaMKII signalling pathway has been studied due to a previous connection to cardiac arrhythmias. There were no changes in expression levels of ADAMTS13 (Figure 3.5.5 A-C) excluding its possible contribution during TC.

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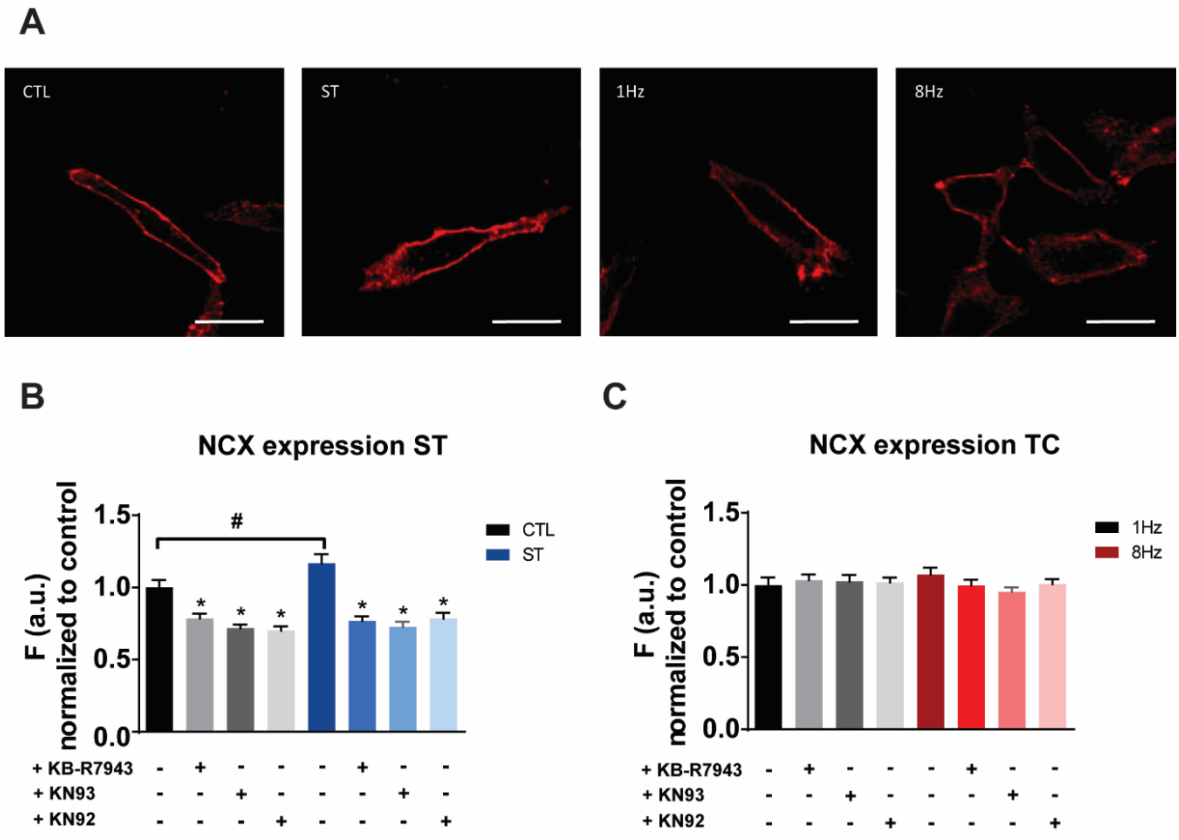

Figure 3.5.3 Differential NCX protein expression between stretch (ST) and tachycardia (TC).

*(**A**) Immunofluorescent staining of representative fields from bio-flex and 35mm dishes, scale bar = 20 µm. (**B**) Increased NCX expression after 24 h of stretch is independent of the CaMKII signaling pathway. (**C**) Tachycardia (8 Hz, 3 h) does not alter NCX expression. $^{*}p<0.05$ vs. control/ vs. ST, $^{\#}p<0.05$. Average cell count n=500 - 600 per group,(This Figure was adapted from Djalinac et al, JCMM, 2020 Ref (295) with permission of the publisher (Wiley)*

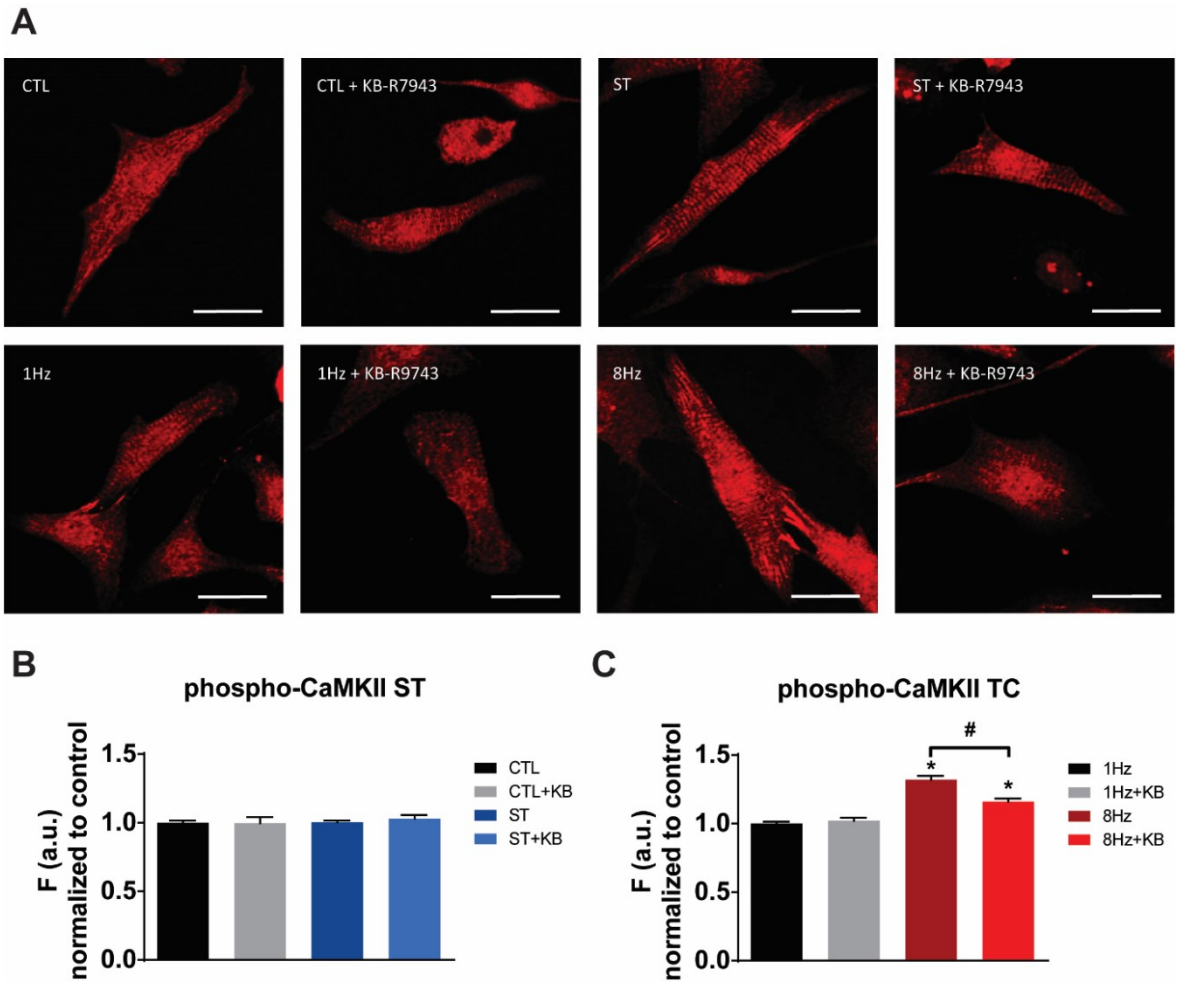

Figure 3.5.4 Effect of stretch (ST) and tachycardia (TC) on CaMKII phosphorylation at T286.

*(**A**) Immunofluorescent staining of representative fields from bio-flex and 35mm dishes, scale bar = 20 µm. (**B**) Unchanged phosphorylation of CaMKII after 24 h of ST. (**C**) Increased phosphorylation of CaMKII upon 3 h of tachycardia (8 Hz). *p<0.05 vs. control, #p<0.05. Average cell count n =1000 – 1500 per group. (This Figure was adapted from Djalinac et al, JCMM, 2020 Ref (295) with permission of the publisher (Wiley)*

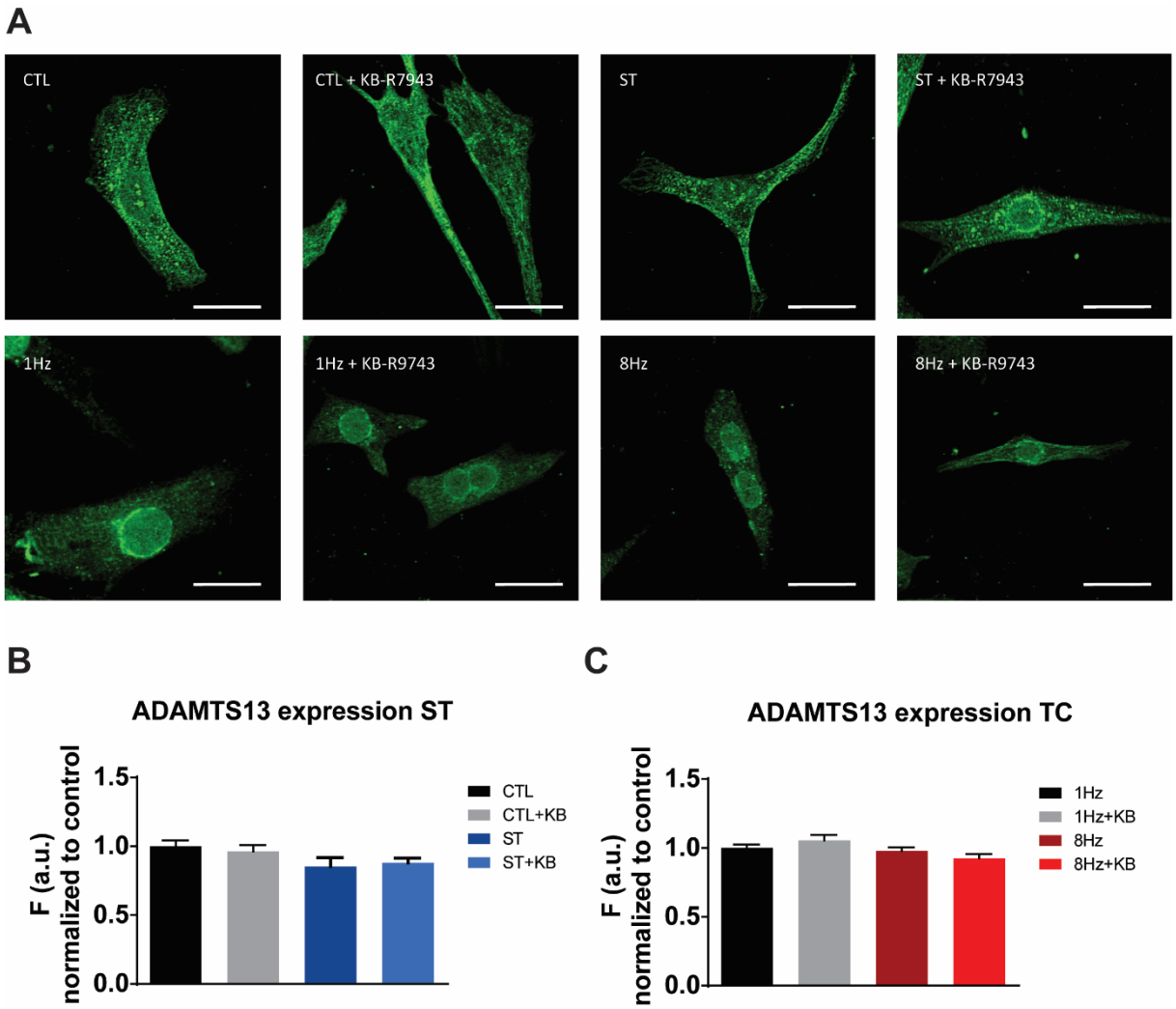

Figure 3.5.5 Expression levels of ADAMTS13 after 24 h of stretch (ST) and 3 h of tachycardia (TC).

(A) *Representative immunofluorescent staining of MC from bio-flex and 35mm dishes, scale bar = 20 µm.* ***(B)*** *Protein levels after 24h of ST.* ***(C)*** *Protein levels upon 3 h of tachycardia (8Hz). Average cell count n=1000 -1500 per group. (This Figure was adapted from Djalinac et al, JCMM, 2020 Ref (295) with permission of the publisher (Wiley).*

3.6 To aim 6: Microarray characterisation discloses the differences in gene regulation upon stretch and tachycardia and reveals contribution of NCX

NCX proved to be a key player during stretch and tachycardia as evaluated in a limited selection of a pro-hypertrophy panel of genes as well as mechanistically. Therefore, NCX inhibition was used to acquire deeper understanding of all downstream targets and determine transcriptional interplay between stretch and tachycardia by means of microarray hybridisation. The total number of genes regulated via the stretch intervention sums up to 1953 genes while in case of tachycardia this number is 826. The shared fraction consists of 59 genes regulated by both stretch and tachycardia (Figure 3.6 A). These results lead to the conclusion that stretch and tachycardia mainly show a different gene expression profile with only 2% of genes being shared. As for the conditions under NCX inhibition (Figure 3.6 B) we can say that 2610 genes are regulated via NCX dependently or independently of stretch whereas 1538 genes are regulated via NCX in a setting where tachycardia is inflicted (again both in a dependent or independent manner). The shared portion of genes equal to 282 out of which 175 genes are solely regulated via NCX (independently of stretch or tachycardia). In summary this makes a total of 6% of genes that depend on NCX. To further elucidate the stretch or tachycardia dependent gene expression differences that rely on NCX we looked into different group intersections of the Venn diagram (Figure 3.6 C). First, stretch alone is responsible for regulation of 1156 genes. Inhibition of NCX results in altered expression of 1737 genes independently of stretch. However, 535 genes that are expressed under stretch conditions are found to have altered expression after NCX inhibition (27%). Therefore, NCX is involved in mediating the expression levels of these genes when cardiomyocytes are subjected to stretch. On the other hand, tachycardia alone is responsible for altering expression levels of 579 genes whilst under NCX inhibition this number amounts to 1000. The intersection showing tachycardia dependent genes that are mediated through NCX compromises of 141 genes (17%). From these findings we can conclude that more genes are mediated via NCX in the stretch model than the tachycardia model, however most genes overall show an independent regulation either by the trigger applied or the treatment with the inhibitory substance. The smallest fraction is comprised of commonly triggered genes that show alterations upon NCX inhibition. These are the following: *Slpil3, Efnb2, Kif14, Ccna2* and sequence *TL0ACA36YA15*. From the known genes *Kif14, Ccna2* are involved in

the regulation of the cell cycle while *Efnb2* is studied in cardiovascular disease. *Slpil3, Efnb2* and the *TL0ACA36YA15* transcript show the same pattern of regulation while *Kif14* and *Ccna2* differ in expression direction between stretch and tachycardia.

