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Linking molecular targets to hypertrophic cardiomyopathy using genome-edited human induced pluripotent stem cell cardiomyocytes

Submitted by

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Due to the impact of COVID-19 on this thesis, the original scope of the work was reduced to accommodate time limitations. As such, the number of targets investigated was narrowed down from four to one to allow the collection of data from a variety of different assays. This decision was reached in discussion with my supervisory team.

Dedication

I dedicate this thesis to my family members who saw me start this journey but did not get the chance to see me complete it. Nana, Grandad Butterworth, Auntie Jane and Grandad Hossein, I miss you dearly. I know you would all be immeasurably proud of me if you were still here.

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Abstract

Hypertrophic cardiomyopathy (HCM) is the most common cardiomyopathy, estimated to affect 1:250 people, resulting in impaired cardiac function due to thickening of the left ventricle. Once disregarded as genomic junk, long non-coding ribonucleic acids (lncRNA) have emerged as key regulators of human disease, including cardiovascular disorders. Work prior to this thesis developed an hiPSC-CM model of HCM, introducing the pathological R453C mutation into the *MYH7* sarcomeric gene. RNA sequencing on the lines of the model uncovered four lncRNAs that may be involved in the progression or prevention of HCM.

Current transfection methods pose a barrier to the full potential hiPSC-CMs offer. In absence of a suitable transfection method and the criticality of efficient transfection prior to the phenotyping assays, a new method was optimised specifically for this thesis. Using a cationic reagent and through systematic optimisation of several conditions, a high transfection efficiency without compromising cardiomyocyte purity and with little effect on cell viability was achieved.

Expression modulation systems were harnessed for the probing of the phenotypic effects of the lncRNA targets on HCM, with the aim of providing a new avenue for pharmacological therapeutics. CRISPR/Cas9 technology was implemented for the creation of stable knock outs of the lncRNAs in each of the isogenic lines. One of the lncRNAs, *MINCR*, revealed novel characteristics upon knock out in hiPSCs.

A monolayer cardiac differentiation protocol was applied to the three lines of the HCM model, after which hiPSC-CMs were transfected with overexpression vectors in addition to small interfering RNAs (siRNA) to achieve knock down. Functional phenotyping was performed on hiPSC-CMs with up or downregulated lncRNA target expression. Assessing brain natriuretic protein (BNP) as a marker of hypertrophy, suggested that the lncRNAs in question act as gene modifiers, promoting or preventing HCM depending on the *MYH7* cell line. Probing the lncRNA involvement in mitochondrial respiration did not reveal any immediate lncRNA involvement thus far. Finally, assessing the switch between the two

isoforms of myosin heavy chain, a hallmark of HCM, revealed the potential involvement of lncRNAs in regulating their expression, with further optimisation of the methods required.

The work in this thesis optimised a transfection protocol for successful hiPSC-CM transfection which is critical for the investigation of disease mechanisms in cardiomyocytes. In addition, results from phenotyping lncRNA expression modulated hiPSC-CMs of the HCM model highlights the role of the lncRNA targets in acting as gene modifiers that impact HCM in a cardioprotective or disease-causing manner.

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Abbreviations

Abbreviation	Description
ADP	Adenosine diphosphate
ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
AVC	Arrhythmogenic ventricular cardiomyopathy
BMP	Bone morphogenic protein
BNP	Brain natriuretic protein
BOS	Bosentan
BrdU	Bromodeoxyuridine
CAR T	Chimeric antigen receptor T cell
CTD	C-terminal domain
CMs	Cardiomyocytes
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRa	CRISPR activation
cTnT/I/C	Cardiac troponin T/I/C
CVD	Cardiovascular disease
DCM	Dilated cardiomyopathy
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DSB	Double stranded break
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EHT	Engineered heart tissue
eIncRNA	Enhancer lncRNA
ET1	Endothelin 1
EtBr	Ethidium bromide
FBS	Fetal bovine serum
GFP	Green fluorescent protein
gRNA	Guide RNA
HA	Homologous arm

HBSS	Hanks balanced salt solution
HCM	Hypertrophic cardiomyopathy
hESC	Human embryonic stem cell
hiPSC	Human induced pluripotent stem cell
hiPSC-CM	Human induced pluripotent stem cell derived cardiomyocyte
KO	Knock out
LHA	Left homology arm
lncRNA	Long non-coding RNA
LVNC	Left ventricular non compaction
mESC	Mouse embryonic stem cell
MHC	Myosin heavy chain
miRNA	Micro RNA
mNET-Seq	Mammalian native elongating transcript sequencing
mRNA	Messenger RNA
MT	Matrigel
MUT	Mutant
NGS	Next generation sequencing
OCR	Oxygen consumption rate
OE	Overexpression
PAC-MAN	Prophylactic antiviral CRISPR in human cells
PBS	Phosphate buffer solution
Pol II	Polymerase II
PPIE	Peptidylprolyl isomerase E
RCM	Restrictive cardiomyopathy
RIP	RNA immunoprecipitation
RNA	Ribonucleic acid
RNA-Seq	Ribonucleic acid sequencing
RHA	Right homology arm
RT	Room temperature
SCD	Sudden cardiac death
SEM	Standard error of the mean
SERCA	Sarcoplasmic reticulum ATPase

sgRNA	Single guide RNA
shRNA	Short hairpin RNA
SINE	Short interspersed repeat elements
siRNA	Short interfering RNA
sncRNA	Short non-coding RNA
SNP	Single nucleotide polymorphism
SP34	Stem pro-34
SpCas9	Streptococcus pyogenes Cas9
TALE	Transcription activator like effector
TALEN	Transcription activator like effector nucleases
TrIP-Seq	Targeted RNA immunoprecipitation sequencing
VN	Vitronectin
WNT	Wingless/INT protein
WT	Wild type
ZFP	Zinc finger proteins
ZFN	Zinc finger nucleases
α-MHC	Alpha myosin heavy chain
β-MHC	Beta myosin heavy chain

1. Introduction

1.1 Thesis Rationale

Cardiomyopathies are a heterogeneous group of diseases with complex underlying mechanisms and overlapping phenotypes. The most prevalent of the cardiomyopathies, hypertrophic cardiomyopathy (HCM), is the most frequent cause of sudden cardiac death in afflicted patients [2].

Hypertrophic cardiomyopathy is a complex heart condition characterised by hypertrophy of the left ventricle, primarily in the interventricular septum. With an autosomal dominant pattern of inheritance, hypertrophic cardiomyopathy is primarily a familial disease. Mutations are most frequent in sarcomere or sarcomeric protein coding genes, such as *MYH7*, which is associated with severe clinical characteristics and outcomes. There is a vast spectrum of disease, which encompasses being asymptomatic to suffering from dyspnoea, arrhythmias and finally heart failure. Even within families harbouring the same mutation, variable penetrance and expression in addition to other factors including environmental and genetic background lead to different outcomes between members [3].

Soon after the completion of the human genome project in 2003, non-coding RNAs were referred to as ‘dark matter,’ thought to have no biological function [4]. Research over the last two decades has disproven that original hypothesis and long non-coding RNAs (lncRNA) have emerged as modifiers of many diseases, including cardiovascular pathophysiology, development and metabolism [5]. Many studies have been conducted in animal models but owing to poor lncRNA conservation between species, these studies can only indicate possible functions that lncRNAs in general may possess. Studies using human samples and human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CM), provide an invaluable source of material to investigate lncRNA function and mechanisms in HCM [6]. hiPSCs provide an unlimited source of material, and the ability to harness gene editing for the creation of isogenic controls allows the creation of models of disease with the same genetic background.

Previously, Dr Diogo Mosqueira created an HCM model of *MYH7* to recapitulate phenotypes of the disease for their functional and molecular analysis [7], RNA sequencing in these model lines revealed four lncRNA targets that may progress or prevent HCM disease. In this thesis, the involvement of those four novel lncRNA transcripts in HCM progression or prevention are explored, by harnessing cutting edge CRISPR/Cas9 technology and other expression modulating systems. Initially, the design and synthesis of the constructs for expression modulation are described. This is followed by the optimisation of a transfection protocol for the hiPSC-CMs used in this thesis in the expression of lncRNA targets. Finally, the remaining chapter revolves around the phenotyping of lncRNA expression modulated hiPSC-CMs, including novel revelations about the role of one lncRNA in hiPSCs. The workflow is illustrated in **Figure 1.1**.

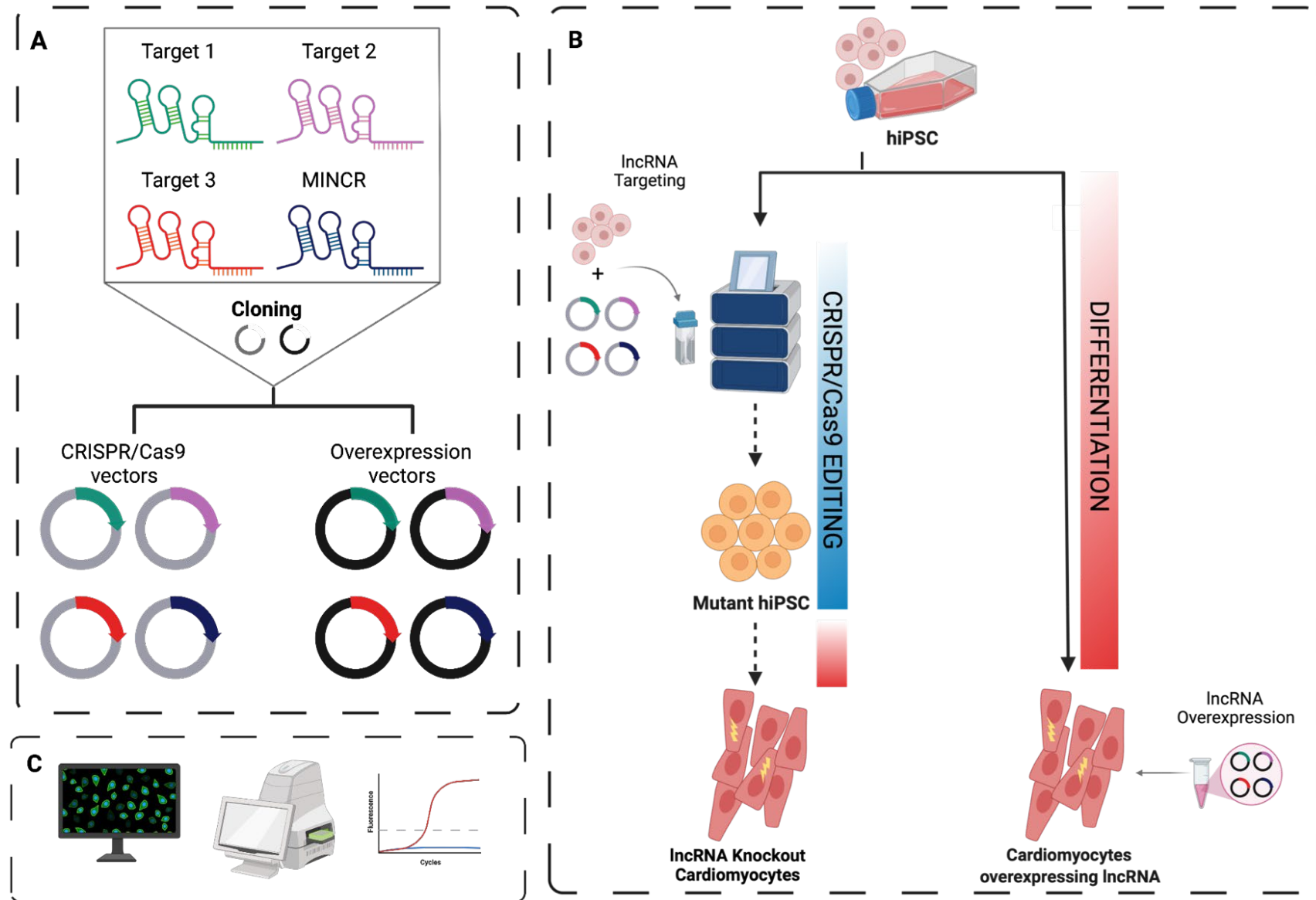


Figure 1.1 – PhD Workflow. **A)** Starting with the four lncRNA targets, vectors were constructed for CRISPR/Cas9 gene editing of lncRNA knockouts and for the overexpression of the lncRNA targets (Number of vectors created for CRISPR/Cas9 editing is not reflected by the figure). **B)** hiPSCs undergo CRISPR/Cas9 gene editing to create lncRNA target knock out lines which are then differentiated to hiPSC-CMs. Overexpression was conducted in hiPSC-CMs. hiPSCs are directly differentiated to hiPSC-CMs which are then transfected with lncRNA target overexpression vectors. **C)** The hiPSC-CMs with lncRNA knockouts or lncRNA overexpression then functionally underwent phenotyping to evaluate mitochondrial activity, hypertrophy and myosin heavy chain isoform switch. Created using Biorender.com 2022.

1.2 Cardiac Disease

1.2.1 Incidence and cost

Cardiovascular diseases (CVDs) are the leading cause of death worldwide; an estimated 17.9 million people died from a CVD in 2019, accounting for 32% of global deaths [8]. The majority of these deaths, three quarters, occurred in low- and middle-income countries. The lack of access to early detection and/or the high costs associated with healthcare results in premature deaths and contributes to poverty, as a result of increased spending on healthcare or lost wages because of illness. To conquer these issues, many countries will require a reformation of their healthcare programmes. In 2015 UK healthcare costs associated with CVD were estimated to be £12 billion, and non-healthcare costs just over £8 billion [9]. Direct costs include in-patient care, medication, and follow-ups while non healthcare costs include economic losses due to mortality and morbidity.

Globally, since 1990 the number of people living with CVD has increased by 93% and projections show that this number is set to increase. Environmental risk factors such as air pollution have a significant impact on CVD risk, along with modifiable factors such as lifestyle choices. The 2020 COVID-19 pandemic had a huge impact on those living with comorbidities and led to the adoption of unhealthy habits. International research has shown that people with a CVD were 3.9 times more likely to suffer more severe outcomes and 2.7 more likely to die than those not living with a CVD after contracting COVID-19 [10]. CVD was also found to be the most common comorbidity in those suffering from severe COVID-19 infections [11], 45% of death certificates in England in spring 2020 mentioned CVD [12]. It is without a doubt that the impact of the pandemic will be felt for many

years to come, highlighting the unfulfilled need to improve diagnostics and treatments of CVD, which begins at understanding the molecular mechanisms that underlie disease.

1.2.2 Cardiomyopathies

The first person to make a link between a structural defect of the heart and resulting heart failure was Richard Lower in 1669 [13]. A defining moment in the field was the conceptualisation of the term cardiomyopathy in 1957, which distinguished coronary diseases from (at the time) ‘idiopathic’ cardiac disease [14]. Initially, cardiomyopathies were thought to be uncommon, which soon turned out to be inaccurate and similarly the discovery of mutated genes meant that some cardiomyopathies were no longer idiopathic [15, 16]. Presently, estimates of incidence and prevalence of cardiomyopathies are based on screening studies of a certain population to assess cardiovascular risks. Global estimates are therefore likely to be underestimated as only individuals who meet the criteria for screening are included. The impact of this is relevant as many individuals will be carrying disease causing mutations but will have incomplete penetrance of the mutations and/or delayed onset of symptoms and are unaware of the risks they carry [17].

Cardiomyopathies are a heterogeneous group of diseases, which complicates their classification. There have been several revisions and two expert panels, the American Heart Association (AHA) and European Society of Cardiology (ESC). The panels do not fully agree on the terms of classification, specifically on the inclusion of ion channelopathies as a cardiomyopathy, the latter against the inclusion [18, 19]. The most recent AHA statement in 2006 proposed the following definition: *“Cardiomyopathies are a heterogeneous group of diseases of the myocardium associated with mechanical and/or electrical dysfunction that usually (but not invariably) exhibit inappropriate ventricular hypertrophy or dilatation and are due to a variety of causes that frequently are genetic. Cardiomyopathies either are confined to the heart or are part of generalized systemic disorders, often leading to cardiovascular death or progressive heart failure-related disability [18].”* Primary cardiomyopathies include hypertrophic cardiomyopathy (HCM, dilated cardiomyopathy (DCM), arrhythmogenic ventricular cardiomyopathy (AVC),

restrictive cardiomyopathy (RCM) and left ventricular non-compaction (LVNC). These can be subdivided into genetic, mixed and acquired.

HCM is the most common inherited cardiomyopathy with mutations identified in many genes, mostly encoding sarcomeric proteins and is inherited in an autosomal dominant manner [20]. Due to vast genetic heterogeneity and variable penetrance, the disease spectrum, in terms of age of onset, disease course and severity of symptoms, is extremely broad. HCM is characterised by hypertrophy, in particular of the left ventricle [21]. Previously estimated to affect 1:500 people, this figure is likely underestimating the prevalence due to the improvement of detection using sensitive imaging methods such as MRI rather than echocardiogram. Prevalence is now estimated to be around 1:250 [22]. The most frequently involved genes are myosin binding protein C (*MYBPC3*) and β -myosin heavy chain (*MYH7*), accounting for 40% and 15-25% of cases, respectively.

DCM is one of the main causes of heart failure, characterised by left or biventricular dilation and systolic dysfunction in the absence of any ischemic conditions [23]. Prevalence is estimated to be 1:250 in the population with heart failure and 1:2500 in the general population [24]. DCM can be familial or nonfamilial. Mutations that arise in genes encoding structural components of the sarcomere and desmosome have been identified having a role in DCM. Studies have shown that in 30-40% familial cases a causative mutation is present [25]. Of the 12 most frequently mutated genes in DCM, truncating mutations in *TTN* occur in 25% of familial cases [26].

AVC contains a spectrum of phenotypes that overlaps with other cardiomyopathies, in particular DCM, by presentation of arrhythmia associated with ventricular dilation and systolic dysfunction [27]. The hallmark however is the replacement of the ventricular myocardium with fibrofatty tissue [28]. AVC is a genetically heterogeneous disorder, most frequently caused by mutations in the desmosome, a protein complex that lends structural support to tissues [29]. The prevalence has been estimated between 1:1000 to 1:5000. The discordance may stem from initial manifestation of AVC being SCD, therefore undiagnosed patients could be an additional 30% of the population [30].

Characterised by stiffness of the ventricular walls, RCM leads to diastolic dysfunction, raised end diastolic pressure and dilated atria, despite no change in left ventricular wall thickness [31]. It is the least common of the cardiomyopathies, mutations in sarcomeric proteins have been associated with RCM. Like DCM, it can be familial or nonfamilial.

LVNC is characterised by a thick trabeculated non-compacted myocardial layer with adjacent deep intra-trabecular recesses [18]. Similar to other inherited cardiomyopathies, it is genetically heterogeneous and has been linked to mutations in genes encoding sarcomeric proteins, Z disc, cytoskeleton proteins and mitochondria [32]. Prevalence is estimated to be 1:5000 in the general population [33].

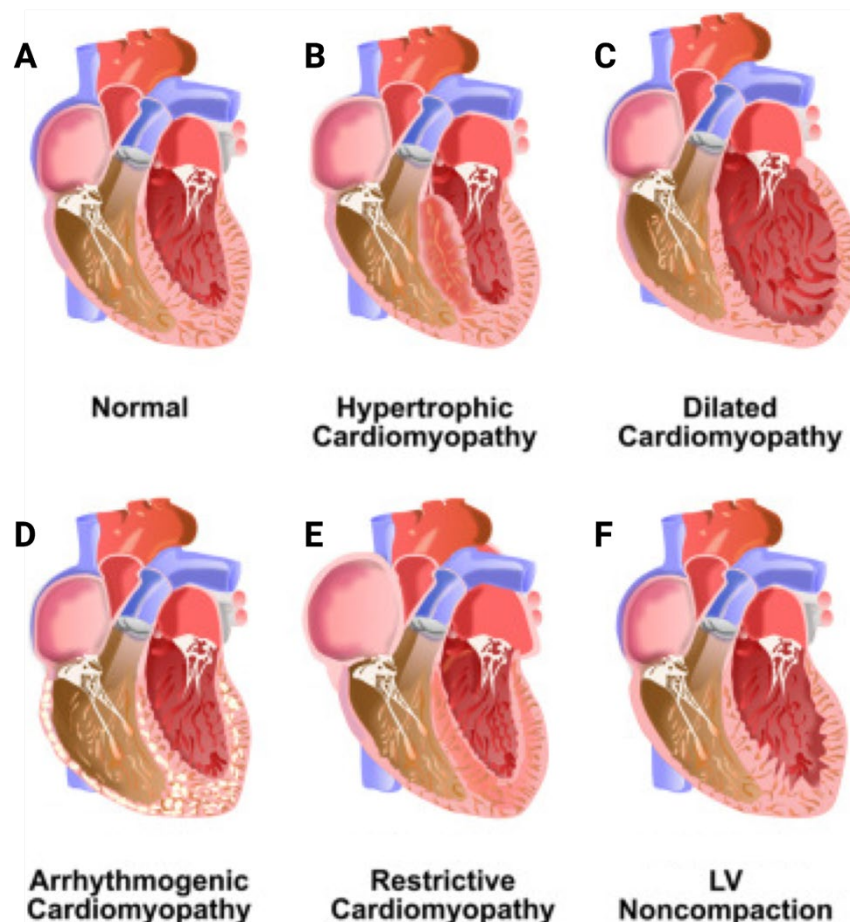


Figure 1.2 – Schematic of a normal heart vs those of primary cardiomyopathies. A) Shows the structure of a healthy heart, allowing unobstructed normal outputs. B) The heart in HCM is characterised by thickening of the interventricular septum. C) Dilated cardiomyopathy presents as the dilation and impairment of ventricular function. D) AVC, fibrous fatty tissue is within the heart

muscle, impairing normal contractile function, resulting in arrhythmias. **E)** RCM is characterised by stiffness of the ventricular walls, leading to diastolic dysfunction. **F)** LVNC occurs due to malformation of the left ventricle. The tissue is spongy as opposed to smooth and firm. Adapted from [34].

1.2.3 Complexity of genotype - phenotype relationships

The terms used to describe the two most prominent cardiomyopathies, HCM and DCM, are historical terms that arose from autopsies and echocardiography decades ago [35, 36]. These terms remain in use despite the overlap in phenotypes across the cardiomyopathies. For example, patients presenting with HCM can transition to present DCM phenotypes due to maladaptive pathways, which in the presence of sarcomeric mutations is associated with poor outcomes [37]. This highlights the complexity of these diseases.

Thousands of mutations have been associated with cardiomyopathies and it has become apparent that it is not possible to assign a disease course based on a single variant [38]. The overlap in genetics termed **genetic heterogeneity** is when mutations in the same genes cause different cardiomyopathies. For example, mutations in *ACTC1*, *MYH7* and *TNNT2* are present in HCM, DCM, RCM and LVNC and mutations in *TTN* are present in HCM, DCM, RCM and AVC [39]. In a 2021 genome-wide association study (GWAS) using 1,733 HCM cases and 5,521 DCM cases, Tadros *et al.* identified new HCM associated loci that had been previously associated with DCM [40]. **Phenotypic heterogeneity** is used to describe the phenomenon where cardiomyopathy phenotypes vary within the same genetic mutations [41]. Analysis of three missense DCM mutations in *ANKRD1* revealed different pathway alterations leading to different phenotypes [42]. Clinical outcomes can vary within a family which have the same causal mutation as a result of **variable penetrance** and **variable expressivity**. As an example of this, a study by Purevjav *et al.* looking at a family carrying an RCM mutation Q529X-MYPN found that the parent who carried the mutation did not present any RCM characteristics, while both offspring did [43]. The ultimate phenotype is thought to be influenced by factors such as genetic background, comorbidities, and lifestyle, but ultimately the complexity surrounding these factors has not been completely unravelled.

1.3 Hypertrophic Cardiomyopathy

1.3.1 Clinical and histological characterisation

Clinically, the HCM spectrum is vast and can range from asymptomatic to a relatively benign course with symptoms such as dyspnoea to severe cardiac dysfunction. Overall HCM is characterised by asymmetrical left ventricular hypertrophy with preserved or increased ejection fraction. Hypertrophy usually presents in the basal interventricular septum, leading to left ventricular outflow obstruction [44]. Left ventricular diastolic dysfunction is also associated with HCM. The presence of multiple factors that may cause cardiac hypertrophy can make it difficult to distinguish between primary HCM from secondary hypertrophy, as a result of other conditions (termed phenocopy conditions) which may account for 5 to 10% of diagnosed HCM cases [45]. HCM is usually diagnosed in late adolescence and is the leading cause of sudden cardiac death (SCD) in those aged under 35 [46]. Screening is fundamental for preventative care and understanding the risks one carries, emphasised in a study by Velzen *et al.* Over a 31 year period, 777 relatives of 209 HCM patients were screened for HCM mutations, 30% were diagnosed with HCM after the first screen while 16% later developed HCM during the follow up period [47]. Survival rates were high due to the use of preventative ICD's and surgery, highlighting the impact of screening.

Histological features of HCM are cardiomyocyte hypertrophy and disarray and the presence of interstitial fibrosis [48]. These phenotypes suggest that hypertrophy is an adaptive response to increased stress relating to impaired cardiac function. It is important to note that there is a distinction between pathological hypertrophy, associated with HCM and physiological hypertrophy linked to exercise or pregnancy. Cardiac hypertrophy in response to exercise or pregnancy is reversible and is not associated with the structural and molecular profile of pathological hypertrophy which is not reversible [49].

1.3.2 Genetics of hypertrophic cardiomyopathy

A family had been studied by Paré and colleagues with the conclusion that there was a familial link between the cases of HCM within the family [50]. In 1990, pioneers of HCM research underlying the molecular genetics of HCM, Christine

and Johnathan Seidman, discovered the R403Q mutation in the family aforementioned [15]. HCM was no longer classed as an idiopathic cardiomyopathy and subsequent HCM genetic discoveries stemmed from this initial finding.

The relevance of HCM mutations is embedded in the process of cardiac contraction. Fluxes of calcium across cell membranes are responsible for the generation of cardiac impulse by pacemaker cells. The impulse is generated in the sinoatrial node and conducted to the atrioventricular node, where it then splits into the left and right bundle branches of Purkinje fibres. The wave of depolarisation then travels down to the apex and then up alongside the left and right ventricles causing simultaneous ventricular contraction [51]. Cardiac tissue is composed of cardiomyocytes arranged in series. Myofibrils are composed of myofilaments displaying repeating sarcomeres, the basic contractile unit. Sarcomeres are composed of thick and thin filaments - myosin and actin, which slide over each other during muscle contraction and relaxation as a result of an action potential. Other proteins involved in cardiac contraction are troponins (T, I and C) and tropomyosin. In a relaxed state, tropomyosin covers the myosin-binding site on actin, but on arrival of an action potential, an influx of Ca^{2+} from the sarcoplasmic reticulum alters the structure exposing the actin-binding site by binding to troponin-C [52]. Myosin binds to actin forming a cross-bridge and the myosin is pulled along the actin - a power stroke, releasing adenosine diphosphate (ADP) and an inorganic phosphate that was bound to the myosin head. The length of the actin and myosin structures do not change, contraction is simply due to the myosin pulling the actin along its length, shortening the sarcomere [53]. A new molecule of adenosine triphosphate (ATP) binds to the myosin head causing the cross-bridge to detach and the ATP is hydrolysed to ADP and phosphate by ATPase, returning the myosin to a relaxed state – a complete contraction cycle (**Figure 1.3**). The functioning of the sarcomere is ultimately responsible for cardiac contraction, and any changes to this can be detrimental.

To date, there are a total of 29 genes associated with HCM. Nine of those are established genes, while the rest remain likely causal. The nine established genes all encode sarcomeric proteins and generally consist of missense mutations; *MYH7* and *MYBPC3* are responsible for 50% of familial cases, troponins T and I

(*TNNT2* and *TNNI3*) and α -tropomyosin (*TPM1*) account for less than 10% familial cases and *ACTC1*, *MYL2*, *MYL3* and *CSRP3* although established, are uncommon causes of HCM [3]. The sarcomeric gene mutation has been used as a successful tool for the prediction of the outcome of disease, according to a study with data from 4591 HCM patients, those with a sarcomeric mutation have a two-fold greater risk of severe consequences than those without [54]. The other 40% of cases are classed as nonfamilial, where there is no underlying sarcomeric mutation present and no family history of HCM. A study by Ingles and colleagues determined this type of HCM is associated with the male sex, older age and less severe clinical outcomes than those with familial HCM [55].

Studies thus far have not completely elucidated the molecular mechanisms that occur because of *MYH7* mutations. It was generally accepted that *MYH7* mutations cause calcium cycling impairment [56] and energy imbalance [57], and many studies suggest a hypothesis of HCM induced hypercontractility. A recent study using a mouse myoblast system (allowing the expression of the human predominant ventricular β -MHC (*MYH7*)) assessed the impact of four *MYH7* mutations (R249Q, H251N, D382Y and R719W) on myosin activity, determining that those mutations lead to a significant increase in myosin heads available for actin binding [58]. This in turn can lead to hypercontractility and was a fundamental addition to *MYH7* mutation knowledge. Mutations in *MYH7* are generally associated with more severe disease and greater penetrance than other mutations. Bioinformatic analysis has revealed over 300 mutations have been identified in *MYH7* relating to HCM disease [59], with the position of the mutation within the gene bearing a significant impact on the severity of HCM. The R453C mutation is known to cause severe phenotypes of HCM, with high penetrance. The positioning of the mutation, between the nucleotide-binding pocket and actin-binding site on myosin, positions it well to perturb many activities of myosin [60]. In conflict with this notion of higher penetrance of *MYH7* mutations, a 2021 whole-exome sequencing (WES) study on a pedigree of suspected familial HCM, revealed a novel R671C mutation in *MYH7* with significant genetic and phenotypic heterogeneity [61]. Of the three family members studied, one was a survivor of SCD, one presented with ventricular diastolic dysfunction and the other did not present any cardiac dysfunction.

All considered, HCM is an extremely complex disease with various manifestations of disease and clinical outcomes. Studies have pushed forward the understanding of the molecular mechanisms of the mutations underlying HCM, however, the efforts are hampered by the variable genetic and phenotypic penetrance.

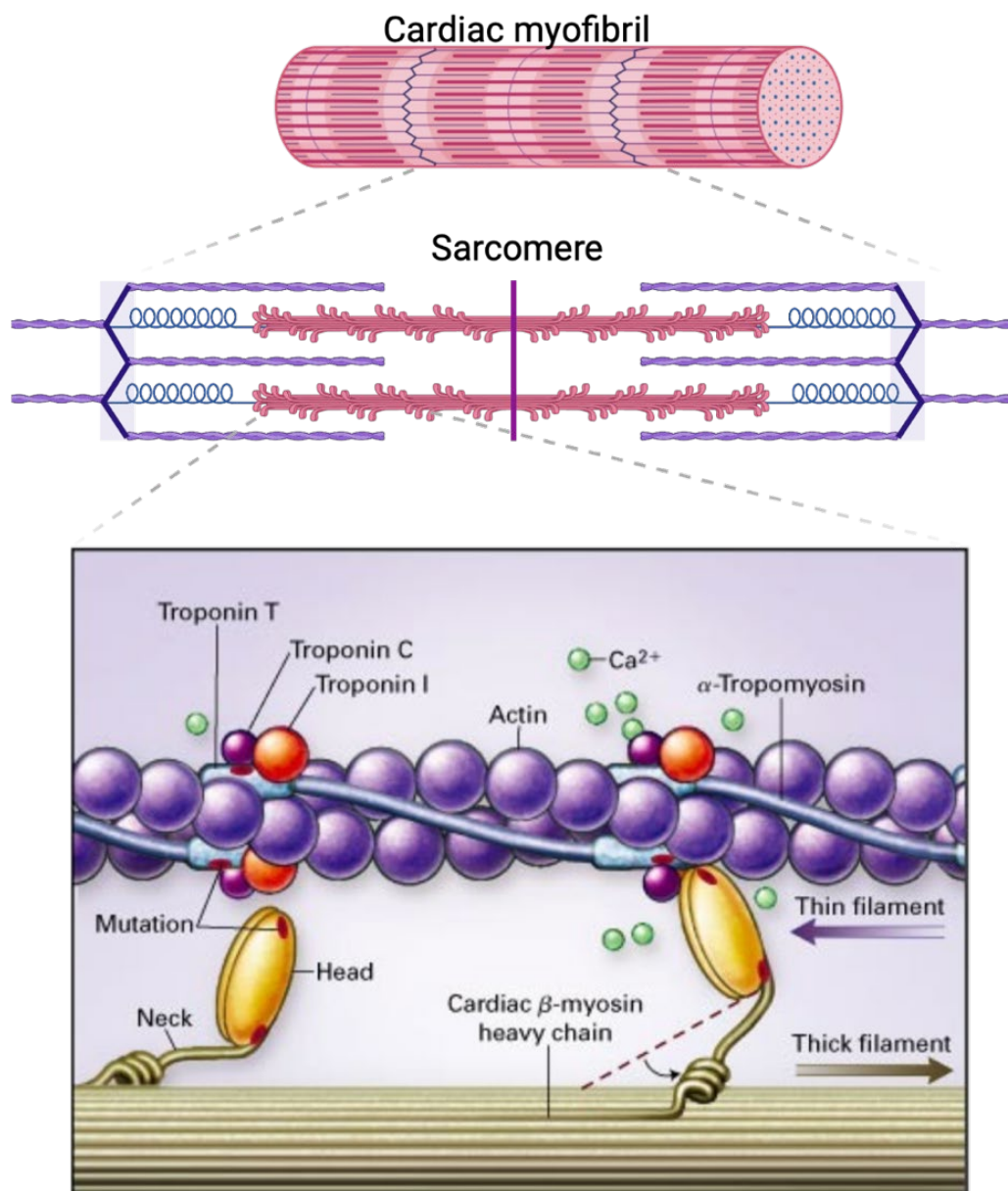


Figure 1.3 – Sarcomeric structure and generation of a power stroke. Adapted from [62]

1.3.3 Mechanisms of Disease

Hypertrophy is a compensatory response to altered contractility and/or physiological stress whereby the cardiomyocytes increase in volume without undergoing hyperplasia and have enhanced protein synthesis to cope with a

pressure overload. In HCM, hypertrophy predominantly occurs in the basal interventricular septum and the free wall of the left ventricle. The thickening of the myocardium increases energy demands as more sarcomeres are produced, similarly, hypertrophy is also associated with increased fibrosis. Several micro RNAs have been identified as biomarkers for both hypertrophy and fibrosis [63]. Cardiac hypertrophy is inextricably linked to foetal gene expression.

Histologically, **fibrosis** is one of the most obvious features of HCM. Of the five cell types compromising the heart, 30% are fibroblasts [64]. Arrhythmias are associated with fibrosis of the heart muscle which is strongly correlated with SCD [65] due to the non-conductive fibrotic tissue, the degree of fibrosis correlates with impairment of cardiac relaxation. Poor prognosis has been linked to greater extents of fibrosis in a recent study assessing patterns of fibrosis in HCM patients [66]. The molecular trigger between sarcomeric mutations and cardiac fibrosis has not been elucidated, but increased expression of several profibrotic molecules are involved, including collagens. Irregular collagen turnover can lead to adverse remodelling in HCM, procollagen molecules are produced in the endoplasmic reticulum of fibroblasts, the cleavage of these molecules results in the formation of mature collagen fibres [67]. Using serum biomarkers, it was found that collagen type I synthesis leads to left ventricular hypertrophy. Possible mechanistic drivers include impaired energetics, discussed below. So far, fibrosis has also been attributed to the apoptotic death of cardiomyocytes and the subsequent expansion of the interstitial matrix. In mice and humans carrying mutations in *MYH7*, there was constant proliferation of fibroblasts [68]. Fibroblast proliferation leads to increased secretion of extracellular matrix proteins, leading to adverse cardiac remodelling. Due to these effects, matrix metalloproteinases (MMPs) have also been suggested as useful biomarkers for fibrosis [69]. TGF- β signalling leading to the activation of fibroblasts was also reported in patients with HCM [70], while the treatment with a neutralising antibody led to the reduction of proliferation of non-cardiomyocytes [71]. There is also a known role of angiotensin II as a promoter of TGF- β , since treatment with a receptor antagonist, prevented the development of fibrosis.

Under conditions of cardiac remodelling, the adult heart returns to the **foetal gene program**, whereby genes that became quiescent after birth, resume expression and adult genes become downregulated. At a certain point, the adaptive shift to the foetal gene program can no longer support cardiac function and is therefore a prevalent feature during heart failure [72]. Such foetal genes that are commonly expressed in this shift are atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), β -myosin heavy chain (β -MHC), α -skeletal actin and foetal troponins. Metabolically speaking, foetal hearts derive most of their energy from carbohydrates, while postnatally fatty acid oxidation is relied on. After birth, the switch in substrates used for energy production is accompanied by a change in gene expression towards adult isoforms. In response to stress, changes in metabolic fluxes trigger an adaptive response by shifting expression toward foetal expression [73]. Similarly, changes occur in sarcomeric protein expression postnatally. In an attempt to rescue pathological conditions in the heart, there is a switch in myosin isoform expression to maintain the contractile function [74]. The upregulation of β -MHC and concomitant downregulation of α -myosin heavy chain (α -MHC) is a hallmark of HCM [75]. The lower ATPase activity of β -MHC results in reduced contractile capability.

Mutations in critical sarcomeric proteins lead to altered sarcomere function by the incorporation of mutated proteins, affecting the normal force generation process - **disturbed biomechanical stress sensing**. These alterations may lead to a hypertrophic response to cope with the mechanical load. The M-line and Z-disc are fundamental components of the sarcomere and have been implicated in the sensing and transduction of biomechanical stress [76]. Cardiac muscle LIM proteins (Z-disc) [77] and myozenin-2 [78] (a modulator of a pro-hypertrophic pathway) mutations have also been implicated in HCM. Mutations in titin which stretches from the M-line to the Z-disc and have a mechanical and structural role, have also been linked to HCM [79]. These mutations lead to altered mechanical function by affecting multiple parameters; force of contraction, cross bridge connections, ATPase activity, calcium sensitivity and relaxation properties [80]. Assessments in human skeletal and myocardial tissues assessing *MYH7* mutations showed the functional effects on the above parameters vary by each mutation [81]. Furthermore, the term allelic imbalance has been used to explain

alterations to the force-generating mechanism, whereby unequal amounts of mutated and WT myosin exist in the sarcomere, causing contractility imbalances [82]. This leads to the energy depletion hypothesis which is discussed further in Chapter 5.

The heart has extraordinary energetic demands in comparison to any other organ. Reserves of ATP within the heart are a limited resource and are not sufficient to keep up with the energy demand required for normal cardiac function and maintenance of cell viability. Therefore, cardiomyocytes must continuously synthesise ATP, predominately by oxidative phosphorylation (>95%) in the mitochondria, to maintain contractile function of cardiomyocytes and intracellular ion homeostasis [83]. The heart has the ability to metabolise a variety of substrates; in a healthy heart, the primary source of energy is fatty acid oxidation (two thirds) and the secondary source is glucose oxidation and lactate (one third) [84]. A healthy heart can adapt its substrate utilisation based on physiological states e.g., exercise or starvation, while in the context of HCM, **metabolic shifts** are a maladaptive response to cardiac dysfunction as a result of sarcomeric mutations. Studies have shown that in patients with HCM, the reduced pool of phosphocreatine, which is the main reserve source of ATP production stress, results in insufficient ATP replenishment [85]. As a result, fatty acid oxidation metabolism acts as a compensatory response, whereby many ATP molecules are produced at a greater oxygen expenditure [86]. At a later stage of the disease, a metabolic shift from fatty acid oxidation to glucose oxidation ensues, whereby less ATP is produced at a faster rate, however, the energy output cannot keep up with demand, resulting in cardiac failure [87].

Cardiomyocyte function relies on the coordinated movement of Ca^{2+} between the cytosol and sarcoplasmic reticulum. The movement of Ca^{2+} is mediated by crucial proteins and transporters which together are responsible for cardiac rhythm and regulation of excitation-contraction coupling [88]. **Impaired calcium cycling and sensitivity** changes in expression or phosphorylation of key proteins (as a result of mutations) to calcium cycling such as sarcoplasmic/endoplasmic reticulum ATPase 2 (SERCA) (regulated by phospholamban (PLN)) and ryanodine receptor 2 can cause Ca^{2+} reuptake defects or diastolic Ca^{2+} leaks leading to altered

contractility, [89, 90]. In HCM, sarcomeric mutations cause dysregulated calcium cycling which can be used as an early indicator of disease [91]. Upregulation of calcium signalling in cardiomyocytes was found to drive cardiomyocyte hypertrophy *in vivo* and *in vitro* [92, 93], suggesting calcium dysregulation is a driver of HCM. Correction of impaired calcium cycling has emerged as a promising approach for the treatment of heart failure.

Mechanisms of disease in HCM are interwoven, highlighting the true complexity of HCM. **Figure 1.4** provides an overview of the mechanisms discussed above.

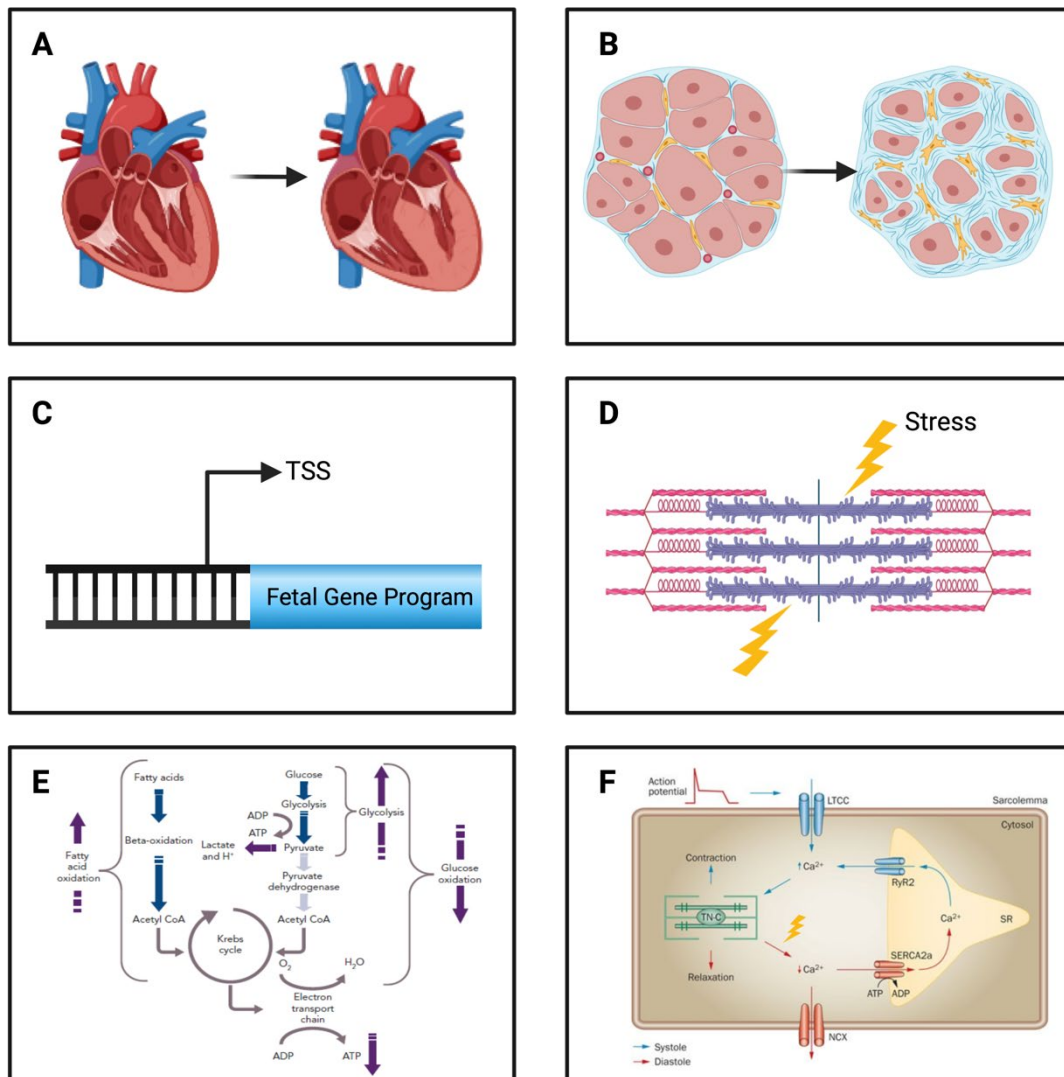


Figure 1.4 – Mechanisms of Hypertrophic Cardiomyopathy. **A)** Cardiac hypertrophy. **B)** Cardiac fibrosis. **C)** Fetal gene expression. **D)** Disturbed biomechanical stress sensing. **E)** Metabolic shift. **F)** Altered calcium cycling. Adapted from [94, 95]. Created using Biorender.com 2022.

1.3.4 Management of HCM

Treatment options for HCM patients are not extensive. Most often medications are prescribed for the treatment of symptoms, as opposed to the underlying mechanism of disease. Inotropic agents such as beta-blockers and calcium channel blockers are often prescribed. Patients who have a significant SCD risk often undergo invasive therapeutic treatments including pacemaker implantation, placement of an implantable cardioverter-defibrillator and in patients with end-stage heart failure the last resort is heart transplant [3]. Pharmacological treatment of HCM is the optimal method for disease management but this is hampered by at least two factors: i) current understanding of HCM molecular mechanisms is poor, preventing direct mechanism targeting and ii) much of the drug development and testing is conducted in animal models which in turn end up being cardiotoxic in human trials, thus not making it to market. Of the drugs that have passed clinical trials, 61 have been removed from the market due to cardiotoxicity between 1950 and 2017 [96]. There is a drive for using human models including hESC-CMs for better prediction of cardiotoxicity [97].

Understanding the mechanisms of disease is crucial for the development of targeted therapies especially when considering the phenotypic heterogeneity.

