LONG NON-CODING RNAS AND GATA4-FOG2 INTERACTION CARDIOMYOCYTE HYPERTROPHY

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Tiivistelmä/Referat – Abstract

Left ventricular hypertrophy (LVH) takes place when cardiomyocytes respond to excessive stress by growing in size. Cardiomyocytes have a very marginal capability to proliferate, which is why hypertrophic growth is almost their only option to meet the requirements of increased workload. In the long run, however, LVH leads to further problems, such as cardiac failure and an increased risk of myocardial infarction. Hypertension is the most prevalent cause of LVH, and its current treatment relies on antihypertensive drugs. They decrease the workload of the heart and therefore alleviate symptoms but have very little effect on the built damage and remodeling. Understanding the details of cellular level signaling pathways and genetic expression in LVH is crucial for future drug development. Regulation of gene expression is a very complex process, which involves more than just DNA being translated into a protein. In this project, two types of factors participating in this regulation were in focus: long non-coding RNAs (lncRNA) and transcription factors GATA4 and FOG2.

LncRNAs are RNA sequences of more than 200 nucleotides that do not code for any protein final products themselves but are involved in chromatin remodeling as well as transcriptional and post-transcriptional gene regulation. They are highly organ-selective, which makes them potential targets for drug development. Our group has previously found a selection of cardiomyocyte-selective lncRNAs, which share a similar expression pattern in neonatal mouse hearts. In this project, three of them were silenced in a primary cardiomyocyte culture while simultaneously hormonally inducing hypertrophy. The goal was to see whether these lncRNAs have an effect on the hypertrophic response and apoptosis in the cardiomyocytes.

Transcription factors are proteins with partially similar activities to lncRNAs; they regulate, which genes are expressed under certain circumstances. GATA4 is an important transcription factor in the heart as it targets various developmental and functional genes in cardiomyocytes. FOG2 is a cofactor of GATA4; interaction between them regulates the activity of GATA4. Our group has recently developed a selection of compounds that affect protein-protein interaction between GATA4 and NKX2-5, another important transcription factor. The second part of the project was to set up and optimize a compound screening assay for GATA4-FOG2 interaction.

The results showed no change in hypertrophic response when the lncRNAs were silenced. Other experimental designs could still reveal if they have effects that could not be seen with these protocols. The silencing had no effect on apoptosis. As for the GATA4-FOG2 interaction experiments, transfecting COS-1 with GATA4 and FOG2 plasmids in a ratio of 10:1 resulted in a signal suitable for compound screening. Initial compound screening results indicated the compounds may have an effect on GATA4-FOG2 interaction, but further studies are needed before drawing conclusions.

Avainsanat – Nyckelord – Keywords

left ventricular hypertrophy, cardiomyocyte, long non-coding RNA, GATA4, FOG2

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Tiivistelmä/Referat – Abstract

Sydämen vasemman kammion hypertrofia (engl. left ventricular hypertrophy, LVH) on seurausta sydänlihassolujen, eli kardiomyosyyttien koon kasvamisesta vastineena kohonneeseen työtaakkaan. Kardiomyosyyteillä on häviävän pieni jakautumiskyky, ja siksi hypertrofinen kasvu on käytännössä niiden ainoa tapa vastata suuremman työtaakan vaatimuksiin. Pitkällä tähtäimellä LVH kuitenkin aiheuttaa suurempia ongelmia, kuten sydämen vajaatoimintaa ja kohonnutta sydäninfarktin riskiä. Kohonnut verenpaine on yleisin LVH:n aiheuttaja, minkä vuoksi lääkehoito nojautuukin pitkälti verenpainelääkkeisiin. Niillä voidaan vähentää sydämen työtaakkaa ja siten lievittää oireita, mutta ne eivät korjaa syntyneitä vaurioita. Solutason signalointijärjestelmien ymmärtäminen on olennaista tulevaisuuden lääkekehityksessä LVH:ta vastaan. Geenien ilmentymisen säätely on monimutkainen prosessi, johon kuuluu muutakin kuin pelkästään DNA:n translaatio proteiiniksi. Tässä erikoistyössä keskityttiin kahteen tähän ilmiöön vaikuttavaan tekijään: pitkät ei-koodaavat RNAt (engl. long non-coding RNA, lncRNA) sekä transkriptiotekijät GATA4 ja FOG2.

LncRNA:t ovat yli 200 nukleotidiä pitkiä RNA-sekvenssejä, jotka eivät itsessään koodaa mitään tiettyä proteiinia, vaan hienosäätävät muiden geenien ilmentymistä ja toimintaa. Tiettyä lncRNA:ta esiintyy pääasiassa vain tietyssä elimessä tai kudoksessa, minkä vuoksi ne ovat kiinnostavia lääkekehityksen kannalta. Tutkimusryhmämme on aiemmin tunnistanut joukon kardiomyosyyttispesifisiä lncRNA:ita, joiden ilmentyminen vastasyntyneillä hiirillä noudattaa samanlaista kaavaa syntymää seuraavina päivinä ja viikkoina. Tässä erikoistyössä niistä valikoitiin kolme, joiden ilmentymistä hiljennettiin vastasyntyneiden hiirten kardiomyosyyteissä samanaikaisesti altistamalla niitä hypertrofiselle stimulaatiolle. Tavoitteena oli tutkia, onko näillä lncRNA:illa vaikutusta apoptoosiin ja hypertrofiseen vasteeseen kardiomyosyyteissä.

Transkriptiotekijät ovat proteiineja, joiden toiminnot ovat osin samanlaisia kuin lncRNA:iden: ne säätelevät kohde-elimensä geenien ilmentymistä eri olosuhteissa. GATA4 on tärkeä transkriptiotekijä sydämessä, sillä sen kohteisiin lukeutuu monia kardiomyosyyttien kehitykseen ja toiminnan ylläpitämiseen liittyviä geenejä. FOG2 on GATA4:n kofaktori; niiden yhteisvaikutus säätelee GATA4:n aktiivisuutta. Tutkimusryhmämme on aiemmin kehittänyt kymmeniä GATA4:n ja NKX2-5:n, toisen sydämessä keskeisen transkriptiotekijän, väliseen interaktioon vaikuttavia yhdisteitä. Erikoistyön toisessa osassa pyrittiin asettamaan ja optimoimaan koeasetelma yhdisteiden vaikutuksien GATA4:n ja FOG2:n vuorovaikutukseen seulomiseksi.

Tuloksien perusteella hypertrofinen vaste ei muuttunut lncRNAita hiljennettäessä. On kuitenkin mahdollista, että kaikkia vaikutuksia ei voinut havaita käytetyillä menetelmillä, minkä vuoksi muiden koeasetelmien kokeileminen voisi tuottaa lisätietoa aiheesta. Tutkittavilla lncRNA:illa ei ollut vaikutusta apoptoosiin. GATA4-FOG2-interaktiokokeiden perusteella transfektoimalla COS-1 soluja suhteessa 10:1 GATA4- ja FOG2-plasmideilla saadaan yhdisteiden testaamiseen sopiva signaali. Alustavien tulosten perusteella yhdisteillä saattaa olla vaikutusta GATA4-FOG2-interaktioon, mutta ennen johtopäätösten tekemistä on tehtävä lisää tutkimuksia.

Avainsanat – Nyckelord – Keywords
vasemman kammion hypertrofia, sydänlihassolu, pitkä ei-koodaava RNA, GATA4, FOG2

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ABBREVIATIONS

ACE	angiotensin convertase enzyme
ANP	A-type natriuretic peptide
ATI	angiotensin I
ATII	angiotensin II
ATR	angiotensin (II) receptor
BNP	B-type natriuretic peptide
BSA	bovine serum albumin
Bvht	braveheart
Chaer	cardiac-hypertrophy-associated epigenetic regulator
Chast	cardiac hypertrophy associated transcript
CHRF	cardiac hypertrophy related factor
CPR	cardiomyocyte proliferation regulator
CSFM	complete serum free medium
CVD	cardiovascular disease
DAPI	2-(4-amidinophenyl)-1H -indole-6-carboxamidine
DCM	dilatative cardiomyopathy
DMEM	Dulbecco’s modified Eagle medium
DMEM/F-12	Dulbecco's modified Eagle medium/Ham's F12 nutrient mixture
DMSO	dimethyl sulfoxide
DOX	doxorubicin
E	embryonic day

ECG	electrocardiography
Echo	echocardiography
ESC	embryonic stem cell
ET-1	endothelin-1
FBS	fetal bovine serum
IEG	immediate early response gene
iCLM	induced cardiac- like myocyte
iPSC	induced pluripotent stem cell
lncRNA	long non-coding RNA
lncRNP	lncRNA-associated ribonucleoprotein complex
LVH	left ventricular hypertrophy
MEF2C	myocyte specific enhancer factor 2C
mRNA	messenger RNA
MI	myocardial infarction
miRNA	micro RNA
MHC	myosin heavy chain
Mhrt	myosin heavy-chain-associated RNA transcript
MyBP-C	myosin-binding protein C
MYH	myosin heavy chain
NaP	sodium pyruvate
ncRNA	non-coding RNA
nls	nuclear localization sequence
P	postnatal day

PenStrep	penicillin-streptomycin
PBS	phosphate-buffered saline
PFA	paraformaldehyde
rBNP	rat BNP
RAS	renin-Angiotensin System
siRNA	small interfering RNA
Sirt1	silent information regulator factor 2 related enzyme 1
SNP	single nucleotide polymorphism
T_3	triiodothyronine
TAC	transverse aortic constriction
TAD	topologically associating domains
TE	tromethamine - ethylenediaminetetraacetic acid
tRNA	transfer RNA
UCR	ultra-conserved region

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1 INTRODUCTION

When the heart, like any organ or system, faces excessive stress, it has to adapt to the new circumstances. However, due to their lack of proliferation capability cardiomyocytes start growing in response to increased stress (Grossman et al. 1975). This phenomenon is called hypertrophy, and in pathological cases usually more accurately left ventricular hypertrophy (LVH) based on its location (Campbell et al. 1991). LVH is both the cause and result of other cardiovascular diseases. Hypertension is the most common background for it, and advanced LVH increases the risk for cardiac failure and myocardial infarction among others (Devereux et al. 1987; Levy et al. 1988).

Current treatment of LVH and cardiac failure is based on antihypertensive drugs (Hypertension, Käypä hoito recommendations 2014; Cardiac failure, Käypä hoito recommendations 2018). This is logical considering that hypertension is a significant risk factor for LVH. However, antihypertensive drugs do not repair the damage; they only alleviate symptoms and slow down the progression of the condition. A recent key question among researchers have been why the cardiomyocytes are only able to grow but not proliferate.

Understanding the cellular and molecular mechanisms behind both hypertrophy and proliferation are crucial for finding new, innovative treatment options. Long non-coding RNAs (lncRNAs) present an example of a new target of interest. All non-coding RNAS (ncRNAs) are RNA sequences that do not directly code for any protein but fine-tune transcriptional, post-transcriptional and epigenetic regulation of other genes (Kapranov et al. 2007). Because of their high organ-selectivity they present significant potential for medical research (Ramos et al. 2013). Transcription factors, which also adjust gene transcription and have very organ-selective functions, are another important target (Estella et al. 2012). They are special proteins that bind to DNA promoter regions, enabling them to enhance or inhibit the expression of certain genes.

In the first experimental part of this project, neonatal mouse cardiomyocytes were transfected with LNA GapmeRs that silence lncRNAs, which out group has previously

found to have similar expression patterns during the first postnatal days and weeks in neonatal mice. The cardiomyocytes were exposed to endothelin-1 (ET-1) to stimulate hypertrophy, and their response to the stress was studied. In the second part, a compound screening assay for protein-protein interaction between GATA4 and FOG2 transcription factors was set up and optimized. Then, the assay was tested with five compounds, which our group has earlier shown to alter the interaction of GATA4 with one of its other cofactors, NKX2-5, to see if they also affect GATA4-FOG2 interaction.

2 LITERATURE REVIEW

2.1 Left ventricular hypertrophy

The human heart is normally about the size of a fist. In cardiac hypertrophy the walls of the ventricles have thickened, which with the exception of certain genetic diseases signals that the heart is trying to function with excessive hemodynamic stress, with the exception of certain genetic diseases such as hypertrophic cardiomyopathy (Grossman et al. 1975). The systemic circulation requires a continuous supply of blood, but the heart falls behind from the demands. However, the heart does not have many ways to compensate. Cardiomyocytes, which are the contracting unique muscle cells in the heart, have a nearly nonexistent regeneration capability, but they can grow in size. In practice, hypertrophy is almost synonymic to left ventricular hypertrophy (LVH), because the highest number of cardiomyocytes is localized in the walls of the left ventricle (Campbell et al. 1991). This is logical considering that the left ventricle is responsible for the systemic circulation (Guyton and Hall 2006). Right ventricular hypertrophy exists, too, but it is usually a cause of lung-related conditions, such as pulmonary hypertension (Parikka 2016). The idea of this compensatory mechanism is understandable; thicker ventricular walls can push a bigger volume of blood to the aorta. This strategy, however, quickly reaches its limits. Simultaneously, fibrosis and death of millions of cardiomyocytes take place, and hypertrophy cannot compensate for all of that (Kajstura et al. 1996).

Diagnostics rely on electrocardiography (ECG) and clinical examination of the patient (Hypertension, Käypä hoito recommendations 2014). ECG measures the electric

impulses in the heart, therefore making it possible to draw a curve of the contractions and detect abnormalities. Echocardiography (Echo), which uses ultrasound to gain information of the structure of the heart, is an option in unclear or otherwise difficult cases (Devereux et al. 1986). The results from Echo are more reliable than the ones obtained from ECG but also the usage is much more expensive. The reliability of ECG can be increased by using certain validated criteria, known as Cornell and Sokolow criteria (Okin et al. 2000). Medical research usually opts for Echo because of the higher number of quantities that can be measured: thickness and mass of the walls and volume of the ventricles among others (de Simone et al. 2008). Among hypertensive patients ECG finds approximately 5 – 10% to have LVH (Kaplan 1998). The Framingham Heart study, which covered nearly 5 000 patients, published even smaller numbers, 3%, while Echo detected 16 – 19% LVH cases (Kannel et al. 1969; Levy et al. 1988). Eventually, no matter how precise the method being used to diagnose the patient, the line between a normal and enlarged mass of the heart is not sharp. The results must be individually set in proportion to height, weight and gender among other factors before drawing conclusions.

2.1.1 Background and outcomes of LVH

Arterial hypertension is the most common condition in the background of LVH, increasing the risk up to 5-fold (Devereux et al. 1987; Kumpusalo et al. 2001). Hypertension, however, is treatable, whereas the effects of LVH cannot be entirely cured. Additionally, not only does hypertension risk developing LVH, as also the other way around LVH can cause hypertrophy. Ageing, gender, myocardial infarction (MI), obesity and valvular heart diseases increase the risk, too (Levy et al. 1988; Jula and Karanko 1994; de Simone et al. 1994; Benjamin and Levy 1999). de Simone et al. (1994) found that the significances of these factors may vary between genders, as men were more likely to develop LVH with hypertension and women with excess weight. A follow-up study by Hubert et al. (1983) suggested obesity is a significant risk for LVH and other cardiovascular diseases (CVD) even with no other common risk factors. In addition to the previous common risk factors, additional examples such as smoking, diabetes, insulin

resistance, alcohol consumption, urine sodium levels and lack of physical activity have been linked with LVH (Benjamin and Levy 1999).

Both systolic and diastolic functionality can be significantly reduced because of LVH. This means that the heart is unable to perform its most fundamental task: pumping blood. When this inability develops further it is called cardiac failure; a condition that affects about one to two percent of the population worldwide (Mosterd and Hoes 2007). Shortness of breath and fatigue especially during physical activity are the most common symptoms and are caused by blood accumulating in pulmonary circulation as the heart does not have the power to push the incoming blood forward (Lommi 2018). Liquid starts to accumulate in the body and particularly legs get easily swollen. Patients with difficult cases of cardiac failure often find their symptoms worse when lying down because it allows blood in the veins of the legs re-enter bloodstream. In cardiac failure the heart may not be able to keep up with the re-entering blood, which again starts to accumulate in the pulmonary circulation. The swelling can also be seen on the scale and patients can experience rapid weight gain.

Cardiac failure is a common cause of death: in 2013 it was reported to be at least one factor in every 9th case of death in the United States (Mozaffarian et al. 2016). Additionally to its lethality, cardiac failure decreases the quality of life of the patients, leads to several hospital admissions and therefore consumes lots of money. In 2012 the treatment costs for cardiac failure were 700 million dollars in the United States only and are expected to rise in the future. Because of the strong correlation, a heart with LVH is considered an intermediate form between a healthy heart and cardiac failure. In addition to cardiac failure, LVH is a direct risk for MI, arrhythmias and sudden cardiac deaths (Levy et al. 1990).

In the case of MI, millions of cardiomyocytes are lost and the damage starts building immediately. This type of remodeling differentiates from hypertension-induced LVH. Cardiac healing following MI is a complex system, which begins with a triggered inflammatory phase, during which the necrotic myocardium is replaced with granulation tissue (Fraccarollo et al. 2012). Second, fibrogenesis results in scar tissue formation. The

scar is not able to contract like cardiomyocytes but it is a crucial part in MI recovery, as it prevents the left ventricle from rupturing (Brown et al. 2005; Dobaczewski et al. 2010). In contrast, fibrosis in areas remote from the infarct site results in significant increase in myocardial stiffness (Brown et al. 2005). Hypertrophy and many other structural changes take place during the following weeks and months. The phases of cardiac remodeling in case of MI is presented in Figure 1.

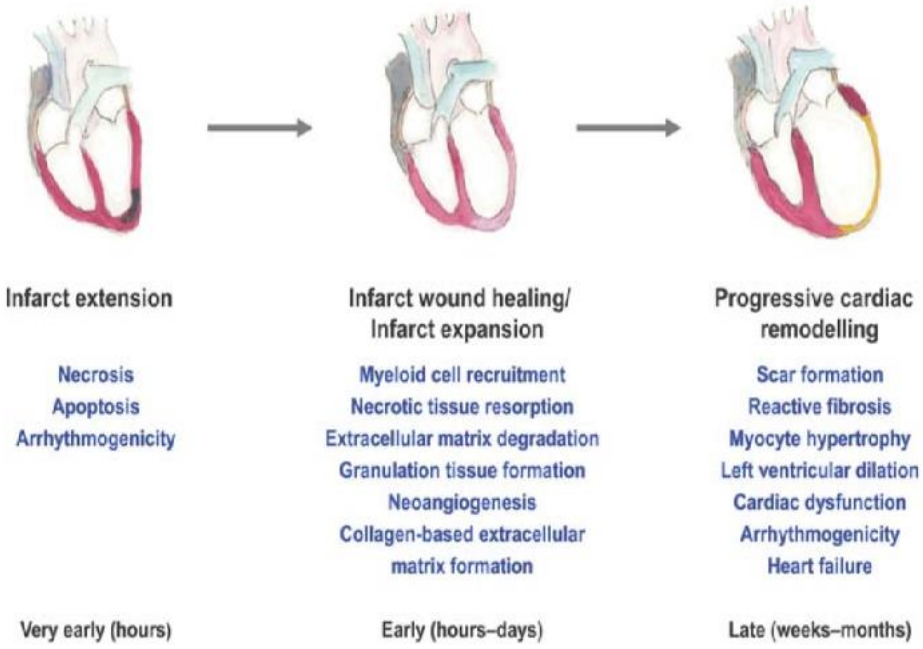

Figure 1: Phases of cardiac remodeling following a myocardial infarction (Fraccarollo et al. 2012).

2.1.2 Classification of hypertrophy

Hypertrophy is not necessarily bad - in fact, it is essential for normal growth, adaptation and maintenance. There are two ways to categorize hypertrophy: physiological and pathological hypertrophy, and concentric and eccentric hypertrophy (Weeks and McMullen 2011; van Berlo et al. 2013b) (Fig 2). Physiological hypertrophy is a healthy, normal phenomenon. A classic cause for this includes substantial physical activity, for example (Boström et al. 2010; Weeks and McMullen 2011). The volume of ventricles and the thickness of cardiomyocytes grow proportionally, which increases pumping

efficacy and reduces the pressure falling upon the walls. This kind of hypertrophy is benign and allows the heart to maintain its functionality – or even increase it as the volume of the ventricle is increased in relation to the thickness of the wall (Fig 2). Neonates and pregnant females experience the same phenomenon as their hearts face increased pressure and start to grow (Weeks and McMullen 2011).

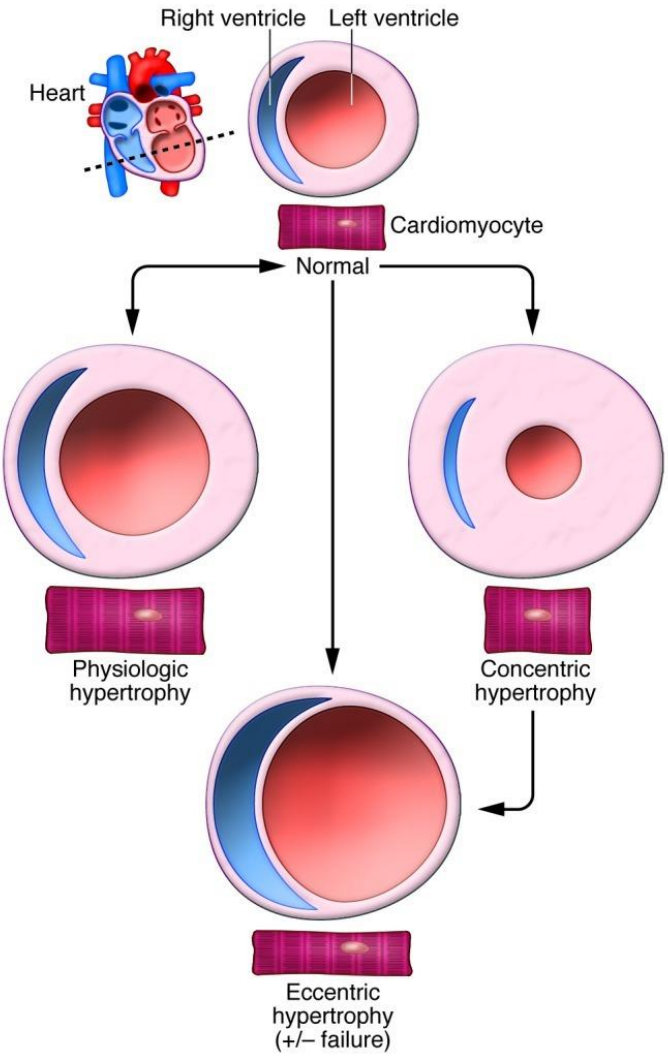

Figure 2: Different types of cardiac hypertrophy. In physiologic hypertrophy cardiomyocytes grow proportionally in length and width so that the ventricular volume is increased together with the thickness of the wall. In concentric remodeling cardiomyocytes grow significantly more in width compared with length. This results in thickening of the walls and a loss of chamber volume. In eccentric growth cardiomyocytes grow longer but not wider, leading to excessive chamber enlargement with thinner walls. (van Berlo et al. 2013b).