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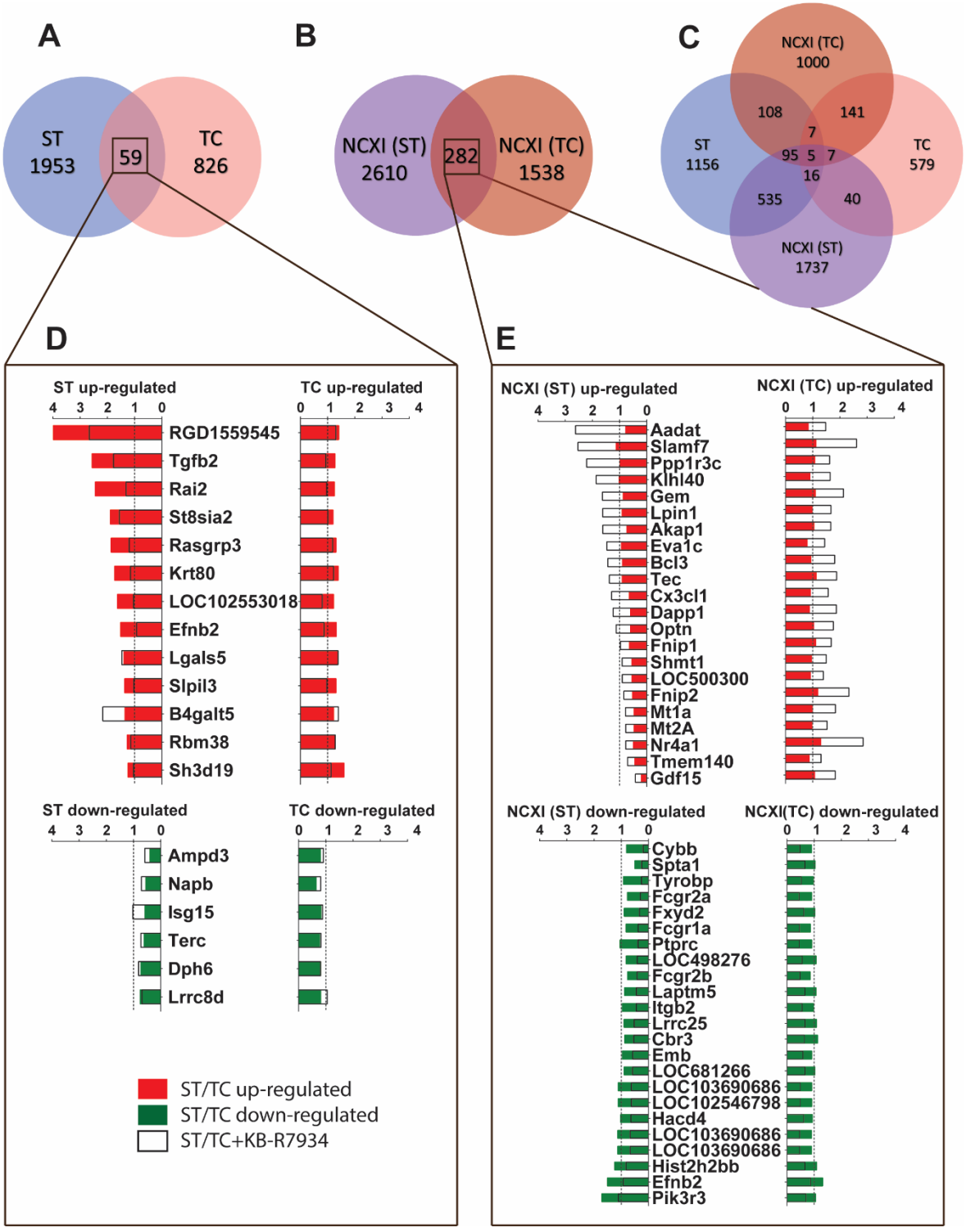


Figure 3.6 Summary of microarray gene expression in NRVCM.

*The hybridization was performed in 6 groups: stretch (ST), control for ST, tachycardia (TC), control for TC, ST combined with NCX inhibition (NCXI) and TC combined with NCXI. (**A**) and (**D**), Genes regulated either via stretch (ST) or tachycardia (TC) stimulation or by both*

*triggers. (**B**) and (**E**), Genes regulated by the NCX inhibitor KB-R7934 (NCXI) in combination with ST or TC. (**C**), Venn diagrams that display group interactions were generated from the microarray dataset thereby considering P < .05 and fold change cut-off from at least 1.2- fold up- or −1.2 down-regulated (0.83 2-ΔΔCT ratio). TC/ST + KB-R7934 (NCXI) was presented normalized to control, (**B**), statistical significance confirmed comparing ST/TC vs ST/TC + KB-R7934 (NCXI). n = 3 biological replicates per group. (This Figure was adapted from Djalinac et al, JCMM, 2020 Ref (295) with permission of the publisher (Wiley)*

Out of the commonly NRVCM regulated genes (Figs. 3.6 D-E) which show significant inhibition or at least a near to significant trend towards inhibition of NCX we can single out *Tgfb2* and *Efnb2* for their known involvement in pre-existing cardiovascular disease conditions. Firstly, up – regulation of *CNN1* (2.28-fold, p = 0.053) followed by NCX inhibition (-2.05-fold, p = 0,076) was observed in the stretch group. The tachycardia stimulated group did not show a prominent difference when being compared to its control group (1.10-fold, p = 0.171). However, under NCX inhibition a significant down-regulation of the *CNN1* gene is present (-1.32-fold, p = 0.009).

3.7 To aim 7: Bioinformatic prediction and functional characterization

3.7.1 Ingenuity Pathway analysis - major findings

Ingenuity pathway analysis (IPA) had been done to link changes in gene expression with activation or inhibition of established molecular pathways involved in canonical biological processes. The calculation of significance is based on the Fisher's exact test. The obtained z score represents the magnitude of activation when dealing with positive values or inhibition in case of negative values.

Major findings dependent on NCX regulation in stretch include first of all cell death (Figure 3.7.1) then proliferation, motility and energy substrate shift. Stretch independently regulated actions include cholesterol biosynthesis and the oxidative stress induced pathway. On the other hand, there is no significant activation or inhibition present in tachycardia alone. However, the NCX inhibition under tachycardia exposure shows the following: increased

lipid metabolic activities, decreased cell death and as opposed to stretch increase in cell motility through activated movement, chemotaxis, homing and migration programs. Upstream regulators unique for KB-R7943 treated cells subjected to field stimulation are p38 MAPK which is activated and FBXO32 which is inhibited. Other regulators such as NFkB, TNF, IL1B, STAT1, VCAN are present also in the KB-R7943 treated cells under stretch.

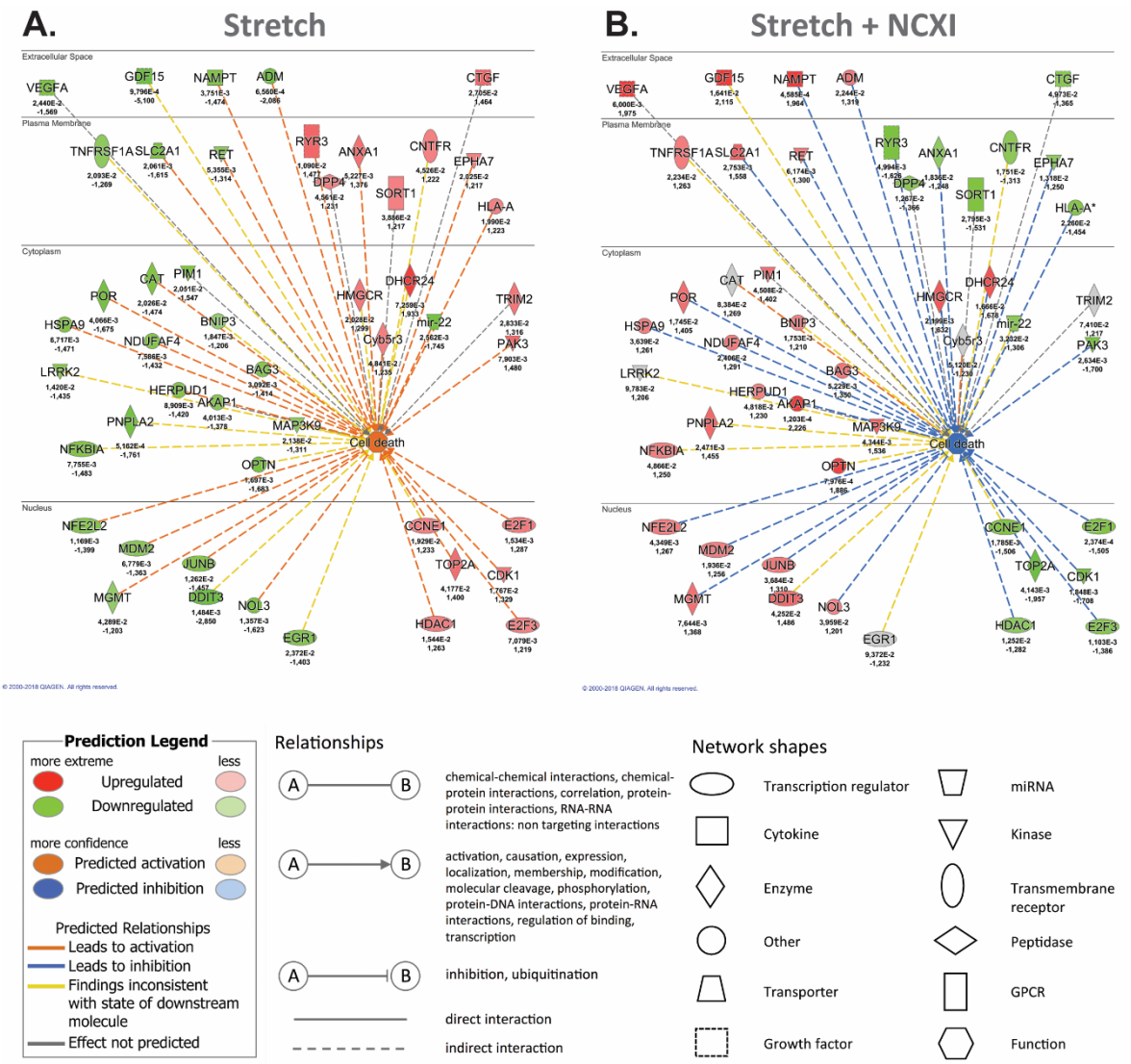


Figure 3.7.1 IPA cell death signaling pathway regulated during stretch and inhibited via NCX.

*(**A**) Expression of genes that control cellular death is in the pattern of promoting activation (**B**) Cell death is significantly inhibited by NCX inhibition. Statistical significance was*

determined with Fisher's exact test. P values are represented as log values and gene expression alterations are presented as relative fold changes. Grey coloured molecular shapes represent signalling molecules that are not significant but within the p < 0.1 cut off.

3.7.2 Stretch dependent regulation of microarray genes

Previous reports show that cholesterol is necessary for preservation of cardiomyocyte contractility (286). Immense stretch activation of pathways involved in cholesterol biosynthesis in our MC culture is even augmented via NCX inhibition. As shown in Rysä et al. there is a significant activation of NRF2-mediated Oxidative Stress Response after 12 h of stretch stimulation (220). However, we report a significant inhibition after 24 h of stretch which is followed by annihilation of the observed effect under NCX inhibition. Additionally a recently published study linked MEF2 and NRF2 with p53 cascade mediated heart failure in an *in vivo* TAC model (287). We found the p53 and NRF2 pathways as well as MEF2D transcriptional factor to be significantly inhibited in early stage cardiac remodelling under stretch stimulation. Upstream regulators that are stretch induced include EPHB1 and PKA.

