Universitätsklinikum Hamburg-Eppendorf

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University of Applied Sciences

Faculty of Life Science

Master of Science degree in Pharmaceutical Biotechnology

Role of microRNAs in Atrial Fibrillation: Establishment of Technological Pipeline

Master Thesis

Date of Submission: 07.05.2018

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Acknowledgments

I am deeply grateful to Prof. Dr. Tanja Zeller for providing me with the opportunity to pursue my master thesis at the University Clinic Eppendorf, Hamburg. I thank her for the guidance and commendable suggestions given throughout the thesis. I would also like to thank Prof. Dr. Oliver Ullrich for supporting me and providing feedback and suggestions in my master thesis

I am extremely thankful to my supervisor Julia Krause, who always her spared precious time to share knowledge and offer me meticulous guidance. I owe a lot to Tim Hartmann for his continuous support and feedback throughout my experiments. I would also like to express my gratitude to the wonderful team and their support that added to the great work atmosphere in the laboratory.

Contents

List of Abbreviations

Abbreviations	**Full-form**
miR, miRs, miRNA	microRNA
nM	Nano Molar
nm	Nanometers
Ct	Cycle threshold
RIN	RNA Integrity Number
FBS	Fetal Bovine Serum
rpm	Rounds Per Minute
RT	Room temperature
µl	Micro liter
ml	Milli litre
siRNA	Short/Small interfering RNA
DMSO	Dimethyl Sulfoxide
PFA	Paraformaldehyde
PBS	Phosphate-buffered saline
DAPI	4´,6-Diamidin-2-phenylindol
min	Minutes
s	Seconds
h	Hours
CTRL	Control

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Abstract

Various important biological processes are governed by microRNAs (miRNAs) which are involved in fine-tuning of gene expression. Any discrepancies in the levels of certain miRNAs during a diseased state, such as arrythmogenesis, affects the pathways associated to cardiomyocyte metabolism and cardiac electrical and structural remodelling. Atrial fibrillation (AF) is a common arrhythmia affecting 1-2% of the population with evident risk of stroke and heart failure. It is necessary to characterize the disease at the molecular level since the involved pathways are not completely understood. In order to study and confirm the role of miRNAs in disease models such as AF, cell culture experimental approaches are commonly used. These experiments need to be established for each cell type and miRNA of interest. Within the symAtrial consortium, several miRNAs had been identified in association to AF, however, their function and mechanism in the pathology of AF is unknown and needs to be further investigated. The goal of this thesis was to establish a technological platform to aid in better characterization of the role of miRNAs in relation to AF. The thesis was divided into two aims: The first aim was to establish a technological pipeline with cell culture experiments to facilitate miRNA characterization. Two cell lines were used, one was the cell line of interest for AF–mouse atrial cardiomyocytes HL-1 cells, which are relatively difficult to culture and experiment with and the second cell line was HEK293, which is an easy-to-handle cell line. First the experimental conditions were established in HEK293 cells and were subsequently applied onto the HL-1 cell line. The transfection experiments included knockdown of a miRNA and measurement of its effect on cell viability and target mRNA levels. All experimental approaches were successfully established in HEK293 cells; however, for the HL-1 cells the similar experimental approaches failed. Consequently, the conditions had to be adapted for the HL-1 cells and a successful transfection was achieved. The second aim was to validate a set of miRNAs – miR-21, miR-100 and miR-483-5p, in samples of AF individuals. A housekeeper–miR-16 was used. However, the results of this thesis revealed that under the diseased condition of AF, miR-16 was deregulated. In conclusion, the technological pipeline using a cell culture platform was successfully established for HEK293 cells. For HL-1 cells, further experiments are required to establish the pipeline. In addition, a miRNA housekeeper stable under the diseased condition of AF is required, which could help identify dysregulated miRNAs in AF individuals. A candidate miRNA could be functionally characterised using the established cell culture platform.

1. Introduction

Atrial Fibrillation (AF) is the most common type of arrhythmia worldwide [1] affecting 1-2% of the general population[2]. Most cases of AF remain unnoticed due to its asymptomatic nature[3,4]. Clinical data has revealed that having AF predisposes the individual to a risk of stroke by five-fold, to heart failure by three fold[5] and is the third common cause of all ischemic strokes[3,6]. A handful of established risk factors include age, sex, hypertension, diabetes mellitus, obesity, smoking and alcohol abuse. In addition, AF is higher among the population with cardiovascular diseases such as myocardial infarction, coronary artery disease, congenital heart disease and cardiomyopathy. A genetic component of AF has also been revealed[7]. The pathophysiological pathways in relation to the development of AF, such as ion-channel modulations, inflammation, atrial fibrosis and cardiac developmental pathways are not completely understood[5,8] and therefore, a better molecular understanding of the mechanisms underlying AF are required to enhance AF risk assessment.

1.1. Pathophysiological remodelling of the heart during Atrial Fibrillation

AF is a diseased condition of the heart where the normal sinus rhythm is compromised by the remodelling of the atrium. The abnormal heart rhythm in AF is characterized by rapid, asynchronous electrical activation (figure 1) in the atrium and loss of atrial contractility[9]. The remodelling of the atria is linked to genetic variations mediated by several regulatory molecules that are predominantly involved in atrial physiology.

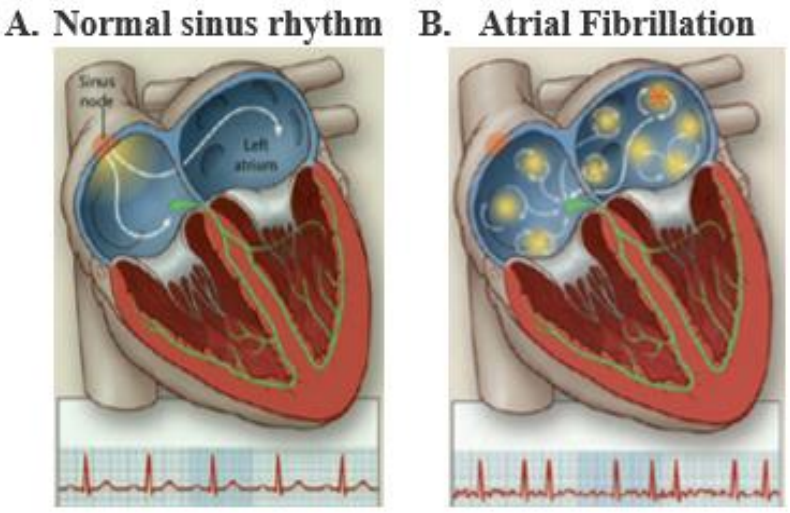


Figure 1 - Pathophysiology of Atrial Fibrillation in heart.
A. normal electrical conduction in a health heart originating from the SA node, contracting the atrium, which then passes through AV node **B.** Rapid multiple electrical impulses rising from various positions in the atrium other than the SA node[10]

Two major ways of remodelling can occur during AF – structural and electrical remodelling.

The structural remodelling in AF is defined as any change in the atrial structure or functions that induces atrial arrhythmias[11]. It consists of atrial enlargement and development of tissue fibrosis as the primary pathological modification in the progression of AF[12,13]. This slow process can be due to underlying cardiac diseases, systemic processes and aging[11]. Atrial fibrosis is irreversible once established and leads to impaired atrial conduction by affecting the sinoatrial and atrioventricular node.

Electrical remodelling in AF is defined as any change in the electrical functioning of the heart. As can be seen in figure 1, rapid electrical impulses or ectopic impulse formations (figure 1, B) produced in the atria overwhelm the normal electrical impulse that arise only from the sinoatrial node (figure 1, A). Atrial structural remodelling plays a role in altering the atrial electrophysiological properties, which includes ion channel modulations, decrease in Calcium (Ca^{2+}) current and abnormal expression of the gap junction connexin hemichannels that connect cardiomyocytes electrically[11].

Other than remodelling of atrium, the changes in the autonomic nervous system, Ca^{2+} handling abnormalities and sodium channel remodelling are also considered as key pathophysiological mechanisms contributing to AF progression.

So far, the mechanisms of AF development have been well established. Ion-channel modulations, atrial fibrosis, atrial conduction abnormalities have been characterized at the mechanistic level. However, the molecular causes, which lead to such extensive remodelling of the atrium for AF, remain unclear. To obtain a clear interpretation of the disease, every level of molecular biology i.e., genome (DNA), transcriptome (RNA, miRNA, lncRNA), proteome and metabolome have to be scrutinized which will thereby help in the assessment and treatment of AF.

1.2. microRNAs

microRNAs (miRNAs) are a class of RNA that are non-coding and highly conserved amongst species. They are short ~22 nucleotide sequences that regulate gene expression by regulating mRNA translation and stability. A single miRNA has many mRNA targets and a single mRNA can be acted upon by several miRNAs[14]. They are initially a ~180 nucleotide hairpin or a stem-loop structure, as shown in figure 2, from which the final ~ 22 nucleotide version of long mature sequence is carved out. The expression of miRNA varies between the circulating miRNAs and miRNAs in cells and tissues. Therefore, it is challenging to understand their effect on mRNA regulation.

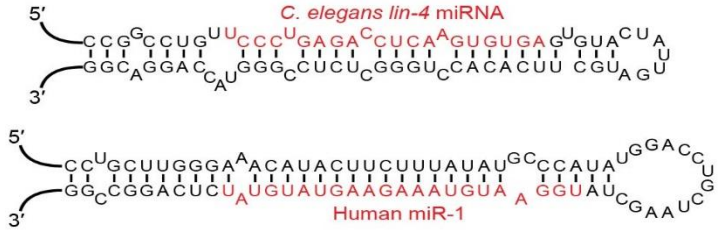


Figure 2 - Pri-microRNA stem-loop structure of C.elegans lin-4 and Human miR-1.
The sequence in red denotes the mature microRNA sequence. The mature sequence is achieved in the cytoplasm after being acted upon by the microprocessor complex and Dicer.[15]

1.3. Synthesis of miRNAs in a cell

miRNAs are present at various locations in the genome, such as in the intergenic region, within introns of coding/non-coding genes and within exons of non-coding genes. miRNAs are mostly transcribed by RNA polymerase II and a subset by RNA polymerase III. The promoters and transcriptional activators for miRNAs are poorly defined. Once transcribed, they are called primary miRNAs (pri-miRNA) and have a hairpin structure due to sequence complementarity (figure 2). Two major cleaving actions are carried out – one in the nucleus and the other in the cytoplasm. A microprocessor complex that consists of Drosha (RNase III enzyme) and Pasha (DiGeorge syndrome critical region 8 gene – DGCR8) then acts upon this pri-miRNA in the nucleus. Pasha, a double stranded RNA binding protein, binds to the miRNA hairpin allowing Drosha to cut the pri-miRNA into 11 bp sequences known as pre-miRNAs. Due to their small size, pre-miRNAs are exported from the nucleus to the cytoplasm by Exportin 5 Ran-GTP complex. In the cytoplasm, RNase III Dicer processes these pre-miRNAs, cleaving them into a ~22 nucleotide duplex. This step also leaves overhangs at the 3´end in each direction. RNA helicases unwind these duplexes, forming the final step of microRNA synthesis.

The sequence with higher thermo-stability and number of targets is incorporated to a RNA Induced Silencing Complex (RISC). RISC binding activates the miRNA to be functionally capable of carrying out its' functions. A pictorial representation is described in figure 3.

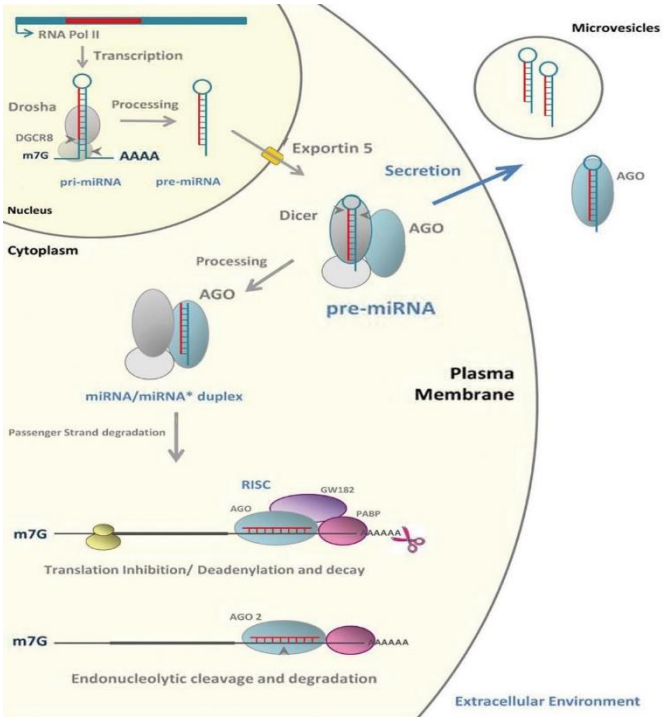


Figure 3- Synthesis, activation and release of microRNA from the cell
Translation by RNA Pol III creates a primary miRNA (stem-loop structure) in the nucleus, which is then acted upon by the microprocessor complex – Drosha and Pasha. After export to the cytoplasm with the help of Exportin 5, Dicer acts to form the final mature 22 nucleotide sequence, which is activated by binding to RISC.[16]

1.4. Function of miRNAs in a cell

The main function of a miRNA is mRNA degradation and translational repression. miRNA regulate gene expression by pairing to the 3´ Untranslated Regions (3´-UTR) of a target coding messenger RNA (mRNA). A single miRNA sequence has many targets to which they bind by Watson-crick base pairing to induce a negative regulation. Since, major mRNA degradation occurs due to miRNA activity, miRNAs play an important role in post-transcriptional control of homeostatic and developmental events[17]. miRNAs are not only present in cells and tissues but are also detectable in body fluids such as total blood, serum and plasma largely due to their small size and high stability. This occurs with the help of exosomes, apoptotic bodies or microvesicles (figure 3) that enclose these miRNAs to protect them from RNase activity. Due to the wide range of targets and association to diseases, miRNAs can be used as therapeutics (tissue specific miRNAs), or as biomarker (circulating miRNAs) for a disease[18].

miRNAs regulate approximately 30% of protein-coding genes and are therefore predicted to participate in all cellular processes[19]. In order to determine the role and function of miRNA in a specific process and/or during a disease, molecular methods such as knockdown/overexpression experiments followed by qRT-PCR and microarray sequencing can be used.

1.5. Role of miRNAs in progression of Atrial Fibrillation

Several miRNAs act as important regulators of AF affecting both the structural and electrical modelling that can be actively detected in the atrial tissue and body plasma. miRNAs help regulate cardiac conduction, excitability, ion-channel modulations, calcium handling, repolarization, cardiac physiology, fibrosis and apoptosis. For example, miR-21 is most widely studied in relation to AF since miR-21 dysregulation leads to increased fibrosis and decreased L-type calcium current handling[18]. Two muscle-specific miRNAs – miR-1 and miR-133 are most abundantly expressed in the heart[20]. miR-1, miR-499, miR-26 and miR-203a play an important role in action potential conduction whereas, miR-133, miR-29 and miR-30 regulate fibrosis associated pathways[17]. It is probable that most of these miRNAs jointly contribute to arrythmogenesis. However, even though various studies exist to study this correlation of miRNAs with AF, they show contradicting results. In order to identify these relations between miRNAs and AF, it is necessary to have certain experimental platforms that allow profiling and understanding of miRNA regulation at various molecular levels.

1.6. The symAtrial consortium – miRNA project

symAtrial – Systems Medicine of Atrial Fibrillation encompasses an integrative multidisciplinary systems-based approach to investigate the development and progression of atrial fibrillation. This consortium is coordinated by the group of Molecular Cardiology, Genomics and Systems Medicine of the Cardiology Department at the University Heart Centre Hamburg (Dr.rer.nat. Tanja Zeller) and entails several sub-projects that aim at identifying novel molecular risk factors for AF by creating a central data integration and systems medicine platform. One such sub-project deals with the identification and molecular

characterization of AF-related candidate genes, respective pathways and their functional assessment. In this, '-omics' data from multiple levels of molecular biology such as transcriptomics, miRNAs, proteomics and metabolomics of the atrial tissue and patient blood-based samples will be acquired and analysed. To facilitate this investigation of the findings of '-omics' analysis, different experimental approaches will be employed to characterize the molecular role of the identified candidate genes.