Chronic overload and stress lead to pathological changes (Grossman et al. 1975). There is a significant difference, though: the cardiomyocytes do not grow proportionally. If pathological hypertrophy is concentric, the volume of the left ventricle shrinks the thicker the walls grow, because the cardiomyocytes are mostly stretched across their diameter but not in length (Pearson et al. 1991) (Fig 2). Concentric pathological hypertrophy is usually a consequence of long-term, gradual increase in blood pressure, but it can also be seen in cases of aortic stenosis (Messerli et al. 1987; Frohlich et al. 1992). Simultaneous fibrosis and scar formation make the structure of the walls very stiff (Grossman et al. 1975). If the constant stress continues long enough, the compensatory mechanisms of the heart slowly start scraping its own functionality.

Another form of pathological growth is eccentric pathological hypertrophy. In this case, the cardiomyocytes grow more in length than across their diameter (Grossman et al. 1975; Ganau et al. 1992). The volume of the left ventricle is increased but the walls become thin in relation to the diameter of the ventricle (Fig 2). One fourth of LVH cases are eccentric hypertrophy (Ganau et al. 1992). This form of cardiomyocyte growth is also known as dilatative cardiomyopathy (DCM), and considering that cardiac remodeling it does not belong under the term of hypertrophy, as the walls do not actually thicken. Examples of conditions often linked with DCM include mitral and aortic valve leakage conditions, MI and cardiac failure (Grossman et al. 1975; Messerli and Aepfelbacher 1995). MI results in death of millions of cardiomyocytes and the heart must find a way to compensate the decreased workforce. Valvular diseases allow blood to flow backwards in the heart, which leads to pathological remodeling to compensate the loss in cardiac output. Despite the functional logic behind increasing the ventricular volume in such cases, the phenomenon significantly upsurges the end-diastolic volume. As the tension upon ventricular walls is higher in the case of eccentric LVH, scientists expected it to have mortality rates as well. However, it was shown to be the exact other way around, indicating that the thickening is even more damaging for the myocardium than ventricular dilation (Koren et al. 1991; Ghali et al. 1998; Akinboboye et al. 2004)

2.1.3 Cardiomyocyte regeneration

Cardiomyocytes make approximately one third of the heart, the contracting tissue myocardium (Nag 1980; Banerjee et al. 2007; Pinto et al. 2016). However, considering the volume of the heart, they occupy up to 90% (Anversa et al. 1980, Tang et al. 2009; Pinto et al. 2016). Other predominant cell types include fibroblasts, endothelial cells and smooth muscle cells. Cardiomyocytes can be further classified into four subtypes (Guyton and Hall 2006). One class of contracting cardiomyocytes is located in the ventricles while another one is in the atriums. The third subtype is pacemaker cells that give rise to electric stimuli and the fourth group conducts it forward.

Neonatal mammals are able to recover from cardiac damage as the cardiomyocytes at the site of injury proliferate and give rise to substitutive cells (Porrello et al. 2011). Their hearts respond in a different manner: hyperplasic growth. Neonatal mice have been proven to be capable of replenishing lost cardiomyocytes in case of an injury (Soonpaa et al. 1996; Porrello et al. 2011). A few years ago, Haubner et al. (2016) were the first to report that a newborn human had fully both structurally and functionally recovered from a major ischemic injury, providing stronger evidence of the regeneration capability than ever before. Because of this special characteristic of neonatal cardiomyocytes, neonatal rodents have a fundamental role in cardiac regeneration research. They are easy to modify genetically and outcomes can be seen relatively quickly (Zaruba and Field 2011). Another common model for cardiac regeneration is the zebrafish, which maintains its capability of cardiac regeneration throughout its lifespan (Poss et al. 2002).

Soon after birth, however, mammal cardiomyocytes lose the ability to proliferate. Only 0.5 – 2% of adult murine and human cardiomyocytes regenerate every year, and it has been estimated that a 75-year-old still has 50% the exact same cardiomyocytes he had when he was born (Parmacek and Epstein 2009; Bergmann et al. 2015). However, not all researchers entirely agree on these renewal rate numbers (Nadal-Ginard et al. 2003; Bergmann and Jovinge 2014). Reliably labeling proliferating cardiomyocytes to differentiate them from non-proliferating ones has been challenging, which plays a part in the varying results (Bergmann et al. 2009; Laflamme and Murry 2011).

Cardiomyocytes carry one or two nuclei, making the task to separate new cardiomyocytes from old ones even more challenging. Soonpaa et al. (1996) found out postnatal cardiomyocytes tend to replicate their DNA and nuclei, but not the cytosol. The cells become binuclear, after which going through a full mitotic cycle is even more unlikely (Li et al. 1996) (Fig 3).

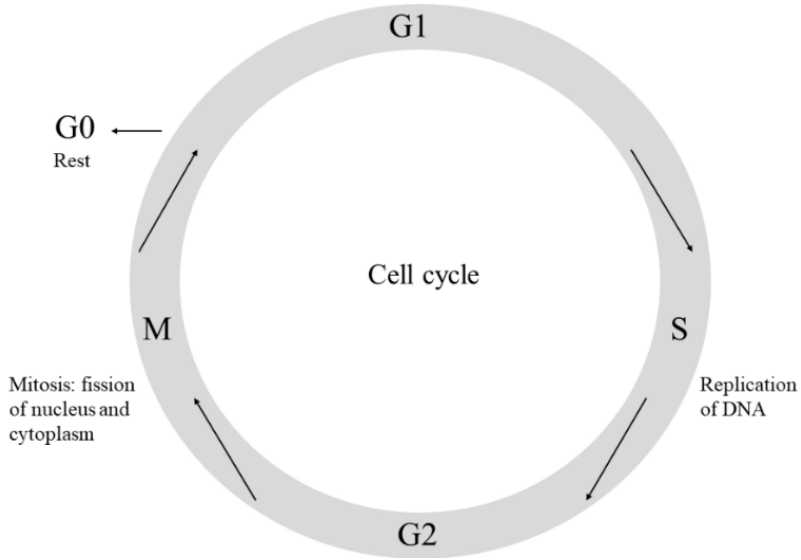

Figure 3: The cell cycle. During phases G1 and G2 the cell is focusing on maintaining homeostasis and carrying out its basic tasks, including cell respiration and protein synthesis, for example. DNA is duplicated in phase S. Cell division takes place in the mitotic M phase. If needed, the cell can also go to a passive resting phase G0, which significantly decreases its energy requirement.

In mice, the loss of regeneration capacity takes place quickly after birth; already at one-week-old it is practically nonexistent (Li et al. 1996; Porrello et al. 2011). Mice younger than that are able to recover from an artificial injury at myocardium nearly perfectly because of their cardiomyocytes' proliferation capacity (Soonpaa et al. 1996). Older mice without this capability undergo scar formation and get different stages of cardiac failure. This phenomenon has been studied by surgically removing tissue from the tip of myocardium (Porrello et al. 2011; Konfino et al. 2015) or blocking circulation from the coronary artery (Porrello et al. 2011; Haubner et al. 2012; Konfino et al. 2015). Rough estimations of the renewal time window in humans have been made, despite not everyone completely agrees on whether the rodent cardiomyocyte renewal window exists in large

animals or in humans in the first place (e.g. Porrello et al. 2011; Mollova et al. 2013, Lázár et al. 2017).

2.1.4 Left ventricular hypertrophy on cellular level

Inside a cardiomyocyte, there are elongated myofibrils organized side by side. The myofibrils are made of recurring sarcomeres, which for their part consist of thin actin- and thick myosin filaments. The earliest publications on the sarcomere structure and function track decades back in time and are still relevant (Huxley 1957). The sarcomere filaments move in an interlocked pattern, and this movement acts as a bow in the contractions (Sequeira et al. 2014). The sarcomeres are outlined by α actinin sheets that attach actin filaments to so called Z-discs. Actin filaments are thin, and they dominate the perimeters of a sarcomere unit. This is called the I-band. The thicker myosin filaments for there are concentrated in the middle of the sarcomere – the A-band. Myosin-binding protein C (MyBP-C) holds the myosin filaments in place. Additionally, six titin proteins cross the sarcomere along the surface of the thick filament and further to the Z-disc, where they interact with α-actinin. The sarcomere structure is presented in Figure 4. During hypertrophy the sarcomere structures lose their organized pattern, and cardiomyocytes change their genetic expression and increase protein synthesis.

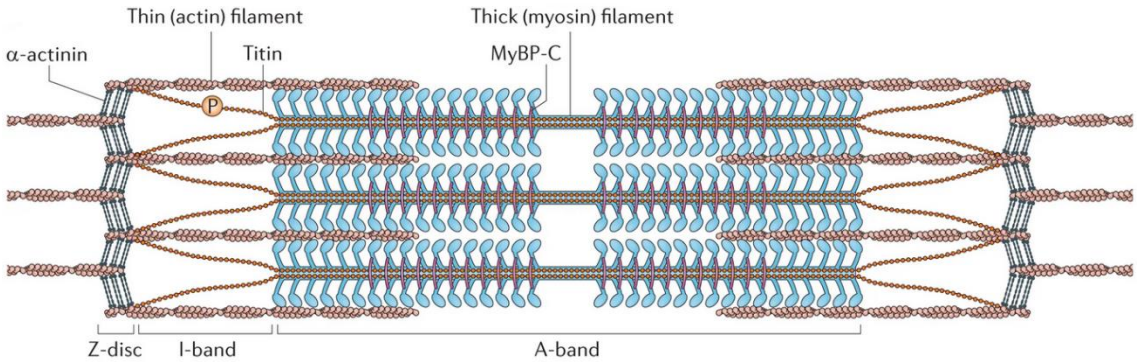

Figure 4: Structure of a sarcomere unit. Z-discs determine the outliers of the sarcomere and consist of α-actinin. Thin actin filaments are attached to the Z-discs and they form the I-bands of the unit. In the middle, thick myosin filaments form the inner A-band. In addition to actin and myosin, the sarcomere structure includes myosin-binding protein C (MyBP-C) that holds the myosin filaments together, and six titin proteins crossing through the entire structure along the myosin filaments (Hwang and Sykes 2015).

Immediate early response genes (IEG) are upregulated within 30 minutes from the beginning of hypertrophic stress (Izumo et al. 1988; Iwaki et al. 1990; Chien et al. 1991).. Particularly A- and B-type natriuretic peptides (ANP, BNP) (*Nppa*, *Nppb*), β-myosin heavy chain (β-MHC)(*Myh7*) and α-actinin (*Acta1*) are activated simultaneously with IEGs (Schwartz et al. 1986; Izumo et al. 1987; Izumo et al. 1988; Chien et al. 1991). Examples of IEGs include c-fos and c-myc protooncogenes as well as a major heat shock protein gene (hsp70). What is interesting is that BNP, β-MHC and α actinin are at their highest activity during embryogenesis, after which their expression decreases in relation to other cardiac genes (Hunter and Chien 1999; van Berlo et al. 2013b). BNP is prevalent in the ventricular walls throughout lifespan, but after birth ANP is usually expressed mostly in the atriums. However, during hypertrophic stimulus, both ANP and BNP are significantly increased in the ventricular walls. The reason why these factors are reactivated is relatively unclear to this day but likely the phenomenon has to do with cardiac protection, since ANP and BNP have been shown to carry anti-hypertrophic effects (e.g. Rosenkranz et al. 2003a, Rosenkranz et al. 2003a). According to some research groups, the induction of fetal genes itself could be harmful and a possible drug target (O'Donoghue and Braunwald 2010).

After IEGs have emerged, the changes take place in other sarcomere protein genes, too. This leads to growth and reorganization of the sarcomere structures (Lee et al. 1988; Long et al. 1989; Pratt and Dzau 2018). It is a natural phenomenon in both physiological and pathological hypertrophy and essential for increasing the contraction force. However, the mechanism of sarcomere growth is different between concentric and eccentric (Hunter and Chien 1999). In concentric pathological hypertrophy proteins are added side by side to the sarcomeres, causing the cell to widen out. In eccentric pathological hypertrophy the proteins are stacked on top of one another, which is why the cells are stretched longer. In addition, contractibility is negatively impacted as hypertrophic stress affects the expression of genes regulating intracellular calcium ions (Kent et al. 1993; Chung et al. 2011; Luo and Anderson 2013; Coppini et al. 2018).

2.2 Treatment of left ventricular hypertrophy and cardiac failure

Currently, there is no curing treatment available for cardiac failure despite about one to two percent of the population worldwide suffers from it (Mosterd and Hoes 2007). Medication aims at lowering the workload of the heart via renin angiotensin system, adrenergic β receptors or diuresis, for example (Cardiac failure, Käypä hoito recommendations 2018). Starting the right combination of medicine at the right time will significantly increase the patient's life expectancy. However, in severe cases and after the syndrome has continued and developed for many years, there is a shortage of options. The treatment of LVH is crucial for the prevention of complications from pathological remodeling (Ruilope and Schmieder 2008).

2.2.1 Medical treatment

Due to its strong association with hypertension, the treatment of LVH is based on blood pressure medication (Hypertension, Käypä hoito recommendations 2014). Patients benefit from these drugs even if they did not have an abnormally high blood pressure to begin with. The most common blood pressure medication classes include adrenergic β receptor blockers (β blockers), selective for type β_1 or both β_1 and β_2, Ca^{2+} receptor blockers, angiotensin convertase enzyme (ACE) blockers, angiotensin II receptor (ATR) blockers and diuretics. The adrenergic system increases heart rate via β_1 receptors, among other effects, which increases the workload of the heart. Preventing stimulus via this track mediates the efficacy of β blockers. Calcium ion influx intermediates Ca^{2+} electric current inside cardiomyocytes and vascular smooth muscle cells, which affects heart rate and the diameter of blood vessels. Diuretics regulate different ion efflux and influx transporters so that more fluid is excreted from the kidneys. A smaller blood volume puts less load on the heart and decreases blood pressure and also helps cardiac failure patients with swelling.

Angiotensin I (ATI) is formed from renin, which is excreted from the kidneys. Angiotensinogen enzyme from the liver converts renin into ATI. ATI is then further converted into angiotensin II (ATII) by ACE. ATII is highly vasoconstrictive when

binding to ATRs. A decrease in the diameter of blood vessels naturally decreases the volume in which all blood is supposed to fit, which then upsurges blood pressure. Additionally, ATII also increases aldosterone secretion from adrenal glands. Aldosterone enhances Na^+, Cl^- and H_2O reabsorption and K^+ secretion, which results in a higher blood volume and further increases blood pressure. ATII also raises sympathetic activity, which stimulates β receptors, and indirectly increases blood volume via the kidneys. This system is known as the renin-angiotensin system (RAS) (Fig 5) and it mediates the efficacy of ACE and ATR blockers. RAS has been shown to be highly activated in hypertrophy (Baker et al. 1992; Sadoshima et al. 1993). Research has revealed that ACE and ATR blockers prevent hypertrophy, supposedly because cardiomyocytes release ATII upon stretching, and this ATII activates internal RAS signaling in the heart (Pfeffer et al. 1982; Baker et al. 1990; Baker et al. 1992; Sadoshima et al. 1993). ATII has also been found to induce hypertrophy independently. Transgenic mice that overexpress angiotensinogen only in the heart developed both left and right ventricular hypertrophy over the course of their development and full-grown mice had approximately 20% larger ventricular walls than wild-type controls (Mazzolai et al. 1998; Mazzolai et al. 2000) These findings further emphasize the role of RAS affecting drugs in LVH.

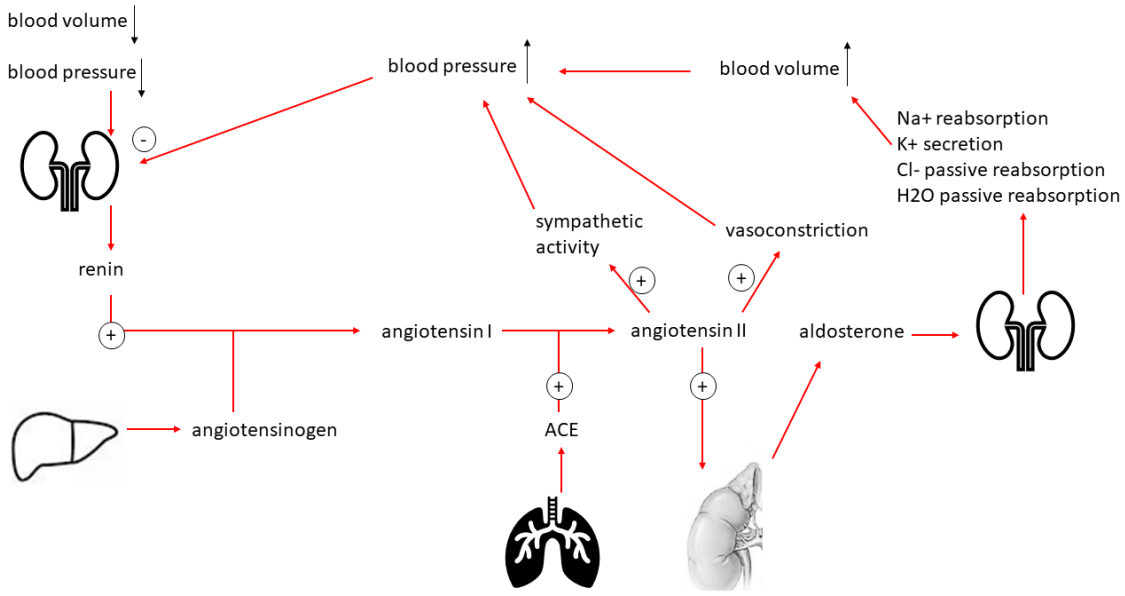

Figure 5: The renin-angiotensin system (RAS). ACE = angiotensin convertase enzyme, Na^+ = sodium ion, K^+ = potassium ion, Cl^- = chloride ion.

Gottdiener et al. (1997) conducted a double-blinded randomized trial with six common antihypertensives administered to 1 105 male patients who all had particularly high diastolic pressure levels. According to their findings, captopril, hydrochloride thiazide and atenolol significantly reduced the mass of the left ventricular wall. Diltiazem, clonidine and prazosin were equally effective in hypertension but had no effect on the ventricular walls. At the same time, Liebson et al. (1995) did a similar setting with five antihypertensive drugs (n = 844) and found that in the long run drug treatment did not have a better positive impact on the ventricular walls than non-medical treatment, such as lifestyle changes. To compare different classes of medication, Schmieder et al. (1998) carried out meta-analysis covering 1 175 patients on placebo (n = 165), diuretics (n = 304), β blockers (n = 367), calcium channel blockers (n = 441) and ACE blockers (n = 438). The results showed the overall duration of medication is important for the obtained results and decrease in systolic pressure significantly reduced the mass of ventricular walls. ACE and calcium channel blockers ranked higher than β blockers and diuretics although they all reduced blood pressure equally in both groups.

The Losartan Intervention For Endpoint reduction (LIFE) studies began in the 1990's as it became more evident that β blockers and diuretics did not benefit patients with hypertension considering morbidity (Dahlöf et al. 1997). Although they reduced blood pressure, patient deaths did not decrease. Overall, the LIFE project covered several clinical studies and only a couple of examples are presented here. Devereux et al. (2004) examined 960 patients having either losartan (n = 457) or atenolol (n = 459) and found losartan reducing the mass in the left ventricular walls more than atenolol. In addition, atenolol increased the diameter of the left ventricle compared to losartan. Another publication from the LIFE study by Dahlöf et al. (2002) got similar results from an even larger group of patients. They double-blinded 9193 participants with hypertension and LVH to receive either losartan (n = 4605) or atenolol (n = 4588) and found that losartan reduced cardiac morbidity compared to atenolol even if blood pressure was reduced equally.

It is worth acknowledging that the classic antihypertensive drugs were developed a couple of decades ago, and their efficacy was studied back then as can be seen in the years of

these publications. However, they maintain their relevancy in treatment recommendations to this day (Hypertension, Käypä hoito recommendations 2014). More recent studies have, instead, examined the benefits of combining two or more antihypertensive drugs. Like the starting point of the LIFE project stated, final health outcomes must be taken into account besides the direct effects on the ventricular walls. Research shows that combining an angiotensin receptor (ATR) blocker, such as losartan, with a thiazide diuretic appears to reduce the risk of stoke more than a β blocker with a thiazide diuretic (Dahlöf et al. 2005; Ruilope and Schmieder 2008). The inferiority of β blockers among LVH patients has also been pointed out in monotherapy, for which ACE, ATR and Ca^{2+} blockers are more beneficial choices (Fagard et al. 2009). For cardiac failure patients diuretics are an essential drug to remove excessive body fluids that accumulate in the lower limbs and lungs as a result from insufficient heart function (Cardiac failure, Käypä hoito recommendations 2018). Depending on the severity of their illness they may also need medication for cardiac arrhythmias, such as fairly usual atrial fibrillation. The European guidelines for LVH medical treatment were summarized from by Mancia et al. (2013) who gathered over 700 studies about hypertension medication under a variability of other conditions that may have an impact on the outcome, such as LHV. Like the examples mentioned here, their final outcome was that the primary medication should be an ACE, ATII or Ca^{2+} blocker, a diuretic or an aldosterone antagonist.

2.2.2 Non-medical treatment and prevention

The first steps that should be taken to decrease the workload of the heart lie within lifestyle choices. Excessive sodium intake, substantial alcohol consumption, unhealthy eating with little vegetables and plenty of saturated fats and lack of physical activity are well established risk factors of hypertension (Marmot et al. 1994; Benjamin and Levy 1999; Appel et al. 1997; Sacks et al. 2001). The exact same factors are also known to enhance LVH of themselves, but as LVH and hypertension are strongly linked together, aiming at a lower blood pressure should be one of the main focuses (Devereux et al. 1987; Levy et al. 1988; Hypertension, Käypä hoito recommendations 2014). Focusing solely on LVH, medical and non-medical treatment are of same efficacy (Liebson et al. 1995).

The average salt intake in Finland is approximately ten-fold to the recommendations (Paturi et al. 2008; Puska et al. 2009; Helldán et al. 2013). Excessive sodium significantly increases the risk of cardiovascular end points and deaths (Alderman et al. 1998; He et al. 1999; Tuomilehto et al. 2001; Strazzullo et al. 2009). In a study by He et al. (2004) cutting salt intake down by six grams a day significantly reduced blood pressure in just four weeks. Reducing salt also improves the efficacy of many antihypertensive drugs, such as ACE, ATR and β blockers and diuretics (Parijs et al. 1973; Erwteman et al. 1984; MacGregor et al. 1987; Singer et al. 1995; Houlihan et al. 2002). Not only is the indirect effect via blood pressure important in the prognosis, reducing salt intake can also directly decrease LVH. Jula and Karanko (1994) conducted a study in which patients (n = 76) were given a salt-restricted diet. Treatment group was also given advice by a dietician while control group did not. After 12 months the treatment group dropped an average of 5.4% of their left ventricle mass, but no change was detected in the control group. The decreased mass could not be explained with other factors, such as weight loss, which led the researchers to believe it was the sodium restriction that caused the difference between the groups.