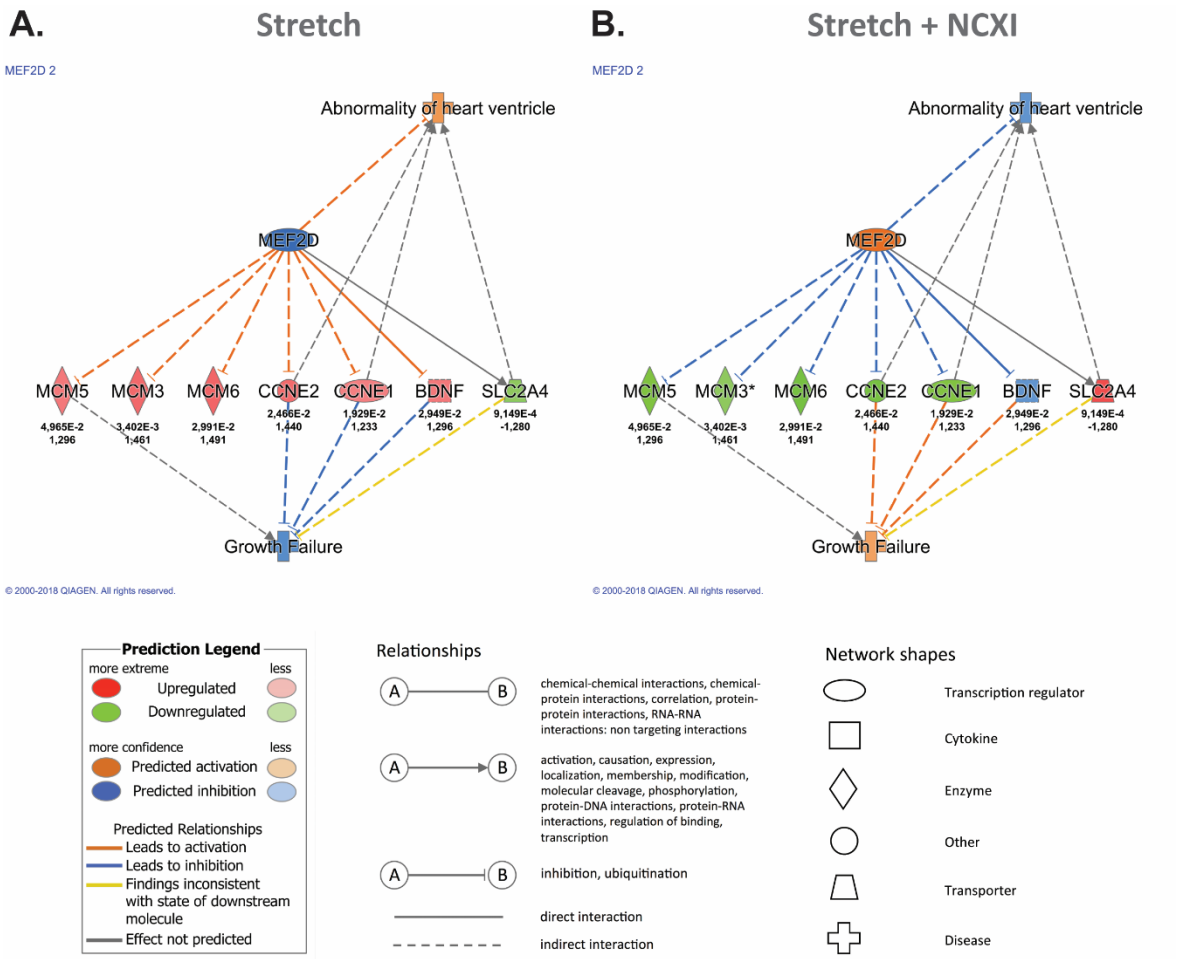


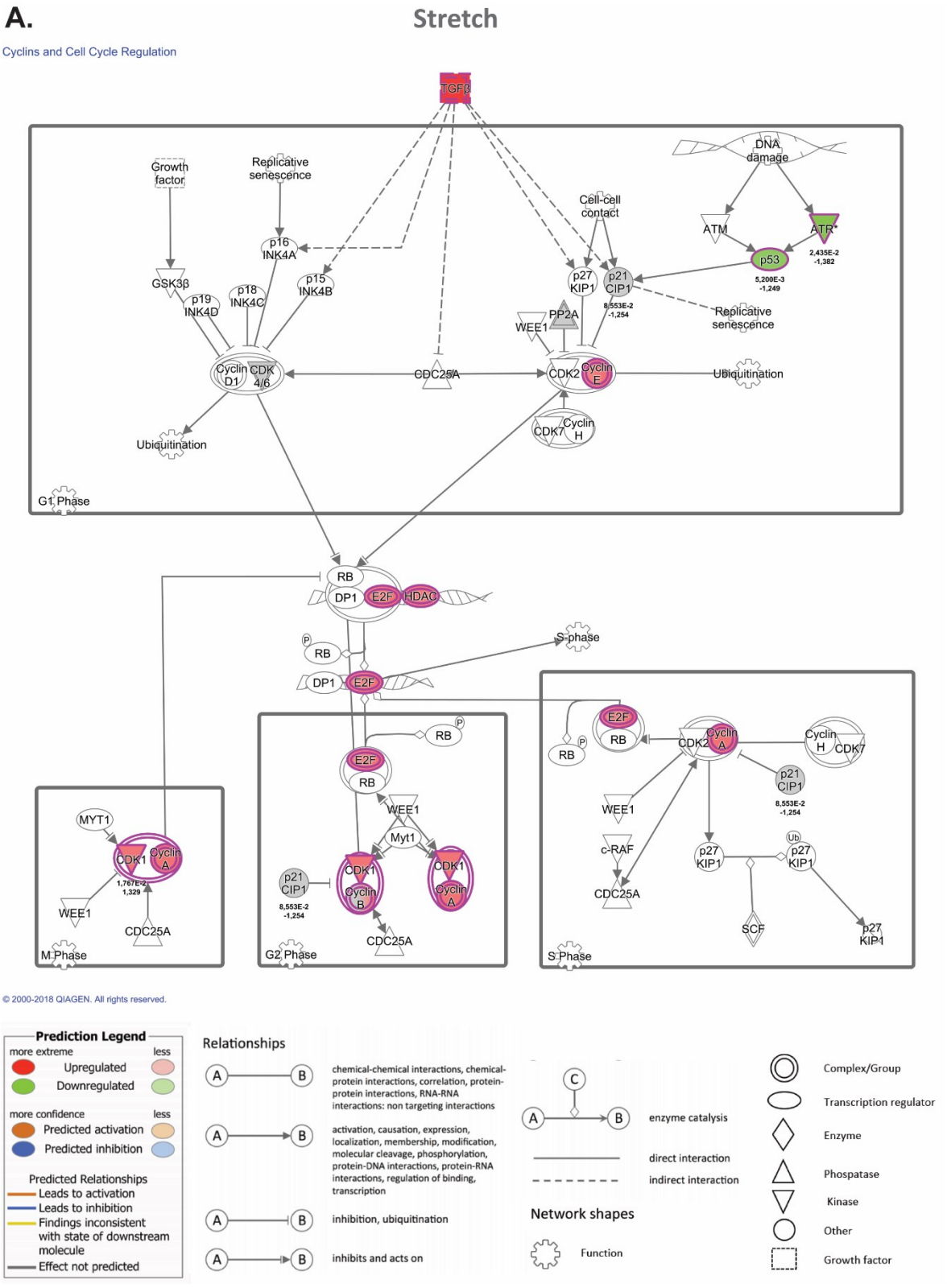
Figure 3.7.2 IPA prediction of activation state transcription regulator MEF2D.

*(**A**) MEF2D is predicted to be inhibited during stretch according to the expression status in the following genes: MCM5, MCM3, MCM6, CCNE2, CCNE1, BDNF, SLC2A4. Inhibition of MEF2D is linked to ventricular abnormalities and promotion of growth (**B**) MEF2D is predicted to be activated upon NCX inhibition with shift in expression pattern from that previously seen in stretch. This initiates growth suppression but also suppresses abnormalities in heart ventricle. Statistical significance was determined with Fisher's exact test. P values are represented as log values and gene expression alterations are presented as relative fold changes.*

3.7.3 Stretch induced regulation of NCX

Studies have shown that stress such as pressure overload *in vivo(288)* or cyclic stretch *in vitro*(289) enables cardiomyocyte proliferation as a coping mechanism. Stretch promotes cell proliferation via increase in cyclins, cell cycle regulation molecules, estrogen-mediated S-phase entry, DNA evaluation checkpoint and CHK protein inhibition. A phenomenon blocked via NCX inhibition. The embryonic heart exhibits a large amount of cyclin expression, therefore, there is an obvious shift to the fetal gene programming (290). P53 and AMT are known regulators of checkpoint integrity and their pathways are inhibited as well.

A.


Stretch
Cyclins and Cell Cycle Regulation
TCFβ
Growth factor
Replicative senescence
DNA damage
Cell-cell contact
ATM
ATR*
p16 INK4A
p53
2,435E-2 -1,382
5,200E-3 -1,249
GSK3β
p19 INK4D
p18 INK4C
p15 INK4B
p27 KIP1
p21 CIP1
8,553E-2 -1,254
PP2A
WEE1
Replicative senescence
Cyclin D1
CDK 4/6
CDC25A
CDK2
Cyclin E
Ubiquitination
Ubiquitination
CDK7
Cyclin H
G1 Phase
RB
DP1
E2F
HDAC
S-phase
P
RB
DP1
E2F
MYT1
CDK1
Cyclin A
1,767E-2 1,329
WEE1
CDC25A
M Phase
RB
E2F
RB
WEE1
Myt1
p21 CIP1
8,553E-2 -1,254
CDK1
Cyclin B
CDK1
Cyclin A
CDC25A
G2 Phase
E2F
RB
RB
CDK2
Cyclin A
Cyclin H
CDK7
p21 CIP1
8,553E-2 -1,254
WEE1
c-RAF
CDC25A
p27 KIP1
Ub
p27 KIP1
SCF
p27 KIP1
S Phase
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Prediction Legend
more extreme
less
Upregulated
Downregulated
more confidence
less
Predicted activation
Predicted inhibition
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted
Relationships
chemical-chemical interactions, chemical-protein interactions, correlation, protein-protein interactions, RNA-RNA interactions: non targeting interactions
activation, causation, expression, localization, membership, modification, molecular cleavage, phosphorylation, protein-DNA interactions, protein-RNA interactions, regulation of binding, transcription
inhibition, ubiquitination
inhibits and acts on
enzyme catalysis
direct interaction
indirect interaction
Network shapes
Function
Complex/Group
Transcription regulator
Enzyme
Phospatase
Kinase
Other
Growth factor

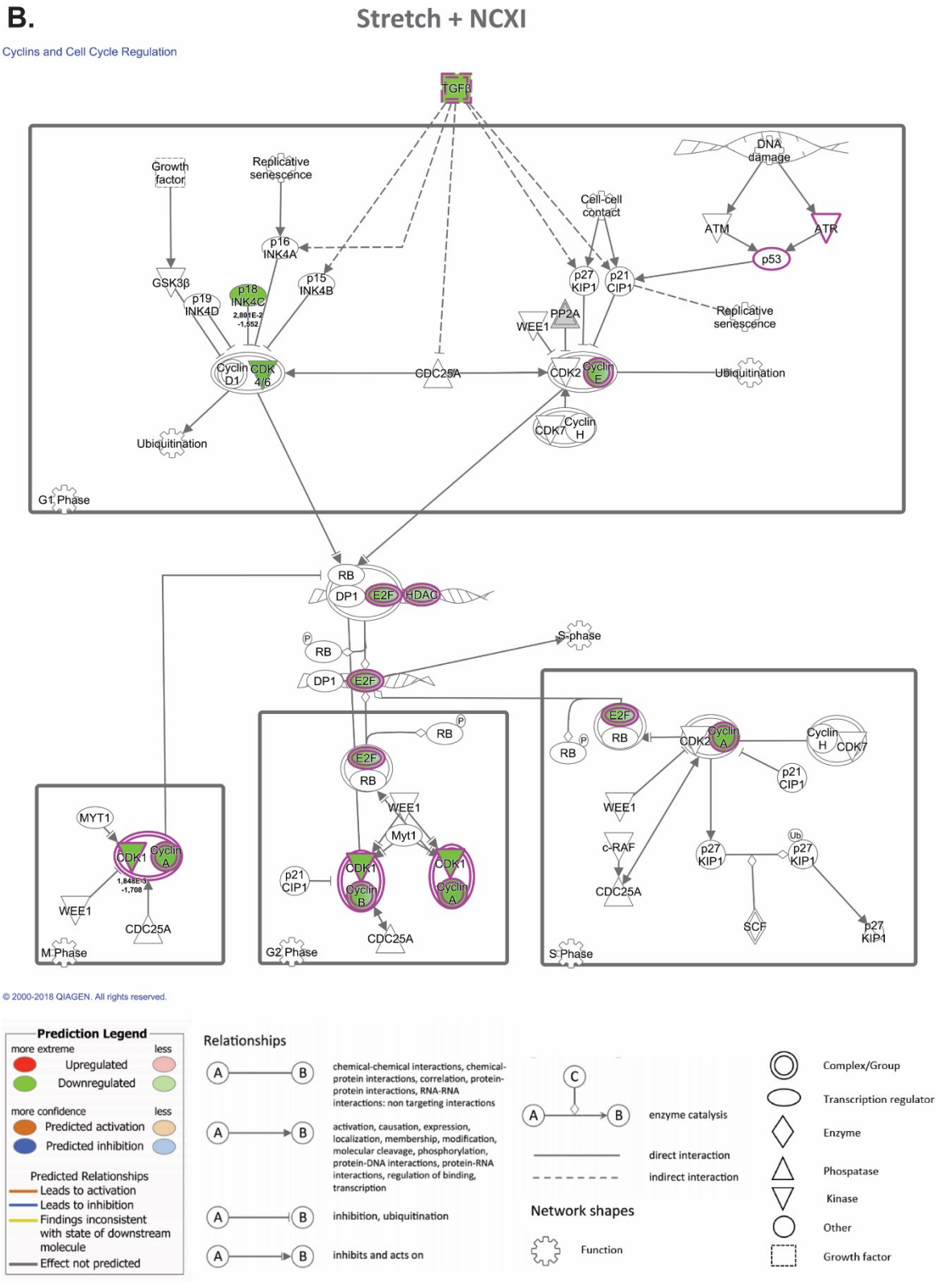

Figure 3.7.3 IPA evaluation of the regulation of cell cycle signaling pathway under stretch conditions.