The miRNA project was derived from the above-mentioned subproject of "molecular characterization of AF candidate genes". A miRNA analysis was carried out in serum samples of 9 AF individuals and 30 age and sex matched controls. Circulating miRNAs were investigated using qPCR-based miRNA cards. This analysis revealed four miRNAs – miR-21 ($p=1.2\times10^{-3}$), miR-100 ($p=8.8\times10^{-3}$), miR-301a ($p=2.1\times10^{-2}$) and miR-103 ($p=2.8\times10^{-2}$) exhibiting differential expression in the AF subjects compared to controls.

These results were translated onto a cohort of AF patients who underwent a bypass operation. Here, in addition to the above-mentioned miRNAs, other literature-based miRNAs such as miR-31-3p, miR-28, miR-29 and miR-107 were also analysed. The clinical cohort consisted of tissue (right atrial appendages) and serum samples of patients who underwent a bypass operation and therefore suffered from a cardiovascular disease. 111 atrial tissue samples were used for this analysis. It was observed that miR-100 showed significant lower expression in patients diagnosed with AF before the bypass operation when compared to patients that never suffered from AF (control).

The preliminary data showed that certain miRNAs were identified in AF individuals. However, this does not explain the molecular role of miRNAs in the pathogenesis of AF. This could be validated through various molecular experimental designs. Experimental approaches in any new project requires intense optimisation of the applied techniques according to the cell line and miRNA of interest because each cell line and miRNA behave differently with respect to the other.

2. Aim of this master thesis

This master thesis is part of the miRNA project embedded in the symAtrial consortium where dysregulated miRNAs – miR-21 and miR-100 were identified in AF patients. An experimental approach was proposed to further analyse the role of miRNA in AF and the cardiovascular system in general, which formed the basis of this master thesis.

The specific aims of the master thesis were

Aim – 1: Establishment of a cell culture platform to investigate miRNAs

Aim – 2: Validate candidate microRNAs in serum and tissue samples from individuals suffering from AF

For aim 1, a cell culture platform with HEK293 and HL-1 cells was established in order to study knockdown/over-expression of a target microRNA. The important transfection model parameters – transfection efficiency, cell viability and target gene expression had to be identified and measured.

For aim 2, candidate miRNAs of the symAtrial miRNA subproject were measured in AF patients and controls by RT-PCR.

The aims and over-all goal are depicted in figure 4.

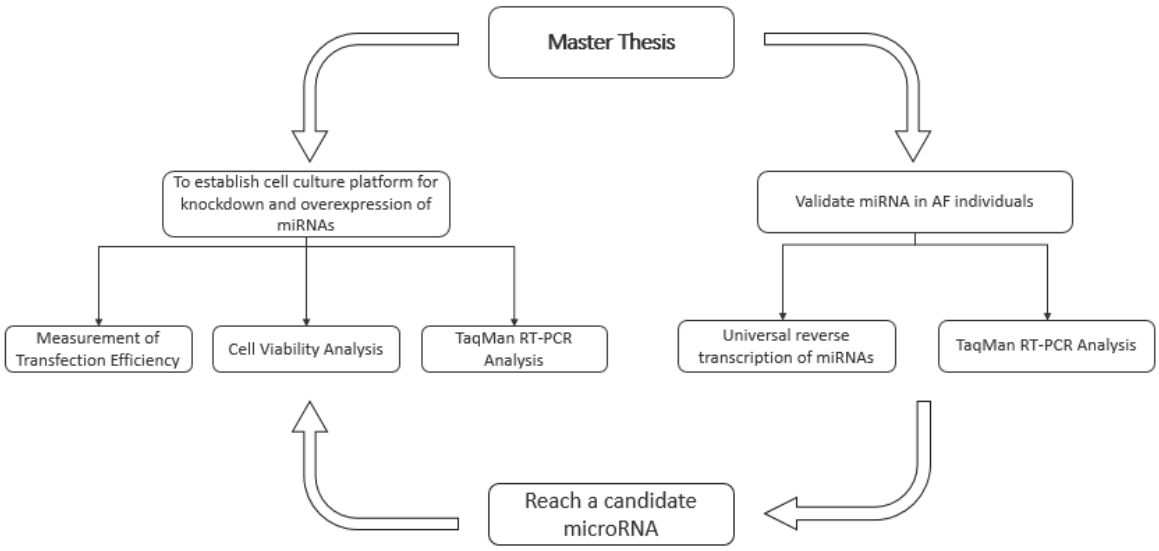


Figure 4 – Aims of the master thesis and the important parameters performed and analysed

3. Materials and methods

3.1. Materials

3.1.1. Laboratory material

Cell culture flasks	TPP® tissue culture flasks, Sigma-Aldrich
Serological pipettes (1ml, 5ml)	Cellstar® Greiner Bio-GmbH
Serological pipettes (10ml)	Sarstedt AG & Co. KG
Serological pipettes (25ml)	BD Falcon™ Franklin Lakes. USA
Reaction tubes (1.5ml, 2ml)	Eppendorf AG
Pipette filter tips (10µl, 20µl, 100µl, 200µl, 1000µl)	Biosphere® Sarstedt AG & Co. KG
x-well slide (8-well slide)	Sarstedt AG & Co. KG
24 – well adherent cell culture plate	Sarstedt AG & Co. KG
96 – well adherent cell culture plate	Sarstedt AG & Co. KG
384 well PCR plates	Sarstedt AG & Co. KG
Optical adhesive cover	Applied Biosystems®, Life Technologies GmbH

3.1.2. Cell culture Medium and chemicals

Dulbecco's Modified Eagle's Medium	Sigma-Aldrich
Claycomb medium	Sigma-Aldrich
Penicillin 100U/ml, Streptomycin 100µg/ml (Pen/Strep)	Sigma
Phosphate Buffered Saline 1X (PBS)	gibco® Thermo Fischer Scientific
Fetal Bovine Serum (Heat inactivated)	gibco® Thermo Fischer Scientific
Fetal Calf Serum	Biochrom
L-Glutamine	gibco® Thermo Fischer Scientific
(±)-Norepinephrine (+)-bititrate salt	Sigma-Aldrich
Fibronectin	Biochrom
Trypsin-EDTA	Biochrom
Preservation solution (freeze medium)	95%FCS + 5%Dimethyl Sulfoxide

3.1.3. Transfection Reagents

Lipofectamin RNAiMAX	Thermo Fischer Scientific
DharmaFECT 1	Dharmacon
Negative Control	Ambion, Thermo Fischer Scientific
*mir*Vana miRNA Inhibitor Let-7c Positive Control	Ambion, Thermo Fischer Scientific
*mir*Vana miRNA Mimic miR-1 Positive Control	Ambion, Thermo Fischer Scientific
ON-TARGETplus GAPD control siRNA	RNAi control reagent, Dharmacon
BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control	Thermo-Fischer Scientific
siGLO Green Transfection indicator	Dharmacon

3.1.4. Chemicals and other Reagents

CellTiter-Blue® Cell Viability Assay	Promega
Hoechst Blue 33342 (P4082)	Cell Signalling Technology
Alexa Fluor® 488 Phalloidin	Invitrogen™, Life Technologies
Paraformaldehyde (PFA-4%)	Sigma-Aldrich (4% PFA in PBS)
β-Mercaptoethanol for molecular biology	Sigma-Aldrich
Ethanol 100% (v/v) for molecular biology	Applichem
RNase Free Water	Invitrogen™, Life Technologies
TBE Buffer (Tris/Borate/EDTA)	Thermo Scientific™

3.1.5. Molecular Biology Kits

RNeasy Mini Kit	QIAGEN GmbH
RNase Free DNase Set	QIAGEN GmbH
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems™, Life Technologies GmbH
TaqMan™ Gene Expression Master Mix	Applied Biosystems, Thermo Fischer Scientific
TaqMan Gene Expression Assays	Applied Biosystems, Thermo Fischer Scientific
TaqMan Fast Advanced Master Mix	Applied Biosystems®, Life Technologies GmbH
TaqMan Advanced miRNA Assay	Applied Biosystems®, Life Technologies GmbH

3.1.6. Equipment

Water bath	Gesellschaft für Labortechnik GmbH
Laminar Air Flow clean bench (steril) Heraeus	Thermo Scientific
Microscope	Leica Microsystems, Cambridge Ltd.
Centrifuge Functional Line 400R	Thermo Scientifc
Mr. Frosty	Thermo Scientific
Pipettes Research®/Reference®	Eppendorf AG
Pipette Pipetus®	Hirschmann Laborgeraete GmbH & Co. KG
CO_2 chamber	Heracell™, Thermo Scientific
Microscope	ZEISS ApoTome
Microplate reader Infinite 200/200Pro	Magellan, Tecan Diagnostics
NanoDrop™ 2000/2000c Spectrophotometer	NanoDrop Technologies Inc. USA, Thermo Scientific
Thermal Cycler, GeneAmp® PCR System 9700	Applied Biosystems™, Life Technologies
Thermal Cycler 7900HT	Applied Biosystems®, Life Technologies GmbH

3.1.7. Software

SDS 2.4	Applied Biosystems®, Life Technologies GmbH
Prism 6.0	GraphPad

3.2. Methods

3.2.1 Cell culture

Two cell lines were used for transfection experiments. Experimentation was first performed with HEK293 cells in order to understand the transfection process and later, the conditions were replicated onto the cell line of interest – HL-1 cells.

HEK293 cells

Human Embryonic Kidney cells 293 (HEK293) are an adherent cell line derived from human embryonic cells grown in tissue culture. Generated by Alex van der Eb in the year 1973 in Leiden, The Netherlands, this transformed cell line originates from an adenovirus 5 sheared culture of human embryonic kidney cells derived from the kidney of an aborted human embryo of unknown parents[21]. The transcriptome pattern of the HEK 293 cells line resembles that of adrenal cells, which have neuronal properties. An embryonic adrenal precursor cell, therefore, seems the most likely origin cell of the HEK293 line and so they should not be as an *in vitro* model for typical kidney cells[22].

HL-1 cells

HL-1 cells are an adherent cell line derived from the AT-1 mouse atrial cardiomyocyte tumour lineage growing in a transgenic mouse. They possess properties that were non-existent in their progenitors, the AT-1 cells, such as that they retain a differentiated cardiac phenotype during continuous passage culture, thus making them different from other immortalized cell line. HL-1 cells retain the gene expression pattern of normal adult mouse myocytes even though they are actively dividing. In addition, this cell line expresses β-MHC and α-skeletal which makes it difficult to revert to an embryonic stage[23].

Cells were cultured in 75cm^2 flasks with the specific cell culture mediums. After passaging or thawing the cells, they were allowed to grow for 2-3 days in an incubator with the conditions being 37°C with 5% CO_2 saturation and 95% air. The specific requirements of individual cell lines such as the cell culture medium for the individual cell lines, its' constituent nutrients and antibiotics, additional reagents to facilitate passaging and freezing of cells, and respective centrifugation speeds and split ratios are depicted in table 1.

Table 1– Specifications for culturing HEK 293 cells and HL-1 cells

<table>
<tr><th></th><th colspan="2">HEK 293T cells</th><th colspan="2">HL-1 cells</th></tr>
<tr><td>Culture medium</td><td colspan="2">DMEM – 12ml
With
10% FBS
1% Pen/Strep</td><td colspan="2">Claycomb medium – 10ml
with
10% FBS
1% L-Glutamine
1% Norepinephrine
1% Pen/Strep</td></tr>
<tr><td>Trypsin-EDTA solution</td><td colspan="2">1X – 5ml</td><td colspan="2">2X – 5ml</td></tr>
<tr><td>Phosphate-buffered Saline (PBS)</td><td colspan="2">1X – 10ml</td><td colspan="2">1X – 10ml</td></tr>
<tr><td>Centrifugation speed</td><td>1800 RPM</td><td>3 min</td><td>1500 RPM</td><td>3 min</td></tr>
<tr><td>Desired Split Ratio</td><td colspan="2">1:10 or 1:20</td><td colspan="2">1:2 or 1:5
For confluent batch – 1:10</td></tr>
<tr><td rowspan="5">Fibronectin coating</td><td colspan="2" rowspan="5">Not required</td><td colspan="2">Required
0.02% Gelatin + PBS</td></tr>
<tr><td>Type</td><td>Volume</td></tr>
<tr><td>75cm^2 Flask</td><td>5 ml</td></tr>
<tr><td>24 well-plate & 8-well slide</td><td>500 µl</td></tr>
<tr><td>96-well plate</td><td>100 µl</td></tr>
<tr><td>Freeze medium</td><td colspan="2">DMSO – 1ml
95% FCS
5% dimethyl sulfoxide</td><td colspan="2">DMSO – 1ml
95% FCS
5% dimethyl sulfoxide</td></tr>
</table>

Passaging

The cell culture medium, PBS, and Trypsin-EDTA were pre-warmed to 37°C in a water bath. The cells were taken out from the incubator and stripped off the old medium. Immediately PBS was added to wash the cells. Next, Trypsin-EDTA solution was added to detach cells from the surface of the flask. Once detached, normal cell culture medium was added directly to trypsin-EDTA solution since serum in the medium will inhibit trypsin. At this stage, cells counting could be performed if required.

Cell counting – Prepare the cell counting chamber (Neubauer Zählkammer). Mix 10 µl of Tryptan blue dye with 10 µl of cells and count under the microscope. equation 1 was used to calculate the seeding cell number.

$$Cell\ number = \frac{sum\ of\ cells\ in\ 4\ squares}{4} \times 2 \times 10^4\ cells/ml$$

Equation 1 - Calculation of cell number for cell seeding

According to the chosen ratio or required number of cells, the volume of cells + rest medium were transferred to the flask.

Medium renewal

The medium was renewed every 2-3 days. The cell culture medium was pre-warmed to 37°C in a water bath. The cells were removed from the incubator, stripped of the old medium and added with fresh medium.

Freezing cells

Mr. Frosty, a preservation container, was brought to room temperature. After detaching the cells as described in passaging, the total volume was transferred to a falcon tube and centrifuged at the speeds according to each cell line (refer to table 1). After centrifugation, supernatant was removed carefully, and the cell pellet was dissolved in freeze medium. After resuspension, the cryo-tube must be placed for 48 h in Mr. Frosty at -80 °C for slow freezing.

Thawing cells

For thawing cells, the desired cell line was thawed in the water bath until a little bit ice was left. Immediately cells were transferred to a falcon tube containing warm medium and centrifuged at respective speeds for each cell line. The supernatant was removed and pellet dissolved in 12 ml culture medium, which was then transferred to a new culture flask.

Cell seeding conditions

The number and health of cells before and after seeding onto a well plate influence the end result. The cells taken must be present in the log phase of growth before seeding, since over grown cultures do not provide best growth characteristics. HEK293 cells have increased growth capacity and thereby increased metabolism, whereas HL-1 cells grow at a slower pace. In order to have a better transfection, the seeding density mentioned in table 2 was employed in the experiments.

Table 2– Cell seeding conditions for HEK293 and HL-1 cells

	HEK293 cells	**HL-1 cells**
8-well slide system	3.5×10^4	3.7×10^4
24-well plate	1.0×10^5	1.5×10^5
96-well plate	1.5×10^4	2×10^4

3.2.2. Transfection

Transfection is defined as the process of deliberately introducing naked or purified nucleic acid into the eukaryotic cells by opening channels in the cell membranes to allow the ease of uptake of the foreign material by the cells[24]. Transfection methods are broadly classified into non-viral and viral, and for the following experiments a non-viral approach was used. This so-called chemical-based transfection is known as Lipid-Mediated gene delivery or lipofection in which the positively charged lipid combines with negatively charged nucleic acid (forming liposomes) that are easily taken up by the cell. Also known as RNA interference technique, miRNAs are knocked down to observe its effects on respective target mRNA level.