Those who exercise substantially have on average lower blood pressure than those with very little physical activity (Huai et al. 2013). According to the U.S. Department of Health and Human Services (2008) 150 minutes of moderately straining endurance training is the most recommendable form of exercise for hypertension patients. It has been shown to reduce blood pressure approximately 8/5 mmHg (Cornelissen and Smart 2013). Among overweight patients losing weight only 4% on a sparse energy diet reduces blood pressure roughly 6/3 mmHg and decreases the need of medical treatment (Neter et al. 2003; Horvath et al. 2008). Combination of weight loss and reduced salt consumption appears to diminish LVH (MacMahon et al. 1986). Similar results can be obtained by increasing exercise, which helps lose weight. However, it is important to emphasize the importance of maintaining the weight loss results and the reduced sodium levels (Fagerberg et al. 1984; Bao et al. 1998). The changes one makes in their lifestyle must be sustainable in order to benefit from them in the long run.

2.2.3 Recent targets of interest in research

Despite antihypertensive drugs benefiting patients with LVH even if they were not hypertensive, the demand for innovative treatments is high. Particularly in the case of MI it would be crucial to prevent the pathological remodeling in an early phase (Fig 1). Researchers are breaching out to find treatments that repair and prevent cardiac remodeling instead of only alleviating symptoms.

Cardiac regeneration has been a hot topic in the field of LVH and cardiac failure treatment for the past decades. Researchers have spent considerable effort and resources to find a way to overcome the immediate hypertrophic response to injury. Stem cells are one of the most studied fields in several diseases and conditions, and the heart is no exception. Animal models and humans have confirmed that cellular replacement, which means stem cells or other types of cells, are injected directly to the site of injury, has modest yet potential effects in cardiac repair (e.g. Fisher et al. 2016; Khan et al. 2016; Park et al. 2019; Fan et al. 2020). However, before drawing any conclusions, it was also shown that the implanted cells seldom retain in the heart and the mechanistic base of cardiac improvement after cell therapy remains undecided. The transient benefit obtained from stem cell implantation is likely due to paracrine signaling between neighboring cells (Mercola et al. 2011).

Another approach has been turning other cardiac cells, such as fibroblasts, into cardiomyocytes by taking advantage of developmental gene regulatory networks (Buckingham et al. 2005; Olson 2006; Srivastava 2006). In theory, such reprogrammed cells can occupy the site of injury before hypertrophy and fibrosis, therefore enhancing cardiac function after MI (Ieda et al. 2010; Qian et al. 2012; Song et al. 2012). Fibroblasts can be reprogrammed into induced pluripotent stem cells (iPSCs), which exhibit the properties of embryonic stem cells (ESCs) after forced expression of pluripotency genes OCT4, SOX2, Krüppel-like factor 4 and MYC – together also known as OSKM factors (Takahashi et al. 2006; Takahashi et al. 2007). Generating iPSC intermediates from patient fibroblasts offers a possibility for patient-specific disease models and better choices of treatment (Yoshida and Yamanaka 2011; Nakamura et al. 2013). Efe et al.

(2011) successfully generated contracting patches of cardiomyocytes from mouse embryonic fibroblasts and adult mouse fibroblasts when using specific culture conditions without the iPSc intermediate stage. However, there are major issues to be solved. First, iPSCs are highly teratogenic, meaning they pose a risk for embryos if the receiver is pregnant. Second, the pluripotency genes are carcinogenic. Additional challenges include low induction rate to iPSCs, inadequacy of iPSCs to further differentiate into cardiomyocytes and failure of exogenous cells to gain a foothold at the site of injury, for example (Yoshida and Yamanaka 2011; Bellin et al. 2012).

Attempts to bypass these hardships have been made, such as implementing the reprogramming directly within the heart without the stem cell intermediate stage. Recently, a deeper understanding of transcription factors and their interactions, which specify cardiac lineage and the differentiation of cardiomyocytes during embryogenesis, has been gained. The same transcription factors carry potential to activate cardiac gene expression in fibroblasts *in vitro*. Ieda et al. (2010) got initial results that combining three transcription factors – GATA4, myocyte specific enhancer factor 2C (MEF2C) and TBX5 (collectively called GMT factors) – could reprogram cardiac and dermal fibroblasts into induced cardiac- like myocytes (iCLMs) in mice, with a yield of ~5−7%. A tiny fraction of these iCLMs were able to contract spontaneously after 4–5 weeks. Nevertheless, the efficacy obtained by Ieda et al. (2010) varies between research groups and some, such as Chen et al. (2012) report the method not successful at all. The variables causing the inconsistency are most likely the need for a specific stoichiometry of reprogramming factors to activate the cardiac gene programme, the heterogeneity of fibroblasts between different studies and the variances in the cell culture conditions used among others. Song et al. (2012) managed to increase the yield to ~20% by adding in a fourth transcription factor, HAND2, and the newly generated iCLMs apparently rise from direct conversion of fibroblasts without an intermediate stem cell state. Additional strategies to generate cardiomyocytes include muscle-specific micro RNAs (miRNAs) and combining this with transcription factor introduction (Efe et al. 2011; Jayawardena et al. 2012). The exact mechanism accountable for direct cardiac reprogramming remains partially unknown. Various combinations of different transcription factors have been proven to directly activate genes encoding cardiac proteins and regulate their own expression patterns (Ieda

et al. 2010; Qian et al. 2012; Song et al. 2012). Seemingly, these factors can switch on endogenous cardiac genes that gradually create a stable cardiac phenotype when they reach their threshold expression levels.

Surprisingly, applying these strategies *in vivo* was a greater success than anticipated after the iCLM yields in *in vitro* studies. Qian et al. (2012) and Song et al. (2012) both delivered GMT factors by retroviral vectors to mouse hearts after left anterior descending ligation and saw major improvement in cardiac function which was still clear a year after surgery. When these iCLMs were examined by isolating and culturing them *in vitro* the sarcomere structures, contractility and cardiomyocyte-like gene expression were similar to mature cardiomyocytes. Encouraging results were also obtained by Jayawardena et al. (2012) who transfected miRNAs with lentivirus vector. Presumably the native surroundings in the living heart improved the results significantly from artificial *in vitro* conditions. These findings were not, however, an operational treatment for humans. Human cells are much harder to reprogramme, likely due to their stable epigenetic modifications that take place over time. Nam et al. (2013) reported initial progress towards reprogramming human fibroblasts into cardiomyocytes *in vitro*. The foremost problem is that these cells do not proliferate, which significantly reduces their potential in therapeutic use where millions of dead cardiomyocytes should be replenished. An additional danger lies in the possible alterations in cardiac architecture, which could quickly become deadly.

Instead of making new cardiomyocytes from other cells, researchers have also studied ways to make cardiomyocytes proliferate again. Positive cell cycle regulators that have been identified include cyclins, cyclin-dependent kinases and proto-oncoproteins, and they are expressed at considerably higher levels in the embryonic heart and downregulated in the adult heart (Pasumarthi and Field 2002; Ahuja et al. 2007). Forcing overexpression of some of these factors, such as cyclin A2, cyclin D2, neuregulin 1 and SV40 large T antigen have been shown to be linked with dedifferentiation and proliferation of mature cardiomyocytes (Chaudhry et al. 2004; Engel et al. 2005; Engel et al. 2006 Kühn et al. 2007; Bersell et al. 2009). However, all experiments have resulted in relatively low yields but a chance for future therapeutics remains.

A third direction is utilizing the same transcription factors as in cell reprogramming, but rather aiming at preventing hypertrophic response. Synergy between transcription factors determines lots of cellular processes, such as which genes are expressed and whether the cell should opt for apoptosis, for instance (Lyons et al. 1995; Molkentin et al. 1997; Horb and Thomsen 1999). Some of these protein-protein interactions are mediators for hypertrophic gene expression. Our research group has focused on synthetizing numerous small molecules that interfere with the interaction between GATA4 and NKX2-5 and was in fact the first one to reportedly succeed (Välimäki et al. 2017). These two factors have been a particular target of interest and other research groups have also acknowledged their potential in future treatments (Ieda et al. 2010; Song et al. 2012; Kang et al. 2015). However, this topic will be covered more profoundly further in this literary survey.

2.3 Long non-coding RNA

During the past couple of decades an increasing number of studies have found long non-coding RNAs (lncRNAs) an important factor in genetic regulation. They belong to a larger group of non-coding RNAs (ncRNAs). All ncRNAs are all indirect regulators of gene transcription despite not coding for any proteins themselves and their duties include transcriptional, post-transcriptional and epigenetic modifications in gene expression among others (Kapranov et al. 2007). They are highly organ-selective and are likely to contribute to both developmental and functional cell events and are therefore an interesting drug target.

The discovery of ncRNAs helped provoke a change in the general understanding of genetics towards the acknowledgement that a single sequence can be read in multiple ways: it can be transcribed into sense, antisense, coding and non-coding transcripts (Carninci et al. 2005; Kapranov et al. 2005). In fact, the number of non-coding RNA in the human genome is more than double compared to protein-coding RNA (NONCODE database version v5.0 (www.noncode.org); GENCODE database release 32 (www.gencodegenes.org/human). Because this newly found system clearly had many more aspects to be considered and had a more complex nature altogether than the simple idea of one sequence resulting in one protein, ncRNAs had to be classified. One crude

classification system was based on their proximity to gene-coding genes, such as overlapping, cis-antisense, bidirectional or intronic ncRNAs (Carninci et al. 2005; Kapranov et al. 2007). The problem with this classification was, however, that very few ncRNAs match only one category. Another way to roughly classify ncRNAs is their size, which is used in the determination of lncRNA. They are characterized by their size of more than 200 nucleotides. On the other hand, other ncRNAs such as micro RNA (miRNA), small interfering RNA (siRNA) and transfer RNA (tRNA) are functionally so different that the size should not be considered an indicator of the nature of the ncRNA.

Focusing on lncRNAs, they come with various functions and structures and interact with different targets, therefore having to do with many endogenous pathways. Depending on their location either in the nucleus or in the cytoplasm, lncRNAs may interfere with transcriptional or post-transcriptional events, or translation of messenger RNA (mRNA). Examples of the functions of nuclear lncRNAs include chromosome scaffolding, chromatin remodeling, alternative splicing, epigenetic modification and transcriptional activation or silencing (Clemson et al. 2009; Hung et al. 2011; Mercer et al. 2011; Derrien et al. 2012; Kugel and Goodrich 2012; Lee 2012). In almost all cases, these functions require lncRNA interacting with RNA-binding proteins, forming nuclear lncRNA-associated ribonucleoprotein complexes (lncRNPs). While cytoplasmic lncRNAs also form lncRNPs, they are generally less understood. Mercer et al. (2011) presented results showing that cytoplasmic lncRNPs can consist of lncRNAs transcribed from nuclear DNA or expressed locally in the cytoplasm from mitochondria. These cytoplasmic lncRNPs can direct cytoplasmic events vital for maintaining cellular structure and functions, such as protein localization and turnover, mRNA translation and stability, obtainability of cytoplasmic factors, and protein scaffolding (Yoon et al. 2013; Rashid et al. 2016).

When discovered in the first place, the massive amount of lncRNAs took researchers by surprise and led them to believe it was only transcriptional noise derived from low DNA polymerase fidelity (Struhl 2007). However, it was soon noticed that expression of many lncRNAs is restricted to specific developmental contexts. In vivo studies carried out with

mice showed large numbers of lncRNAs specifically expressed only during embryonic development or stem cell differentiation (Amaral and Mattick 2008; Dinger et al. 2008). The overall importance of ribonucleic acid, RNA, can be highlighted with s a scientific hypothesis that all life begun with RNA. It is a simple structure – an easy way to store information (Mercer et al. 2009). Presumably, the double-stranded version with a slight extra to its structure, DNA, slowly developed from the single-stranded RNA as an upgraded version. Due to its higher stability, DNA then made it possible to start producing the material for which it contained instructions. As evolution went on, more complex and elaborate organisms were developed. Currently it is known that most of the genome in intricate organisms is regulated by far more steps than simply DNA sequence leading to a certain protein (Carninci et al. 2005; Kapranov et al. 2007). Instead, transcriptional activity is developmentally regulated. After the discovery of ncRNAs it was clear the development of RNA did not stop when first strands of DNA emerged; DNA never actually replaced RNA. It went along handling its own role and territory, which was undiscovered until recent times.

2.3.1 Long non-coding RNA in disease

An important distinctive factor between lncRNAs and protein-coding RNAs is that lncRNAs are more tissue or cell selective (Ramos et al. 2013). This makes them encouraging targets for medical research as they are likely to contribute only to the functions of their specific tissue or organ. Specialization in certain tissues also supports the theory that lncRNAs must have an impact on the embryonic development of the organ and tissue of question. If every possible combination of functions and structures of living organisms was stored solely inside protein-coding DNA, it should be possible to link all genetic diseases to mutations in that code. A codon – a unique set of three nucleotides that determine a single amino acid – can be observed for aberrations (Crick et al. 1961). The results can be used to predict possible outcomes in the order of amino acids being linked together, affecting the structure, folding and function of the final protein and the possible effects taking place in the organ and entire organism. However, the genetic background of various diseases and conditions are yet to be explained despite the human

genome has been sequenced, which suggests coding sequences do not work alone (Wapinski and Chang 2011).

As recently as in the beginning of the century, small- and large-scale mutations in the non-coding regions of the genome were easily labeled unimportant. Examples include chromosomal translocations, copy-number alterations, nucleotide expansions and single nucleotide polymorphisms. Recently, the interest in them has been increasingly growing. It is still challenging to point out, which of these mutations have to do with human disease and how exactly they contribute to them. When mutations take place in the mostly well established, protein coding genome, outcomes are predictable. Instead, when they take place in the vastly unknown non-coding regions, even the most sophisticated estimates are just speculation (Fig 6).

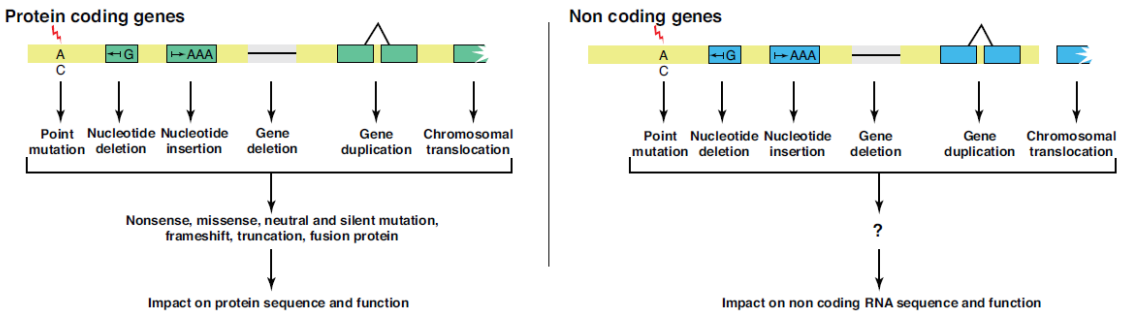

Figure 6: A graph of possible mutations taking place in any regions of the genome and the known effects. When a mutation occurs in protein coding genes, the consequences are well established and even predictable. However, the same does not apply to the non-coding genes. Even if it is possible to detect a change in sequence and function of the gene, the full mechanisms are unclear (Wapinski and Chang 2011).

2.3.2 Cardiac long non-coding RNAs

Matkovich et al. (2014) characterized 321 cardiac-specific lncRNAs by comparing mouse RNA sequencing from the heart, liver and skin. Further on, 52 of them were found abundant and highly cardiac-enriched. Approximately two out of three of these lncRNAs were expressed by cardiomyocytes, whereas cardiac fibroblasts contributed to only 2%. Li et al. (2013) used deep sequencing and microarrays to profile lncRNA expression and identified several upregulated lncRNAs in mouse hearts after treating them with a β adrenoreceptor agonist, which increases heart rate. Another similar study profiled upregulated lncRNAs in mouse hearts after ischemia or reperfusion, also finding a large

number of matches (Liu et al. 2014). An even more intriguing discovery was made by Ounzain et al. (2014) when they identified 1521 novel lncRNAs that were not listed in the mouse genome at the time. Interestingly, almost three in four of these lncRNAs were found in the human genome, suggesting possible conservation in human. Especially ultra-conserved regions (UCRs), defined as DNA stretches at least 200 bp long conserved in human, rat and mouse genome, have been associated with congenital abnormalities in the heart and other organs as well (Baira et al. 2008; Martinez et al. 2010; Liu et al. 2019). These studies and numbers are just examples of how much potential the cardiac research field sees in lncRNAs.

The first evidence of the relevance of lncRNAs in CVDs originate from vast genome studies that identified a susceptibility locus of coronary artery disease on the human chromosome 9p21.53 (Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007). This locus was found to be adjacent to the last exon of a specific lncRNA, which was named ANRIL (or CDKN2BAS). The three research groups independently came to the conclusion that single nucleotide polymorphisms (SNPs) on ANRIL significantly increases the risk of coronary artery disease. Later on, these suspicions turned out to be correct: ANRIL SNPs have been linked to the development of coronary atherosclerosis, peripheral artery disease, carotid arteriosclerosis among other CVDs (Chen et al. 2014). Another discovery connected lncRNAs and MI for the first time, arose from genetic studies showing enriched SNPs in a MI-susceptible locus coding for a lncRNA that was later named MIAT (also known as RNCR2/AK028326/Gomafu) (Ishii et al. 2006).

Despite these achievements in lncRNA and CVD research, the role of lncRNAs is only starting to be acknowledged. Surprisingly few lncRNAs have been reliably associated with hypertrophy when compared to the thousands of upregulated lncRNAs in infarction studies (Matkovich et al. 2014). In addition to disease, lncRNAs regulate and control embryonic cardiac development (Grote et al. 2013; Klattenhoff et al. 2013). An example is Braveheart (Bvht), which was discovered by Klattenhoff et al. (2013) after they used multiple ESC differentiation strategies to find necessary factors for cardiac gene activation. After birth, lncRNAs remain essential for normal cardiac function throughout lifespan. Han et al. (2014) discovered a group of lncRNAs partially overlapping the gene

locus Myh7, coding for β-MHC. These lncRNAs were named myosin heavy-chain-associated RNA transcripts (Mhrt). Their research was one of the first major ones proving the relevance of lncRNAs to cardiac hypertrophy. After inducing cardiac hypertrophy in mice by transverse aortic constriction (TAC) surgery the expression of Mhrt was dynamically downregulated. In addition, TAC has been shown to induce isoform change from Myh7 to Myh6, which is characteristic of cardiomyopathy (Miyata et al. 2000; Krenz and Robbins 2004). Respectively, overexpression of Mhrt after TAC significantly reduced hypertrophy and improved cardiac function in mice (Han et al. 2014).

By today, many research groups have identified different lncRNA strands that contribute to the complex chains of events in cardiac functionality and generation. Nonetheless, no one has been able to take these findings to be tested and developed towards possible treatments for heart illnesses. Viereck et al. (2016) compared the lncRNA transcriptome between TAC and sham operated mice. The group discovered a specific conserved lncRNA cardiac hypertrophy associated transcript (Chast) that was upregulated in hypertrophic cardiomyocytes after it had been activated by pro-hypertrophic transcription factors. In addition, overexpressing Chast was effective enough to promote hypertrophy both in vitro and in vivo with no other hypertrophy-promoting factors on site. Correspondingly, blocking the effect of Chast with GapmeR antisense oligonucleotides prevented cardiac hypertrophic growth after TAC surgery. The researchers also found Chast to be upregulated in aortic stenosis patients.

Another known pro-hypertrophic lncRNA is cardiac hypertrophy associated epigenetic regulator, cardiac-hypertrophy-associated epigenetic regulator (Chaer). Wang et al. (2016) reported Chaer cardiac knockout mice suffered less hypertrophic growth and fibrosis after TAC than wild type control group. Respectively, Chaer overexpression resulted in cardiomyocyte growth. Cardiac hypertrophy related factor (CHRF) was discovered by Wang et al. (2014) when the group profiled ATII -regulated lncRNAs. TAC in murine hearts and human heart failure samples showed upregulation of CHFR in vivo and resulted in increased cardiomyocyte hypertrophy and apoptosis. ATII treatment in vitro had the similar effects.

Silent information regulator factor 2 related enzyme 1 (Sirt1) antisense lncRNA was found by Li et al. (2018) and it significantly promoted cardiomyocyte proliferation both in vitro and in vivo, while the suppression of Sirt1 antisense lncRNA had the opposite effect. Cardiomyocyte proliferation regulator (CPR), on the other hand, appears to block proliferation (Ponnusamy et al. 2019). The expression of CPR is significantly higher in adult hearts than during fetal stage. Moreover, inhibition of CPR restores cardiac function and reduces scarring after MI, and overexpressing CPR in neonatal mouse hearts nearly obliterated their regeneration capacity. Finally, as promising as these couple of examples of cardiac lncRNAs appear, the world has barely revealed the tip of the iceberg. Piccoli et al. (2017) managed to identify over 1400 different lncRNAs deregulated in overloaded mouse cardiomyocytes, suggesting there is plenty of work left for the future. All the examples covered in this chapter are summarized in Table 1.

2.4 Transcription factors

As briefly mentioned above when discussing current targets of interest in cardiac regeneration research, transcription factors are an extensive yet promising direction. According to one definition: “Transcription factors are key proteins that decode the information in our genome to express a precise and unique set of proteins and RNA molecules in each cell type in our body” (Estella et al. 2012). They are proteins holding specific domains that bind to the promoter regions of particular genes. They also contain a domain that interacts with RNA polymerase II or other transcription factors and therefore control the amount of mRNA produced by the gene in question. In this following paragraph I will focus on a couple of cardiac-specific transcription factors that have a significant role in cardiac development and hypertrophy.

Table 1: Examples of studied cardiac-selective long non-coding RNAs (lncRNA) and their effects. SNP = single nucleotide polymorphism.