1.3.5 Disease modelling HCM

Disease models have been created to further the understanding of HCM phenotypes and underlying molecular mechanisms, with the overarching aim to improve therapies. Relevance of the model to human physiology is a paramount feature but the difficulty in obtaining patient samples, and the varying physiology of animal models compels the field to develop better models of HCM. Models of HCM include: i) cardiac muscle strips, ii) myofibrils obtained from skinned heart tissue, iii) isolated cardiomyocytes, iv) purified sarcomeric proteins actin and myosin, v) animal models and vi) hiPSC-CMs.

The first studies to investigate the mechanisms of heart failure were using small muscle strips taken from human hearts which were explanted [98]. The high physiological relevance allows the assessment of many parameters such as fibrosis, hypertrophy and sarcomeric disarray [99]. Studies using such models

indicated that the mechanisms underlying force generation are affected in heart failure [100, 101]. However, cardiac muscle strips are obtained during myomectomy and therefore samples need to be processed immediately and are not a stable source. Similarly, isolated human cardiomyocytes are an excellent source of relevant material and have revealed arrhythmias and alterations in diastolic calcium concentrations in HCM [102]. However, isolated cardiomyocytes dedifferentiate soon after isolation and do not proliferate in culture [103]. Myofibrils from skinned heart muscle from patients with two different *MYH7* mutations have been used to investigate multiple parameters; calcium sensitivity, ATP consumption, energy consumption [104, 105] and isometric tension [106], but they are also difficult to obtain due to their excision relying on HCM patient surgery [107]. Avoiding the limitations in obtaining human biopsies and culture conditions, sarcomeric proteins are easy to obtain and have allowed the analysis of ATPase activity and the understanding of the kinetics of contraction. An *in vitro* motility assay was developed which allowed the tracking of actin filaments over myosin molecules [108]. However, later studies performed on myofilaments produced conflicting results [109]. The limitations of this model are due to the concentration of actin and myosin in the sarcomere as opposed to the production and purification of these proteins for the assay – it cannot reflect the natural physiology [110].

Animal models have been an asset in advancing knowledge of HCM pathophysiology as they provide an accessible and common biological source. The creation of transgenic models has made it possible to recapitulate disease states. Animal models of HCM have been created in rodents, cats, dogs, zebrafish and *Drosophila* [111]. The cost of creating a new transgenic animal and maintaining the population is high, especially when considering large animal models. Perhaps the greatest limitation however is the species difference between human and animal physiology and disease.

A promising and emerging alternative to the above models are human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) [112], which overcome the limitations discussed above (difficulty in obtaining, lack of complexity and species differences). Reprogramming of patient somatic cells to hiPSCs and their

subsequent differentiation to cardiomyocytes results in genetically relevant HCM models, which is further strengthened by gene-editing technology. It is also possible to study the development of the disease, one group induced mechanical stress upon hESC-CMs and recapitulated the hallmarks of HCM including hypertrophy, increased expression of stress biomarkers and foetal gene expression [113]. As with any model, the limitation of hiPSC-CMs is their immaturity in comparison to human adult cardiomyocytes, further explored in section 1.5.3.

1.4 Long non-coding RNA

1.4.1 lncRNA structure and genomic organisation

The completion of the Human Genome Project in 2003 revealed that the majority of the human genome is transcribed into protein-coding and non-coding RNA [114]. Non-coding RNAs have been broadly divided into two groups based on transcript length, small non-coding RNA (sncRNA) and long non-coding RNA (lncRNA). The biology and mechanisms of sncRNAs have been extensively reviewed [115], while much less is known of lncRNAs. lncRNA are typically classed as transcripts that are over 200 nucleotides in length with many similarities to messenger RNAs (mRNA) [116], yet offering very little coding potential and possess high tissue specificity [117].

Annotations of lncRNA are significantly behind those of protein-coding genes. Three possible factors for this are their lower expression level, the lack of understanding between sequence and function relationship, and finally that they are weakly conserved in evolution [118]. Along with improving annotations, another core aim is to identify molecular, biological and disease functions of lncRNAs. Current methods apply data from mRNA to lncRNA [118]. The lack of comprehensive lncRNA annotation hinders experimental and analytical approaches. The advent of improved sequencing methods and RNA capture could theoretically enable the complete annotation of lncRNA, but it is an ambitious prospect that has sceptics. Mercer and colleagues argue that this may not be possible at all. Using RNA-capture sequencing surveying human chromosome 21, the smallest chromosome, the number of lncRNA isoforms was undetermined due

to alternative splicing generating a '*limitless diversity of noncoding variants*' [119].

In absence of a clear understanding between the sequence, structure and function, lncRNAs have been categorised by their genomic position relative to adjacent protein-coding genes [118]. This gives more biologically relevant information than using the arbitrary 'over 200 nucleotides in length' classification which defines most lncRNAs. This categorisation gives greater insights into the heterogeneity of lncRNAs in their biogenesis and function. The categories of genomic position are the following: i) intergenic, ii) intronic, iii) sense and iv) antisense. Intergenic lncRNAs lie between two other genes (coding or non-coding) with no overlap in their exons and under the control of an independent promoter, whereas intronic lncRNAs lie within the introns of protein-coding genes. Sense lncRNAs arise from the same strand as coding exons and often span the whole length of the gene, contrary to antisense lncRNAs which can arise from the antisense strand of a coding gene which it may totally or partially overlap with. It is important to note that a lncRNA can be intergenic and sense or antisense or intronic and sense or antisense. The schematic in **Figure 1.5** demonstrates these positionings.

lncRNAs generally have lower sequence conservation between species, unlike mRNAs which have high conservation, suggesting that they have evolved different roles based on species. Many lncRNAs do not exist within the human genome, for example, the mouse lncRNA *Braveheart* [120]. However, there is a subset of lncRNAs that do exhibit conservation between species which fall into one of three classes of lncRNA conservation, as proposed by Ulitsky *et al.* in 2016 [121]. Class I lncRNAs contain the most conservation whereby intron-exon structure and other sequences are conserved among species. Such examples are the well-studied *XIST*, *NEAT1* and *MALAT1*. Class II conserved lncRNAs maintain the act of transcription and RNA elements on the 5' end, but most intron-exon structures are altered and few splice sites are conserved. Class III lncRNAs have no similarity in sequence other than the promoter sequence or the transcriptional process. Generally, the higher conserved lncRNAs exhibit greater expressivity and have more resemblance to protein-coding genes [122].

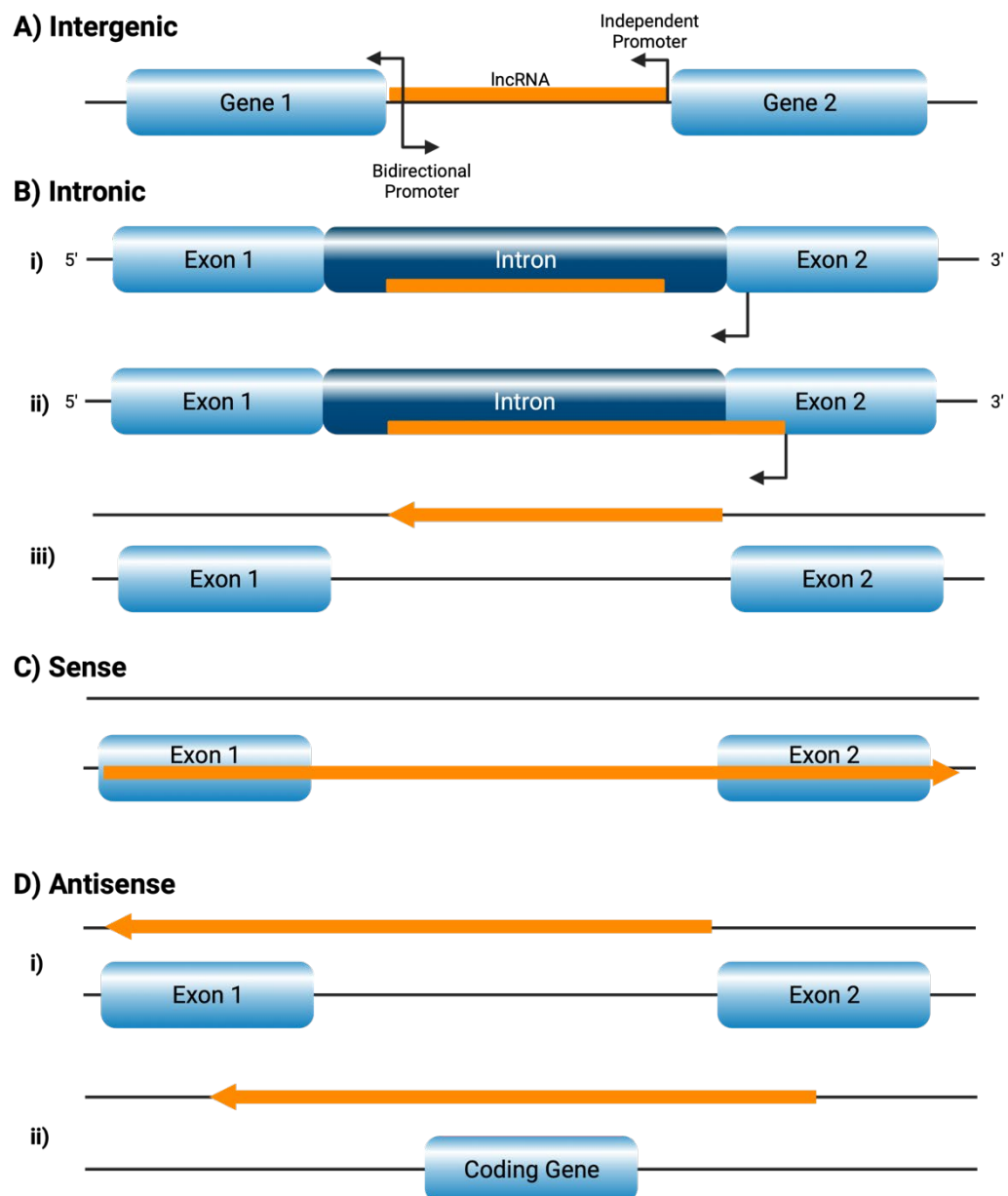


Figure 1.5 – Genomic organisation of lncRNAs. **A)** Intergenic lncRNAs sit between two coding or non-coding genes, with an independent promoter or a promoter that may be shared with other genes. **B)** Intronic lncRNAs sit within the intron of another gene and can overlap with just the intron (i) or overlap into the exon (ii) of the other gene. Intronic lncRNAs may also arise from the antisense strand (iii). **C)** Sense lncRNAs arise from the same coding strand as another gene and usually overlap with the whole gene. **D)** Antisense lncRNAs can partially (i) or fully (ii) cover the sense gene on the opposite strand. Adapted from [123]

1.4.2 lncRNA biogenesis, localisation and function

lncRNA biogenesis and localisation

Most lncRNAs are transcribed by RNA polymerase II (Pol II) leading to 5' capping and 3' polyadenylation, which is a similar feature of mRNAs, therefore it was initially thought that the two nucleic acids underwent the same biogenesis pathway. However, unearthing the distinctions in transcription, processing and export of lncRNAs which determine their localisation and function, highlights the differences from mRNA (**Figure 1.6**). Studies assessing lncRNA localisation have revealed that they are more often localised to the nucleus than cytoplasm compared to mRNAs [117, 124], posing the question; what drives the differences in localisation? Early studies showed that lncRNAs are poorly conserved between species, exhibit low expression levels and contain fewer exons, suggesting that there must be differences in their pre-and post-transcription to mRNAs [125].

A key difference between lncRNA and mRNA is that expression levels of lncRNAs are significantly lower than mRNA. While this is likely due to the presence of repressive histone markers at their promoters, such as H3K9me3 [125], it is feasible that the transcription process they undergo plays a role in this feature. The C-terminal domain (CTD) of Pol II undergoes different phosphorylated states during transcription. Using mammalian native elongating transcript sequencing (mNET-Seq), Proudfoot and colleagues compared the profiles of CTD in transcripts between protein-coding and lncRNAs. The study revealed that lncRNAs undergo poor splicing efficiency and that Pol II pauses inefficiently along lncRNA promoters, whereas in mRNA Pol II pausing is a tightly regulated process [126]. The pausing of Pol II along the lncRNAs leads to transcription termination independent of the polyadenylation signals. The weaker internal splicing signal of lncRNAs also contributes to their inefficient splicing compared to mRNAs. Changes in the expression of splicing regulators can also impact efficiency, for example, the splicing inhibitor peptidylprolyl isomerase E (PPIE) suppresses the splicing of certain lncRNAs in mouse ESCs and hESCs [127]. These transcriptional and processing features are regulators of nuclear retained lncRNAs.

When early studies characterised the first lncRNAs which were chromatin regulators, a precedent was set that lncRNAs are predominantly nuclear [128, 129]. It has emerged that a significant number of lncRNAs are also exported to the cytoplasm [130], which presumably means they share similar export processes to mRNAs. A study by Zuckerman et al. revealed that NXF1 and TREX components were relied on by A/U and G/C rich lncRNA transcripts respectively, for their export to the cytoplasm [131]. Upon arrival in the cytoplasm, lncRNAs are then likely sorted to either remain in the cytoplasm or localise to a specific organelle such as the mitochondria [132] or exosome [133]. The sorting mechanism has not yet been elucidated. A significant study in 2016 revealed that approximately 70% of cytoplasmic lncRNAs are associated with polysomes, a group of ribosomes that are bound to mRNA, thereby possibly resulting in the lncRNA degradation [130]. The action of such ribosomal bound lncRNAs has not yet been determined.

Understanding lncRNA location within a cell can indicate potential molecular mechanisms as they localise to their site of action. Nuclear lncRNAs possess roles in gene expression by regulating transcription through interactions with chromatin. Cytoplasmic lncRNAs mediate signalling pathways and post-transcriptional processing, affecting gene expression.

As discussed above, there is generally poor conservation of lncRNAs across different species. A 2020 study by Guo et al. demonstrated that of lncRNAs that are conserved (defined as $\geq 20\%$ sequence similarity) between mESCs and hESCs, their localisation is different, with more lncRNAs localised to the cytoplasm in hESCs [127]. Interestingly there were no observed differences in conserved mRNA localisation. While it is speculated that different processing in each species is a factor, this suggests that there may be a different functional relevance between species in conserved lncRNAs. This is alarming given animal models are so prevalent in human disease research and provides a strong case for the requirement of human relevant models such as hiPSC-CMs for lncRNA research.

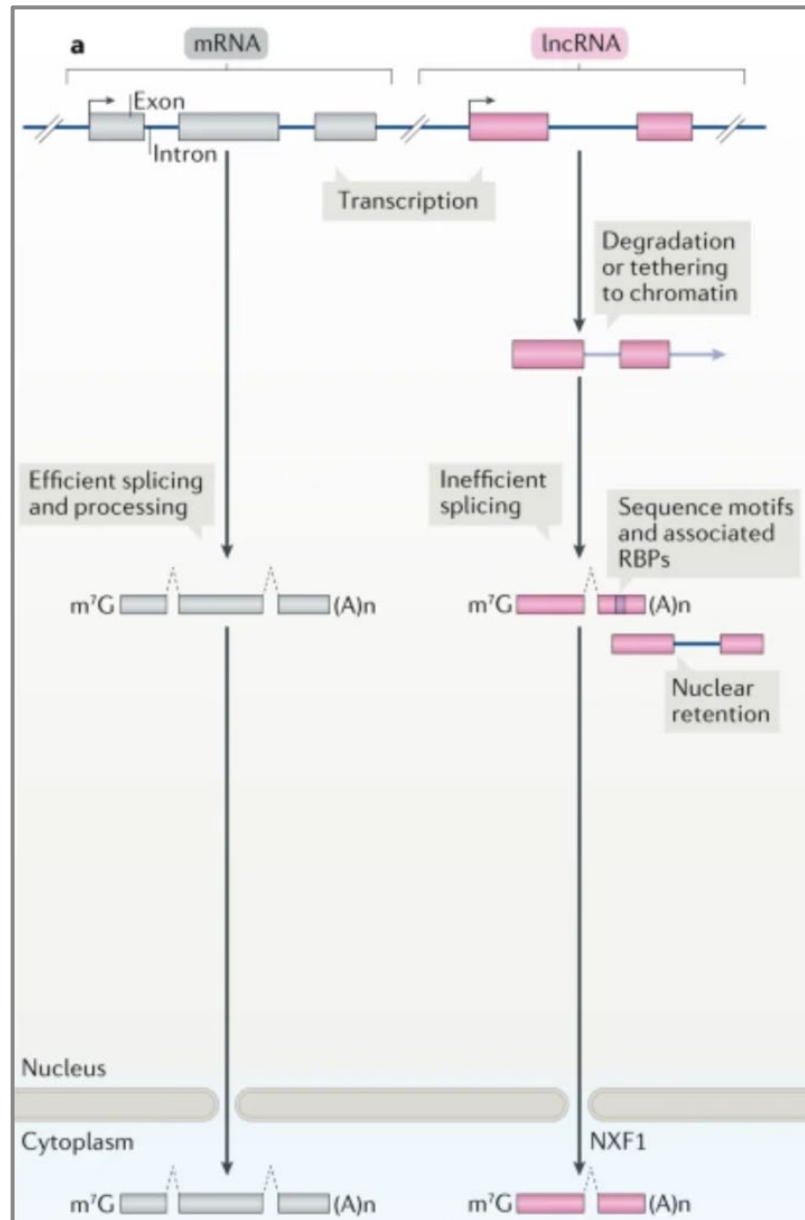


Figure 1.6 – Biogenesis of mRNAs and lncRNAs and their subsequent localisation. Adapted from [134]

Gene Regulation by lncRNAs

lncRNAs interact with other nucleic acids and proteins to exert their roles by modulating chromatin structure and function and transcription of genes in *cis* or in *trans*. They also have less well understood roles in organelle formation and as organisation factors.

Chromatin regulation by lncRNAs can occur through protein-lncRNA interaction or lncRNA-DNA direct interaction. lncRNAs that localise on chromatin can interact with and recruit proteins, enabling their binding and activity with the DNA. Proteins

that bind to and spread along chromatin such as the polycomb repressive complex 2 (PRC2) have been reported to be facilitated by several lncRNAs. Yap *et al.* discovered that the genes *CDKN2A* and *CDKN2B* are regulated through the recruitment of PRC1 and PRC2 to their promoters by the neighbouring lncRNA *ANRIL*, which resulted in the control of their expression and maintenance of their epigenetic state [135]. *ANRIL* is both a *cis* and *trans* acting lncRNA. Alu elements are a family of short interspersed repeat elements (SINE), which are specific to the primate family. Studies have shown that lncRNAs can act in *trans* and regulate distant targets through Alu sequences which drive the recruitment of PRC1 and PRC2 to the distant genes [136]. In addition, other factors including chromatin factors and transcription factors are involved in lncRNA facilitation of PRCs.

A second mode of action that lncRNAs can take to modulate expression is by acting as decoys. *lncPRESS1* was identified as a regulator of a gene network that promotes pluripotency. Deacetylase sirtuin 6 (SIRT6) binding to chromatin represses several pluripotency genes thereby promoting differentiation. *lncPRESS1* acts as a decoy by sequestering SIRT6 from the chromatin, maintaining high levels of histone activity and the expression of pluripotency genes [137].

The ability of lncRNAs to form triple helices or R-loops with DNA can also mediate gene activation or silencing. The development of targeted RNA immunoprecipitation sequencing (TrIP-seq) elucidated the regulation of *SPHK1* by the lncRNA *KHPS1* which activates the expression of the oncogene by forming a triple helix with DNA at its enhancer [138]. In summary, lncRNAs can regulate chromatin by recruiting chromatin modifiers to the target gene promoters, act as a decoy target of chromatin modifiers or interact directly with the chromatin.

Transcription regulation by lncRNAs on neighbouring genes is determined by its relative genomic position in relation to that gene, lncRNAs can silence or activate expression. By interfering with transcription machinery, lncRNAs can suppress gene expression by altering the presence of transcription factors, the activity of Pol II and histone modifications. Studies by Rom *et al.* on the lncRNA *Chaserr* found that decreased levels of *Chaserr* led to an increase of expression of the neighbouring chromatin remodelling gene *Chd2* in mice by the increased accessibility of its promoter [139]. In humans, *CHD2* haploinsufficiency is related

to several neurological conditions [140]. *Chaserr* is highly conserved between human and mouse and further studies by the group revealed that targeting of the human *CHASERR* using Gapmers resulted in similar expression changes seen in mouse suggesting that there may be human therapeutic relevance [139].

lncRNAs transcribed from enhancers known as enhancer associated lncRNAs (e-lncRNA) which can promote the expression of nearby protein coding genes (PCG) due to their proximity to the PCG promoter due to a preformed chromatin loop. An example of this is the e-lncRNA *SWINGN* which recruits chromatin activating molecules to the promoters of nearby PCGs [141].

lncRNAs exert their role through forming interactions with nucleic acids and proteins. Their influence on gene expression continues through to post transcriptional regulation. By base pairing with other RNA molecules, they can recruit mRNA degrading proteins [142]. Similarly, some lncRNAs have miRNA complimentary sites which compete with target mRNAs by acting as a miRNA 'sponge' and thereby reducing gene expression [143]. lncRNA – protein binding to create lncRNA-protein complexes (lncRNPs) leads to altered mRNA splicing and in some cases modulation of signalling pathways [144]. Finally, lncRNAs have been found to be localised to organelles such as mitochondria and exosomes. lncRNAs localised to exosomes are secreted and function as a *trans* regulators between different cells. Effects of such lncRNAs are genomic instability, cellular reprogramming and epigenetic regulation, which are generally cancer related phenotypes [145]. Unsurprisingly, lncRNAs localised to the mitochondria (which can be encoded for by either nuclear DNA or mitochondrial DNA), have a function in mitochondrial metabolism, apoptosis and nuclear crosstalk [146].

1.4.3 lncRNAs in disease

Since their discovery and the realisation of their abundance, lncRNAs have been at the forefront of research in human disease and are of particular interest due to the potential for personalised medicine. Given their significant roles in cell function, their dysregulation opens up the possibility of disease. Identifying causative phenotypes and mechanisms can lead to improved targeting of disease and improved patient treatment. lncRNAs are associated with multitude of different diseases including cancer, cardiovascular, neurological, muscular,

haematopoietic, to name a few [147]. A few major cardiovascular lncRNAs will be mentioned below.

A landmark study in 2013 revealed a cluster of lncRNA transcripts in *Myh7* loci that were involved in heart failure. Termed *Mhrt*, these abundant transcripts are cardiac specific and their suppression leads to hypertrophy and heart failure. The human *MHRT* also derives from the *MYH7* locus featured similar phenotypes in diseased heart, suggesting a conserved function [148]. Recently, the previously unannotated lncRNA *Ahit*, was identified as a cardioprotective lncRNA in mouse hearts, its mechanism predicted and identified using *in silico* prediction and RNA immunoprecipitation. Using human samples of patients diagnosed with hypertrophy, the same function of *AHIT* was observed, identifying a new therapeutic pathway [149]. A summary of lncRNAs associated with CVD in humans to date is shown in **Table 1.1**.

The characteristics of lncRNAs make it difficult to determine their role in human disease. As mentioned, their low expressivity, poor species conservation and lack of protein products pose a barrier to traditional methods of disease investigation. Therefore, to decipher their role one should overexpress them or remove the lncRNA (by KO for example), specifically in human models if investigating human disease. There is an unmet need for tools and processes that allow the characterisation of lncRNAs in disease.

Table 1.1 - lncRNAs in CVD.

lncRNA	Associated disease	Reported function
Anril	Coronary artery disease	Biomarker (in diabetic type II patients)
	In-stent restenosis	Biomarker
	Left ventricular dysfunction	Biomarker
Braveheart	Cardiac lineage development	Regulation of chromatin modifications via PRC2
Carl	Myocardial infarction	Inhibition of mitochondrial fission and cardiomyocyte apoptosis by sponging miR-539
Chaer	Cardiac hypertrophy	Epigenetic modulation of hypertrophic gene expression
Chast	Cardiac hypertrophy	Induction of hypertrophic cell growth and gene expression
Chrf	Cardiac hypertrophy	Sponging of miR-489
	Doxorubicin-induced heart failure	Regulation of TGF- β signaling
Fendrr	Organ development derived from lateral mesoderm	Regulating chromatin modifications via PRC2 and TrxG/MLL
Ftx	Myocardial infarction	Regulation of cardiomyocyte apoptosis by targeting miR-29b-1-5p
H19	Cardiac hypertrophy	Targeting Ca/calmodulin-dependent protein kinase II δ (CaMKII δ)
	Coronary artery disease	Biomarker
	Diabetic cardiomyopathy	Regulation of cardiomyocyte apoptosis by targeting VDAC1
	Ischemia reperfusion injury	Regulation of necrosis by targeting miR-103/107
	Myocardial infarction	Activation of autophagy
Hotair	Cardiac hypertrophy	Interaction with miR-19
Lipcar	Cardiac remodeling and heart failure	Biomarker
	Coronary artery disease	Biomarker
	Left ventricular diastolic function	Biomarker
Malat1	Atherosclerosis	Regulation of inflammation
	Cardiac fibrosis after myocardial infarction	Regulation of TGF- β signaling via miR-145
Mdrl	Ischemia-reperfusion injury	Inhibition of mitochondrial fission and cardiomyocyte apoptosis by sponging miR-361
Meg3	Cardiac fibrosis and diastolic dysfunction	Regulation of TGF- β I induced p53 signaling
	Myocardial infarction	Regulation of cardiomyocyte apoptosis
Mhrt	Cardiac hypertrophy	Regulation of isoform switch Myh6 to Myh7
	Doxorubicin-induced cardiomyopathy	Inhibition of cardiomyocyte apoptosis
	Heart failure	Biomarker
Miat	Cardiac fibrosis after myocardial infarction	Regulation of cardiac fibrosis by interaction with several miRNAs
	Cardiac hypertrophy	Sponging miR-93
		Sponging miR-150
	Left ventricular diastolic function	Biomarker
	Diabetic cardiomyopathy	Regulation of myocardial hypertrophy and apoptosis
	Myocardial infarction	SNP in exon 5 as risk allele for MI
Mirt1	Myocardial infarction	Suppression of NF- κ B signaling
Nron	Heart failure	Biomarker
Sencr	Left ventricular diastolic function	Biomarker
Wisper	Cardiac fibrosis after myocardial infarction	Alternative splicing of Plod2 mRNA by enabling nuclear localization of TIAR

1.5 Human induced pluripotent stem cell cardiomyocytes

hiPSC-CMs overcome many of the limitations presented by animal models and patient samples. Animal models lack physiological relevance and patient samples are difficult to obtain. On the contrary, hiPSC-CMs provide an unlimited source of material owing to the proliferative ability of hiPSCs. The relative ease of gene editing in hiPSCs is also an invaluable tool for disease modelling, as modifications are maintained through the process of differentiation. Other uses of hiPSC-CMs include drug screening, toxicity testing and the study of human development.

1.5.1 Derivation and characterisation of hiPSC

A turning point in modern science was in 1998 when embryonic stem cells (ESC) were derived from blastocysts [150]. Although promising for medical research and regenerative medicine, the ethical issues around the use of human embryos restricted their use. The focus was set to reprogramme differentiated cells to cells of pluripotent ability. Pluripotent stem cells have unlimited self-renewal capability and the ability to differentiate into all three germ layers. The second turning point came in 2007 when Yamanaka and colleagues successfully reprogrammed differentiated human somatic cells to induced pluripotent stem cells (hiPSC) by four factors: Oct-4, Sox2, Klf4 and c-Myc [151]. Characteristically, hESC and hiPSCs express transcription factors OCT4, NANOG and SOX2, cell surface marker stage specific embryonic antigen (SSEA 3/4) and tumour related antigens (TRA 1-60, TRA1-81) [152].

Reprogramming strategies are split into two groups: integrative or non-integrative transfer systems. These can be viral or non-viral transduction / transfection methods. Strategies to improve reprogramming to meet safety and efficiency standards have been at the forefront of reprogramming research since the revolutionary Yamanaka study. Integrative viral vectors include retroviral and lentiviral transduction which were used for the first reprogramming of hiPSCs. While lentiviral remains the most efficient strategy (0.1-1%) [153], insertional mutagenesis is a risk in integrating viruses. To circumvent the limitations of integrative viruses, adenoviruses [154, 155] and the single stranded RNA Sendai-virus [156] have been used to successfully reprogramme hiPSCs at the cost of low

efficiency. Non-viral and non-integrative methods have been investigated, such as messenger RNA (mRNA) [157] and micro RNA (miRNA) [158], but are also hampered by low efficiency.

1.5.2 Differentiation of hiPSC towards cardiomyocytes

Recapitulating Cardiac Development

To effectively differentiate hiPSCs to cardiomyocytes, it is essential to apply the principles of cardiogenesis during human development. Soon after fertilisation, gastrulation occurs when the three germ layers form: endoderm, ectoderm, and mesoderm. Generation of cardiomyocytes from hiPSCs occurs in four stages: i) formation of the mesoderm, ii) formation of the cardiac committed mesoderm, iii) the formation of cardiac mesoderm and iv) maturation of early cardiomyocytes. Mesoderm formation is well studied and is controlled by growth factors such as bone morphogenic proteins (BMP) and wingless/INT proteins (WNT).

Each stage of differentiation can be characterised by the expression of different transcription factors. It is possible to identify cardiomyocytes by the expression of structural proteins such as cardiac troponin T (cTnT) and α -actinin. Typical markers in each of the preceding stages are: mesoderm expression of Brachyury, cardiac mesoderm expression of Mesp-1 and cardiac progenitor expression of Nkx2, Tbx5 and Mef2c [159].

Cardiac Differentiation Protocols

The differentiation of hiPSCs to cardiomyocytes arises after sequential steps of differentiation (mesoderm → cardiac mesoderm → cardiac progenitors → beating cardiomyocytes) are regulated by growth factors such as BMPs, WNTs and fibroblast growth factors (FGF). The protocols that have been developed for this differentiation act by activation or inhibition the signalling pathways that drive cardiac differentiation. There are four main classes of cardiac differentiation protocols; embryoid body formation [160], inductive co-culture [161], guided monolayer culture and guided suspension culture [162]. In this thesis monolayer culture was used for the ease and reproducibility of cardiomyocytes obtained. There are several reports of directed monolayer cardiomyocyte differentiation protocols. The first was developed by Laflamme which yielded >30%

cardiomyocyte purities based on treatment with Activin A followed by BMP4 in RPMI+B27 medium [163]. Perhaps the most significant study was performed by Burridge *et al.*, who sought to determine the most efficient cardiac differentiation protocol using the least number of components. Using small molecule directed differentiation and basal RPMI supplemented with rice derived albumin and ascorbic acid, they were able to achieve 95% cardiomyocyte populations [164]. There are several iterations of this method which revolve around the temporal regulation of WNT using growth factors and small molecules and the use of different media to drive cardiac differentiation [165, 166]. Nevertheless, cardiac differentiation protocols are still hampered by the production of mixed populations composed of the three cardiac subtypes.

1.5.3 hiPSC-CM maturity

hiPSC derived cardiomyocytes are characteristically and functionally immature compared to adult cardiomyocytes, reflecting cardiomyocytes present in foetal stages. For improved modelling of disease and drug screening, the field is pushing forward research to promote the maturation of hiPSC-CMs for a better reflection of adult human heart physiology. The characteristics used to differentiate between hiPSC-CMs and adult cardiomyocytes are broadly; gene expression, composition and organisation of the contractile apparatus, metabolism and electrophysiology [167]. Calcium handling genes do not show the same expression profiles as those of adult cardiomyocytes, even after long term culture *in vitro* [168]. Morphologically, hiPSC-CMs display less multinucleation compared to human adult cardiomyocytes (1% vs 20%) and the rod shape is also less defined [169]. Similarly, although hiPSC-CMs have correct sarcomere biology, their organisation resembles an immature phenotype and their length is typically shorter [170]. Metabolically, hiPSC-CMs rely on the metabolism of glucose, while in the adult heart fatty acids are the primary metabolic resource [171].

Improvement of hiPSC derived cardiomyocyte maturation has several investigative avenues. It is known that human neonatal cardiomyocytes can take up to 10 years to reach the adult phenotype *in vivo* [172]. Applying this knowledge to hiPSC-CMs, long term culture of differentiated cardiomyocytes should result in more mature cells. In 2014, the long term hiPSC-CM culture studies of Lundy and colleagues

over a 120 day period showed markedly more mature characteristics of hiPSC-CMs [170]. While 2D monolayer culture of cardiomyocytes is simple and scalable, they fail to mimic *in vivo* conditions. Engineered heart tissues (EHT) whereby hiPSC-CMs are seeded into 3D structures and subjected to stretching (resembling mechanical loading of the heart) have generated cardiomyocytes with contractile characteristics close to the adult heart [173]. More recently, studies have been looking to recapitulate other conditions of the adult heart. Co-culture with other non-cardiomyocyte that are present in the heart and are responsible for extracellular matrix (ECM) production was shown to result in improved maturation of hiPSC-CMs [174]. A significant recent study revealed that by influencing the metabolism of hiPSC-CMs using maturation media (medium containing levels of glucose and Ca^{2+} that are physiologically relevant to the metabolic needs of cardiomyocytes), fatty acid oxidation was enhanced, along with electrophysiological and mechanical properties [175]. While there has been some success in maturation of hiPSC-CMs, the field is still undergoing efforts for improved approaches. It was once inconceivable for stem cell research to be where it is today, so the interest, time and money that is poured into the field will lead to the continual evolution of heart models. Whether or not there will ever be a perfect model of the human heart is debatable, but considering all that *Homo sapiens* have achieved in 300,000 years, it might not be beyond human bounds [176].

1.5.4 hiPSC-CM modelling in cardiac disease

Although the contribution of animals to human research has been indispensable, the use of animal models in drug metabolism, disease modelling and toxicity studies are not a reliable tool for the predication of a human response due to indisputable physiological differences such as heart size and beat rate. Mice have beat rates 10x faster than human hearts and express predominantly α -MHC in both atria and ventricles while human ventricles express β -MHC [177, 178]. The *in vitro* capabilities of hiPSC-CMs allow more physiologically relevant experiments which can also be conducted in a high throughput manner.

The derivation and differentiation of hiPSC and hiPSC-CMs is already a powerful tool for modelling of diseases. The addition of gene editing technology has allowed

the creation of mutant cell lines that can recapitulate single mutations while maintaining an isogenic control line. The combination of hiPSC-CM and gene editing technology has overcome the issues that had arisen, for example, cells from healthy family members had been used as a control when studying cells from a diseased patient. The ability to create an isogenic healthy cell line by removing the disease-causing mutation prevents any phenotypic changes that might arise from genetic background variation [6].

1.6 Genome editing technology

The ability to modify gene sequences in almost every eukaryotic cell type has given researchers the power to elucidate the role of genetics in disease through the creation of cell and animal models. The applications of gene editing have also proven useful in the fields of biotechnology and biomedical research. The methods are based on engineered or bacterial processes. **Figure 1.7** summarises current gene editing technologies.

1.6.1 Mechanisms of editing

The concept of gene editing is to create double stranded breaks (DSB) and exploit the natural cellular repair mechanisms. Those are homology directed repair (HDR) and non-homologous end joining (NHEJ). HDR requires an exogenous repair template (donor template) which is incorporated into the region of the DSB allowing precise modifications, however it is slow as it is cell cycle dependent. NHEJ relies on using the cellular endogenous repair mechanism which is highly error prone and often results in indels and is therefore often used in gain or loss of function applications. Gene editing can therefore be applied in four ways: i) generate frameshift mutations, ii) deletions of sequences, iii) insert DNA sequences and iv) correct genes.

Gene editing technologies can be used to introduce indels at specific site, resulting in **frameshift mutations and/or gene knock outs (KO)**. This method is useful for the correction of diseases and when the function of a gene is disease causative. Recently, Zhang and colleagues modified two base pairs of the Apolipoprotein 4 (*APOE4*), which is associated with high Alzheimer's disease risk, to the sequence of *APOE2* which carries no risk of Alzheimer's disease [179].

Gene deletion can be performed by excising segments of DNA up to several kilobases long using gene editing applications, through the introduction of DSB. This can be of particular use for disease research where total loss of gene function is to be studied. A region spanning 10 kilobases of DNA was successfully deleted from HEK cells using CRISPR/Cas9, suggesting that precise and simple editing is possible [180]

A donor DNA template can be used for **gene insertion** at the DSB site. HDR is used to repair the DSB, incorporating the DNA of interest due to its flanking DNA being homologous to either side of the break site. Insertion of transgenes into safe harbour loci such as AAVS1 ensure their expected function while avoiding insertional mutagenesis [181]. Similar to insertion strategies-, a donor DNA template containing corrected DNA sequences can be used for precise single base editing to **correct gene mutations**. The template containing the correction is incorporated into the genome by HDR. Wu *et al.* performed gene correction on hiPSC-CMs derived from a patient harbouring a novel mutation in the ion channel gene *KCNH2*, confirming its pathogenicity in long QT syndrome (LQTS) [182].

1.6.2 Targeted nucleases

Zinc finger nucleases (ZNF) are composed of site-specific DNA binding domains, zinc finger proteins (ZFP), attached to a non-sequence specific nuclease, FokI, which requires dimerization to function. Introduction of a DSB requires two ZFNs, each bound to opposite strands of DNA. The ZFP region of the ZFN is composed of an array of Cys₂His₂ fingers, which each recognise 3 base pairs (bp) of DNA – a codon. Generally, ZFN applications employ 3 to 6 ZFPs, enabling each dimer to specify an 18 to 36bp region [183]. To improve the specificity, a recent study used architectural diversification by selecting new linker regions (finger-finger and finger-FokI linkers), increasing the number of ZFN configurations for targeting DNA cleavage by 64-fold [184]. High specificity and low off target effects come at a cost, each ZFN assembly costs 5,000 to 10,000 USD [185] and is a labour-intensive process and does not permit the targeting of multiple loci at once.

Transcription activator like effector nucleases (TALEN) is a protein-based targeting system. Similar to ZFNs, TALEN gene editing utilises the non-specific

endonuclease FokI that requires dimerization to become active and requires two TALENs on opposing strands of the DNA sequence [186]. The difference between these two strategies lies in the higher specificity of TALENs due to each transcription activator like effector (TALE) corresponding to one base pair whereas ZFPs recognise 3bp [187]. Drawbacks lie with the requirement of a T base at the start of the target site [188] which can be a limitation for precise single base editing. Furthermore, complex cloning is required for their production and their large size poses issues for their delivery.

Clustered regularly interspaced short palindromic repeats (CRISPR) were originally discovered in *E.coli* and were later revealed to be part of the adaptive immune response of bacteria and archaea in response to foreign DNA [189]. The system was adapted for use in mammalian cells, whereby a 20 base pair guide RNA (gRNA) that is sequence specific is complexed with the Cas9 protein, resulting in cleavage of the target site. The benefit of this system is that specificity is determined by the gRNA sequence, which is significantly easier to assemble and less laborious than engineering new proteins, as is required in the other two editing systems. Furthermore, as the gRNA and Cas9 protein are not bound, it is possible to multiplex and target multiple sites.

There are three approaches to CRISPR/Cas9 editing; i) the plasmid based strategy [190], ii) intracellular delivery of Cas9 mRNA and gRNA and iii) direct delivery of Cas9 and gRNA, known as a ribonucleoprotein complex [191]. Mindful of mRNA having poor stability and resulting in transient transfection, in this thesis the plasmid based strategy was used as it is easy to assemble, and results in longer expression of the Cas9 protein and gRNA [190] as opposed to the other two methods.

CRISPR/Cas systems are divided into two groups based on their structure and organisation; type I consists of multiprotein effector complexes while type II have a single effector protein [192]. The system used in this thesis was of the type II class CRISPR/Cas9 system which uses a single Cas9 protein derived from *Streptococcus pyogenes*, spCas9 [193]. Each Cas protein has a specific recognition site, which is one limitation to this editing technique, as a protospacer

adjacent motif (PAM) is required near the target site for binding and cleavage by Cas proteins [194]. In this thesis the recognition site for the spCas9 was 5'-NGG-3' (where N can be any nucleotide), however engineering of Cas9 proteins has led to more abundant recognition sites. The most recently engineered Cas9 variants are SpG and SpRY which can target a greater set of 5'-NGN-3' PAM sites and all PAM sites in human cells, respectively, removing PAM site restriction barriers [195].

Along with PAM site restrictions, the high prevalence of off target activity is a major drawback of CRISPR/Cas9 systems. The binding of Cas9 to unintended genomic sites occurs due to the tolerance of up to five mismatches between the gRNA and target site [196]. In order to understand how these mismatches influence target recognition, Singh and colleagues used probes to observe interaction between Cas9, gRNA and DNA in real time [197]. Their observations were that four mismatches in the PAM distal end of the gRNA induces off target effects. To combat this undesirable feature of CRISPR/Cas9 gene editing, *in silico* tools have been designed for the predication of off target effects by aligning gRNA sequences with reference genome sequences based on homology [198]. There also considerations that can be made when designing gRNAs to mitigate off target effects, these are; i) GC content of the gRNA, ii) length of gRNA, iii) truncated gRNA and iv) chemical modifications of gRNA. Studies have shown that a GC content of 40-60% in the gRNA increased on target activity due to the stabilising effects of GC content [199]. Other studies have indicated that gRNA up to 17 nucleotides reveal higher genome editing efficiency than those 18-20 nucleotides long [200]. Combining the gRNA with a nickase Cas9 mutant [201] or incorporating 2'-O-methyl-3'-phosphonoacetate into the backbone of the gRNA reduced off target effect [202], by 50-1500 fold and 40-120 fold respectively. Finally, engineering of Cas9 proteins has been another approach to the issue of off target effects. High fidelity SpCas9 (SpCas9-HF1), a variation of SpCas9, did not recapitulate the large number of off target effects its predecessor did, even at repetitive target sites [203]. The methods discussed are depicted in **Figure 1.7**.

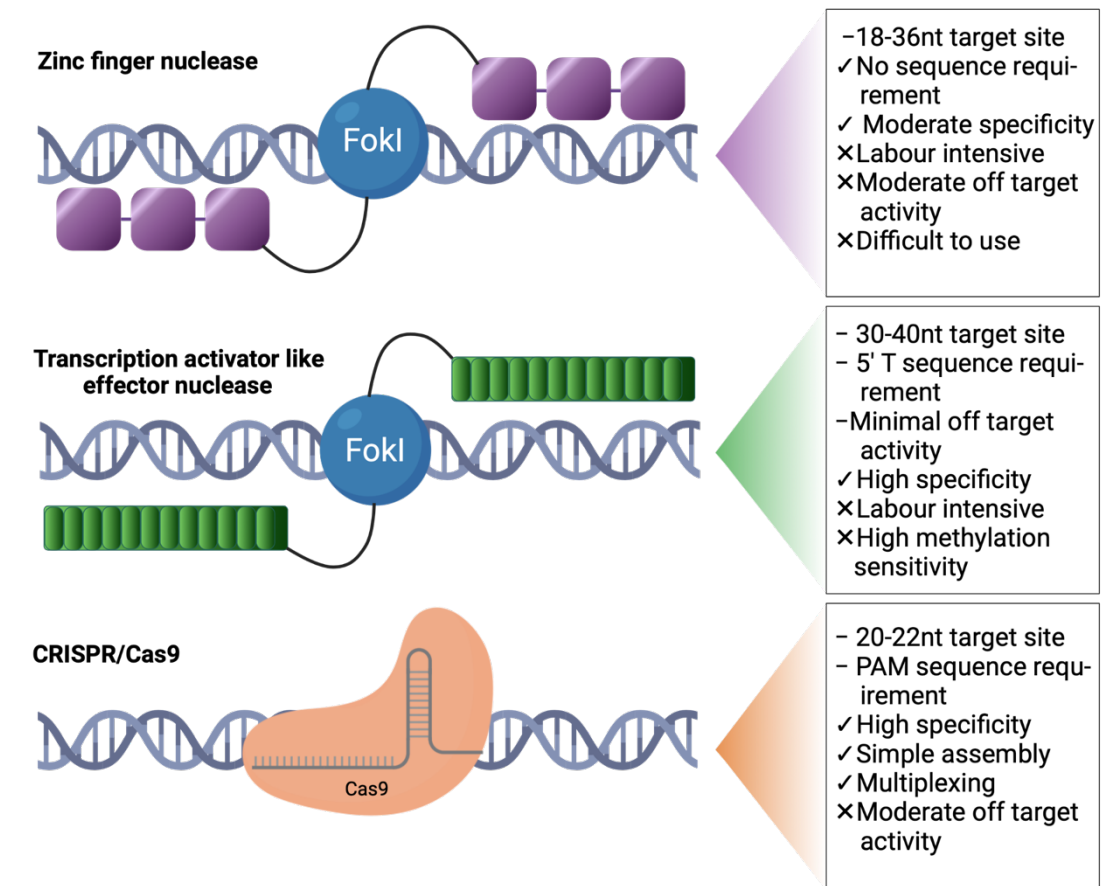


Figure 1.7 – Genome editing technologies. Zinc finger nucleases (ZNF), transcription activator like effector nuclease (TALEN) and CRISPR/Cas9 are the most widely used gene editing technologies. ZNF and TALEN make use of the FokI nuclease for the creation of DSB, while the Cas9 nuclease performs this role in CRISPR systems. Created using Biorender.com 2022.

1.6.3 Applications of gene editing

Gene targeting has a wide range of applications besides the engineering of human disease models. The technologies described above have been used to generate reporter lines, creating new cell therapies, and producing lines for large-scale production of biomolecules for use in the pharmaceutical industry. In terms of disease modelling and gene therapy, editing technologies have been applied to an array of diseases including; cancer, CVD, metabolic, viral, neurodegenerative, hereditary eye conditions and haematological [204].