3.2.2.1. Transfection medium prepared with Lipofectamine® RNAiMAX

The transfection reagent used to deliver siRNA to the cells was Lipofectamine® RNAiMAX. The siRNA dilutions were prepared according to the protocol given by Thermo Fischer Scientific. The first step is to dilute Lipofectamine® RNAiMAX and the positive/negative siRNAs with Opti-MEM separately. The siRNA undergoes 1:50 dilution, whereas Lipofectamine® RNAiMAX undergoes a 1:16.67 dilution. Second step is to dilute Lipofectamine® RNAiMAX with the siRNA in a 1:1 ratio. The final dilution is a 1:10 dilution of this transfection medium with cell culture medium in the well plate. The siRNAs used were a fluorophore control – Block-iT™ Alexa Fluor® Red Fluorescent Control, a positive control – *mir*Vana™ miRNA inhibitor let-7c, and a negative control. The concentration of siRNA employed were 30nM, 10nM, 3nM, 1nM, 0.3nM and 0.1nM. The transfection medium was switched with normal cell culture medium at two time points, 4 h and 24 h. The final cultivation of cells occurred after 2-days of transfection.

3.2.2.2. Transfection medium prepared with DharmaFECT 1

The transfection agent used was DharmaFECT 1. The siRNA dilutions were performed according to the protocol provided by Dharmacon. The backbone of dilutions was essentially similar to section 3.2.2.1. The siRNA and DharmaFECT 1 reagent were first mixed with serum-free medium separately. Here, the siRNA was diluted at 1:20 ratio and DharmaFECT 1 was diluted at 1:25 ratio. Second step was to dilute DharmaFECT 1 solution and siRNA solution in a 1:1 ratio and incubate for 10 min for the siRNA-DharmaFECT 1 complexes to form. Lastly, this complex was diluted with antibiotic-free cell culture medium in the well plate in a 1:5 ratio. The siRNA used were a fluorophore control – siGLO Green Transfection Indicator, two different positive controls – ONTARGETplus GAPD control siRNA, *mir*Vana™ miRNA mimic miR-1 and a negative control. The concentration of siRNA employed were 100nM, 75nM, 50nM and 25nM. This transfection medium was switched with normal cell culture medium at 24 h and the final cell cultivation was at 2-days post transfection.

3.2.3. Measurement of transfection efficiency using a fluorophore control

The success of a transfection experiment can be easily identified with the help of a fluorophore siRNA. This method is widely used to localize the signal of fluorophore and thereby deduct if the transfection agent was capable to optimally introduce the siRNA into the cell. Both the transfection agent and the quality/quantity of siRNA used form the parameters that affect transfection efficiency and so a suitable choice for the respective cell line must be taken. The efficiency of transfection was ascertained by equation 2.

$$\text{Transfection Efficiency} = \frac{\text{number of cells giving the fluorescent signal}}{\text{number of living cells under the microscope area}} \times 100$$

Equation 2 - measurement of transfection efficiency

For HEK293 cells, the cells were seeded normally, whereas for HL-1 cells fibronectin coating was applied to the wells before seeding. After reaching 70% confluence, the cells were transfected the next day with Lipofectamine® RNAiMAX and fluorescent control. **Fixing of cells with 4% Paraformaldehyde (PFA)** – After the categorical transfection period, the cells were washed 1X with PBS, and ~ 300 µl of 4% PFA was added to each well enough to cover the surface. This allows PFA to form covalent chemical bonds between proteins of the cells and its' surroundings. After incubation for 15 min, PFA was removed from the wells. **Hoechst blue stain –** ~400 µl of Hoechst blue stain was added to each well and incubated for 15 min. The stain should be prepared with PBS at a dilution of 1:10,000. **Membrane co-staining –** To get a clearer signal of localization of target siRNA flurophore, the membrane of cells can be stained for F-actin with the stain – Alexa Fluor® 488 Phalloidin. The stain was diluted 1:400 times with PBS. Incubation period was for 90 min after which the wells were washed at least once with PBS. The mounting medium with DAPI was used. At least 2-3 drops of mounting medium were applied over the slide, and immediately a rectangular coverslip was placed on the medium. The slide was allowed to dry in a humid slide box at 37°C for 2 h or at room temperature for overnight and then observed under the microscope.

3.2.4. Cell viability assay

The aim of this method is to measure the viability of cells under the influence of siRNA (positive and negative controls) and compare them to the untreated cells. A living cell is capable of metabolizing the blue coloured compound Resazurin with very less intrinsic fluorescence to a fluorescent pink coloured compound Resorufin. This shift of colour is detectable at the fluorescent range of 579 – 584 nm or at an absorbance maximum of resazurin at 605 nm and resorufin at 573 nm. Fluorescence or absorbance can be used to measure the metabolized compound. A simple workflow is described in figure 5.

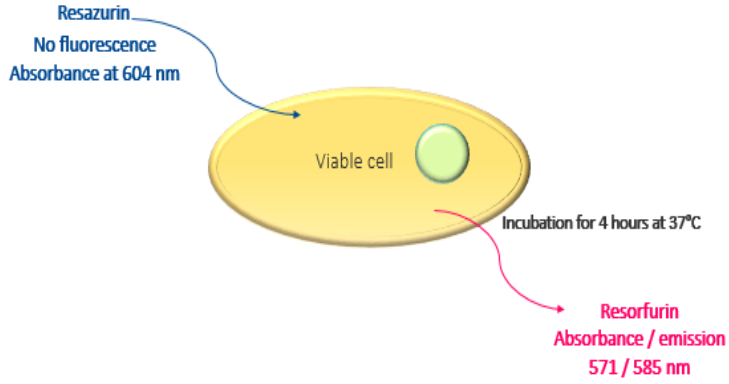


Figure 5 – Mechanism of action of CellTiter® Blue reagent
The blue cell viability reagent is made of concentrated resazurin. This is added to cells. Viable cells metabolize resazurin to resofurin, a pink coloured compound, that is measured at absorbance / emission of 571/585 nm

HEK293 cells were seeded normally in a 96-well plate whereas HL-1 cells required prior coating of the wells. The cells were then transfected with negative and positive control siRNA upon reaching 60-70% confluence. It is important to have untreated cells as control to study the exact effect of the siRNA treatment on the cells. After the 48 h transfection period, 20µl of CellTiter Blue® reagent was added and the cells were incubated for a period of 1-4 h. During this period of incubation, a subtle change of colour from blue to pink occurred as explained in figure 5. The cell culture medium + CellTiter Blue® reagent acts as the blank. At the end incubation period, the plates were observed using the spectrophotometer and software – Magellan by Tecan. The absorbance range is set from 570 nm to 600 nm to study the blue shift. All values are subtracted from the blank to remove effects of cell culture medium. Values will be presented according to the wells selected.

3.2.5. Molecular Biology Techniques

3.2.5.1. RNA isolation

Nucleic acids are negatively charged molecules due to the phosphate groups (PO3-) that are capable of being attracted to polar solvents like water. RNA and DNA precipitate out in the presence of salts and ethanol. RNA isolation kits exploit the properties of nucleic acids to extract purified RNA from cells and tissues.

The protocol followed was according to the guidelines provided by Thermo Fischer Scientific. Immediately post transfection period, RLT buffer was added according to the estimated number of cells in the wells and then the plates were kept in -80°C to freeze. This method of quick freezing will create ice crystals that would disrupt the cell. Later, the plates were thawed and one volume of 70% ethanol was added to the samples. Homogenization causes high molecular weight genomic DNA and other heavy molecules are sheared to create a homogenized lysate. Addition of Ethanol ensures the integrity of RNA.

1. RNA isolation – The complete volume (700µl) of RNA/RLT buffer/ Ethanol mixture was transferred to the spin easy column and incubated for 1 minute, then, centrifuged for 15 s at RT 10,000 RPM and the flow through was discarded.
2. Washing the column – 350 µL of RW1 Buffer was added to the column and centrifuged for 15 s at RT 10,000 RPM and discarded the flow through.
3. DNase treatment – This step is necessary to remove DNA from the column. Useful for RNA application like the TaqMan RT-PCR that is sensitive to even small amounts of DNA contamination. 80 µl DNase Mix and the columns are incubated for 1 h.
4. Wash the column – After DNase treatment, RW1 and RPE buffer were added to the column and centrifuged for 15 s at RT 10,000 RPM and the flow through is discarded after each wash step.
5. Drying the column – A dry run was performed by centrifuging the columns for 2 min at RT 10,000 RPM. After this the columns were allowed to dry by keeping the lids of the columns open for 2-5 min allowing the residual ethanol to vaporize.
6. Elution of the RNA – Since RNA is soluble in water and not ethanol, 30 µL of RNase-free water was added and incubated for 5 min. This property helps in eluting RNA molecules freely. The column is centrifuged at maximum speed for 1 minute at RT to collect the eluate.
7. Measurement of RNA concentration using NanoDrop – For baseline measurements (blank) – 1µl RNase free water was used. 1 µL of RNA sample from the eluate was taken for measurement (peak at 260 nm). Storage of the RNA was done at -80 °C

3.2.5.2. Reverse transcription mRNA – Synthesis of complementary DNA (cDNA)

An mRNA molecule is free from introns and represents the amount of the gene that would be transcribed and translated into proteins. cDNA is synthesized from mRNA template in a reaction catalyzed by the enzymes reverse transcriptase and DNA polymerase. A poly (A) tail, which is used as a primer site, helps in differentiating between mRNA, tRNA and rRNA in the cells. Reverse Transcriptases (RTs) synthesize the first strand of cDNA using the mRNA template and a short primer of oligo deoxy-thymidine nucleotides (*oligo-dT*). This *oligo-dT* is complementary to the 3' poly (A) end of the RNA. RTs also use an RNase H function that degrades the RNA part of the hybrid. After this, only a single stranded cDNA molecule is present, and from this a double stranded DNA is created by DNA polymerase. A lot depends upon the reverse transcription efficiency of the RNA since, Real Time-PCR (RT-PCR) gives out Ct values. Complementary DNA synthesis is a common procedure where RNA is involved. Since the success of the experiment is measured at the mRNA levels, isolated RNA and its' reverse transcribed cDNA prove to be useful molecular biology tools.

All the RNA samples were normalized to 12.5 ng/µl in MicroAmp 8-tube strips. Normalized RNA was subjected to denaturation according to the program mentioned in table 4 by the Applied Biosystems thermal-cycler. The denatured samples are placed on ice and not in room temperature. The 2X Reverse Transcription master mix should be prepared using the High Capacity cDNA Reverse Transcription kit (table 3). The sample and master mix were mixed in a 1:1 dilution. After incubation of 10 min, the samples were subjected to a second cycle in the thermal-cycler with the conditions listed in table 5. The final step is to dilute the cDNA sample 1:5 times with double distilled water. This provides the end concentration of cDNA to 1.25 ng/µl. The samples can be frozen at -20°C for longer periods and thawed when used for TaqMan analysis.

Table 3 - Contents and specifications for Master Mix preparation for 1 reaction

Product	**Concentration**	**Final Concentration**	**Volume**
RNA Template	12.5 ng	1.25 ng	20 µl
RT Buffer	10X	1X	4 µl
RT Random Primers	10X	1X	4 µl
dNTP Mix	100mM	4mM	1.6 µl
MultiScribe Reverse Transcriptase	50 U/µl	2.5 U/µl	2 µl
RNase Free Water	-	-	8.4 µl
Total Master Mix Volume			20 µl

Table 4 – Part 1 of cDNA synthesis-cycler program

Step	**Temperature**	**Time**
RNA denaturation	70°C	10 min
Hold	4°C	∞

Table 5 – Part 2 of cDNA synthesis-cycler program

Step	**Temperature**	**Time**
Primer Annealing	25°C	10 min
cDNA synthesis	37°C	120 min
Inactivation of enzyme	85°C	5 min
Hold	4°C	∞

3.2.5.3. Reverse transcription miRNA – complementary DNA (cDNA)

The Advance miRNA assays are pre-formulated with primer and probe sets that work cohesively to allow quantification of mature miRNA expression levels. The main goal of this assay is to develop amplified amounts of cDNA of microRNAs from the whole RNA sample. As such, multiple miRNAs from a single sample, also from samples that are limited in quantity, can be reverse transcribed into cDNA. The protocol followed was provided by the manufacturer. The RNA template was normalized to 5 ng/µl. This normalized RNA was subjected to poly (A) tailing reaction spanning 55 min. This poly (A) tail acts as a 'tag' to identify miRNA in the sample. To this, an adaptor molecule ligation reaction spanning 60 min was performed. This adaptor acts as the forward primer binding site for the Reverse Transcription reaction (20 min). Here, miRNAs with adaptor molecules are reverse transcribed selectively. This product was amplified in the next step of miR-Amp reaction (30 min). This product is then diluted 1:10 times with 0.1% TRIS-Borate-EDTA (TBE) buffer in order to be used for PCR.

3.2.5.4. TaqMan Real-Time PCR (RT-PCR) – Gene expression analysis

TaqMan RT-PCR is optimal for detection and quantification of low abundance RNAs in a sample. TaqMan utilizes the simple 5´ – 3´ endonuclease activity of *Taq* polymerase to amplify our target gene in the cDNA sample by extending the primers on the template strand in a 3´-5´ direction. The TaqMan probe is a hydrolysis probe that consists of two labels, one fluorescent at the 5'-end and another a quencher at the 3'-end coupled with a minor groove binder, which helps us quantify the amplified product. The mechanism followed is based on the phenomenon of FRET – Fluorescent Resonance Energy Transfer.

The cDNA templates were thawed and kept on ice at all times. Either a 96 well plate or a 384 well plate can be used for the TaqMan analysis in the Applied Biosystems thermal cycler. 2 µl of cDNA template was added to the wells according to a pre-defined layout. 8µl of Master Mix (table 7) was added to the 2 µl of cDNA template and the plate was covered with an optical adhesive cover. The plate was centrifuged to remove air bubbles and settle the mix at the bottom of the tubes. For HEK293 cells, *GAPDH* (table 6) was used as the housekeeper whereas for HL-1 cells two housekeepers – *Gapdh* and *Cdkn1b* (table 6) were used. The cycler program (table 8) is set-up using the software SDS 2.4 onto the device Applied Biosystems 7900HT Fast Real-Time PCR System. This system contains laser and detection systems that can detect the fluorescent signal from the amplified product (FAM).

Table 6 – Gene Expression assays used for TaqMan gene expression analysis

Assay ID	Species and Gene Symbol	Gene Name
Hs04397751_m1	human *HMGA2*	High mobility group AT-hook 2 – homo sapiens
Hs02786624_g1	Human *GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase
Mm04183367_g1	mouse *Hmga2*	High mobility group AT-hook 2 – Mus musculus
Mm99999915_g1	mouse *Gapdh*	Glyceraldehyde-3-phosphate dehydrogenase
Mm01598981_g1	Mouse *Twf1*	Twinfilin, actin-binding protein, homolog 1
Mm00438167_g1	Mouse *Cdkn1b*	Cyclin-dependent kinase inhibitor 1B

Table 7 – Preparation of TaqMan gene expression master mix per reaction

Component	Concentration	Final Concentration	Volume
TaqMan™ Gene Expression Master Mix	2X	1X	5 µl
TaqMan Gene Expression Assay	60X or 20X	3X or 1X	0.5 µl
RNase Free Water	-	-	2.5 µl
Total Volume of reaction			8 µl

Table 8 - TaqMan real-time PCR program for gene expression analysis

Step	Temperature	Time	Repetitions
Uracil-N-glycosylase activation	50°C	2 min	1X
Initial denaturation	95°C	10 min	1X
Denaturation	95°C	15 s	40X
Annealing and elongation	60°C	1 min	

3.2.5.5. Real-time PCR using TaqMan Advanced microRNA assays

The isolated total RNA was treated to selectively reverse transcribe only the miRNAs in the sample as described in section 3.2.5.3. This technique has advantages such as it provides increased specificity for microRNAs in the sample and the isolated total RNA allows further investigations through other analysis techniques. The product from miR-Amp reaction were thawed on ice, vortexed and centrifuged to homogenize the solutions. The miR-Amp product had to be diluted 1:10 times with 0.1% TBE buffer for use in RT-PCR. The RT-PCR reaction mix is prepared according to table 10. The miRNA assays used are mentioned in table 9. The housekeeper used was miR-16 (table 9). An optical adhesive cover was used to seal the TaqMan reaction Plate. Using the SDS 2.4 software, the program (table 11) can be set onto the Applied Biosystems 7900HT Thermal Cycler.