Name	Single / group	Function in the heart	Ref.
MIAT	single	Normal function prevents myocardial infarct, SNPs increase risk. Adjacent to the locus of a susceptility gene.	Ishii et al. 2006
ANRIL	single	Normal function prevents coronary artery disease; SNPs significantly increase risk. Exon adjacent to the locus of a susceptility gene.	Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007
Myosin heavy-chain-associated RNA transcript (Mhrt)	group	Hypertrophic stress causes downregulation. Partially overlapping *Myh7* locus, which is why these lncRNAs can affect myosin heavy chain synthesis. Restoring levels to pre-stress levels protects heart from hypertrophy and failure.	Han et al. 2014
Cardiac hypertrophy related factor (CHRF)	single	Upregulation causes cardiomyocyte hypertrophy and apoptosis. Stress induces expression, regulated via sngiotensin II.	Wang et al. 2014
Braveheart (Bvht)	single	Essential for for cardiac gene activation and embryonic development of the heart.	Klattenhoff et al. 2016
Cardiac hypertrophy associated transcript (Chast)	single	Activating with pro-hypertrophic transcription factors causes upregulation in cardiomyocytes. Capable of promoting hypertrophy on its own both in vitro and in vivo.	Viereck et al. 2016
Cardiac-hypertrophy-associated epigenetic regulator (Chaer)	single	Strongly hypertrophic. Myocardial inhibition results in less hypertrophy. Overexpression promotes cardiomyocyte growth.	Wang et al. 2016
Silent information regulator factor 2 related enzyme 1 (Sirt1) antisense lncRNA	single	Promotes cardiomyocyte proliferation.	Li et al. 2018
Cardiomyocyte proliferation regulator (CPR)	single	Inhibits cardiomyocyte proliferation. Overexpression in neonatal hearts significantly decreases regeneration capability, while inhibition in adult hearts restores function and reduces scarring after myocardial infarct.	Ponnusamy et al. 2019

2.4.1 GATA family

The family of GATA transcription factors consists of six members (GATA1-6), all of which bind to DNA nucleotides with their two zinc finger structures (Ko and Engel 1993; Merika and Orkin 1993) (Fig 7). Their name origins from their binding to (A/T)GATA(A/G) sequence. Functions of GATA transcription factors cover cell differentiation and proliferation among many others throughout lifespan from infancy to old age. The first subfamily, GATA1, -2 and -3 affect mostly blood cells (Molkentin 2000). GATA1 regulates the development of red blood cells, mast cells, megakaryocytes et cetera. GATA2, on the other hand, has an effect on endothelial cells in blood vessels, fibroblasts, monocytes and red blood cell progenitors to some extent, among others. Targets of GATA3 include T lymphocytes, mast cells, the brain and kidneys (Patient and McGhee 2002). The second subfamily – GATA4, -5 and -6 are located particularly in internal organs: liver, intestines, lungs and heart, for instance (Arceci et al. 1993; Kelley et al. 1993; Laverriere et al. 1994; Morrisey et al. 1996; Morrisey et al. 1997). GATA4 and -6 are myocardium-specific in the heart, whereas GATA5 is mostly located in the endocardium (Charron and Nemer, 1999). In each organ GATA transcription factors control genetic expression, function and development of that specific type of tissue. This survey will focus especially on GATA4 due to its cardiac functions. In addition to the heart, it can be found in the gastrointestinal tract, the pancreas, the liver, the bladder and the gonads to some extent (Grépin et al. 1994; The Human Protein Atlas – GATA4).

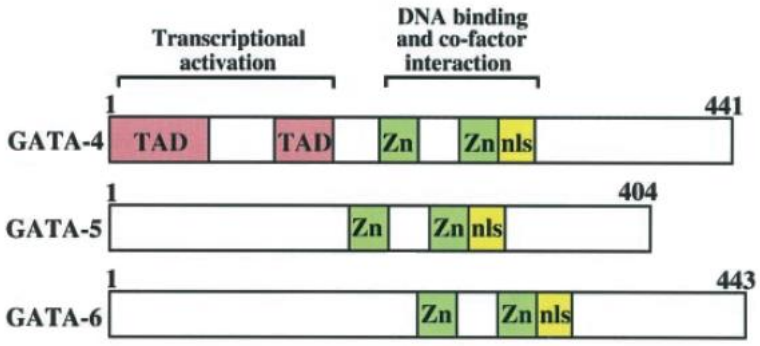

Figure 7: Functional structures of GATA4, -5 and -6. The zinc finger structures (Zn) and nuclear localization sequence (nls) are responsible for binding to DNA and cofactors. Topologically associating domains (TAD) activate transcription of target genes (Molkentin 2000).

Pressure-induced overload has been shown to stimulate both expression and DNA-binding of GATA4 and -6 in mice (van Berlo et al. 2013a). Excessive expression has also been reliably linked with increased hypertrophy in vitro and in vivo (Liang et al. 2001; van Berlo et al. 2013a). Oka et al. (2006) and van Berlo (2013a) have demonstrated how reducing GATA4 expression in mouse heart reduces hypertrophy caused by pressure overload, and GATA6 deletion also showed decrease in ATII and phenylephrine based hypertrophy. These results indicate GATA4 and -6 have a great significance in LVH.

GATA4 regulates the expression of many sarcomere protein coding genes, such as *Myh7*, troponins I and C and natriuretic peptides *Nppa* and *Nppb* (Molkentin 2000). It affects many proteins related to hypertrophy and cardiac function in general: endothelin-1 (ET-1), muscarine receptors and A1 adenosine receptor to name a few. Hypertrophy and hypertrophic factors, such as ATII, stimulate GATA4, which further stimulates transcription of ATRs and Myh7 (Hasegawa et al. 1997; Herzig et al. 1997). GATA4 stimulation, however, does not necessarily mean an increase in the total amount of GATA4 – it can also mean an improvement in its affinity to DNA. This happens by changes in the post-translational modifications of GATA4, such as phosphorylation, acetylation and protein-protein interactions (Katanasaka et al. 2016).

GATA6 is a less known hypertrophic transcription factor but evidence of its necessity for hypertrophic response has been found (van Berlo et al. 2013a). It works by the side of GATA4 and can take over its duties in some cases. Cardiac differentiation is an example of a process in which the tasks of GATA4 and GATA6 overlap in many respects (Pikkarainen et al. 2004; Zhao et al. 2008, van Berlo et al. 2013a). This explains why GATA4 knockout animal models can still express some target genes of GATA4 (Pu et al. 2004; Zeisberg et al. 2005; Bisping et al. 2006).

2.4.2 Protein-protein interactions

Knocking GATA4 out of the genome could seem a suitable way to target LVH as it so strongly enhances hypertrophy, but this would lead to severe issues considering that GATA4 is also needed for cardiac development and maintenance. Several studies have

shown homozygous GATA4 knockout mice suffering severe cardiac developmental disorders (Pu et al. 2004; Zeisberg et al. 2005; Oka et al. 2006). Most die during embryonic stage and the ones that survive express clear symptoms of cardiac failure (Pu et al. 2004; Rojas et al. 2008; Mohammadi et al. 2017). Rather than labeling GATA4 good or bad, one must take a deeper look at its interactions with cofactors. Other transcription factors can bind to GATA4 and regulate its activity. This is called protein-protein interaction, as it involves at least two different proteins working together in a unique pattern. Some of them increase the expression of hypertrophic genes while some induce cell cycle and proliferation. Abnormal protein-protein interactions cause various diseases, which has led many scientists to searching for an inhibitor against these interactions (Scott et al. 2016). Apart from LVH, examples include cervical cancer, leukemia, infection and neurodegenerative conditions (Ryan and Matthews 2005).

Our research group has done previous work on the interaction between GATA4 and its cofactor homeobox protein NKX2-5. Like GATA4 it is essential for normal cardiac development and homozygous knockout mouse models die during embryonic phase (Lyons et al. 1995). NKX2-5 has a high affinity for T(C/T)AAGTG sequence (Chen and Schwartz 1995) and it alone can induce hypertrophic genes, such as natriuretic peptides (Akazawa and Komuro 2003). The heterodimer complex of GATA4 and NKX2-5 is a highly effective activator of hypertrophic genes. A study by Pikkarainen et al. (2003) showed that GATA4-NKX2-5 interaction induces the expression of BNP in stretched cardiomyocytes, for example. Previously, our group has discovered a selection of small molecules that affect the synergy between GATA4 and NKX2-5 (Välimäki et al. 2017). Chemical structures of hit compounds are seen in Figure 8. No previous inhibitors have been published apart from one compound that prevents GATA4 from binding to DNA (El-Hachem et al. 2011). Isoxazole compound 3i-1000 showed the highest potential for a GATA4-NKX2-5 interaction inhibitor, so our group studied the chemical space around the molecule and created more 200 new potential derivates from it (Välimäki et al. 2017; Jumppanen et al. 2019).

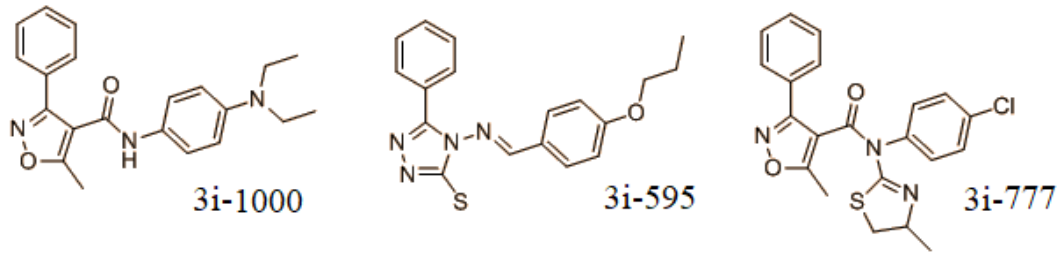

Figure 8: Chemical structures of three hit compounds for GATA4-NKX2-5 synergy modifiers by Välimäki et al. (2017). Compounds 3i-1000 and 3i-777 inhibited the interaction, whereas 3i-595 enhanced it.

2.4.3 Friend of GATA 2 – FOG2

The zinc finger transcriptional regulator Friend of GATA2 (FOG2, also known as ZFPM2) is a regulative protein that lacks the ability to bind to DNA by itself, but instead pairs up with other transcription factors to regulate the transcription of specific genes. It is most highly expressed in cardiac and nervous tissues. In the heart, the most essential roles of FOG2 are found in the development and morphogenesis of coronary vessels (Tevosian et al. 2000). Fog2-/- knockout mouse embryos die around embryonic day (E)13.5 with severe deficiencies in cardiac development: large ventricular septal defect, thin myocardium, overriding aorta and grave underdevelopment of the coronary vascular plexus. Under normal conditions, epicardial cells go through a transition to mesenchymal cells, giving rise to the subepicardial coronary vasculature. Despite an intact epicardial layer forms and epicardium-specific genes are expressed in Fog2-/- heart, no markers of cardiac vessel development are detected. Expression of FOG2 under the control of Myh6 promoter, which is restricted to cardiomyocytes only, partially recovered the damage in cardiac development, but the other deficits remained.

In the myocardium the most important co-factor for FOG2 is GATA4 (Cantor and Orkin 2005). Transfecting FOG2 transiently in primary cardiomyocytes has been shown to either enhance or inhibit GATA4 transcriptional activity (Lu et al. 1999; Svensson et al. 1999). Whether it functions as an enhancer or an inhibitor depends on the cell line and the promoter in which they interact. Lu et al. (1999) investigated how FOG2 affects the interaction of GATA4 with α-MYH promoter and discovered that in COS-1 cells it enhanced this interaction, whereas in neonatal rat cardiomyocytes the effect was

inhibitory. Crispino et al. (2001) reported that a single amino acid mutation in Gata4 inhibits the interaction between GATA4 and FOG2 in mice. They develop similar developmental deficits as Fog2-/- knockout mice and die by embryonic day (E) 13.5. The mutation did not impact protein expression or DNA binding properties of the transcription factors. The effects of FOG2 in the adult heart were investigated by Zhou et al. (2001). Inhibition of myocardial FOG2 in adult mice did not result in structural deficits as in embryos, but they developed severe cardiac failure symptoms and died within a couple of months. These results suggest GATA4-FOG2 interaction is necessary for cardiomyocyte development and therefore an interesting target to look at in cardiac drug development.

In a recent study, Liu et al. (2019) carried out experiments on P19 cells, which are mouse embryo-derived teratocarcinoma cells. A lncRNA with ultra-conserved elements (UCEs), named uc.245, was either overexpressed or silenced in the P19 cells. The group transfected the cells with recombinant pGPU6/GFP/Neo-FOG2 or pGPU6/GFP/NeoshRNA FOG2 vectors to up- or downregulate FOG2 gene. The group hypothesized uc.245 would influence P19 proliferation and apoptosis via FOG2. The results showed that when uc.245 was overexpressed, sh-FOG2 further repressed proliferation but induced apoptosis. However, overexpressing FOG2 obviously weakened proliferation inhibition and apoptosis driven by uc.245. These results provided evidence that uc.245, an example of a cardiac lncRNA with UCEs, functioned closely related to FOG2.

3 AIM OF THE STUDY

This project aims at gaining a deeper understanding of the effects of three types of lncRNAs – here named lncRNA-J, -C and -F – in neonatal mouse ventricular cardiomyocytes. The phenomena in focus include doxorubicin- (DOX) induced apoptosis and hypertrophy after ET-1 induced stress on the cardiomyocytes. Our group has previously discovered that lncRNA C and F are upregulated and J downregulated in the neonatal mouse myocardium but soon to be leveled out during the following weeks, which is the same time gap where cardiomyocytes lose their cardiac regeneration

capability (Fig 9; Pohjolainen et al. unpublished). In addition, they have been shown to be mostly expressed in cardiomyocytes instead of other cell types, such as endothelial cells. The lncRNAs of interest will be silenced in neonatal mouse ventricular cardiomyocytes using antisense LNA GapmeRs. Then, known hypertrophic and apoptotic markers will be immunostained and their fluorescence signals will be used to analyze the cardiomyocytes.

Additionally, previous hit compounds for GATA4-NKX2-5 interaction will be tested for their effects on GATA4-FOG2 interaction, using a similar COS-1 cell luciferase assay as described in Jumppanen et al. (2019). As the method has already proven to be functional with GATA4 and NKX2-5 plasmid transfections, it is worth seeing if some of these compounds could, in fact, have efficacy in manipulating interactions of GATA4 with more than just one of its cofactors. This part of the study will be comprised of first setting up optimizing the GATA4-FOG2 luciferase assay and then exposing the cells to the test compounds.

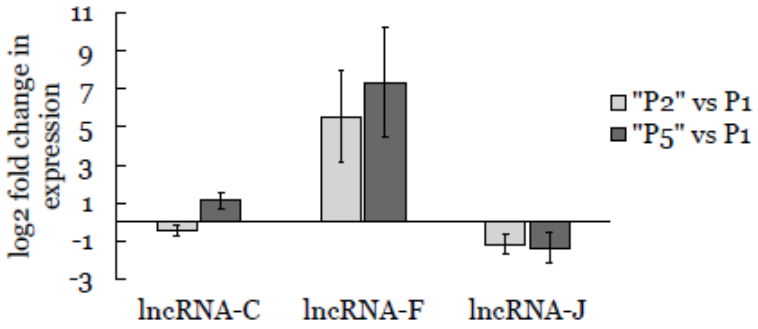

Figure 9: Three LncRNAs go through significant changes in their expression during the first postnatal days in neonatal mouse ventricular cardiomyocytes. The cardiomyocytes were isolated on postnatal day 1 (P1) and cultured for one (P2) or four (P5) days. The results are shown as means ± the standard error of the mean (SEM) (n=3-7) (Pohjolainen et al. unpublished).

4 MATERIALS AND METHODS

4.1 Reagents and other compounds

Dulbecco's modified eagle medium/Ham's F12 nutrient mixture (DMEM/F-12; 31330-038), penicillin-streptomycin (PenStrep) and Fetal Bovine Serum (FBS) used in neonatal cardiomyocyte cultures were purchased from Gibco Life Sciences (Paisley, UK). Dimethyl sulfoxide (DMSO), ET-1, insulin – transferrin - sodium selenite solution, sodium pyruvate (NaP), triiodothyronine (T3) and bovine serum albumin (BSA) were from Sigma (Darmstadt, Germany). DOX for cardiomyocyte apoptosis assays was from Tocris Bioscience (Bristol, UK).

LNA GapmeRs were purchased from Qiagen (Hilden, Germany). Lipofectamine 3000 and P3000 reagents for LNA GapmeR transfections were from Thermo Fisher Scientific. Tromethamine - Ethylenediaminetetraacetic acid (TE) buffer was purchased from Integrated DNA Technologies (Coralville, Iowa, USA).

Immunofluorescent staining antibody monoclonal anti-α-actinin (sarcomeric) antibody produced in mouse (A7811) and DNA-staining 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma. CellEvent™ Caspase-3/7 Green Detection Reagent was from Invitrogen (Carlsbad, California, USA). Alexa Fluor™ 488 goat anti-mouse IgG (A-11029), Alexa Fluor™ 647 goat anti-mouse IgG (A21236) and Alexa Fluor™ 647 phalloidin (A22287) were obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

Antifoam 204 for plasmid-containing bacteria cultures was from Sigma. FuGENE6 reagent, which was used for plasmid transfections in COS-1 cells was from Promega (Madison, Wisconsin, USA). Test compounds for GATA4-FOG2 interaction screening were synthetized in the Faculty of Pharmacy, University of Helsinki.

4.2 Neonatal mouse ventricular cardiomyocytes

Neonatal mouse cardiomyocytes were harvested from maximum 48 h old C57BL/6JOlaHsd mice. The mice were decapitated. Hearts were isolated surgically, and the ventricles were separated from other tissue and atrium in a dish with phosphate buffered saline (PBS). The ventricular cardiomyocytes were dissociated into a suspension using Miltenyi Biotec Neonatal (Mouse) Heart Dissociation Kit (reference number 130-098-373, Macs Miltenyi Biotec, Bergisch Gladbach, Germany). Cardiomyocytes were isolated with Miltenyi Biotec Neonatal Cardiomyocyte Isolation Kit, mouse (reference number 30-100-825, Macs Miltenyi Biotec). The cardiomyocytes were suspended and cultured in DMEM/F-12 supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. To enhance attachment, 96-well plates were coated with gelatin: 0.1% gelatin in purified H_2O incubated at +37 °C, saturated air humidity, 5% CO_2 for 30 – 60 min. Cardiomyocytes were plated on the coated 96-well plates at densities varying between 30 000 and 50 000 cells/well. After plating the cardiomyocytes were incubated at +37 °C, saturated air humidity, 5% CO2 for 24 h. After 24 h, those cardiomyocytes that were to be transfected 72 h after plating had their medium changed into complete serum free medium (CSFM): DMEM/F-12 supplemented with 5 µg/ml of insulin, 5 µg/ml of transferrin, 5 ng/ml of selenium, 2.8 mM NaP, 1 % BSA, 0.1 nM T3, 100 U/ml of penicillin and 100 µg/ml of streptomycin.

4.3 Exposure to endothelin-1 and doxorubicin

Cardiomyocytes were exposed to ET-1 or 1% BSA in DMEM, which was the solvent of ET-1, diluted to 100 nM in CSFM 24 h or 72 h after plating. Exposure times were 24 h at +37 °C, saturated air humidity, 5% CO_2, and transfections were carried out within 30 min from the initiation of the exposures.

DOX was added for two purposes: a positive control for apoptosis and combined with the antisense LNA GapmeRs to see if they affect the cytotoxicity of DOX. For preliminary experiments, cardiomyocytes were exposed to 3 and 1 µM of DOX. When combined with antisense LNA GapmeRs only 1 µM concentration was used. Exposure time was 23 h at

+37 °C, saturated air humidity, 5% CO_2, and transfections were carried out within 30 min from the initiation of the exposures. An equal number of wells was exposed to DMSO diluted to CSFM in the same ratio as DOX.

4.4 LNA GapmeR transfections

The target lncRNAs, lncRNA C, F and J, were silenced with antisense locked nucleic acid (LNA) GapmeRs. LNA GapmeRs are oligonucleotides comprised of three parts. In the middle, there is a DNA sequence antisense to the target RNA. The LNA parts increase binding affinity and protect the GapmeR from being deactivated by nuclease enzymes. When LNA GapmeR finds its target RNA, they are attached to one another with the antisense sequence and the GapmeR catalyzes degradation of the RNA by RNAse (Fig 10).

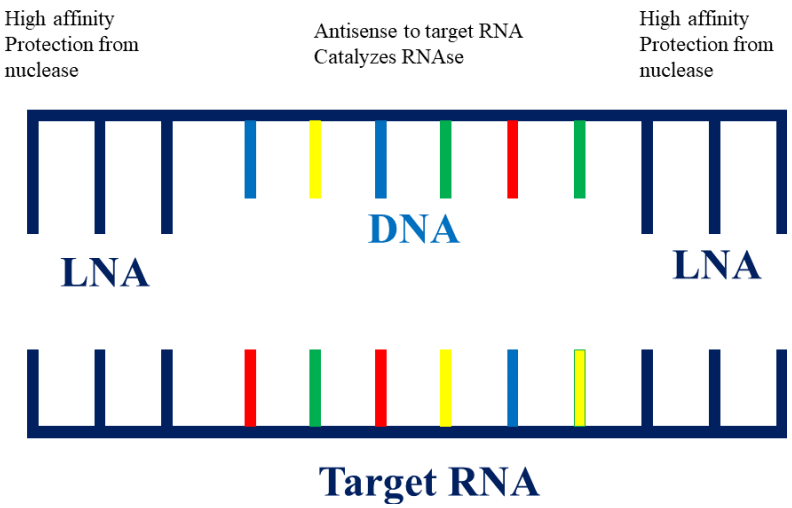

Figure 10: The mechanism of silencing RNA by antisense LNA GapmeRs.

Cardiomyocytes were exposed to 100 nM GapmeRs 1-3 for lncRNA J, GapmeRs 3 and 6 for lncRNA F, and GapmeR 3 for lncRNA C. Later, they will be referred either as GapmeRs J-1, J-2, J-3, F-3, F-6 and C-3 or lncRNA J/F/C GapmeRs. A larger group of LNA GapmeRs has been previously tested and these six were chosen based on preliminary results by Pohjolainen et al. (unpublished).

In lncRNA J GapmeR experiments the transfections were carried out 24 h after plating the cardiomyocytes, whereas in lncRNA F and C GapmeR trials the cardiomyocytes were incubated for 72 h after plating (Fig 11). The time points were based on the natural expression pattern of the lncRNAs in neonatal mouse ventricular cardiomyocytes (Pohjolainen et al. unpublished). First, the GapmeRs were diluted with DMEM/F-12. In the case of all other GapmeRs but C-3, 2 µl/µg DNA reagent lipid P3000 (Thermo Scientific) was added too. The DNA in LNA GapmeRs is usually negatively charged, and P3000 enhances the transfection by coating the negative charge. Lipofectamine 3000 reagent (Thermo Scientific) was diluted with DMEM/F-12 and mixed 1:1 with the GapmeR dilution. The GapmeR – reagent – solution was incubated for 10 – 15 min at room temperature (~ 22 °C) and added to cells so that the final concentration of the GapmeRs was 100 nM. After transfections the cardiomyocytes were incubated at +37 °C, saturated air humidity, 5% CO_2 for 23 (apoptosis assays) or 24 h (hypertrophy assays). For each experiment, negative control groups were transfected either with GapmeR negative control A alone or with P3000 reagent.

4.5 Immunofluorescence staining and high content screening

4.5.1 Caspase-3/7 reporter

Cardiomyocytes were stained with 7 µM CellEvent™ Caspase-3/7 Green Detection Reagent in 5 %FBS in PBS (later: caspase reporter). The reporter becomes fluorescent and binds to DNA if caspase-3 and caspase-7 –common biomarkers for apoptosis – are expressed and activated in the cells. This was carried out 23 h after the transfections (Fig 11). Cardiomyocytes were incubated with the caspase reporter at +37 °C, saturated air humidity, 5% CO_2 for 60 min.