*(**A**) Stretch promotes cell cycle re-entry through activation of TGFß. This promotes an increase in expression of cyclins and direct DNA modulation through E2F and HDAC transcription regulators. (**B**). Inhibition of NCX leads to cell cycle arrest via reduction in activity and expression of the corresponding molecules that were previously affected by stretch alone. Statistical significance was determined with Fisher's exact test. P values are represented as log values and gene expression alterations are presented as relative fold changes.*

In the process of hypertrophic remodelling it has been shown that there is a shift from lipid to glucose metabolism as a main source of energy (291). Indeed, in our dataset there is a subtle but significant decrease in oxidation of fatty acids. Interestingly under NCX inhibition fatty acid oxidation is significantly activated.

Actin cytoskeleton signalling can promote formation of stress fibbers and influences cell motility. These changes of the cellular cytoskeleton are mediated through a class of G proteins known as Rho GTPases that can act through integrin receptors (292). Another signalling pathway involved in cell motility is the paxillin pathway (293). All of these pathways have under stretch conditions a trend towards significance and a z score that implicates an activation state. All of the pathways are as well significantly down-regulated under NCX inhibition.

Integrin linked pathways have been associated with cyclic stretch induced hypertrophy (294). Indeed, the Integrin linked kinase pathway (ILK) is activated with a significant z score under stretch conditions. As per IPA this effect is reversed via NCX inhibition.

The VEGF pathway is activated according to IPA in stretch. Additionally, VEGF is the target of the Mir208a also found to be up-regulated under stretch conditions and down-regulated via NCX inhibition (Online Supplementary Table S2 (295)). VEGF, a major player in angiogenesis and hypertrophy, has been shown to promote *Ephb2* expression during shear stress (296). *Ephb2* expression is significantly up-regulated in both stretch and tachycardia and interestingly down-regulated via NCX inhibition in both cases.

Comparing the disease and functions associated to stretch and NCX we can conclude that stretch initiates cell death both via necrosis and apoptosis a process successfully reversed

by inhibiting the NCX reverse mode. Predicted initiation of morphological changes due to stretch includes formation of cellular protrusions, branching and sprouting which is inhibited via NCX. Changes in cellular assembly involve microtubule, cytoskeleton and cytoplasm reorganization and follow the same pattern. Finally, increase in fatty acid metabolism under stretch conditions is predicted to be reversed upon NCX inhibition.

3.7.4 NCX dependent gene program

We have found a large fraction of genes that are not regulated in response to either stretch or tachycardia. All of the genes have in common a regulation dependent on the NCX activation state, however, a majority of them do not appear in both experimental settings. According to the DAVID functional annotation tool a small gene fraction in common for different trigger settings shows enrichment of pathways involved exclusively in inflammation. When all NCX dependent effects are taken into account without regards to the experimental setting, we can observe activation of pro-inflammatory cytokines in response to NCX inhibition specifically TNF, IL1B and IFNG. A prominent activation of STAT1 and EGLN1 is also present. Additionally, NCX regulates the PI3K-Akt, Ras, Rap1 and the Bacterial invasion of epithelial cells pathway but through different set of genes in stretch versus tachycardia.

4 Results part II (human myocardium)

4.1 To aim 1: Stretch and tachycardia induced differential and common patterns of gene expression

Muscle strips (trabeculae) isolated from the right atrial appendage of heart surgery patients (Table 4) were subjected to two triggers of early remodelling: Sustained stretch with increased pre- and afterload for 6 h or sustained tachycardic stimulation (2,5 Hz) for 6 h. Microarray screening was performed with 5 patient samples for each of these four groups: 1. Stretch 2. Control for stretch 3. Tachycardia 4. Control for tachycardia.

Table 4. Baseline characteristics of patients that underwent surgical interventions that provided atrial muscle strip specimens

Demographic parameters/ Medical history		Medication (%)	
Gender m/f (%)	56,6/44,4	**ß-blockers**	44,4
Age (y ± SD)	66,8 ± 9,4	**ACE Inhibitors**	44,4
BMI (% ± SD)	29 ± 6,4	**AT1 Antagonist**	22,2
SR (%)	100	**MRA**	0
AFib	0	**Statins**	77,7
Paroxysmal AFib	0	**Ca^{2+} Antagonist**	44,4
EF (% ± SD)	54,9 ± 9,4		
CABG (%)	77,8		
AVR (%)	11,1		
MVR (%)	11,1		

Abbreviations: BMI – Body mass index, SR – sinus rhythm, AFib – atrial fibrillation, EF – ejection fraction, CABG – coronary artery bypass grafting, AVR – aortic valve replacement, MVR – mitral valve replacement, ACE - angiotensin-converting-enzyme, AT1 – angiotensin 1, MRA - mineralocorticoid receptor antagonist. n = 9.

The analysis for protein coding and non-coding genes with $p < 0.05$ showed a largely independent regulation by stretch *versus* tachycardia as demonstrated by 1305 transcripts regulated solely after stretch and 1837 solely after tachycardia (Figure 4.1.1 A), which also revealed that tachycardia resulted in a 40% higher number of significantly regulated genes than stretch. Out of the 1305 stretch regulated genes, 683 were found to be upregulated and 622 downregulated. Similarly, tachycardia resulted in an equal ratio of 922 upregulated and 915 down-regulated transcripts. The list containing all significantly regulated genes can be found in Online Supplemental Table S1 (295).

Among the protein coding genes *ACTA1* was the highest up-regulated gene after 6 h of pathological stretch (Figure 4.1.1 C), while *TRAJ22* and *SNRPN* the highest upregulated genes after sustained tachycardia (Figure 4.1.1 B). It became obvious, that in tachycardia, several of the downregulated genes were associated with inflammation, such as *PI15*, *IL1A, IL1B, VCAM1, KIR3DL3* and *CCL4*. Notably, regulators of the extracellular matrix – *MMP1, MMP10* along with several zink finger proteins were also among downregulated genes. Most of these genes are part of an identical network as well as the predicted master regulator Lymphotoxin. qPCR assessment of a selection of best –up and –down regulated genes on the microarrays confirmed unidirectional regulation of array and qPCR expression (Figure 4.1.1 D).

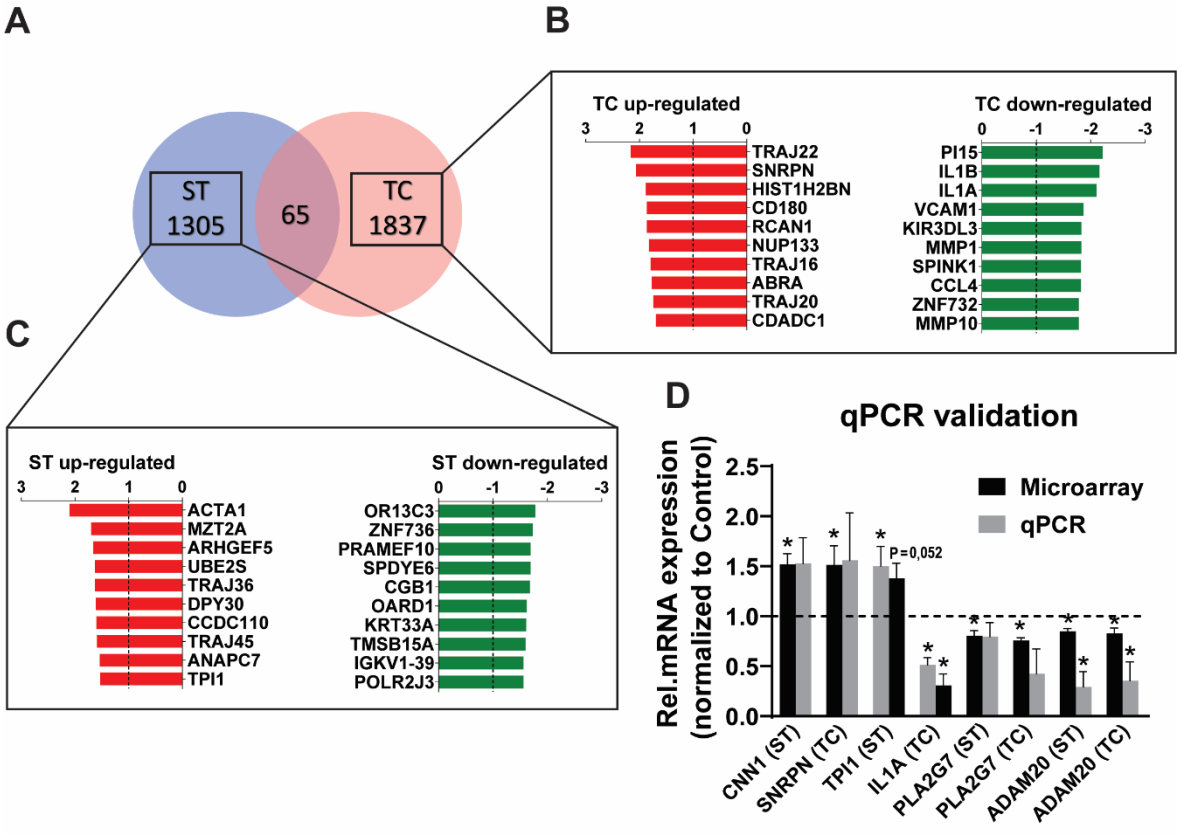

Figure 4.1.1 Genome wide expression characterization of stretch (ST) and tachycardia (TC) in human atrial trabeculae.

A. *Microarray Venn diagram showing all genes regulated by ST or TC with $p < 0.05$ and fold-change cut-off of at least 1.2-fold up or -1.2 down-regulated, n = 5 hearts per group.* ***B - C*** *A Zoom-In of the top 10 genes (only protein coding ones) up-regulated (red) or down-regulated (green) after 6h of either stretch or tachycardia stimulation.* ***D.*** *qPCR validation of some of the strongest regulated protein coding microarray genes with a determined function. Statistical difference determined by One-Sample T-Test. * $p < 0.05$, n = 4-5 hearts per group.*

Despite the predominantly independent gene regulation between stretch and tachycardia, there were 65 transcripts that were commonly regulated by both triggers (Figure 4.1.2 A): The larger fraction (39 genes) displayed unidirectional regulation (Figure 4.1.2 B) while the smaller fraction (26 genes) was regulated in opposite direction (Figure 4.1.2 C) between both triggers. The majority of the common genes with identical direction were upregulated and not downregulated in their expression. Among characterized transcripts with a known biological function, a microRNA - *MIR1183* had the highest expression level,

whereas *MIR 4501*, *ADAM20* and *PLA2G7* were the most downregulated common transcripts.

Bioinformatical estimations via ingenuity pathway analysis (IPA) provided information on molecules and associated networks, canonical pathways, disease and functions, upstream regulators and causal networks which are summarized in Djalinac et al, Online Supplemental Table S2 (295). By these means we also determined potential upstream regulators of the affected target molecules on our arrays: For stretch this included inhibition of PARP1 and BRD4 and activation of MAP2K1/2. In contrast, tachycardia pointed to a larger spectrum of potential upstream regulators which included activation of TP53, RBM5, JAG2, PDGF BB, NR3C2, SPP1, IL1RN. And an inhibitory effect of tachycardia was suspected for these putative regulators: TNF, JUN, IKBKB, IL4, TLR4, IL1A, CD40, IL18, PRKCD, P38 MAPK, Lymphotoxin, PTGS2, CD14, C5, Gm-csf, MBD2, Fcer1, IL17A, Jnk, ERK1/2, SAA, TP63, ECSIT, NDRG1, ESR1 and Mek.

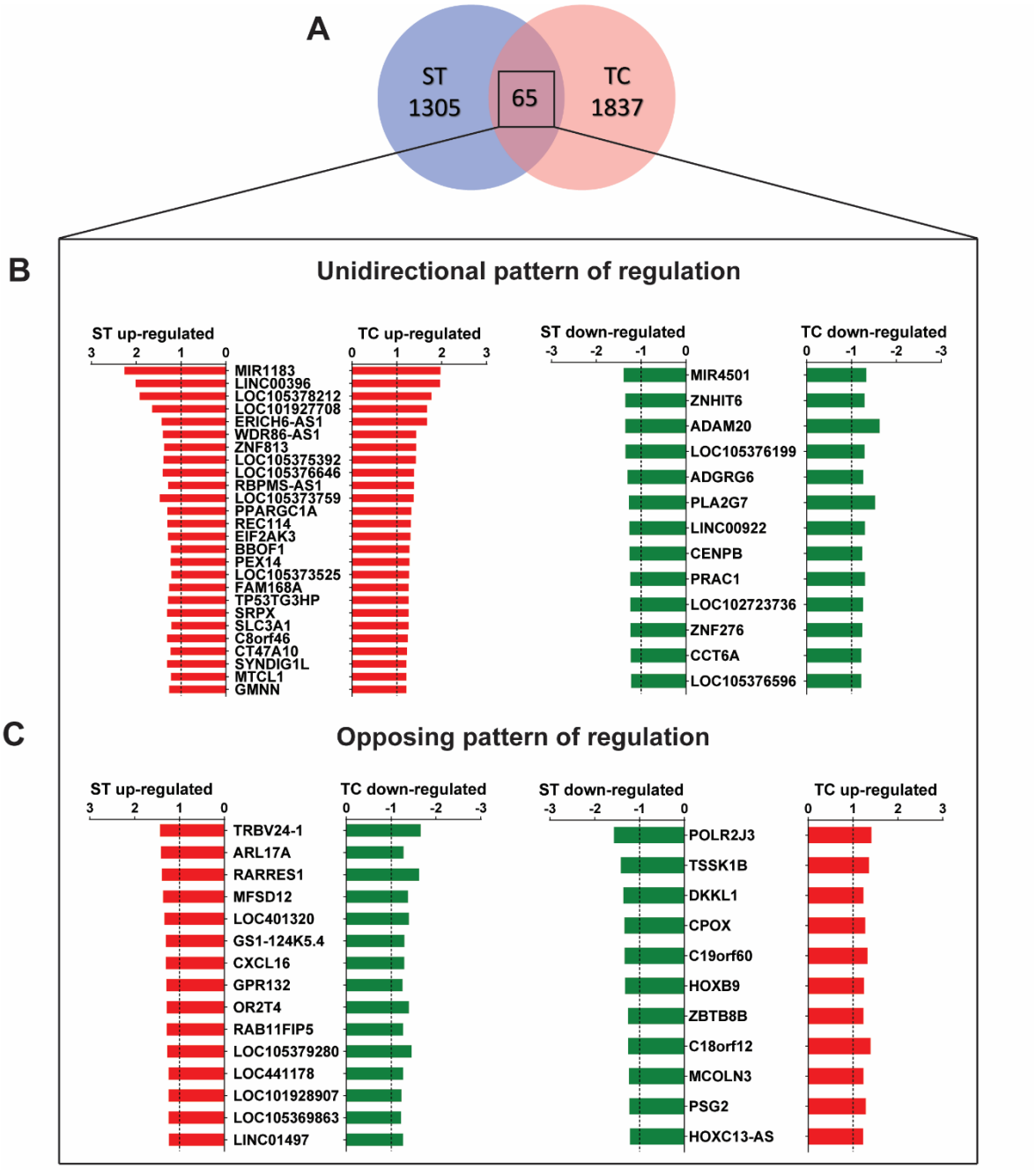

Figure 4.1.2 Commonly regulated genes by stretch (ST) and tachycardia (TC).