Table 9 - TaqMan Advanced miRNA assays for microRNA analysis

Assay ID	microRNA Name	miRBase Version
477860_mir	hsa-miR-16	MI0000070
477975_mir	hsa-miR-21-5p	MI0000077
478224_mir	hsa-miR-100-5p	MI0000102
478122_mir	hsa-miR-483-5p	MI0002467

Table 10 - List of reagent to prepare TaqMan real-time PCR reaction mix

Component	Concentration	Final Concentration	Volume per reaction
TaqMan Fast Advanced Master Mix	2X	1X	10 µl
TaqMan Advanced miRNA Assay	20X	1X	1 µl
RNase Free Water	-		4 µl
Total PCR Reaction Mix volume			15µl

Table 11 - Program for Applied Biosystems PCR Thermal-Cycler

Step	Temperature	Time	Repetitions
Enzyme activation	95°C	20 s	1
Denature	95°C	3 s	40 cycles
Anneal / Extend	60°C	30 s	

3.2.6. Clinical Cohort – AFHRI-B

AFHRI-B stands for 'Atrial Fibrillation in High Risk Individuals – Biopsy' and is a cohort from the University Heart Centre Hamburg. Patients with AF are recruited into this study. Tissue samples from the right atrial appendages and serum samples of the patients who had undergone a bypass operation and therein suffered from a cardiovascular disease are collected and stored at -80°C/liquid nitrogen.

This cohort was divided based on the occurrence of AF with respect to the bypass operation. The phenotypes were

- Controls – Patients with no AF were taken as controls
- Pre-operative AF (Pre-OP) – Patients suffering from AF before the bypass surgery but not after.
- Post-operative AF (Post-OP) – Patients suffering from AF after the bypass surgery but had not been suffering before.

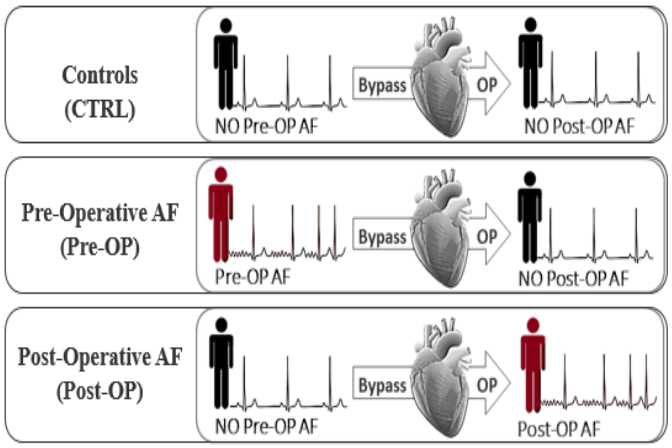


Figure 6 - Division of different phenotypes in AFHRI-B cohort
AF individuals that underwent a bypass surgery were chosen for the cohort. The individuals were categorised according to the occurrence of AF with respect to the bypass surgery. Individuals with no AF throughout were taken as controls.

The advantage of the AFHRI-B cohort for this project was that the level of circulating miRNA levels in serum samples could be directly compared to the tissue-specific miRNA levels. Therefore, this cohort samples were helpful in identifying and validating the previously characterized miRNAs from the symAtrial miRNA project at the tissue and serum level in AF individuals.

3.2.7. Analysis of RNA quality by RIN

The quality of RNA is determined using RNA Integrity Number (RIN). RIN is an algorithm that assigns a number from 1-10 based on the integrity of the RNA in sample, with 10 being the highest value. Agilent Bioanalyzer and Agilent 2100 software computes the RIN for a sample using characteristics of the electropherogram trace based on the total RNA ratio between 18S and 28S RNA peaks. These peaks are typically obtained using capillary electrophoresis, where even small amounts of a nucleic acid sample can be run in a thin tube. In order to generate a peak of the 28S or 18S rRNA, a detector is maintained in the machine. The 28S peak degrades quickly than 18S and so, a large value for 28S is desired because it is the most prominent species of rRNA. The larger the 28S peak, the better is the RNA integrity. RIN is calculated using equation 3.

$$Total\ RNA = \frac{\text{area under the 18S and 28S rRNA peaks}}{\text{total area under the graph}}$$

Equation 3 - RIN analysis-calculation of RNA Integrity number

3.2.8. Statistical analysis

3.2.8.1. Statistical analysis for cell culture experiments

For every experiment, each concentration of siRNA is employed in triplicates and the siRNA-treated cells are compared to untreated control cells. All analysis for significance and graph plots were performed using GraphPad Prism 6.0 software. Graph plots are shown with standard error of the mean (SEM). The comparisons were performed between the control and treated groups by Kruskal-Wallis test with one-way ANOVA and Dunn's multiple comparisons test as post-hoc test.

3.2.8.2. Statistical analysis for microRNA analysis

For microRNA analysis, the t-test by the non-parametric Mann-Whitney test was used to determine significance between the diseased and control phenotypes of the clinical cohort. Kruskal-Wallis test with Dunn's multiple comparisons test as post-hoc test was performed for the phenotypes. The significance was performed using GraphPad Prism 6.0 software. The threshold for significance was observed at $p \leq 0.05$.

4. Results

The aims of this master thesis were aligned to meet the goals of the miRNA project embedded in the symAtrial consortium. The over-all goal was establishing a cell culture platform to analyse candidate miRNAs in relation to AF. In order to meet the first aim of the master thesis, two cell lines – HEK293 and HL-1 cells were used. Of these, HL-1 cells are the cell line of interest since they are mouse atrial cardiomyocytes. Transfection experiments for miRNA knockdown in these cell lines were pursued. In order to achieve the second aim of the master thesis, miRNA reverse transcription and RT-PCR analysis was performed to validate candidate miRNAs in AF patient samples at the tissue level. The samples were available through the AFHRI-B cohort.

4.1. Establishment of cell culture platform for miRNA analysis.

For establishing a cell culture platform, HEK293 cells and HL1 cells were used. On them, transfection with a lipofection transfection agent was carried out to deliver miRNA into the cells. The molecule to be transfected were siRNAs. The siRNA used were of the fluorophore control, positive and negative control. The flow of experiments is described in figure 7.

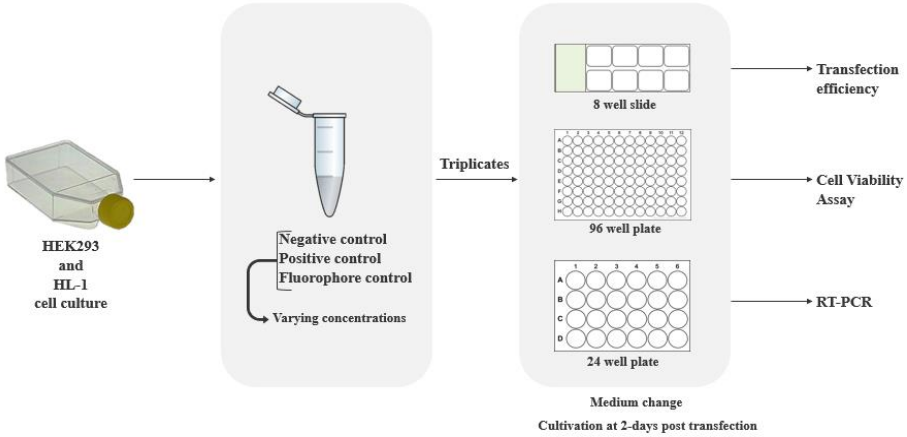


Figure 7 – Flow of experiments to meet the first aim: establishment of a technological platform using cell culture
The HEK293 and HL-1 cells were successfully cultured in 75cm^2 flasks. The experimental approach was divided into three sets. The first set of cells were taken to test the transfection efficiency (fluorophore control-siRNA) using an 8-well slide. Second set, a 96-well plate was used for the cell viability test when treated with the positive and negative controls. The third set was used to study the target mRNA expression levels by TaqMan RT-PCR using a 24-well plate. The fluorophore control, positive control and negative control were all added at the same concentrations and conditions as mentioned in section 3.2.2.1. For HL-1 cells, the conditions were adapted to suit the cell line (section 3.2.2.2) because HL-1 cells did not react well to the conditions that worked for HEK293 cells.

4.1.1. Platform with HEK293 cells

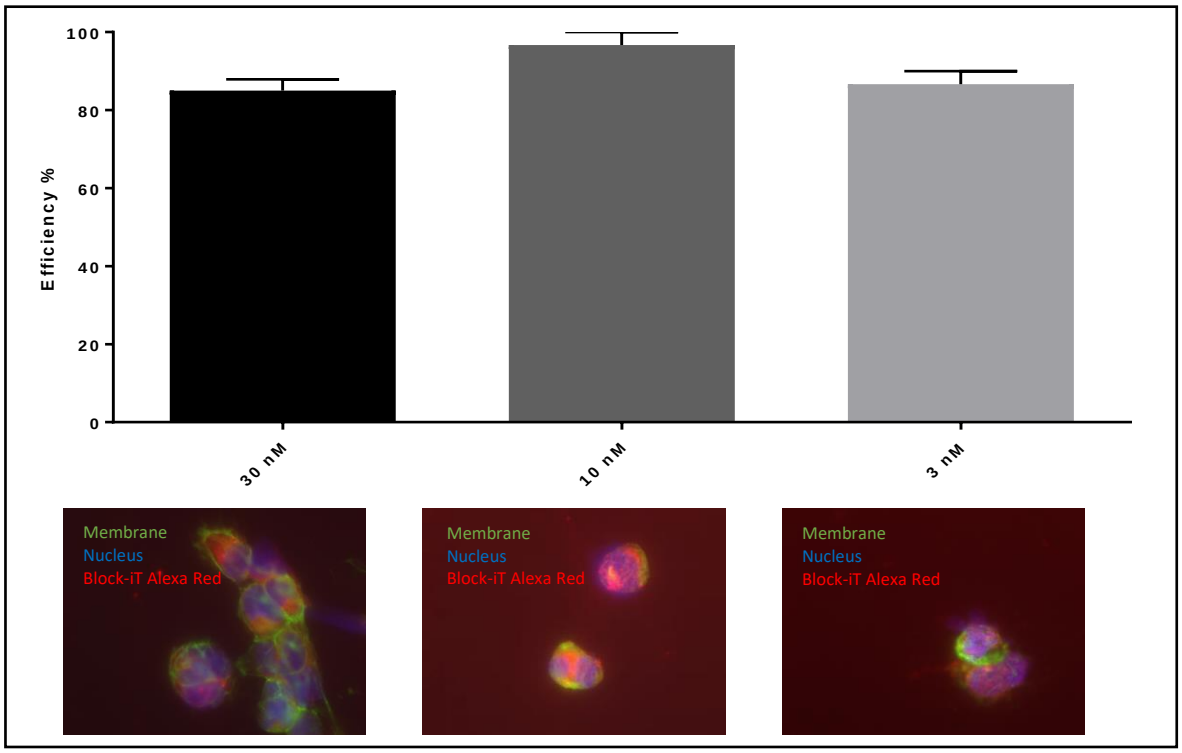
Human Embryonic Kidney 293 (HEK293 cells) were used first. HEK293 cells were successfully cultured in the lab and used for transfection experiments. In this section, results of experiments performed with HEK293 cells in cell culture to get a clear and detailed picture of miRNA transfection are shown.

Measurement of transfection efficiency

A fluorophore indicator used at similar concentrations to the positive control-siRNA could help assess the siRNA uptake by the cells. As mentioned in section 3.2.3, the number of cells fluorescing at the different concentrations will provide with a mirror of how well the positive control-siRNA will be taken up by the cells. Six concentrations of fluorophore control (30nM-0.1nM) tested for two time points (4h and 24h) were the determining factors of the best transfection efficiency.

The following results were obtained by using Lipofectamine® RNAiMAX to transfect HEK293 cells with Block-iT™ Alexa Fluor® Red Fluorophore (fluoresces red). All siRNA-concentrations were taken in triplicates. The cells, after the treatment as mentioned in section 3.2.3, were observed under a multi-channel microscope. To ease observational understanding, the cells were co-stained for nucleus (blue) and membrane (green), and the overlaid figures are shown below.

A.



B.

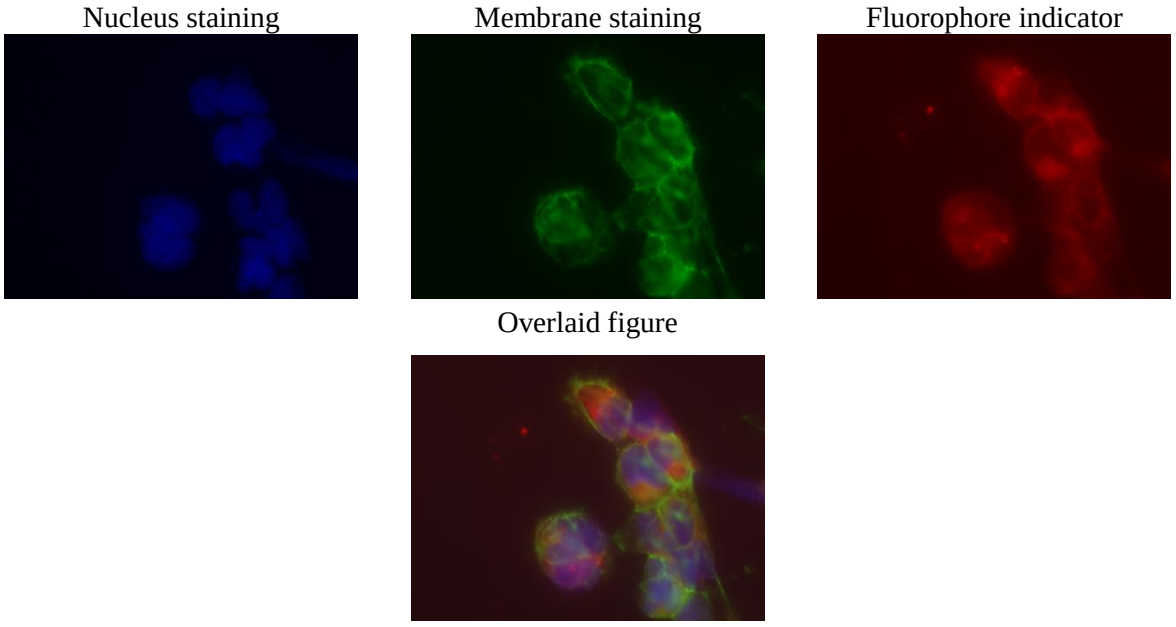


Figure 8 – Measurement of transfection efficiency – HEK293 cells
Measurement of transfection efficiency of HEK293 cells transfected with Lipofectamine® RNAiMAX and indicator Block-iT Alexa Red Fluorophore is shown. All siRNA-concentrations were taken in triplicates. **A.** a graphical representation of efficiency percentage and their concentration corresponding cell images, as observed under the microscope, are shown below the bar graph. Only the three higher concentrations transfected for 24 h-transfection period are presented from which 30nM and 10nM showed the best transfection efficiency. **B.** Pictures from individual channels for 30nM fluorophore control clearly show that the whole cell is fluorescing red. 10nM concentration showed near 100% transfection efficiency with a clearer signal in all cells observed under the microscope. The signal became weaker and unclear as the concentrations decreased from 30nM to 0.1nM.

The transfection efficiency was tested for two time points – 4 h and 24 h. At 4 h post transfection, the fluorescent signal was 30-40% lower than that observed at 24 h (pictures not shown). The signal decreased significantly over the fluorophore concentration range (30nM-0.1nM), the brightest fluorescent signal being from the highest concentration of siRNA fluorophore (30nM and 10nM). In addition, number of cells fluorescing and fluorescence was better at 10nM rather than at 30nM. The red fluorescent signal, signifying the fluorophore control, is emanating from within the cells. Lipofectamine® RNAiMAX is suitable for being used as the siRNA transfection agent for HEK293 cells, showing effective transfection signal at 24 h. This was proof that Lipofectamine® RNAiMAX is compatible with HEK293 cells.

Cell Viability and RT-PCR analysis – HEK293 cells

HEK 293 cells were used first in order to establish a cell culture platform for miRNA transfection. miRNA transfection experiments were carried out in different well-plate formats as shown in figure 7. It was important to analyse whether the cell viability and target mRNA levels were influenced by the positive control-siRNA – miRNA inhibitor let-7c. A successful transfection of miRNA inhibitor let-7c in HEK293 cells would provide the experimental parameters to be followed, in case a suitable candidate miRNA is found.