4.5.2 Immunofluorescence staining

Plates were washed 2 x 5 min with PBS 24 h after transfections and in apoptosis assays 60 min after applying caspase reporter (Fig 11). The cardiomyocytes were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature (~ 22 °C). The wells were again washed 3 x 5 min with PBS. The cardiomyocytes were permeabilized with 0.1% Triton X-100 in PBS for 10 min. Then, they were washed 2 x 5 min with PBS. Non-selective binding sites were blocked with 4% FBS in PBS for 45 – 60 min at room temperature. Primary antibody dilution was prepared to 4% FBS in PBS and added to cells. The cardiomyocytes were incubated for 60 min in a shaker (300 rpm). The primary antibody solution for both apoptosis and hypertrophy assays included anti-α-actinin at a dilution of 1:600. After incubation, cardiomyocytes were washed 3 x 5 min with PBS. Secondary antibody dilutions were prepared to 4% FBS in PBS and added to cells. The cardiomyocytes were incubated for 45 min in a shaker (300 rpm) protected from light. For apoptosis assays, the secondary antibody solution contained AlexaFluor™ 647 nm anti-mouse at 1:200 and 0.1 mg/ml DAPI at 1:50. For hypertrophy assays the secondary antibody solution contained AlexaFluor™ 488 nm anti-mouse at 1:200, AlexaFluor™ 647 nm phalloidin at 1:50 and 1 µg/ml of DAPI. Finally, the cardiomyocytes were washed 3 x 5 min with PBS. The cardiomyocytes were left in PBS and the plates were stored at +4 °C protected from light.

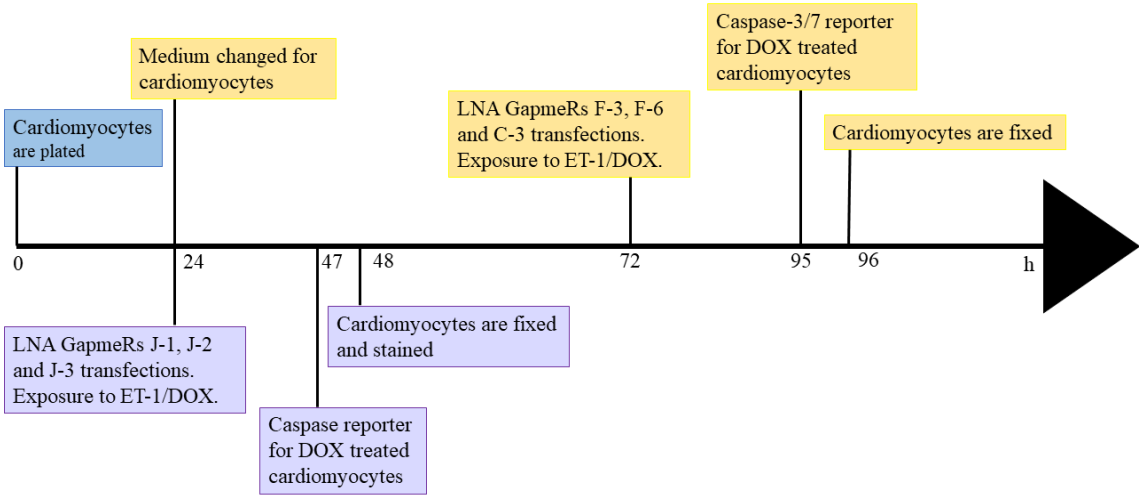

Figure 11: Cardiomyocyte treatment flow chart. Yellow boxes mark lncRNA C and F GapmeR experiments and purple boxes lncRNA J GapmeR experiments.

4.5.3 High Content Screening

Immunostained cardiomyocytes were imaged and analyzed with ThermoFisher CellInsight CX5; a high content screening imaging instrument, using a 10x objective. In Compartmental Analysis protocol, plates were imaged for DAPI (at 386 nm), caspase-3/7 (at 485 nm) and α-actinin (at 650 nm). Cells were identified by their nuclear staining with DAPI. Cardiomyocytes were differentiated from other cell types with the presence of α-actinin. DAPI, caspase reporter and α-actinin intensities inside the nuclei were measured. Additionally to intensity measurements, in preliminary experiments a fluorescence intensity threshold for caspase-3/7 positive and negative cells was manually set as described by Karhu et al. (2018). Morphology Explorer protocol imaged the plates for DAPI (at 386 nm), for α-actinin (at 485 nm) and phalloidin (at 650 nm). The cells were again identified with DAPI and cardiomyocytes were selected with α-actinin. In addition, only cells with 1 or 2 nuclei were included to further specify the differentiation from other cell types. From α-actinin and phalloidin images the number of fibers detected, their area and their alignment were measured in addition to intensity measurements. Fiber alignment was measured as the standard deviation of angles formed between individual fibers. Therefore, a higher fiber alignment value means the fibers are less aligned, and in theory, perfectly parallel fibers would have a value of zero.

4.6 GATA4-FOG2 compound screening optimization

4.6.1 COS-1 cultures

Frozen COS-1 cells were thawed from liquid nitrogen containers and cultured in DMEM supplemented with 10% FBS and 1% PS. They were cultured in 75 cm^2 Easy Flasks (Thermo Fisher Scientific) and kept at +37 °C, saturated air humidity, 5% CO2. The cells were passaged 1:10 twice a week. For transfections, COS-1 were plated on Isoplate 96-well plates with white walls (PerkinElmer, Waltham, Massachusetts, USA) at a density of 10 000 cells/well.

4.6.2 Plasmid production and purification

The empty expression plasmid MT2 (pMT2, 4976 bp) and mouse GATA4 expressing plasmid (pMT2-GATA4, 4976 + 1700 bp) were donations from DB Wilson (Department of Pediatrics, St. Louis Children's Hospital) (Arceci et al. 1993). The mouse FOG2 expression plasmid (pCS2-FOG2, 4095 + 5477 bp) was a gift from M Heikinheimo (Children´s Hospital, University of Helsinki) (Tevosian et al. 1999; Anttonen et al. 2003). The luciferase reporter plasmid NP-112 with rat BNP (rBNP) minimal promoter region attached with a -90 tandem binding site for GATA4 has been previously described by Grépin et al. (1994) and Kinnunen et al. (2015). The plasmids were produced in bacterial culture. Bacteria containing the desired plasmid was added to lysogeny broth supplemented with 0,1% ampicillin and 0,1% antifoam 204 first diluted 1:10. Bacteria was cultured overnight at + 37 °C in a shaker, 250 rpm.

Plasmids were purified with NucleoBond® Xtra plasmid purification kit midi according to the instructions (reference number 740410.10/.50/.100, Macherey-Nagel, Düren, Germany). DNA density was measured with NanoDrop 1000 -spectrophotometer (Thermo Scientific). Then, the plasmids were diluted with sterile H2O to the desired concentration and stored at + 4 °C.

4.6.3 Plasmid transfections

Each transfection group per experiment was designed so that the total amount of DNA was equal between groups. In practice, this meant altering the amount of the empty pMT2 plasmid in ratio with plasmids pMT2-GATA4 and pCS-FOG2. NP-112 was not changed between groups and was adjusted to the amount pMT2-GATA4 so that NP-112:pMT2-GATA4 ratio was 4:1 or 5:1. FuGENE6 was added at a reagent:DNA ratio of 3:1. DMEM supplemented with 10% FBS and 1% PS was aspirated from COS-1 cells and replaced with 80 µl/well of serum-free DMEM. Then, 20 µl/well of DNA-FuGENE6 mixture was added to the cells. The plate was incubated at +37 °C, saturated air humidity, 5% CO_2 for 24 h.

4.6.4 Luciferase measurement

Luciferase was activated with neolite assay kit as described in the instructions by the manufacturer (reference number 6016716, PerkinElmer). The bottom of the plate was covered with a white plastic sticker, and luminescence signal was measured with Victor2 1420 Multilabel Counter (PerkinElmer).

4.7. Statistical analysis

Statistical analyses were carried out with SPSS statistics 25 software (SPSS, Chicago, Illinois, USA). LNA GapmeR and exposure results were analyzed with one-way ANOVA and Tukey HSD post-hoc test. All groups were compared to each other and to the negative control group. Results were considered statistically significant when $p<0.05$.

5 RESULTS

5.1 Silencing long non-coding RNAs in neonatal mouse ventricular cardiomyocytes

5.1.1 Effects of silencing the long non-coding RNAs on hypertrophic response

In the first experiments, high content screening was used to analyze hypertrophic markers in cardiomyocytes and to see if the GapmeR transfections affect these markers. DAPI was used to identify nuclei and the amount of nuclei per cell, whereas α-actinin and F-actin were the screened hypertrophic phenotype markers. Representative images from the hypertrophy assay are shown in Fig 12.

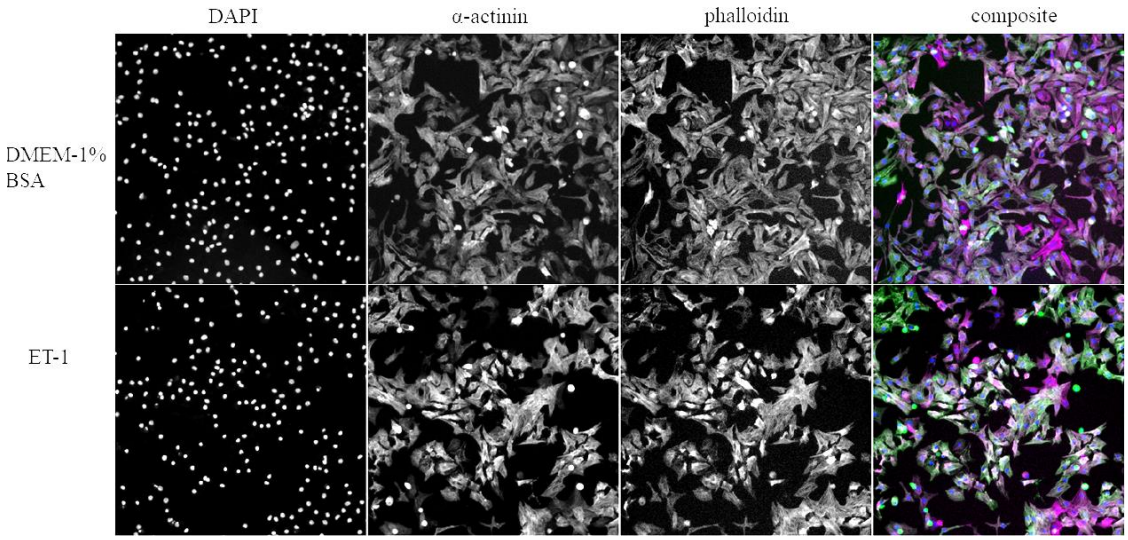

Figure 12: Representative images of neonatal mouse ventricular cardiomyocytes from the hypertrophy assay, with and without exposure to 100 nM endothelin-1 (ET-1). The cardiomyocytes were stained for α-actinin with a specific antibody, F-actin with phalloidin and the nuclei with DAPI. On the top row, cardiomyocytes had not been exposed to ET-1 and the bottom row shows cardiomyocytes exposed to ET-1 for 24 h. In the composite image DAPI is blue, α-actinin staining is green and phalloidin is magenta.

First, DAPI intensity was measured to identify nuclei. In GapmeR C-3 experiments ET-1 had no effect on DAPI intensity, whereas in lncRNA F GapmeR experiments an increase of 10–20% could be seen with the exposure (Fig 13A, 13B). In lncRNA J GapmeR experiments ET-1 decreased the intensity with a few percentages (Fig 13C).

These differences were, however, very moderate and not statistically significant. The GapmeR transfections did not affect DAPI intensities.

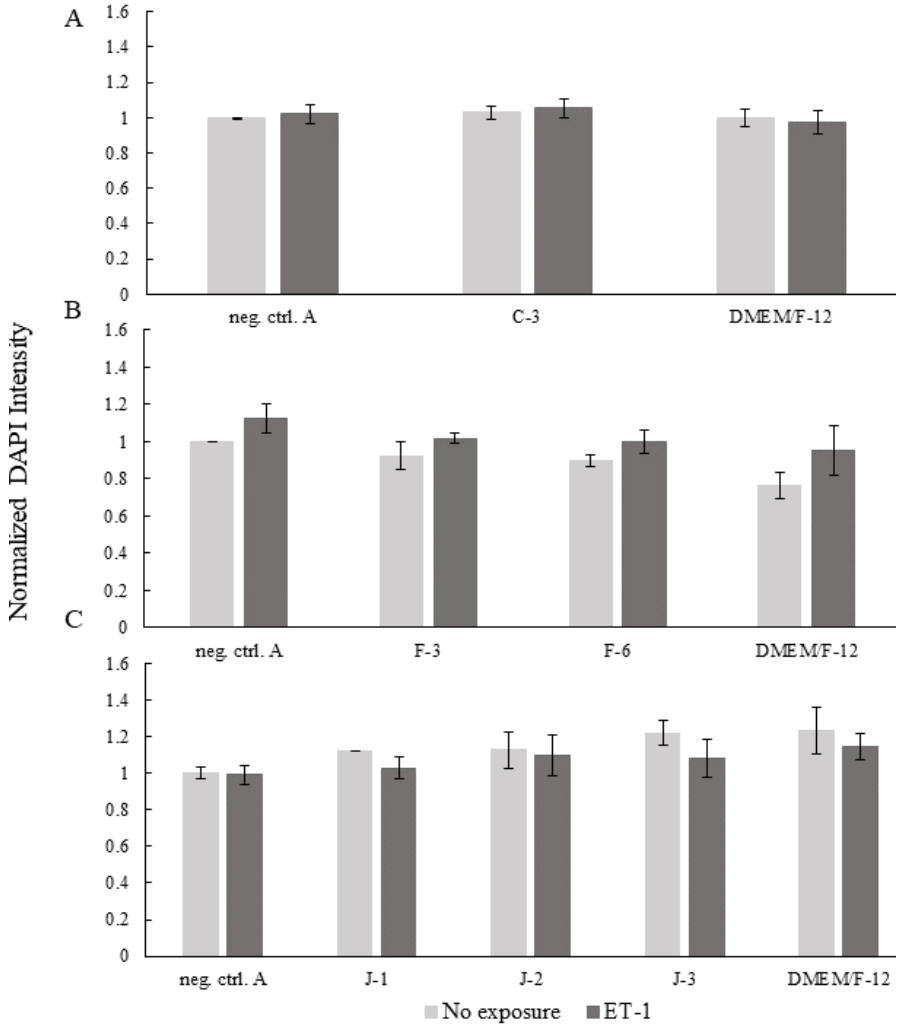

Figure 13: Effects of GapmeR C-3 against lncRNA C (A), GapmeRs F-3, F-6 against lncRNA F (B) and GapmeRs J-1, J-2 and J-3 against lncRNA J (C) and endothelin-1 (ET-1) exposure on nuclear DNA staining intensities in neonatal cardiomyocytes. The results are shown as mean intensity values normalized to negative control A without ET-1 exposure from 3 (A) or 4 (B and C) independent experiments ± standard error of the mean (SEM).

Intensities of α-actinin and F-actin staining were quantified to measure hypertrophy in the cardiomyocytes. In all experiments ET-1-exposed cardiomyocytes had an approximately 5–10% higher α-actinin intensity than their unexposed controls (Fig 14). The only statistical differences with ET-1 were in GapmeRs J-2 ($p=0.016$) and J-3 ($p=0.01$) compared to their unexposed controls. The GapmeRs had no effect on α-actinin intensity.

In lncRNA F and J GapmeR experiments ET-1 exposure increased F-actin staining intensity approximately 20–30% but the differences were not statistically significant (Fig 15). In GapmeR C-3 experiments the increase with ET-1 was not present at all. The GapmeRs had no effect on F-actin staining intensity (Fig 15).

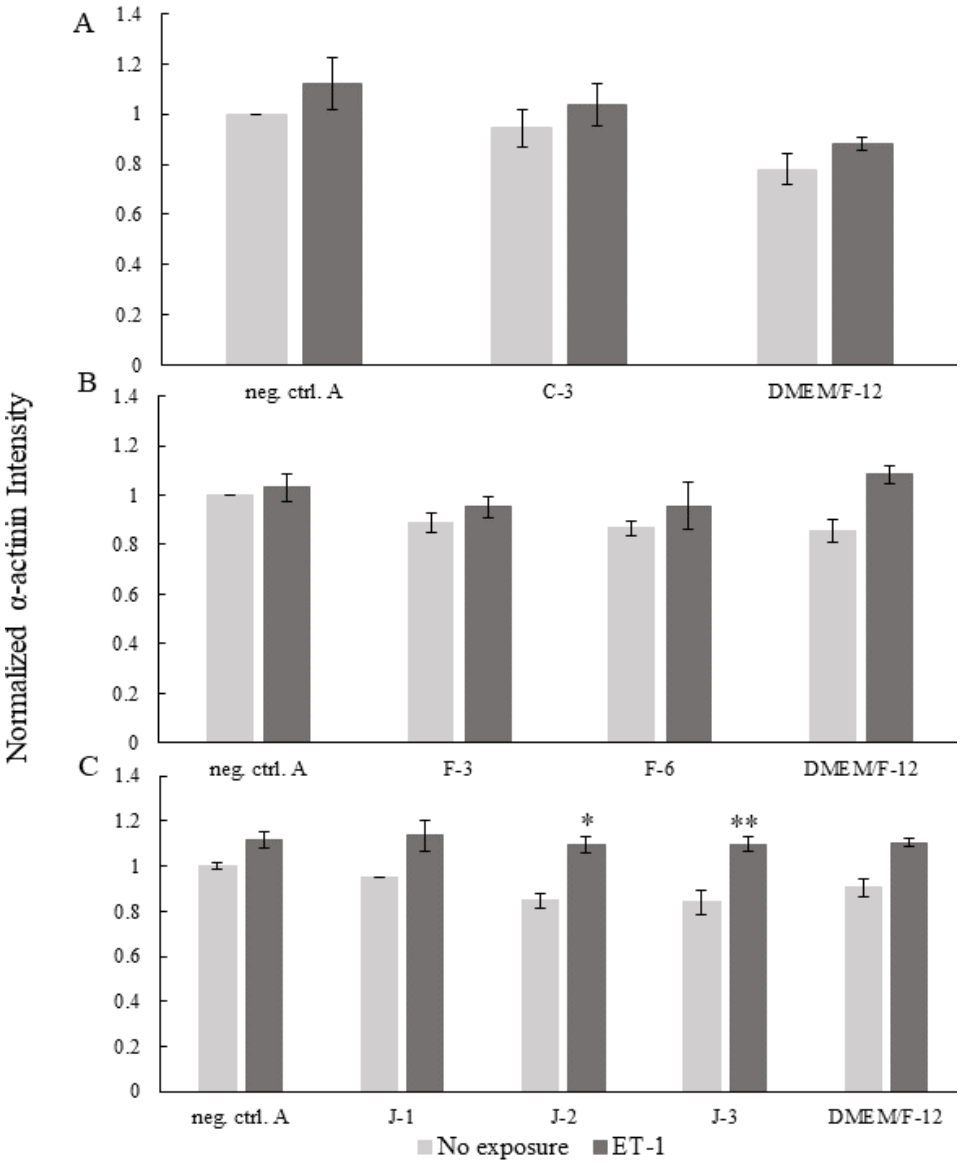

Figure 14: Effects of GapmeR C-3 against lncRNA C (A), GapmeRs F-3, F-6 against lncRNA F (B) and GapmeRs J-1, J-2 and J-3 against lncRNA J (C) and endothelin-1 (ET-1) exposure on α-actinin average intensities in neonatal mouse ventricular cardiomyocytes. *p<0.05 vs. no exposure, **p<0.01 vs. no exposure (one-way ANOVA, Tukey HSD). The results are shown as mean intensity values normalized to negative control A without ET-1 exposure from 3 (A) or 4 (B and C) independent experiments ± standard error of the mean (SEM).

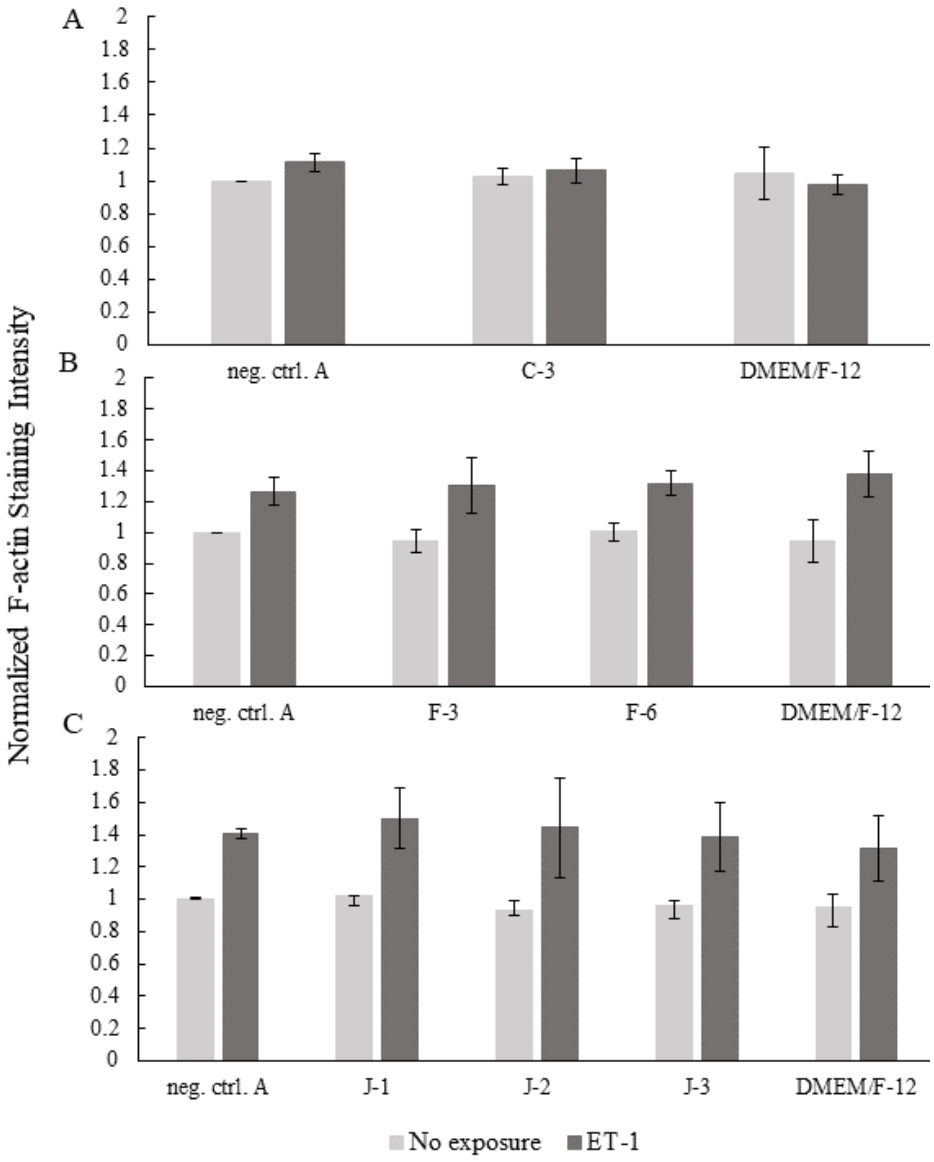

Figure 15: Effects of GapmeR C-3 against lncRNA C (A), GapmeRs F-3, F-6 against lncRNA F (B) and GapmeRs J-1, J-2 and J-3 against lncRNA J (C) and endothelin-1 (ET-1) exposure on F-actin staining total intensities in neonatal mouse ventricular cardiomyocytes. The results are shown as mean intensity values normalized to negative control A without ET-1 exposure from 3 (A) or 4 (B and C) independent experiments ± standard error of the mean (SEM).