*(**A**) Microarray Venn diagram with cutoff values as specified in figure 1. There are 65 regulated genes by both, ST and TC. (**B**) Out of this group there are 39 transcripts with the same direction and similar pattern of regulation. (**C**) 26 transcripts with opposing directed regulation.*

4.2 To aim 2: MicroRNA expression profile and detection of *MIR1183* as strongest and commonly regulated target

After evaluation of all protein coding genes, we generated a list of all significantly regulated miRNA genes (Figure 4.2 A). Small RNAs, including miRNAs were present in a close to equal relative expression of 13 % and 14 % from total regulated stretch and tachycardia regulated transcripts, respectively. Interestingly, stretch showed an almost exclusive upregulation of affected miRNAs as compared to tachycardia. Conversely, tachycardia stimulation induced a balanced response concerning miRNA expression (Figure 4.2 A). Among the hits, *MIR1183* was the top regulated transcript not only among miRNAs but also when considering overall array gene expression. More importantly, *MIR1183* was the highest regulated transcript that involved both stretch and tachycardia (Figure 4.2 A). Accordingly, qPCR evaluation confirmed the upregulation in both, stretch and tachycardia (Figure 4.2 B).

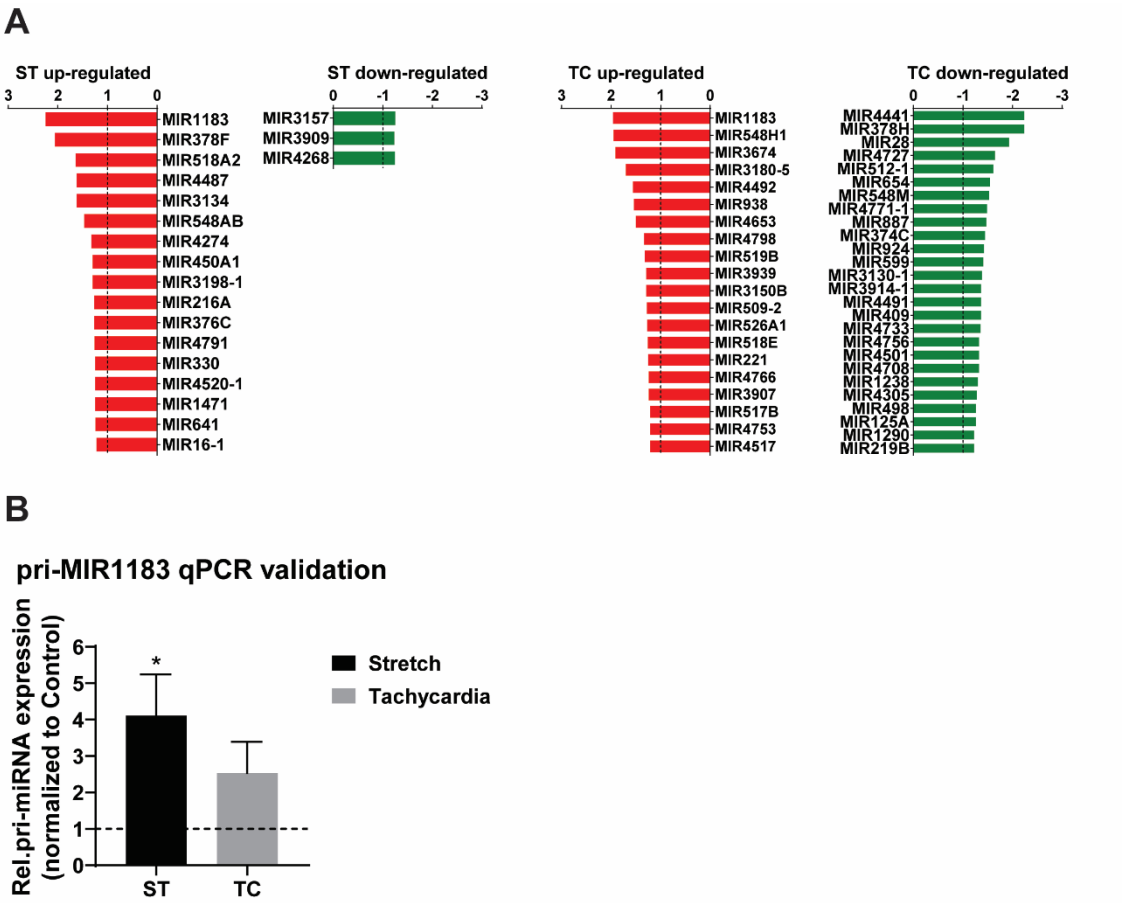

Figure 4.2 MicroRNA expression profile of ST and TC in human atrial trabeculae.

*(**A**) Microarray expression profile for precursors of miRNA in human atrial muscle strips regulated by ST or TC with $p < 0.05$ and fold-change cut-off of at least 1.2-fold up or -1.2 down-regulated, $n = 5$ hearts per group. (**B**) pri-MIR1183 expression showed the highest fold change and was validated by qPCR in human atrial muscle strips. Two-way ANOVA for microarrays and Paired Sample T-Test for qPCR, $^{*}p < 0.05$ $n = 5$ hearts per group.*

4.3 To aim 3: *MIR1183* is a commonly expressed biomarker in human cardiac tissue of different etiology

Considering the novelty of microarray findings, we further investigated whether *MIR1183* might serve as a tissue biomarker. Therefore, we evaluated chronic gene expression levels in samples of both atrial and ventricular source. Samples from patients suffering from documented long-term atrial fibrillation were compared to those with stable sinus rhythm (Table 5).

Table 5. Baseline characteristics of sinus rhythm and atrial fibrillation groups of patients that underwent surgical interventions and provided atrial muscle strip specimens.

Groups	**SR**	**AFib**
Demographic parameters		
Gender m/f	1/5	4/2
Age (y ± SD)	66 ± 10	73,7 ± 9,8
BMI (% ± SD)	29,2 ± 3,5	24,8 ± 2,9
Cardiac function		
EF (%± SD)	60,8 ± 9,2	56,2 ± 1,5
LVEDD	normal	normal
RVEDD	normal	normal
IVS	slight hypertrophy	hypertrophy
LA major axis	slightly enlarged	very enlarged
Comorbidities (%)		
Hypertension	83	100
Diabetes	17	0
CABG	17	67
AVR	17	17
MVR	100	100
Medication (%)		
ß-blockers	67	83
ACE Inhibitors	50	33
AT1 Antagonist	17	33
MRA	17	17
Statins	50	33
Ca^{2+} Antagonist	17	0

Main groups: SR – sinus rhythm, AFIB – atrial fibrillation –Parameters: BMI – Body mass index, , EF – ejection, LVEDD – left ventricular dilation diameter, RVEDD – right ventricular dilation diameter, IVS – interventricular septum, LA – left atria, CABG –

coronary artery bypass grafting, AVR – aortic valve replacement, MVR – mitral valve replacement, ACE - angiotensin-converting-enzyme, AT1 – angiotensin 1, MRA - mineralocorticoid receptor antagonist. n = 12.

Additionally, ventricular samples from patients suffering from end-stage dilated cardiomyopathy (DCM) were compared to samples from non-failing donor hearts (NF) and to samples from patients with only diastolic dysfunction (dD) (Table 6).

Table 6. Baseline characteristics of failing and non-failing ventricle donors.

Groups	NF	DCM	dD
Gender m/f (%)	50/50	100/0	57,1/42,9
Age (y ± SD)	63 ± 16,3	59 ± 14	60 ± 14,2
BMI (% ± SD)	25,4 ± 5,1	25,8 ± 2	26,6 ± 2,9
SR (%)	75	33,3	100
AFIB (%)	25	66,7	0
EF (%± SD)	58 ± 0,13	22,5 ± 6,12	59,7 ± 4,9
CHD	0	16,7	14,28
Hypertension	25	83,3	71,42
Diabetes	0	33,3	0
Hyperlipidemia	0	50	14,28
Paroxysmal AFIB	25	33,3	0
CABG	0	0	0
AVR	0	0	0
MVR	0	0	0
ß-blockers	25	33,3	0
ACE Inhibitors	25	50	0
AT1 Antagonist	0	0	0
MRA	0	50	0
Statins	0	16,7	0
Ca^{2+} Antagonist	0	0	28,57

Main groups: NF – non failing left ventricles, DCM – dilated cardiomyopathy left ventricles, dD - non-failing left ventricles with diastolic dysfunction. Parameters: BMI – Body mass index, SR – sinus rhythm, AFIB – atrial fibrillation, EF – ejection fraction, CABG – coronary artery bypass grafting, AVR – aortic valve replacement, MVR – mitral valve replacement, CHD – coronary heart disease, ACE - angiotensin-converting-enzyme, AT1 – angiotensin 1, MRA - mineralocorticoid receptor antagonist. n = 24.

Atrial myocardial samples from patients with chronic atrial fibrillation showed a significant increase in pri-MIR1183 levels compared to patients with normal sinus rhythm (Figure 4.3 A).

Samples from patients with dilated cardiomyopathy (DCM) also showed a significant upregulation compared to samples from non-failing donor hearts. In contrast, samples from patients with diastolic dysfunction and preserved systolic function (dD) had no significant effect (Figure 4.3 B).

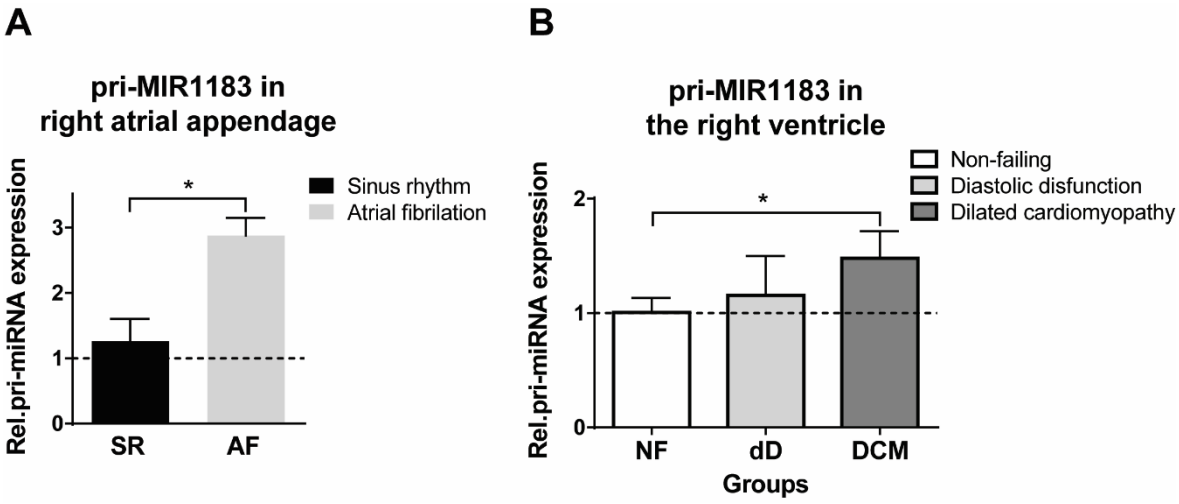

Figure 4.3. Chronic expression of pri-MIR1183 in healthy versus diseased atrial and ventricular human myocardium.

*(**A**) Increase in relative mRNA expression of pri-MIR1183 in right atrial appendage tissue from patients with normal sinus rhythm versus patients with persistent atrial fibrillation. (**B**) Increase in mRNA levels of pri-MIR1183 in right ventricular myocardial samples from the following heart groups: non-failing donor hearts (NF), hearts with diastolic dysfunction but preserved systolic function (dD) and hearts with dilative cardiomyopathy (DCM). The dashed line represents the baseline expression. $^{*}p < 0.05$, $n = 4 – 7$ per group. Statistical difference determined by One Sample T-Test.*

4.4 To aim 4: *MIR1183* induced downstream consequences

Investigation of *MIR1183* mechanism of action involves looking into target molecules upon which *MIR113* exerts its gene silencing properties. Thus, we used the tool TargetScan7 to assess *MIR1183* target genes. Out of the entire list of possible target genes (3539) with a different cumulative weighted context ++ score which predicts efficacy of miRNA binding to complementary mRNA sites (297) 1337 were expressed on the microarray. We looked solely at downregulated genes since downregulation reflects direct miRNA effects on mRNA. Out of these target genes 24 downregulated genes emerged under tachycardic pacing (Figure 4.4 A) whereas 13 showed significant downregulation during the stretch intervention (Figure 4.4 B). The most interesting candidate mRNA targets include the stretch and tachycardia commonly expressed genes *ADAM20* and *PLA2G7* (Figure 4.4 C). Additionally, both *ADAM20* and *PLA2G7* are the protein coding genes with the highest common downregulation detected on the microarray with convincing regulation of *ADAM20* expression assessed via qPCR.