Cells grown without any treatment (untreated) were taken as control. For the treated cells, the transfection medium was replaced to normal medium after the intended siRNA-transfection periods 4h and 24h. All siRNA-concentrations were taken in triplicates. The cells were cultivated at 2-days post transfection. For cell viability assay, the CellTiter Blue reagent® was added. For RT-PCR, cells were subjected to total RNA isolation and cDNA synthesis. Six concentrations of positive and negative control-siRNA (30nM – 0.1nM) and two time points (4 h and 24 h), as mentioned in section 3.2.2.1, were the two determining parameters through which the optimal experimental conditions to be administered were identified.

The cell viability analysis showed that there was significant difference in cell health between the two transfection periods-4h and 24h ($p < 0.05$) but not within the siRNA-concentration range 30nM – 0.1nM. Positive control miRNA inhibitor let-7c is expressed in many species and its' target mRNA – *HMGA2* is upregulated upon let-7c knockdown. The mRNA levels were normalized to a housekeeper – *GAPDH*, and the relative mRNA expression ($2^{-\Delta Ct}$) is shown in figure 9. The negative control-treated cells showed similar regulation as untreated cells indicating that the negative control-siRNA has no sequence similarity and does not affect gene expression. At the 4 h siRNA-transfection period, the relative mRNA expression seems to be similar between the untreated and treated cells. There is no significant regulation at 4h of siRNA-treatment. At 24 h siRNA-transfection period, the positive control showed significant upregulation at 3nM concentration ($p < 0.01$) when compared to untreated cells. The 10nM concentration of siRNA was also close to the range of 3nM but had no significance according to one-way ANOVA test.

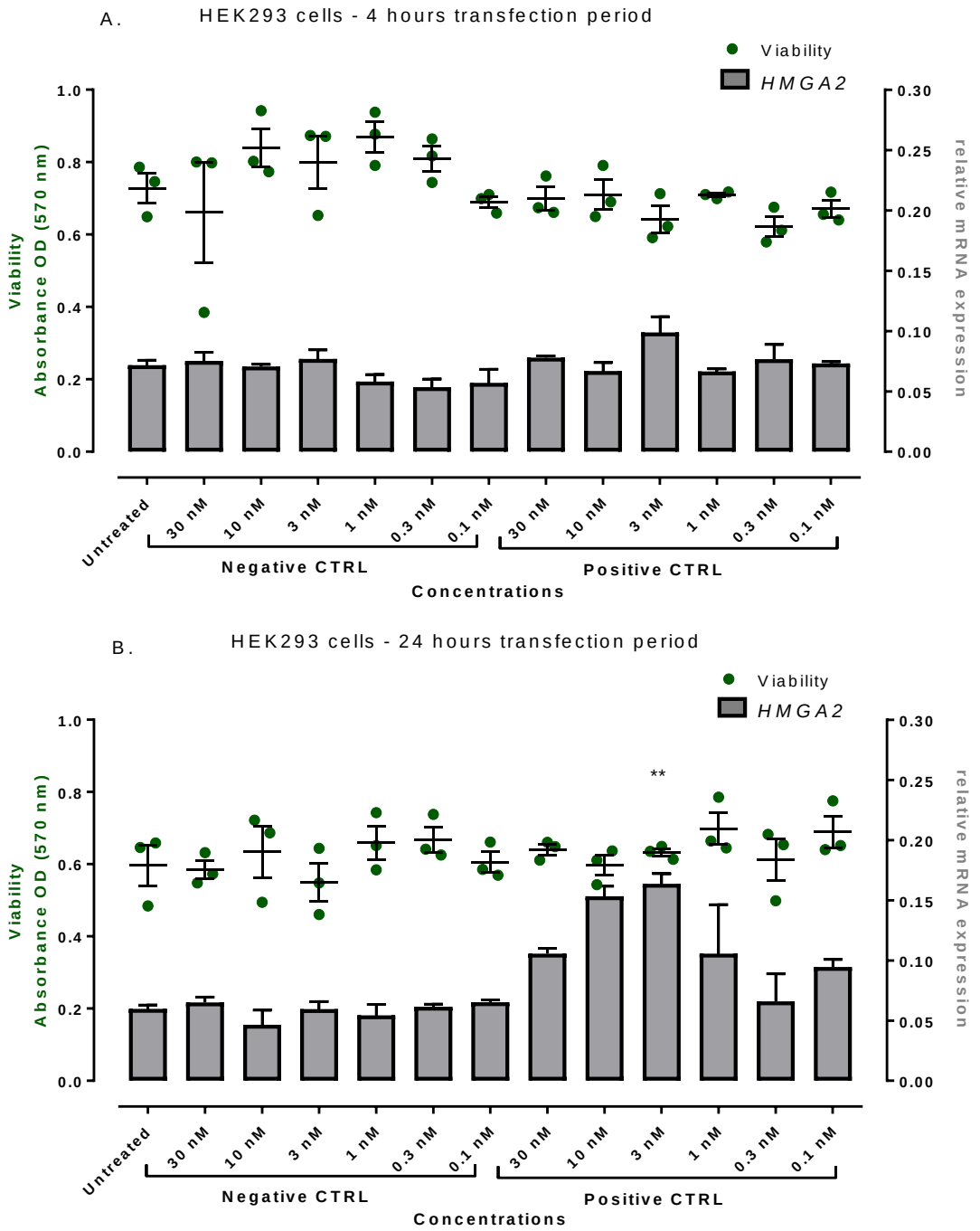


Figure 9 – Treatment of HEK293 cells with miRNA inhibitor let-7c positive control – Analysis of cell viability and RT-PCR

The cell viability is shown on the left Y-axis (green) and the relative target mRNA-*HMGA2* expression ($2^{-\Delta Ct}$) is observed on the right Y-axis (grey) for HEK293 cells treated with the transfection medium for **A.** 4 h and **B.** 24 h. All siRNA-concentrations were taken in triplicates. At the two time points-4 h and 24 h, the positive control-treated cells were compared to the untreated cells over six concentrations of siRNA between 30nM-0.1nM. The cell viability decreases significantly between the time points-4h and 24h ($p < 0.05$) but not within the siRNA-concentration range 30nM-0.1nM. At 4h-transfection period, the treated and untreated cells show similar mRNA expression. At 24h-transfection period, significant upregulation in *HMGA2* expression at the mRNA level can be seen at 3nM siRNA-concentration ($p < 0.01$). The lower concentrations of positive control-siRNA and the negative control-treated cells show similar expression of *HMGA2* as untreated cells. The mRNA expression levels were normalized to the housekeeper – *GAPDH*. Data is shown as mean ± SEM; n=3.

The cell culture platform for miRNA characterization using HEK293 cells was established. HEK293 cells reacted well to the treatment and showed logical regulation based on the quantity of siRNA employed. Transfection efficiency was optimal at 10nM fluorophore siRNA concentration. It was evident that the siRNA treatment does not affect cell viability, hence depending on the time when cell culture medium resources were replenished. It was established that 10nM or 3nM siRNA-concentration with Lipofectamine® RNAiMAX could be used for transfecting HEK293 cells for further experiments.

4.1.2. Platform for HL-1 cells

HL-1 cells were the cell line of interest in the thesis. They are mouse atrial cardiomyocytes and hence, would provide great insight into establishing a cell culture platform for miRNA analysis. The flow of experiments is described in figure 7.

Successful miRNA transfection was obtained for HEK293 cells evident from the target mRNA levels (figure 9). The experimental conditions used for the successful HEK293 transfection were applied onto HL-1 cells. The following section presents results obtained from HL-1 cell culture experimentations.

4.1.2.1. Transfection of HL-1 cells using Lipofectamine® RNAiMAX

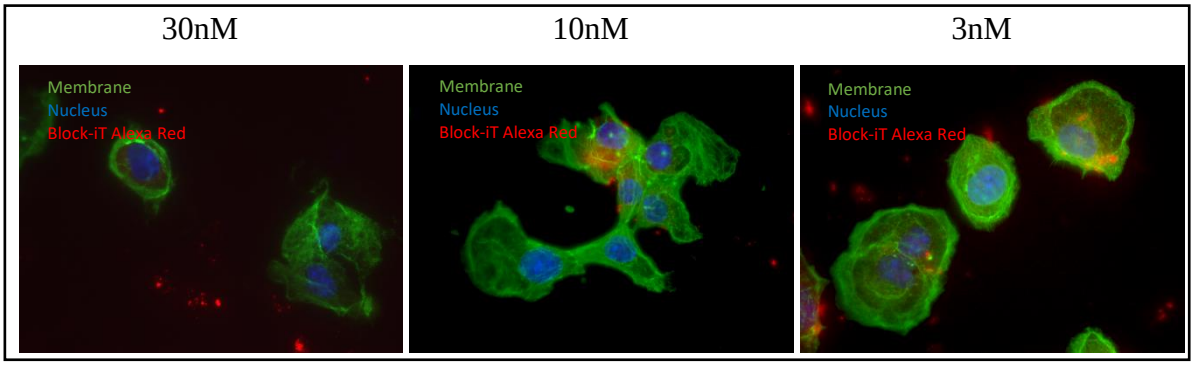
In an attempt to translate the success of HEK293 cells onto HL-1 cells, the experimental conditions used were identical. Lipofectamine® RNAiMAX was used as the transfection agent. The following section presents the results of HL-1 cells treated with the method described in section 3.2.2.1.

Measurement of transfection efficiency

A fluorophore indicator used at similar concentrations to the positive control siRNA will provide with a mirror of how well the positive control siRNA will be taken up by the cells. Six concentrations of fluorophore control (30nM-0.1nM) tested for two time points (4h and 24h) were the determining factors of best transfection efficiency.

The following results were obtained by using Lipofectamine® RNAiMAX to transfect HL-1 cells with Block-iT™ Alexa Fluor® Red fluorescent control (fluoresces red). The cells, after the treatment as mentioned in section 3.2.3, were observed under a multi-channel microscope. All siRNA-concentrations were taken in triplicates. To ease observational understanding, the cells were co-stained for nucleus (blue) and membrane (green), and the overlaid figures are shown below.

A.



B.

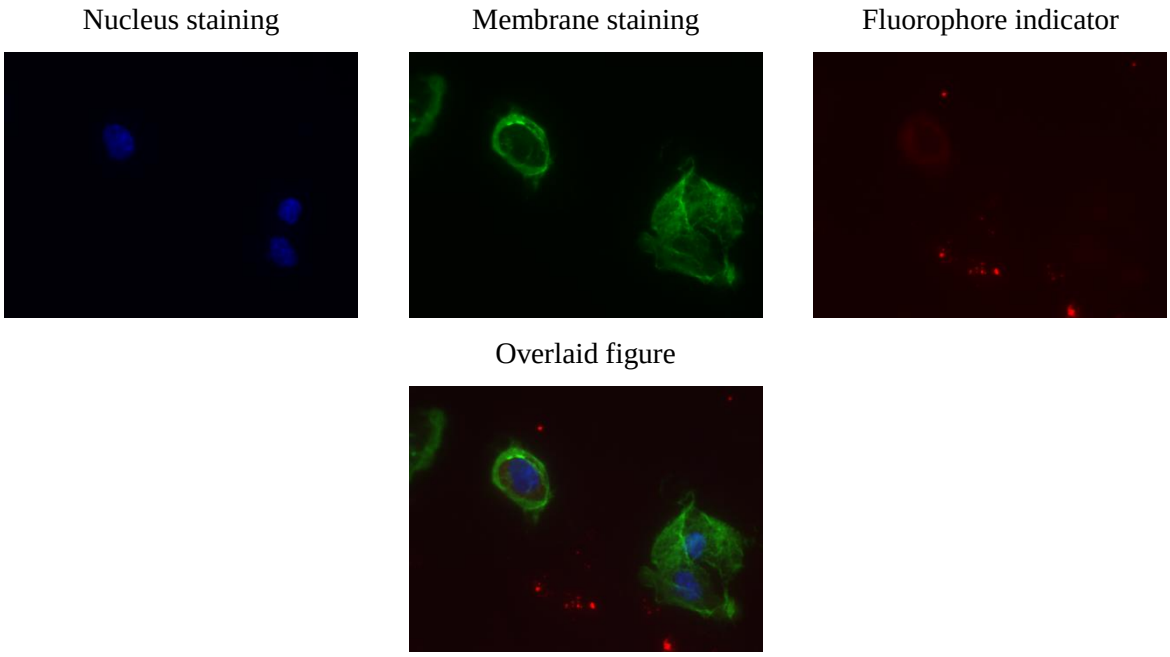


Figure 10 – Measurement of transfection efficiency – HL-1 cells
Analysis of HL-1 cells when transfected with Block-iT™ Alexa Fluor® Red fluorescent control using Lipofectamine® RNAiMAX. All siRNA-concentrations were taken in triplicates. **A.** Cell images of the highest three concentrations of fluorophore control employed – 30nM, 10nM and 3nM. Null fluorescing cells are observed at the all concentrations for both time points – 4 and 24 h (all images not shown). The cells appear to be unaffected by the fluorophore siRNA-treatment. **B.** Images for 30nM fluorophore siRNA from individual channels identifying the nucleus, membrane and the fluorescent indicator show that the signal remained on the membrane or outside the cells and did not emanate from within.

Even though identical conditions that were used for HEK293 cells were applied to HL-1 cells, the conditions did not provide the affirmation of a successful transfection of the fluorophore siRNA into the HL-1 cells. Figure A and B demonstrate the inability of Lipofectamine® RNAiMAX to deliver the fluorescent compound into the cells. The signal did not appear to emanate from within the cell but from outside, proving Lipofectamine® RNAiMAX is not compatible with HL-1 cells.

Cell Viability and RT-PCR analysis

HL-1 cells were cultured and transfected with positive and negative control-siRNA in different well-plate formats as described in figure 7. The transfection agent used was Lipofectamine® RNAiMAX. This agent was used to transfect positive (miRNA inhibitor let-7c) and negative control-siRNA. The effect of the siRNA on cell viability and target mRNA levels were tested and their results are shown in this section.

Cell without any siRNA treatment (untreated) were taken as controls. All siRNA-concentrations were taken in triplicates. The cells were switched to normal medium after 4 h and 24 h of siRNA-transfection period and cultivation of cells was performed at 2-days post transfection. For cell viability assay, the cells were added with CellTiter® Blue reagent whereas for RT-PCR analysis, the cells were subjected to RNA isolation and cDNA synthesis. TaqMan RT-PCR was used to determine the relative mRNA expression levels for mouse *Hmga2*. The target mRNA *Hmga2* expression is supposed to be upregulated upon the knockdown of let-7c.

The cell viability decreased significantly over the two time points – 4 h and 24 h ($p < 0.0001$) but not within the siRNA-concentration range (30nM-0.1nM). The target mRNA expression is the similar for both time points (4h and 24h) and between the treated and untreated cells (Ct value average 37). No significant regulation of the target *Hmga2* was observed according to one-way ANOVA.