Gene editing has prospects in cancer immunotherapy. Due to the ability of CRISPR/Cas9 strategies to target multiple genes as opposed to ZFNs and TALENS, it has been used to create chimeric antigen receptor (CAR) T cells from an

allogeneic source with anti-tumour function by knocking out three genes associated with rejection, simultaneously [205]. This research holds great value for patients for whom autologous cell transfer is not an option, for example the elderly or newborn babies. Testing *in vivo* and *in vitro* on murine models revealed that gene editing CAR T cells is applicable and holds great potential for cancer therapy.

Other recent applications of gene editing include proof of concept designs including an antiviral strategy that arose due to the COVID-19 pandemic. Abbot and colleagues developed a CRISPR/Cas13 strategy named prophylactic antiviral CRISPR in human cells (PAC-MAN) for the viral degradation of SARS-CoV-2 RNAs [206]. Targeting human lung epithelial cells before COVID-19 infection, the strategy inhibited the infection of the target cells by the recognition of CRISPR associated RNAs (crRNA) of SARS-CoV-2 RNA transcripts, resulting in their cleavage. The authors hypothesise that this method could reduce viral loads after transfection, but that effective and safe *in vivo* delivery methods are required.

Although what has been achieved using gene editing so far is monumental, there is still a way to go for the successful clinical application of gene editing technologies. The greatest barriers include the unpredictability of the effects on nearby gene expression, the delivery of the therapeutic genes (safety issues using viral methods) and improvements to the efficiency of repair pathways. Once solutions to these barriers are found, gene editing will hold the key for personalised medicine. Considering the improvements to the CRISPR system that have happened in just one decade since its first use in the human genome [207], in not much time at all researchers will overcome the above impediments.

1.7 Hypothesis and overarching aims of the PhD project

The overarching aim of this project was to **develop the tools** required for the **modulation of lncRNA expression**, in a previously established hiPSC HCM model which harnesses a severe mutation in the *MYH7* gene. The hypotheses of this work were:

- I. The identified lncRNA targets act as gene modifiers in a model of *MYH7* based on a c.C9123T-*MYH7* (p.R453C- β MHC) substitution

- II. The use of hiPSC-CMs to study lncRNAs in the context of human disease is more reliable than animal models considering the lack of lncRNA conservation between species
- III. Functional analysis of lncRNA modulated hiPSC-CMs across the model of HCM would elucidate whether the target lncRNAs have a role in disease prevention or progression

To achieve the goals:

- I. Create CRISPR/Cas9 and overexpression vectors for the modulation of lncRNA expression
- II. Optimise a transfection protocol for high transfection efficiency of modulation vectors
- III. Perform phenotypic assays for the assessment of lncRNA function in HCM progression or prevention

2. Materials and Methods

2.1 Cell Culture

2.1.1 hiPSC Culture

hiPSCs were cultured in a type II Biological Safety cabinet and kept in a humidified incubator at 37°C and 5% CO₂. hiPSCs were maintained in E8 medium (Life Technologies, #A1517001) in T25 flasks (Nunc) coated with 1:100 matrigel (MT) (BD #356235). Cells were passaged every three days by first washing once with Ca²⁺/Mg²⁺ free Phosphate Buffer Saline (PBS, Gibco #14190-094) followed by incubation with cell dissociation solution (CDS), made from trypsin-EDTA 0.25% (Gibco #25300054), RPMI 1640 basal medium (Gibco #21875034) and cell dissociation buffer (Invitrogen #1315104), at a volume ratio of 1:2:2, for 4 minutes at 37°C. CDS was quenched by washing the cells with DMEM-F12 (Gibco #11320033) basal medium supplemented with 15% Knockout Serum Replacement (Gibco #10828028), 1% Non-Essential Amino Acids (NEAA, Gibco #11140050), 1% GlutaMAX (Gibco #35050061), 100 µM β-mercaptoethanol (Sigma #63689) and basic Fibroblast Growth Factor (Peprotech 100-18B, 8ng/ml), followed by centrifugation at 160xg for 4 min. Afterwards, hiPSCs were resuspended in E8 supplemented with 10µM Y-27632 (ROCKi, Tocris Bioscience #1254/10,) and seeded into new MT-coated T25 flasks (Nunc) at approximately 30,000 cells/cm². Medium was changed every day.

2.1.2 Monolayer Cardiac Differentiation of hiPSC

hiPSC differentiation was performed as above, however flasks were seeded with a range of densities from 10,000-30,000 cells/cm². E8 media was changed the following morning and the pre-conditioning step was performed the day after by adding a MT overlay (MT diluted 1:100 in StemPro™ 34 Serum Free Medium (SP34) Gibco #10639011), supplemented with 1ng/mL BMP4 R&D #314-BP-050). Approximately 12-16 hours later, medium was replaced by SP34 supplemented with 8ng/ml ActA (Life Technologies #PHC9564) and 10 ng/ml BMP4. 48 hours later, medium was changed RPMI 1640 supplemented with B27 minus insulin (-INS, Life Technologies #A1895601) and the small molecules (SM)

KY0211 (R&D #4731) and XAV939 (R&D #3748), both at 100µM. Two days later the medium was changed to RPMI 1640 with insulin (+INS, Life Technologies #0080085-SA) supplemented with the same SM as before. From there on medium was changed every two days using RPMI B27 +INS until day 15 of differentiation.

2.1.3 hiPSC Cardiomyocyte Dissociation

hiPSC-CMs obtained by monolayer differentiation were dissociated using a protocol previously described, using Collagenase II [208]. In brief, cells were washed twice in Ca²⁺ and Mg²⁺ free Hank's Balanced Salt Solution (HBSS, Life Technologies #14175095). Cardiomyocytes were then incubated for 3.5h at 37 °C in 200U/ml Collagenase II (Worthington #LS004176) in Ca²⁺ and Mg²⁺ free HBSS supplemented with 1mM HEPES (Sigma #H4034), 10µM Y-27632 and 30nM N-Benzyl-p-toluenesulfonamide (BTS, TCI #B3082), all of which were diluted 1:1000. Thereafter dissociated cardiomyocytes were collected from the flasks and washed with RPMI supplemented with 24µg/mL Deoxyribonuclease II (DNaseII – Sigma #D8764). Post incubation, dissociated cells were collected from the flasks and washed in RPMI supplemented with 24µg/ml DNase and centrifuged at 100xg for 15 min. Subsequently the cells were resuspended in warm RPMI and passed through a 100µm cell strainer (VWR# 89508-840) and collected. Cells were counted using an automated CEDEX HiRes counter (Roche) and the cells centrifuged again as before. Afterwards, the cardiomyocytes were resuspended in RPMI B27 +INS and seeded into Vitronectin-N (VN, Lifetech #A14700) coated 96 well plates (Clear – Nunc or black – CellCarrier, Perkin Elmer) at densities according to the assay to be performed.

2.2 CRISPR/Cas9

2.2.1 Guide RNA Design and Assembly

A dual guide RNA strategy was to disrupt target lncRNA sequences in the hiPSC HCM model encoding the R453C- β-MHC substitution. The design involved deleting a segment of the sequence and inserting either a blasticidin or puromycin antibiotic resistance cassette, to ensure disruption of gene expression. A WT Cas9 plasmid (Sigma Aldrich, CAS9P-1EA) with a CMZ promoter was used. sgRNAs were identified using the bioinformatics website <http://crispr.mit.edu>, by searching for

NGG PAM sites. ACCG and AAAC sequences were added to the 5' and 3' ends of the respective complementary sequences, which were then annealed and cloned into the BsaI digested U6-gRNA backbone using T4 DNA ligase (New England Biolabs, NEB), according to manufacturer's instructions. Ligated vectors were then transformed into competent TOP10 *E.coli* (Invitrogen) and plasmid DNA was extracted using the Wizard *Plus* SV Minipreps DNA Purification System (Promega).

2.2.2 Targeting Construct Design and Assembly

The donor template targeting construct consisted of an antibiotic resistance cassette (blasticidin or puromycin) flanked by 500bp homology arms. Two pUC57 vectors (Eurofins), containing either blasticidin or puromycin antibiotic cassettes, were used as a backbone for the targeting constructs. The homology arms were amplified using *MYH7*^{WT/WT} DNA using the primers listed in **Table 2.1**, using Phusion DNA polymerase (NEB) following the manufacturer's instructions. The PCR conditions were: 98°C for 30s, 98°C for 30s, 64°C for 30s and 72°C for 30s for 35 cycles and 72°C for 10min. Amplified DNA was then run at 80V for 1h in a 1% agarose gel (w/v) using Ethidium Bromide (EtBr, Sigma) as a fluorescent dye, and then visualised in a Fujifilm LAS-4000 System and software (GE Healthcare). PCR products were purified using the QIAQuick Gel Extraction kit (Qiagen). The left homology arm (LHA) underwent SacI/Ascl digestion and the right homology arm (RHA) (KpnI/HindIII digestion).

Table 2.1 - Primers used for the amplification of 500bp homology arms.

IncRNA	PCR Reaction	Forward Primer Name (F) ¹	Reverse Primer Name (R) ²	Primer Sequences 5' → 3'
Target 1	LHA ³	Pos1_KO_Left arm_FWD	Pos1_KO_Left arm_REV	F: TACAGCTGATGGGATCCA R: CCTAATCCAGGAGGGT
	RHA ⁴	Pos1_KO_Right arm_FWD	Pos1_KO_Right arm_REV	F: TTCCGAGAGAGCGCGGCC R: AAAAATAAGCTCATGCA
Target 2	LHA	Pos2_KO_Left arm_FWD	Pos2_KO_Left arm_REV	F: AGTAATGAGCTCAAACTAATGATTAATGAT R: TATCAGGCGCGCCGTAATATGCATGTAA
	RHA	Pos2_KO_Right arm_FWD	Pos2_KO_Right arm_REV	F: TGCTCAGGTACCATTTTAAAAATTTGTAT R: GTGATGAAGCTTAGCACAGTCAGGAGAAAG
Target 3	LHA	Neg1_KO_Left arm_FWD	Neg1_KO_Left arm_REV	F: TAAAAAGAGCTCTGTCTTTATCACACAAT R: GTACCTGGCGCGCCAGCAGAACATATCAGC
	RHA	Neg1_KO_Right arm_FWD	Neg1_KO_Right arm_REV	F: CCTCCCGGTACCCGAGGATTATAGGTGTGA R: AGGATTAAGCTTTTATAAGCAAATCCAAG
MINCR	LHA	Neg4_KO_Left arm_FWD	Neg4_KO_Left arm_REV	F: GCTTCCGGTCTGTTTGGT R: GTGGAAGGGCATGGA
	RHA	Neg4_KO_Right arm_FWD	Neg4_KO_Right arm_REV	F: AAGCGCGTGGGACAGCAG R: TCACAGGGTTATGGGGC

¹Forward ³Left homology arm
²Reverse ⁴Right homology arm

The PCR generated and digested HAs were subsequently cloned into the pUC57 templates in a two-step double digestion cloning strategy. The templates were first digested with SacI/Ascl and purified as above. Afterwards the SacI/Ascl digested LHA was cloned into the pUC57 SacI/Ascl digested vector and assembled as in 2.2.1. Diagnostic digestion was performed with BsaI to confirm correct insertion of the LHA. Following, the templates containing the LHA underwent KpnI/HindIII digestion and the RHA was inserted into the vectors using the same protocol as the LHA insertion. As before, diagnostic digestion was performed using BsaI and completed vector Sanger Sequencing was outsourced to Eurofins Genomics. All enzymes were purchased from NEB and all digestions were performed at 37°C for 1h.

2.2.3 Antibiotic Kill Curve

To determine the concentration of antibiotics for the selection of positive clones, a kill curve was performed. hiPSCs were seeded into MT coated 6 well plates (Nunc) as previously described, at an approximate density of 26,000 cells/cm². Fresh medium was added the day after seeding and selection began the following day. Antibiotics were made up in E8 and delivered to the cells at a range of concentrations daily until no cells remained. Concentrations tested were 0-15µg/mL blasticidin (Invitrogen) and 0-9µg/mL puromycin (Invitrogen). The kill curve determined the optimal concentrations were 3µg/mL for blasticidin and 7.5µg/mL for puromycin.

2.2.4 hiPSC Editing Transfection, Selection and Genotyping

Transfection was performed using the Primary cell 4D Nucleofector X Kit L (Lonza). Three days prior to transfection, hiPSCs were seeded into T25 flasks as previously described. On the day of transfection, cells were dissociated from the flasks and quenched as mentioned in 2.1.1. Post quenching, cells were resuspended at 1 million cells/mL and each 1mL was aliquoted to 1.5mL microcentrifuge tubes (Eppendorf) and centrifuged at 160xg for 4 min. Each transfection condition used 1 million cells. The BGK was aspirated, and the cells were resuspended 100µL P3 buffer. 1µg of each gRNA, Cas9 and donor plasmid was added to the cell/P3 mixture which was then transferred to Amaxa cuvettes. Nucleofection was performed using the Amaxa 4D Nucleofector (Lonza) using program CA137, then

cuvettes were incubated at 37°C for 5 min. Following, 900µL E8 supplemented with 10µM Y- 27632 was added to each cuvette, gently pipette up and down before being added to a fresh MT coated 6 well plate (Nunc). Medium was changed 28 and 48h later.

72h post transfection, cells were re-seeded sparsely at 52 cells/cm² into a MT coated 6 well plate and antibiotic treatment was performed using the concentrations previously determined, until colonies appeared. Colonies were picked using manual dissection once they were approximately 0.5-1mm in diameter. The cells were washed once with PBS and 1mL of 1mM ethylenediaminetetraacetic acid (EDTA (Gibco, 1500054) was added to each well and incubated at 37°C for 4 min. EDTA was aspirated and 3mL E8 supplemented with 10µM Y- 27632 was slowly added to the well. Under a stereomicroscope built into a class II safety cabinet and using a P20 Pipette (Eppendorf), colonies were dissected. Clones were then expanded and cultured into monolayers and genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen), following manufacturers guidelines. Genotyping PCRs were then performed using Phusion polymerase as described using the primer sequences seen in **Table 2.2**. Amplified DNA was loaded in a gel and visualised as already described.

MINCR KO Clone 5 was dissected into one well of a 48-well plate (Nunc). In absence of proliferation, 48h after dissection the clone was dissociated into two wells of a 96-well plate (Nunc). Upon visual suggestions of apoptosis (cell debris and shrinkage) and lack of proliferation, one well was washed with PBS and fixed with 4% paraformaldehyde (PFA) for 15 mins at RT, then again rinsed and left in PBS. The other well was lysed and RNA extracted as described in section 2.5.1.

Table 2.2 - Primers used for genotyping genome edited clones

IncRNA	PCR Reaction	Forward Primer Name (F) ¹	Reverse Primer Name(R) ²	Primer Sequences 5' → 3'	Expected PCR Product
Target 1	LHA ³	G5_IncRNApos1_genotyping_FWD	EF1alpha_REV	F:GTCAGGGAAGTCTAAACCCAGCAAC R:GCGATCTCTGGGTTCTACGTTAGTG	792bp
	RHA ⁴	PolyA_FWD2	G3_IncRNApos1_genotyping_REV	F: ACTGCATTCTAGTTGTGGTTTGTCC R:CTGTTTTCAGTTTGCTCTTCTATAG	606bp
	Whole	G5_IncRNApos1_genotyping_FWD	G3_IncRNApos1_genotyping_REV	F:GTCAGGGAAGTCTAAACCCAGCAAC R:CTGTTTTCAGTTTGCTCTTCTATAG	⁵ Blast:3.4kb ⁶ Puro:3.6kb
Target 2	LHA	G5_IncRNApos2_genotyping_FWD	EF1alpha_REV	F:ATTTCTGCTTATTAGAGACTTTTAG R:GCGATCTCTGGGTTCTACGTTAGTG	735bp
	RHA	PolyA_FWD2	G3_IncRNApos2_genotyping_REV	F:ACTGCATTCTAGTTGTGGTTTGTCC R:AGCACATCAGTCTTTGTCATATTAG	981bp
	Whole	G5_IncRNApos2_genotyping_FWD	G3_IncRNApos2_genotyping_REV	F:ATTTCTGCTTATTAGAGACTTTTAG R:AGCACATCAGTCTTTGTCATATTAG	Blast:3.7kb Puro:3.9kb
Target 3	LHA	G5_IncRNAneg1_genotyping_FWD	EF1alpha_REV	F:GTATCCAATCAAGCTAACTATGGTG R:GCGATCTCTGGGTTCTACGTTAGTG	837bp
	RHA	PolyA_FWD2	G3_IncRNA neg1_genotyping_REV	F:ACTGCATTCTAGTTGTGGTTTGTCC R:AGCAAGTAAACACCAACAATGTTG	704bp
	Whole	G5_IncRNA neg1_genotyping_FWD	G3_IncRNA neg1_genotyping_REV	F:GTATCCAATCAAGCTAACTATGGTG R:AGCAAGTAAACACCAACAATGTTG	Blast:3.6kb Puro:3.8kb
MINCR	LHA	G5_IncRNAneg4_genotyping_FWD	EF1alpha_REV	F:ACCATTATCTTATGAATCCGCTCG R:GCGATCTCTGGGTTCTACGTTAGTG	843bp
	RHA	PolyA_FWD2	G3_IncRNA neg4_genotyping_REV	F:ACTGCATTCTAGTTGTGGTTTGTCC R:TTGGAATTTCAAGCCCAACTCTACA	727bp
	Whole	G5_IncRNA neg4_genotyping_FWD	G3_IncRNA neg4_genotyping_REV	F:ACCATTATCTTATGAATCCGCTCG R:TTGGAATTTCAAGCCCAACTCTACA	Blast:3.5kb Puro:3.7kb

¹Forward ³Left homology arm ⁵Blasticidin
²Reverse ⁴Right homology arm ⁶Puromycin

2.3 Knock Down Silencing of *MINCR*

2.3.1 siRNA Design and Transfection

siRNAs for the knockdown of *MINCR* were purchased commercially (Thermofisher) which targeted exons 1 and 3 (**Table 2.3**). siRNAs were delivered to hiPSC-CMs using the Viafect transfection protocol described in **2.4.2**. Delivery was performed individually and in pairs; siRNA1+ siRNA3, siRNA1+ siRNA4, siRNA2+ siRNA3 and siRNA2+ siRNA4 to determine the optimal combination for *MINCR* knockdown.

Table 2.3 - siRNA target location and sequence for *MINCR* knock down

siRNA Name	siRNA ThermoFisher ID	Target Exon	siRNA Location (bp)	Sequence 5'→3'
siRNA 1	n542660	1	124	F:GGUUCCCAACUUCGCAGAAtt
				R:gCCAAGGGUUGAAGCGUCUU
siRNA 2	n551693	1	162	F:CUAGAGACCUCAGACCGCAtt
				R:gGAUCUCUGGAGUCUGGCGU
siRNA 3	n489973	3	565	F:CAGAGACAUUAAUACUCAtt
				R:aaGUCUCUGUAAUUAUUGAGU
siRNA 4	n489974	3	650	F:CCAUGCUACUUAUGUAUtt
				R:gtGGUACGAUGUAUAUCAUA

2.4 lncRNA Overexpression

2.4.1 Construct Design and Assembly

lncRNA target overexpression constructs were built using the vector AAVS1-GECO plasmid containing a CAG promoter as a backbone. lncRNA target sequences were amplified from *MYH7^{WT/WT}* DNA (*MYH7^{WT/WT}* cDNA for target 3) with Phusion DNA Polymerase (NEB) using the primers listed in **Table 2.4**. The PCR conditions were: 98°C for 30s, 98°C for 30s, 64°C for 30-60s and 72°C for 30s for 35 cycles and 72°C for 10min. Amplified DNA was then run at 80V for 1h in a 1% agarose gel (w/v) using Ethidium Bromide (EtBr, Sigma) as a fluorescent tag, and subsequently visualised in a Fujifilm LAS-4000 System and software (GE Healthcare). PCR products were purified using the QIAQuick Gel Extraction kit (Qiagen). Sequencing of the amplified DNA was outsourced to Eurofins Genomics using primers in **Table 2.5**. Enzymatic sites had been included in the amplification primers, therefore the lncRNA DNA was digested with BsrGI and ClaI, run in a gel, visualised and purified as above.

The GECO protein was excised from the backbone plasmid by enzymatic digestion of BsrGI and ClaI. The digested lncRNA fragments were cloned into the vector using T4 ligase (NEB) according to manufacturer's guidelines. Ligated vectors were then transformed into competent TOP10 *E.coli* (Invitrogen) and plasmid DNA was extracted using the Wizard *Plus* SV Minipreps DNA Purification System (Promega).

To remove the IRES-Puro nucleotide sequence from the now CAG-lncRNA vector, it was enzymatically digested with ClaI and PstI. This resulted in a partial deletion of the PolyA tail. To replace the lost PolyA nucleotides, complementary DNA oligonucleotides replacing the missing sequence (**Table 2.6**) were annealed and cloned into the ClaI/PstI digested CAG-lncRNA vector using T4 ligase, transformed and extracted as above. Completed vectors were outsourced to Eurofins Genomics for sequencing using the same primers.

Table 2.4 - Primers for the amplification of lncRNA targets

lncRNA	Product Size	Forward Primer Name (F)	Reverse Primer Name (R)	Primer Sequences 5' → 3'
Target 1	5.6kb	Pos1_primer_FWD	Pos1_primer_REV	F: GTTCTCTGTACAGCAGTGGTCTGAAATTGA R: AAATAAATCGATTCTTAGTGAAGTAACTTT
Target 2	659bp	Pos2_primer_FWD	Pos2_primer_REV	F: GTGGCTTGACACCATACAACAATGTTTC R: ACGCAAATCGATTCTATCATCAATGTTAT
Target 3	645bp (cDNA)	lncRNAeg1_OE_cDNA_FWD 2	lncRNAeg1_OE_cDNA_REV 2	F: CATCCTCATGTACATCATGTAGCGCCAGCCA R: ACCATTATCGATTATTAACGGAGTAATGTTG
MINCR	1.5kb	Neg4_primer_FWD	Neg4_primer_REV	F: TCCGGTTGTACACTCAGGCTTCCGGTCTGT R: AAGTTTATCGATTCTTCAGTTCACAAGCGT

Table 2.5 - Primers used for sequencing of lncRNA PCR amplified products and the completed CAG-lncRNA overexpression vectors

Target	PCR Reaction	Primer Name	Primer Sequence 5' → 3'
Sequence OE Vectors	Sequence lncRNA from CAG promoter	CAG Sequencing FWD	GGCTCTAGAGCCTCTGCTAACC
	Sequence from PolyA to check correct linker DNA insertion	PolyA Sequencing REV	GGACAAACCACAACCTAGAAATGC

Table 2.6 - Linker DNA oligonucleotide sequences of the PolyA tail

Name	Forward Oligo Name (F)	Reverse Oligo Name (R)	Oligo Sequences 5' → 3'
Linker DNA	Poly A Linker FWD	Poly A Linker REV	F: CGATAACTTGTATTATGACGCTTA R: ATTCGACGTTATTGTTCATA

2.4.2 Viafect™ Transfection

Dissociated hiPSC-CMs were plated according to the assay to be completed in VN coated 96-well vessels. On the morning of 72h post dissociation, medium was changed to RPMI B27+INS supplemented with 10% foetal bovine serum (FBS) (Gibco, A4766). Transfection was completed using the Viafect™ Transfection Reagent (Promega) according to manufacturer's guidelines. In brief, Viafect and CAG-lncRNA DNA were complexed at a ratio of 6:1 Viafect:DNA at room temperature (RT) for 20min, after which 10µL of complex was added to each well and incubated at 37°C and 5% CO₂ for 48h. GFP was transfected as a control to measure transfection efficiency (TE). Media was then changed to RPMI B27+INS and 72h post transfection, TE was estimated by imaging GFP transfected hiPSC-CM with the Operetta high content imaging system (Perkin Elmer). Refer to Chapter 4 for in depth method.

2.5 Molecular Biology

2.5.1 RNA Extraction and cDNA Synthesis

Total RNA was extracted from cell pellets using the Zymo Direct-zol RNA Microprep kit (Zymo Research). For samples to be run by real-time qPCR, RNA was reverse transcribed using Superscript III Reverse Transcriptase kit (Invitrogen) according to manufacturer's guidelines.

RNA from *MINCR* KO Clone 5 pellet was extracted using the Nucleospin RNA XS Microkit for RNA purification (Machery Nagel) and reverse transcribed using Superscript IV (Invitrogen) using the manufacturers protocol.

2.5.2 Real Time qPCR

Real time qPCR was performed using the TaqMan Gene Expression Assay (Applied Biosystems). TaqMan master mix (#4369016) along with probes of interest (*TNNT2*- Hs00165960_m1, *MYH7*- Hs01110632_m1, *MYH6*- Hs01101425_m1, *MINCR*- Hs004406557_g1, *LOC339975*- Hs03657543_m1, *LOC101927151*- Hs04404968_m1, *HIF1α*-AS2- Hs04275422_s1 or *GADPH*- 4333764f) were added to a MicroAmp fast 96 well plate (#4346907). cDNA prepared from 250ng of RNA was added to the plate which was then sealed with a plastic cover (Applied Biosystems). Amplification was performed by the ABI 7500 fast Real Time qPCR

machine (Applied Biosystems, #4311971). Expression data was normalised to the cardiac gene *TNNT2* and relative quantification was calculated using the average of the untransfected hiPSC-CMs of each line.

2.5.3 Immunocytochemistry (ICC) and Imaging

Immunostaining was performed on hiPSC-CM or hiPSC previously fixed by first washing with PBS then using 4% paraformaldehyde (PFA) for 15 min at RT in 96 well plates. Cells were washed with twice with PBS then permeabilised with 0.1% Triton-X (Sigma) in PBS for 15 minutes and washed three times with PBS. Blocking solution made from 4% Goat Serum (Sigma) in PBS was added to cells for 1h at RT, after which cells were incubated with primary antibodies diluted in blocking solution (**Table 2.7**) and left overnight at 4 °C. The following day, cells were washed three times with 0.1% Tween-20 (Sigma) in PBS and secondary antibodies added for 1 hour at RT. The cells were washed three times, 5 mins per wash with 0.1% Tween-20 and then incubated with DAPI (1:500) (Sigma, D9542) for 10 mins at RT. Following this, they were washed and stored in PBS at 4 °C. Image acquisition was performed using the Operetta high content imaging system (Perkin Elmer) and analysed using the Harmony high content imaging software.

Table 2.7 - Antibodies used for immunostaining

Primary Antibody	Dilution	Secondary Antibody	Dilution
α -actinin (Sigma, A7811)	1:800	Alexa Fluor 488	1:400
Cardiac Troponin T (Abcam, 45932)	1:500	Alexa Fluor 647	1:400
ProBNP4 (Abcam, 13115)	1:500	Alexa Fluor 647	1:400
β -MHC (Sigma, M8421)	1:400	Alexa Fluor 488	1:400
α -MHC (BioGenes, custom product)	1:200	Alexa Fluor 647	1:400
OCT4 (Millipore, MAB4419)	1:300	Alexa Fluor 488	1:400
Caspase 3 (Abcam, 32042)	1:250	Alexa Fluor 647	1:400

2.6 Phenotyping lncRNA Modulated hiPSC and hiPSC-CMs

2.6.1 BNP Assay

BNP assay was performed by seeding dissociated hiPSC-CMs at 1093 cells/mm² in VN coated 96 well plates (CellCarrier, Perkin Elmer). Cells were transfected as previously described in **2.4.2**. 72h post transfection, cells were treated with 10nM Endothelin-1 (ET1 (Sigma, E7764)) or 100nM Bosentan (BOS (Sigma, SML1265))

for 15h, whereby 1µg/mL Brefeldin A (BFA (Sigma, B7651) was added and incubated for 3h at 37°C and 5% CO₂. Cells were then fixed and immunostained as described in **2.5.3**, using cardiac troponin T (cTnT) and ProBNP4 as primary antibodies.

2.6.2 Seahorse Analysis of Mitochondrial Respiration

hiPSC-CMs were dissociated as per section **2.1.3** and seeded into VN coated XF96 plates (Agilent), at densities 3301, 3773 and 4716 cells/mm². Cells were transfected as previously described in **2.4.2**. Three days after transfection, medium was changed to XF Base Media (Agilent Technologies #102353) supplemented with 10mM glucose (Sigma #G7528), 2mM L-glutamine (Life Technologies #25030-081) and 1mM sodium pyruvate (Sigma #S8636) approximately 1h prior to the assay. Inhibitors were injected in the following sequence during the assay; 1.5µM oligomycin (Sigma, 75351-5MG) , 0.4µM FCCP (Sigma, C2920-10MG) and 1µM rotenone (Sigma, R8875-1G). The cells were fixed as previously mentioned. OCR Values were normalised to the number of cells in each well, calculated by 1:500 DAPI in PBS staining and imaging using the Operetta high content imaging system (Perkin Elmer).

2.6.3 α-MHC and β-MHC Isoform Switch

The MHC isoform switch assay was performed by seeding dissociated hiPSC-CMs at 1090 cells/mm² in VN coated 96 well plates (CellCarrier, Perkin Elmer). Cells were transfected as previously described in **2.4.2**. 72h post transfection, cells were fixed and immunostained as previously described (**2.5.3**) using α-MHC and β-MHC as primary antibodies.

2.6.4 Caspase 3 Staining *MINCR* Clone 5

MYH7^{MUT/MUT} clone 5 hiPSCs were split between two wells of a 96 well plate (split 1:1 from the 48 well). At the same time, healthy *MYH7*^{MUT/MUT} hiPSCs were seeded into several wells of a 96 well plate at low density, to roughly recapitulate the density of clone 5 cells (clone 5 was not counted to prevent further loss of cell death and loss). Negative control wells were treated with normal hiPSC culture conditions while the positive control wells were treated with 4µg/mL camptothecin in E8 medium for 24h. Clone 5 cells were fixed 24h post seeding, while the

negative and positive controls were fixed at the end of the 24h camptothecin treatment. The cells were immunostained using methods described above, Oct4, Caspase 3 and DAPI were primary antibodies. The stained cells were imaged using high content imaging.

2.7 Statistical Analysis

All values in figures and text are standard error of mean (SEM of at least two independent experiments, unless stated otherwise in the figure legend. Statistical analysis was performed using GraphPad Prism Software (V9). Differences were considered significant when * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ and **** $p < 0.0001$.

3. Generation of lncRNA expression modulation systems

3.1 Introduction

As discussed in Chapter 1.2 and 1.3, HCM is a complex disease associated with mutations in genes encoding sarcomeric proteins. The vast genetic heterogeneity and variable penetrance presents a challenge in understanding HCM and the development of treatments. For improved comprehension of HCM progression and genotype – phenotype relationships, an HCM model was previously generated using CRISPR/Cas9 by Dr Diogo Mosqueira, by introducing a c.C9123T-MYH7 (p.R453C- β MHC) substitution. This gave rise to three variants; *MYH7*^{WT/WT}, *MYH7*^{WT/MUT} and *MYH7*^{MUT/MUT} [7].

Since the development of this model, progress has been made in elucidating the phenotypes associated with the genetic background of HCM. However, there is still no consistent association between the HCM genotype and phenotype in human patients. Furthermore, with advances in NGS, it has been demonstrated that the genetic basis of HCM is more complicated than previously thought. Current known mutations only account for half of cases, while the remaining cause of the cases are unknown [209]. Thus, there is a need for further investigations into other causes that would explain those cases with unknown causes.

One such avenue to consider is that of lncRNA involvement. The question of their functionality has not been fully answered, but studies have linked around 20 lncRNAs to cardiac disorders, including HCM [1]. To obtain new insights into HCM, global transcriptomic analysis of hiPSC-CM cultured in 2D and 3D formats derived from the *MYH7* variants was performed [7]. After cross comparison of the results to available data from literature on HCM and non-HCM hearts, previously undefined lncRNAs emerged as top hits. The 15 lncRNA candidates of interest were narrowed down to four through validation. The expression, whether an increase or decrease, of the lncRNAs in the *MYH7* lines was validated using qPCR. For targets that consisted of multiple isoforms, provisional work was completed using RT-qPCR to determine which isoforms are expressed in the *MYH7* variant

lines. Of the four validated targets, it was found that two were upregulated and two were downregulated in the *MYH7* variant lines, leading to a theory that they may have roles in HCM disease progression or prevention.

The aim was to modulate the expression of the validated target lncRNAs in the *MYH7*^{WT/WT}, *MYH7*^{WT/MUT} and *MYH7*^{MUT/MUT} lines, through either overexpression or knockout (KO)/down (KD) and analyse the phenotypic outcomes to determine whether the lncRNAs have an impact on HCM disease progression or prevention. A successful discovery could pave the way for improved pharmacological targets and biomarkers for improved treatment and identification of HCM.

3.1.1 CRISPR/Cas9 Gene Targeting of lncRNA in hiPSC

The development of gene editing has revolutionised research into single gene contributions to disease. Of the three major technologies, ZNF, TALENs and CRISPR/Cas9, the latter was selected for this project due to the ease of assembly and high efficiency. To investigate the effect of loss of function of the lncRNAs in the *MYH7* HCM model, a lncRNA KO strategy was devised whereby the lncRNA deletion would be repaired via the HDR pathway to integrate an antibiotic cassette which would effectively disrupt the gene. This approach was used due to a concern that some targets, specifically *MINCR*, have multiple isoforms that arise from alternative splicing. By inserting a large cassette into the *MINCR* gene, transcription will be disrupted, resulting in a lack of alternative splicing and no gene expression.

3.1.2 Knock Down of *MINCR*

An alternative method of reducing the expression of lncRNA targets in the *MYH7* HCM model was by knockdown utilising four predesigned siRNAs targeting two of three exons. The siRNAs were delivered singularly and in pairs using the same transfection method previously optimised for use in the overexpression of lncRNAs in hiPSC cardiomyocytes, which is discussed in Chapter 2.4.2.

3.1.3 Overexpression Targeting in hiPSC Cardiomyocytes

Gene overexpression is a powerful and straight forward process to study the biological function of the target gene products. In this project, overexpressing the target lncRNAs in hiPSC-CMs was performed by modifying a targeting cassette containing a strong promoter (CAG) to overexpress the lncRNAs of interest. By delivering the target vectors to the hiPSC-CMs with a specialised transfection protocol, it was possible to transfect with very high efficiencies.

3.1.4 Chapter Aims and Objectives

The overarching aim of this chapter was to create the tools needed to modulate the expression of lncRNAs to assess their effects on the phenotypes of the *MYH7* HCM model. A schematic overview of CRISPR/Cas9 mediated genome editing is shown in **Figure 3.1**, while an overview of the overexpression strategy is shown in **Figure 3.2**. Knockdown of lncRNA was implemented as a reserve measure.

Progress was made on all four targets in both KO and overexpression constructs. KO constructs were completed for targets 1, 3 and 4 and overexpression constructs were completed for targets 1, 2 and 4. After the generation of all constructs for targets 1, 2 and 3, no further investigation was performed due to time constraints. Target 4, from here on known as *MINCR*, was selected for full characterisation as it was the most likely target to yield interesting data due to having greater annotations.

Due to the impact of the COVID-19 pandemic on time, the number of targets to complete the modulation pipeline had to be narrowed down to one. Although this approach carries greater risk, it allowed a broader range of phenotyping assays to be completed. These decisions were reached through discussion with my supervisor, while rationalisation of projects was an expectation of the university to gain access to funds to extend the PhD.

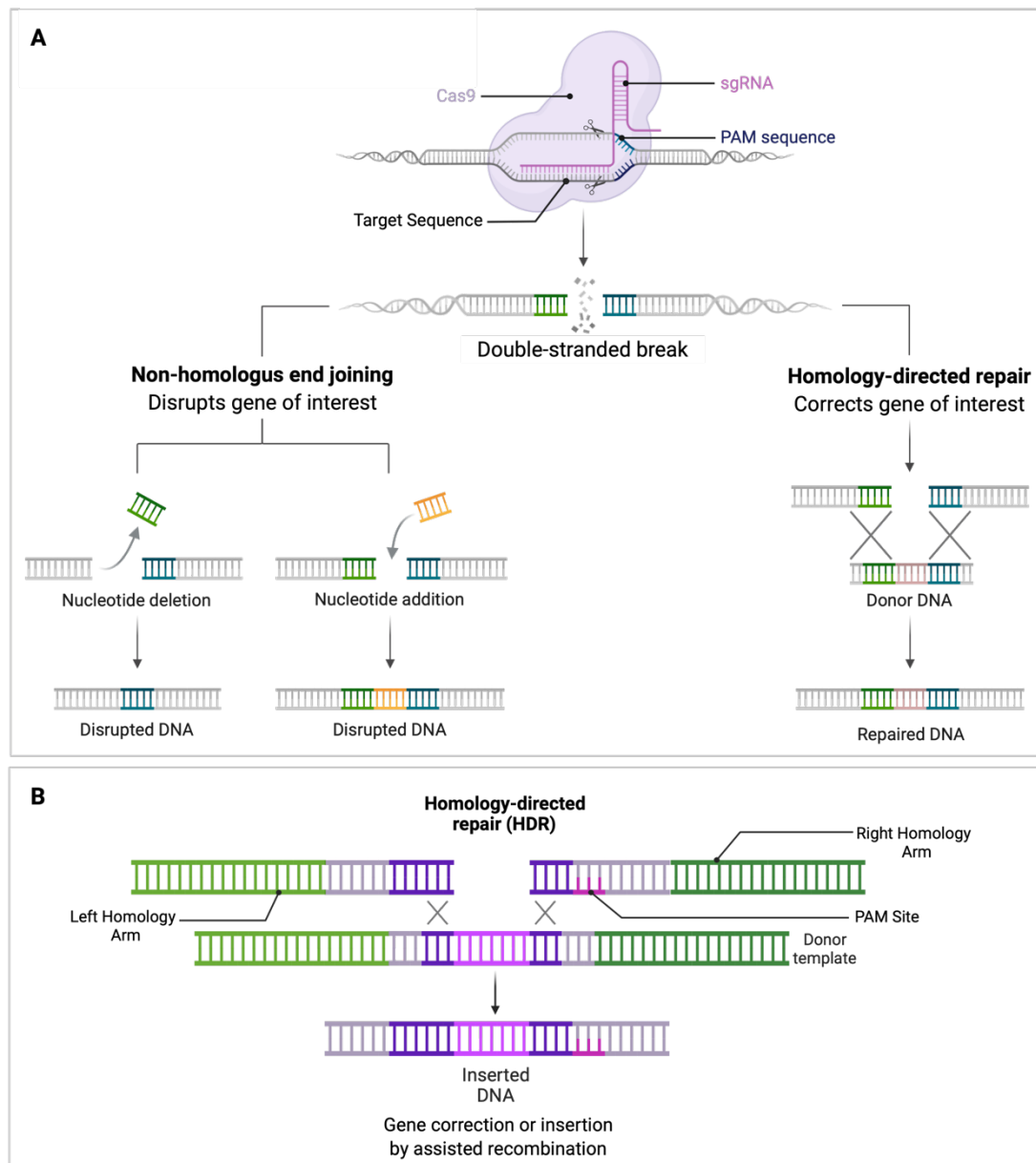


Figure 3.1 – CRISPR/Cas9 gene editing. **A)** DSB are induced via a complex of gRNA and Cas9 nuclease which binds upstream of the PAM site. DSB can be repaired in one of two ways; via the NHEJ using endogenous DNA repair machinery resulting in indels, or via the HDR pathway. **B)** DSB repair via HDR pathway uses an exogenous repair template ie. donor template, to introduce precise modifications at the target locus. Graphic adapted from “CRISPR/Cas9 Gene Editing”, using BioRender.com 2021.

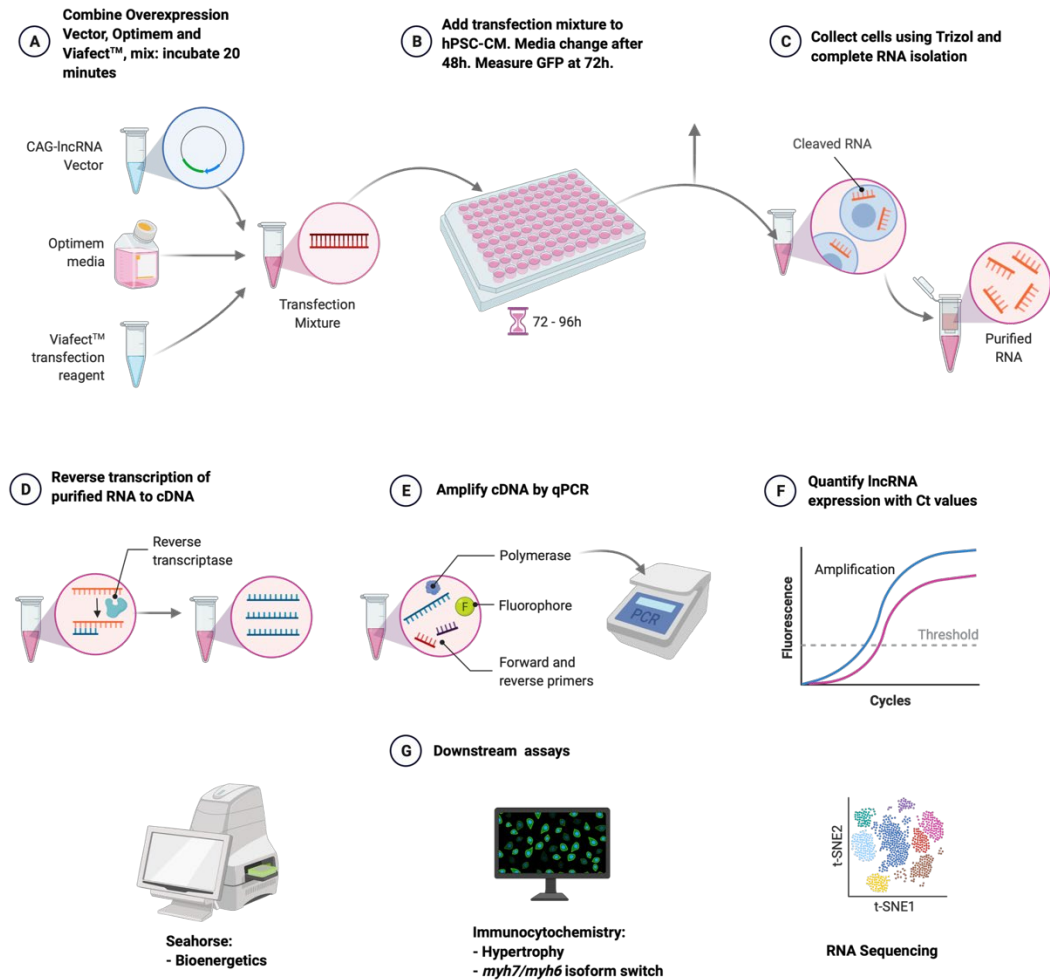


Figure 3.2 – Overview of overexpression of lncRNA strategy. To assess the effect of overexpressing lncRNAs in hiPSC-CMs, **A)** the transfection mixture containing Viafect™, lncRNA/CAG plasmid and medium was added to the cells. **B)** GFP was transfected as a measure of efficiency - fluorescence measured after 72h. **C)** 96h after transfection samples were collected using Trizol and RNA isolation was performed and **D)** reverse transcribed to cDNA then **E,F)** lncRNA expression was quantified using qPCR. **G)** Downstream phenotyping assays were performed to assess the phenotypic results of lncRNA overexpression. Created using Biorender.com 2022.

3.2 CRISPR Knockout Results

3.2.1 Design Strategy

In pursuance of understanding the phenotypic effects of lncRNA KO on HCM phenotypes, a CRISPR/Cas9 strategy was designed (**Figure 3.3**). The approach included deleting a portion of the lncRNA sequence using a dual gRNA procedure and inserting an antibiotic cassette resistance using the HDR pathway. This would ensure complete disruption to the gene. The strategy was the same for each target, but from hereon, only *MINCR* will be discussed.

The lncRNA *MINCR* has seven transcripts (**Figure 3.3A**), therefore PAM sites were selected to ensure that most transcripts would be targeted. The chosen gRNAs targeted all but one transcript with a deletion of 306bp (**Figure 3.3B**). Transfection was performed with four vectors; i) Cas9, ii) gRNA1 & iii) gRNA2 composed of annealed oligonucleotide sequences cloned into pU6 vectors, and iv) donor template containing 500bp homology arms flanking antibiotic resistance cassettes blastocycin or puromycin, cloned into a pUC57 vector (**Figure 3.3C**). The insertion of these 2.1kb (blastocidin) and 2.3kb (puromycin) cassettes via HDR ensured complete disruption to the transcripts and enabled antibiotic selection of positive clones.

Transfection was mediated by electroporation, after which hiPSCs were selected using antibiotic resistance of puromycin or blastocidin, picked manually and expanded then genotyped using PCR. PCR positive clones would then be sequenced and karyotyped for confirmation (**Figure 3.3D**).