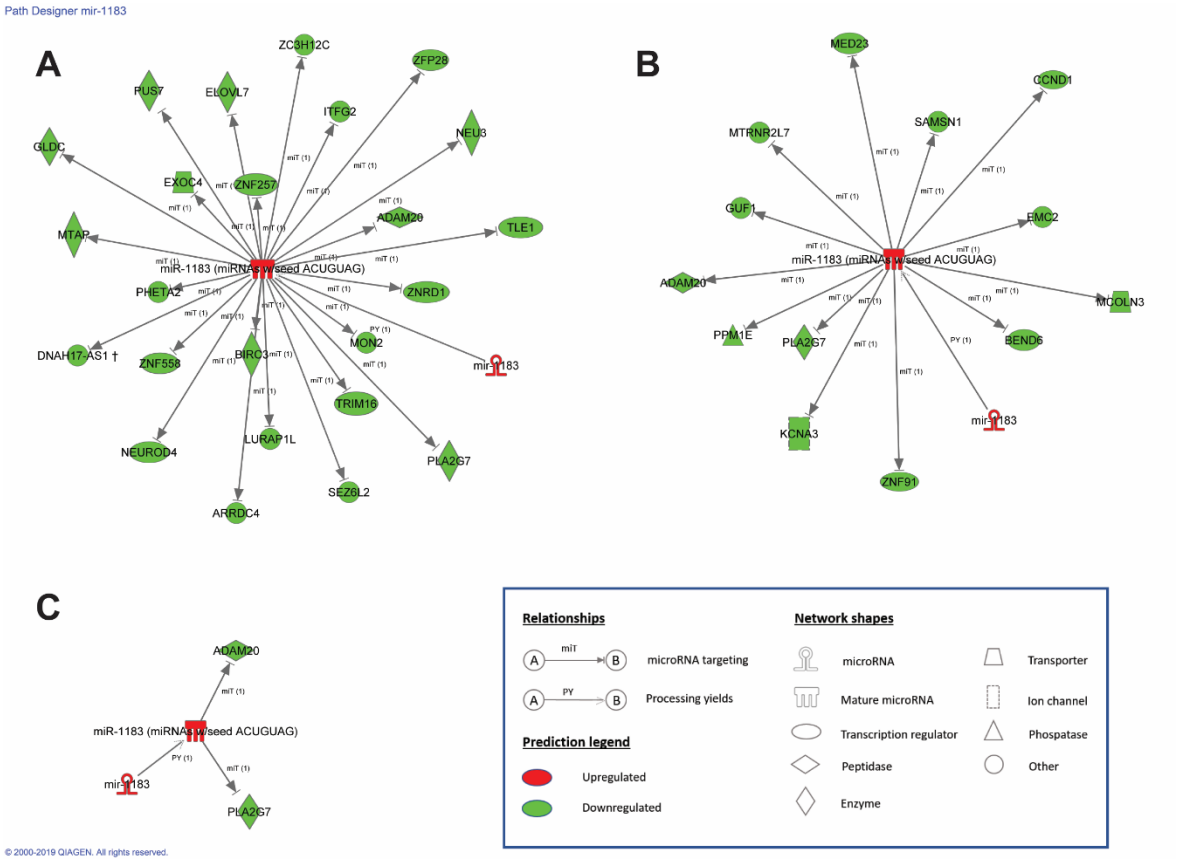

Figure 4.4 microRNA – mRNA network displaying the direct regulation of target genes by MIR1183.

*Up-regulation of MIR1183 and down-regulation of target genes following (**A**) Tachycardia (**B**) Stretch. (**C**) Commonly regulated MIR1183 and target genes ADAM20 and PLA2G7.*

5 Discussion

Studies on molecular mechanisms behind initial stages of cardiac disease development are of major interest since progression towards heart failure remains highly irreversible. For the first time we made a comparative study of two potent triggers: stretch and tachycardia in context of early cardiac remodelling and in two independent studies. We found that differential expression patterns underlie tachycardia and stretch mediated cardiac remodelling in rat in part I of this thesis, but also in human myocardium, which was part II of this thesis. In part I: The NRVCM cell culture was used to gain insight into molecular mechanisms and determine cell specific actions. Novel observations include myocyte specific gene alterations and hypertrophy independent of hemodynamic changes. Unique expression of specific genes with pathological connotation – *ACTA1* and *RCAN1* either in stretch or in tachycardia. Other novelties are stimulation and rate dependent structural changes initiated by tachycardia in myocytes and the mere presence of morphological hypertrophy in non-myocytes; the majority of which we characterized as fibroblasts. We performed transcriptomic microarray profiling not only under specific stimuli but also in conditions where the sodium-calcium exchanger (NCX) is inhibited. Via NCX inhibition we were able to finally distinguish which genes, specific for a single trigger or in either conditions, hold potential to reverse the remodelling process. Overall, it was shown that early remodelling initiated with stretch largely depends on NCX expression and reverse mode activation while NCX contribution in tachycardia is less but still relevant. We additionally determined that phosphorylation of CaMKII happens downstream of NCX when a tachycardic stimulus is applied. As the second part of this thesis performed in the before mentioned human myocardium our findings suggest that a non-coding miRNA *MIR1183* displays consistent expression between stretch and can act as a biomarker. We were able to confirm this in acute and chronic conditions on tissue level. Clinical implications of these two studies are therefore of importance to patients with different cardiomyopathy etiology such as HFpEF and atrial fibrillation (AFib).

5.1 NRVCM trigger dependent early cardiac remodelling

Cyclic stretch applied to primary cell culture has been described as a relevant model for studying changes *in vitro* (28, 29) which are complementary to systemic pressure overload. In contrast, the mechanisms induced by tachycardia alone have not been directly assessed. Instead, few investigations have been made to elucidate the pathophysiology behind mechanoelectrical (ME) coupling. These events involve stretch-related signalling mediators as initiators of tachycardia and indirectly cardiac remodelling (298). Other findings suggest that the presence of left ventricular hypertrophy (299) and moreover the degree of hypertrophy is in correlation with the occurrence of non-sustained tachycardia (300). To summarize, tachycardia is currently investigated more as a consequence of an already active trigger such as hemodynamic overload rather than a direct cause to cardiac remodelling. The use of cell culture is therefore specifically important in describing early remodelling on gene expression level, but also in gaining a clearer mechanistic insight of either stretch or tachycardia induced excitation transcription (ET) coupling. By applying either stretch or tachycardia *in vitro* we were able to mimic cardiac stress in its early stages.

5.1.1 To aim 1: Stretch and tachycardia initiate morphological cardiac remodelling in cardiomyocytes and non-myocytes

Cardiac composition has been thoroughly studied over time (301) as it represents an important factor that influences remodelling through mediators of signalling between neighbouring cells either via paracrine secretion or cell contact mechanisms (302). Cardiac remodelling in terms of structural changes can be quantified at best by evaluating the degree of present hypertrophy.

In this thesis, we concentrated firstly on morphological assessment of single cardiac cell hypertrophy followed by expression of defined pro-hypertrophic marker genes. Stretch induced cell size increase became evident at the 72 h time point of stimulation. A slight difference was already present after 48 h, however, it did not reach significance. After 72 h the increase was 10% and highly significant, so we can be sure that the achieved hypertrophy is relevant. Other studies report hypertrophic response between 10 – 40 % after

similar experimental settings. The variability in stretch response could be attributed to the form of stretch applied (biaxial or uniaxial) as well as frequency of the stretch stimulus. Another significant co-variant might be the extracellular matrix, since different coatings are available and chosen based on cell attachment and survival. In the course of this thesis laminin, gelatine, prolactin, collagen type I and collagen type IV were tested and the best performance was achieved by using collagen type IV coating. Application of biaxial stretch in other studies varies between 10 – 20% of the flex membrane dimensions. We did not observe any statistical difference or trend with 10% biaxial stretch while application of 20% biaxial stretch resulted in significant cell size increase. Due to equipment limitations, however, stretch stimuli with this strength could only be applied at 30 cycles per minute.

On the other hand, by using field stimulation we were able to show for the first time a relation between different frequency rates and degree of hypertrophy after 48 h. We evaluated 1 Hz, 5 Hz and 8 Hz frequencies to correlate gene expression results with morphological data. We assume that 1 Hz frequency is considered as prevention of atrophy when compared to unstimulated cells and preservation of contractile units (303). Frequency of 5 Hz can be considered as a tachycardia near the physiological upper limit, and 8 Hz as a supra-physiological tachycardic frequency.

To investigate cell specific actions we subjected a pure non-myocyte cell culture to same degrees of stretch or tachycardia as in myocytes. Previous studies on cardiac cell interaction defined the direction of paracrine signalling from myocytes to non-myocytes as a requirement for non-myocyte cell growth. For instance, canine fibroblasts showed an increase in cell size after treatment with HL1 myocyte conditioned media and subsequent 5 Hz field stimulation (304). Also fibroblast remodelling is achieved *in vivo* after chronic atrial pacing in a pig model of LV failure (305). Here we report for the first time tachycardia induced non-myocyte hypertrophy, which is myocyte independent. Additionally, as we managed to show by means of flow cytometry and immunocytochemistry, hypertrophy of non-myocyte refers specifically to fibroblasts. A question remains as how pure fibroblasts sense tachycardia without the presence of a contractile apparatus and in absence of myocyte coupling: Although fibroblasts are non-excitable cells, they do express a number of ion channels during their remodelling. The emerging role of voltage gated sodium and potassium channels during atrial fibrillation in fibroblasts isolated from human patients (306) and a chronic tachypacing animal model (307) makes them attractive candidates to explain the findings in NMC in this thesis. Another mechanism of calcium entry could involve NCX

reverse mode activation through local increase of sodium and consequently intracellular calcium influx. Additionally, TRP/Orai channels govern store operated calcium entry (SOCE) (308), regulate fibroblasts during AFib (309) and are functionally coupled with NCX (310). K_{ir} channels were also shown to facilitate SOCE in AFib fibroblasts (307). Mir26a is identified as an important downstream regulator of both K_{ir} and TRPC3 channels (307, 309) while NFAT is accountable for targeting Mir26a (309). We speculate that due to the similarity in experimental models these are the most likely mechanisms of tachycardia induced NCM hypertrophy.

5.1.2 To aim 2: Characterisation of neonatal rat NMC composition

While there is an agreement that the mammalian heart compromises of approximately 30-40% myocytes, conflicting reports exist regarding the exact proportion of other cellular types termed non-myocytes (311). A recent study on non-myocyte cellular composition highlighted the abundance of endothelial cells over other cell types such as resident mesenchymal cells (RMC), vascular smooth muscle cells (VSMC) and immune cells. The caveat in most studies dealing with non-myocytes is the great number of fibroblast subpopulations that form a large part of RMCs followed by a lack of fibroblast specific markers. Pinto et al made use of MEFSK4 as a new highly specific fibroblast marker which is suitable for flow cytometric measurements (312). However, the primary focus of this study was on the composition of the isolated mouse heart and the findings therefore remain to be verified in other species. By using flow cytometry we identified RMCs as the majority of non-myocytes and used more specific immunofluorescent staining to identify fibroblasts as the prevailing cell type that constitutes the neonatal rat heart. Similarly, another study (313) that used neonatal rat hearts as well as adult rat hearts identified fibroblast as the main cellular group. More importantly the study made it clear that substantial differences exist when comparing rat and mouse hearts for cellular composition. Therefore, the species specific cellular contribution must be taken into account when discussing about the myocyte – non-myocyte interplay.

5.1.3 To aim 3: Marker gene evaluation uncovers a crucial time window for manifestation of early remodelling events

We aimed to investigate early changes in gene expression inflicted by pathological signaling pathways that derive from biomechanical or tachyarrhytmic insult *in vitro*. Mechanical stretch that mimics pressure overload as well as filed stimulation mimicking tachycardia were applied independently to myocytes and non-myocytes. For the first time we show that stretch and tachycardia differentially regulate gene expression of marker genes related to cellular hypertrophy.

Our time dependent gene expression profiling in cyclic stretch marked 24 h as a peak time point for transcriptional activities. With the exception of *RCAN1* all genes showed upregulation in myocytes with *ACTA1* being the most pronounced marker gene. Previously, other authors evaluated the effects of stretch on NRVCM after 24 h as well and found specific stretch related induction of *ACTA1, NppA, NppB, FHL1* and *BNIP3* which, excluding the *BNIP3,* is reproducible in our own measurements (219, 314). The same authors focused in a separate study on cell proliferation and correlated it with expression of *Rnd1* after 24h, which is a gene that is also upregulated in our array dataset (315). In another NRVCM study (220) *ANP* and *BNP* expression peaked at 24 h cyclic stretch and contributed to high expression of *CNN1* gene determined with microarray, which we also observed. Isolated adult rat cardiomyocytes were also assessed for *VEGF* expression and production after 24 h of stretch (316). In summary different studies, confirm the reproducibility of stretch related gene expression changes within 24 h of stimulation.

Expression profiling of tachycardia up to 24 h at 5 Hz versus 1 Hz frequency revealed that the significant time-dependent change happens early on within the first 3 h of stimulation. Frequencies up to 4 Hz are considered physiological (317) and show a positive FFR in neonatal rats (318) This might explain why 5 Hz showed an only moderate increase in gene expression. However, with 8 Hz we observed a significantly prominent gene upregulation at the given time point. In conclusion, high frequency pacing from physiological, through moderate to highly tachycardic frequencies shows clearly a rate-dependent effect. In addition, cells were stable at this time point with continuous synchronized beating and normal viability excluding confounding effects that might have occurred at later time points. Notably a distinct pattern of the aforementioned marker gene

regulation emerged with tachycardia when compared to the stretch model. Here *RCAN1* was the best marker gene which along with *FHL1* showed both a stimulation and rate-dependent effect. The 3 h time point as a marker of early remodelling is supported by findings of other studies that show that during 4 h of rapid short term atrial pacing there is an increase in mRNA levels of specific voltage-gated potassium channels in a frequency dependent manner. The study was done in isolated rat hearts and the frequency at the given expression peak was 20 Hz (319). The same authors also emphasize significance of early induction of gene expression modifications and influence on electrical remodelling. The latter can also be observed for the L-type calcium channel α1c and potassium channel Kv4.3. Both show a decrease after 3 h (320) of rapid pacing and remain downregulated at 24 h in rat atrial myocytes, which might be mediated via activation of p38 MAPK and ERK signaling pathway (321).