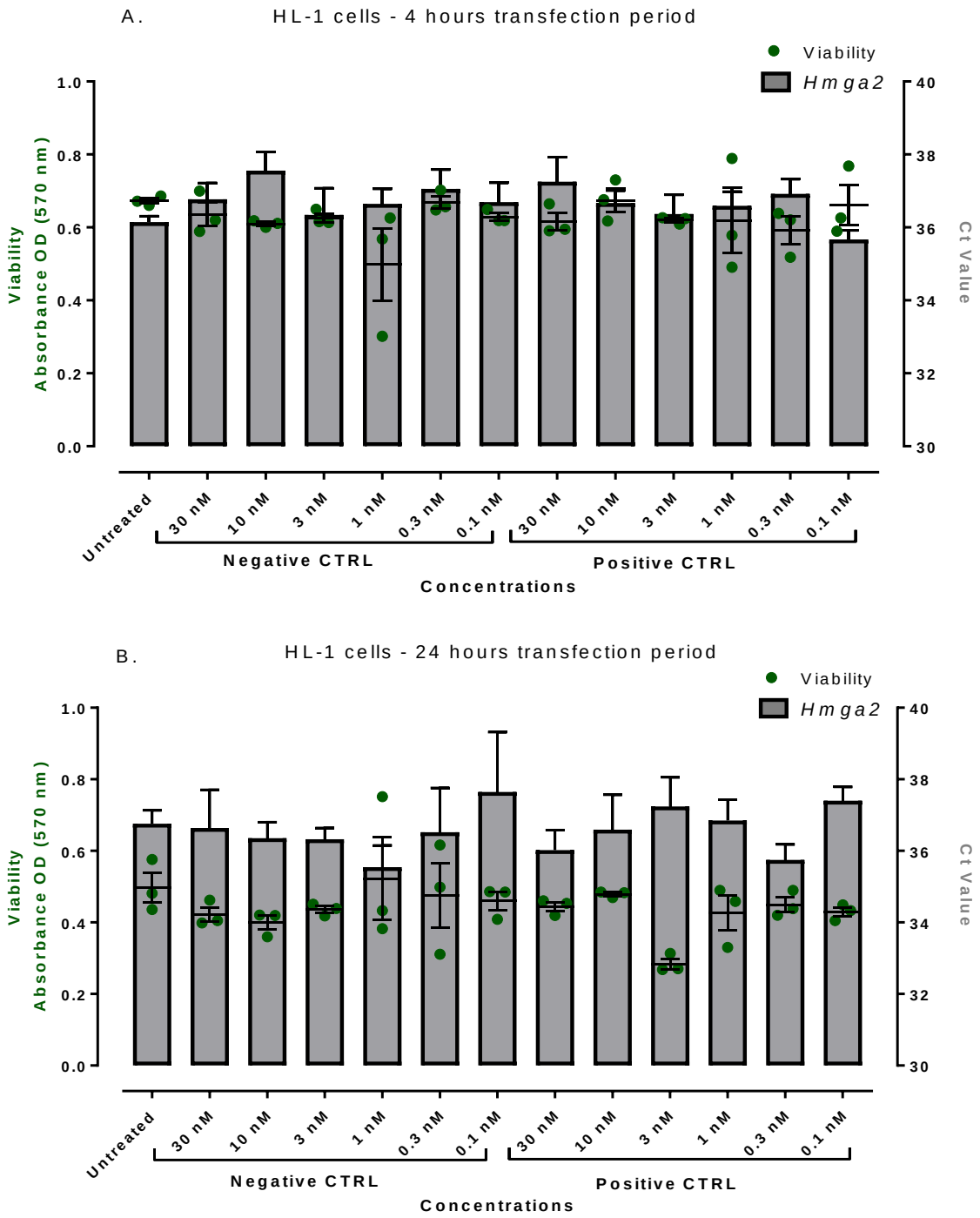


Figure 11 – Treatment of HL-1 cells with miRNA inhibitor let-7c positive control – Analysis of cell viability and RT-PCR

The cell viability is presented on the left Y-axis (green) and the raw Ct value of mRNA-*Hmga2* expression on the right Y-axis (grey) for HL-1 cells when treated with siRNA-transfection medium for **A.** 4 h and **B.** 24 h. All siRNA-concentrations were taken in triplicates. The cell viability differed significantly ($p<0.0001$) over the two siRNA-transfection periods 4h and 24h, but not within the siRNA-concentration range 30nM-0.1nM. The *Hmga2* expression was not observed in HL-1 cells indicated by the raw Ct values average at ~37 Ct. There was no difference in target mRNA regulation in either the untreated cells or the negative and positive control-treated cells. mRNA expression levels were not normalized to any housekeeper, raw Ct values are shown here. Data is a representation of mean ± SEM; n=3.

HL-1 cells did not show any regulation of *Hmga2*, neither in the treated nor untreated cells. The siRNA treatment had no effect in the cells because the target mRNA – *Hmga2* is not expressed in adult mouse cells, except under cancerous conditions. Adult mouse cardiomyocytes do not express *Hmga2* but embryonic cells do so abundantly. Neither the concentration range of siRNA nor the time points were determining factors to understand the miRNA transfection conditions for HL-1 cells.

In conclusion, the transfection agent – Lipofectamine® RNAiMAX was not compatible with HL-1 cells. The knockdown of miRNA inhibitor let-7c positive control could not be studied since its recommended target – *Hmga2* is not expressed in adult mouse cells. All experimental conditions that worked with HEK293 cells could not translated onto HL-1 cells. The results were incomparable and so the experimental conditions were changed.

4.1.2.2. Transfection for HL-1 cells using DharmaFECT 1

To achieve the first aim of the master thesis, it was important to establish a cell culture platform for HL-1 cells. However, as was observed in section 4.1.2.1, the experimental conditions did not provide a successful cell culture transfection platform for HL-1 cells. Therefore, HL-1 cells were subjected to different experimental conditions that are described in section 3.2.2.2.

First, the transfection agent was replaced from Lipofectamine® RNAiMAX to DharmaFECT 1. Second, the fluorescent control was changed to siGLO Green Transfection Indicator since it is compatible with DharmaFECT 1. Third, the positive control was changed. Instead of miRNA inhibitor let-7c, two new positive controls were chosen – *mir*Vana™ miRNA Mimic miR-1 and ONTARGETplus GAPD control siRNA. Fourth, only one time point of siRNA-transfection period was chosen – 24 h. The negative control and cell viability assay reagent remained the same.

In the following section, results from transfecting HL-1 cells with DharmaFECT 1 are shown. Here, the positive controls – miR-1 mimic, GAPD siRNA and the negative control-siRNA treated cells were compared to untreated cells (control). The flow of experiments is described in figure 7.

Measurement of Transfection Efficiency

The fluorescent control is designed in such a way that it has the same length and charge as that of the positive control-siRNA. Therefore, after transfection, if a cell is fluorescing, it provides with a positive indication that the transfection agent (DharmaFECT 1) is capable of delivering the siRNA into the cells and hence compatible with the cells.

siGLO Green Transfection Indicator

DharmaFECT 1 was used to transfect siGLO Green Transfection Indicator into HL-1 cells. Three concentrations were tested (50nM, 30nM and 20nM) for one time point (24 h). All siRNA-concentrations were taken in triplicates. After 24 h, the cells were fixed and mounted with coverslip.

siGLO Green Transfection Indicator fluoresces green as was observed using the multi-channel microscope. The cells were also stained for the nucleus (Hoechst Blue). The overlaid figures are shown below.

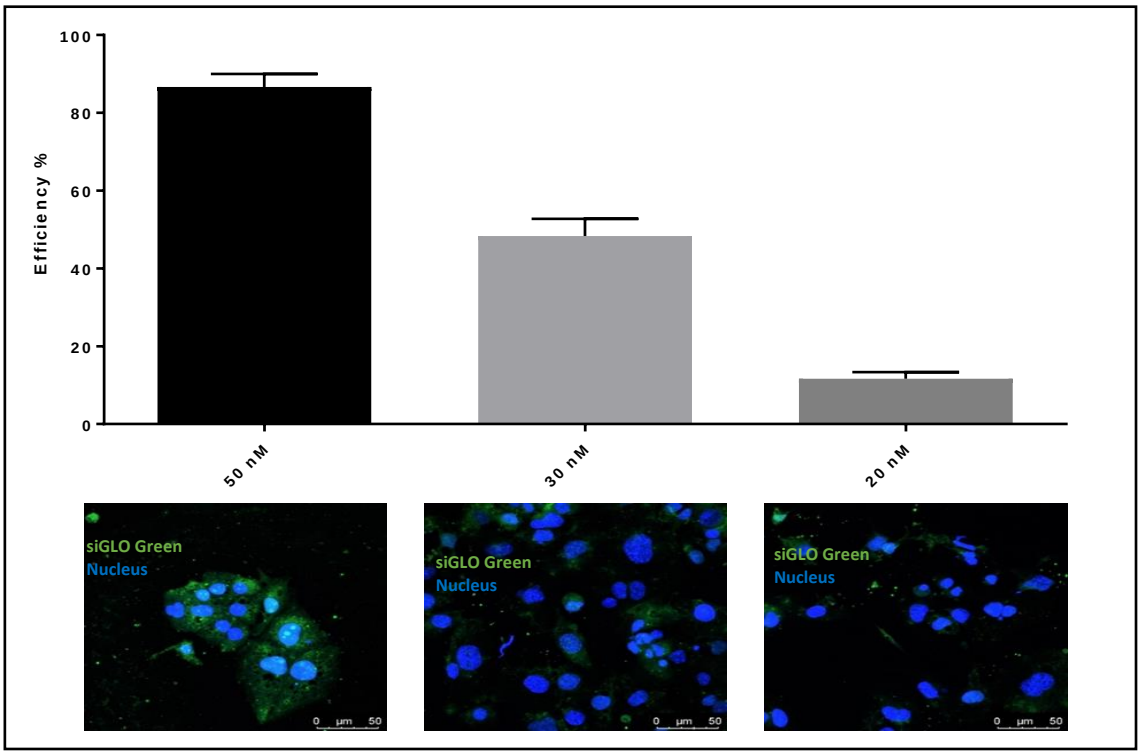


Figure 12 – Measurement of transfection efficiency with adapted conditions – HL-1 cells
HL-1 cells were transfected with the fluorophore control – siGLO green Transfection indicator for 24h. The transfection agent tested was DharmaFECT 1. All siRNA-concentrations were taken in triplicates. The fluorescent signal was the strongest at the highest fluorophore siRNA-concentration. A stark decrease can be seen in the green fluorescence signal over the fluorophore concentrations. Signal emanates from within the cells and evenly distributed as can be seen at 50nM concentration. Data is shown as mean ± SEM; n=3.

The green signal from the fluorescent control seems to be evenly distributed within the cell, implying DharmaFECT1 is compatible with HL-1 cells to deliver the siRNA. This is in contrast to the results as seen in figure 10 with Lipofectamine® RNAiMAX, where it was clear that the cells did not take up the siRNA, evident from null red fluorescence from the cells. Here, based on fluorescence signal, 50nM showed a good concentration to obtain a clear signal. This was proof that the DharmaFECT 1 was compatible with HL-1 cells and could be used for further transfection experiments.

Cell viability and RT-PCR analysis

The experimental approach using the previous conditions failed in establishing a cell culture platform using HL-1 cells. The transfection was not successful using Lipofectamine® RNAiMAX and as seen in figure 10. In addition, the target of miRNA inhibitor let-7c – *HMGA2* was not expressed by mouse atrial cardiomyocytes. Therefore, the experimental conditions were changed for HL-1 cells in order to establish the cell culture platform for miRNA analysis. During experimentation, the cells were treated with two different positive controls and were compared to one set of negative control and untreated cells.

***mir*Vana miRNA Mimic miR-1 positive control treatment**

miR-1 mimic was chosen as a positive control because miR-1 is abundantly present in heart. The knockdown of miR-1 could be easily detected on the mRNA levels on one of its target – PTK9 or *Twf1*.

The HL-1 cells were transfected with a miR-1 mimic positive control at the concentrations 100nM, 75nM, 50nM and 25nM, which were compared to untreated cells (control). All siRNA-concentrations were taken in triplicates. The cells were cultivated 2-days post transfection and subjected to cell viability assay and RT-PCR. CellTiter Blue reagent was added for the cell viability analysis. For RT-PCR, the cDNA synthesis was performed from the isolated RNA samples of treated and untreated cells. These cDNA samples were used for TaqMan RT-qPCR analysis of the target gene *Twf1* for mouse. The target *Twf1* should be downregulated upon the knockdown of miR-1. The negative control-treated cells were the same for experiments with miR-1 mimic and GAPD siRNA.

Cell viability analysis revealed that the miR-1 mimic positive control-treated cells were lower than the untreated cells but showed no significant effect on HL-1 cell viability. Negative control-siRNA treatment had a significant impact on the cell viability (green, $p < 0.05$) at 75nM when compared to the untreated cells. The relative target mRNA levels ($2^{-\Delta Ct}$) of *Twf1* showed significant downregulation (grey, $p < 0.0001$) for all positive control-siRNA concentrations (100nM-25nM) implying successful knockdown of miR.1. However, here too, a significant upregulation of *Twf1* was observed in the negative control-treated cells at 100nM and 50nM (grey, $p < 0.05$).

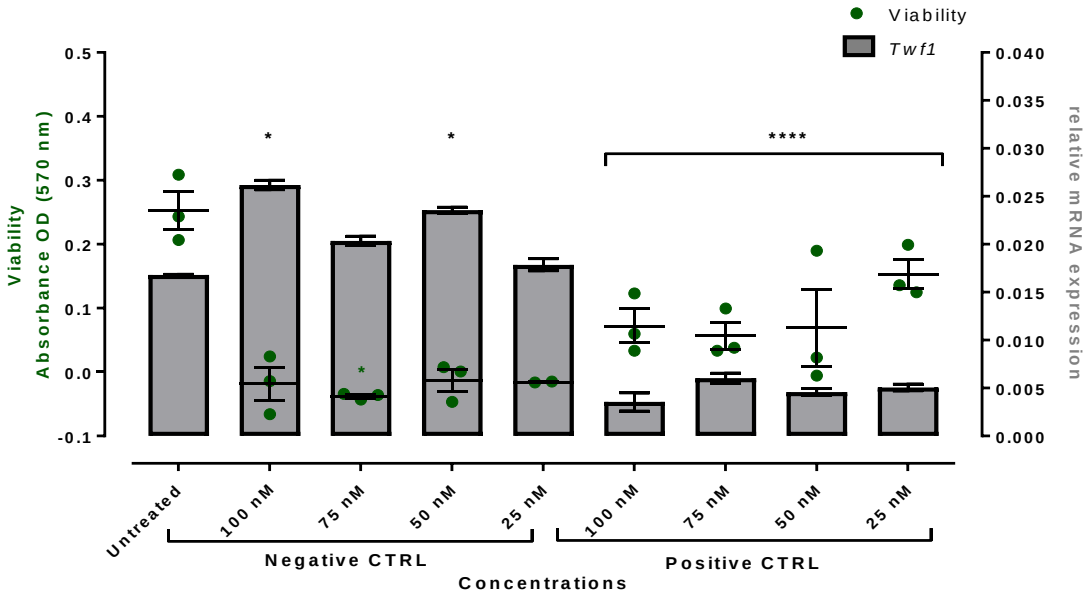


Figure 13 – Treatment of HL-1 cells with miR-1 mimic positive control – Analysis of cell viability and RT-PCR
All siRNA-concentrations were taken in triplicates. Cell viability analysis – The viability of positive control-treated cells was lower than untreated cells, however, this was not significant. This implies that the positive control-siRNA did not have significant impact on cell viability. At 75nM of negative control-siRNA, significant decrease in cell viability was observed ($p < 0.05$). RT-PCR analysis – *Twf1* mRNA levels were significantly downregulated ($p < 0.0001$) when compared to the untreated and negative control-treated cells. Negative control-treated cells showed a certain upregulation of target mRNA-*Twf1* at 100nM and 50nM siRNA-concentration ($p < 0.05$). mRNA expression levels were normalized to the housekeeper *Cdkn1b*. Data is represented as mean ± SEM; n=3

The HL-1 cell viability was not affected by the miR-1 mimic positive control treatment. In addition, the cells showed significant downregulation of the target mRNA *Twf1* implying miR-1 can be used as a positive control for HL-1 cells. The miRNA concentration range can be between 100nM-25nM for further experiments.

ON-TARGET plus GAPD positive control treatment

GAPD is ubiquitously present in all cells and is non-essential to cell health. The knockdown of GAPD can be easily studied at the mRNA levels of the target – *Gapdh* and was therefore chosen as a positive control in the experiments.

HL-1 cells were seeded at densities mentioned in section 3.2.1 and were treated with GAPD siRNA positive control at concentrations 100nM, 75nM, 50nM and 25nM using DharmaFECT 1. All siRNA-concentrations were taken in triplicates. The negative control-treated cells were the same for experiments miR-1 mimic and GAPD siRNA. CellTiter® Blue reagent was added to test the effect of positive control-siRNA on the cell viability. For RT-PCR, treated and untreated cells were subjected to RNA isolation and cDNA synthesis. RT-PCR was performed for the target mRNA – *Gapdh* regulation measurement. The target – *Gapdh* was downregulated upon knockdown of GAPD.