In addition to intensities, α-actinin and F-actin fiber alignments were measured to study how hypertrophy and the GapmeRs affect fiber organization. In lncRNA J GapmeR experiments ET-1 decreased F-actin alignment value 5–10% and a few percentages of α-actinin, too, but the differences were not statistically significant (Fig 16–17). Nevertheless, DMEM/F-12 had approximately 10% higher value in α-actinin and F-actin

alignments with ET-1 exposure, which is contradictory to the other groups and DMEM/F-12 controls. The GapmeRs had no effect on fiber alignments.

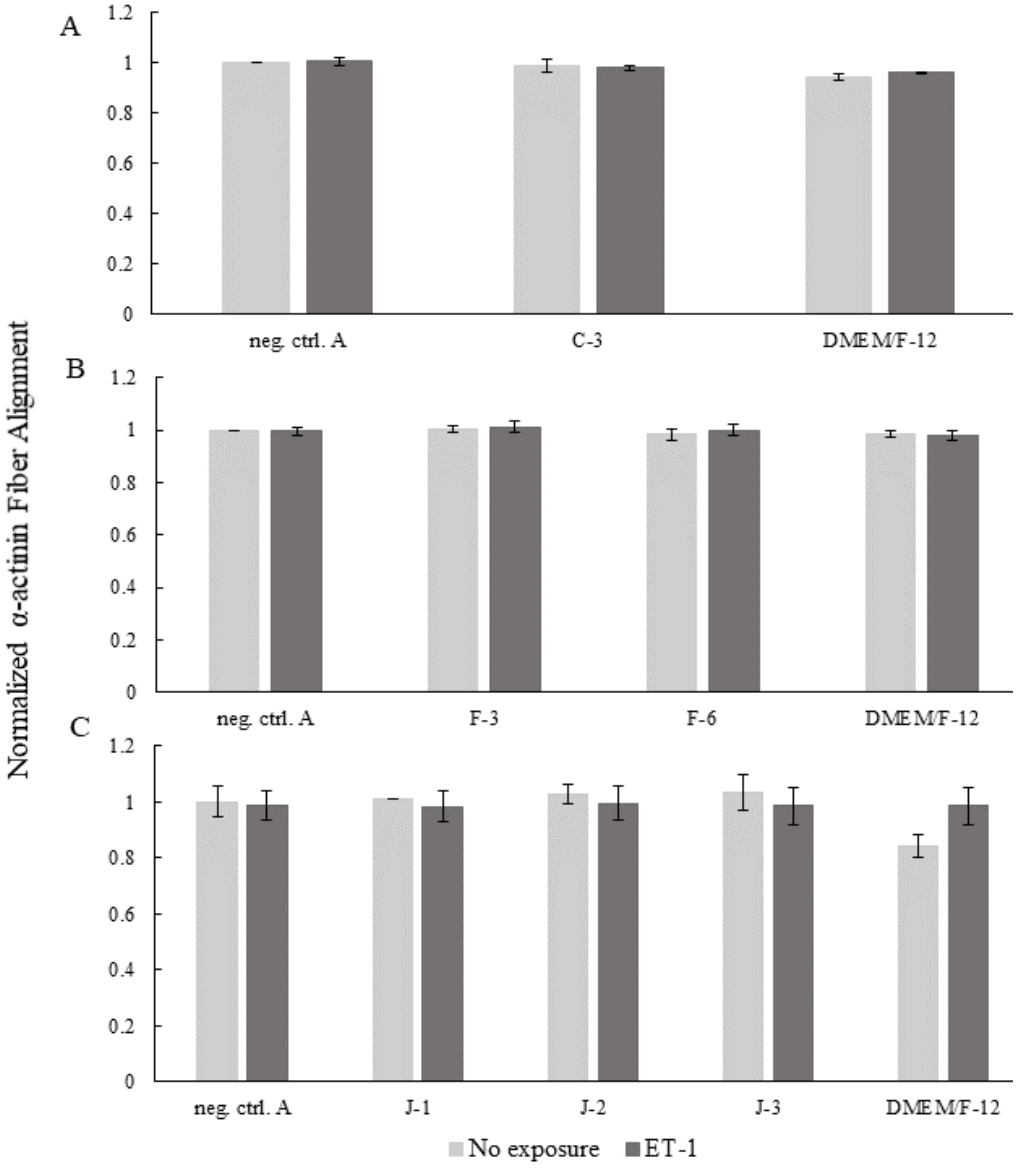

Figure 16: Effects of GapmeR C-3 against lncRNA C (A), GapmeRs F-3, F-6 against lncRNA F (B) and GapmeRs J-1, J-2 and J-3 against lncRNA J (C) and endothelin-1 (ET-1) exposure on nuclear α-actinin fiber alignment in neonatal mouse ventricular cardiomyocytes. The results are shown as mean intensity values normalized to negative control A without ET-1 exposure from 3 (A) or 4 (B and C) independent experiments ± standard error of the mean (SEM).

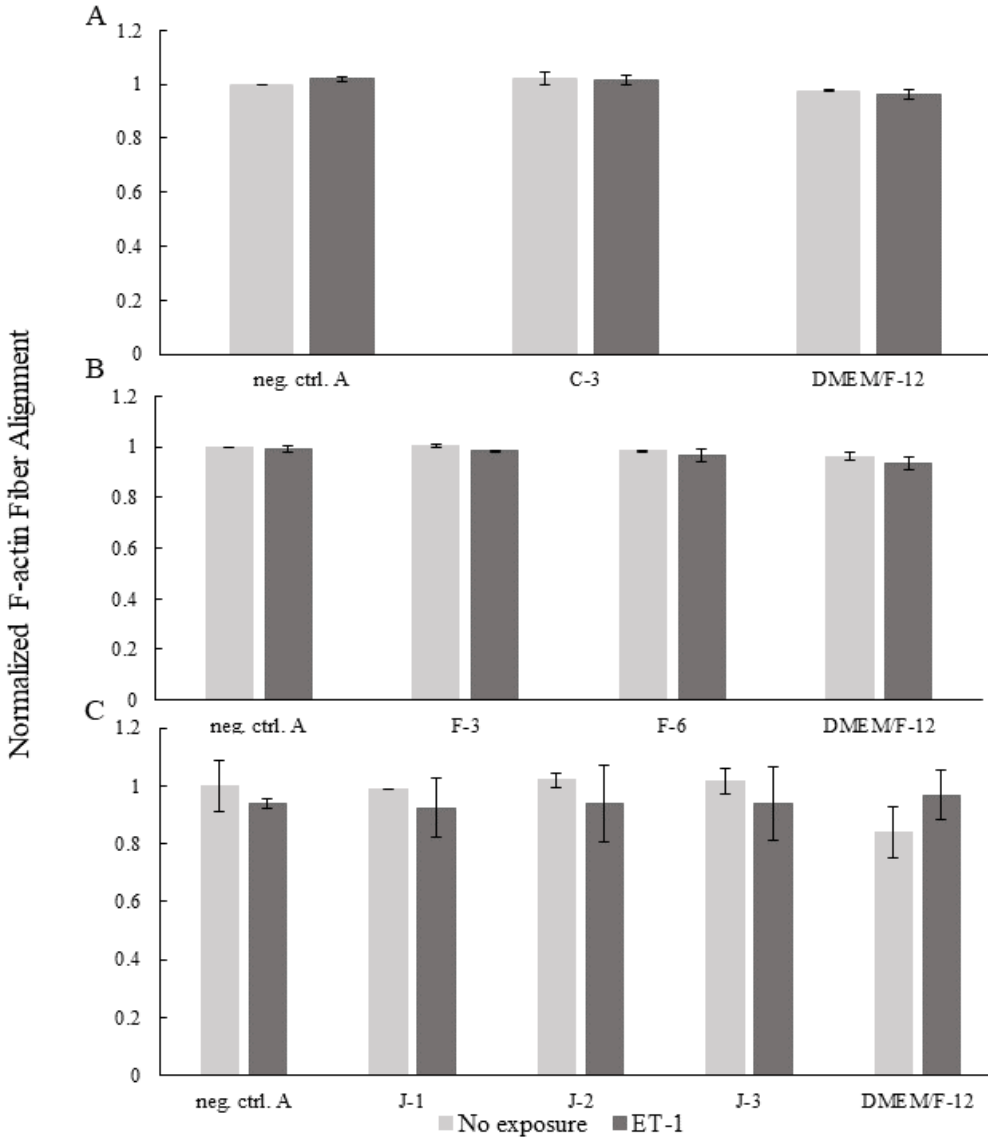

Figure 17: Effects of GapmeR C-3 against lncRNA C (A), GapmeRs F-3, F-6 against lncRNA F (B) and GapmeRs J-1, J-2 and J-3 against lncRNA J (C) and endothelin-1 (ET-1) exposure on nuclear F-actin fiber alignment in neonatal mouse ventricular cardiomyocytes. The results are shown as mean intensity values normalized to negative control A without ET-1 exposure from 3 (A) or 4 (B and C) independent experiments ± standard error of the mean (SEM).

Another way to measure hypertrophic effects on α-actinin and F-actin fibers was the fiber average area. In all transfection groups the average area of α-actinin fibers increased approximately 5–10% with ET-1 treatment compared unexposed cardiomyocytes in lncRNA F and J GapmeR experiments, but these differences were not statistically significant (Fig 18). ET-1 had no effect in lncRNA C GapmeR experiments. In lncRNA F GapmeR experiments ET-1 increased F-actin fiber average area 10–20%. However, in

the case on DMEM/F-12 control in lncRNA F GapmeR experiments the increase was up to 50 % (Fig 19B). There were no statistically significant differences in F-actin fiber areas between individual groups, but the increase in lncRNA F experiments was significant when comparing ET-1 to DMEM-1% BSA alone (p=0.004). In lncRNA C and J GapmeR experiments this ET-1-induced increase was not present. The GapmeRs did not affect α-actinin or F-actin fiber area (Fig 18–19).

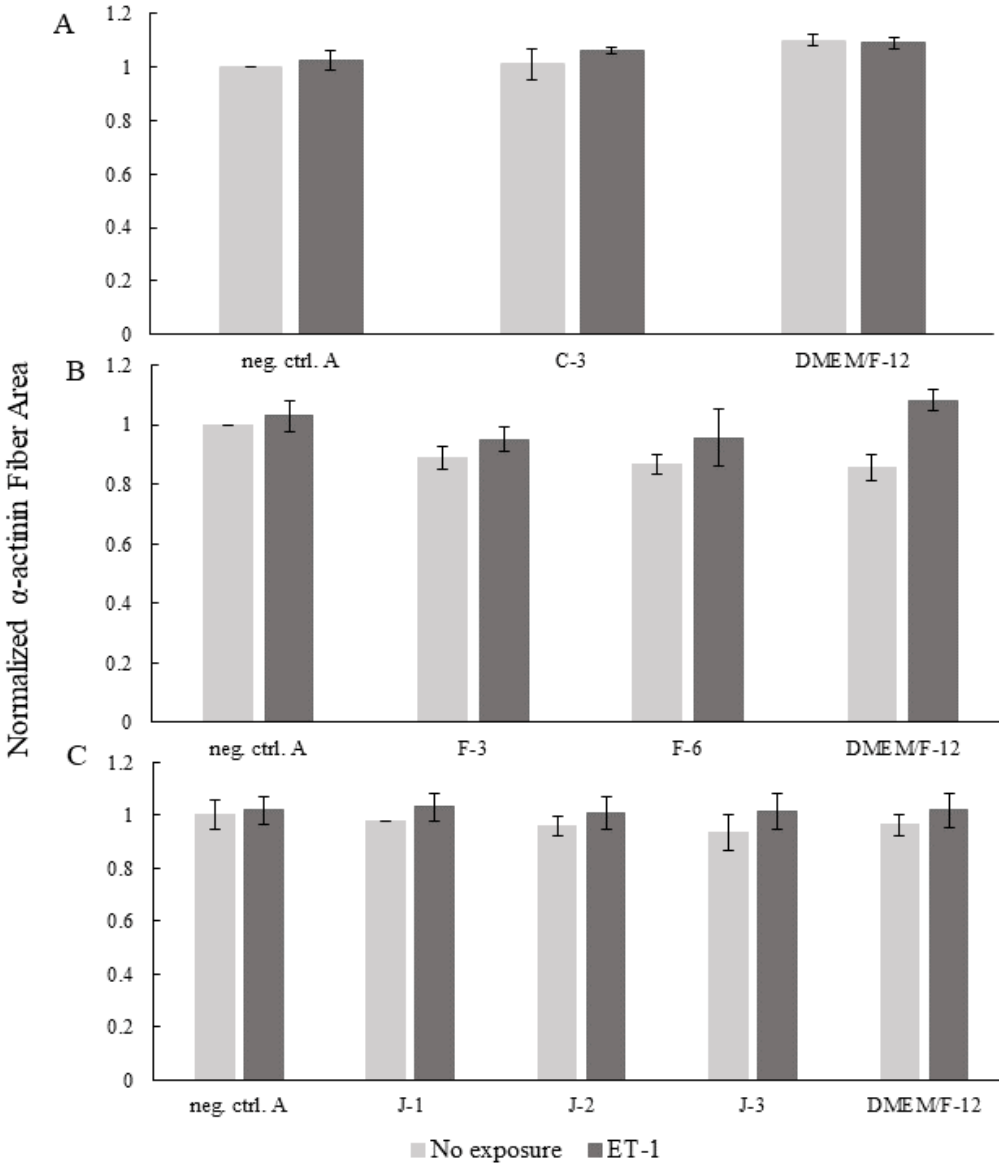

Figure 18: Effects of GapmeR C-3 against lncRNA C (A), GapmeRs F-3, F-6 against lncRNA F (B) and GapmeRs J-1, J-2 and J-3 against lncRNA J (C) and endothelin-1 (ET-1) exposure on nuclear α-actinin fiber average area. The results are shown as mean intensity values normalized to negative control A without ET-1 exposure from 3 (A) or 4 (B and C) independent experiments ± standard error of the mean (SEM).

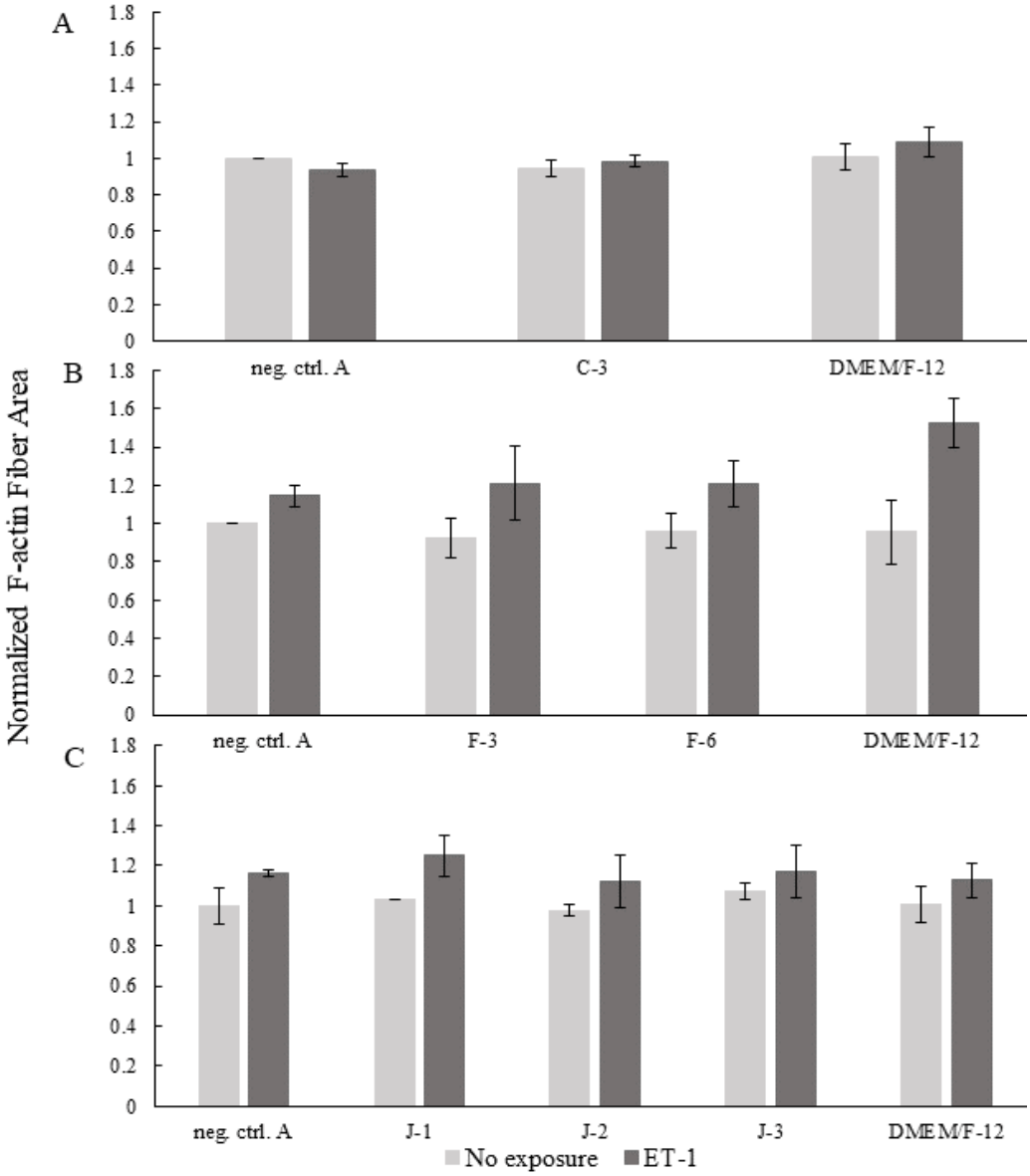

Figure 19: Effects of GapmeR C-3 against lncRNA C (A), GapmeRs F-3, F-6 against lncRNA F (B) and GapmeRs J-1, J-2 and J-3 against lncRNA J (C) and endothelin-1 (ET-1) exposure on nuclear F-actin fiber average area in neonatal mouse ventricular cardiomyocytes. The results are shown as mean intensity values normalized to negative control A without ET-1 exposure from 3 (A) or 4 (B and C) independent experiments ± standard error of the mean (SEM).

To recap the results from the hypertrophy assays, silencing lncRNAs C, F and J with these chosen LNA GapmeRs had no effect on α-actinin and F-actin in neonatal mouse ventricular cardiomyocytes with or without ET-1 exposure. Some marginal differences between groups could be seen but they were not statistically significant. ET-1 stimulation

affected the measured parameters, but the effect was mostly insignificant and sometimes contradictory between groups.

5.1.2 Effects of silencing the long non-coding RNAs on cardiomyocyte apoptosis

In the apoptosis assays the GapmeRs were studied for effects on DAPI, caspase-3/7 reporter and α-actinin first comparing to DOX and then combining with DOX. These experiments gave insight on whether the GapmeRs increase or decrease cardiomyocyte apoptosis and if they are capable of protecting cardiomyocytes from DOX-induced apoptosis. Representative images of cardiomyocytes from the apoptosis assay are shown in Figure 20.

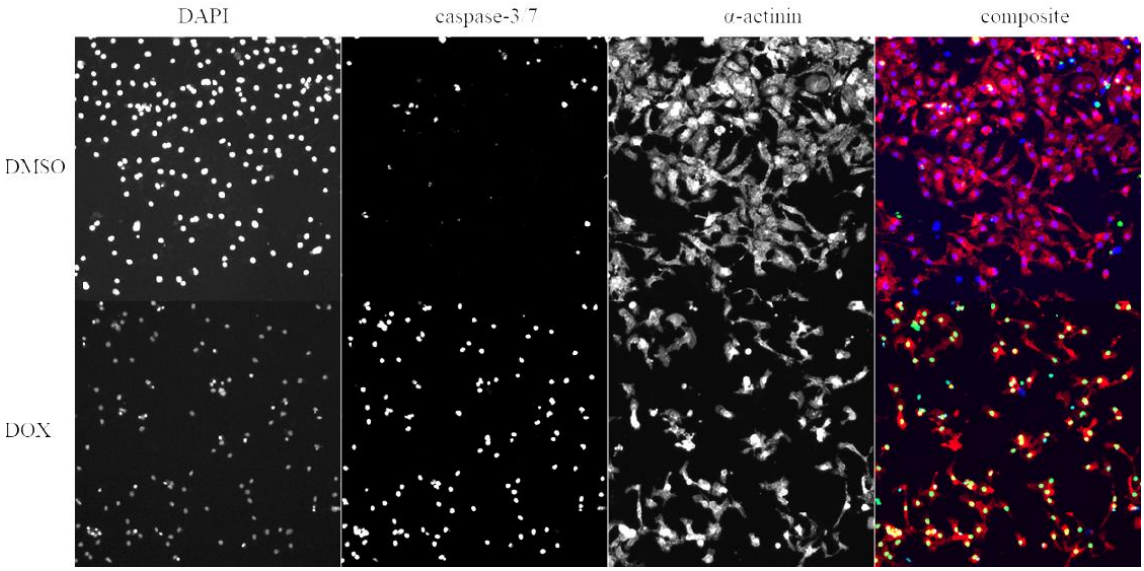

Figure 20: Representative images of neonatal cardiomyocytes from the apoptosis assay, with and without exposure to 1 µM doxorubicin (DOX) for 23 h. The cardiomyocytes were stained with a specific fluorescent antibody for α-actinin, caspase-3/7 reporter and the with DAPI. The top row shows cardiomyocytes without exposure to DOX and the bottom row with the exposure. In the composite image DAPI is blue, caspase-3/7 reporter is green and α-actinin is red.

There was no sign of increased cardiomyocyte apoptosis after silencing lncRNAs C, F or J. DOX-exposed cardiomyocytes had a significantly lower DAPI intensity than those transfected with the GapmeRs (Fig 21). This is due to one of the mechanisms of actions

of DOX; degradation of the DNA double helix structure. When cardiomyocytes were transfected with the GapmeRs together with DOX exposure, the obtained results were similar. In all experiments exposure to 1 µM DOX for 23 hours reduced the intensity of DAPI approximately 60% compared to corresponding control groups in DMSO, which was statistically significant in lncRNA J GapmeR experiments ($p<0.001$) (Fig 21). The GapmeRs did not affect DAPI intensity.