To summarize: induction of stretch and tachycardia in NRVCM resulted in differential time dependent gene expression. 24 h marked the peak of stretch induced gene regulation while field stimulation showed an earlier peak effect. Another interesting finding is that the gene *ACTA1* is prominently upregulated during stretch conditions while *RCAN1* is only regulated under tachycardia. The assumption is that these genes belong most likely to different signaling pathways.

5.1.4 To aim 4: Cardiomyocytes and Non-myocytes contribute to trigger specific marker gene expression

The marker genes *ACTA1, NppA, NppB, FHL1* and *RCAN1* were selected based on their known regulation during pathological stimuli such as endothelin-1 and phenylephrine-mediated hypertrophy *in vitro* (322) as well as *in vivo* models of pathological hypertrophy (323). So each of these genes has its own specific relevance in remodelling:

Skeletal alpha (α)-actin or *ACTA1* is involved in regulation of muscle contraction (324) as well as cytoskeleton organization (325). *ACTA1* expression in the cardiovascular systems can serve as a marker of pathological hypertrophy (326-330), hypertrophy associated cardiac diseases (331, 332) and ultimately heart failure as an end result of the before mentioned conditions.

The natriuretic peptides *NppA* and *NppB* are essential during the developmental phase and are a part of the fetal gene program (333, 334). Their levels are subjective to changes upon hypertrophic stimuli (335), hypertension (328, 336) and progression to heart failure (337).

FHL1 is also a member of the fetal gene program and is found to be expressed upon biomechanical stretch along with the rest of the marker genes (219). The *FHL1* gene shows a stable expression during this time while other genes incline towards down-regulation. This is of relevance since during time the degradation of the myofilaments will determine the contractility of the cell (338).

RCAN1 is linked to the calcineurin/NFAT signalling pathway (339-341). *RCAN1* inhibits Calcineurin by binding to its catalytic domain and therefore represses pathological hypertrophy (342). High expression of *RCAN1* is part of a coping mechanism against sudden insult in the brain (343) and lung (344) and is highly involved in regulating pathological cardiac remodelling either directly as mentioned previously or through interaction with other signalling mediators (345).

With the exception of the natriuretic peptides non-myocytes show absence of significant gene regulation upon stretch. This could be a consequence of a later gene up-regulation onset in non-myocytes, but more likely, the hypertrophic responses are mediated through different downstream signalling pathways. As mentioned previously the *NppA* and *NppB* genes encoding ANP and BNP natriuretic peptides are reliable pro-hypertrophic markers (346, 347). Interestingly studies on co-cultured cardiac cells reveal the absence of ANP and BNP in a pure myocyte cell culture but confirm expression when all cell types are present (348). This suggests that myocyte expression of natriuretic peptides is dependent on the non-myocyte presence. This is also supported by our findings were pure non-myocyte show minor but significant increase in *NppA* and *NppB* expression while this effect is highly pronounced in the myocyte enriched culture. Therefore, we can conclude that non-myocytes influence myocyte *NppA* and *NppB* expression by means of paracrine signalling. In addition, we can conclude that the major increase in mRNA levels derives from myocytes which are therefore mainly responsible for stretch induced effects. In any case, the investigated genes in our given period can be considered as selective, or at least preferential, markers for myocyte hypertrophy.

When we compared the expression pattern of myocytes under tachycardia to that of non-myocytes undergoing the same experimental procedure we saw an interesting correlation. Namely, three marker genes followed the myocyte gene expression pattern when stimulated with 8 Hz frequency out of which only *RCAN1* showed a rate-dependent effect. Meaning that the expression pattern at 8 Hz frequency is not only myocyte specific. However, by looking into the proportion of cell distribution in the culture we can say that the overall effect on gene regulation is still myocyte derived.

5.1.5 To aim 5: Underlying sarcolemma and intracellular remodelling mechanisms behind acute stretch and tachycardia triggering

It is known that stretch alters the activity of calcium handling proteins promoting pathological changes in myocytes. This is mediated from one side through the activation of mechanosensors, respectively, sarcolemma ion channels such as L-type Ca^2 channel (349-353), Na^+/H^+ exchanger (354-356), Na^+/Ca^{2+} exchanger (357-359), TRPC channels (360, 361) as well as through activation of the renin-angiotensin-aldosterone-system (RAAS) mainly in an autocrine manner (362, 363), via the AT receptors (364) or through an ATII independent mechanism (365). Intracellular regulators of calcium homeostasis, namely CaMKII (366, 367) and Calcineurin (368, 369), also play a vital role in the development of pathological hypertrophy. Based on these known mechanisms we performed a screening of inhibitory compounds after 24 h of stretch in an attempt to suppress the established marker gene expression upregulation. The TRP channel, Calcineurin, CaMKII and the Na^+/Ca^{2+} exchanger (NCX) showed significant contribution to gene regulation. Out of these, the selective inhibition of the reverse mode NCX had the most robust influence on the marker genes.

The relevance of NCX in ET-coupling can be attributed to several factors. As a sarcolemma transporter NCX is essential in maintaining calcium homeostasis in an unstimulated cell via forward mode activity (370). This is achieved through bi-directional transport of sodium and calcium (371).The NCX also interacts with other transporters such as the NHE (372) and TRPC3 (166) in various contexts. By influencing calcium fluctuations the NCX also has impact on two notable intracellular signalling molecules: Calcineurin (373) and CaMKII (374). This relation is specifically relevant during tachycardia (375).

Finally, the downstream targets of NCX include transcriptional factors MEF2 and SIK1 through which NCX might mediate gene regulation as a final step in its ET-coupling cascade (374).

Contribution of NCX reverse mode to calcium regulation is minor in physiological conditions, however, it is known that in cardiac remodelling the NCX has an increased ability and probability to act in its reverse mode, i.e. the mode where calcium influx is present (376). The underlying causes are increase in $[Na]_i$ and prolongation of the AP which enables activation of the reverse mode as a compensatory mechanism at first. Subsequently an activated NCX reverse mode leads to impaired functional responses such as altered SFR (377) and diastolic dysfunction (378) and promotes heart failure (379, 380) through increased $[Ca]_i$. In accordance, in few cases such as heart failure with preserved ejection fraction (HFpEF) there is a possibility to prevent the remodelling process by inhibiting the NCX (381).

NCX reverse mode inhibition in our dataset prevented stretch and tachycardia mediated up-regulation of marker genes which enabled us to conclude that NCX is crucial in both triggering events through changes in its activity status. Next, we observed that along with changes in activity NCX also shows an increase in protein expression following stretch. Interestingly, we observed that tachycardia on the other hand shows only activity change without protein expression increase within the given time frame. Therefore, protein expression increase in not essential for a change in NCX activity post tachycardia. This, however does not exclude protein expression changes in a later time point.

We were also interested in broadening our understanding of signaling pathways downstream of NCX. Therefore, we applied KN93 and its inactive analog KN92 to investigate whether KN93 effects on gene expression are specifically mediated through the CaMKII signaling pathway. We determined that KN93 acts in a CaMKII independent way by displaying a similar inhibitory potential as KN92. This effect coincides with a previous finding that showed unspecific inhibition of I_{kr} channels by KN93 which were also inhibited by KN92 (382). In light of this findings we decided not to use KN93 as a proof of CaMKII activity. However, we did not exclude CaMKII involvement but only the inability to detect it by KN93. Instead, we measured CaMKII activation by determining its phosphorylation levels and found an absence of activation in stretch and a strong activation in tachycardia mediated via NCX, which leads to the conclusion that CaMKII plays a mechanistic role only

in tachycardia induced cardiac remodelling. Additionally, we investigated whether a recently established regulator of CaMKII – ADAMTS13 could play a role in tachycardia induced CaMKII activation. ADAMTS13 is a protease which regulates CaMKII phosphorylation thereby increasing propensity towards arrhythmogenesis (383) and is therefore a very attractive candidate in a setting of accute tachycardic triggering. We found however that the expression levels of ADAMTS13 remained consistent between normal and high frequency pacing groups precluding its effect on CaMKII phosphorylation and additionally excluded any NCX mediated effects.

Based on the provided rationales we chose NCX as a target for whole genome evaluation in stretch and tachycardia induced cardiac remodelling. Inhibition of NCX was accomplished using KB-R7934 within the reverse mode specificity concentration range (384).

5.1.6 To aim 6: Differential gene expression of stretch and tachycardia downstream targets

A number of transcriptomic studies (385-387) that have been performed so far were mainly focused on *in vivo* stretch induced changes that already manifest a pathological phenotype. Few studies (388, 389) focus on early stages of cardiac remodelling and to our knowledge none dissect the discrepancy between different types of wall stress that can act simultaneously and contribute to further stages of disease progression. In addition, we report here for the first time the genome wide expression changes under NCX inhibition.

Using microarray technology, we were able to distinguish between larger portions of genes regulated separately, either by stretch or tachycardia, and a smaller portion regulated in both cases. Based on this evidence we can draw a conclusion that within the investigated time period stretch and tachycardia manifest a trigger specific program with very little overlap.

By evaluating the consequences of NCX inhibition for the first time on genome wide level we were able to show that in 40% cases stretch-related gene alterations are prevented as well as around 20% of tachycardia related alterations, which further confirmed the crucial involvement of NCX reverse mode activity during acute triggering. Based on the previously

presented evidence it is highly probable that a certain number of genes which are regulated specifically by tachycardia do depend on the activation state of CaMKII. Despite the discrepancy in stretch and tachycardia gene regulation, the individual genes from the commonly regulated fraction can be considered as valuable markers of impaired ET coupling. The following genes are found to be significantly regulated by both triggers and additionally dependent on NCX reverse mode:

Efnb2 which was previously described as highly expressed after myocardial infarction *in vivo* and has a possibility to act through the VEGF signaling pathway (390), *CCNA2* - a gene involved in hyperplastic growth (391) and *KIF14* a significant contributor to cellular proliferation (392). Both of these genes are modulators of cell cycle properties.

Other genes of interest would be *TGFß2*, *Rasgrpr3* and *Isg15*. *TGFß2* is commonly associated with mitral valve disease and other cardiac abnormalities (393). *Rasgrpr3 is* upregulated during mitral regurgitation (394). Additionally, it is an activator of the Ras-GTP signaling while beneficial effects upon its inhibition include attenuated proliferation of tumor growth (395). *Isg15* is mainly involved in inflammation-induced cardiomyopathy but also dilated cardiomyopathy (DCM) (396), however with species-dependent regulation (397, 398). These genes were found to be commonly regulated by stretch and tachycardia and show a trend towards NCX inhibition.

Genes such as *GDF15* and *Tmem140* are strongly affected by NCX inhibition and involved in pathological cardiovascular events in upregulated expression state (399-402). These genes are in fact reversed to normal baseline expression levels when downregulated by the main trigger, which could be interpreted as initial coping mechanism against the pathological insult. *Fnip1* (403), *Fnip2* (404), *AKAP1* (405) downregulation promotes hypertrophy and heart failure. *Akap1* additionally mediates mitochondrial impairment (406-408). Therefore, gene expression alterations through decrease in NCX activity could be seen as a rescue mechanism. The mentioned genes are found to display actions through the mTOR-AMPK signaling (409, 410).

5.1.6.1 Comparative analyses to other transcriptional studies

In order to draw conclusions regarding data reproducibility we made a correlation between our findings and datasets from publications of other authors obtained in similar circumstances. The first observation is that there is a limited overlap ranging from 15 % to 20 % (219, 220). The observed difference in overlap can be attributed to several factors. Biological sex is an important cofounding variable as it has been shown recently (411). As stated in Rysä et al. the gender effect had not been controlled within their study which is also a limiting factor in our case. The extracellular matrix influences cell contractility, as previously discussed, and therefore may contribute to the magnitude of observed effects. The differences in the activation state of signalling pathways for e.g the NRF2-mediated Oxidative Stress Response pathway in Rysä et al. can be contributed to a different time point of evaluation .

Nevertheless, another NRVCM microarray profiling study (219) showed increased expression of *ACTA1, FHL1, NppA* and *NppB* after 24h of stretch which is in correlation with our qPCR marker gene assessment. Aditionally, as a novelty, NCX inhibition decreases the expression levels of *ACTA1, FHL1* and *NppA* in our own study. Another comparable microarray study was performed in a myocyte-enriched culture and also in a non-myocyte cell culture (412). The findings of this study made it clear that the differentially regulated genes involved in cholesterol biosynthesis represent the top regulated genes in the CM-enriched culture, but play no role after 24 h of cyclic stretch in fibroblasts. According to IPA cholesterol biosynthesis and associated pathways are the biological pathways with the highest z score in our stretch group. Interestingly inhibition of NCX significantly augments the expression of genes involved in the mentioned pathways. An interesting gene candidate described in the before mentioned study done by Rysä et all is *CNN1* found to be among the three genes with the highest regulation within the 24 h time frame. Through careful examination of our dataset, we can draw a conclusion that *CNN1* possesses a translational potential since it shows a significant or near to significant unidirectional expression in both NRVCM and human arrays. The *CNN1* gene encodes Calponin 1, a protein most notably known for its role in modifying smooth muscle contraction by regulating actin filaments through a so far undefined mechanism of action (413).A recently published study took interest in elucidating the role of *Rnd1* after 24 h of stretch. The microarray hybridisation was used as a redout for increased *Rnd1* expression and it is reproduced within our own

dataset. Therefore, although there is discrepancy between similar transcriptomic studies and our own study the overlapping genes and molecular pathways are highly valuable due to their consistent expression independent of the cofounding effect.