Knockdown of GAPD in a cell does not affect cell viability. This was observed in cell viability analysis where the values of positive control-siRNA treated cells showed no significant difference when compare to untreated cells. However, the cells treated with negative control-siRNA showed significant decrease in cell health at 75nM (green, $p < 0.05$). In RT-PCR analysis, strong significant downregulation of the relative target mRNA *Gapdh* levels ($2^{-\Delta Ct}$) can be seen (grey, $p < 0.0001$) at all siRNA concentrations (100nM-25nM) indicating a successful knockdown of GAPD. However, here too, the negative control-siRNA concentration – 100nM showed a significant upregulation in the target *Gapdh* levels when compared to untreated cells.

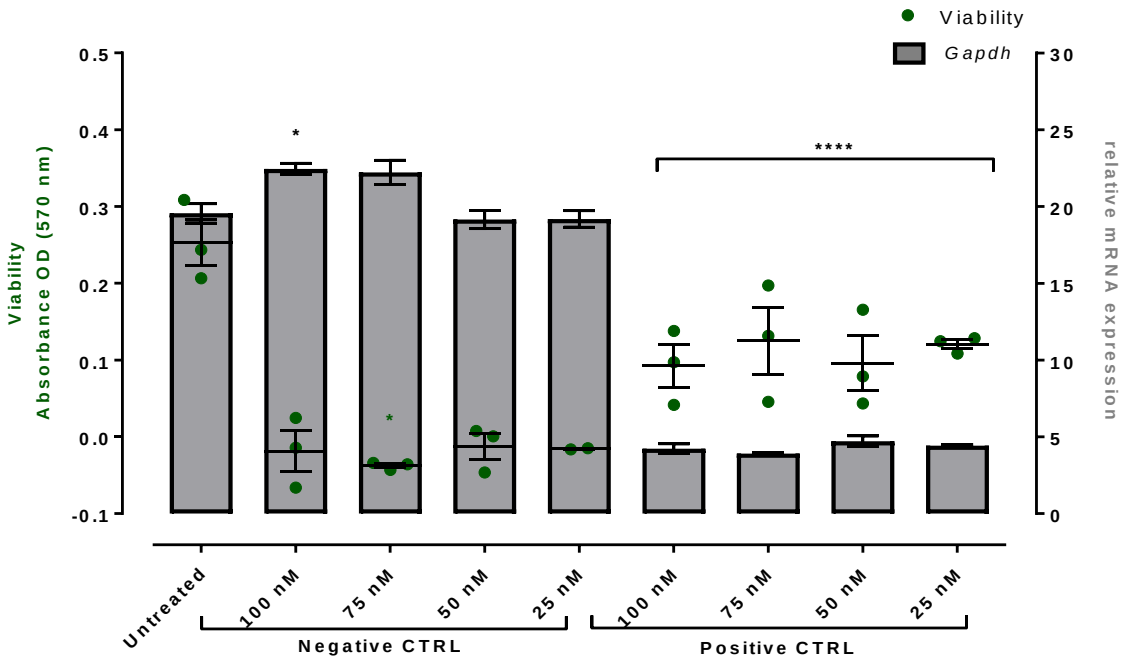


Figure 14 – Treatment of HL-1 cells with GAPD siRNA positive control – Analysis of cell viability and RT-PCR

All siRNA-concentrations were taken in triplicates. Cell viability is shown on the left Y axis (green) and relative mRNA expression ($2^{-\Delta Ct}$) is shown on the right Y axis (grey). Cell viability analysis shows a significant decrease in cell viability for HL-1 cells treated with negative control at 75nM, whereas GAPD siRNA did not have any significant impact on the cell viability when compared to untreated cells. RT-PCR analysis – target *Gapdh* showed highly significant downregulation ($p < 0.0001$) for all the concentrations (100nM-25nM) when compared to negative control and untreated cells. Since the negative control showed some impact on cell viability, significant upregulation at 75nM negative control-siRNA concentration was observed ($p < 0.05$). mRNA expression levels were normalized to the housekeeper – *Cdkn1b*. Data are shown as mean ± SEM; n=3

The HL-1 cells were not significantly affected in cell viability when treated with the positive control – GAPD siRNA for 24h. In addition, the target mRNA levels of *Gapdh* was significantly downregulated as seen by the RT-PCR analysis. This implies that GAPD siRNA can be used as a good positive control in knockdown experiments with HL-1 cells using DharmaFECT 1 as the transfection agent. The miRNA levels to be administered can be in the range of 100nM-25nM for further experiments.

In conclusion, based on the experiments of the HL-1 cell line, the cell culture platform could not be established using Lipofectamine RNAiMAX. In addition, the positive control *mir*Vana miRNA inhibitor let-7c did not show any expression at target mRNA levels in HL-1 cells using RT-PCR analysis. This led to adaptation of conditions for HL-1 cells. The transfection agent DharmaFECT 1 was compatible with HL-1 cells, the new positive controls used miR-1 and GAPD were successfully transfected as was observed at their respective target mRNA levels using RT-PCR. Therefore, HL-1 cell culture platform could be established using DharmaFECT 1 and miR-1, GAPD positive controls at the concentration range 100nM-25nM.

4.2. Validation of candidate microRNA

The second aim of the master thesis was to validate selected candidate miRNAs in the AFHRI-B cohort to assess their relation to AF.

Isolated RNA samples from the right atrial appendages were available for miRNA analysis. First, the available RNA samples were subjected to RIN analysis to test the quality of RNA. Subsequently, for microRNA analysis, these RNA samples were reverse transcribed by Advanced TaqMan miRNA assay, which resulted in a cDNA ready for TaqMan RT-PCR.

4.2.1. RIN analysis – RNA Quality check of AFHRI-B cohort samples

RIN analysis was performed to test the quality of RNA samples in the cohort. This was performed to exclude samples with a degraded RNA quality that might affect the results. RIN analysis of 120 AFHRI-B cohort samples revealed no significant difference in the quality of RNA between the phenotypes of the cohort. RIN for the samples under the CTRL phenotype averaged near 7.2, Pre-OP phenotype near 7.5 and Post-OP phenotype near 7.1. Majority of samples have a RIN 6 and above indicating good RNA quality and reliability of the obtained results.

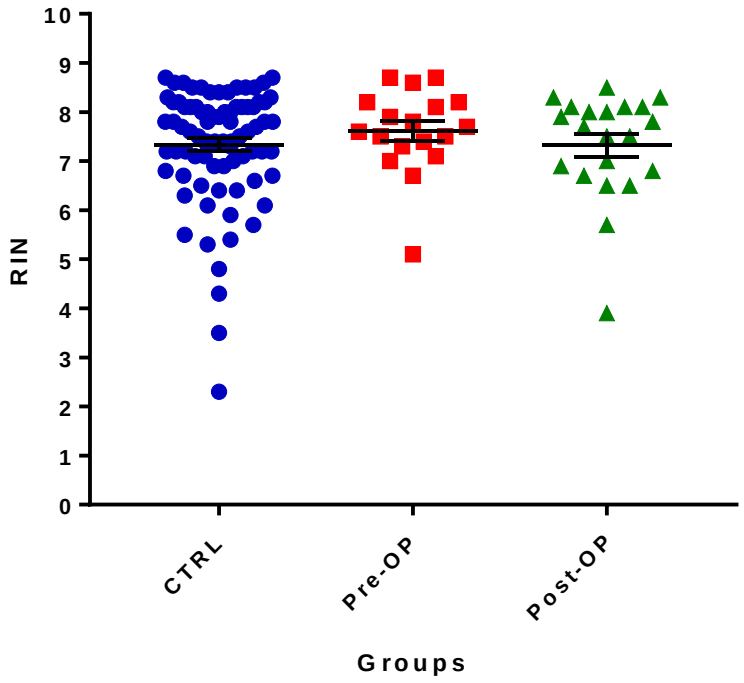


Figure 15 – RIN analysis of RNA samples from AFHRI-B cohort
RNA Integrity Number of samples from AFHRI-B cohort average around 7.5, which indicates a higher integrity of RNA. Data are represented as mean ± SEM; n=120.

RIN for majority samples was good and acceptable. All samples with a RIN of 6 and above were included for miRNA analysis whereas the RNA samples with a RIN of < 6 were excluded because of the degraded quality.

4.2.2. Housekeeper miR-16

The analysed miRNAs are miR-21, miR-100 and miR-483-5p. The raw Ct values of the candidate miRNAs need to be normalized to a housekeeper because normalizing the samples to a reference standard, which is stable during a diseased state, helps to overcome any pipetting error or error of measurement in samples that may have occurred. In the laboratories, the miRNAs are usually normalized to miR-16 and this is the most common housekeeper used for all miRNA analysis so far.

After the measurement of the miR-16 housekeeper in the AFHRI-B cohort samples, raw Ct values showed a significant deregulation between the control and pre-operative AF ($p < 0.001$).

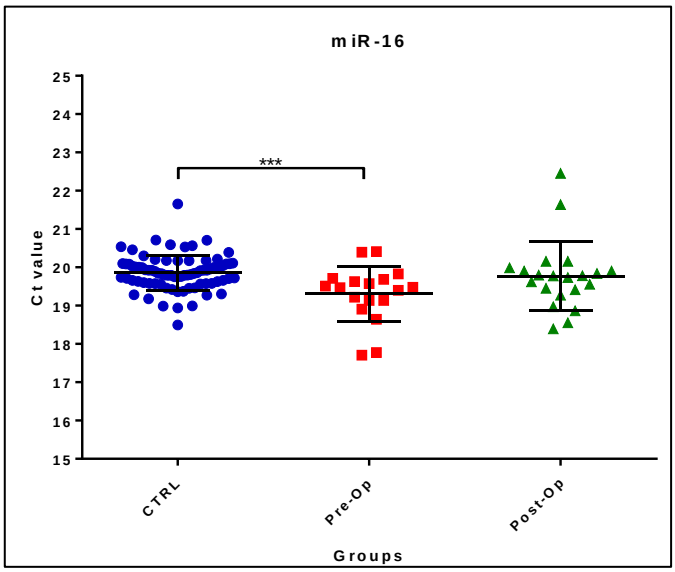


Figure 16 – Regulation of housekeeper miR-16 between the phenotypes of AHFRI-B cohort
Analysis of miR-16 using TaqMan RT-PCR was performed. miR-16 was used as the housekeeping miRNA for validating a candidate miRNA. Raw Ct values show a clear significant regulation between the control and Pre-OP phenotypes ($p < 0.001$). This shows that miR-16 is unstable and other miRNAs cannot be normalized to it. Data is shown as mean ± SEM; n=128.

The data indicates that miR-16 is not a stable housekeeper and is regulated by AF status. Normalization of miR-21 and miR-100 to this unstable miR-16 would provide a false significant regulation of the candidate miRNA.

Therefore, miR-16 was not used as the reference gene in the candidate miRNA analysis. Instead, only raw Ct values were used for an initial analysis in this master thesis.

4.2.3. miRNA analysis in the AFHRI-B cohort

The RNA samples with good RIN were taken for analyzing miRNAs -21, -100 and -483-5p. These miRNAs were analyzed using TaqMan microRNA assays. A quantitative Ct value was obtained and miR-16 was used as the housekeeper.

The raw Ct values were taken for the miRNA analysis and these values were not normalized to the housekeeper miR-16. Raw Ct values of all the miRNAs analysed show that miR-21 regulated significantly between the control and Pre-Op phenotypes ($p < 0.01$). miR-100 showed no regulation between the phenotypes. The microRNA 483-5p was expressed at an early stage in TaqMan RT-PCR and showed a Ct value of ~ 6, or remained undetermined for most samples.

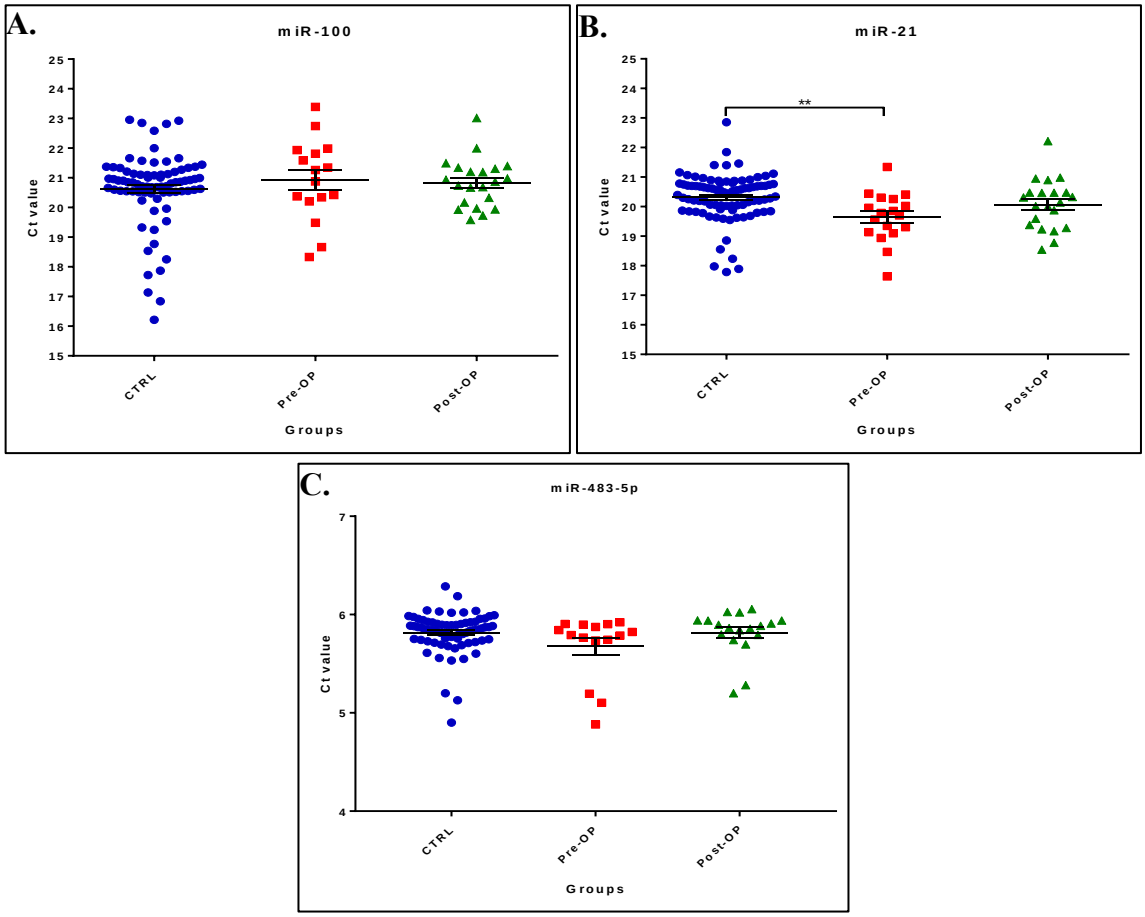


Figure 17 – miRNA analysis on AFHRI-B cohort
miRNA analysis was performed using TaqMan RT-PCR. **A.** microRNA analysis of miR-100 – no significant regulation was observed between the phenotypes. **B.** microRNA analysis of miR-21 – raw Ct values of miR-21 show a clear significant regulation between the CTRL and Pre-OP phenotypes was seen ($p < 0.01$). **C.** microRNA analysis of miR-485-5p – raw Ct values averaged around 6 and most of the samples returned undetermined in the TaqMan RT-PCR analysis. Data is shown as mean ± SEM; n=128.

The raw Ct values obtained from RT-PCR were analysed for the miRNAs. miR-21 showed a regulation between the control and Pre-OP phenotypes whereas miR-100 and miR-483-5p did not show any regulation. These values are not normalized to any housekeeper.

miR-21 showed regulation between the Pre-OP and CTRL phenotypes, indicating that individuals suffering from AF prior to the bypass surgery have increased expression of miR-21. The other two miRNAs, miR-100 and miR-483-5p did not show any regulation between the phenotypes when analysed with the raw Ct value. However, the housekeeper used – m miR-16 showed a regulation between the control and Pre-OP phenotypes. This indicates that the housekeeper, which is supposed to express in a stable manner irrespective of the disease, is regulated by AF or by the cardiovascular disease itself. Since the quality of samples was good, the quality of RNA was not the reason for the regulation found in the housekeeper. Therefore, the miR-16 cannot be used as a housekeeper to normalize the other miRNAs. Instability of the housekeeper played a decelerating role in determining and validating a candidate miRNA. In addition, the results obtained from the symAtrial consortium in this cohort were false positives as they were normalized against housekeeper miR-16.