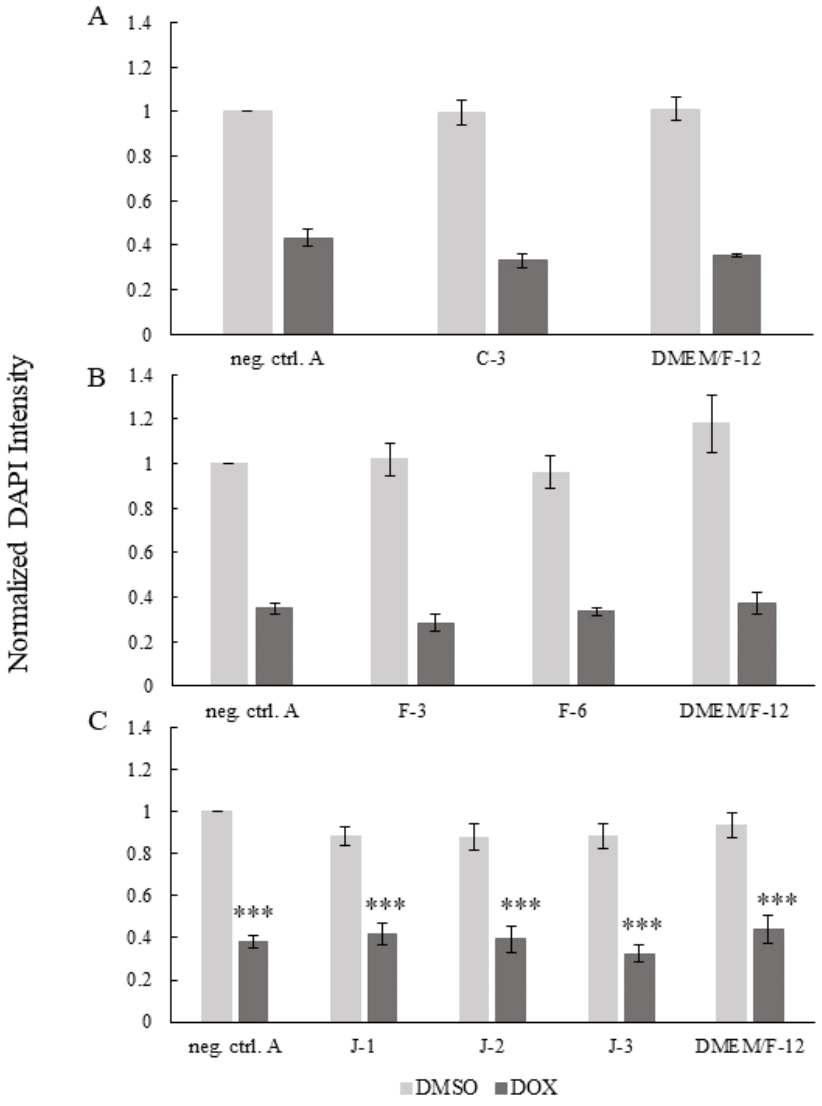

Figure 21: Effects of GapmeR C-3 against lncRNA C (A), GapmeRs F-3, F-6 against lncRNA F (B) and GapmeRs J-1, J-2 and J-3 against lncRNA J (C) and doxorubicin (DOX) on nuclear DNA staining intensity in neonatal mouse ventricular cardiomyocytes. ***$p<0.001$ vs. DMSO (one-way ANOVA, Tukey HSD). The results are shown as mean intensity values normalized to negative control A without DOX exposure from 2 (A and B) or 3 (C) independent experiments ± standard error of the mean (SEM).

Caspase reporter intensity was significantly increased in DOX treated cardiomyocytes. In the first experiments, where DOX and lncRNA J GapmeRs were applied individually, the cells were divided into caspase positive and negative groups by setting a threshold in the intensity range. Then, the percentage of caspase positive cells was measured. The results showed nearly no apoptotic cells at all in the LNA GapmeR transfected groups (<5%), while in DOX treated cardiomyocytes the rate more than 95% (Fig 22). To further highlight the effect of DOX, cardiomyocytes exposed to 3 µM had a nearly 20-fold higher caspase intensity compared to DMSO controls (Fig 23). There was a clear dose-dependent effect, as 1 µM increased the intensity only 5-fold.

The difference between DOX and DMSO was also seen when cardiomyocytes were transfected with the GapmeRs together with DOX exposure (Fig 24). In lncRNA J GapmeR experiments the effect of DOX was significant with J-3 and DMEM/F-12 (p=0.021). The GapmeRs had no effect on caspase reporter intensity (Fig 23–24).

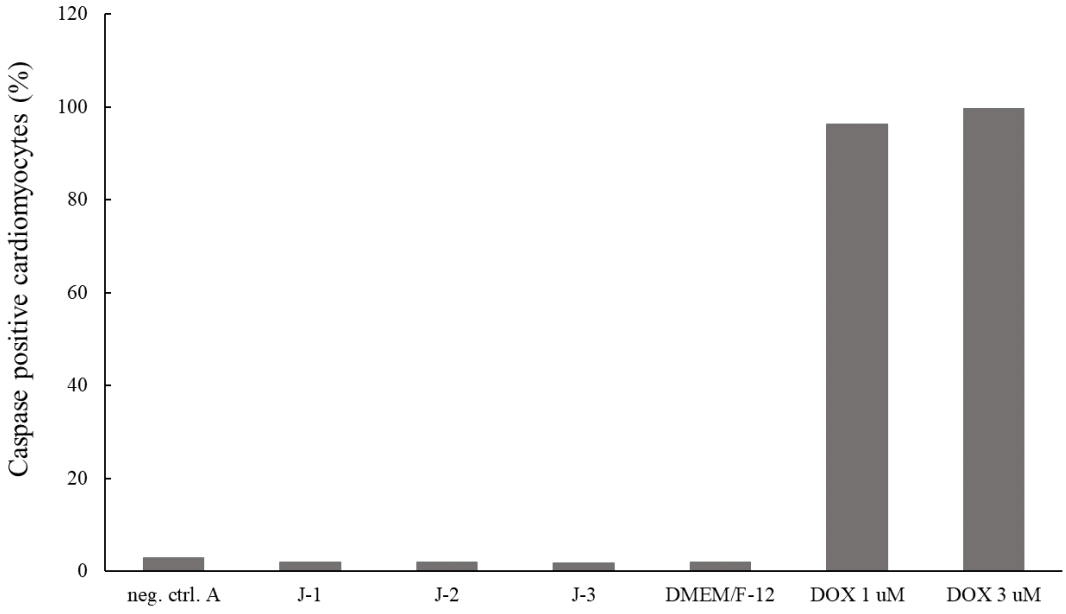

Figure 22: Effects of GapmeRs J-1, J-2 and J-3 against lncRNA J and doxorubicin (DOX) exposure on the percentage of neonatal mouse ventricular cardiomyocytes with caspase-3/7 activity. DOX exposed cardiomyocytes were > 95% caspase positive, whereas in the other groups the value was < 5%. The results are shown as percentages of caspase positive cardiomyocytes from one individual experiment.

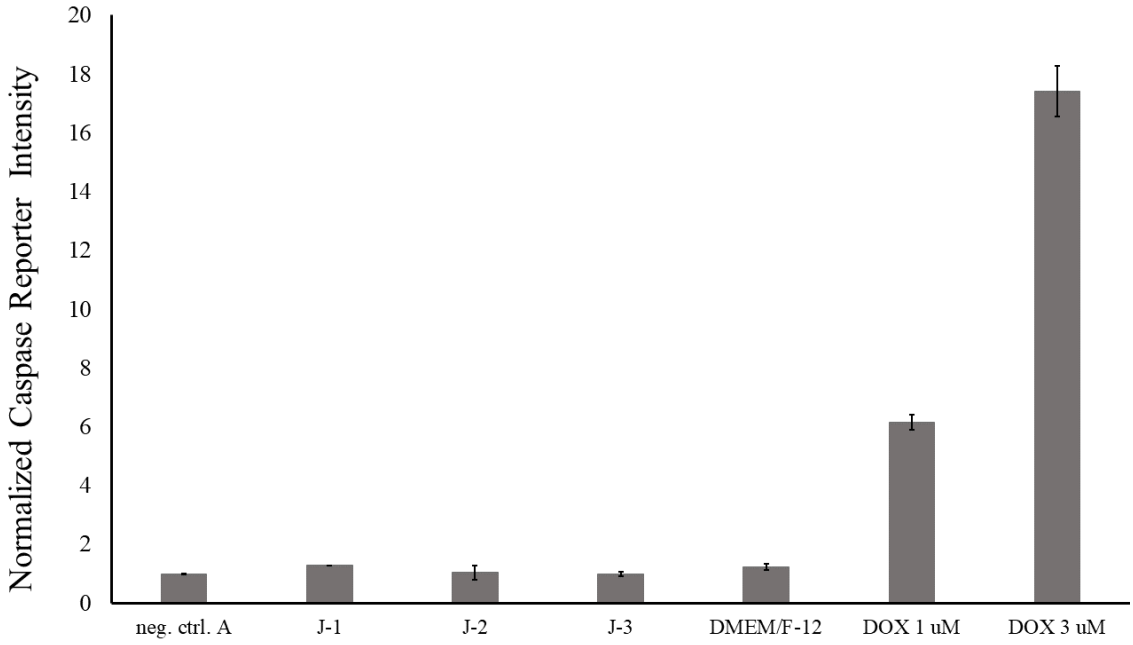

Figure 23: Effects of GapmeRs J-1, J-2 and J-3 against lncRNA J and doxorubicin (DOX) exposure on nuclear caspase-3/7 intensity in neonatal mouse ventricular cardiomyocytes. The results are shown as intensity values normalized to negative control A from two individual experiments ± standard error of the mean (SEM).

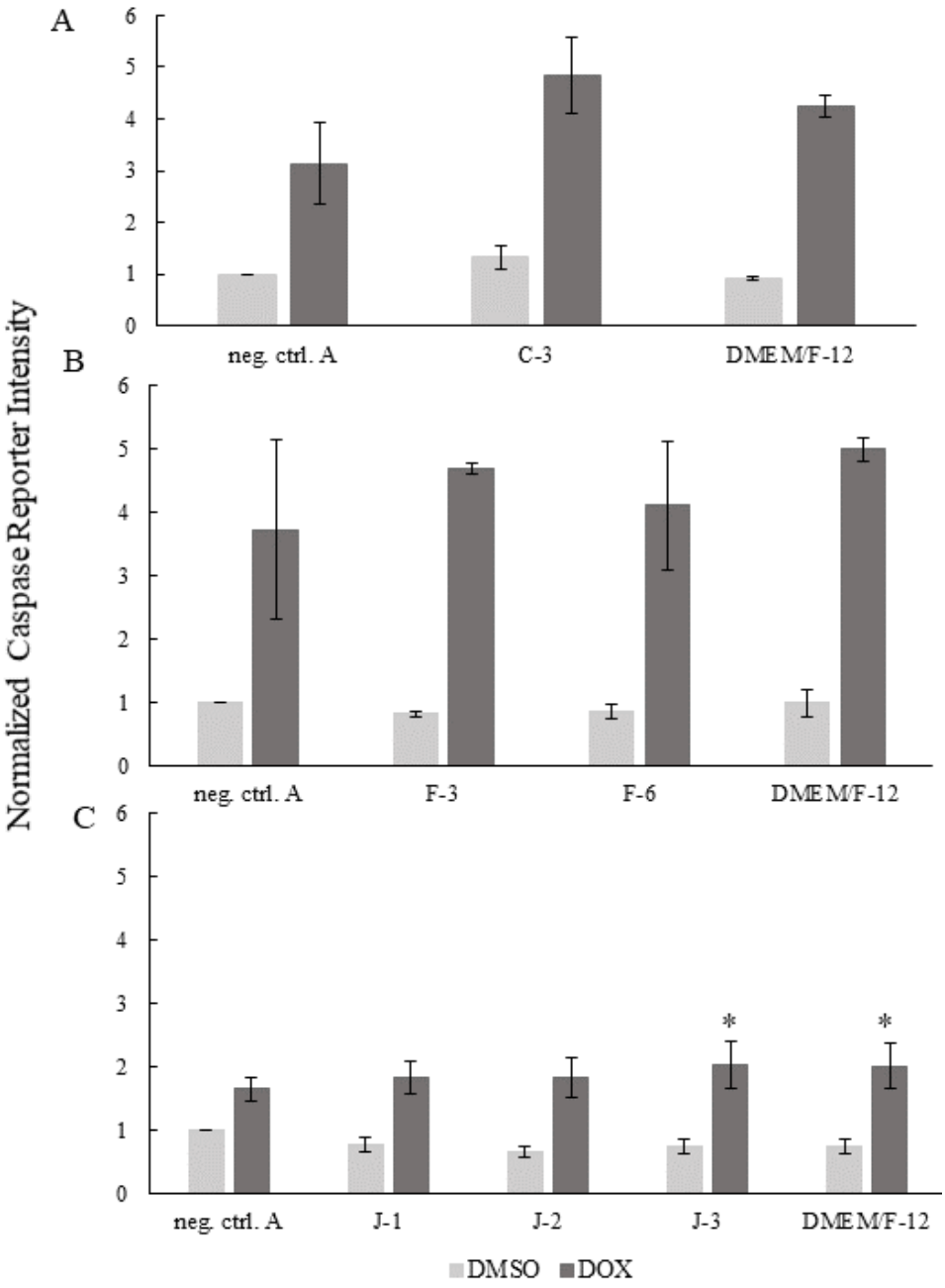

Figure 24: Effects of GapmeR C-3 against lncRNA C (A), GapmeRs F-3, F-6 against lncRNA F (B) and GapmeRs J-1, J-2 and J-3 against lncRNA J (C) on nuclear caspase-3/7 reporter intensity in neonatal mouse ventricular cardiomyocytes with and without doxorubicin (DOX) exposure. *$p<0.05$ vs. DMSO (one-way ANOVA, Tukey HSD). The results are shown as mean intensity values normalized to negative control A without DOX exposure from 2 (A and B) or 3 (C) independent experiments ± standard error of the mean (SEM).

DOX exposure also increased nuclear α-actinin intensity. This moderate rise could first be seen in the experiments where lncRNA J GapmeRs and DOX were studied independently, although the effect was not statistically significant (Fig 25). In the following studies that combined GapmeR transfections and DOX exposure the difference was shown again. In lncRNA J and F GapmeR experiments DOX increased the α-actinin intensity approximately 10–35 % (Fig 26). In GapmeR C-3 experiments the increase was less than 10% but overall, none of the differences between individual groups were statistically significant. The tested GapmeRs had no effect on α-actinin intensities (Fig 25–26).

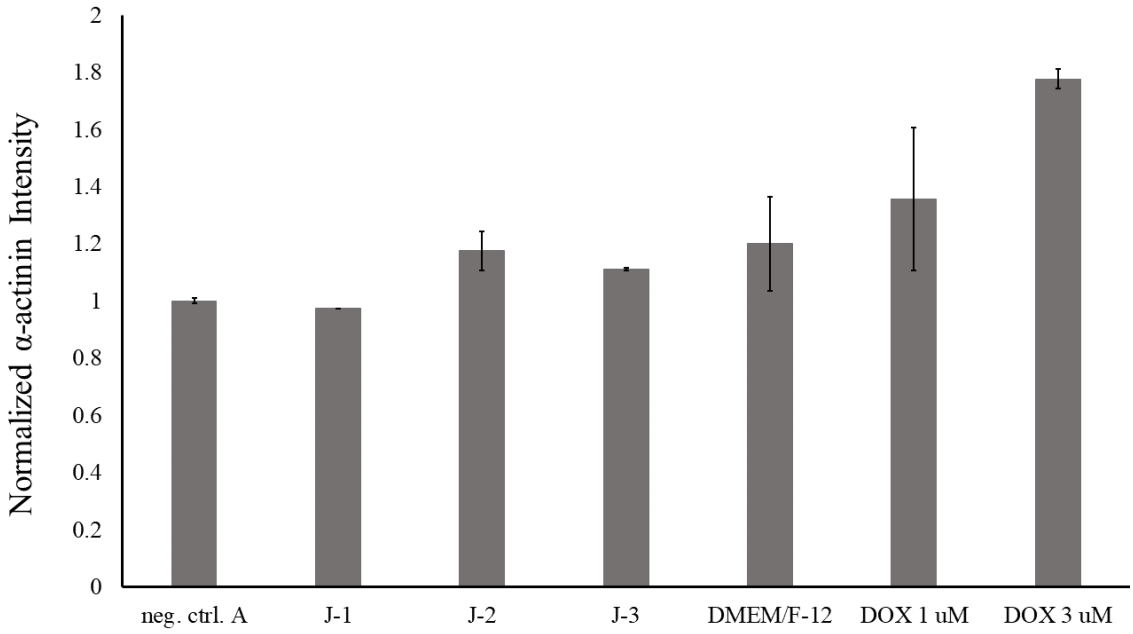

Figure 25: Effects of GapmeRs J-1, J-2 and J-3 against lncRNA J and doxorubicin (DOX) exposure in nuclear α-actinin intensity in neonatal mouse ventricular cardiomyocytes. The results are shown as intensity values normalized to negative control A from two individual experiments ± standard error of the mean (SEM).

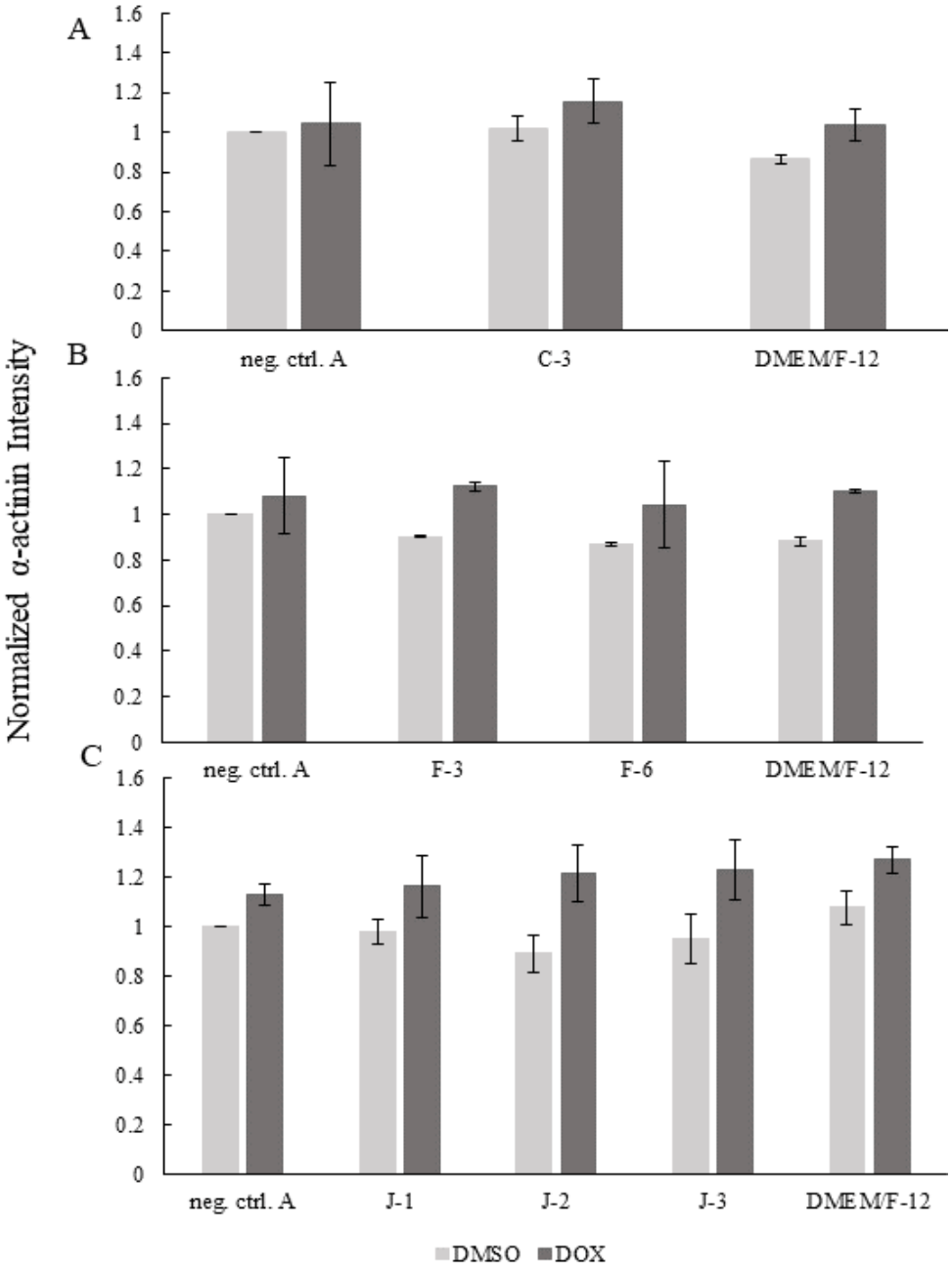

Figure 26: Effects of GapmeR C-3 against lncRNA C (A), GapmeRs F-3, F-6 against lncRNA F (B) and GapmeRs J-1, J-2 and J-3 against lncRNA J (C) on nuclear α-actinin intensity in neonatal cardiomyocytes with or without doxorubicin (DOX) exposure. The results are shown as mean intensity values normalized to negative control A without DOX exposure from 2 (A and B) or 3 (C) independent experiments ± standard error of the mean (SEM).

Overall, these results showed that the tested LNA GapmeRs did not increase apoptosis in neonatal mouse ventricular cardiomyocytes compared to or combined with DOX exposure. On the other hand, they neither had any protective effect on cardiomyocytes in

DOX-induced apoptosis. Treating cardiomyocytes with 1 or 3 μM DOX significantly reduced the intensity of DAPI, which indicated degradation of DNA, and increased the intensity of caspase-3/7 activity reporter. In addition, an increase in α-actinin increase could be seen because of apoptosis-induced degradation of the cytoskeleton in cardiomyocytes.

5.2 Setting up and optimizing a compound screening assay for GATA4-FOG2 interaction

The second experimental part of the project aimed at setting up a luciferase assay to screen for compounds that affect the protein-protein interaction between GATA4 and FOG2. This begun by finding a suitable ratio between the two transfection factors combined with the GATA4-dependent reporter plasmid NP-112. In the first experiments, the amount of pMT2-GATA4 was fixed to a solid 25 ng/well together with 100 ng/well of NP-112 and testing concentrations between 10 and 45 ng/well of pCS2-FOG2. There was not much variation between these groups, but control group with only pMT2-GATA4 gave a seven-fold signal compared to those wells transfected with both pMT2-GATA4 and pCS2-FOG2, suggesting the GATA4-inhibitory effect of FOG2 is indeed strong in this in vitro culture (Fig 27). In addition, the average signal from wells transfected with pMT2-GATA4 and pCS2-FOG2 was not higher than that from empty plasmid pMT2 control group.

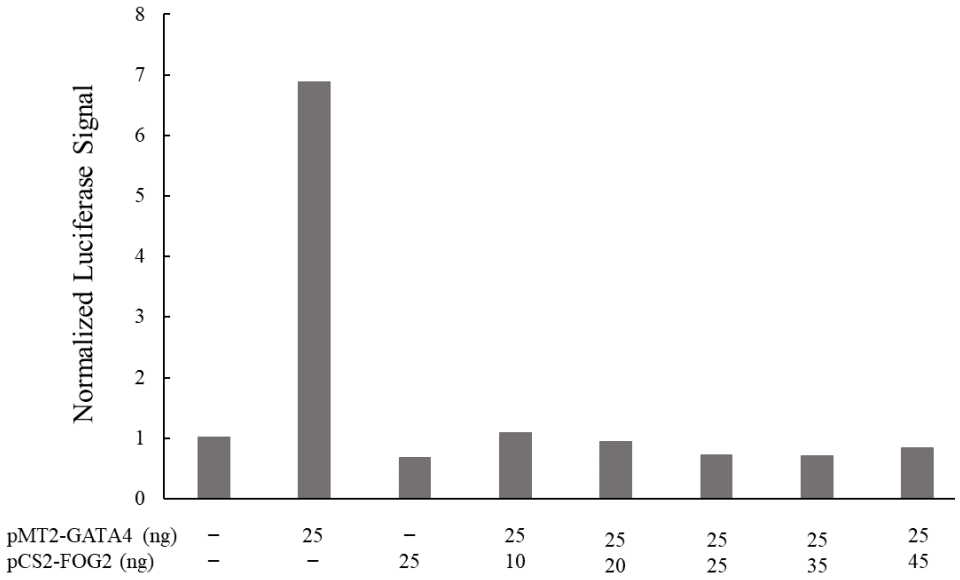

Figure 27: Luciferase signals from different ratios of combinations of pMT2-GATA4 and pCS2-FOG2 in COS-1 cells. The numbers in each group stand for nanograms of the plasmid in question per well. These groups had 100 ng/well of luciferase reporter vector NP-112. The results are shown as mean luciferase signal values from one experiment normalized to the empty plasmid group.