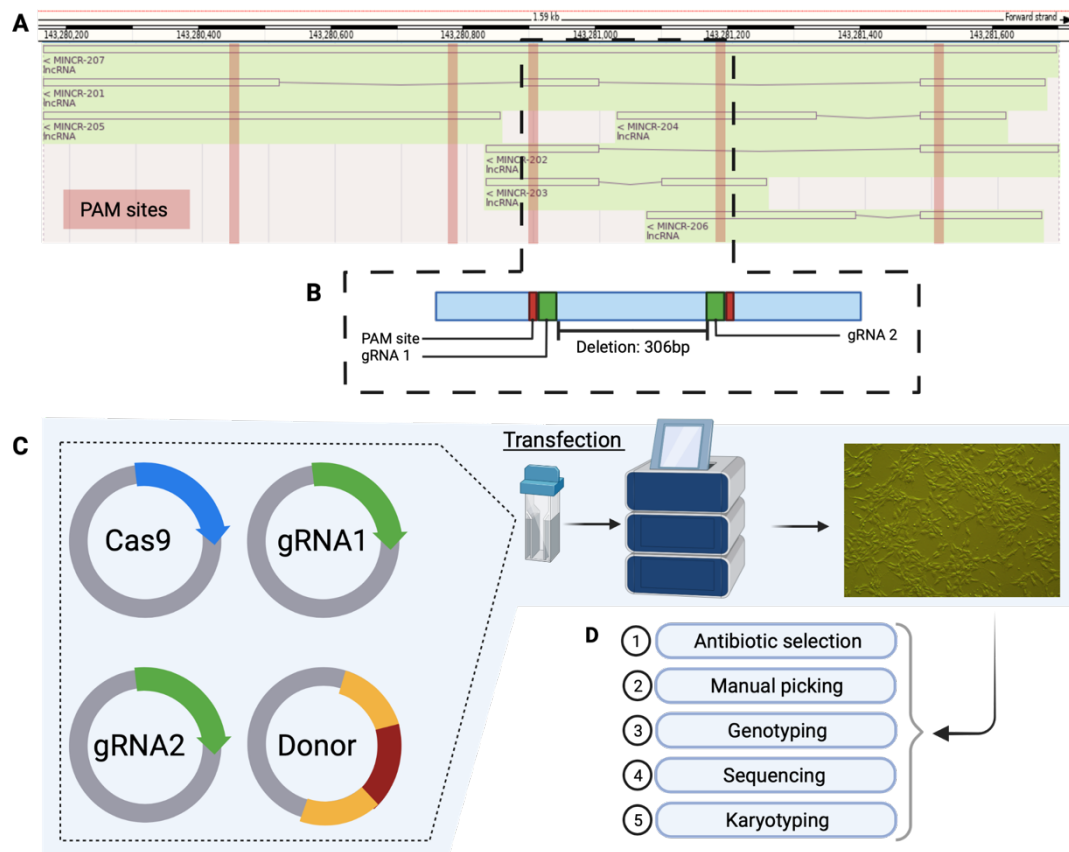


Figure 3.3 – Overview of CRISPR/Cas9 system for *MINCR* knock out. **A)** Structure of lncRNA *MINCR* which has seven transcripts due to alternative splicing. Using a CRISPR design tool, five potential PAM sites were identified (highlighted in red) with minimal off target effects. **B)** To ensure complete disruption of the gene, two PAM sites were selected covering most of the transcripts, between which would be a deleted. **C)** The strategy was to transfect the cells with vectors for Cas9, gRNA and a donor template to facilitate HDR and insertion of a cassette in place of the deleted segment. **D)** Transfected cells would then undergo antibiotic selection, colony picking, genotyping, sequencing, and karyotyping.

3.2.2 Guide Design

The two gRNA vectors used in this thesis were intended to create two DSB in the *MINCR* gene, which would target six out of seven transcripts. It was not possible to target all transcripts due to the location of the PAM sites. **Figure 3.4** provides an overview of the gRNA synthesis process. gRNA sequence oligonucleotides provided by the MIT CRISPR tool were ordered with BsaI overhangs once annealed to allow ligation into the BsaI digested pU6 vector (**Figure 3.4A,B**). Diagnostic digestions using EcoRI were performed, success is determined as the unique site is removed upon successful cloning of the gRNA oligos (**Figure 3.4C**). gRNA plasmids were confirmed by Sanger sequencing (**Figure 3.4D**).

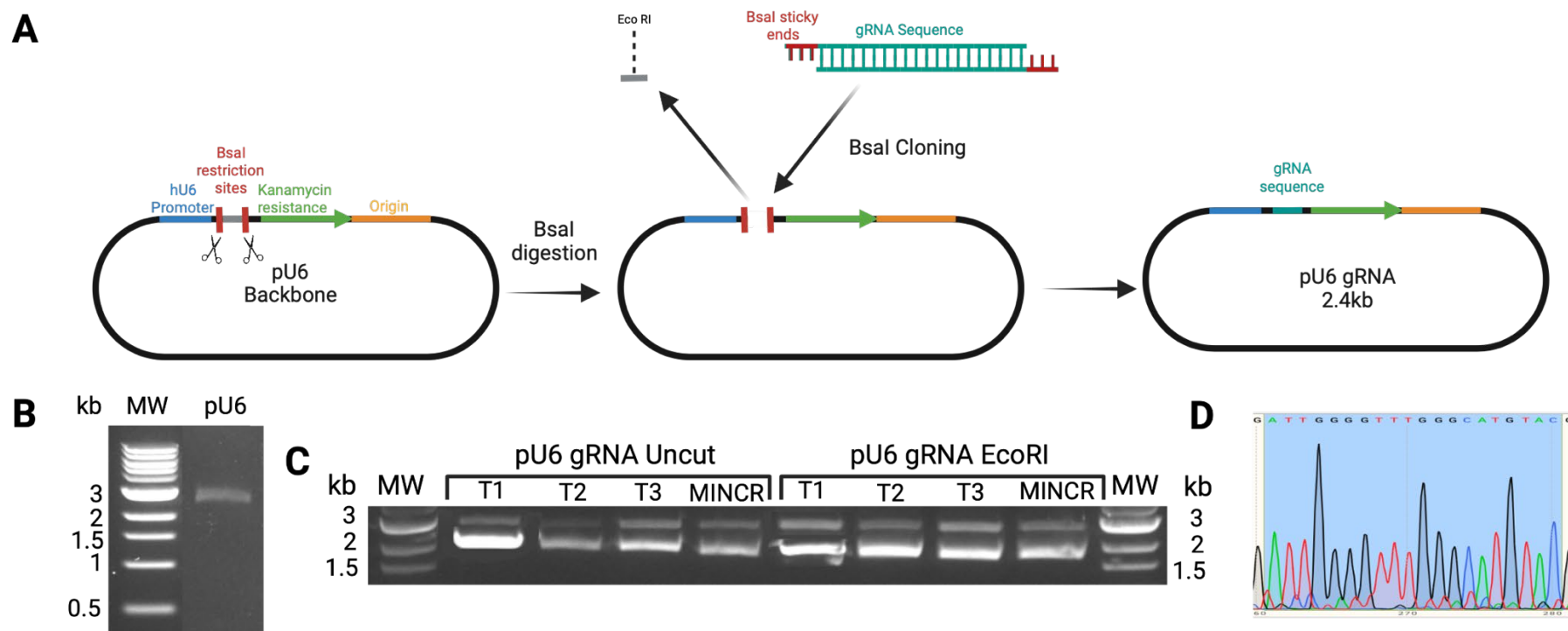


Figure 3.4 – CRISPR/Cas9 gRNA synthesis. **A)** gRNA oligonucleotide DNA was cloned into a pU6 backbone vector using BsaI restriction site ligation. **B)** pU6 was digested with BsaI and purified before the gRNA sequence was ligated in. **C)** gRNA plasmids were then confirmed by diagnostic enzymatic digestion using EcoRI. There should be no change between uncut and cut, as if successful the EcoRI site is removed by BsaI digestion. **D)** Further confirmation was obtained by Sanger sequencing.

3.2.3 Target Vector Construction

An overview of the target vector synthesis is shown in **Figure 3.5**. In short, the process to create the vectors consisted of the following steps: i) digest templates pUC57 blasticidin and pUC57 puromycin with *SacI* and *Ascl*, ii) insert left homology arm (LHA), iii) digest templates containing LHA with *KpnI* and *HindIII*, iv) insert right homology arm (RHA), v) confirm sequence with diagnostic digestion and Sanger sequencing. From hereon the template sequences will be referred to as pUC57 blast and puro.

The templates for the targeting vectors were two pUC57 plasmids, with either puromycin antibiotic resistance or blasticidin antibiotic resistance cassettes. Each lncRNA target had a targeting vector of each antibiotic cassette, in total 8 vectors, and 6 were completed. The use of two vectors with different antibiotic cassettes allowed a more comprehensive transfection and selection strategy.

Homology arms were designed flanking 500bp upstream and 500bp downstream of the PAM sites, providing recognition sites to the target locus and the facilitation of the introduction of the antibiotic cassettes via HDR (**Figure 3.5A**). These homology arms were amplified by PCR using primers designed to have enzymatic overhangs complimentary to the overhangs in the backbone pUC57 vectors post enzymatic digestion (**Figure 3.5B,C**). The targeting vectors were produced in a two-step cloning strategy; the enzymes used were constant between pUC57 blast and puro. Initially, the templates were digested with *SacI* and *Ascl* (**Figure 3.5D**), and the LHA was cloned in by restriction site ligation. Correct insertion of the homology arm was reviewed by diagnostic digestion with *BsaI* and Sanger sequencing. Subsequently, pUC57 blast and puro containing the LHA were digested with *KpnI* and *HindIII* and the RHA inserted using restriction site ligation and cloning (**Figure 3.5E**). Completed vectors were linearised using *BsaI* digestion (**Figure 3.5F**) and confirmation of the correct integration of the homology arms was concluded by Sanger sequencing.

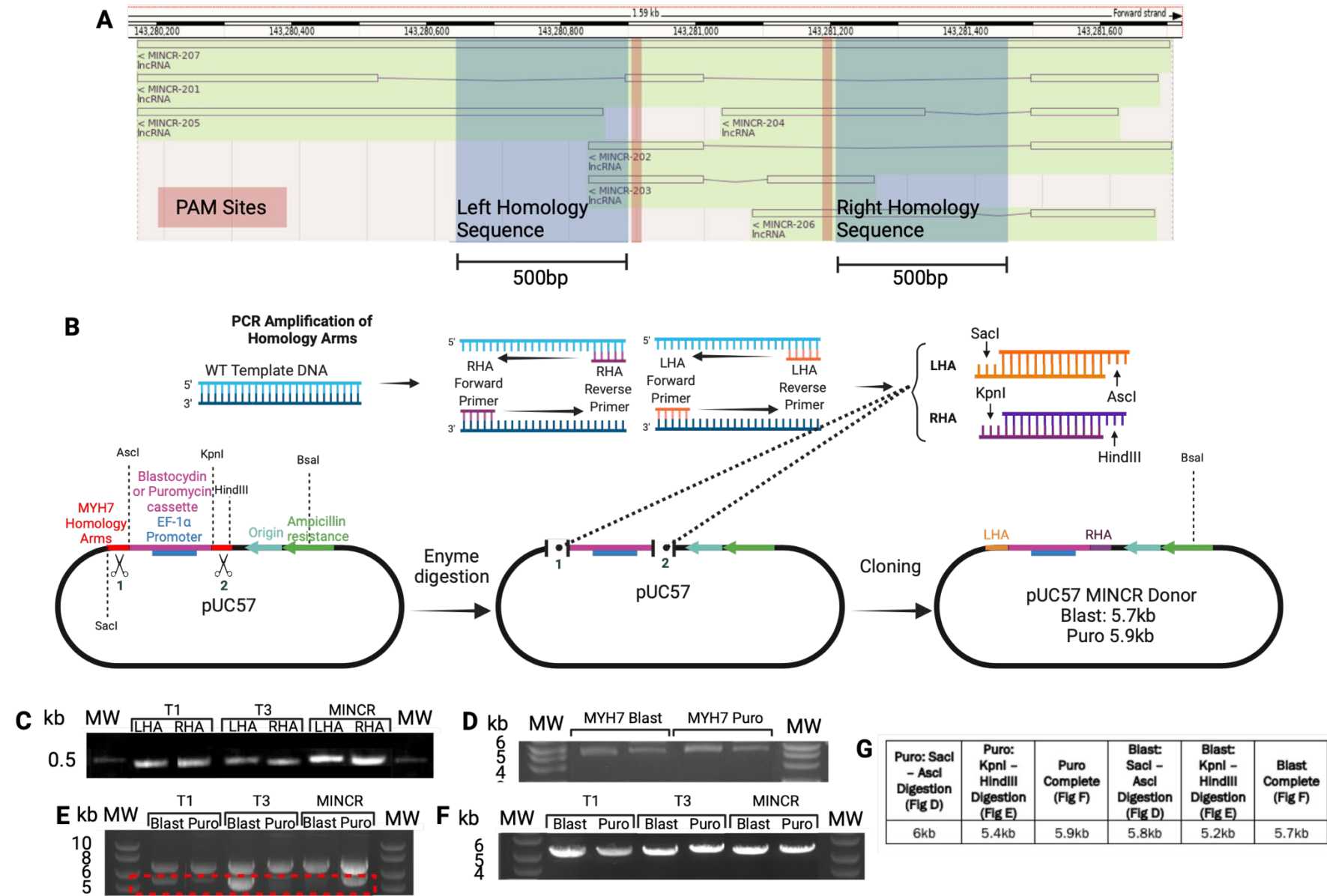


Figure 3.5 – CRISPR/Cas9 donor template synthesis. **A)** Homology arms were designed flanking the PAM sites by 500bp. **B)** An overview of the cloning strategy. The 500bp homology arms were amplified using WT template DNA and primers designed to introduce enzymatic sticky ends once digested with the respective enzyme **(C)**. The pUC57 vector with either a blastocycin or puromycin resistance cassette was used as a template, with a two-step digestion and insertion of the homology arms. Firstly, pUC57 blast and puro were digested with *SacI* and *Ascl* resulting in 5.8kb and 6kb products respectively. **(D)**. The LHA was then cloned in via restriction site ligation. Following this, the pUC57 Blast and Puro plasmids were digested with *KpnI* and *HindIII*, producing 5.2kb and 5.4kb bands **(E)**. The RHA was cloned in using restriction site ligation. The final donor plasmids were diagnostically digested with *BsaI*, for final products of 5.7kb pUC57 Blast and 5.9kb pUC57 Puro and sent for Sanger sequencing **(F)**. **G)** Table showing expected band sizes at each stage.

3.2.4 hiPSC Transfection and selection

Upon the completion of all vectors and gRNAs to target *MINCR*, transfection was performed using the Lonza nucleofection kit. The four vectors required for targeting were complexed and delivered to the cells by nucleofection (**Figure 3.6A**). Post nucleofection, cells were selected using antibiotic resistance using 3µg/mL blastocidin or 7.5µg/mL puromycin treatment, the concentrations had previously been determined by an antibiotic kill curve (Chapter 2.2.3). Once colonies had formed, they were isolated using EDTA dissociation and manually picked under a microscope using a pipette (described in detail in Chapter 2.2.4). Once picked, the clones were expanded into monolayer format through successive splitting from 48 well plates through to T25 format (**Figure 3.6B**).

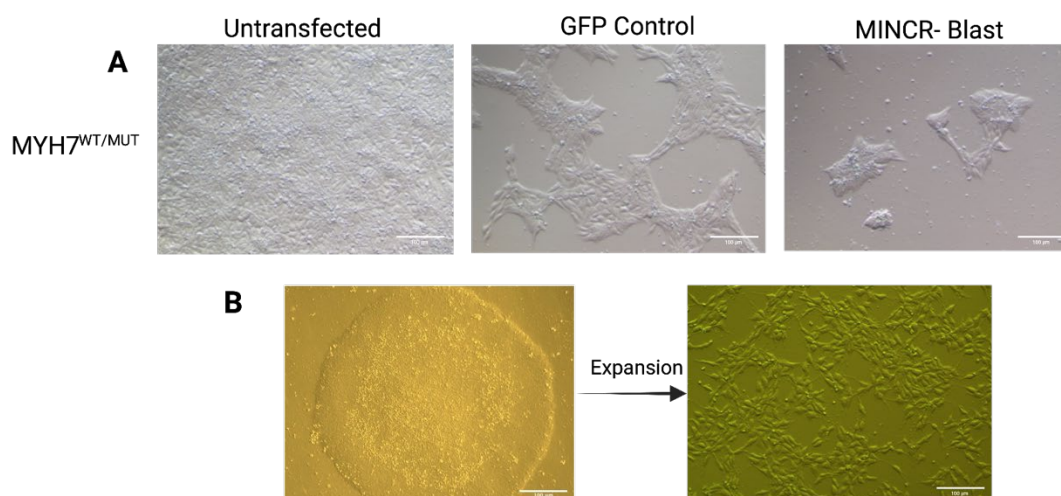


Figure 3.6 – hiPSC Transfection and Selection. **A)** Transfection was performed using the Lonza nucleofection protocol, brightfield images taken 24h post nucleofection in the *MYH7*^{WT/MUT} line. **B)**

Following antibiotic selection, clones were picked then expanded and genotyped. Scale bars=100µm.

3.2.5 Genotypic characterisation

To determine whether targeted clones contained the intended deletion and insertion of the homologous arms and antibiotic cassette, genotyping was performed using three PCR reactions (**Figure 3.7**).

PCR reaction 1 and 2 were designed to amplify the homology arms and would only be successful if the clone was successfully edited. The forward primer of the LHA reaction and reverse primer of the RHA were 150bp up/downstream from the target sequence. This ensured that any indels near the gRNA target site would not impact on primer recognition and that amplification would occur only if the clone was positive, as the remaining primer for each reaction was within the antibiotic cassette.

The third PCR reaction amplified the whole insertion cassette. Using the forward and reverse primers from reactions 1 and 2 respectively, it would be possible to distinguish between a positive and negative clone based on the resulting band size. In the case of *MINCR*, if the clone was positive, a band of 3.5kb or 3.7kb, depending on whether the donor template contained blast or puro, would be amplified. A negative clone would amplify the target region without the insertion and the band would be 1.6kb.

Of all the clones that were expanded into monolayers and genotyped, PCR reactions 1 and 2 did not amplify, and PCR reaction 3 only amplified the *MINCR* sequence without a cassette inserted – 1.6kb. There were several clones that could not be expanded sufficiently to extract genomic DNA and perform genotyping. A hypothesis was proposed that *MINCR* might have a role in the survival and/or proliferation of hiPSCs. It was possible to fix and RNA extract a sample of *MYH7^{WT/MUT} MINCR KO* Clone 5 which was characterised. This is discussed in Chapter 5.2.

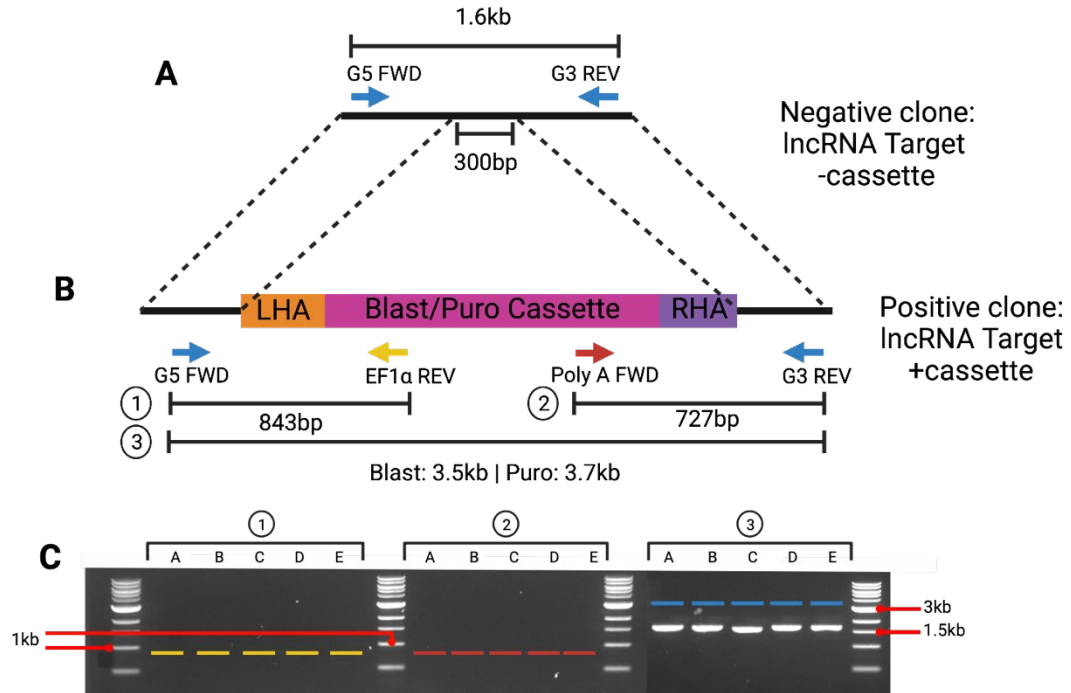


Figure 3.7 – hiPSC Clone Genotyping *MINCR*. Three PCR reactions were designed to amplify the components of the inserted cassette: 1) the LHA, 2) the RHA and 3) the whole sequence including the blast or puro antibiotic resistance cassette. Using primers outside of the homology arms ensured that amplification is only successful if the clone is positive. **A)** A negative clone would not amplify either of the homology arms as one primer for each is within the sequence of the antibiotic cassette. The PCR reaction for the whole cassette would amplify the region of the lncRNA between the primers including the deletion site, 1.6kb for *MINCR*. **B)** A positive clone would be successful in all three PCR reactions. The LHA and RHA would now be amplified as the primer binding sites would be present within the inserted cassette. **C)** Gel image showing negative clones for each PCR reaction for *MINCR* Knockout in hiPSC. Neither of reactions 1 or 2 have amplified the expected LHA and RHA bands. Expected bands are shown in yellow (LHA) and red (RHA). The bands in PCR 3 suggest a negative clone as the size corresponds to ~1.6kb which is expected if there is no inserted cassette. The blue band shows what would be expected in a positive clone.

3.3 Knock Down Results

3.3.1 Knock Down Strategy and Cardiomyocyte Transfection

Due to the possibility that the lncRNA *MINCR* might have a role in cell proliferation, a knock down strategy was designed to reduce expression (**Figure 3.8**). Four commercial siRNAs were purchased which targeted exons 1 and 3. Of the seven *MINCR* transcripts, six were targeted by the siRNAs in at least one exon. To determine the optimal siRNA or siRNAs for *MINCR* knockdown, hiPSC-CMs were transfected with each individual siRNA and a pair of siRNAs: 1+3, 1+4, 2+3 and 2+4 (**Figure 3.8A**). The siRNAs were delivered using the previously optimised Viafect™ transfection protocol, with the intention to reduce the expression level of *MINCR* (**Figure 3.8B,C**). Transfection efficiency was assessed using GFP (**Figure 3.9**).

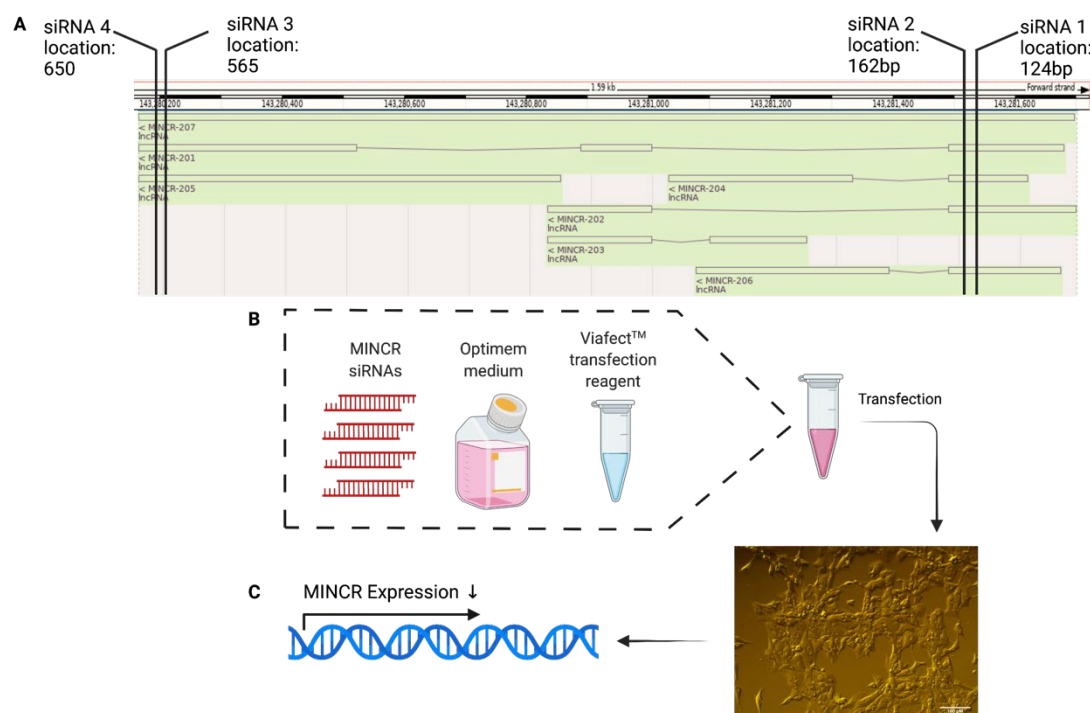


Figure 3.8 – Overview of the strategy to knock down *MINCR* in hiPSC-CMs. **A)** siRNAs purchased commercially targeted exons 1 and 3. **B)** The Viafect™ transfection method already optimised for Overexpression was used to deliver the siRNAs to hiPSC-CMs, **C)** for the purpose of down regulating *MINCR* expression. Phenotyping was then performed. Scale bar =100µm.

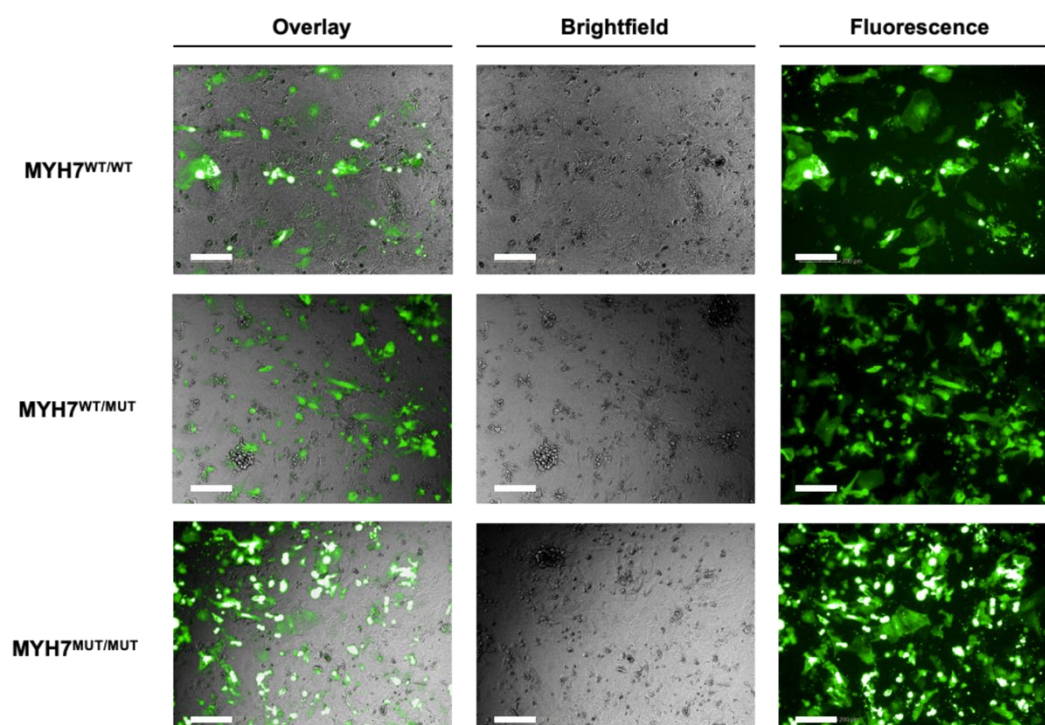


Figure 3.9 – GFP efficiency of hiPSC-CM transfection with siRNA. Viafect™ mediated transfection efficiency was approximated using a GFP plasmid and Operetta imaging. Scale bars =200µm.

3.4 Overexpression Results

3.4.1 Design Strategy

To modulate the expression of the lncRNA targets for their upregulation, the approach was to use a strong promoter, CAG, to drive the expression of the lncRNAs. This promoter is a hybrid between the viral promoter CMV and chicken β actin promoter, which has greater resistance to silencing in many cell types [210]. A CAG vector containing the lncRNA target was delivered to the hiPSC-CMs via transfection with Viafect™ (Figure 3.10A). The resulting upregulation of the lncRNA targets was then phenotypically assessed (Figure 3.10B).

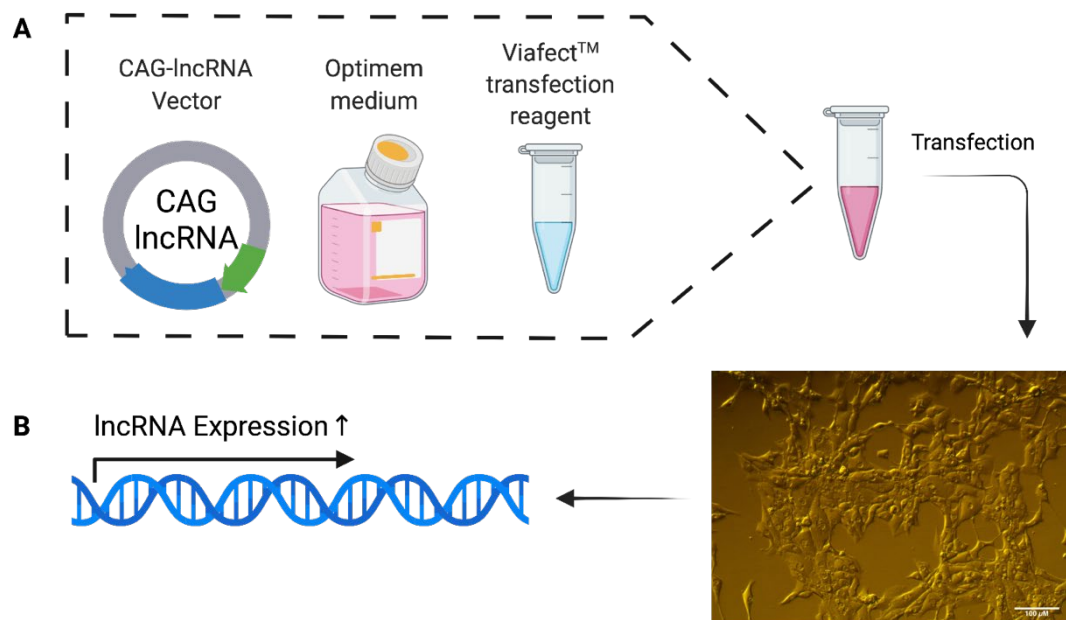


Figure 3.10 – Overview of the strategy to overexpress lncRNAs in hiPSC-CMs. A) A plasmid containing the CAG strong promoter and the lncRNA target was delivered to hiPSC-CMs using Viafect™ transfection reagent, B) with the intention to upregulate lncRNA expression.

3.4.2 Plasmid Design and Construction

A summary of the CAG-lncRNA plasmid design is demonstrated in **Figure 3.11**. Synthesis of overexpression vectors commenced with the PCR amplification of the lncRNA targets. Using *MYH7^{WT/WT}* DNA as a template, primers were designed to amplify the lncRNA target and add BsrGI and ClaI restriction sites once digested with the respective enzymes, to allow cloning into the backbone vector (**Figure 3.11A,D**).

The plasmid AAVS1-GECO with a CAG promoter was used as a backbone (**Figure 3.11B**). The GECO gene was removed via restriction digestion with BsrGI and ClaI and the lncRNA digested PCR product was successfully cloned in via restriction site ligation (**Figure 3.11C**). The IRES/Puro sequences were removed from the vector to avoid potential interference with the lncRNA expression, due to their location between the lncRNA sequence and poly A tail. This was done by restriction digest with ClaI and Psil which deleted around 20bp of the poly A tail (**Figure 3.11E**). Oligonucleotide sequences with ClaI and Psil overhangs were ordered, annealed, and ligated into the plasmid via restriction site ligation to replace the missing section of the poly A tail. The final vectors were confirmed by diagnostic digestion with unique cutters KpnI and NotI (**Figure 3.11F**) and Sanger sequencing.

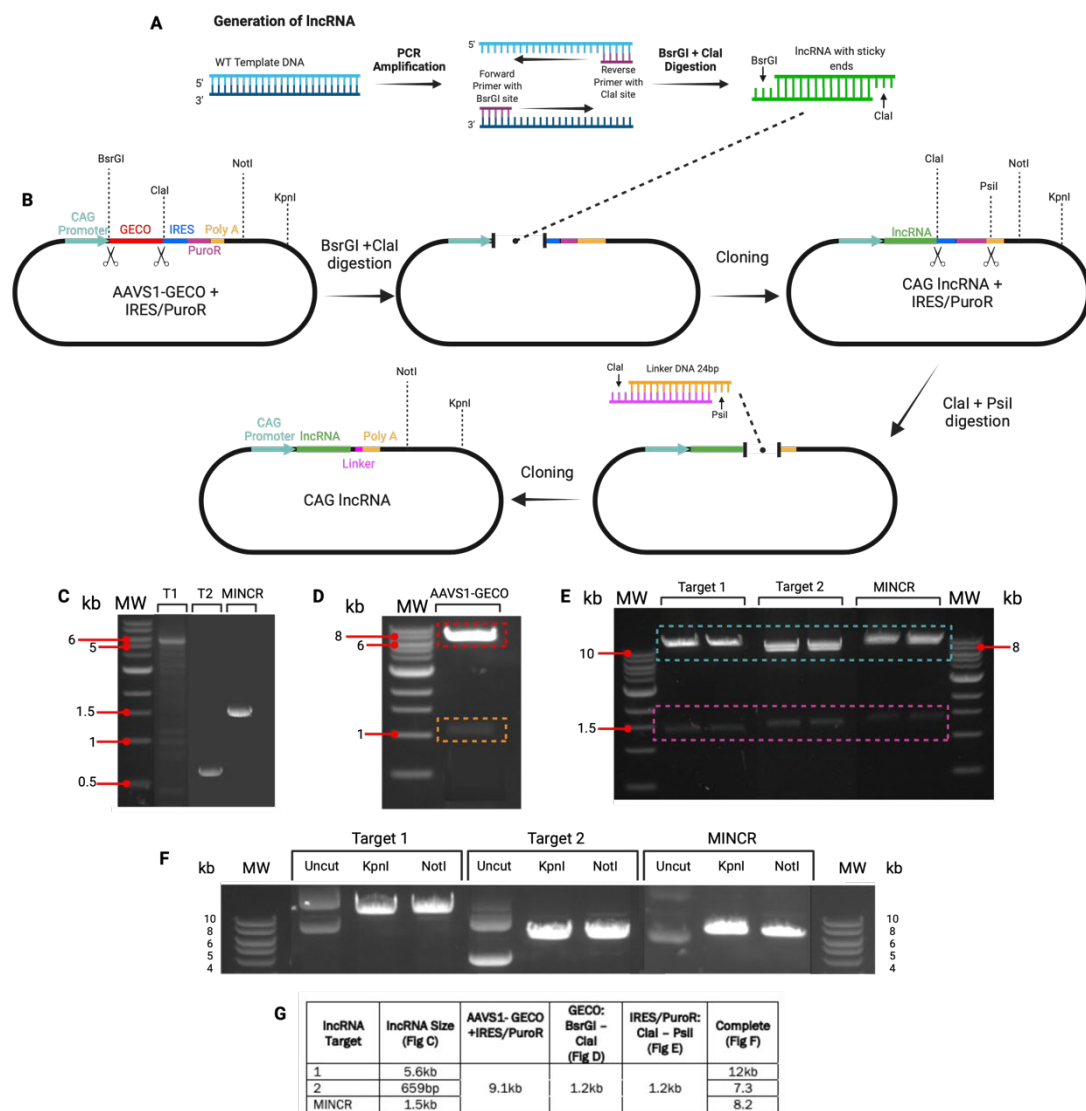


Figure 3.11 – Overview of overexpression plasmid design. A&C) The lncRNA sequences of Targets 1,2 and *MINCR* were amplified using PCR. **B)** A vector containing a strong promoter was used as a backbone and a two-step digestion and cloning strategy was used to complete the vectors. **D)** The GECO sequence (orange box) was removed using enzymatic digestion with BsrGI and ClaI, and the PCR amplified lncRNA sequences were cloned into the backbone vector (red box) by restriction site ligation. **E)** To remove the IRES/Puro components (pink box), the vectors were then digested with ClaI and PstI (blue box), and **F)** a linker sequence made up of oligonucleotides was cloned in using ClaI and PstI restriction site ligation to replace the removed nucleotides of the Poly A tail. **G)**

3.4.3 Amplification of Target 3

Target 3 presented a challenge for the overexpression strategy due to its length. At 35kb, it was not possible to PCR amplify using the same approach as the other lncRNA targets (**Figure 3.12A**). To overcome this issue, two methods were devised that involved amplifying the exons of the lncRNA, due to the majority of the lncRNA being made up of introns.

The principal design used *MYH7*^{WT/WT} DNA as a template to amplify the cDNA of target 3 (**Figure 3.12B**). The amplification primers were designed to engineer in BsrGI and ClaI restriction sites to the cDNA to facilitate restriction-based ligation into the CAG lncRNA backbone vector.

An alternative approach was conceived and kept in reserve. This method amplified each of the exons in separate reactions using *MYH7*^{WT/WT} DNA as a template (**Figure 3.12C**). The primers used to amplify the exons were designed to engineer in restriction sites to the end of each exon to allow the stitching of exons together and subsequent cloning into the CAG lncRNA backbone vector. As above, BsrGI and ClaI restriction sites were used for cloning into the CAG vector, by their addition to exons 1 and 3 respectively. Exons 1 and 2 were stitched using EcoRI and exon 3 was added using NotI ligation. This approach would have led to alterations in the lncRNA sequence and therefore was not the desired approach.

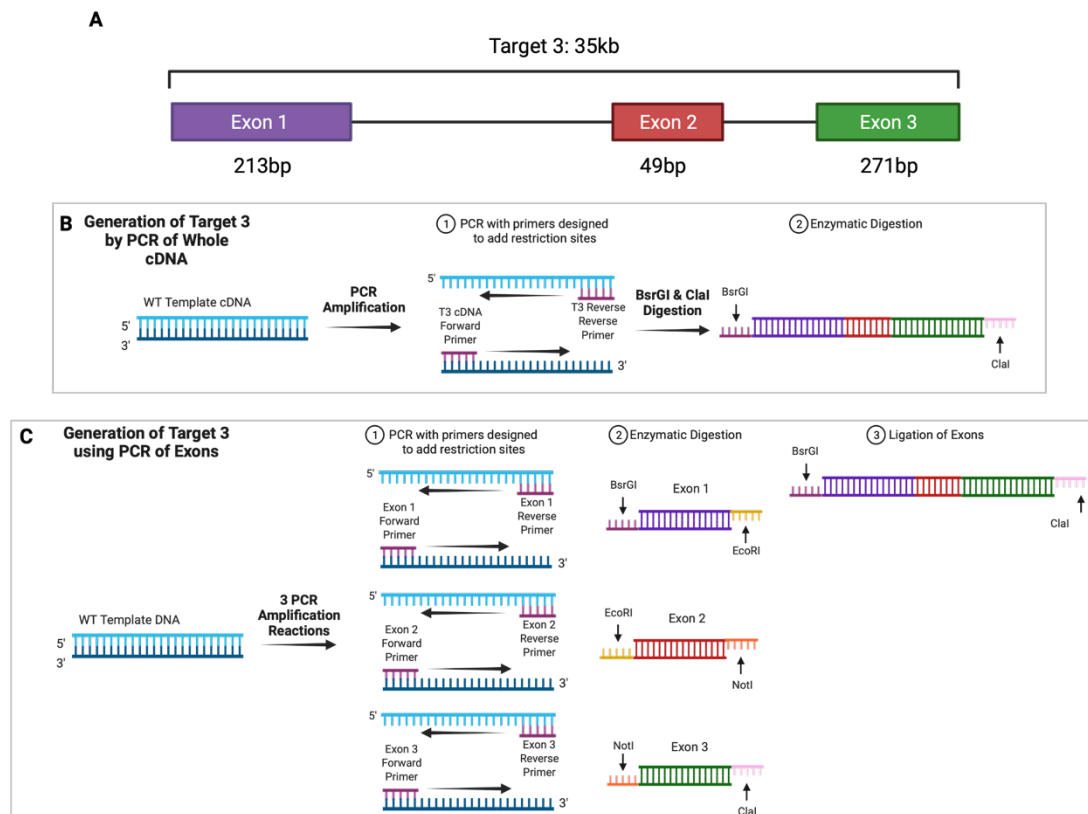


Figure 3.12 – Amplification of target 3. **A)** Target 3 spans 35kb, predominantly made up of introns. Two methods were attempted to amplify the coding regions. **B)** The preferred strategy was to use *MYH7^{WT/WT}* as a template for the amplification of target 3 cDNA. Primers were designed to add restriction site BsrGI and ClaI to the cDNA for subsequent restriction site based cloning into the CAG lncRNA backbone vector. **C)** A secondary method used *MYH7^{WT/WT}* as a template for amplification of the individual exons, engineering in restriction sites EcoRI to exons 1 and 2 and NotI to exons 2 and 3 to enable their stitching together. As in **B**, BsrGI and ClaI were added to exons 1 and 3 respectively, to facilitate ligation into the CAG lncRNA backbone vector.

3.4.4 hiPSC-Cardiomyocyte Transfection

Completed CAG lncRNA vectors were delivered to hiPSC-CMs using the Viafect™ protocol as described in Chapter 2.4.2. Transfection efficiency was measured using GFP by imaging on the Operetta High Content System (Figure 3.13).

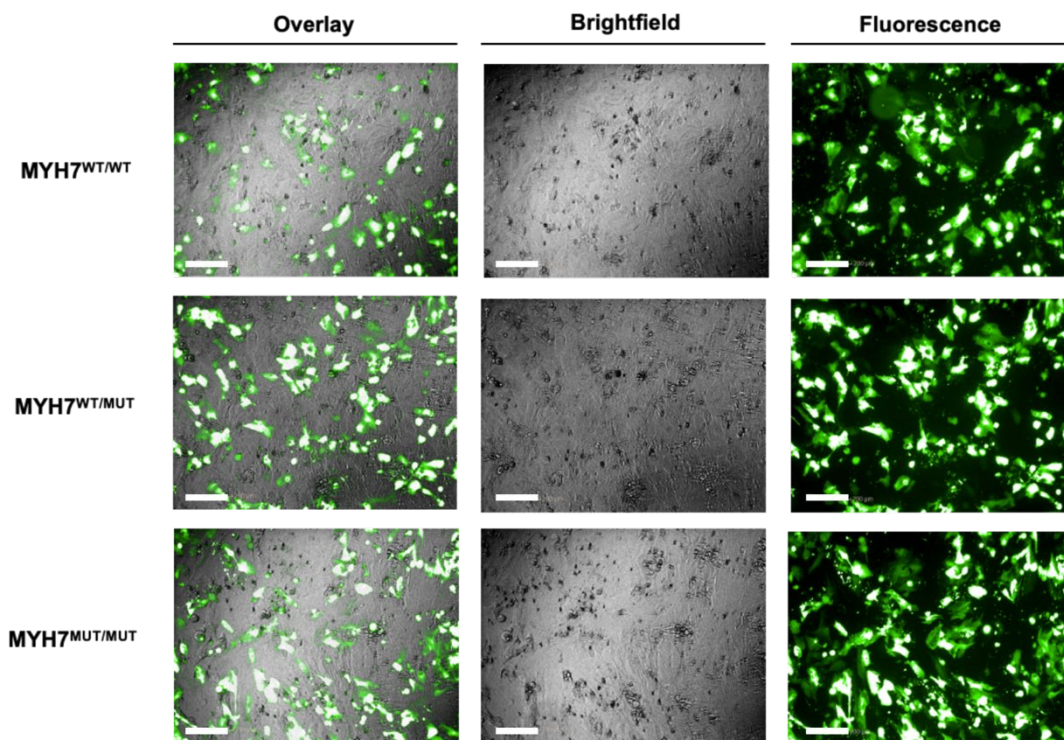


Figure 3.13 – GFP efficiency of hiPSC-CM transfection with overexpression system. Viafect™ mediated transfection efficiency was approximated using a GFP plasmid and Operetta imaging. Scale bars =200µm.

3.5 Discussion

This chapter described the design and construction of plasmids used for modulating lncRNA expression in the *MYH7* HCM model line. To understand the function of lncRNAs on the molecular level, three approaches used to modulate their expression: CRISPR/Cas9 knockout, siRNA knockdown and overexpression.

3.5.1 Strengths and Weaknesses of CRISPR/Cas9 KO Strategy

This thesis implemented a plasmid based CRISPR editing system. While there are some advantages of other approaches, such as RNP complex editing, for this thesis the plasmid strategy was overall more advantageous. One of the advantages of RNP complexes over plasmid-based strategy is non-reliance on cell machinery to transcribe and translate Cas9 and gRNA prior to them forming a complex [211], which can result in varied editing efficiencies based on the levels of expression. Nevertheless, HDR templates transfected with an RNP complex are usually much shorter and with shorter homology arms [212]. In this thesis a large 3.5kb or 3.7kb antibiotic cassette was inserted for the selection of positive clones, which would not have been possible using an RNP complex strategy due to the HDR template limitation. Finally, although plasmid DNA is toxic to cells, reducing the number of vectors during transfection may improve the survival of cells and therefore positively targeted clones. In future experiments this could be achieved by integrating the Cas9 and its promoter within the gRNA vector, without creating a plasmid too large to cause toxicity itself [213]. Electrotransfer (electroporation) causes the reduction of cell viability when large plasmids are used for transfection. At 4104bp, Cas9 could be cloned into the same plasmid as the 20bp gRNA with independent promoters and not cause toxicity during electrotransfer.

Vector synthesis was commercially available with an estimated delivery period of 8 weeks, but realistically the delivery period was expected to be longer and therefore the vectors were constructed manually. The uptake of CRISPR/Cas9 in many biological applications due to its simplicity has culminated in greater demand of CRISPR products, and the lead times for commercial vectors has significantly decreased.