5.1.7 To aim 7: Relevant bioinformatical relationships and findings

Based on the degree of significance and direction of the regulation of stretch, tachycardia and/or NCX specific genes we gained several relevant predictions through Ingenuity Pathway Analysis (IPA). These predictions included information about signalling pathway activation states, specific gene regulators within pathways as well as biological functions that are influenced by pathway activation. Analysis of stretch-regulated genes managed to show: that stretch promotes activation of cholesterol biosynthesis pathways, necessary for contractility preservation (286). A surprising finding was the inhibition of the NRF2-mediated Oxidative Stress Response previously reported to be activated under similar experimental conditions, however within different time frames (287, 389). Stretch significantly down-regulates the p53 encoding gene *TP53* and MEF2D. Loss of p53 contributes to cardiac hypertrophy and p53 deficient myocardium shows down-regulation of MEF2D (414) Stretch significantly activates VCAN which in turn encodes versican - a glycoprotein important in early cardiac development (415) and affects cell proliferation (416). Stretch also affects cell cycle re-entry which is supported by *in vivo* findings where stress conditions such as pressure overload enable cardiomyocytes to re-enter the cell cycle (288). Following stretch levels of PPARG gene regulator are reduced. PPARG activation exhibits cardioprotective properties mainly through anti-oxidative properties, and inhibition of cardiac differentiation and proliferation (417). Stretch also activates SIRT2, presumably as a coping mechanism since SIRT2 is an important regulator of the cell cycle and manifests a protective role against cardiac hypertrophy (418).

Tachycardia is often described as a condition secondary to an already ongoing inflammation (419-421). Interestingly our data show inhibition of pathways related to inflammation. Persistent supraventricular tachycardia does show increase in inflammatory marker expression (422, 423) however, there is no evidence on acute tachycardia effects and inflammation.

Further array evaluation made a distinction between highly regulated gene programs that are dependent on the NCX reverse mode activity. These include the mentioned cholesterol pathway that shows even stronger activation after NCX inhibition. Pathways involved in the regulation of cell cycle are largely dependent on NCX in stretch and are inactivated. As mentioned adult cardiomyocytes are terminally differentiated and unable to proliferate unless a condition such as stretch is present. Furthermore, cardiomyocyte terminal differentiation and proliferation arrest is associated with downregulation of versican (424). As described stretch significantly activates VCAN while NCX inhibition massively down-regulates it. NCX also restores the stretch downregulated levels of PPARG. Interestingly stretch induced SIRT2 activation is even augmented via NCX inhibition. Based on these observations we can conclude that NCX is an important contributor in maintaining normal cardiac function by maintaining cell proliferation and differentiation under control. Biological functions highly dependent on stretch and NCX include cell death, morphology, movement, contractile apparatus properties and energy substrate shift.

Tachycardia manifested exclusively NCX independent pathways. However, NCX inhibition under tachycardia conditions shows the following: increased lipid metabolic activities, decreased cell death and as opposed to stretch increase in cell motility, suggesting a trigger independent program. In addition, several upstream gene regulators such as p38 MAPK were predicted to be activated. P38 MAPK inhibition plays a role as well in enabling cell proliferation (425). Therefore, NCX is important in controlling proliferation trigger dependently as seen in stretch but also trigger independently. Also FBXO32 affected gene program is inhibited which implies that NCX reverse mode may be implicated in muscle atrophy since this enzyme is considerably upregulated under the mentioned condition (426) a tendency that is also observed in stretched cells. Common traits of NCX mediated effects irrespective of the trigger suggest inflammation suppression. As seen with the IPA upstream analysis upon NCX inhibition various cytokines such as TNF, IL1B and IFNG are activated.

We can **conclude** that we successfully addressed key features of early excitation transcription coupling. Using pro-hypertrophic marker genes we were able to describe changes in gene expression and compare their abundance and overlap in all cell types. Next, we determined that NCX reverse mode activity is responsible for changes in ion handling followed by largely independent transcription alterations. Furthermore, stretch contributes

to an increase in NCX protein expression that further initiates the reverse mode activity and gene expression. On the other hand, activation of NCX alone is sufficient to promote CaMKII phosphorylation and produce expression changes during tachycardia. Stretch application for 72 h and tachycardia for 48 h are sufficient to induce morphological hypertrophy of not only cardiomyocytes but also fibroblasts, which we identified as the prevailing non-myocyte cell type in NRVCM. This study provided new insights in characterising excitation transcription coupling in its early stages which is a step forward in dealing with reversible cardiovascular conditions such as HFpEF.

5.2 Remodelling in human atrial myocardium

Atrial remodelling in human hearts can be caused by a variety of insults linked to either stretch or tachycardia as a triggering mechanism. The comparison of the acute remodelling effects of these triggers has not been yet characterized. In line with the aims of the study we characterized a largely independent gene regulation when applying either isometric stretch or sustained tachycardia of 2.5 Hz in isolated atrial human trabeculae for 6 hours. Only a small fraction of genes was regulated in common between stretch and tachycardia but contained the strongest regulated gene *MIR1183*. Finally, we confirmed the presence of chronic *MIR1183* expression at different stages of cardiac disease progression, in AFib and DCM, respectively.

5.2.1 To aim 1: Microarray gene expression uncovers independent stretch and tachycardia gene regulation

The microarray transcripts are classified in four major groups as protein coding, non-coding and transcripts with a predicted function which is either protein coding or non-coding. Additionally, the array provides information on sequences that are mapped within the genome, however have not an assigned function. As indicated by the total number of regulated genes tachycardia stimulation had an overall of 40% higher impact on early gene expression than stretch did. Stretch as pressure overload *in vivo* leads to atrial dilation and therefore indirectly contributes to atrial fibrillation while tachycardia directly influences

electrical remodelling. Therefore, it is probable that stretch-dependent gene regulation requires a more chronic stimulus in comparison to tachycardia, which would explain the discrepancy in the number and assignment of regulated genes. 56% of stretch regulated transcripts represent protein-coding genes while 64% are found to be regulated upon tachycardia, with a very limited presence (2% and 3% respectively) of predicted sequences. The abundances of non-coding genes, the majority of which are classified as either pseudogenes or small RNAs is distributed to a similar extent in stretch (18%) and tachycardia (22%). The reminder of the sequences within each group are unclassified (unassigned function).

Trigger-specific actions include stretch mediated upregulation of *ACTA1, CNN1, TPI1* and tachycardia mediated upregulation of *SNRPN* and *RCAN1.* The functional role of *ACTA1*, *RCAN1* and *CNN1* was discussed in part I of this thesis and these genes therefore show a degree of translational potential from NRVCM to human. Interestingly, the trend towards differential expression between *ACTA1* and *RCAN1* exists in the human dataset although the results lack qPCR reproducibility. On the other hand *CNN1* is up-regulated in human atrial muscle strips with a significant p value in stretch (as in the NRVCM array) and with borderline significance ($p = 0.07$) in tachycardia.

The gene *TP1* is stretch regulated and only in human. The gene encodes the triose-phosphate isomerase enzyme - a participant in processes of glycolysis and gluconeogenesis. Clinical manifestations following TPI deficiency include skeletal muscle myopathy due to impaired mitochondria function (427). It is also implied that oxidative modification of TPI can affect metabolic regulation in type 2 diabetes hearts (428).

Tachycardia regulated *SNRPN* is a human gene that encodes the small nuclear ribonucleoprotein polypeptide N. Although the gene function was primarily described in developmental medicine (429, 430), it also shows relevance in the cardiovascular field. Single nucleotide polymorphism of the introne region within *SNRPN* is found in COPD patients and participants of the Framingham study (431).

Although the IPA was not able to predict any highly relevant signalling pathways which are tied to either stretch or tachycardia gene regulation it is interesting to mention that tachycardia seems to lead to downregulation of a number of genes involved in the process of inflammation such as *IL1A, IL1B, VCAM1, KIR3DL3* and *CCL4*. As determined by the GO molecular function interleukin 1 alpha and beta are one of the major pro-inflammatory

cytokine family. *KIR3DL3* is involved in antigen processing, *VCAM1* is an adhesion molecule whose expression is additionally modulated by *IL1B* (432) and *CCL4* is a chemokine for a number of inflammatory cells.

Following the observation that stretch and tachycardia show a distinctive pattern of gene regulation, we next concentrated on finding individually expressed genes that would link stretch and tachycardia triggering events in both models. Stretch and tachycardia do share a small in common regulated gene fraction with genes regulated in a predominantly unidirectional manner and it is among these genes that we identified a non-coding *MIR1183* precursor gene which stands out as the top upregulated array transcript with a predefined function.

5.2.2 To aim 2: *MIR1183* as a novel tissue marker in right atrial remodelling

MIR1183 displayed the highest expression fold change among defined sequences in both stretch and tachycardia and was the top regulated miRNA precursor gene in stretch as well as in tachycardia. Therefore, due to this increased and uniform expression, *MIR1183* stands out as an attractive biomarker candidate from the common gene fraction: This miRNA is relatively new in terms of its association with cardiovascular disease which warrants caution when interpreting its clinical relevance but also presents an opportunity to identify a novel role of this potential biomarker. The relevance of increased *MIR1183* expression levels in cardiovascular disease has been previously established in a cohort of patients suffering from rheumatic heart disease (433). The authors observed high levels of plasma *MIR1183* and a positive trend regarding tissue expression. Additionally, they also emphasized its potential biomarker role and speculated as well on possible implications during hypertrophy. Another study found that *MIR1183* is among the top up-regulated miRNAs in plasma of patients diagnosed with essential hypertension (434). However, direct assessment of *MIR1183* levels in the human myocardium have not been reported yet nor was the behaviour upon an acute insult such as in our settings. Additionally, *MIR1183* has no known orthologues in lower species, which might provide explanation behind the lack of previous studies. Microarray gene chip can detect a gene encoding the precursor of the mature form of *MIR1183* and so we aimed to verify this finding via qPCR and found a strong correlation with the chip findings.

Our further aim was to distinguish between acute and chronic effects of stretch and tachycardia on *MIR1183* levels. For this purpose, we used atrial tissue samples from patients undergoing mitral valve replacement surgery with normal ventricular heart rate and persistent atrial fibrillation which reflects both, atrial increased wall stress (i.e. cellular stretch) and atrial tachycardia on long term. We observed a significantly increased *MIR1183* in the right atrial appendage, which coincides with a previous study that found some other differentially expressed miRNAs in left and right tissue samples of atrial fibrillation patients (435).

5.2.3 To aim 3: *MIR1183* in atrial versus ventricular remodelling

We were also interested in seeing if the chronic changes in atrial myocardium are similar in a diseased ventricle and compared ventricular human samples at different stages of heart disease to each other. The groups consisted of healthy – non-failing hearts (NF), hearts displaying signs of diastolic dysfunction (dD) measured via echocardiography and hearts with present dilated cardiomyopathy (DCM). A trend towards *MIR1183* upregulation was observed in dD hearts while a prominent expression change was seen in the DCM group. Stretch and tachycardia normally lead to progression towards DCM, therefore we can say that pri-*MIR1183* is detectable in the early phases or during acute triggering events and it stays present in failing myocardium. This finding confirms its value as a biomarker in the early onset of the disease and its role in the pathological signaling that results in end-stage heart failure.

5.2.4 To aim 4: Predicted *MIR1183* mechanism of action and future perspectives

We performed a TargetScan search for all potential *MIR1183* targets and looked up those with suppressed gene expression on the array. We found two of those targets in the common fraction of stretch and tachycardia regulated genes: *PLA2G7* and *ADAM20*.

PLA2G7 encodes for the protein LP-phospholipase 2 (LPA2) which is found to be commonly down-regulated in foam cells (436). LPA2 is known as a biomarker and is mediating cardiac effects through the inflammatory response (437). It has been revealed that

the LPA2 levels significantly decrease following myocardial infarction and stroke (438). As other genes encoding inflammation mediators are downregulated as well, we can speculate that cardiomyocytes display a coping mechanism mediated through the LXR/RXR nuclear pathway down-stream as indicated by IPA.

ADAM20 is a member of the disintegrin and matrix metalloprotease domain family which are involved in processes of adhesion and proteolysis and are important for cell to cell and cell to matrix communication (439). So far, *ADAM20* was found to have a role in managing cell fusion during reproduction (440) but a possible relationship with cardiovascular disease is not defined.

It would be of great interest to evaluate the potential of *MIR1183* as a circulatory biomarker specifically when we have shown the presence of increased tissue expression during chronic atrial fibrillation. A patient cohort with such clinical manifestations would highly benefit from establishing additional diagnostic and prognostic parameters. In addition, it would be interesting to inspect a possible correlation between other established biomarkers such as NT-proBNP and *MIR1183*. What remains to be investigated as well is the exact molecular mechanism of action by which pressure overload and cardiac arrhythmias simulate *MIR1183* overexpression followed by detailed characterisation of *MIR1183* targeted effects. Upon stimulation with the brain-derived neurotrophic factor, human endothelial progenitor cells show increased expression levels of several miRNA among which is *MIR1183* (441) which raises the question of heterogeneous expression among different cell types. Specific cell types accountable for *MIR183* secretion and synthesis are therefore in domain of future investigations. A limitation in gaining mechanistic insight is the inability to investigate MIR1183 *in vivo* due to human oriented expression.

Conclusion: in the scope of this thesis, I managed to gain insight into stretch and tachycardia mediated early remodelling by gene expressions changes on genome wide level, identified a *MIR1183* precursor gene with the highest upregulation shared by both triggers, verified its upregulation status in acute and chronic remodelling, and end stage heart failure. Thereby we helped setting ground for future investigation of its role as a biomarker.

6 Bibliography

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