5. Discussion

Within the symAtrial consortium, which deals with identifying and characterizing the molecular mechanism of AF-related genes, a project to identify and analyse miRNAs in relation to AF was proposed. This miRNA project identified several circulating miRNAs in relation to AF. In this thesis, the over-all goal was to establish a cell culture platform to provide a more detailed functional analysis of miRNA and to validate the results obtained from the symAtrial project regarding the candidate miRNA.

Overall, the results of this thesis showed:

1. Successful establishment of a cell culture platform based on HEK293 cells in regards to cell viability and RT-PCR analysis.
2. Based on the HEK293 cell platform, the adaptation of this platform to HL-1 cells could not be optimized. Therefore, the conditions for HL-1 cells were changed.
3. Candidate tissue miRNA – miR-21 was validated based on raw Ct values.
4. The housekeeper miR-16 seems to be regulated by AF, and therefore cannot be used for normalization of miRNA Ct values.

5.1. Successful establishment of HEK293 cell culture platform

The HEK293 cell culture platform was successfully established. This was observed based on three parameters – transfection efficiency, cell viability and target mRNA RT-PCR analysis. HEK293 cells are easy to transfect cell line due several characteristics such as their fast reproduction rate, easy maintenance, better survival, ease of transfection using wide variety of methods and high transfection efficiency[25]. HEK 293 cells have proven to be reliable hosts in experiments where inducible RNA interference systems[26] had to be studied. In the thesis, HEK293 cells produced a good transfection efficiency at 10nM fluorophore-siRNA concentration using a lipofection method of transfection. This shows the ease of accommodation of HEK293 cell towards the transfection method. The HEK293 cell viability had decreased in the experiments when the transfection medium was changed at 4 hours and 24 hours, but this was not an effect of siRNA but simply exhaustion of cell culture medium resources. At the mRNA levels, due to successful transfection efficiency demonstrated by the lipofection agent, it was expected that an upregulation of the target *HMGA2* mRNA would be visible. This was positively confirmed as an upregulation was visible at the positive control-siRNA concentrations 10nM and 3nM. Thus, these first results showed that HEK293 cells could be used to study miRNA function.

5.2. Adaptation of HL-1 cells

Although HEK293 cells are widely used for transfection experiments, in this thesis, the focus was to study miRNAs in HL-1 cells. HL-1 cells are atrial cardiomyocytes and provide insight about miRNA regulation in atrial cells. Consequently, the main aim was to establish the cell culture platform HL-1 mouse atrial cardiomyocytes. HL-1 cell line have already been used in studies relating to cardiac hypertrophy, mature cardiomyocyte-specific genes, testing of novel cardio-therapeutic drugs and treatments, and production of cardiac proteins[23].

Various studies have also used HL-1 cells as their model cell line to investigate AF. Mace LC et al., 2009[27] were able to stimulate HL-1 cells to obtain the transcriptional profile associated with atrial myocyte remodelling. They identified 758 genes that were significantly altered concluding that rapidly stimulated HL-1 myocytes are concordant with human AF. Another study by Climent et al., 2015[28] used HL-1 cells to study the remodelling mechanisms related to AF. Other studies have also used HL-1 cells to study AF in relation to miRNAs. A study by Ling TY et al., 2017[29] investigated the potential role of miR-499 in AF by microarray analysis of atrial tissues and then targeting the specific miRNA target gene in HL-1 cells. miR-21, the potential biomarker for AF has also been

analysed for AF using HL-1 cell lines in a study by Barana A et al., 2014[30]. Hence, for the thesis it was evident to use HL-1 cells to establish a technical platform to study the role of microRNA knock down / over expression in relation to AF.

Even though HL-1 cells have been widely used, they are indeed difficult to culture, maintain and use for transfection experiments. This was observed during this master thesis in the experiments with lipofection agent – Lipofectamine® RNAiMAX. The measurement of transfection efficiency did not yield in fluorescing cells indicating the lipofection agent was incapable of delivering the siRNA. This test was performed thrice with Lipofectamine® RNAiMAX ending with the same results. Cell viability analysis of HL-1 cells with Lipofectamine® RNAiMAX and miRNA inhibitor let-7c and negative control showed no significant regulation between the negative and positive control-treated cells but a difference was visible between the time points. Nevertheless, this significant difference between the time points also occurs for the untreated cells thus implying that the siRNA-treatment itself has no effect on cell viability. In addition, the positive control – miRNA inhibitor let-7c used to measure the target mRNA levels of *Hmga2* gene using RT-PCR showed an average Ct value of 37. The untreated, negative control and positive control-treated cells had no difference or detectable mRNA expression. A review of literature identified that the gene *Hmga2* is only expressed during the embryonic stages and not during adult stages[31,32]. This helps understanding that the HL-1 cells, being from adult atrial cardiomyocytes of mouse, might not address the *Hmga2* gene. The experiment for RT-PCR analysis was repeated on a small scale for only two concentrations (10nM and 3nM) showing similar results. Other target mRNA regulated by miR-let-7c were found through literature (RAS) but their expression profile was not clear. Therefore, in order to meet the first aim of the thesis, which was to establish a cell culture platform to analyse miRNAs in relation to AF, the platform for HL-1 cells was optimised by changing the transfection reagents and testing the new conditions.

DharmaFECT 1 was later chosen as it was shown to be affective with HL1- cells by a study[33]. siGLO Green Transfection indicator was compatible with DharmaFECT 1 and so the fluorescent indicator was also changed. In lieu of the RT-PCR results obtained with miRNA inhibitor let-7c, the positive controls were changed to mirVana mimic miR-1 positive control, Ambion Thermo Scientific and ONTARGETplus GAPD siRNA positive control reagent, Dharmacon. DharmaFECT 1 proved to be a compatible transfection agent

since the transfection efficiency was proved successful as the cells were fluorescing green. Cell viability of HL-1 cells remained unaffected by the positive control-siRNA. Since miR-1 and GAPD are ubiquitously present, they proved to be convenient positive controls. Their knockdown caused a stark downregulation of their respective mRNA targets. Though these new reagents were identified and found to be successful in establishing a cell culture platform for HL-1 cells, further optimization is required. This is because all the positive control-siRNA concentrations tested showed similar downregulation and there was no significant difference of regulation between the highest and lowest siRNA-concentrations. Therefore, this would require more experiments to attain a particular optimal siRNA concentration.

5.2.1. Negative control-siRNA – effect on cell viability and target mRNA levels

After the transfection reagents were adapted for HL-1 cells, a positive transfection efficiency was obtained with the new lipofection agent DharmaFECT 1. Next, the cells were experimented to test the effect of the positive control-siRNAs on cell viability and target mRNA level expression. Upon experimentation, the results for cell viability analysis revealed a significant decrease in HL-1 cells treated with negative control-siRNA, whereas the HL-1 cells treated with the positive controls for GAPD and miR-1 showed no effect on cell health. In addition, TaqMan RT-PCR analysis revealed significant upregulation of the targets *Gapdh* and *Twf1* in the negative control-treated cells. Though it is widely stated that a negative control does not have sequence specificity to any known gene and should not affect cell viability or gene expression, the results shown in the cell viability and TaqMan analysis in 4.1.2.2 are certainly different. The negative control used in this case was the same vial used for previous experimentation with the previous transfection reagents. This aberration can be attributed to four reasons:

a. Effect of transfection agent – First, because DharmaFECT1 was not tested on HEK293 cells, it could not be said if this is a factor affecting the cell viability. Cell viability was good with Lipofectamine® RNAiMAX but not DharmaFECT 1, so this parameter could not be compared. In order to rule out the assumption that the transfection agent might have affected the cell health, HL-1 cells were subjected to Lipofectamine® RNAiMAX (1:16.67) and two different dilutions of DharmaFECT 1 (1:25 and 1:50) treatment for 24 h without any siRNA. A one-way ANOVA analysis showed significant difference between the groups ($p < 0.05$). The treatment from Lipofectamine® RNAiMAX and DharmaFECT 1 (1:50) dilution were same and showed no difference in cell viability when compared to the untreated cells.

However, DharmaFECT 1 (1:25) showed a slight decrease in cell viability, though not significant. In the thesis, 1:25 dilution of DharmaFECT 1 was used. This implies that the 1:25 dilution of DharmaFECT 1 treatment might have had an effect on cell viability and this was the dilution used in the experiments.

b. False seeding conditions – HL-1 cells require time to adjust to a new seeding and sufficient confluence might not have been achieved even after ~24h of seeding. Though it is recommended to wait for the confluence to be achieved, the cells were not left for too long (< 24h) during these experiments. Though, a ~70% confluence was available before transfection, the cells might not have grown at normal rate thereby affecting the results.

c. Contamination of the negative control – In cell viability analysis, the cell treated with negative control showed significant decrease in cell viability. In addition, the significant upregulation observed for negative control in RT-PCR analysis was not ideal when compared to untreated cells. Probable reason could be contamination of the negative control used, in which case the experiment must be repeated. Consequently, the experiment for cell viability was repeated with a fresh vial of negative control and was compared with untreated cells. The positive controls miR-1 and GAPD were also repeated in this experiment. Here, the negative control showed no significant effect on the cell viability and the values were close to the untreated cells. TaqMan RT-PCR analysis could be not performed, however in order to confirm if this was only the case of contamination, a RT-PCR of the targets could also be observed.

d. Regulation by negative control – the negative control itself might have some effect on cell viability therein having some sequence similarity. Though this case is improbable, the negative control must be changed in order to obtain clear results.

5.3. Candidate validation and housekeeper miR-16

The transfection platform using cell culture was designed to be useful for when a candidate miRNA to be pursued is identified. Therefore, in parallel to establishing the technological cell culture platform, candidate miRNA analysis was performed on the RNA samples (tissues) from the AFHRI-B cohort. First, the integrity of RNA samples used had to be analysed. This was performed to rule out the possibility of using degraded RNA samples that might affect the miRNA analysis later. RIN helped segregate between good and degraded samples and only the good samples were included within the thesis work. Later, the RNA samples with good RIN were reverse transcribed into a template ready for PCR. TaqMan RT-PCR analysis was performed for miR-21, miR-100 and miR-483-5p on these

cDNA samples. The housekeeper used here was the miR-16. The miR-100 was never studied in relation to AF and therefore was an intriguing factor to be investigated.

The housekeeper used for miRNA analysis in the laboratory is miR-16. In the thesis, analysis of the raw Ct values of miR-16 exhibited significant regulation between the control and Pre-OP phenotypes. This showed that the housekeeper is not stable in AF. A housekeeper is a gene that remains unregulated under all diseased conditions with no exceptions. Despite huge number of studies being performed to establish an association of miRNAs and cardiac diseases, no unified consensus on the best housekeeper for miRNA analysis has been achieved. An investigation by Masè et al., 2017 on reference genes revealed miR-16 to be among the worst housekeeper in relation to AF[34]. This is in contrast when compared to a study of miRNAs with cancer by Rinnerthaler G et al., 2016 where they found miR-16-5p to be a stably expressed housekeeper in cohort with metastatic tumour samples[35]. This shows that a miRNA that could be stable in one disease might not be in another. This uncertainty calls for a better housekeeper within the same class of RNA to achieve results that are not divergent of the actual biological picture. Other classes of RNAs such as SNORD or U6 could not be used since they were beyond the scope of this thesis. In addition, using other classes of RNAs for normalization of miRNAs does not provide a clear correlation of results since every class of RNA is different in their expression profile.

The results obtained from the preliminary data of symAtrial could not be validated in the thesis. The expression of miRNA candidates – miR-21, miR-100 and miR-483-5p analysed by RT-PCR could not be verified because normalization of their expression to the housekeeper miR-16 could not be performed. The miRNA analysed were evaluated based on the raw Ct values obtained from RT-PCR. Raw Ct values indicate that miR-21 was regulated between the Pre-OP AF individuals and controls. Various studies have established miR-21 to play a significant role in AF individuals such as in pathogenesis of AF, increased expression in atrial myocytes during AF[30], downregulation of calcium channels[30] and formation of AF substrate[36]. In the miRNA analysis, miR-100 was not regulated between the phenotypes. If the results could be validated, miR-100 would serve as an interesting candidate for miRNA analysis as it is not well characterized in relation to AF. miR-100 regulates the natriuretic peptide receptor 3 (NPR3), which is a clearance receptor for cardiac natriuretic peptides, thereby playing an important role in cardiovascular homeostasis[37] and has shown to regulate apoptosis in cardiomyocytes through association with insulin-like

growth factor 1 receptor (IGF1R)[38]. In addition, several studies have identified miR-100 to be upregulated in heart failure[39] and dysregulated in AF individuals[40]. However, no study is available establishing a definite role of miR-100 with AF. The miR-483-5p had Ct values of ~6 and could not be validated in the thesis. In literature it is observed that miR-483-5p was detected in post-operative AF individuals but its' prognostic value could not be demonstrated[41] however, it has shown potential to become a biomarker.

This indicates that the results obtained from the symAtrial consortium were false positives as they were normalized to the housekeeper miR-16. Therefore, the miRNAs identified obtained in the preliminary data were not significant.

5.4. Conclusion

Since miRNAs serve as potential biomarkers and their investigation can provide detailed insights into the molecular mechanisms during a disease, establishment of a platform to analyse target miRNA in cell culture system became the primary goal of this master thesis. HEK293 and HL-1 cells were used to perform transfection experiments in which various technical factors such as cell seeding density, siRNA transfection agent, and choice of positive controls and quantity/quality of siRNA were dealt with. A successful cell culture platform was established for HEK293 cells but not for HL-1 cells. The second aim of the thesis was to validate miRNAs in tissue samples from AF patients. Here, the candidate miR-21 showed regulation when analysed using raw Ct values. However, normalization of these raw Ct values failed because the potential housekeeper miR-16 showed regulation by the AF status. The results obtained from symAtrial consortium could not be validated in this master thesis.

In conclusion, investigation of miRNAs for specific cell types in AF research is not simple because the cell line of interest HL-1 cells were not easy to handle. On the other hand, the housekeeper miR-16, which seemed to be stable for other cardiovascular diseases, did not seem to be stable for AF. Hence, a good housekeeper is prerequisite in understanding the correlation of miRNA expression to AF.

6. Summary and Outlook

Atrial fibrillation is the leading cause of morbidity however, the pathophysiological mechanisms of AF are not well investigated. Molecular mechanisms to understand the underlying genetic aetiologies still needs to be identified. Various studies have concluded a definite relation between miRNAs and AF. miRNAs are substances that can be easily measured in available body fluids like blood and serum, making them perfect candidates for biomarker analysis. The method to establish the relationship between the miRNA and the disease begins with a wide range microarray search that might provide insights about dysregulated miRNAs. AF, as a disease, has other cardiovascular diseases as its risk factors and this arises complications in identifying a miRNA biomarker specific to AF only. The targets of these miRNAs are analysed through miRNA databases such as TargetScan, miRBase, etc. Based on a pathway analysis and possible role in heart function, a miRNA can be established as a personalised global biomarker for AF.

On the experimental level, a new reference gene for normalization of PCR results has to be established to obtain the exact correlation between the miRNAs and AF. Other stable miRNAs can be identified through review of literature, which can be tested on the serurm or tissue samples available from AF individuals. In addition, microarray analysis could be performed to bring out a set of miRNAs that are ubiquitously present in all diseases but not affected by the disease. Another method would be to perform high-throughput sequencing to achieve information on any potential housekeeping miRNA.

For atrial fibrillation research, cell culture platform can also be established using primary cell culture of human atrial appendages and human pluripotent stem-cell derived atrial-tissue specific models[42,43]. Better seeding conditions, wider range of siRNA transfection period and less vigorous treatment of lipofection agent over a broader range of siRNA concentrations might lead to establishment of HL-1 cell culture platform suitable for the project. In addition, once the candidate miRNA has been tested on the cell culture platform, the experiments could be translated onto animal models as most studies use C57BL/6 inbred mice for research[44–46]. The candidate miRNAs can be tested in the serum as biomarkers and if a candidate is obtained and functionally validated, it could be further analysed as a potential RNAi therapeutic.

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Declaration

I hereby declare that I wrote this thesis without any assistance and used on the aids listed. Any materials from other works, either as a quote or as idea have been indicated under ‘References’.

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07.05.2018, Hamburg