Successful recombination between the target site and donor template is key for HDR. Homology arms are generally between 500-1000bp long, where longer homology arms more suitable when there is a high degree of homology between the target site and off target sites. Longer homology arms have also been associated with greater efficiency and robustness of recombination than shorter homology arms and facilitate the insertion of larger sequences during repair [214, 215]. For the above reasons, the large 3.5kb and 3.7kb inserts necessitated longer homology arms. On the contrary, longer homology arms increase the molecular weight of the HDR template vector used during nucleofection. Consequently, fewer copies of the template are introduced to the target cells, possibly affecting the efficiency of HDR. For this reason, homology arms of 500bp were used in this work.

Although no positive clones were identified from the genotyping strategy, there is the possibility that there were heterozygous clones screened. The third PCR reaction where the whole insertion cassette is amplified could be favouring amplification of the non-targeted allele (1.6kb product) over the targeted allele (3.6kb product), despite using an appropriate extension time. It is known that the primers used for this reaction are functional as it is possible to amplify the sequence from the isogenic control line *MYH7^{WT/WT}* which was used as a negative control. The same cannot be said for the primers used in PCR reactions 1 and 2 which intended to amplify the homology arms. These two reactions may preferentially amplify smaller wild type products as opposed to more complex and longer blast/puro products. Walsh and Higuchi demonstrated that preferential PCR amplification may occur due to significant GC% differences between alleles and the conditions of the reaction favouring the denaturation of one allele over the other due to this [216]. In addition, they showed that shorter products are preferred for amplification. Future experiments could modify the reaction conditions using a GC buffer with higher temperatures and an increased number of cycles.

The complicated nature of lncRNA genomic loci, extensively discussed in Chapter 1.4 requires knowledge on; i) their positioning in the genome, ii) whether they have regulatory roles and iii) if they perform their role in *cis* or *trans* to correctly utilise

CRISPR editing for knockouts, without inadvertently affecting neighbouring or overlapping genes. A genome wide analysis sought to determine the 'CRISPRability' of lncRNAs and found that 62% of the total lncRNAs were determined non-'CRISPRable' due to the complex organisation of lncRNA loci [217]. The methods generally used are CRISPR excision, CRISPR homology repair and CRISPR mutagenesis. Rarely used for the KO of lncRNA is CRISPR mutagenesis as small mutations are not sufficient to disrupt lncRNA expression to the extent of silencing it. Most studies employ CRISPR excision, homology repair or a combination of both, the latter used in this thesis [218-220]. Considering the possible complications that could arise, the approach to excise of a small segment of lncRNA genome and insert a large cassette meant that firstly there was less risk in unwittingly deleting uncharacterised elements leading to false phenotypes and secondly the lncRNA would be sufficiently interrupted leading to no expression.

3.5.2 Strengths and Weaknesses in *MINCR* Knockdown by RNAi

As discussed in Chapter 1.4, few lncRNAs have been functionally characterised and the localisation of *MINCR* has not been elucidated to in hiPSC or hiPSC-CM. lncRNA modulation by knockdown could be improved by understanding the subcellular localisation of the target lncRNAs in the *MYH7* HCM model used in this thesis. Studies have shown that lncRNAs localised to the cytoplasm, nucleus, or both are more effectively suppressed by siRNAs, ASOs and a mix of both, respectively [221]. Localisation can be performed by RNA fluorescence in situ hybridisation (FISH) [117]. To determine the location of targets 1 and 3, this approach would be sufficient due to their length. lncRNA targets 2 and *MINCR* are relatively short in comparison, and the requirement of a single probe set to contain 25 oligos 18-22nt in length makes it unlikely for successful binding to the target lncRNA sequences. In this case, prediction methods using bioinformatics may be a more pragmatic approach [222-224].

Assessing the transfection efficiency after siRNA transfection for the knockdown of *MINCR* could be improved by labelling the siRNAs with common fluorescent labels such as FAM or Cy3, as opposed to transfecting cells separately with GFP. Previous studies have demonstrated that adding such labels has minimal impact on the silencing ability of the siRNA [225], and there are now commercially

available kits making it accessible and cost effective. Furthermore, labelled siRNAs would increase confidence in results if a time course of gene silencing was performed, with the additional benefit of understanding the subcellular localisation of the siRNA. The ability to localise and track *MINCR* silencing may also provide some answers to the proposed hypothesis of its involvement in proliferation and/or survival of hiPSCs.

It would not, however, be a flawless approach. Transfection conditions are highly optimised per cell line, not taking into consideration the macromolecules being delivered. This was highlighted by Han *et al.* who demonstrated that small RNAs were degraded in the presence of serum in culture media during transfection [226]. If labelled siRNAs were to be used in future experiments, it would be necessary to re-optimize the Viafect™ transfection method for hiPSC-CM, as at present it uses FBS.

3.5.3 *MINCR* Clone 5 Considerations

The characteristics observed in clone 5 resulting from *MINCR* knockout prompted a hypothesis, guided by studies that linked *MINCR* expression to cell cycle regulation and apoptosis in cancer. The observations and literature suggested that clone 5 may be positive for the knockout of *MINCR* and characterisation was performed in place of genotyping results from lack of genomic DNA. Further analysis and discussion of clone 5 is in Chapter 5.

3.5.4 Strengths and weaknesses of lncRNA overexpression approach

To overexpress the lncRNA targets in this work, a plasmid-based approach was opted for due to the ease of assembly and the benefits of expression. Other methods to induce of gain of function such as CRISPR activation (CRISPRa) or an inducible system, require laborious development. Furthermore, by targeting the lncRNA gene loci via CRISPRa, transcription is initiated by the lncRNA promoter which may not result in significant overexpression and requires colony selection. In addition, it may result in off target effects by interacting with nearby promoters [227], further highlighting that overexpression of lncRNAs requires knowledge of their positioning. Oppositely, transfection of a lncRNA transcript via a plasmid permits the use of a promoter of choice, such as CAG in this work, to drive higher

gene expression, which can lead to a more obvious phenotype. It was possible to quickly determine the TE by transfecting hiPSC-CMS with GFP and visualising expression by fluorescent microscopy, culminating in simpler and more efficient experiments.

The ability of lncRNAs to exert their roles in *cis* and in *trans* further complicates selecting a method to determine their role through overexpression. As mentioned above, they can be targeted directly at the gene level or at the RNA transcript level. Neither manipulation technique is perfect, CRISPRa cannot discriminate between *cis* and *trans* acting lncRNAs, nor differentiate between isoforms to overexpress. On the contrary, vector transfection of the lncRNA gives control over which isoform to overexpress but does not reproduce activity of the lncRNA in *cis*.

3.5.5 Considerations for modulating lncRNA expression

The complex and unique biology of lncRNAs requires significant consideration before electing which modulation methods to use. This is especially difficult when modulating undefined lncRNAs as performed in this thesis. However, the continuously expanding knowledge of lncRNAs and the ever-evolving field of gene editing will result in superior bioinformatic prediction and refined editing methods that may eventually have the power to evade the complexity of lncRNA biology. Preceding this, at present to modulate lncRNAs the following factors must be considered; i) lncRNA position in the genome, ii) whether the lncRNA acts in *cis* or *trans* and iii) subcellular location of the lncRNA.

4. Development of an optimised protocol for high efficiency transfection of hiPSC-CMs

4.1 Introduction

4.1.1 Importance and uses of transfection

Transfection is the introduction of macromolecules such as DNA (plasmid DNA or DNA oligonucleotides), siRNA, mRNA, protein and ribonucleoprotein (RNP) complexes into eukaryotic cells [228]. Transfection is a powerful tool that enables the study of gene expression, gene functions and the production of recombinant proteins. Some of the first protocols date back to the 1960s when Pagano and colleagues were using the cationic polymer DEAE-Dextran to transfect African green monkey kidney cells and Hela cells with viral RNA particles [229]. Since then, numerous other methods have been established and it has become a powerful tool used in disease modelling. This Chapter will first consider the approaches available for delivering macromolecules to cells. Next, it will explain the steps that led to an optimised protocol for high-efficiency transfection of hiPSC-CMs, which was published in Methods & Protocols and used in the work described in Chapter 5 of this thesis.

Different types of transfection are required for distinct experimental objectives. Stable transfection is required for the creation of stable cell lines, large scale protein production [230] and gene therapy, while transient transfection is used for short term studies of gene expression modulation on a small scale. Transfection *in vitro* using human-derived cells such as hiPSC-CMs complements previous *in vivo* animal models and is more biologically relevant. The outcome of this is improved modelling leading to improved diagnostics and treatments. Ideal methods should have high transfection efficiency, low cell toxicity, minimal effects on cell physiology and be reproducible. Efficiency can depend on cell health, confluency, DNA quality and conditions of transfection require optimisation for each macromolecule as they have individual characteristics [231]. In principle, once all of these factors have been taken into account during optimisation, high transfection efficiency is possible in most cases and experimental outcomes can

be very valuable. For some cell types, especially hiPSC-CMs, transfection remains a problematic technique.

In the 1980s and 1990s, the ability to deliver genetic material to cells in culture and *in vivo* gave rise to the field of gene therapy. Whilst still experimental, research focusing on recessive gene disorders, acquired genetic diseases and viral infections [232], there have been some important successes. For example, Flotte *et al.*, researching cystic fibrosis was one of the first to use AAV vectors in the pursuit of gene therapy, deducing that it enabled stable integration and expression [233]. Transfection methods utilised in gene therapy are predominantly viral transduction due to its efficiency. A huge breakthrough in gene therapy treatments came with the conditional authorisation of Roctavian by the European Medicine Agency for the treatment of haemophilia A in the European Union. The therapy uses adeno-associated virus (AAV) harbouring the gene for factor VIII required for clotting of blood. In clinical trials, 85% of patients experienced reduced bleeding rates and one infusion of Roctavian was sufficient [234]. More comprehensive data is required, including longer term studies to assess side effects and the number of treatment cycles required for successful treatment. Regardless, Roctavian is at the forefront of a revolution in human healthcare.

4.1.2 Methods of transfection

Transfection of DNA is either stable or transient (**Figure 4.1**). **Stable transfection** results in continuous expression of the gene of interest as the transfected DNA integrates into the host genome and is passed onto the progeny [235]. Stable transfection is a more complex method to perform and can be unpredictable. For example, if using plasmid DNA containing the transgene, random regions of the plasmid not containing the gene of interest can become integrated into the genome [236]. Stable transfection begins with transient transfection but then using selectable markers such as antibiotics, cells that have successfully integrated the foreign material can be expanded, while other non-stable cells will die [237]. Stable transfection can be hindered by the toxicity of the transgene or by silencing by gradual epigenetic mechanisms [238-240]. In the case of lncRNAs, an example where stable transfection has been used was in the creation of the isogenic genome-edited hiPSCs used in this thesis as a model of HCM. The

introduction of the pathological R453C SNP mutation into healthy hiPSCs was performed by CRISPR/Cas9-nickase editing [7].

In contrast, the outcome of **transient transfection** is a shorter expression window due to the non-integration of material into the host genome. Rather than using selection of clones, reporter genes (e.g., GFP) can be used to assess gene expression 48-96h post-transfection [241]. Cell proliferation and nuclease degradation can result in dilution and loss of transfected DNA, respectively, hence reducing expression [242]. However, since these experiments are often a fast turnaround, for most experimental needs this is not an issue. The use of reporter genes to assess efficiency as opposed to selecting positive colonies which then require downstream assessment to check correct integration makes transient transfection a faster process. The downside to this is that this method is not suitable for studies using vectors with inducible promoters as expression will be lost.

The research objective determines which approach to take, taking into consideration cell type. Typically, transient experiments require harvesting of the cells soon after transfection (24-96h) to analyse the effect of gene expression, to mitigate the loss of gene expression if left for longer. Stable transfection leads to sustained long term expression of a transgene. For both methods, the consensus is that at the time of transfection it is ideal that cells are actively proliferating, as they are more receptive to transfection than quiescent cells due to the disassembly of the nuclear membrane during mitosis enables the entry of DNA.

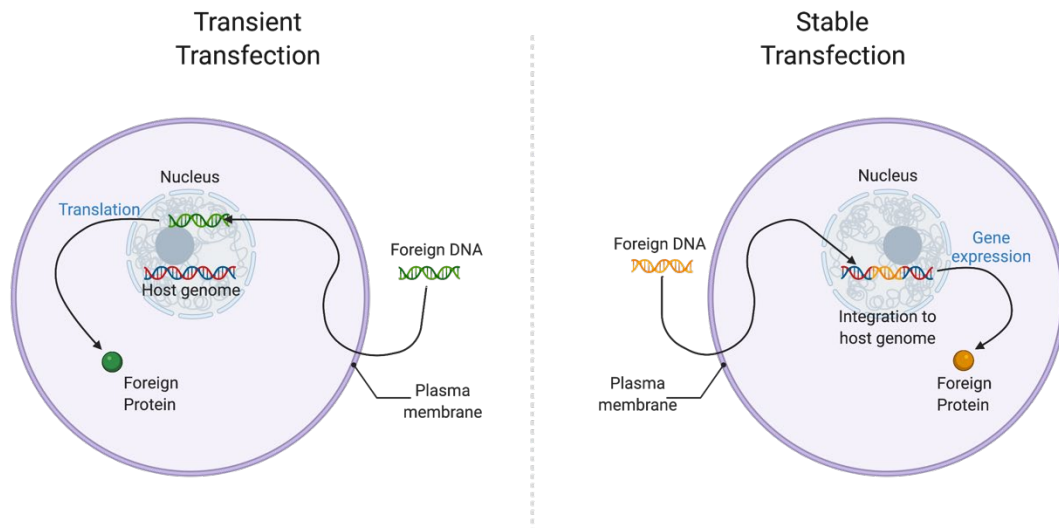


Figure 4.1 – Schematic showing transient and stable transfection. The difference between transient and stable transfection lie in whether the foreign DNA is integrated into the host cell genome. Created using Biorender.com 2022.

Once the transfection type has been decided, the next stage is to establish which methods of transfection to use. Transfection methods are categorised into three groups: biological, physical, and chemical. **Table 4.1** gives an overview of possible techniques. The main challenge surrounding transfection is overcoming the electrostatic conflict; negatively charged DNA must enter a negatively charged plasma membrane. Each technique has been developed to overcome this issue.

Biological methods refer to those using viruses to deliver the genetic material to the cells, also known as transduction. Viral vectors are hazardous to the operator and can have mutagenic effects in the target cells due to unintended insertional mutagenesis [243, 244] and therefore carry greater risks to the user than other transfection techniques. Adeno-associated virus (AAV) has been used to investigate micro RNA regulation of cardiomyocyte proliferation *in vitro*. In 2021, Bian *et al.* transduced hiPSC-CMs with an AAV coding for microRNA 199a and grafted them into murine hearts, to avoid genomic integration of the microRNA [245]. When direct *in vivo* microinjection of AAV coding for microRNA 199a had been performed previously, many animals passed away from arrhythmias, likely associated with uncontrolled expression of the miRNA and viral mutagenesis [246]. Although viral transduction has high efficiency, in addition to transduction complications, it entails producing substantial quantities of virus containing the

transgene, which itself is a complex process. Furthermore, viral production requires high biological safety for the user, inducing risks and costs.

Adenoviral transduction is a highly efficient method to achieve transient transfection of transgenes up to 8kb [247]. Adenoviruses are double-stranded DNA viruses that bind to the Coxsackievirus and adenovirus receptor (CAR) on the cell membrane and enter the cell via endocytosis. Host machinery replicates the virus resulting in expression but also an immune response from the host cell, which is of greater concern for *in vivo* applications. Adeno-associated viruses (AAV) carry single-stranded DNA and their transfection carries lower toxicity than adenoviral transfection, but their reduced packaging capacity (5kb) limits their application [233].

Lentiviral transduction enables stable gene transfer *in vitro* and *in vivo* using recombinant lentiviral vectors such as HIV. Kosaka *et al.* were able to achieve a transfection efficiency of 42% using HIV lentiviral vectors in mouse embryonic stem cells (mESC) [248]. In hiPSCs, using a hanging drop lentiviral transduction of GFP, flow cytometry demonstrated 91% GFP positive cells [249]. Once the viral RNA has entered the cell, it is reverse transcribed by reverse transcriptase to form double-stranded DNA which then enters the nucleus. The transgene is incorporated into the genome by the lentiviral integrase enzymes and results in lower toxicity than adenoviral transfection [250].

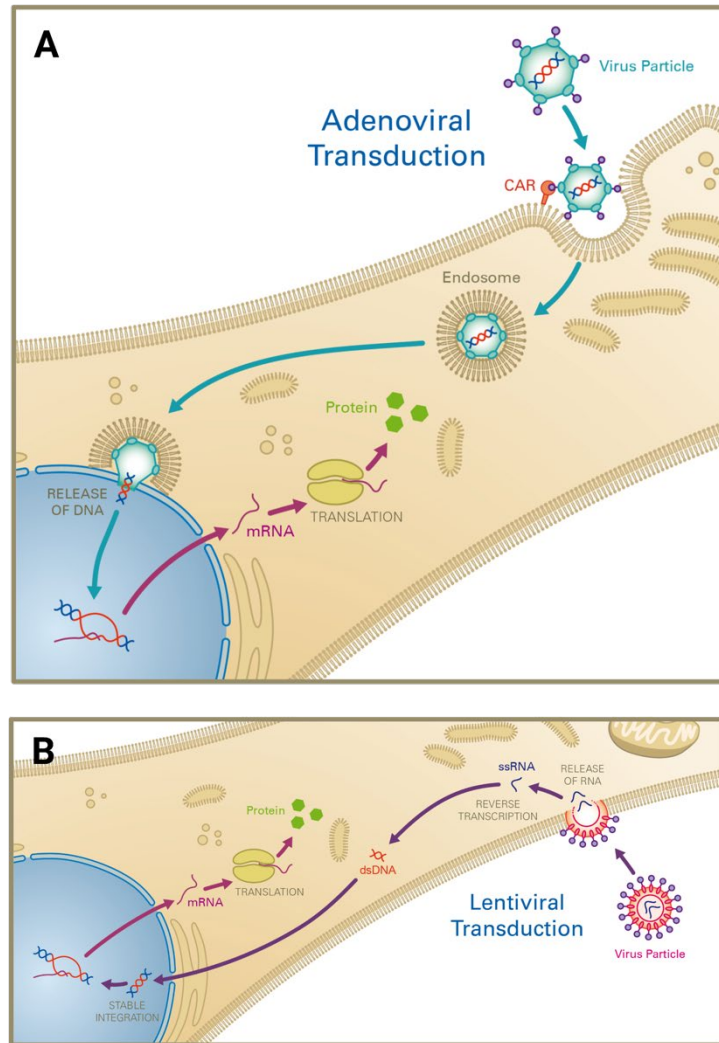


Figure 4.2 – Schematic showing viral transduction. A) Adenoviral transduction of DNA relies on the interaction of the virus particle with surface receptors on the plasma membrane. **B)** Lentiviral transduction results in stable transfection of the reverse transcribed RNA. Reproduced with permission from ibidi GmbH [251].

The second group uses **physical methods**. Physical methods overcome the electrostatic conflict by forcing a hole in the membrane by either puncture or electrical pulses.

Microinjection is a precise transfection method that involves the direct injection of DNA into the host cell nucleus, in principle with an efficiency of 100% [252]. It allows the immediate integration of injected DNA into the endogenous genome, however, it is low throughput and a high skill method that requires an expensive setup consisting of a microinjection system and powerful microscope. Microinjection is used for the creation of transgenic animals, *in vitro* fertilisation

and transfection of challenging cells [253]. Shubeita and colleagues attempted to use microinjection for the transfection of rat cardiomyocytes, but with an efficiency of only 1% due to the resulting loss of viability post-transfection. Attempts to modify the conditions of the experiment were fruitless, as long term viability was raised to just 2.5% post microinjection [254].

Electroporation involves exposing the cells and DNA to an intense electrical field, resulting in cell membrane destabilisation. The cell membrane becomes permeable, allowing the entry of surrounding DNA [255]. This results in stable or transient transfection and is a reliable and easy method once. Despite this, electroporation requires large cell numbers due to high cell death and is not suitable for sensitive cells, such as hiPSC-CMs [256]. It also requires the purchase of specialist equipment and optimisation of the electric pulse, which will vary in each cell line [257]. Nucleofection uses the basis of electroporation, with the addition of buffers that mediate DNA transfer into the cell while the membrane is porous [258]. Cell viability can be compromised, but with sufficient starting material, it provides an alternative for transfection of cells that are not responsive to lipid-based methods. Hamm *et al.* were able to transfect human primary cells and human MSCs with efficiencies between 28.9-45.3%, while viability was between 11-75% [259].

It does not come as a surprise that electroporation of excitable cells is a challenge. Cardiomyocytes achieve their contractility through ion channels and exchangers that regulate the flow of Ca²⁺ ions. This process, further discussed in Chapter 5, is highly sensitive and external factors can be disruptive. During electroporation, parts of the sarcolemma are exposed to an electric field resulting in ion-channel activation and abnormal ion flux, leading to apoptosis [260, 261]. One study was able to achieve efficiencies of 5% with a viability of 13% in mouse cardiomyocytes [262]. While this may be an efficient method for other cell types such as hiPSC, at present for cardiomyocytes this is not a suitable option.

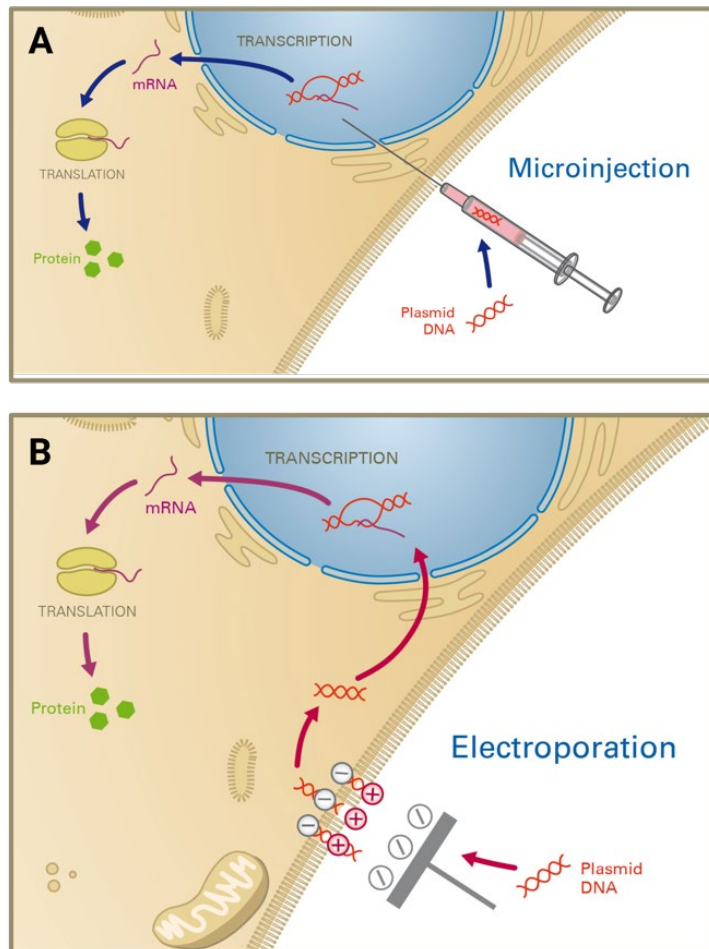


Figure 4.3 – Schematic showing physical methods of transfection. A) Microinjection of DNA directly into the host cell nucleus. **B)** Electroporation uses electrical pulses to create holes in the cell membrane, allowing the entry of DNA into the cell. Reproduced with permission from ibidi GmbH [251].

Transfection via magnetoporation was initially proposed in 1996, whereby DNA is delivered to the cell using a magnetic field. Exogenous DNA is complexed with a magnetofection reagent to form a magnetic biomolecule [263]. Using a magnetic field, the biomolecule is drawn into the cell by endocytosis and pinocytosis [264]. The reagents initially used for this process are highly toxic, but improvements have been made by using iron oxides and carbon nanotubes which increased efficiency and reduced toxicity [265, 266]. In cardiomyocytes, magnetoporation achieved a meagre 18% transfection efficiency in cardiomyocytes, significantly lower (-44%) than the control HEK cells used in comparison [267].

Laser mediated transfection uses the same principles as electroporation, but rather than a current to create membrane pores, a laser is used to form transient pores allowing the DNA to enter the cell [268]. It requires an expensive set-up and

is a low throughput method. Biolistic particle delivery has a similar principle in which subcellular sized particles coated with DNA are transferred into a cell at high velocity using a pulse of helium gas [269]. It can be used for *in vivo* and *in vitro* transfection but necessitates expensive instruments and can result in cell death in 2D monolayer cultures. Physical methods were not fit for the purposes of hiPSC-CM transfection in this thesis, due to resulting poor cell viability, toxicity and low throughput processing.

Finally, **chemical methods** are categorised by liposomal or non-liposomal based. These methods allow the passage of negatively charged DNA into the cell by forming a layer around it and neutralising the charge. For successful entry into the cell, the positive charge contributed by the cationic lipid should be equal to or greater than the negative charge of the DNA. This raises additional optimisation requirements. In 2014, Chan and colleagues demonstrated the importance of optimising serum content when using cationic lipid delivery products [270]. Serum contains a mixture of components, negatively charged serum components can bind to the positively charged cationic-DNA complex, reducing the interactions of the complex and the cell membrane, which can lead to reduced uptake into the cell [271].

In 1987 the first liposome-based transfection protocol was developed by Felgner and colleagues [272]. Liposomes containing N-[1-(2,3- dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) interacted with plasmid DNA, facilitating the fusion of the complex with the cell membrane, allowing the entry into murine cell lines by endocytosis. Lipofection, transfection using cationic lipids, has evolved since then and is a popular transfection method. Reagents often include a polycationic lipid and a neutral lipid for improved transfection [273]. Lipofection is easy and reproducible for the transfection of DNA and RNA into the host cell, resulting in either transient or stable transfection. This method can affect the expression of the transfected DNA resulting in lower efficiency and can result in reduced cell viability due to toxicity in some cell types.

Calcium phosphate has been used as a transfection method for three decades. The principle of calcium phosphate transfection involves the binding of the negatively charged DNA, positively charged calcium and phosphate buffer to form

a precipitate that is taken up by the cell via endocytosis [274]. Transfection can be stable or transient. Although a relatively inexpensive transfection method, toxicity is high in many cell types [275], and Guo and colleagues demonstrated that extensive optimisation of the conditions, buffer, particle concentration, vortex strength and pH is required for efficient transfection [276].

Cationic polymers are positively charged and form complexes with negatively charged nucleotide backbones in the DNA [277]. Uptake into cells is by endocytosis and results in only transient transfection. Efficiency is low in comparison to other methods, and cationic polymers can have high cytotoxicity in some cell types. Studies that transfected hiPSC-CMs using cationic lipids reported efficiencies of 30-40% and 56% [278, 279].

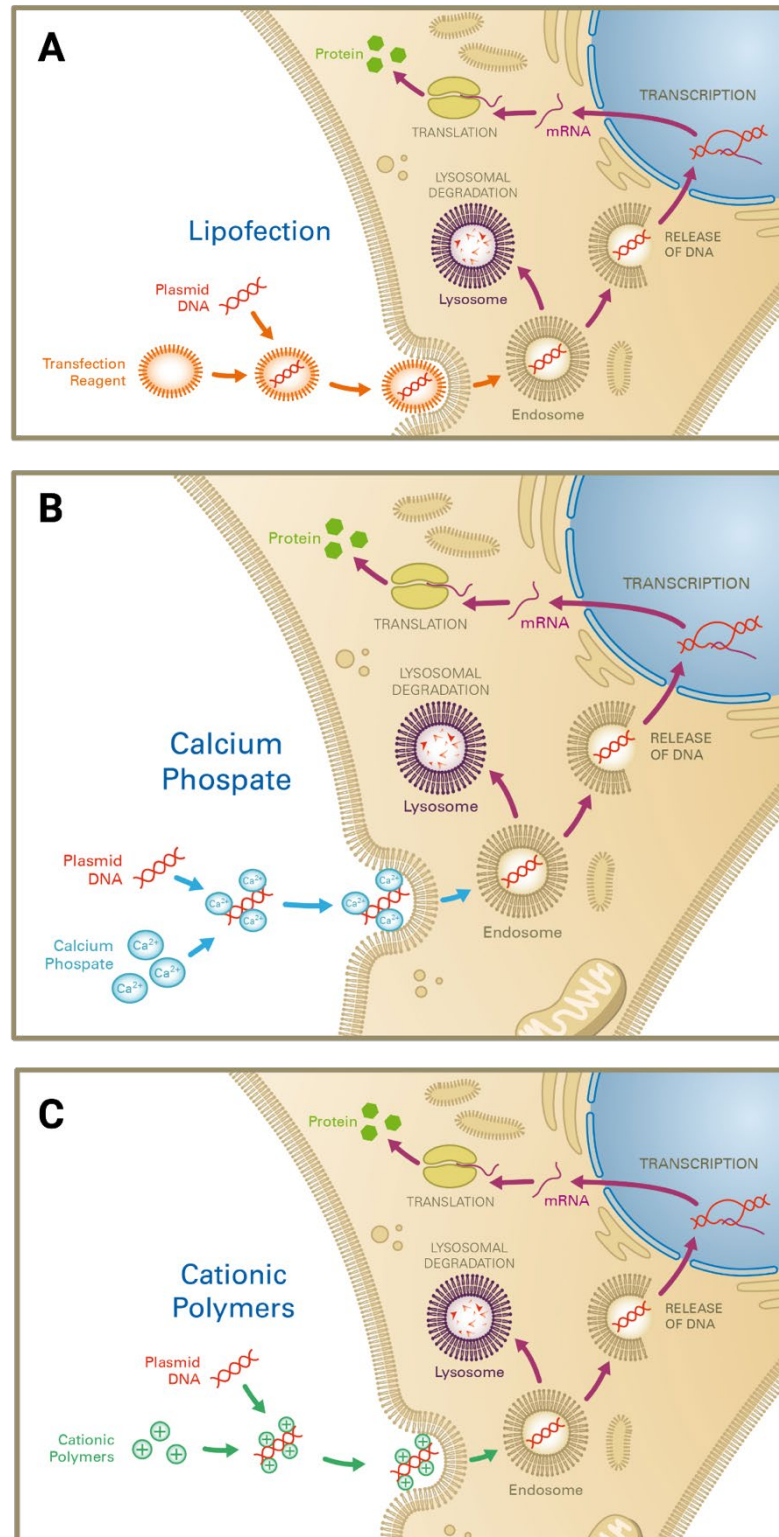


Figure 4.4 – Schematic of chemical transfection methods entering the cell by endocytosis. A) Lipofection, **B)** calcium phosphate and **C)** cationic polymers Reproduced with permission from ibidi GmbH [251]

Table 4.1 - A summary of transfection methods. Adapted from [280]

Method	Cell type	Effectiveness	Cost	Introduced molecule	Advantages	Disadvantages
Instrument-based methods of transfection						
Microinjection	Any <i>in vitro</i> cell	Close to 100%; dependant on injected material	> \$1,000 Grows with process automatization level	Any (DNA, RNA, spermatozooids, proteins, peptides, drugs.)	High efficiency; Precise dosing of injected material; selective delivery; low cytotoxicity	Maximum of 100–200 cells transfected in single treatment; Laborious process
Biolistic transfection	<i>In vitro</i> and <i>in vivo</i> ; e.g., primary leukocytes–lymphocytes, macrophages, and splenocytes	High	High cost of necessary equipment; low cost of utilization	DNA RNA	Possible transfection through physical barriers like epidermidis; possible cotransfection of more than one DNAs in a single use; time efficient	High cost of gene gun; Tissue damage when transfecting small cells
Electroporation	<i>In vitro</i> and <i>in vivo</i> ; See above	Low to moderate	> \$1,000	Plasmids; Oligonucleotides; mRNA; siRNA	High efficiency; Proven efficiency for use on tissues <i>in vivo</i>	High toxicity
Optical transfection	<i>In vitro</i> cells	Comparable to other physical methods	High cost of necessary equipment	DNA, RNA and larger objects	Ability to transfect single cells; Possible transfection with large objects	Diverse efficiency depending on technique
Virus-based methods of transfection						
Adenoviruses	Dividing and non-dividing cells	Expression levels are very high at the beginning, but they quickly weaken in a matter of weeks	\$500–\$1,000	DNA	No integration with the host cell chromosome; Easy viruses' amplification; vectors stability in prolonged storage	Cannot induce prolonged expression; tendency to inducing a strong host immune response; Use possible only in laboratories with Biosafety Level 2 or higher
Adeno-associated virus	See above	See above	\$500–\$1,000	DNA	No integration with host genome; weaker immunogenicity than adenoviruses	Cannot induce prolonged expression
Retroviruses	Dividing and non-dividing cells	Stable expression	≈ \$1,000	RNA	Stable transfected gene expression	Possible retroviral genotoxicity
Chemical transfection methods						
Calcium phosphate	<i>In vitro</i> cells	High	<1,000\$	DNA	Inexpensive; high efficiency; applicable to wide range of cell types; allow to transient and stable transfection	transfection efficiency is influenced by small changes of pH; consistency of precipitate
Cationic lipids	<i>In vitro</i> and <i>in vivo</i>	High	<1,000\$	DNA, RNA, siRNA, and proteins	high efficiency; easy procedure; DNA, RNA and proteins may be introduced; allow to transient and stable transfection	does not work with certain cell types
DEAE-Dextran	<i>In vitro</i> cells	Moderate	<1,000\$	DNA and RNA	Inexpensive; quick and easy method; wide range of cell types may be transfected; DNA and RNA may be introduced	toxicity of DEAE-dextran high concentrations; only for transient transfection; proteins may not be introduced
Magnetic beads	<i>In vitro</i> cells	High	<1,000\$	DNA and RNA	simple method; high efficiency; DNA and RNA may be introduced	only adherent cells may be transfected; cells in suspension must be immobilized

4.1.3 Importance of efficient transfection of hiPSC-CMs

Cardiovascular diseases accounted for 31% of deaths worldwide in 2017 [281]. Despite the huge burden of cardiac disease globally, disease mechanisms are poorly understood, especially in the cardiomyopathies [7]. hiPSC-CMs are considered by many to be one of the best biologically relevant resources to model cardiac disorders, complementing preceding *in vivo* models [7, 282, 283] and promote cardiac repair [284, 285]. Their culture has also been used for providing further mechanical insights into cardiac diseases which will eventually lead to the development of improved treatments. The downregulation of antihypertrophy lncRNA *Ahit* was achieved through transfection and identified interacting partners of the lncRNA and novel disease mechanisms in HCM [149]. Using AAV short hairpin RNA (shRNA) direct injection into mouse hearts, *Ahit* expression was reduced by 43% and resulted in severe hypertrophy. In addition, sustained overexpression of genetically encoded calcium or voltage sensors can be used to establish reporter lines of hiPSC-CMs [286, 287] and their modulation by overexpression can be used to promote more efficient differentiation and maturation [288]. Taking into consideration all the possibilities the use of hiPSC-CMs allows, it is abundantly clear that efficient transfection of hiPSC-CMs is of great benefit to the cardiac research community.

Regardless of their power as a model of human heart disease, historically the transfection of hiPSC-CMs has been very poor using non-viral methods, the best non-viral efficiencies for gene delivery were between 20-56%. Viral gene transfer methods are successful in hiPSC-CMs (90-95% efficiencies) [289], but often cause cell toxicity, entailing technical complexity of viral engineering and biosafety risks. Thus, there is an unmet need for the improvement of non-viral transfection protocols which currently acts as a barrier to fulfilling the potential they have to offer. **Figure 4.5** reflects a literature search conducted on PubMed which revealed a severe deficiency in the number of publications referring to hiPSC-CM transfection (85 publications) in relation to the total of 2133 publications on hiPSC-CMs from the last 20 years. In comparison, there are 12,419 publications referring to HEK-293 cells, and 4,602 publications on HEK-293 transfection.

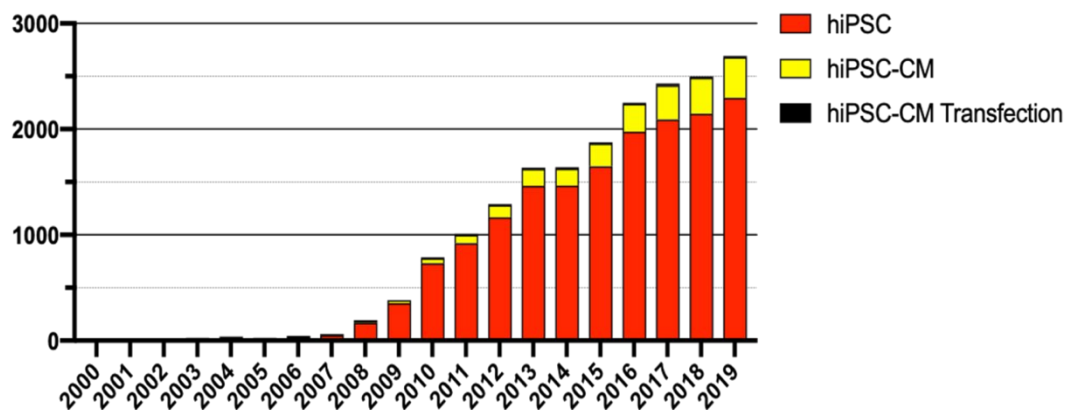


Figure 4.5 – Publications per year based on hiPSC-CM content. The three subject matters; i) hiPSC, ii) hiPSC-CM and iii) hiPSC-CM transfection. Adapted from [290].

4.1.4 Chapter aims and objectives

Within the field of hiPSC-CMs, there was an unfulfilled requirement for improved transfection protocols that achieve high transfection efficiency without compromising cell viability. Current protocols would have been insufficient to carry out the transfection of hiPSC-CMs for the purposes of this thesis, so an optimised method was developed specifically for the transient transfection of cardiomyocytes derived from *MYH7* HCM model lines. This was achieved by systematic optimisation using Viafect™, a cationic lipid delivery reagent, in cardiomyocytes derived from an in-house cell line previously described [7]. hiPSC-CMs quiescence prevented the dilution of DNA over time [291], which maintained the longevity of the transfected material. The conditions optimised were: i) serum content, ii) reagent to DNA ratio and iii) seeding density.

4.2 Materials and Methods

Materials and Methods have been adapted from [290].

4.2.1 Materials

- Vitronectin–Recombinant Human Protein, 500 µg/mL in PBS, (Gibco™, Thermofisher, Loughborough, UK, Cat. No: A174700)
- Phosphate Buffer Saline (PBS, Gibco™, Thermofisher, Loughborough, UK, Cat. No: 14190–094)
- Nunc 96-well Flat Bottom, Delta Coated (Thermofisher, Loughborough, UK, Cat. No: 167008)

- B-27™ Supplement (50X), custom (Gibco™, Thermofisher, Loughborough, UK, Cat. No: 0080085SA)
- RPMI 1640 medium (Gibco™, Thermofisher, Loughborough, UK, Cat. No: 21875034)
- Fetal Bovine Serum (FBS, Gibco™, Thermofisher, Loughborough, UK, Cat. No: 11573397)
- Viafect™ (Promega, Southampton, UK, Cat. No: E4981 1X 0.75 mL, E4982 2X 0.75 mL)
- Opti-MEM™ (Gibco™, Thermofisher, Loughborough, UK, Cat. No: 31985070)
- Axygen™ MaxyClear Snaplock Eppendorff tubes, 1.5 mL (Axygen™, FisherScientific, Loughborough, UK, Cat. No: 11326144)
- Concentrated plasmid DNA of interest (100ng per well of a 96 well plate is required); if using more than one plasmid divide by the number of plasmids to ensure total of 100ng per well
- pmaxGFP from Lonza™ P3 Primary Cell 4D-Nucleofector™ X Kit L, (FisherScientific, Loughborough, UK, Cat. No: 11326144), or any other eGFP reporter plasmid high content imaging
- Cellavista® (Synentec, BPES, Kent, UK) or any other fluorescence plate imaging system

4.2.2 Method

1. Prepare a solution of Vitronectin at 5µg/mL in PBS (1:100 dilution from stock).
2. Coat a Nunc 96-Well Flat Bottom Delta plate with 50µl of Vitronectin solution at 5µg/mL. Incubate at room temperature (RT) for 1h.
3. Supplement RPMI 1640 with a B27 supplement (RB27), aliquot enough for 75µL/well and let it reach RT.

OPTIONAL STEP – it is possible to use other culture media and extracellular matrix proteins provided they have been optimised for cardiomyocyte culture

4. Aspirate the Vitronectin and wash with 50µl PBS. Aspirate PBS and add 75µL RB27 per well.

5. Replate dissociated cardiomyocytes in a single cell suspension at a density ranging from 3.0×10^4 - 4.0×10^4 per well (~ 95 - 125K/ cm^2) in 75 μl RB27. Allow the cells to settle for 30 mins at RT before placing at 37 °C, 5% CO₂.
6. Replace medium with 100 μl /well RB27 every other day for 3 days post plating.
7. On the morning of transfection, replace media with 90 μl /well RB27 + 10% FBS.
8. Calculate transfection mixture:
 - i. Volume of plasmid(s) = $\frac{100 \text{ ng/well} \times \text{number of wells}}{\text{Plasmid concentration (ng/}\mu\text{l})}$
 - ii. Volume of Viafect (μl) = 6 $\times$ total plasmid mass in μg
 - iii. Volume of OptiMEM (μl) = (number wells $\times$ 10) – V_{plasmid} – V_{Viafect}
9. On the afternoon of transfection, add Volumes calculated in step 8 to a 1.5mL Eppendorff tube.

▲ CRITICAL STEP - Flick tube to homogenise transfection mixture (do not vortex or pipette mix). Incubate at RT for 20min.
10. Add 10 μl of transfection mixture to each well, directly into the medium without touching the cells.
11. Place cells in an incubator at 37 °C and 5% CO₂. Replace media the following day with RB27 with or without FBS (as per standard culture practises) and then every second day from thereon until performing desired phenotypic assay.

4.3 Results

4.3.1 Serum content and Viafect™:DNA Ratio affect transfection efficiency

The first stage of optimisation of the protocol sought to determine optimal serum supplementation and ratio of Viafect™ to DNA. Initially, high content fluorescent imaging of cells transfected with the reporter plasmid pmaxGFP was assessed, enabling the detection of positively transfected cells (**Figure 4.6A**). A 96 well plate containing hiPSC-CMs was transfected with different ratios of Viafect™:DNA (ranging from 6:1 to 25:1) while the supplementation with serum ranged from 0-20% (**Figure 4.6B**). The rationale for the ranges investigated was to keep serum content as low as possible. Although using a ratio of Viafect™:DNA lower than 6:1 may have reduced the need for serum, transfection efficiency could be

compromised by less complex formation between the DNA and reagent if they were balanced. Quantification of the transfection efficiency using fluorescence imaging of GFP transfected hiPSC-CMs, demonstrates that the highest transfection efficiencies, ~95%, are reached in the conditions Viafect™:DNA 6:1 + 10 and 20% serum and Viafect™:DNA 8:1 + 10% serum (**Figure 4.6C**). To reduce the serum content and volume of Viafect™ needed per transfection, Viafect™:DNA 6:1 + 10% serum was the chosen condition for future experiments.

To assess how long expression of vectors is maintained in hiPSC-CMs post Viafect™ transfection, the cells were transfected with the CAG-lncRNA overexpression vectors derived in Chapter 3 and RNA was harvested at either 4 or 7 days post transfection. qRT-PCR was performed on untransfected and CAG-lncRNA transfected cells, revealing expression fold changes up to 30,000, 8000 and 12 in target 1, *MINCR* and target 2, respectively. Expression was not compromised 7d post transfection, despite the transient nature of transfection. Further discussion of the results is detailed in Chapter 5.