Because of these results, in order to see a clear signal from the combination groups as well, the amounts of pMT2-GATA4 had to be increased. Obviously, the amount of pCS2-FOG2 had to be very small compared to pMT2-GATA4 but decreasing that amount from the previous trial would have been impossible to pipet. For the next experiments three groups of pMT2-GATA4 were added – 30 ng, 60 ng and 100 ng per well – to see if at some of these levels saturation point would be reached and would therefore be useless for screening. In the same experiment, all these three levels of pMT2-GATA4 were combined with a one tenth, one third or an equal amount of pCS2-FOG2. Additionally, 30 and 60 ng/well of pCS2-FOG2 were tested alone. Each group had 150 ng/well of NP-112. Luciferase signals were still significantly higher when pMT2-GATA4 was not disturbed with the inhibitory pCS2-FOG2 (Fig 28). Because 30 ng/well of pMT2-GATA4 resulted in a higher signal than 60 or 100 ng/well, it was safest to opt for the combinations with 30 ng/well, too, in order to avoid saturation of NP-112. The highest signal from 30 ng/well of GATA4 combined with pCS2-FOG2 was the group with 3 ng/well of the latter one. Therefore this ratio was for compound screening (Fig 28).

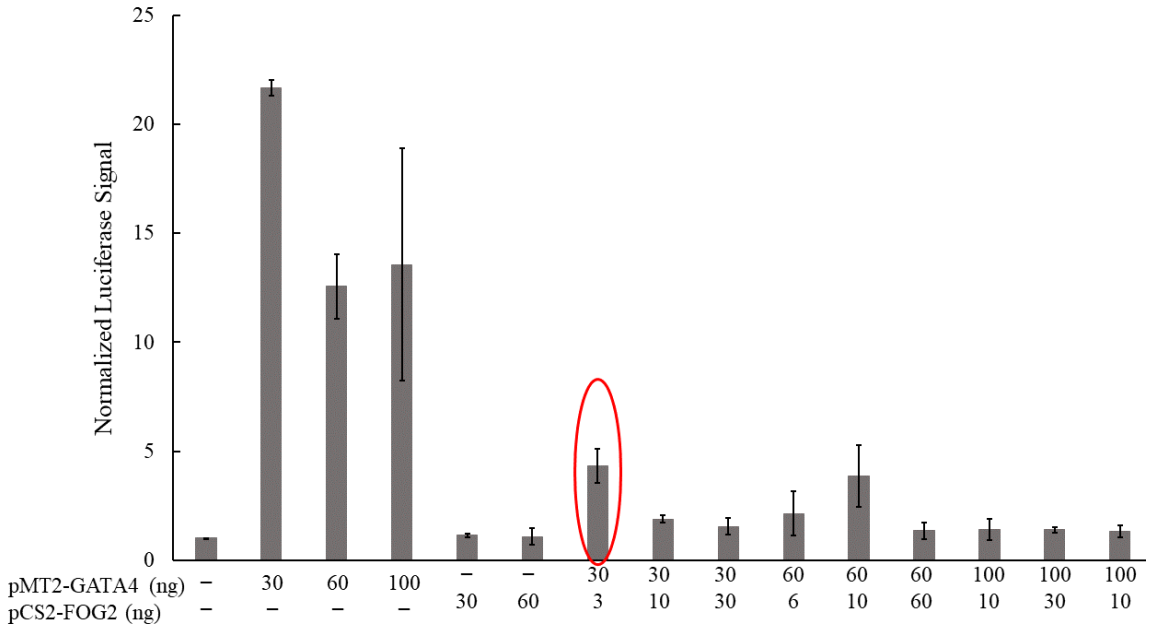

Figure 28: Luciferase signals from a variety of rational combinations of pMT2-GATA4, pCS2-FOG2 and empty pMT2 plasmids in COS-1 cells. The numbers in each group stand for nanograms of the plasmid in question per well. These groups had 150 ng/well of luciferase reporter vector NP-112. The results are shown as mean luciferase signal values from three independent experiments normalized to the empty plasmid group ± standard error of the mean (SEM).

Test compounds 3i-1000, 3i-777, 3i-1027, 3i-1157 and 3i-1083 were chosen for this assay, and COS-1 cells were exposed to them for 24 hours at concentrations of 3 µM and 10 µM. The results shows again that pMT2-GATA4 alone produces by far the strongest signal (Fig 29A). The highest signal of test compounds resulted 10 µM of 3i-777 but it was not remarkably higher than control group with 30 ng of pMT2-GATA4 + 3 ng of pCS2-FOG2 without compound exposure (Fig 29B). All the other compounds and concentrations reduced the signal. Most compounds yielded dose-dependent results; a higher signal with a higher concentration. However, the results were in opposite order for 3i-1027.

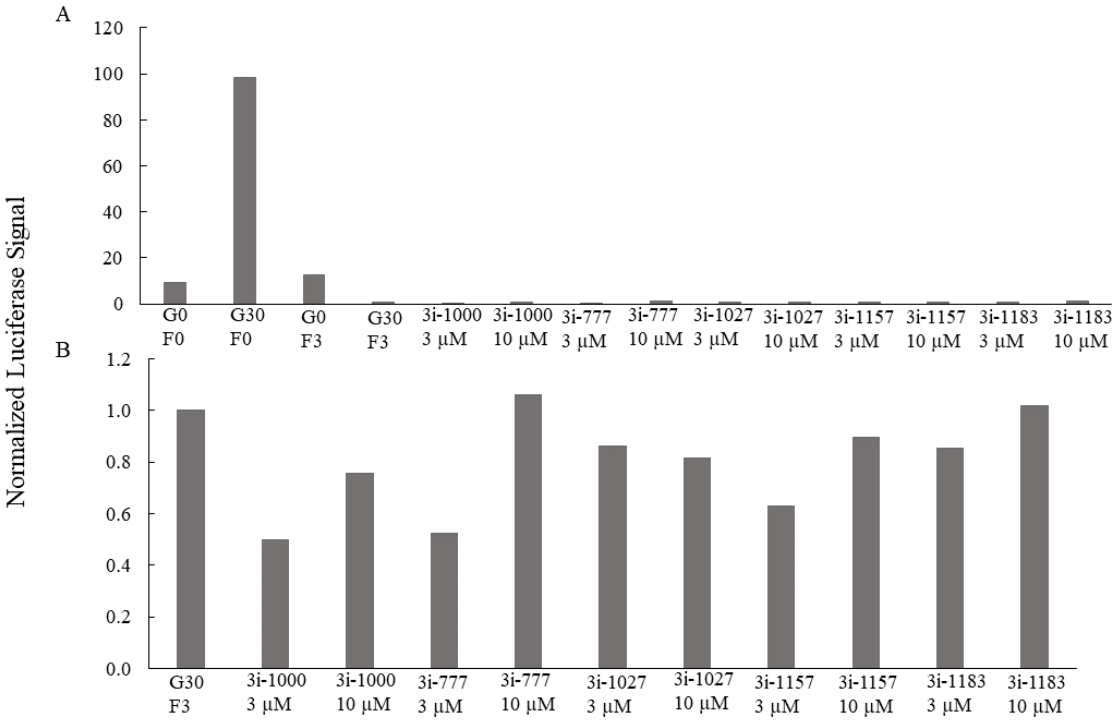

Figure 29: Luciferase signals from COS-1 cells transfected with pMT2-GATA4 and pCS2-FOG2 and exposed to protein-protein interaction affecting compounds. Capital G stands for pMT2-GATA4 and capital F for pCS2-FOG2. The number after letters stands for nanograms of the plasmid in question per well. (A) Luciferase signals from all test and control groups revealed a 100-fold higher signal from G30F0 compared to G30F3. (B) Luciferase signals from only the test groups and their non-exposed control group G30F3 showed the effects of the compounds. These groups had 150 ng/well of luciferase reporter vector NP-112. The results are shown as mean luciferase signal values from one experiment normalized to G30-F3 group.

6 DISCUSSION

Studies all over the world have identified cardiac-selective lncRNA sequences, and as lncRNAs broadly regulate genetic expression and thus the diverse functions of cell they carry a remarkable potential for medical treatments to be developed. In addition, they are highly tissue selective, which can be beneficial in applying them to therapeutic use. The aim of this study was first of all to identify if our three lncRNAs from our group's selection have an effect on hypertrophic responses in neonatal mouse cardiomyocyte *in vitro* cultures and to examine if they increase apoptosis.

Looking at the results from the hypertrophy assays, most differences are between ET-1 and DMEM-1% BSA. This project did not aim at studying the effects of ET-1 as they are already well established (Archer et al. 2017). ET-1 is a vasoconstrictive peptide, which increases blood pressure both independently and by stimulating RAS (Haque et al. 2013). Therefore the increase in α-actinin and F-actin intensities and fiber areas was predictable. Because hypertrophy degrades the organization of sarcomere units, it would be predictable that fiber alignment decreases with ET-1 exposure. In these results, however, ET-1 increased α-actinin and F-actin fiber alignment for the most part albeit the differences were only 1–8 % and not statistically significant. On the other hand, it is impossible to conclude the effect of ET-1 on F-actin fibers alignment in particular from these results as they were contradictory between different sets of experiments.

Overall, it is noteworthy to look into the variation between control groups from different sets of experiments. Basically, negative control and DMEM/F-12 groups were similar in all three experimental sets. The only ones differing were lncRNA J GapmeR experiments as they had a separate time point. LncRNAs C and F GapmeR experiments, on the other hand, should have had similar control groups. However, their results did not align in most cases, indicating that something else than ET-1 the GapmeRs only was also affecting the cardiomyocytes. Assuming that is the case, this factor would also have had an impact on ET-1 and GapmeR results. On the other hands, some experiments were only carried out twice, so carrying out more repetitions could possibly level out the control groups.

The GapmeRs had very little if any impact on the hypertrophic factors investigated, suggesting that silencing the lncRNAs of interest have little effect on hypertrophy. The chosen lncRNAs were selected based on findings by Pohjolainen et al. (unpublished), which indicated these lncRNAs were cardiomyocyte-selective and go through significant changes in their expression patterns in neonatal mouse cardiomyocytes during the first postnatal days. Based on the results of this project, their role in the cardiomyocytes may cover other duties than stress response and growth or contributing to them via other pathways. On the other hand, it is also possible that the GapmeRs were inefficient in silencing their target lncRNAs. In that case the lncRNAs may contribute to hypertrophy but the changes were not detected due to defective GapmeRs. Therefore, further studies

with more LNA GapmeRs can produce different results, and lncRNAs C, F and J are still worth investigating.

In this project, high content screening was used to detect α-actinin and F-actin. Despite their relevance in hypertrophy, no previous data shows these lncRNAs would regulate their expression in particular. Assuming the GapmeRs were functional, it could be that their target lncRNAs do not explicitly affect α-actinin and F-actin, which in these experiments were biomarkers for phenotypic change. Another approach to the subject could be analyzing how the lncRNAs effect cardiac genes on chromatin level. Han et al. (2014), for example, took this approach with their newly discovered cardiac lncRNA. First, they discovered that their newly found cluster of lncRNAs interacts the gene locus *Myh7* and only after that started analyzing the proteins they affect. On the other hand, as α-actinin and F-actin were biomarkers for phenotypic change instead of proteins we aimed at quantifying, more parameters could have been measured from their intensities. In addition to only fiber area, the area of the cardiomyocytes could have been measured, for example.

Understanding how lncRNAs affect hypertrophic factors and the expression of hypertrophic genes is a key point in investigating their functions. Another way to detect if the lncRNAs in question have to do with hypertrophy is to see if their natural expression is affected by hypertrophic stimuli – such as ET-1 or ATII. ET-1 and ATII are known to induce hypertrophic proteins, such as ANP and BNP, and the expression of hypertrophic genes (Hu et al. 1988; Magga et al. 1998) and would therefore likely be reliable markers in lncRNA research too.

What was alarming, LNA GapmeR negative control A, which should not cause any kind of effect, was often the most active one when compared to DMEM/F-12 medium controls although the differences were not statistically significant. The high importance of a functional negative control was thoroughly explained by Lipsitch et al. (2010), who described the fundamental role of negative controls: “The essential purpose of a negative control is to reproduce a condition that cannot involve the hypothesized causal mechanism but is very likely to involve the same sources of bias that may have been

present in the original association". In this case, negative control A must be re-evaluated with other assays and studied for possible gene-regulatory effects. If the positive result from negative control was only present in one set of experiments, the reason behind would likely be an error in pipetting or cell conditions among other factors, but the same phenomenon appears in all lncRNA GapmeR assays and even in both hypertrophy and apoptosis assays. On the other hand, like discussed previously in the differences between similar control groups, if one experiments with GapmeRs against one lncRNA showed different levels between negative control A and DMEM/F-12, the same difference was likely not present in experiments with GapmeRs against another one – comparing lncRNA F and J experiments on the same parameter, for example. In that case, repeating the experiments could level out the differences.

Results from the apoptosis assays imply the used GapmeRs definitely do not increase apoptosis in cardiomyocytes. That suggests further that silencing the lncRNAs of interest does not increase it either. The positive control for apoptosis assays, DOX, also works as supposed, increasing the reliability of the results. DAPI intensities were significantly lower in DOX exposed cardiomyocytes, whereas caspase-3/7 was highly active in those wells. In addition to caspase-3/7, there was also a higher α-actinin intensity in the nuclei of DOX exposed cardiomyocytes. This is likely not because of a sudden higher expression of α-actinin, but accumulation of it around the nucleus as the cytoskeleton of the cardiomyocyte collapses and they become rounded (Kang et al. 2000). The GapmeRs were also studied in DOX-induced apoptosis, in which they appear not to have inhibitory but neither protective effects.

Working with neonatal mammal cardiomyocytes includes plenty of risks that can alter the results, too. From detaching the hearts to plating the cardiomyocytes there are various steps during which wrong conditions could kill a part of them. Cardiomyocytes are highly sensitive to changes in the temperature or using too much force during pipetting, among others. Several well plates had to be discarded during the project as the cardiomyocytes looked visibly ill under the microscope. This had to do with improper gelatin coating of well plates, wrong temperatures during the isolation process and uneven cell densities on. Despite attempting to only choose viable- and healthy-looking cardiomyocytes for high

content screening, there is more than what meets the eye: it is impossible to detect all the tiny effects changes in the environment may have caused to the cells.

The second part of this project was to set up a compound screening assay for GATA4-FOG2 interaction in COS-1 cells utilizing a GATA4-dependent luciferase reporter plasmid NP-112. This required optimizing the transfection ratio between GATA4- and FOG2-transcripting plasmids in order to see the effect of the interaction yet get a clear luciferase signal to be able to use the reporter assay for compound screening. Unlike cardiomyocytes, COS-1 are quick to attach and divide rapidly. The search for the optimal ratio started with using a fixed 25 ng/well of pMT2-GATA4 plasmid and from 10 to 45 ng/well of pCS2-FOG2. It was immediately clear that FOG2 strongly inhibits GATA4 in COS-1 cells as the luciferase signal dropped to approximately one seventh when even the smallest amount of FOG2 was added. This was neither a surprise nor really presupposed, as previous studies have found that FOG2 can both inhibit and enhance the effects of GATA4 (Lu et al. 1999; Svensson et al. 1999). Next, pMT2-GATA4 was upscaled, pCS2-FOG2 was tested in ratios between 1:10 to 1:1 in relation pMT2-GATA4, and NP-112 was increased to 150 ng/well. From these results a suitable option for compound screening was found. Compound screening was carried out with a NP-112:pMT2-GATA4 ratio of 5:1 (150 and 30 ng/well) and pMT2-GATA4-pCS2-FOG2 ratio of 10:1 (30 ng and 3 ng) (Fig 30).

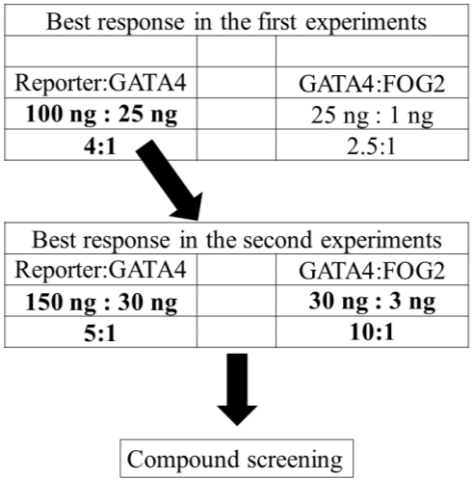

Figure 30: Optimizing the ratios of luciferase reporter and transcription factor plasmids. The first results indicated transfecting reporter and GATA4 plasmids 4:1 was a suitable combination for a clear luciferase signal. Different ratios of FOG2-plasmid in relation to GATA4 were tested in the second experiments. The best response was achieved with reporter:GATA4 ratio 5:1 and GATA4:FOG ratio 10:1.

In previous studies by our group 3i-1000 has been shown to inhibit and 3i-777 to enhance the interaction of GATA4 with its other known cofactor, NKX2-5 (Välimäki et al. 2017). Later on, as more compounds were developed, a new analog 3i-1157 was found to be the most potent synergy inhibitor when using a very similar luciferase assay as in this project (Jumppanen et al. 2019). Instead, 3i-1183 did not affect GATA4-NKX2-5 interaction. In addition to synergy inhibition, 3i-777 and 3i-1157 significantly decreased the expression of BNP in cardiomyocytes. In the GATA4-FOG2 compound screening in this project, 10 µM 3i-777 was only compound inhibiting GATA4-FOG2 interaction, although the difference was rather marginal. Apart from that compound and concentration, all other test groups moderately enhanced the interaction or had no effect at all. However, these results were from a single experiment only. Repetitions need to be carried out before drawing any conclusion on how the compounds affect GATA4-FOG2 interaction.

Despite COS-1 cells being easier to handle than cardiomyocytes, the transfection procedure in particular was prone to errors. It required pipetting very small amounts (<0.5 µl), which is demanding to do precisely. This could explain why there was a relatively large range of variation in these experiments. One way to tackle this problem would be upscaling the amount of plasmids even more, but in this case the empty plasmid pMT2 would need to be upscaled, too, and the total amount of DNA would become quite high. Despite not coding for any protein, the overall amount of empty pMT2 can result in a small luciferase signal as well. If the total amount of DNA per well continuously grew, this fault signal, would grow in the same ratio and interfere with the results. Another reason why upscaling cannot be endlessly used is saturation: at some point, pMT2-GATA4 will yield in such high amounts of GATA4 that the binding sites at NP-112 luciferase become saturated and so the increased GATA4 no longer increases the signal.

Previous experiments with a luciferase reporter and GATA4-FOG2 interaction have been carried out in studying how the transcription factor complex affects certain genes (e.g. Lu et al. 1999, Jia and Takimoto 2003). These studies have also shown how much the results can vary between cell types and promoters, making it difficult to generalize any obtained results. Targeting GATA4-FOG2 interaction has plenty of potential as it plays an important role in the development of cardiomyocytes and other cells types as well.

However, organs are formed of more than one cell type, and based on the results by Lu et al. (1999) or Jia and Takimoto (2003), for instance, a beneficial effect in one cell type could potentially be harmful in the neighboring one. The results obtained from this study show that in COS-1 cells FOG2 inhibits GATA4 from binding to the rBNP promoter in NP-112 luciferase reporter, but this could be very different in cardiomyocytes and different promoter regions, for example. Hypothesizing 3i-1000, for instance, was shown to be both an efficient inhibitor of GATA4-NKX2-5 interaction and an enhancer of GATA4-FOG2 interaction, it could become a groundbreaking drug in cardiac regeneration: LVH could be decreased and cardiomyocyte proliferation increased. Nevertheless, if the enhancement of GATA4-FOG2 was strong in other cardiac cells, such as endothelial cells and fibroblasts, the final outcome could be less ideal. Therefore, the next step should be optimizing a similar assay in cardiomyocytes and see what FOG2 and the compounds do to GATA4 in them.

7 SUMMARY AND CONCLUSIONS

LVH is a noteworthy heart condition both economically and considering the quality of life of the patients. It increases the risk of various other cardiovascular diseases, such as MI or cardiac failure. LVH is caused by pathologically growing cardiomyocytes in the left ventricular wall, which results in thickening of the wall. At the same time, fibrosis increases. Scar tissue lacks the ability to contract, and the malign growth of the cardiomyocytes breaks the organization of their sarcomere structures, resulting in decreased efficacy. Elevated blood pressure is the most common factor behind LVH, and the current treatment is focused on antihypertensive medication. They do not, however, repair the damage. If the condition has already developed into cardiac failure, there prognosis is particularly weak. Current medication options only alleviate symptoms, but the patients often depend on them for the rest of their lives.

The main reason why these types of damages cannot be repaired is that cardiomyocytes have a nearly nonexistent regeneration capability. Science has attempted to overcome this obstacle with a variety of strategies, but so far none of them have made it to actually benefitting patients. The aim of this project was for one thing to determine if a group of

cardiac-selective lncRNAs previously discovered by our group could affect the way neonatal mouse cardiomyocytes responded to ET-1-induced stress, particularly focusing on their α-actinin and F-actin filaments. The second goal was to set up and optimize a compound screening assay for GATA4-FOG2 interaction.

These results showed that at least in this set-up, the lncRNA GapmeRs did not affect the hypertrophic response to stress in neonatal cardiomyocytes. This could either mean that the lncRNAs of interest are not involved in cardiomyocyte hypertrophy, or the silencing with the GapmeRs was inefficient. In addition, these experiments used only two structural proteins as biomarkers for phenotypic changes in hypertrophy. Further studies and different experimental designs could reveal results that were not seen here. The negative control A GapmeR must also be re-evaluated and studied more closely, as in certain cases it appeared to induce the strongest positive result despite not statistically significant.

In the GATA4-FOG2 interaction experiments, the hardship of transfecting these two turned out to be the extremely strong GATA4-inhibitory effect of FOG2. According to the results, transfection ratios NP-112:pMT2-GATA4 5:1 and pMT2-GATA4:pCS2-FOG2 10:1 produce a signal high enough to see if a test compound affects it. Based on the compound screen conducted in this project the compounds have tendency to enhance GATA4-FOG2 interaction but considering that only a single experiment was carried out, further research is needed to draw conclusions.

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