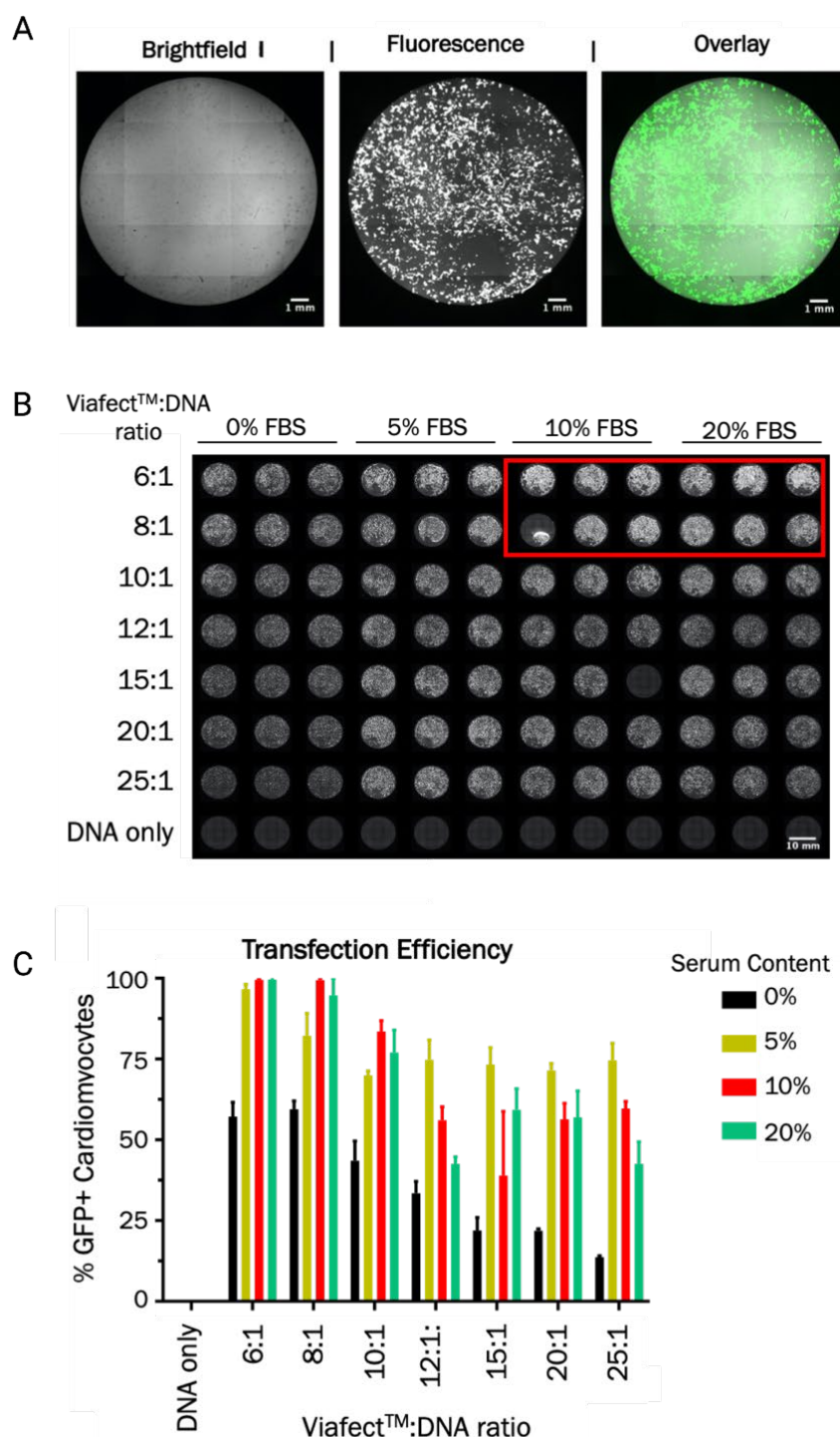


Figure 4.6 – Assessing effects of serum content and Viafect™:DNA ratio on transfection efficiency. hiPSC-CMs were replated into 96 well plate formats and transfected with the reporter plasmid pmaxGFP at a variety of serum concentrations and Viafect™:DNA ratios. **A)** High content fluorescence microscopy enabled the determination of hiPSC-CMs successfully transfected with GFP. **B)** Optimisation of serum content and Viafect™:DNA ratio in a 96 well plate, live by high content fluorescent imaging. **C)** Transfection of hiPSC-CMs using Viafect™ yields the greatest

transfection efficiency with 10% serum (FBS) and a 6:1 Viafect™:DNA ratio. Scale bars A) 1mm B) 10mm.

4.3.2 Seeding density affects transfection efficiency

The experimental format destined for this transfection method was 2D cell culture which relies on cell clusters or monolayers. Therefore cell confluency was an important consideration to prevent reduced cell membrane exposure from curtailing the uptake of nucleic acids or affecting GFP expression. Optimal cell density to achieve high transfection efficiency was determined by transfecting cells seeded at densities between 312 and 1562 cells/mm². **Figure 4.7** shows high content fluorescence imaging of GFP expression 72h post transfection. Results show that highest transfection efficiency occurs when hiPSC-CMs are transfected between 937 and 1250 cells/mm² cells per well of a 96 well plate, translating to approximately 80% confluence. Higher density cultures (1562 cells/mm²) of hiPSC-CMs often result in the peeling and detachment of cells from the well and lower density does not provide an adequate pool of cells for experiments. The conclusion from section 4.3.1 is that a ratio of 6:1 Viafect™:DNA and 10% serum content are the ideal transfection conditions. As a result, phenotyping experiments were performed on hiPSC-CMs transfected with 6:1 Viafect™:DNA in 10% serum and seeded between 937 and 1250 cells/mm² per well of a 96 well plate.

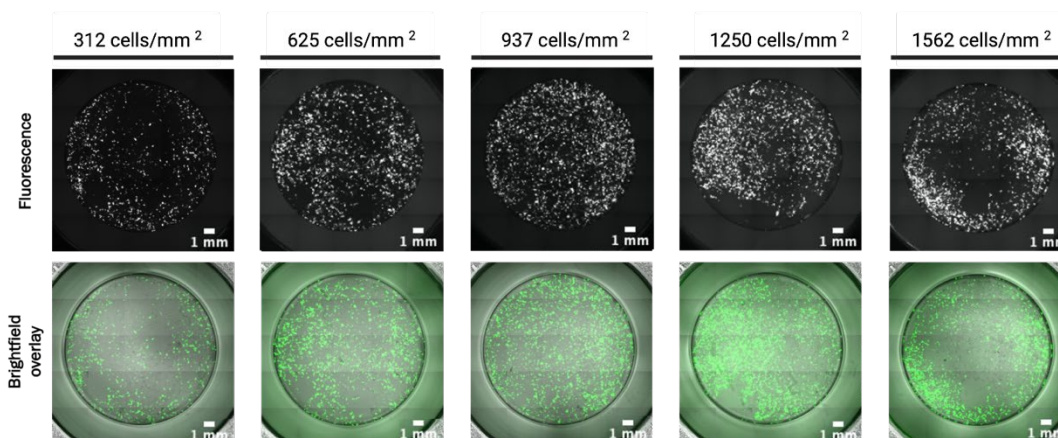


Figure 4.7 – Seeding density affects transfection efficiency. hiPSC-CMs were replated into 96 well plates at a range of densities from 312 to 1562 cells/mm². Three days post seeding, they were then transfected with the pmaxGFP vector using the Viafect™:DNA 6:1 and 10% serum conditions previously determined. Images taken live by high content fluorescence 72h post transfection. Upper panel fluorescent only, lower panel fluorescent with brightfield overlay. Scale bar = 1mm

4.3.3 Derivation of hiPSC-CMs and viability post transfection

Transfection can have unintended effects. For the assays to be performed post-transfection, high hiPSC-CM purity is required. To ensure that Viafect™ transfection did not influence hiPSC-CM purity, it was quantified using high content imaging of α -actinin stained hiPSC-CMs. In brief, hiPSC-CMs were transfected with pmaxGFP and fixed on the day of fluorescence imaging (72h). Control and transfected hiPSC-CMs were stained with α -actinin, cell mask and DAPI (Figure 4.8A-C). Quantification shows no significant difference in hiPSC-CM purity post transfection with Viafect™ (Figure 4.8D), confirming this method is suitable for the downstream phenotyping applications.

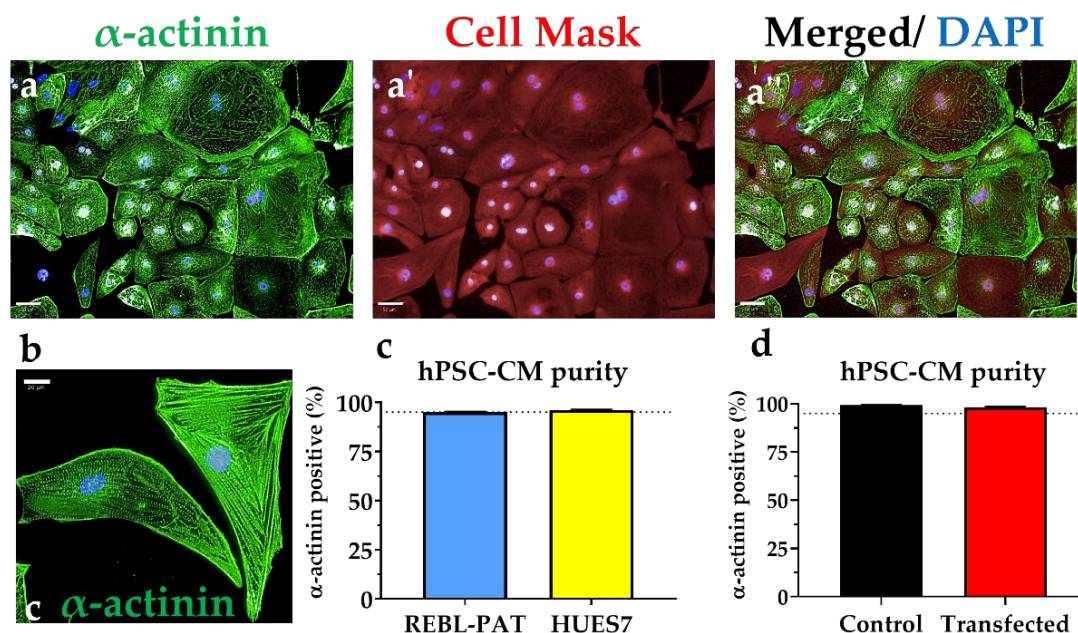


Figure 4.8 – Determination of the purity of human pluripotent stem cell- cardiomyocytes (hiPSC-CMs) used in this study. a–a'') A high-content imaging method was used to quantify the percentage of cells (counterstained by a cell mask) expressing the cardiomyocyte marker α -actinin, labelled by immunostaining (scale bar = 50 μ m). **b)** Sarcomeric banding exhibited by the cardiomyocytes immunostained for alpha-actinin (scale bar = 20 μ m). **c)** Purity of cardiomyocytes resulting from the cardiac differentiation of hiPSC-CMs is typically $\geq 95\%$ (dotted line). **d)** hiPSC-CMs retain high purities upon transfection with Viafect. Data: mean $\pm$ SEM, N=5 biological replicates performed across 8 wells. Adapted from supplementary information from [290]

A successful transfection protocol is not as simple as just achieving high transfection efficiency. High transfection efficiency is inconsequential if the cells lose viability post transfection. Subsequently, it was determined if transfection with Viafect™ bears any impact on hiPSC-CM viability. The cells were fixed on the

day of GFP imaging and immunostained with DAPI and the cardiac marker cardiac troponin T (cTnT). Using high content imaging, the number of cells expressing cTnT in control or transfected hiPSC_CMs was determined. Quantification shows that there was a 33% decrease in hiPSC-CM viability in transfected cells compared to the control (**Figure 4.9**). A viability of 66% is satisfactory for phenotypic assays and as shown in Chapter 5, it was possible to perform all assays desired.

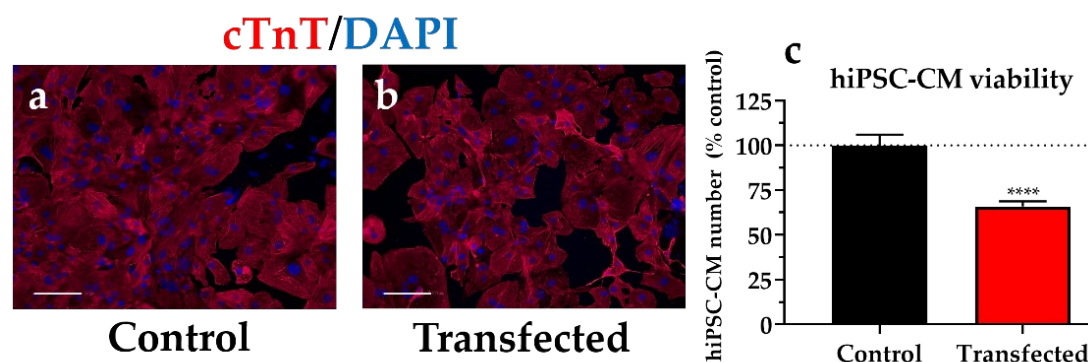


Figure 4.9 – Effects of transfection on hiPSC-CM viability and phenotypic assays. High content imaging of cells stained with the cardiac marker cTnT. **a–c)** Transfected hiPSC-CMs showed a ~34% reduction in cell numbers relative to the untransfected controls, indicating a ~66% cell viability, which still ensured compatibility with the phenotypic assays. **** $p < 0.001$, unpaired Student's t test. Data: mean $\pm$ SEM, N=5 biological replicates performed across 8 wells (scale bar = 100 μ m); cTnT – cardiac Troponin T. Adapted from supplementary information from [290]

4.4 Discussion

This work focussed on optimising a quick and easy transfection protocol for hiPSC-CMs reaching the high efficiencies possible with viral transduction (~95%) without the associated biosafety risks and complexity of viral engineering. In summary, the optimal conditions were: medium supplementation with 10% FBS, a ratio of Viafect™:DNA at 6:1 and a seeding density between 937 and 1250 cells/mm² per well of a 96 well plate. It was also observed that the optimal time to transfect for high efficiencies was three days post dissociation (seeding). Transfecting too soon after plating was detrimental to the efficiency, as were longer timelines.

4.3.1 Addition to field and application in this thesis

Efficient transfection protocols are necessary to fully exploit the potential hiPSC-CMs possess. They are a crucial tool for bridging the gap between molecular mechanisms and *in vivo* animal studies with human biological relevance. As discussed in the introduction, current transfection methods are lacking. While

previous studies using liposomes achieved greater cardiomyocyte viability upon transfection (80-92%) [279], the transfection efficiency was much lower (56%) than reported in this protocol (95%). Furthermore, although transfection with Viafect™ did reduce the viability of the cardiomyocytes to 66%, the cell loss upon transfection did not prevent further phenotypic assays.

Viafect™ transfection does not require the purchase of specialised equipment required for the physical methods of transfection and functions without the tediously long pre-complexation incubation periods. Therefore, for short term studies that intend to assess the effect of gene modulation, this transfection method allows the quick turnaround of results with fewer associated costs. It should be mentioned that while the key parameters optimised are generally applicable across hiPSC-CM lines, the protocol should be optimised for each cell line as there are many hiPSC-CM culture methods [292] which could affect the outcome.

The optimised method was applied in this thesis for the transfection of vectors into hiPSC-CMs, resulting in the overexpression of lncRNA targets and assessment of the phenotypic effects that ensued. Discussed in greater detail in chapter 3, this vector-based transfection approach using Viafect™ negated the laborious development of a CRISPRa or inducible system and permits higher expression levels. However, depending on the desired research outcomes, hiPSC gene editing might be a more efficient way to overcome hiPSC-CM transfection difficulties, as the resulting differentiated cells will continue to express the transgene. For the creation of lncRNA knockouts in this thesis, hiPSC gene editing was performed to ensure complete disruption to their expression using a previously optimised nucleofection protocol. With optimisation, it would have been possible to use Viafect™ to also create stable lncRNA knockout lines.

4.3.2 Other Considerations

For most experiments that utilise transient transfection, the cells are collected 24-96h post transfection as the transgene expression is short lived. This is due to factors such as cell division, half-life of gene expression and protein turnover [293]. The previously accepted notion that there are a fixed number of

cardiomyocytes in the human adult heart has been questioned and within the field there are two opposite views held. One side believes that the number of cells within the heart remains constant over life while the other believes that there is continuous cell turnover. There have been studies conducted using carbon-14 dating of cardiomyocytes [294, 295] and others using visualisation and quantification of individual cardiomyocytes [296]. These studies arrived at different conclusions about the rates of proliferation in human adult hearts, which suggested at the time that the main barrier to determining cardiomyocyte proliferation rates comes down to the requirement of a better technique. A more recent study in 2019 composed a novel technique to sort hiPSC derived cardiomyocytes populations enriched for mitotic events, using molecular beacons, single cell RNA sequencing and fluorescence activated cell sorting [297]. Using this technique, it was possible to separate cells in different events i.e., binucleated, polyploid and true cytokinetic cardiomyocytes, assessing the relative expression of two cell cycle genes. The authors found that hiPSC-CMs transitioned from cytokinetic to acytokinetic as differentiation progressed. Limited cell division in hiPSC-CMs is likely the reason behind the sustained expression of the transgene in the method derived in this thesis. Mitotic events did not result in the dilution of foreign DNA, consequently the only limiting factor was the degradation of DNA over time.

The cell culture technique used in this thesis avoided the use of serum supplementation in the medium due to potential masking effects of hypertrophy [298], which would affect experimental outcomes. Despite this, the optimisation process determined that the use of serum improved transfection efficiency. hiPSC-CMs were exposed to serum for a short period of time as medium was changed the day following transfection. There were no obvious observed detrimental effects such as toxicity to the cells or changes in morphology, surmising that the restricted use of serum during transfection avoids compromising experimental outcomes. An even lower ratio of Viafect reagent to DNA could negate the need of serum. However, whether the reduction of cationic reagent would impact the efficiency of transfection is unclear. A further experiment would be required to determine this. The majority of cationic delivery reagents advise against the use of serum due to effects on the DNA-Lipid complex, reducing transfection efficiency

[299]. The composition of Viafect™ is proprietary, however it could be speculated that serum does not affect the lipid charge and therefore it is an excellent alternative for cells that cannot be cultured without the use of serum.

5. Phenotyping impact of lncRNA modulation in hiPSC and hiPSC cardiomyocytes

5.1 Introduction

The complexity of HCM makes it difficult to predict disease phenotypes resulting in treatment based on patient mutations. As discussed in the introduction (section 1.4), the possible effect of secondary alterations, essentially disease modifiers, having a role in disease progression is becoming greatly accepted [300].

5.1.1 Assessing Hypertrophy in HCM

Cardiac hypertrophy is characterised by the enlargement in size of cardiomyocytes, not an increase in number (hyperplasia), leading to a thicker ventricular wall and reduced interior dimension of the chamber [301]. Hypertrophy is a hallmark of HCM, in response to altered contractility and/or physiological stress, the cardiomyocytes enlarge in an attempt to maintain cardiac output. In this sense, HCM is compensatory as it normalises stress.

The eventual outcomes of the compensatory nature of HCM are heart failure and death, as a result of the numerous structural and functional changes that occur due to changes in the underlying mechanisms governing cardiac function. These changes include reactivation of the foetal gene program leading to hypertrophy and altered energy efficiency and changes in metabolic function. These changes are described in the following sections.

5.1.2 Molecular changes in HCM

Two natriuretic peptide hormones are expressed by mammalian hearts, atrial natriuretic factor (ANF) and B-type natriuretic protein (BNP). The expression of the genes encoding these factors are downregulated after birth. In response to cardiac stress imposed by HCM and heart failure stress, the foetal gene program is reactivated [73]. Reactivation of BNP has widely been used as a marker of hypertrophy *in vitro* and *in vivo* [302]. Using an *in vitro* mouse stretch model which recapitulated the increased loading effects that result in BNP activation, endothelin-1 was implicated in the upregulation of BNP expression [303].

Conversely it was found the receptor antagonist of endothelin-1, bosentan, reduced the expression of BNP. More recently and more biologically relevant to humans, a high throughput assay using hiPSC-CMs as a model of hypertrophy was developed to demonstrate that exposure of endothelin-1 led to robust BNP expression [304]. This high content imaging-based method is especially useful when assessing multiple cell lines and parameters such as in this thesis and provides a simple and quick assay base to perform the required phenotyping.

There are two isoforms of myosin heavy chain, α -MHC and β -MHC, which share a high degree of sequence homology, yet remarkably different enzymatic properties. β -MHC, coded for by *MYH7*, has lower ATP activity and is more energy efficient in comparison to the α -MHC isoform, encoded by *MYH6* [178]. During human development, α -MHC is the predominant isoform during foetal development whereas β -MHC takes over as primary isoform in late development. Cardiac damage (for example resulting from HCM) can revert MHC expression to the foetal like expression pattern (β -MHC $\rightarrow\alpha$ -MHC) [305]. The opposite is true of rodent hearts. During rodent cardiac development, β -MHC is the predominant isoform, switching to α -MHC immediately before birth [306]. Cardiac damage in rodents also causes a return to foetal MHC expression (α -MHC $\rightarrow\beta$ -MHC). It has recently been discovered that *lncMYH7b* regulates the expression of β -MHC to α -MHC [307].

5.1.3 Energy depletion hypothesis in HCM

The human heart has the greatest oxygen requirement in the body, at rest requiring 8mL O₂/min/100g, a 2.6 fold increase than the requirements of the brain [308]. The heart also consumes more energy per gram of tissue than any other organ, primarily using oxidative phosphorylation of the mitochondria to produce ATP [309]. The sliding filament theory is the basis of sarcomeric contraction. An increase of cytoplasmic Ca²⁺ from the sarcoplasmic reticulum on arrival of an action potential triggers the binding of myosin heads to actin, forming a cross bridge. Contraction occurs by the shortening of the sarcomere; the myosin heads pull the actin filaments inwards (known as a power stroke) using ATP as a source of energy. The power stroke results in the release of ADP and an inorganic

phosphate. With the binding site of ATP now available on the myosin, a new ATP molecule attaches, and the cross bridge detaches, **Figure 5.1**. Sodium-calcium exchange pumps then restore the Ca^{2+} concentration and the cytoplasm relaxes [53]. High rates of ATP synthesis are required to maintain the balance between cardiac systolic (contractile) and diastolic (relaxed) function. (**Figure 5.2**).

The energy depletion hypothesis in HCM suggests that inefficient usage of ATP underlies cardiac dysfunction [310]. This can occur due to an increase of and in incorporation of mutant proteins to the sarcomere. In HCM caused by mutations in *MYH7*, the cycling rate of myosin heads is altered, thereby compromising the actin-myosin interaction as the detachment of mutant bridges occurs before wild type [311] (**Figure 5.1 ii and iii**). This can lead to ATP hydrolysis before the generation of force, which imposes metabolic stress and increased mitochondrial respiration to cope with the energetic demands.

In pursuance of understanding whether modulation of *MINCR* expression affects mitochondrial respiration, the oxygen consumption rate (OCR) of untransfected and *MINCR* transfected hiPSC-CMs was measured using the Seahorse XF platform. As shown in **Figure 5.3**, the sequential addition of electron transport chain modulators reveals the parameters of mitochondrial respiration, those modulators are added in the following order; **1**) oligomycin, injected following basal measurements, inhibits ATP synthase, revealing cellular ATP production. **2**) FCCP, an uncoupling agent that disrupts the mitochondrial membrane potential, uncovering spare respiratory capacity. **3**) Rotenone, an inhibitor which halts mitochondrial respiration.

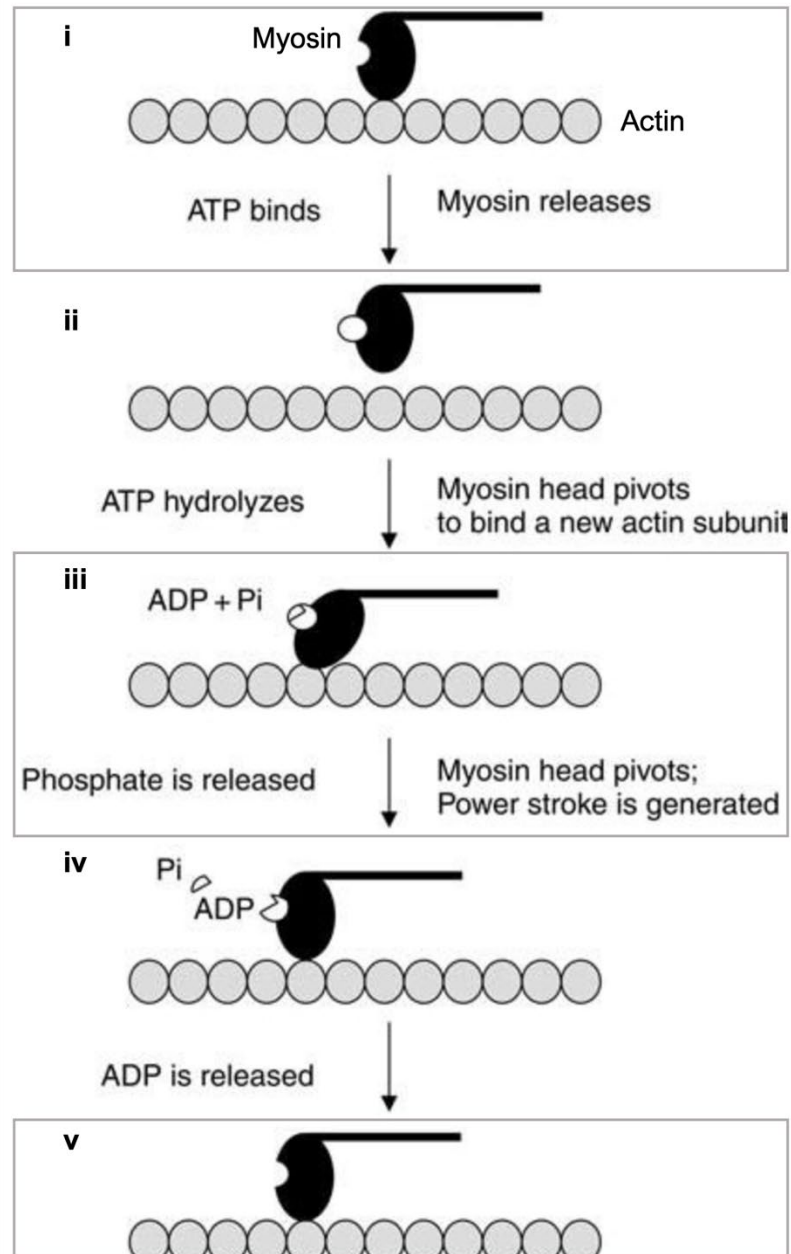


Figure 5.1 – Schematic of actin/myosin power stroke. A simplified version of actin and myosin power stroke where actin is represented by grey circles and myosin as a club shaped monomer. A more detailed depiction of sarcomeric components can be found in **Figure 1.3**. **i)** After the previous cycle, the myosin is bound to actin with weak forces known as rigor confirmation. **ii)** ATP binding to myosin causes a conformational change in the myosin binding site on actin, causing myosin to detach. **iii)** and **iv)** ATP binding causes the myosin neck to bend further along the filament and the ATP molecule is hydrolysed. The myosin head makes contact with the actin filament and the ADP and inorganic phosphate molecules are released, triggering a power stroke. **v)** Myosin returns to its original confirmation and the cycle is ready to repeat upon the binding of a new ATP molecule.

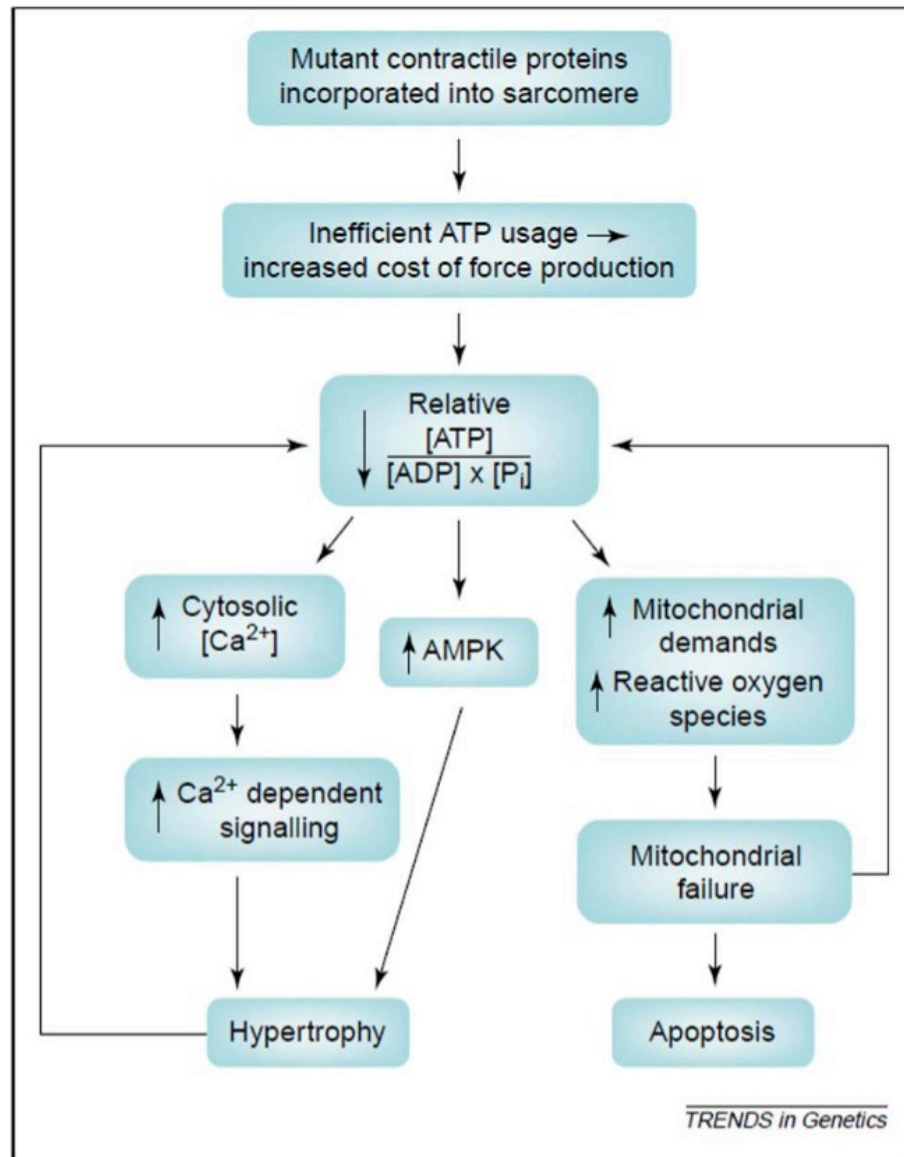


Figure 5.2 – Energy depletion hypothesis as a result of sarcomeric mutations in HCM. Inefficient ATP utilisation is caused by sarcomeric HCM mutations. This leads to an increased cost of force production, resulting in an energy deficit. If the energy supply cannot match the demand, homeostasis of functions such as Ca²⁺ are affected resulting in arrhythmias. Increased production of reactive oxygen species due to increased mitochondrial demands leads to apoptosis. Adapted from [312] with permission of the publisher.

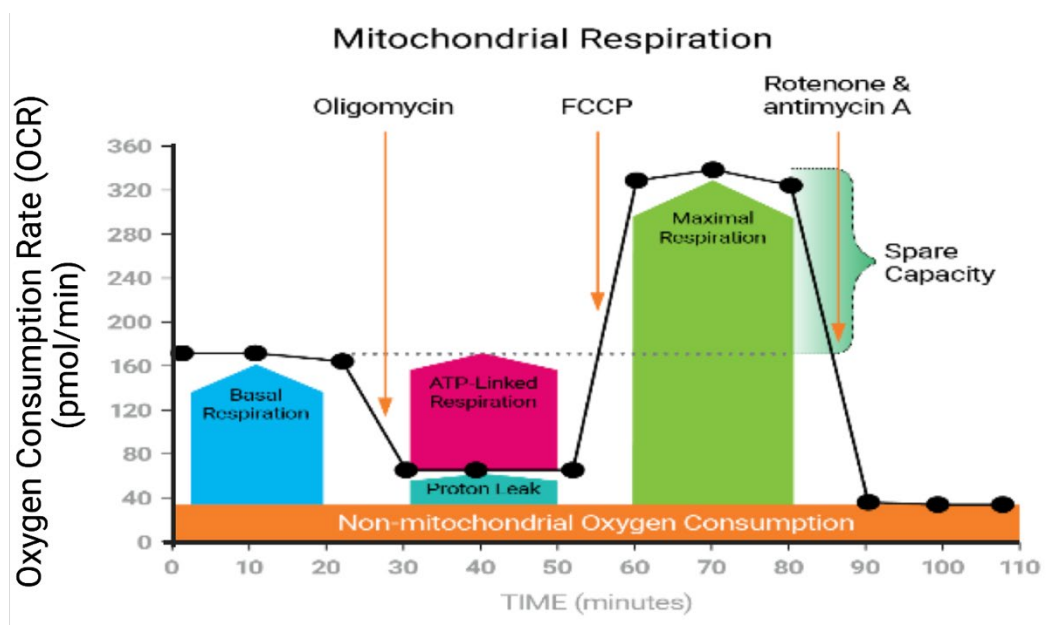


Figure 5.3 – Analysis of mitochondrial respiration using the Seahorse platform. A test profile of the parameters of mitochondrial respiration that can be assessed. Sequential compound injections facilitate the measurement of basal respiration, ATP production, proton leak, maximal respiration spare capacity and non-mitochondrial respiration. (Adapted from Agilent user manual).

5.1.4 *MINCR* role in hiPSC survival

As mentioned in Chapter 3.2.5, the observations of clone 5 led to a hypothesis that *MINCR* may be involved in hiPSC proliferation and/or survival. An exploration of the literature in the field of cancer research corroborated this hypothesis, as *MINCR* knock outs had been associated with reduced cellular proliferation (further discussion in section 4.7). With the little sample material available, the expression of *MINCR* was quantified in clone 5 using qRT-PCR. With a fixed sample, traditional proliferation assays were not an option. Instead, apoptotic levels were assessed by staining cleaved caspase 3.

5.1.5 Chapter Aims and Objectives

This chapter examines the effects of applying the modulation systems devised in Chapter 3 to the HCM model by assessing the phenotypic outcomes. In this chapter, assays to assess cardiac hypertrophy, mitochondrial respiration and the expression of *MYH7/6* were performed in lncRNA target overexpression and knockdown systems in the HCM model. Preliminary characterisation was also performed on a *MINCR* knockout clone.

5.2 *MYH7*^{MUT/MUT} *MINCR* KO Clone 5 Characterisation

5.2.1 Brightfield Images

As described in Chapter 3.2.4, transfected cells were selected, dissected, and expanded from small well formats up to T25 flasks. This requires the cells to actively proliferate, which reseeding into a larger culture vessel from a confluent vessel should prompt. Viewing clone 5 *MINCR* knockout in *MYH7*^{MUT/MUT} under a light microscope led to the observation of unusual characteristics such as accumulation of aggregates on top of the cells and a lack of proliferation. Furthermore, whilst all other clones from the same batch had been expanded into T25 flasks, clone 5 was split from a 48 well plate to two wells of 96 well plate to prompt proliferation. **Figure 5.4** depicts clone 5 24h prior to and post seeding, demonstrating from an observational perspective the poor health of the cells. Due to the difficulties in maintaining clone 5, it was not possible to extract genomic DNA for genotyping, nor was it possible to perform a cell proliferation assay. It was possible to fix one well for ICC and lyse the other for RNA extraction using a specialised kit for little starting material, to determine whether *MINCR* expression was reduced and if *MINCR* knockout resulted in impaired proliferation in hiPSCs.

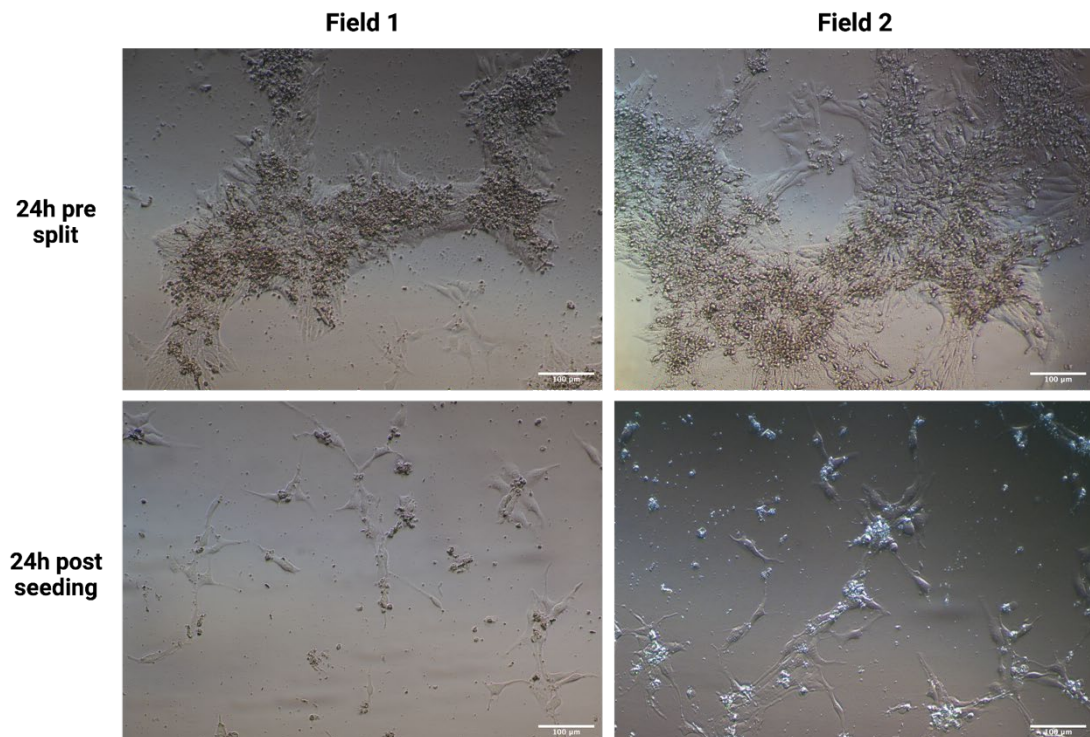


Figure 5.4 – Re-seeding of *MYH7*^{MUT/MUT} *MINCR* KO Clone 5. Brightfield images of clone 5 in two fields of view, 24h pre and 24h post re-seeding. Scale bar = 100µm.

5.2.2 qRT-PCR and Immunocytochemistry

To determine whether *MINCR* knockout is involved in promoting apoptosis, the expression of cleaved caspase 3 was measured by immunostaining. Using 4µg/mL camptothecin, a topoisomerase inhibitor which induces apoptosis through DNA damage, the expression levels of cleaved caspase 3 in clone 5 and +/-VE controls were assessed. hiPSCs were co-stained with DAPI, Oct4 and anti-cleaved caspase 3 (**Figure 5.5**).

To quantify the cleaved caspase 3 expression, an image analysis sequence was developed for high content imaging, demonstrated in **Figure 5.6A**. The algorithm determines the intensity of cleaved caspase 3 in the whole cell region and uses the total cell population (DAPI) to calculate the percentage of cells expressing cleaved caspase 3. The cells were stained with DAPI, anti-cleaved caspase 3 and Oct4 as a positive hiPSC marker. DAPI identifies cell nuclei followed by removal of nuclei clumps. The cytoplasm is found using cleaved caspase 3 expression and the algorithm determines cells expressing cleaved caspase 3 by signal intensity. Quantification showed that a greater percentage of hiPSCs were expressing cleaved caspase 3 in clone 5 compared to the negative control (**Figure 5.6B**), suggesting that the removal of the *MINCR* gene is involved in arresting the cell cycle and promoting apoptosis.

An RNA sample of clone 5 was obtained by lysis with Trizol, processing with the Nucleospin RNA XS Microkit and reverse transcription using Superscript IV. This yielded enough material for qRT-PCR to assess the level of *MINCR* expression compared to untargeted MYH7^{MUT/MUT} hiPSCs. The qRT-PCR analysis showed a 99% reduction in fold change of *MINCR* expression in clone 5 compared to untargeted hiPSCs, strongly suggesting that *MINCR* had been knocked out (**Figure 5.6C**). Although verification using the genotyping strategy would be a superior method to confirm this (also a greater number of replicates), the specificity of Taqman probes provides some reassurance of the observed result.

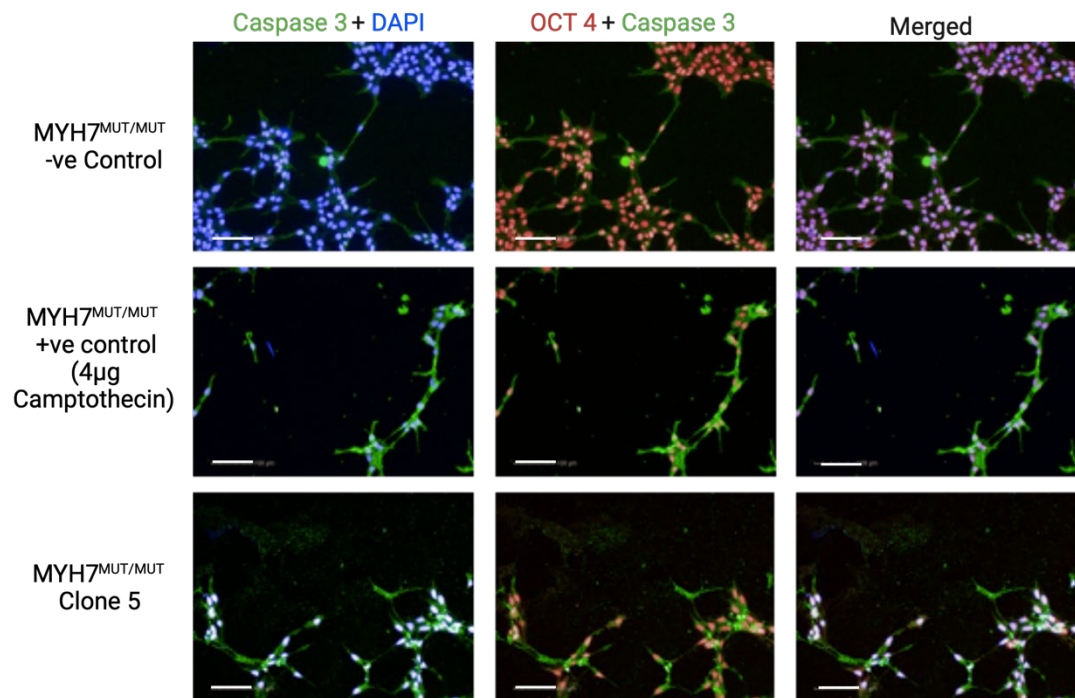


Figure 5.5 – Cleaved caspase 3 expression in untargeted hiPSC, untargeted camptothecin treated hiPSC and *MINCR* KO hiPSC. Representative fluorescent micrographs of MYH7^{MUT/MUT} untargeted control, 4μg/ml camptothecin treated untargeted control and MYH7^{MUT/MUT} clone 5 stained with anti-cleaved caspase3/Oct4/DAPI. Scale bar=100μm.

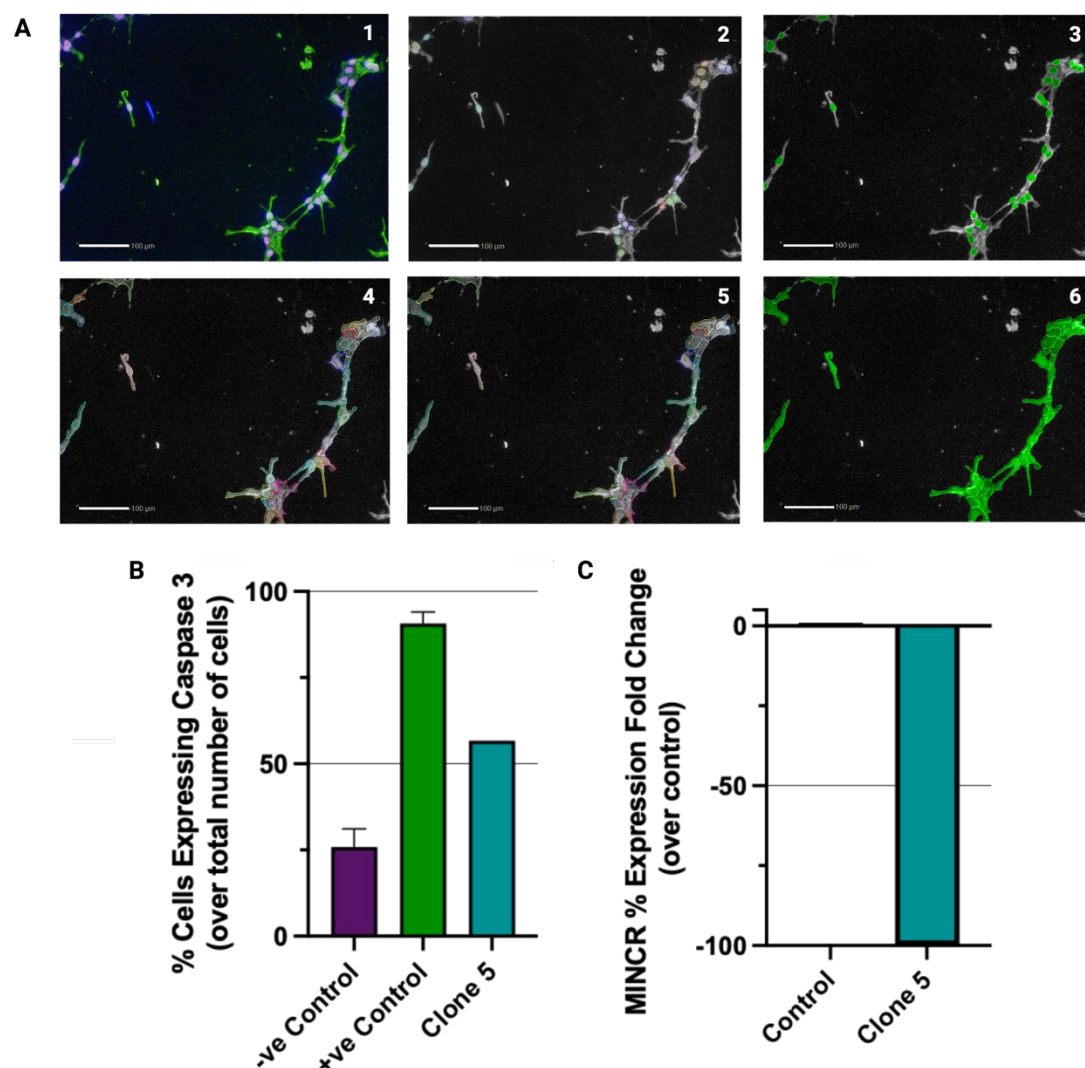


Figure 5.6 – High content imaging analysis sequence for the quantification of caspase 3 expression and characterisation of *MYH7*^{MUT/MUT} *MINCR* KO Clone 5. **A)** Input images of **1)** Merged DAPI/Caspase 3/Oct4. **2)** Find nuclei using DAPI channel, **3)** remove nuclei clumps if present. **4)** Find cytoplasm using caspase 3, **5)** find cell area, **6)** select caspase 3 positive cells (in green) using signal intensity. Percentage cells expressing caspase 3 calculated by dividing total cells expressing caspase 3 by cell number (DAPI), multiplied by 100. Scale bar = 100µm. **B)** Quantification of cleaved caspase 3 expression in *MYH7*^{MUT/MUT} untargeted hiPSCs (-ve control), *MYH7*^{MUT/MUT} hiPSCs treated with 4µg/mL camptothecin (+ve control) and *MYH7*^{MUT/MUT} *MINCR* KO clone 5. (Controls N=2, clone 5 N=1). **C)** qPCR analysis of *MINCR* expression in clone 5 showed reduced expression in comparison to untargeted *MYH7*^{MUT/MUT} (N=1). More biological replicates are required to confirm this observation.

5.3 lncRNA Overexpression Results

5.3.1 Assessing overexpression vector capability

In Chapter 4, an effective combination cell density, serum concentration and Viafect™:DNA ratio was defined to provide transfection efficiencies of *circa* 95% in the hiPSC-CMs used in this thesis. Prior to deploying the phenotyping assays described above, it was first necessary to define when expression peaked after transfection using the CAG overexpression vectors described in Chapter 3. Therefore, hiPSC-CMs derived from the *MYH7*^{WT/WT}, *MYH7*^{WT/MUT} and *MYH7*^{MUT/MUT} lines were transfected with CAG-lncRNA vectors. Samples were harvested at 4 days and 7 days post-transfection. These timepoints were chosen based on the known phenomenon of cell proliferation and nuclease degradation leading to reduced expression of the transgene. RNA was extracted and subjected to qRT-PCR analysis using Taqman assays to enable the ratio of lncRNA target relative to the housekeeping gene, *TNNT2*, to be defined. The rationale for using *TNNT2* is that it is uniquely expressed in the heart [313], and shows low variability between samples under the experimental conditions.

Figure 5.7 displays the results obtained by normalising all results to *TNNT2* and determining the fold change in comparison to each cell line's respective untransfected control. Transfection using target 1 vector led to a fold change between 5000-30,000 greater than the untransfected controls in both timepoints (**Figure 5.7A**). Similarly, the *MINCR* overexpression elicited fold changes between 3000-8000 (**Figure 5.7C**). On the other hand, target 2-fold change was negligible compared to untransfected in all lines and across both timepoints (**Figure 5.7B**).

A possible reason for the differences in overexpression between target 1 (5.6kb) and *MINCR* (1.5kb) relative to target 2 (659 bases) is that the size of the RNAs causes differential harvesting with the extraction kit used. The data in **Figure 5.3 A-C** were produced using kit 1, which is most effective at harvesting transcripts of greater than 200 bases. Therefore, hiPSC-CMs from line *MYH7*^{WT/MUT} were transfected with the target 2 overexpression vector and RNA was harvested 4 days later using a Trizol based RNA extraction approach (kit 2), which is designed to capture RNAs as short as 17 bases. The data in **Figure 5.3D** shows the ratio of

target 2 overexpression was elevated by 10-fold using the Trizol approach. However, this was still substantially lower than the 3,000 to 30,000 ratios observed for target 1 and *MINCR*.

In summary, these data demonstrated that the optimised transfection protocol from Chapter 4 was effective at permitting overexpression of targets 1 and *MINCR* in all three isogenic hiPSC-CM lines, with similar levels observed at both day 4 and day 7 post transfection. Therefore, day 4 was used for future experiments to provide a more rapid turnaround for phenotyping results. Since not all data are significant, despite the high fold change in expression there may be some variability which can be combatted with a greater data set of biological replicates. In contrast, overexpression of target T2 could not be demonstrated effectively, even when an RNA isolation approach designed for short transcripts was used. However, within the context of this thesis, there was insufficient time to explore whether this was an issue of transfection, expression, transcript stability or the isolation method.

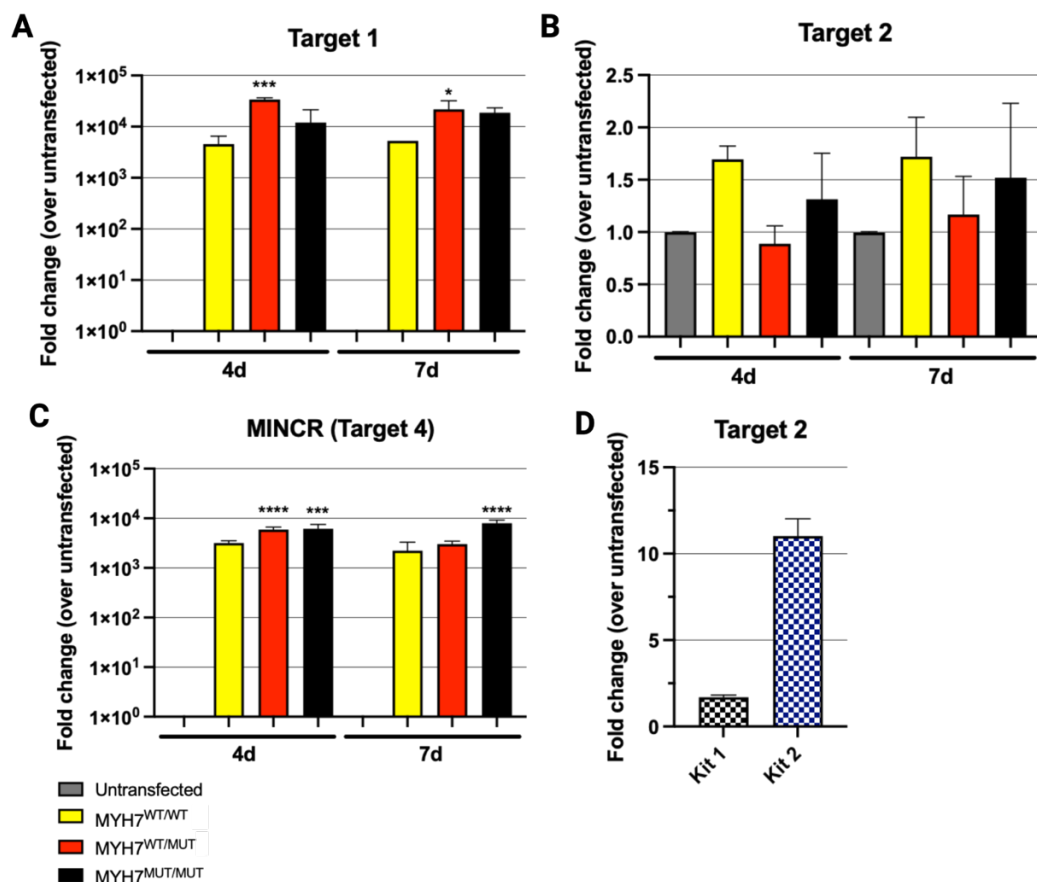


Figure 5.7 – Expression of targets in *MYH7* lines lysed 4d and 7d post transfection with overexpression vectors. qRT-PCR analysis showing increased levels of expression in comparison

to untransfected cells in **A**) target 1 (*MYH7*^{WT/WT} 7d, N=1), **B**) target 2 and **C**) *MINCR*. **D**) shows increased expression of target 2 at 4d when using an alternative RNA extraction method (N=2). N=3 unless stated otherwise. *TNNT2* was used as a housekeeping gene.

5.3.2 Hypertrophy Analysis

Cardiac hypertrophy is associated with increased expression of cardiac markers, frequently analysed is BNP. To assess the effect of overexpressing the lncRNA targets on hypertrophy, the isogenic trio of hiPSC-CMs were transfected with the overexpression vectors for target 1, target 2 and *MINCR*. After 4 days post transfection, secretion of BNP from the cells was blocked using Brefeldin A, a protein transport inhibitor. This enabled immunostaining and subsequent high content imaging analysis of the BNP signal in untreated controls and endothelin-1 (maximises BNP expression) and bosentan (reduces BNP expression) treated cells (**Figure 5.8**).

To quantify the BNP expression, an image analysis sequence was developed for high content imaging, demonstrated in **Figure 5.9**. The algorithm depends on determining the intensity of BNP in the perinuclear region. This is accomplished by co-immunostaining BNP with DAPI and cTnT; the DAPI signal identifies cell nuclei, and the cardiac marker cTnT establishes the cell cytoplasm. The algorithm then selects hiPSC-CMs based on the signal intensity of cTnT, defines the perinuclear region and selects BNP positive cells using signal intensity.

Figure 5.10 shows the results of BNP staining quantified using the high content imaging algorithm described above. In the *MYH7*^{WT/WT} line, there was a significant reduction of 20-25% in BNP expression in all lncRNA target transfected cells treated with endothelin-1 compared to the control (**Figure 5.10A**). In contrast, overexpression of any of the lncRNAs had no significant impact on BNP expression when the hiPSC-CMs were either untreated or treated with bosentan.

These findings further contrast with the observations for the mutant *MYH7*^{WT/MUT} and *MYH7*^{MUT/MUT} hiPSC-CMs, where the many combinations of target 1, target 2 and *MINCR* transfected lncRNAs with no treatment, endothelin-1 treatment or bosentan treatment caused significant reduction in BNP expression by 10% to 70%. The exception to this was target 1 overexpression in *MYH7*^{MUT/MUT} hiPSC-

CMs in untreated and bosentan treated, wherein no significant change was observed (**Figure 5.10B and C** respectively).

These results are intriguing as endothelin-1 induces expression of BNP, mimicking HCM, yet it appears that overexpression of the lncRNA targets, in particular target 2 and *MINCR*, rescue this phenotype when compared to the untransfected control. Treatment with the endothelin-1 receptor antagonist bosentan decreases BNP expression and, in conjunction with lncRNA target 2 and *MINCR* overexpression, the phenotype is reduced. The *MYH7*^{MUT/MUT} line exhibited the most pronounced differences between the expression of BNP when maximised and rescued with drug treatment with overexpressed lncRNA targets. Since no change is observed in BNP expression in the healthy untreated and bosentan treated cells with lncRNA overexpression, yet in the diseased lines changes occur in all conditions, this could be evidence that these targets are acting as gene modifiers.

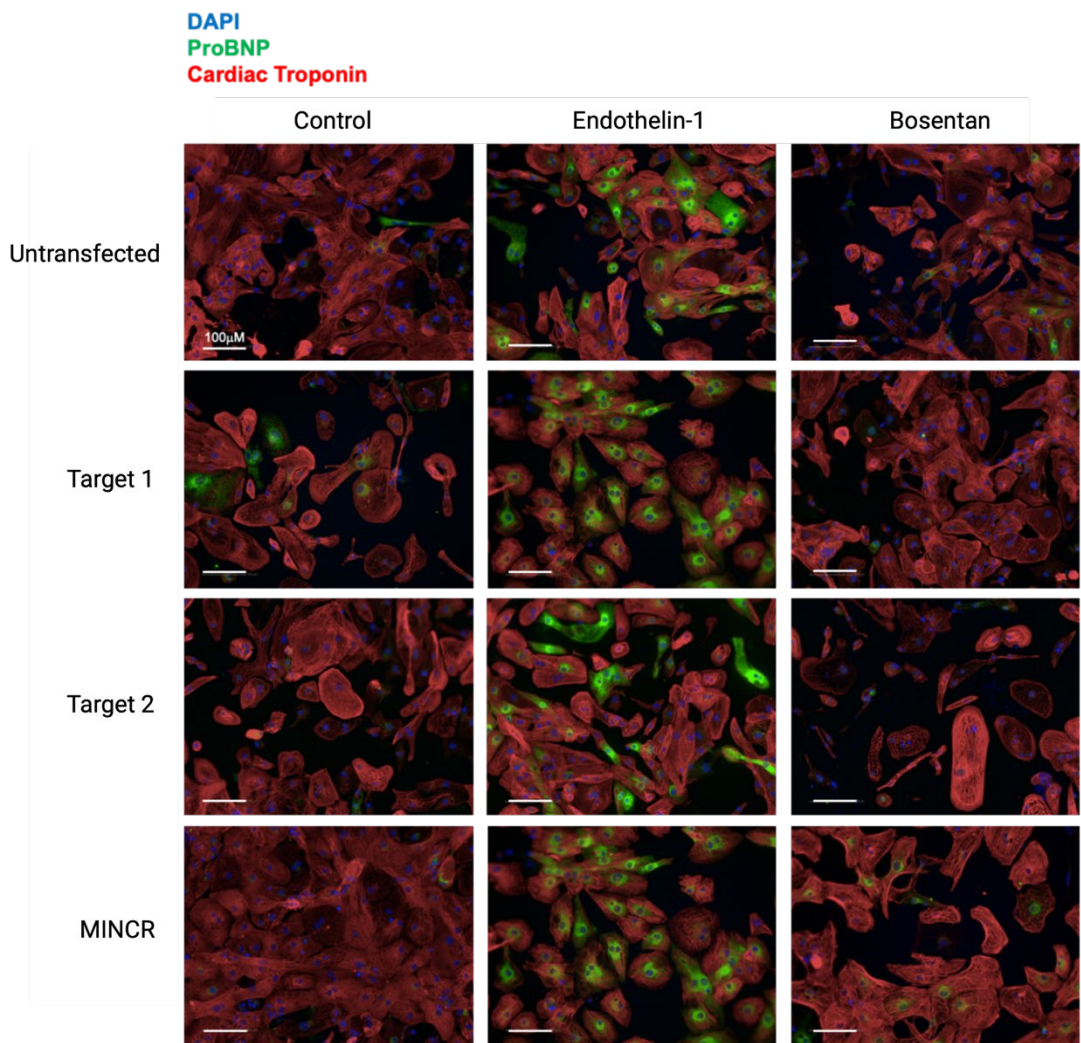


Figure 5.8 – Expression of hypertrophic marker BNP in hiPSC-CMs transfected with overexpression lncRNA targets. Representative fluorescence micrographs in of BNP/cTnT/DAPI stained *MYH7^{WT/WT}* derived hiPSC-CMs transfected with overexpression vectors, then treated with 10nM endothelin-1 or 100nM bosentan. Scale bars=100µm.

DAPI/ProBNP/cTnT

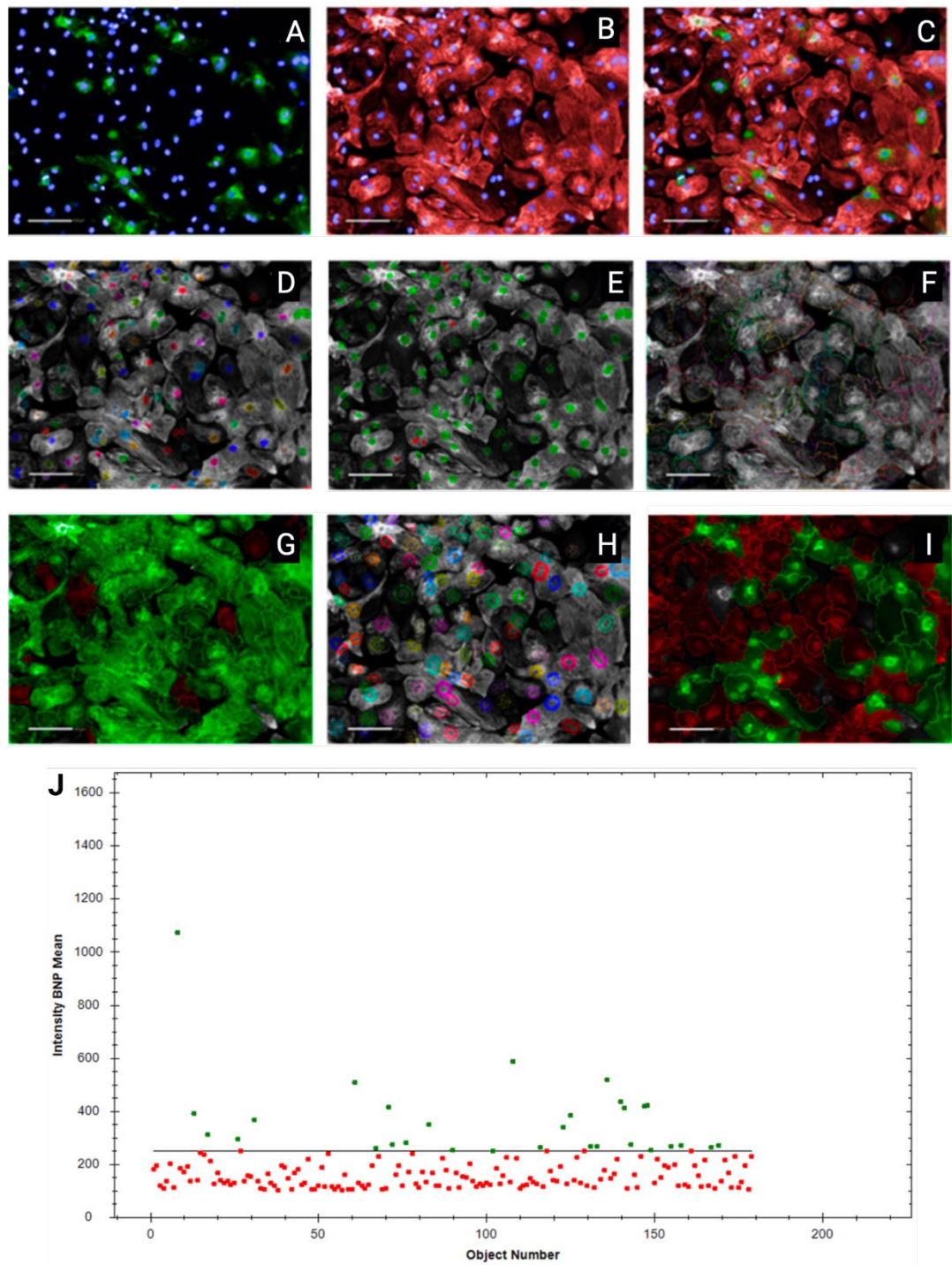


Figure 5.9 – High content imaging analysis sequence for the quantification of BNP expression. Input images of **A)** DAPI/ProBNP, **B)** DAPI/cTnT and **C)** merged DAPI/ProBNP/cTnT. **D)** Find nuclei using DAPI channel, **E)** remove nuclei clumps (red), **F)** find cytoplasm using cTnT cardiac marker, **G)** select cardiomyocytes using cTnT intensity, **H)** define the perinuclear region, **I&J)** select BNP positive cells (in green) using signal intensity. Adapted from [314].

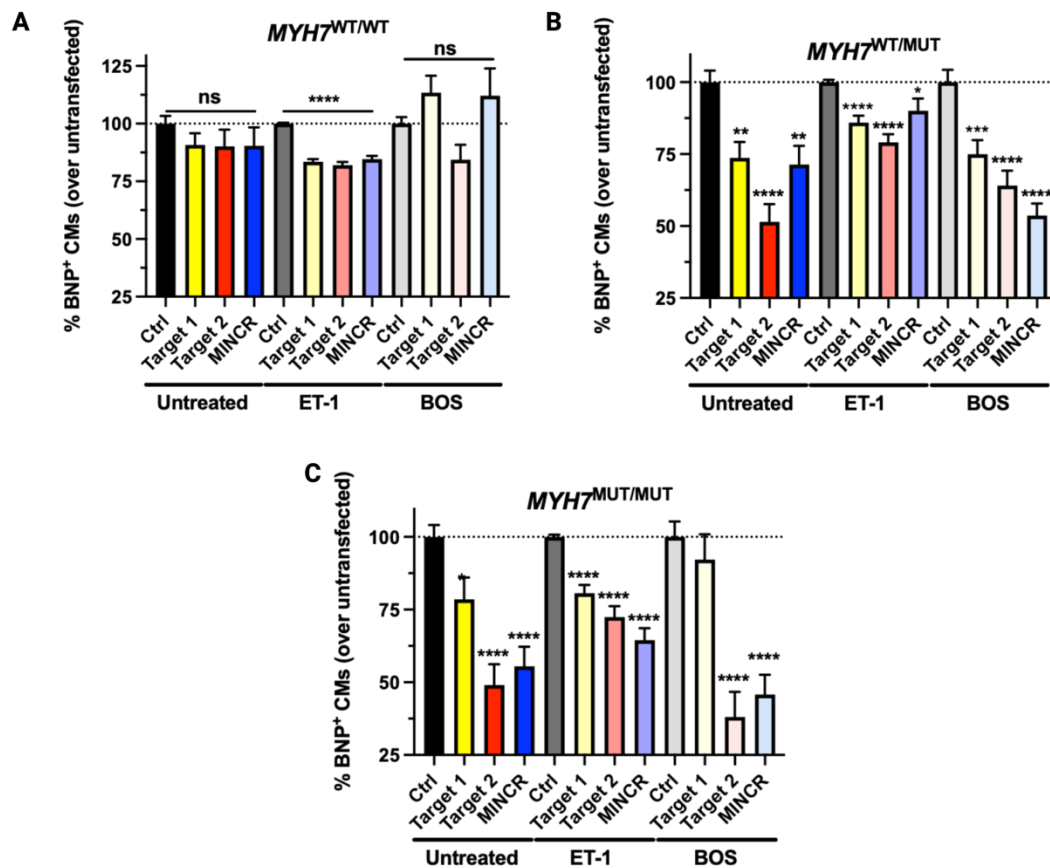


Figure 5.10 – Quantification of percentage of hiPSC-CMs transfected with overexpression vectors expressing BNP. **A)** In the healthy *MYH7^{WT/WT}* line, endothelin-1 treatment in the CAG transfected had significantly less BNP expression compared to its respective untransfected control. **B)** All *MYH7^{WT/MUT}* hiPSC-CMs transfected with lncRNA overexpression vectors displayed reduced BNP expression in comparison to their untransfected controls under all conditions. **C)** The *MYH7^{MUT/MUT}* hiPSC-CMs transfected with overexpression vectors also appeared to reduce BNP expression in all conditions, particularly targets 2 and *MINCR* under bosentan treatment. Data plotted as mean $\pm$ SEM, N=6. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.0001$, between lncRNA target overexpression transfected and respective untransfected controls.

5.3.3 Mitochondrial Respiration

As discussed in the introduction to this chapter, the energy depletion hypothesis suggests that inefficient ATP usage underlies cardiac dysfunction. Assessing mitochondrial respiration by measuring the oxygen consumption rate (OCR) can indicate whether there ATP respiration is occurring abnormally. In order to test whether *MINCR* overexpression in the hiPSC-CM cause a shift in the mitochondrial respiration, the OCR was assessed using the Seahorse XF platform. hiPSC-CMs derived from the *MYH7*^{WT/WT}, *MYH7*^{WT/MUT} and *MYH7*^{MUT/MUT} lines were transfected with the CAG-*MINCR* overexpression vector using the transfection protocol developed in Chapter 4. Three days after transfection, OCR was measured on the Seahorse platform using the sequential addition of 3 electron transport chain modulators: oligomycin, FCCP and rotenone. Upon completion of the assay, the cells were carefully washed, fixed, and subsequently stained with DAPI for the normalisation by cell number.

Analysis of mitochondrial respiration in *MYH7* lines exposed no significant differences of OCR in *MINCR* overexpressed hiPSC-CMs in comparison to the untransfected in any of the lines (**Figure 5.11**). Using OCR values across the assay, it is possible to calculate the parameters of mitochondrial respiration: basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity and non-mitochondrial respiration (**Figure 5.3**). Analysis into the parameters of mitochondrial respiration in *MINCR* overexpressing cells also reveal no significant differences in the overexpression of *MINCR* compared to untransfected (**Figure 5.12**). This suggests that overexpression of *MINCR* might not have any role in altering the function mitochondrial respiration in the HCM model.

Differences between lines were not analysed as they have been previously reported [7]. The lack of any obvious change stemming from lncRNA expression could be due to the slightly different conditions each cell line was exposed to, as seeding was performed so that each line was in a separate plate. It is possible that changes in OCR were not detectable by the Seahorse XF analyser and that perhaps a more sensitive platform is required. Further discussion is in section 5.6.3.

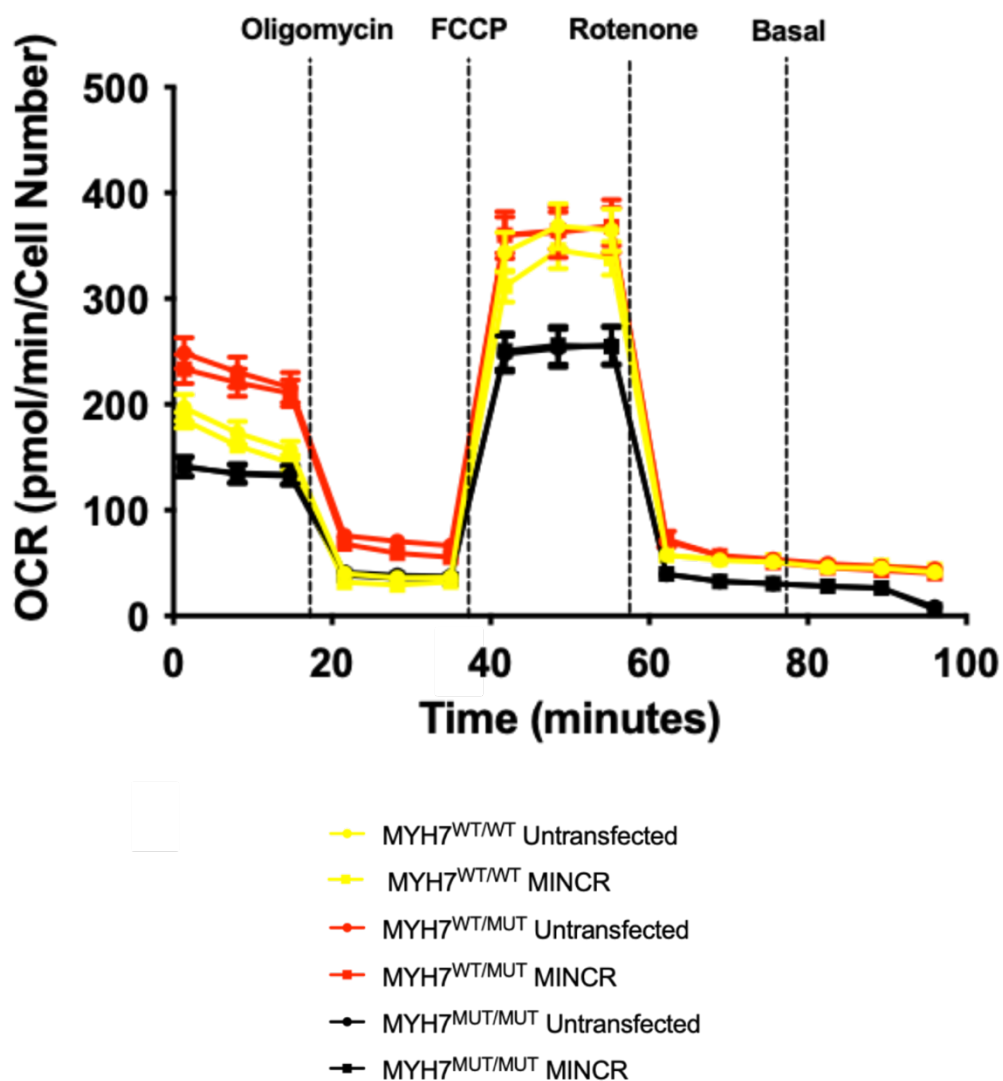


Figure 5.11 – Analysis of mitochondrial respiration in *MYH7* lines overexpressing *MINCR*, using the Seahorse platform. The respiration profiles show no significant difference between untransfected controls and *MINCR* overexpression.

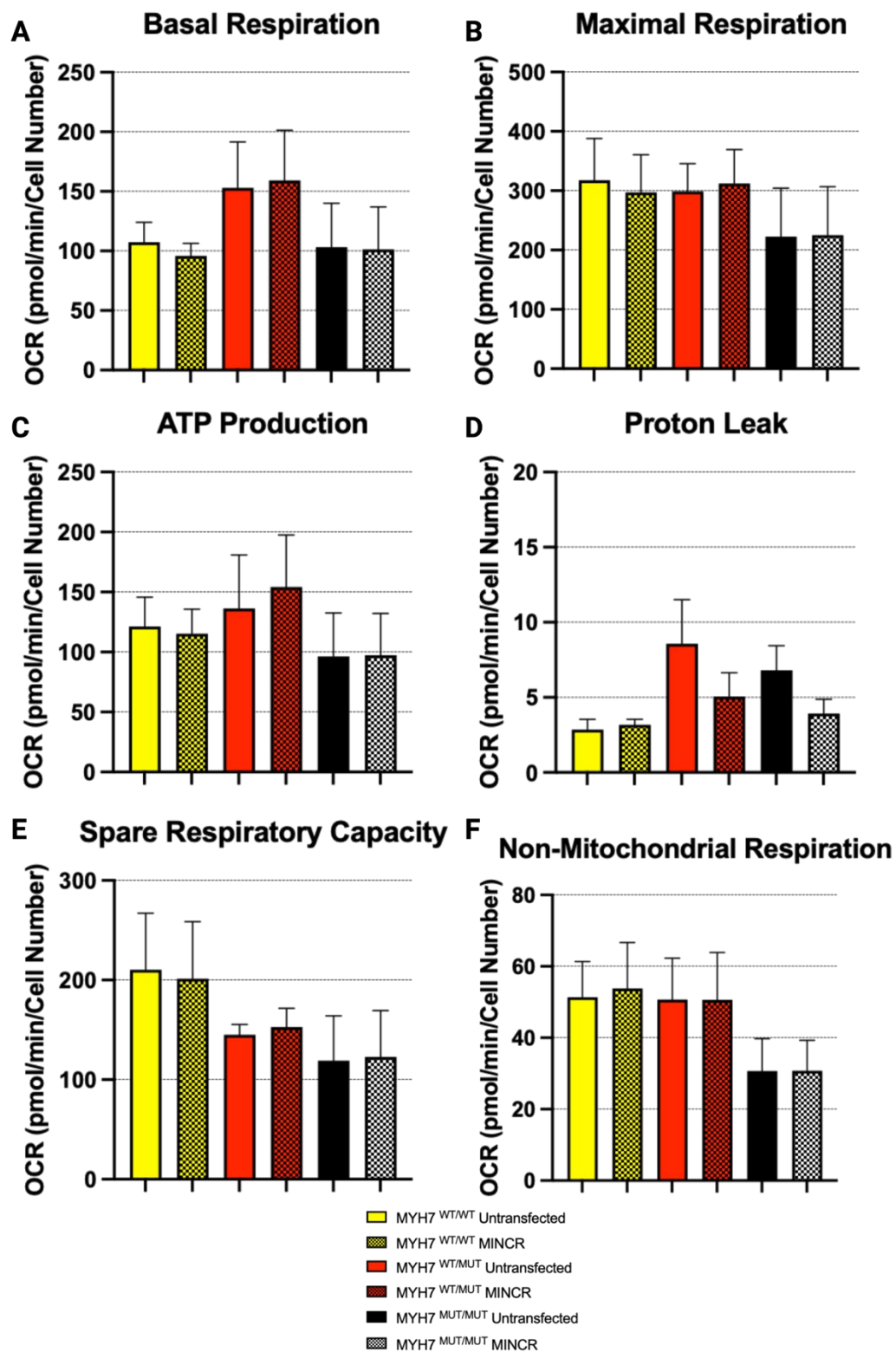


Figure 5.12 – Mitochondrial respiration parameters of *MYH7* hiPSC-CMs overexpressing *MINCR*.
A-F) No significant difference in any of the parameters between the *MINCR* overexpressed and untransfected controls in each line.

5.3.4 *MYH7/MYH6* Expression

As discussed in the introduction, the balance of α -MHC and β -MHC expression is tightly regulated. A shift in the ratio of β -MHC/ α -MHC, where α -MHC expression is reduced and β -MHC expression symbiotically increases, is associated with HCM. To assess whether overexpressing the lncRNA targets had any effect of the ratio of β -MHC/ α -MHC mRNA expression, hiPSC-CMs from the MYH7 HCM lines were transfected with the three lncRNA overexpression vectors. The MHC ratio was measured at 4d and 7d using qRT-PCR with *TNNT2* as a housekeeping gene and expression fold change calculated against the untransfected control (**Figure 5.13**).

Results show that overexpression of target 1 causes a 20-60% reduction in the ratio of *MYH7/MYH6* expression in all lines while target 2 leads to a decrease in the ratio up to -80% (**Figure 5.13A and B**). A reduction in the ratio of *MYH7/MYH6* is a result of *MYH6* expression increasing and *MYH7* expression decreasing. When there is cardiac dysfunction in the human heart, *MYH6* expression is reduced while *MYH7* increases. Therefore, these data suggest that the overexpression of targets 1 and 2 are HCM disease cardioprotective. *MINCR* overexpression follows the same pattern as the other targets in the healthy *MYH7*^{WT/WT} and *MYH7*^{WT/MUT} but has the opposite effect in the *MYH7*^{MUT/MUT} line as the ratio of *MYH7/MYH6* increases. This suggests that *MINCR* is disease causing in diseased lines.

In addition to qRT-PCR analysis of the mRNA content level. the isoform switch was assessed based on protein levels by immunocytochemistry, hiPSC-CMs from each of the three lines were seeded into 96 well plates and three days later transfected with the CAG-*MINCR* overexpression vector. Three days later the cells were fixed, and immunostaining of α -MHC and β -MHC was performed on untransfected and transfected cells, **Figure 5.14**. α -MHC and β -MHC were co-stained alongside DAPI and for the purpose of determining the purity of hiPSC-CMs, separate wells were stained with α -actinin and cTnT. To quantify α -MHC and β -MHC expression, an image analysis sequence was developed for high content imaging, demonstrated in **Figure 5.15**.

To determine purity, cells were co-stained with DAPI and α -actinin or DAPI and cTnT. The DAPI signal identified cells, while the α -actinin or cTnT signal identified positive cardiomyocytes, based on signal intensity (**Figure 5.15A and B**). Purity was above 80% across the three lines. To assess α -MHC and β -MHC expression, the cells were co-stained α -MHC, β -MHC and DAPI; the DAPI signal identifies cell nuclei, MHC stains identified the cytoplasm. The algorithm then selects hiPSC-CMs expressing α -MHC and β -MHC based on the signal intensity of each marker (**Figure 5.15C**).

Figure 5.16 depicts the expression of α -MHC (*MYH6*) and β -MHC (*MYH7*) in the three model lines. The total number of cells was calculated using total nuclei counts. To calculate the percentage expression of each isoform, the number of nuclei expressing each isoform was divided by the total number of cells. In each of the lines there is not a significant change in isoform expression when overexpressing *MINCR*. Given that when analysed by qRT-PCR there is a shift in isoform expression, the results in Figure 5.16 are likely due to the difficulties in quantifying MHC expression using the high content imaging software used. Further discussion in section 5.6.4.

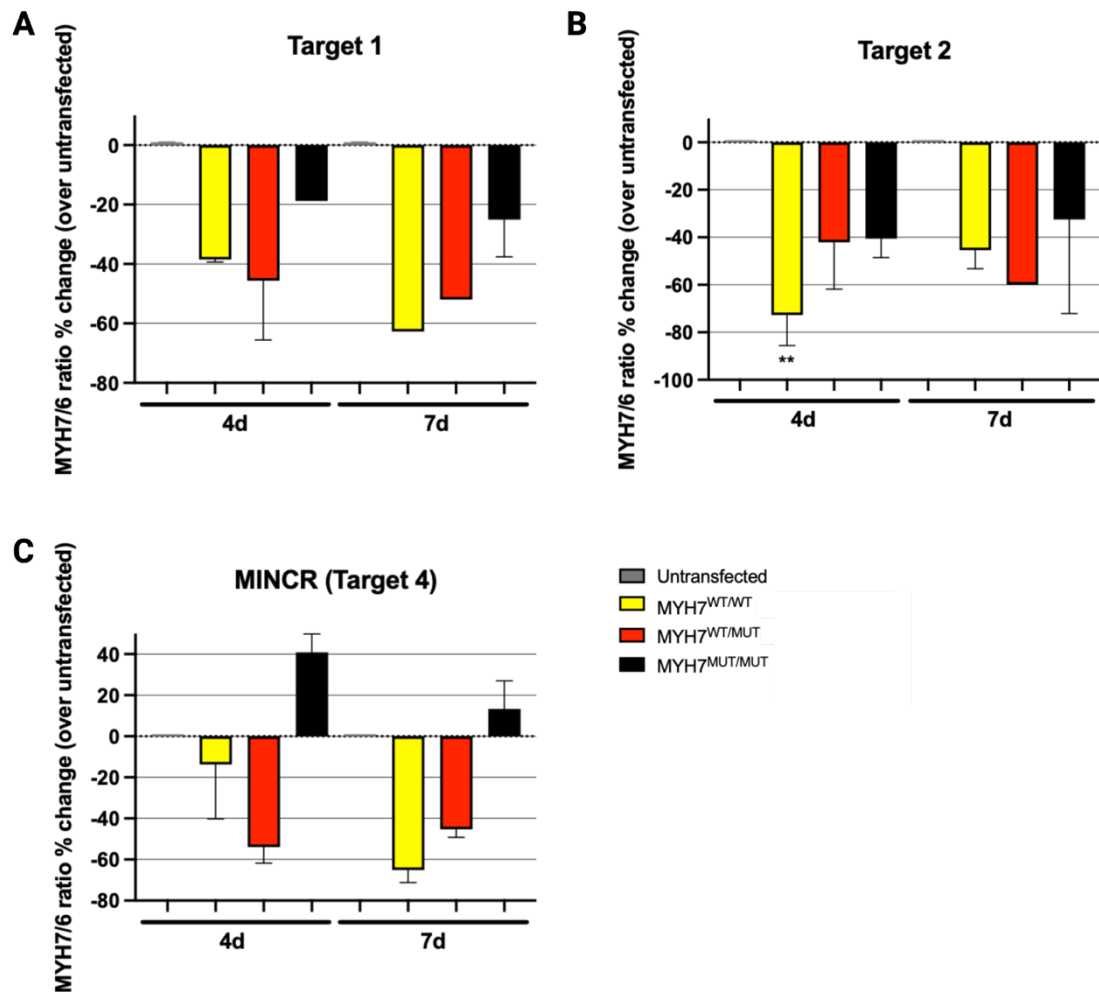


Figure 5.13 – Quantification of the percentage ratio change of *MYH7/6* in R453C *MYH7* hiPSC-CM lines. qRT-PCR analysis shows the % change in the *MYH7/6* ratio in cells hiPSC-CMs transfected with overexpression vectors, compared to the untransfected controls. **A, B)** Targets 1 and 2 showing a decrease in the ratio in all lines and **C)** *MINCR* showing a decrease in the ratio except in the *MYH7^{MUT/MUT}* line. (N=3, with exception to Target 1 4d *MYH7^{MUT/MUT}*, 7d *MYH7^{WT/WT}* + *MYH7^{WT/MUT}* and Target 2 7d *MYH7^{WT/MUT}*). +/- SEM, ** p<0.01.

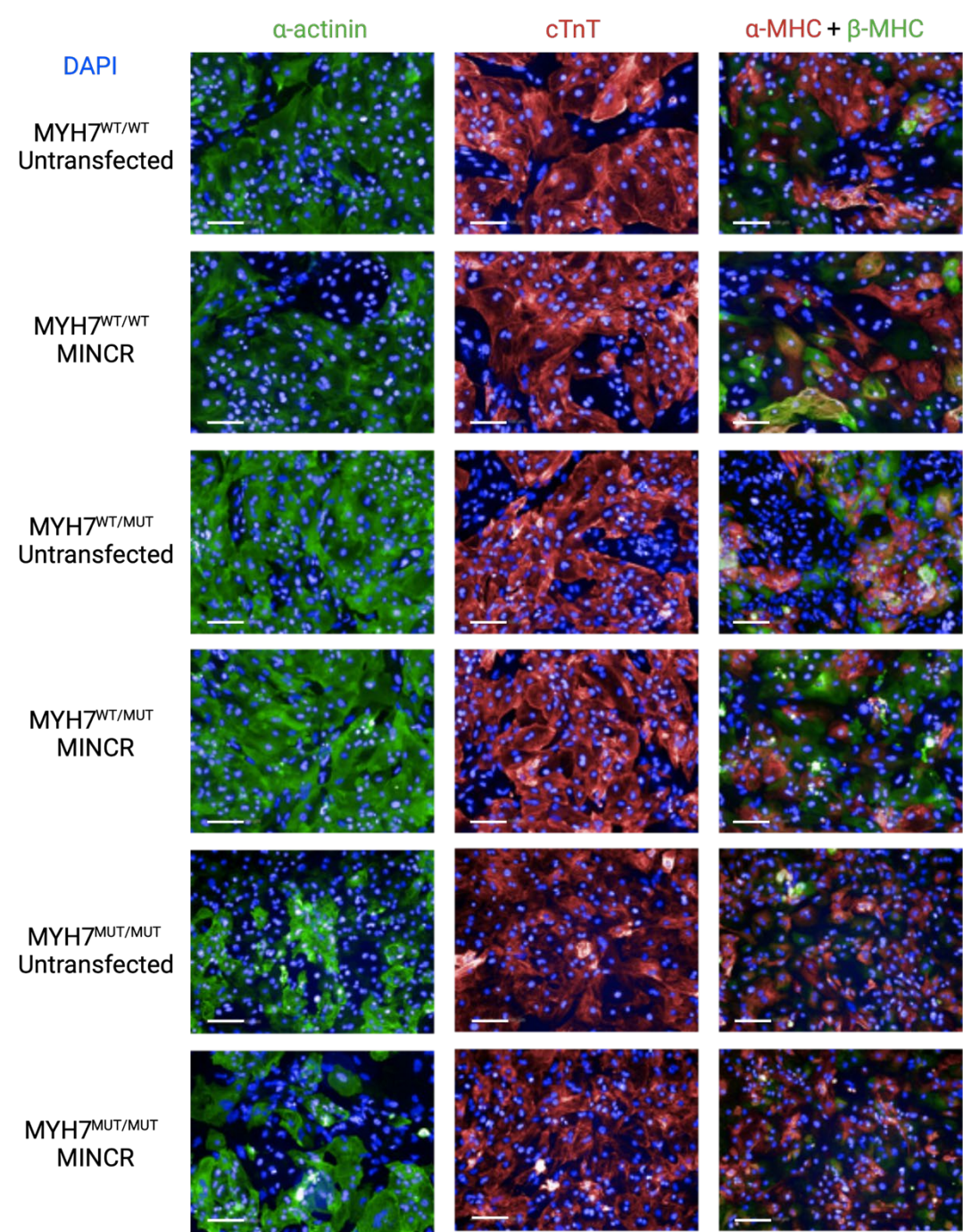


Figure 5. 14 – Expression of α -MHC and β -MHC in hiPSC-CMs overexpressing *MINCR*, to assess the MYH7/6 isoform switch. Representative fluorescent micrographs of α -MHC/ β -MHC/DAPI, α -actinin/DAPI and cTnT/DAPI stained hiPSC-CMs, overexpressing *MINCR*. Scale bar=100 μ m.

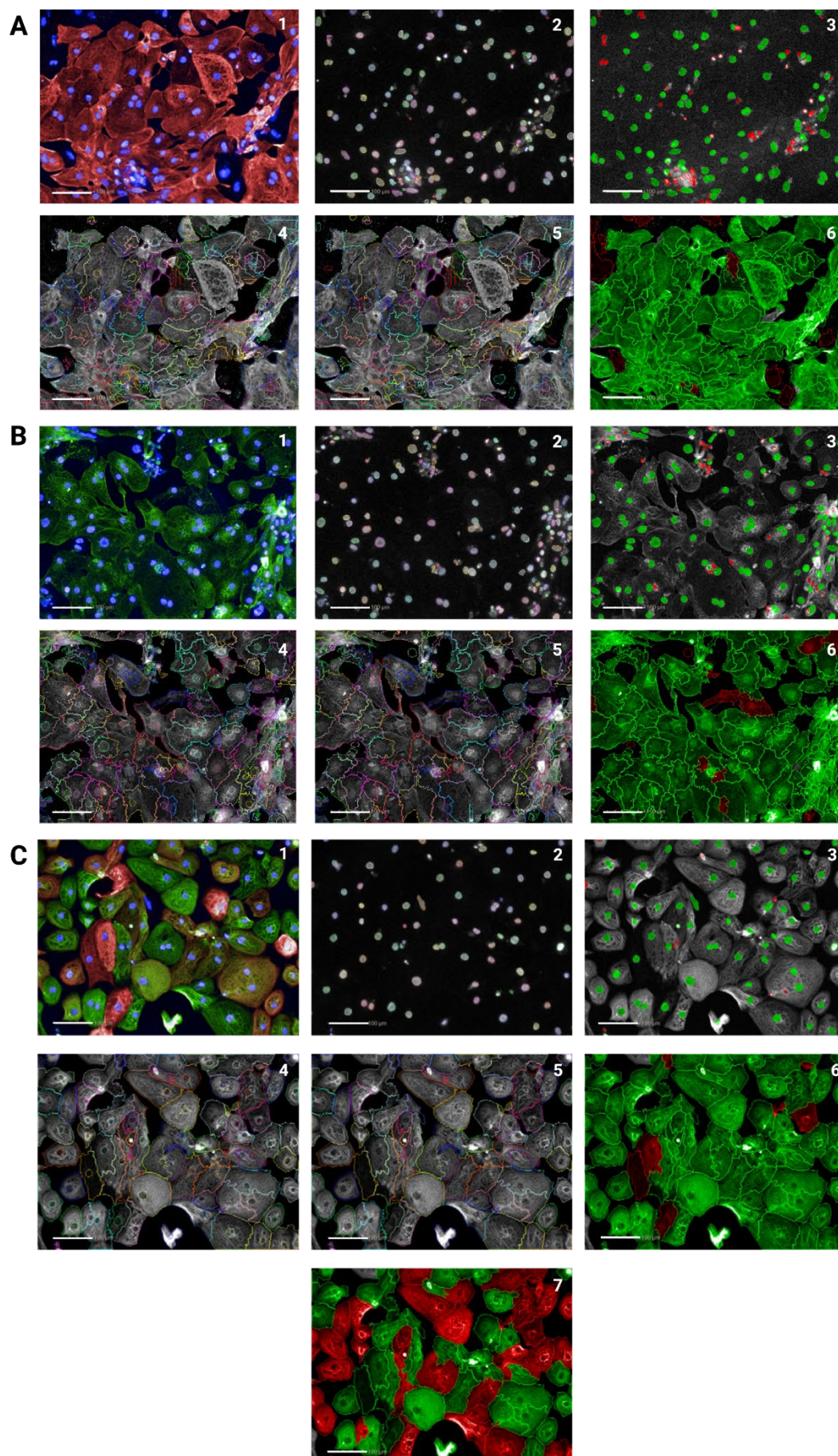


Figure 5.15 – High content imaging analysis sequences for the quantification of *MYH7/6* ratio expression change in hiPSC-CMs overexpressing *MINCR*. Input images of **A)** Algorithm for quantifying purity using cTnT. Input image of **1)** Merged DAPI/cTnT. **2)** Find nuclei using DAPI channel, **3)** remove nuclei clumps if present. **4)** Find cytoplasm using cTnT, **5)** find cell area, **6)** select hiPSC-CMs (in green) using signal intensity. **B)** Algorithm for quantifying purity using α -actinin. Input image of **1)** Merged DAPI/ α -actinin. **2)** Find nuclei using DAPI channel, **3)** remove nuclei clumps if present. **4)** Find cytoplasm using α -actinin, **5)** find cell area, **6)** select hiPSC-CMs (in green) using signal intensity. **C)** Algorithm for quantifying ratio of β -MHC/ α -MHC expression. Input image of **1)** Merged DAPI/ β -MHC/ α -MHC. **2)** Find nuclei using DAPI channel, **3)** remove nuclei clumps if present. **4)** Find cytoplasm using β -MHC or α -MHC, **5)** find cell area, **6)** select β -MHC positive hiPSC-CMs (in green) using signal intensity and **7)** select α -MHC positive hiPSC-CMs (in green) using signal intensity.

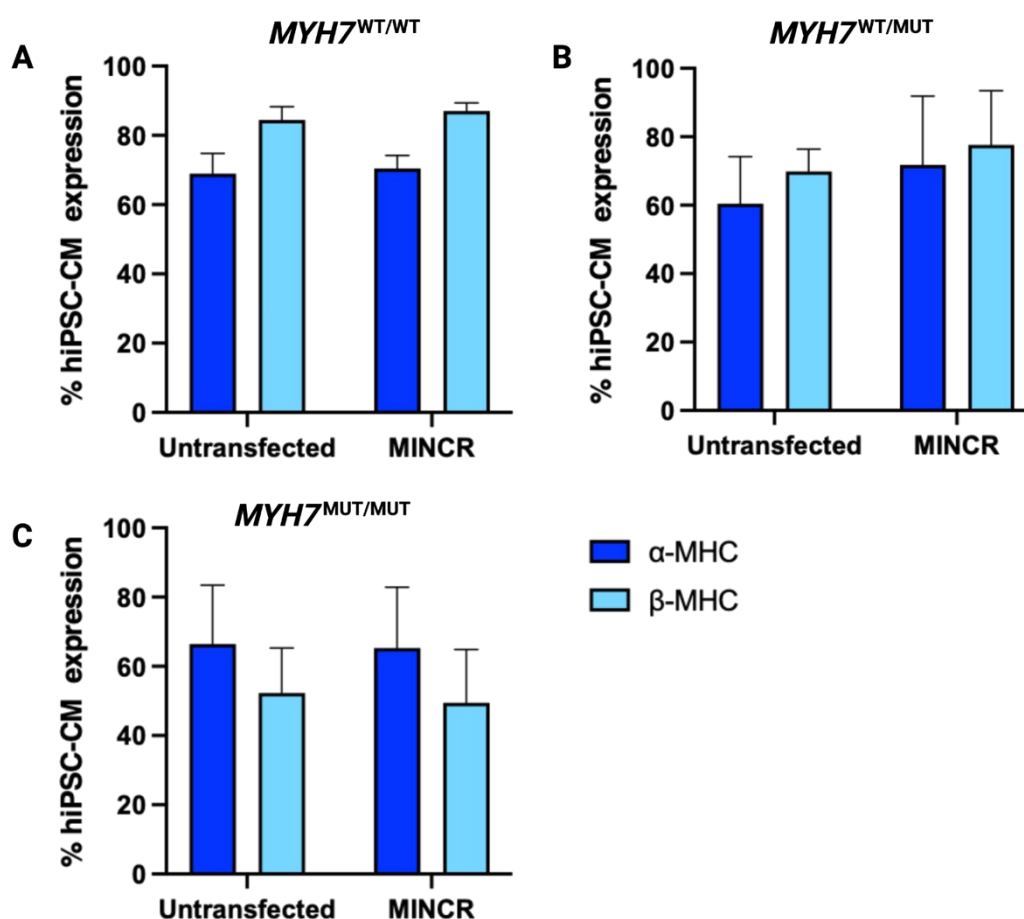


Figure 5.16 – Quantification of α -MHC and β -MHC expression in hiPSC-CMs overexpressing *MINCR*. Quantification by high throughput imaging algorithms reveals the percentage expression of α -MHC and β -MHC in untransfected and *MINCR* overexpressing hiPSC-CMs in **A)** *MYH7*^{WT/WT} **B)** *MYH7*^{WT/MUT} and **C)** *MYH7*^{MUT/MUT}. (N=3) No statistical changes observed.

5.4 *MINCR* Knockdown Results

In the absence of positive knock out clones of *MINCR*, an alternative method was required to reduce the expression of *MINCR*. Unlike the CRISPR/Cas9 approach which would have led to almost complete disruption of gene transcription, knockdown approaches could not recapitulate the extent of gene silencing. However, given the circumstances with time constraints, this was the next logical approach. Predesigned siRNAs to target *MINCR* were purchased and used for the knockdown of expression in the *MYH7* lines. Knock down hiPSC-CMs were phenotypically characterised for mitochondrial respiration and MHC isoform switch to determine whether this modulation had different effects to overexpression.

5.4.1 siRNA knockdown efficiency by qRT-PCR

Defining the functional relevance of lncRNAs, in this case *MINCR*, requires the ability to effectively modulate lncRNA expression levels. Understanding the functional role may pave the way for therapeutic benefit if the lncRNA is associated with disease. Similar to the overexpression strategy, the fold change in expression of *MINCR* using siRNA knockdown was assessed in the HCM model lines. The siRNAs resulting in the greatest fold change would be used for further phenotyping. hiPSC-CMs were transfected with siRNAs singularly and in pairs to determine which siRNA(s) resulted in the best knockdown. GAPDH siRNAs were used as a positive control for the transfection of *MINCR* siRNAs (At the time of purchase, *TNNT2* delivery times were extensive). qRT-PCR analysis showed variable siRNA effectiveness in knocking down *MINCR* across the lines (**Figure 5.17**). The ideal minimum percentage fold change reduction would be at least -70%. The knockdown fold change from the siRNAs was not satisfactory. In the healthy *MYH7*^{WT/WT} line, siRNA combination of 2+4 appeared to be the most successful in reducing *MINCR* expression with a 42% reduction, whereas in the *MYH7*^{WT/MUT} and *MYH7*^{MUT/MUT} lines, siRNA 4 (-35%) and siRNA 1 (-10%) resulted in the greatest reduction in fold change, respectively (**Figures 5.17A,B and C**). Further biological replicates are needed in all lines for greater confidence, greater discussion below in chapter 5.6.5.

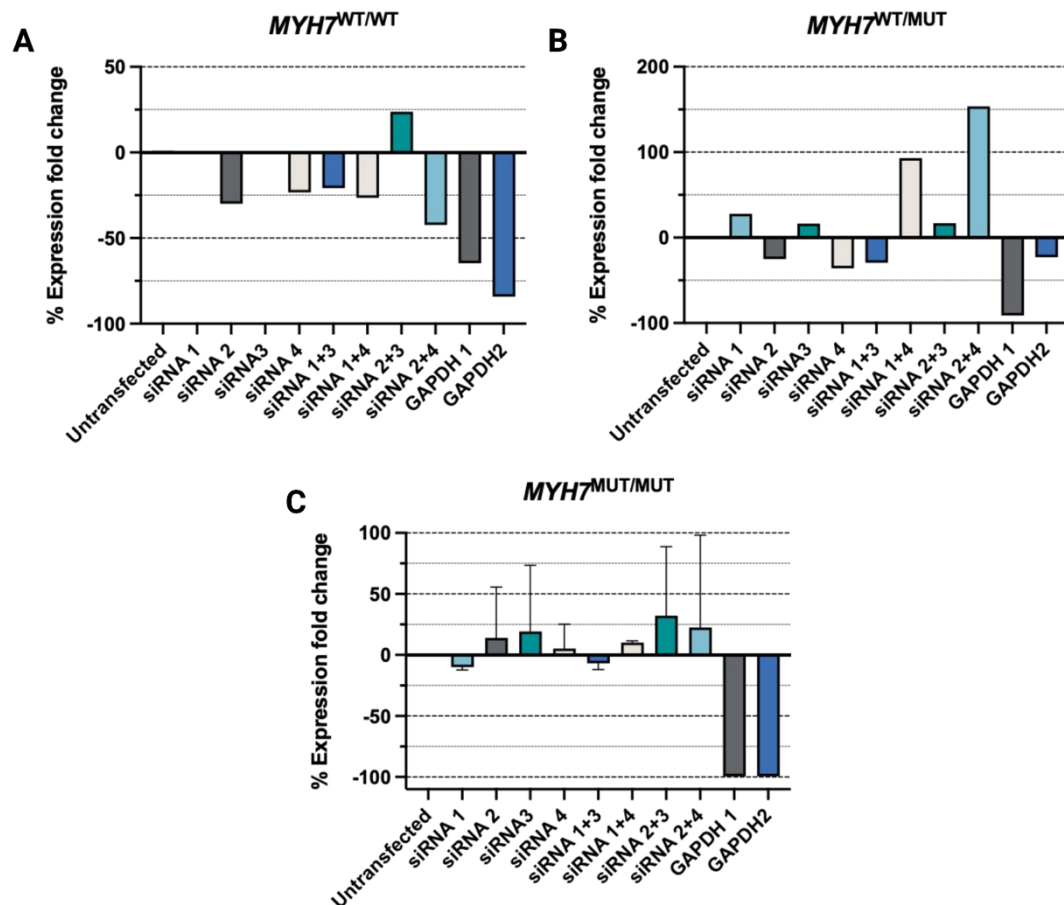


Figure 5.17 – Expression of *MINCR* in *MYH7* lines post transfection with siRNAs targeting exons 1 and 3. qRT-PCR analysis showing expression fold change of *MINCR*, using siRNAs transfected singularly and in pairs, in comparison to the untransfected controls. A) *MYH7*^{WT/WT} line, B) *MYH7*^{WT/MUT} line and C) *MYH7*^{MUT/MUT} line (N=2). *GAPDH* siRNAs were transfected as a positive control. Results were normalised to *TNNT2*.

5.4.2 Mitochondrial Respiration

Altered mitochondrial respiration can be indicative of cardiac dysfunction. To determine whether opposite modulation (knock down as opposed to overexpression) affects mitochondrial respiration, the Seahorse XF platform was once again used for the measurement of the OCR in the trio *MYH7*^{WT/WT}, *MYH7*^{WT/MUT} and *MYH7*^{MUT/MUT} lines where *MINCR* had been knocked down. Based on the results of the qRT-PCR analysis to determine the optimal siRNA for the knockdown of *MINCR* in each cell line (section 5.4.1), *MYH7*^{WT/WT} was transfected with siRNA2+4, *MYH7*^{WT/MUT} with siRNA 4 and *MYH7*^{MUT/MUT} with siRNA 1. The Seahorse assay was performed 3 days post transfection with siRNAs (for continuity between the two modulation systems), using the same electron transport train modulators already described. Post assay the cells were fixed and stained with DAPI for normalisation of OCR values against the total cell number.

Due to a complication with the Seahorse platform, it was unfortunately not possible to obtain the results from the healthy isogenic control line (*MYH7*^{WT/WT}). The results showed no relationships between *MINCR* knockdown and alterations in mitochondrial respiration (**Figure 5.18**). Further evaluation of the parameters of mitochondrial respiration also showed no difference between *MINCR* knockdown and untransfected controls (**Figure 5.19**).

Initially, these data suggest that a reduction in *MINCR* expression bears no impact on mitochondrial respiration. However, as with the overexpression mitochondrial respiration results, it is possible that there is an impact, but the Seahorse platform is not sensitive enough to detect it.

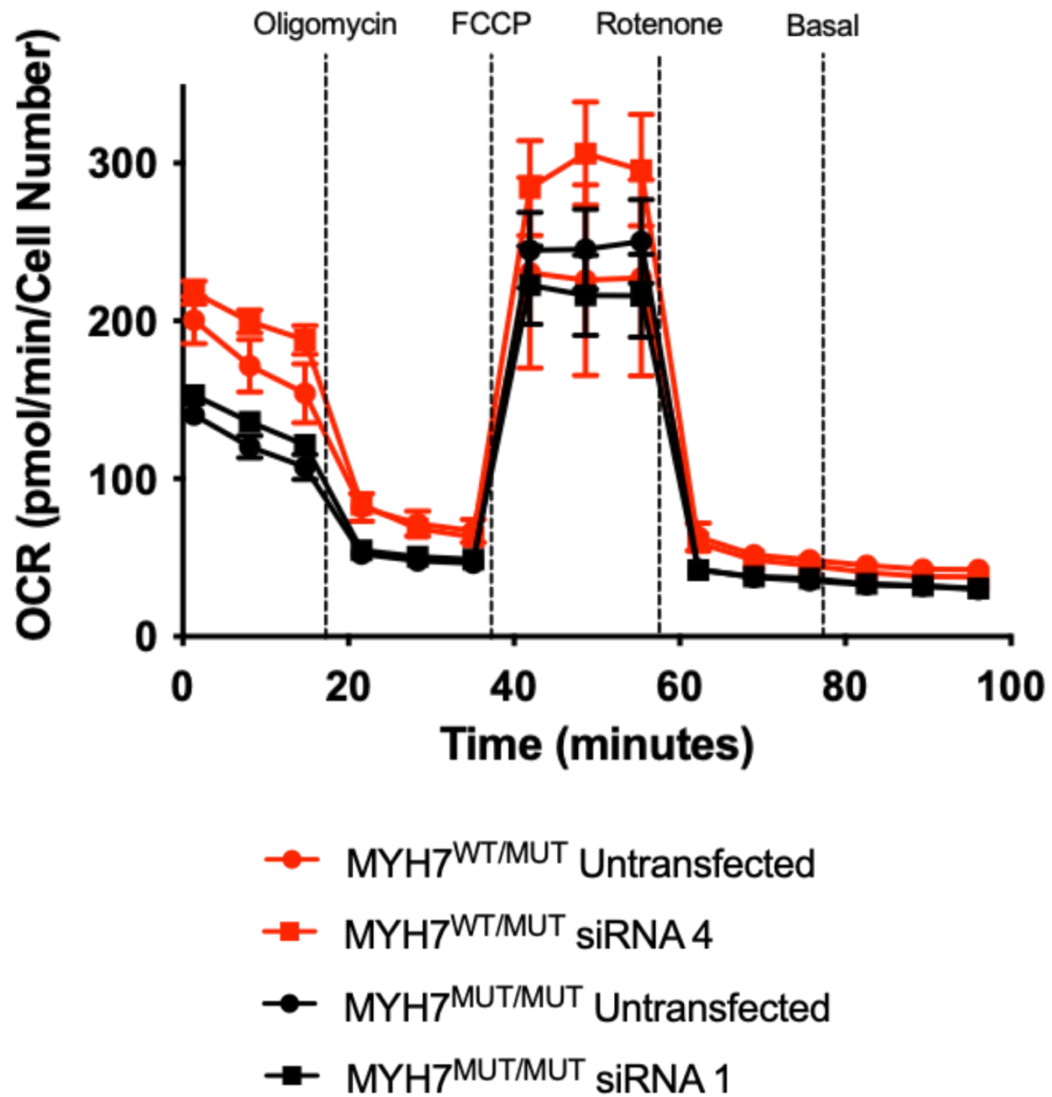


Figure 5.18 – Analysis of mitochondrial respiration in *MYH7* lines with *MINCR* knockdown by siRNA transfection, using the Seahorse platform. *MYH7* hiPSC-CMs with *MINCR* knockdown show no significant difference in respiration profiles between untransfected and knockdown. (^{WT/MUT} N=1, ^{MUT/MUT} N=2).

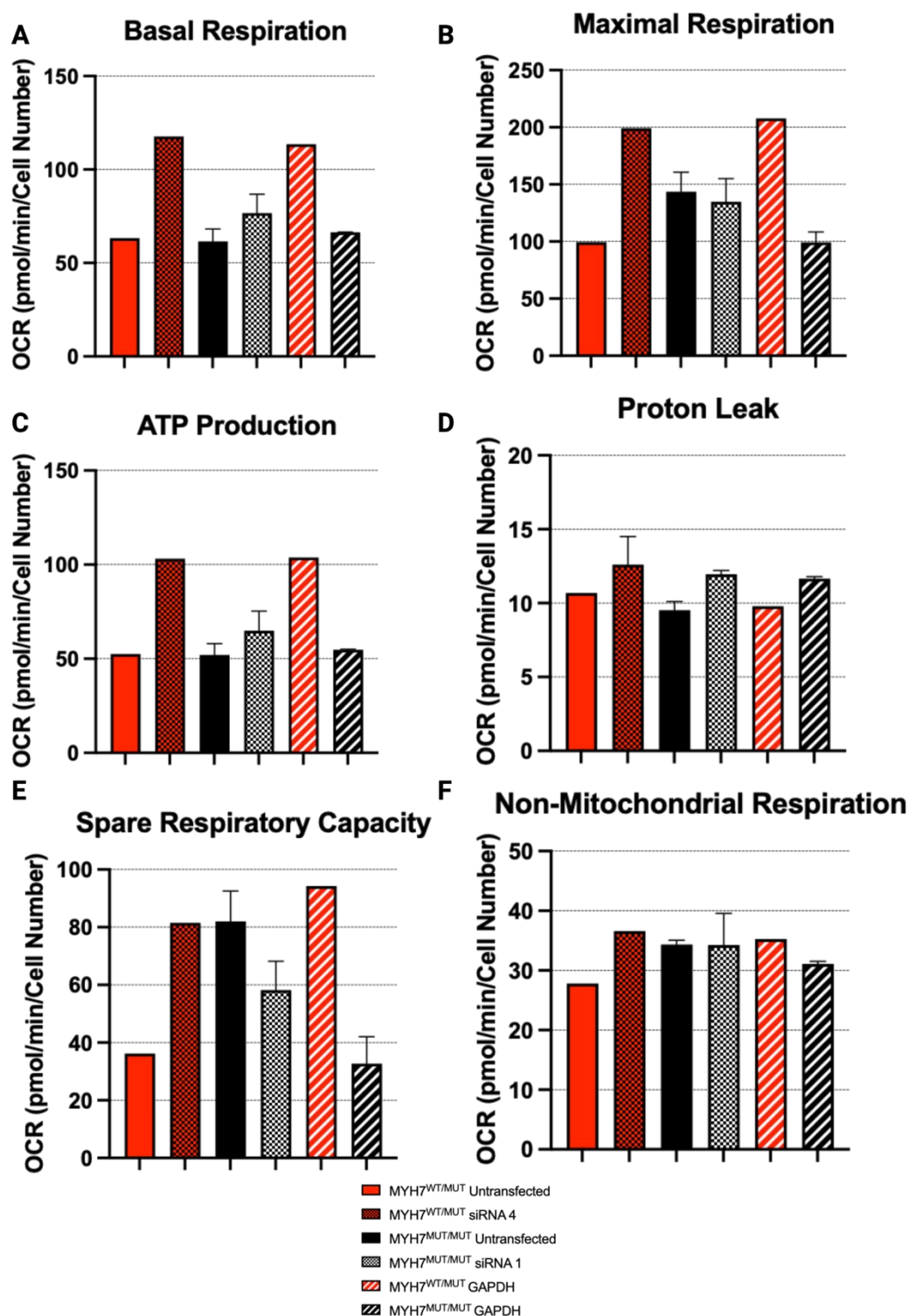


Figure 5.19 – Mitochondrial respiration parameters of *MYH7* hiPSC-CMs with *MINCR* knockdown.
A-F) No significant difference in any of the parameters between the *MINCR* knockdown and untransfected controls in each *MYH7* line. (*MYH7*^{WT/MUT} N=1, *MYH7*^{MUT/MUT} N=2).

5.4.3 *MYH7/MYH6* Expression

To identify whether knocking down *MINCR* expression had a reverse effect on the expression of β -MHC and α -MHC compared to the overexpression results, the immunostaining of the two isoforms was repeated. This would give another indication whether it is involved in HCM progression or prevention as a disease modifier. A switch in the levels of MHC expression indicates a return to the foetal gene program, a feature of HCM that is compensatory. To assess this, hiPSC-CMs from all three *MYH7* lines were transfected with *MINCR* knock down siRNAs (previously determined for each line, section 5.4.1). Three days after transfection the cells were fixed and immunostaining was performed as described in section 5.3.4. **Figure 5.20** shows representative images of β -MHC and α -MHC expression after *MINCR* knock down.

Using the same analysis sequence as in section 5.3.4 (**Figure 5.15**), the purity of the cardiomyocytes was assessed using cardiac markers α -actinin and cTnT. Similarly, the protein expression level of the MHC isoforms was determined, while DAPI intensity was used to normalise the values of each isoform.

Figure 5.21 shows the expression of α -MHC (*MYH6*) and β -MHC (*MYH7*) in the three model lines. The total number of cells was calculated using total nuclei counts. To calculate the percentage expression of each isoform, the number of nuclei expressing each isoform was divided by the total number of cells. In cell lines *MYH7*^{WT/MUT} and *MYH7*^{MUT/MUT}, there are no changes in isoform expression when overexpressing *MINCR* compared to the untransfected control. In the line *MYH7*^{WT/WT}, when overexpressing *MINCR*, α -MHC becomes the predominantly expressed isoform. However, this is based on only one biological experiment, and without qRT-PCR data to guide, and is preliminary data. Further replicates are required for a better understanding of the role of *MINCR* in the isoform switch.

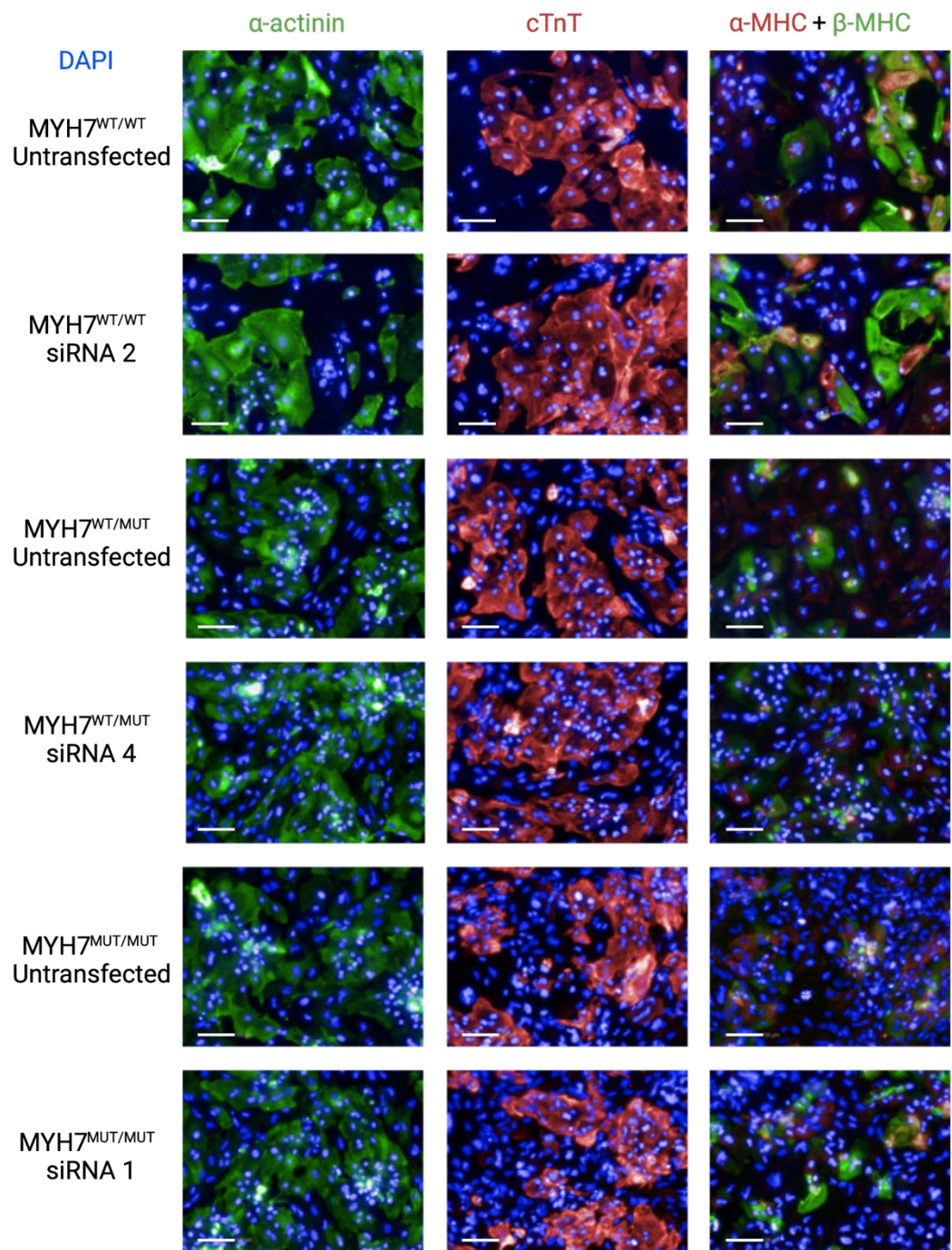


Figure 5.20 – Expression of *MYH7* and *MYH6* in hiPSC-CMs with *MINCR* knockdown, to assess the *MYH7/6* isoform switch. Representative fluorescent micrographs of α -MHC/ β -MHC/DAPI, α -actinin/DAPI and cTnT/DAPI stained hiPSC-CMs transfected with siRNAs to knockdown *MINCR*. Scale bar=100 μ m.

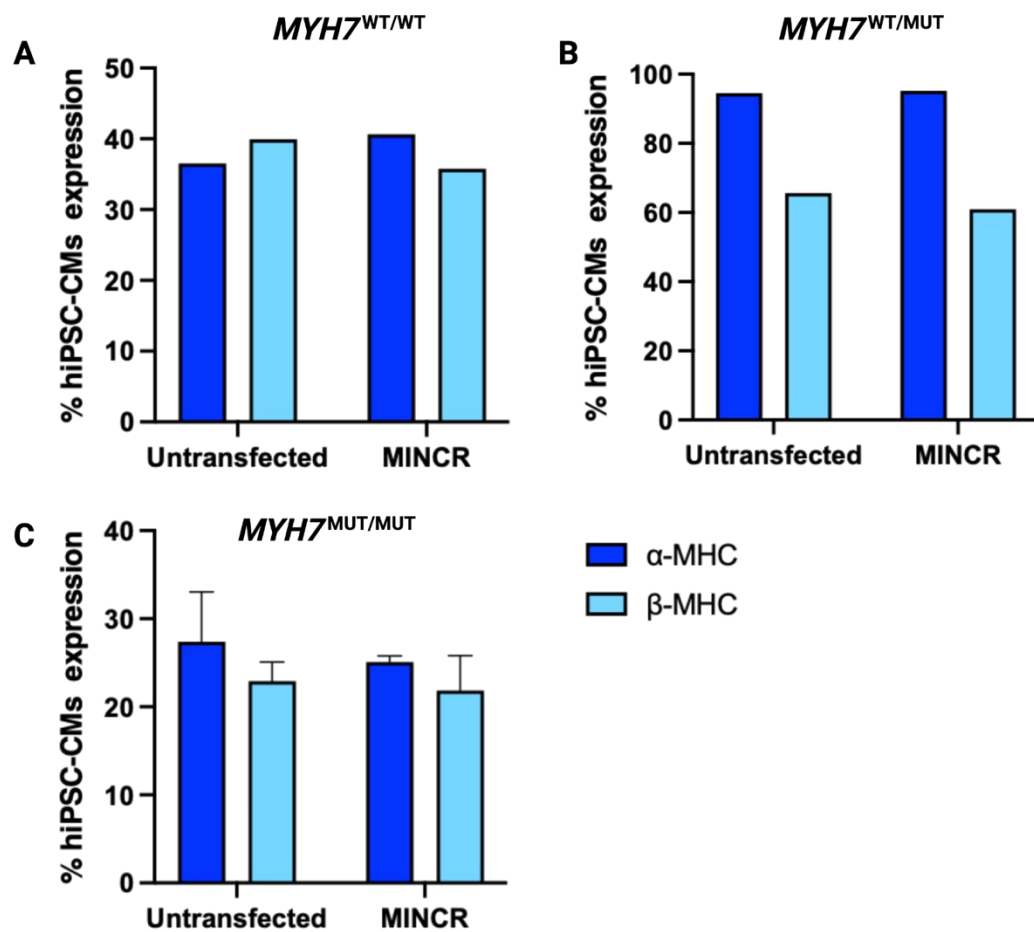


Figure 5.21 – Quantification of α-MHC and β-MHC expression in hiPSC-CMs with *MINCR* knockdown. Quantification by high throughput imaging algorithms reveals the percentage expression of α-MHC and β-MHC in untransfected and *MINCR* overexpressing hiPSC-CMs in A) *MYH7*^{WT/WT} B) *MYH7*^{WT/MUT} and C) *MYH7*^{MUT/MUT} (N=2). (N=1)

5.5 RNA sequencing analysis

To assess whether the overexpression of lncRNA target *MINCR* had impact on the gene expression of HCM associated genes, RNA sequencing was performed on all *MYH7* lines. This data intended to give a greater understanding of the impact of *MINCR* on HCM at the gene level, complementing the phenotypes observed in the other assays and possibly identifying mechanisms to be further investigated. Differential gene expression analysis across a range of genes involved in HCM hallmark processes were: i) apoptosis, ii) autophagy, iii) calcium handling, iv) foetal gene program, v) fibrosis and vi) hypertrophy.

To complement the results observed in section 5.2, that *MINCR* has a role in proliferation and apoptosis in hiSPCs, differential gene expression of key cell cycle genes was also considered.

hiPSC-CMs for each of the isogenic lines were transfected with the CAG-*MINCR* overexpression vector for a total of three biological repeats per line. Untransfected controls were maintained in the same plate. To understand the expression of HCM associated genes at the time when experimental assays were performed, hiPSC-CMs were harvested three days post-transfection using a Trizol based RNA extraction kit. The *MINCR* overexpressing hiPSC-CMs were sequenced by Novogene. The analysis is still ongoing due to the complexity of the data.

5.6 Discussion

5.6.1 *MINCR* Involvement in hiPSC proliferation and survival

As a result of the quantification of *MINCR* expression and the quantification of cleaved caspase 3 expression in clone 5 after targeting with the knockout system, it is presumed that *MINCR* played a role in either hiPSC proliferation and/or cell survival. Turning to the field of cancer, in 2015 Doose and colleagues selected *MINCR* from 13 lncRNAs that might be regulated by MYC in cancer. By performing RNAi mediated knockdown of *MINCR*, an abundant number of genes involved in the cell cycle were downregulated [315]. The effects were recapitulated in another experiment when they specifically knocked down cell cycle genes (without targeting *MINCR*). DNA analysis of cells where *MINCR* was knocked down showed an increase in cells in the G0/G1 phase and measurements of intracellular caspases 3 and 7 were increased, suggesting impaired cell viability. Since then, *MINCR* upregulation has been implicated in various other cancers [316-322], and unsurprisingly *MINCR* downregulation has been associated with neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS) [323]. At present, there is no literature about *MINCR* function/s in hiPSCs or CVD including cardiomyopathies and the exact function of *MINCR* has not been elucidated yet. Excitingly, the observations of clone 5 in this thesis may have uncovered some interesting questions in the future of HCM and hiPSC-CM, discussed below.

Transcriptomic analysis of the changes observed after *MINCR* knockdown in the cancer field suggest that *MINCR* can modulate the expression of genes involved

in cell cycle progression. This raises three questions in the context of this thesis; 1) Has the knockout of *MINCR* in clone 5 recapitulated the observations in the cancer field? 2) Would the overexpression of *MINCR* result in the division of hiPSC-CMs, a largely quiescent cell type? 3) Does transcriptomic analysis of the overexpression of *MINCR* in the HCM model show any alterations in the expression of cell cycle genes? Questions 1 and 2 can be answered by further experimental investigation, questions 2 and 3 are contemplated in section 5.6.6.

While the literature and results in this thesis support the hypothesis about the function of *MINCR* in clone 5, if there had been less urgency with the sample, alternative methods for characterisation would have been implemented. It would have been preferential to measure the proliferative and apoptotic rates of clone 5 using well-published assays such as tetrazolium dye (MTT)/Bromodeoxyuridine (BrdU) assays for proliferation or DNA fragmentation/plasma membrane integrity assays for apoptosis, to name a few [324]. Whilst the above may be more effective assays at assessing proliferation and apoptosis than simply staining activated caspase 3, they require live cells.

In the cancer papers mentioned above, *MINCR* knockdown was performed as opposed to knockout. To generate future *MINCR* downregulated hiPSCs to continue investigating whether the phenomenon observed in clone 5 is reproducible, it would be important to develop robust and effective siRNAs to target *MINCR*. Rather than knockout, knockdown of *MINCR* may not cause complete down-regulation of important cell cycle genes to grant enough time to perform proliferation assays.

5.6.2 Hypertrophy analysis and lncRNAs as gene modifiers

Gene modifiers can modify the phenotypic outcome of the primary disease-causing variant, in this thesis the *MYH7* SNP mutation in the HCM model used. They are not disease-causing themselves but can promote or rescue disease phenotypes [325]. The BNP assay results in this thesis demonstrate gene modifying behaviour when the lncRNAs were overexpressed. The fact that the lncRNAs did not reduce BNP expression in the healthy line in the untreated condition, yet reduced BNP expression alongside endothelin-1 treatment is

indicative of this. This is further validated in the diseased where lncRNA overexpression rescues the hypertrophy phenotype in all conditions.

There is increasing evidence that lncRNAs act as regulators of cardiac hypertrophy and other pathophysiological mechanisms such as calcium handling and metabolism, the latter discussed in section 5.6.5. lncRNAs can exert their regulation in a disease-causing or disease-preventing manner, by a multitude of different methods, discussed in depth in the introduction. The lncRNA *Chaer* promotes hypertrophy by interacting with the PRC2 complex to inhibit histone methylation relating to genes of cardiac hypertrophy [326]. Other pro hypertrophic lncRNAs include *Chast* and *CHFR* which negatively regulate hypertrophy by downregulating proteins or miRNAs that play anti-hypertrophy roles [327, 328]. The results from the BNP staining therefore contribute to this growing body of evidence that target 2 and *MINCR* may be regulators of hypertrophy in HCM, yet the mechanisms of how the lncRNA targets in this thesis exert their role are not understood.

With the number of lncRNAs being characterised and mechanistic links found between their function and HCM, they could be an essential therapeutic target of HCM. Current treatments focus on alleviating the symptoms of HCM, rather than the underlying mechanisms. This will lead to targeted therapies that will increase patient outcomes.

Using flow cytometry to determine cell volume would complement the BNP assay performed in this work. The cell volume of hiPSC-CMs with modulated lncRNA target expression would be calculated by comparing them to a calibration curve established using beads of a known size. An effect of lncRNA modulation on cell volume of HCM model lines would be indicative of a direct HCM mechanism, as HCM is characterised by thickening of the ventricle due to increased cell volume, not division (5.1.1). Comprehensive evaluation of cell volume would therefore be of great interest.

5.6.3 Metabolic analysis

The Seahorse assay results suggest that *MINCR* overexpression or knockdown does not impact mitochondrial respiration in the HCM model. Stated above, the

knockdown efficiency was poor and the metabolism results stem from one biological replicate, meaning further experiments are required before a conclusion can be reached. On the other hand, the overexpression results represent three biological replicates and yet there is no statistical difference between *MINCR* overexpression vs untransfected of each hiPSC-CM line. The initial qRT-PCR results in Figure 5.7 depict a fold change of between 3000-6000 in *MINCR* expression in the HCM model lines transfected with the overexpression vector. It is conceivable that any potential metabolic effects of overexpressed *MINCR* are imperceptible as the fold change after transfection is not great enough given the low endogenous expression levels of lncRNAs.

Moving forward, an alternative method could be used to assess mitochondrial respiration after *MINCR* overexpression or knockdown. One desirable quality of the Seahorse analyser is the ability to perform high throughput assays, for this some level of reliability is sacrificed and variability between replicates is common [329]. In general, higher throughput assays are completed first before using more specific techniques that are lower throughput but with greater accuracy. It could be that modulating *MINCR* creates minor effects in metabolism that cannot be detected using the Seahorse. The Oroboros O2k high respirometry platform produces high-resolution oxygraphs meaning that even low rates of OCR can be trusted [330], with the ability to measure multiple aspects of mitochondrial physiology [331]. On the other hand, with only two chambers, this is a low throughput method that would require a larger volume of cells.

If the lncRNA targets are acting as gene modifiers as suggested by the hypertrophy analysis performed in this thesis, then perhaps additional stressors on the cells are required for the lncRNAs to act in a cardio-protective or disease-inducing manner. Future experiments could apply lncRNA expression modulation in combination with further dysregulation of mitochondrial respiration prior to performing the Seahorse assay. Other alterations to the experiment to induce phenotypes could include optogenetic pacing [287], however, this would be required to take place at the same time as the Seahorse and such a platform does not yet exist.

A recent study using angiotensin II treated mouse cardiomyocytes showed that the lncRNA Plscr4 influences mitochondrial homeostasis by regulating the expression of Mfn2, a molecular target of miR-214 for which Plscr4 acts as a sponge [332]. Its role in HCM was multifaceted, the original discovery was its involvement in hypertrophy through sponging of the miR-214. Such studies which identify multiple roles of lncRNAs in HCM are compelling for the continued investigation of *MINCR* and the other lncRNA targets in this thesis for further phenotyping. Investigations using the HCM model in this thesis are crucial to phenotyping, as it is superior to other hiPSC models without an isogenic control and the many animal studies, mostly conducted in mice which are biologically distinct from human hearts [305, 333].

5.6.4 Considerations for assessing the level of isoform switch between

Two methods were implemented for the assessment of the *MYH7/MYH6* expression. Initially, qRT-PCR was used to determine the expression levels of each isoform. Pooling of different wells for each condition means there is a highly heterogeneous population being assessed in one qRT-PCR run. To attempt to overcome this, staining of each isoform was performed to look at the relative protein expression of each isoform on the single-cell level. This also allowed the confirmation of hiPSC-CM purity by the staining of α -actinin and cTnT in wells of the same plate. Ideally, purity staining or a cell mask to determine cell cytoplasm would have been used to confirm the cell area, however, triple staining can result in channel overlapping of the secondary antibodies leading to bleeding which would make it difficult to distinguish between signals. There are a few issues with this method. Firstly, quantification is based on human input to design the analysis sequences and secondly it is not possible to distinguish between cells expressing a mixture of the isoforms, and those that express just α -MHC or just β -MHC. Weber and colleagues quantified isoform expression based on the sarcomeric banding intensity of each antibody at the single-cell level [334, 335]. Between each batch of hiPSC-CMs, they saw large variability and indeed their analysis also required human input to define the scoring system. However, looking at the single-cell level could improve the quality of data in this work, although this requires additional software that was not available at the time.

Another method that can be used for assessing protein levels is western blots, however, the high degree of sequence homology between *MYH7* and *MYH6* makes it difficult to distinguish between the two bands. This technique was used in 2000 by Miyata and colleagues and required further specialised analysis, highlighting that it is not an optimal method in this context [305]. Further refinement of the immunostaining protocol is required to obtain data that can indicate the expression of each isoform.

In assessing the level of *MYH7*/*MYH6* in the context of human HCM, a decrease of *MYH7* and increase of *MYH6* (i.e., the ratio goes down) indicates reactivation of the foetal gene program. The re-expression of the foetal isoform is a compensatory mechanism for an increased workload. As discussed in the introduction, β -MHC becomes the predominant isoform after birth as α -MHC levels decrease. This analysis cannot be performed in murine models where the opposite is true, giving greater weight to hiPSC-CMs in modelling HCM.

Further analysis of contractility using EHTs, which have the added benefit of being more representative of the human adult heart in terms of mature physiology, or using patch-clamp methods would give greater insights into the functional effects of the isoform switch.

5.6.5 *MINCR* knockdown in hiPSC-CM

The RNAi mediated knockdown of *MINCR* in this thesis was not fully effective. The siRNAs targeted the beginning of exon 1 and the end of exon 3. They were delivered singularly (target E1 or E3) or in pairs (target both exons). As non-protein-coding genes, lncRNAs may retain their function if active fragments remain after cleavage. lncRNA length and the location of target sites is therefore a factor that should be considered when designing siRNAs [336]. At 1.5kb in length, targeting the start of exon 1 and/or the end of exon 3 may have resulted in active fragments remaining, resulting in a less effective knockdown. Due to time constraints as a result of the pandemic, manufacturer pre-designed siRNAs were purchased instead of custom siRNAs using sequences previously published [315]. To conduct future experiments studying the proliferative and apoptotic events after the knockdown of *MINCR* expression, effective siRNAs are a necessity. The

importance of effective siRNAs is confounded by the fact that endogenous lncRNA expression is lower than that of coding genes [337]. As discussed in chapter 3, the localisation of *MINCR* in hiPSCs has not been performed. Perhaps the poor knockdown efficiency was due to *MINCR* being a nuclear lncRNA, better targeted by ASOs [338]. Therefore, localisation determination is crucial for further *MINCR* investigation.

As discussed in chapter 3, an improved fluorescent labelling system would be the optimal method of assessing transfection efficiency. In this thesis siRNA knockdown of *GAPDH* was used as a positive control, with effective knockdown of *GAPDH* expression suggesting that the transfection method was successful. *GAPDH* is a much more well-studied gene [339], therefore siRNA design by the manufacturer could be more rigorous. Despite this, there was no optimisation of the transfection protocol for the use of siRNAs, owing to time constraints. Transfection using Viafect™ was optimised specifically for the hiPSC-CM HCM line. While another lipid-based transfection protocol may be the most effective method for the delivery of siRNAs [280], this is redundant if the health of the cells or transfection efficiency is compromised. As previously mentioned in Chapter 4, optimising the day of transfection, siRNA:Viafect™ and serum presence would ensure that transfection efficiency is not a factor contributing to poor knockdown effectiveness.

5.6.6 RNA sequencing analysis

RNA sequencing is a powerful tool that can be used to detect known and novel transcripts. Sequencing can be performed across multiple genes and samples, as opposed to a method such as qPCR which is used for looking at fewer target sequences of known variants. To further the understanding of the effects of *MINCR* overexpression on genes associated with hallmarks of HCM, RNA sequencing was performed on all three *MYH7* lines. To complement the observations of *MINCR*'s potential role in cell proliferation and apoptosis in hiPSCs, cell cycle progression genes are also being investigated. In addition, looking at cell cycle genes could give some insights into hiPSC-CMs proliferation. In section 5.6.1, it was discussed how the data posed a few questions. The following builds upon those questions in the context of RNA-Seq.

Would the overexpression of *MINCR* result in the division of hiPSC-CMs, a largely quiescent cell type? As detailed in the many cancerous cell lines [316-322], knockdown of *MINCR* is involved in reduced expression of cell cycle genes, leading to reduced cell viability. The preliminary data in section 5.2 suggests the pathways may be replicated in hiPSCs where *MINCR* is downregulated. If this is the case, then it would be interesting to determine if overexpressing *MINCR* causes upregulation of cell cycle genes and can promote proliferation. This can be assessed by performing proliferation assays, but to understand if the genes involved are upregulated, the RNA sequencing results hold the answer.

Does transcriptomic analysis of the overexpression of *MINCR* in the HCM model show any alterations in the expression of cell cycle genes? Similarly to the answer to question 2, the RNA sequencing results will inform whether there is a shift in cell cycle genes. To complement this data, future RNA sequencing should be performed on *MYH7* hiPSC-CMs with downregulated *MINCR* expression, to recapitulate what was found in section 5.2.

The data is still undergoing analysis at the time of writing, but once completed, the results could reveal *MINCR* as a regulator of cell cycle progression in cardiomyocytes and harness new methods to determine if cells are quiescent and a mechanism to leverage.

5.6.7 Other considerations

The minimal increase in fold change in target 2 expression might suggest that it is localised to and carries out its function in the cytoplasm. As discussed in Chapter 3, modulation methods are much more effective if they target the site in which the transcript exists. Performing localisation studies would confirm the site of action of target 2 and enable better modulation strategies.

Engineered heart tissues (EHT) more closely resemble physiological conditions and would be especially insightful into the effects of lncRNA knockouts on contractility [340]. To assess overexpressed lncRNAs in EHTs would require the

development of an optimised transfection strategy to ensure uniform overexpression throughout the tissue.

6. General Discussion

The COVID-19 pandemic had a significant impact on the experimental endeavours of this thesis. Time limitations and restricted access to the facilities meant that several more phenotypes of interest could not be assessed. The long-term nature of hiPSC-CM experiments meant that the focus was turned to one lncRNA, *MINCR*, to enable a wider collection of data. Other experiments would have assessed lncRNA modulation effects on contractility, calcium transients and electrophysiology, detailed in section 6.5.

The contributions of this work revolve around the creation of new gene-editing tools for the investigation of novel target lncRNAs. The work in this thesis harnessed the power of hiPSC/hiPSC-CMs, CRISPR/Cas9 gene editing and the development of a transfection method to deliver new insights into the impact of lncRNAs on HCM. The original aspiration was to complete overexpression and knockouts and subsequent functional characterisation of all four lncRNA targets in all lines of the HCM model. Ultimately this was achieved using only one target, *MINCR*, due to time constraints and further biological replicates are still required to confirm the metabolic data. The constructs for targets 1, 2 and 3 were completed during this thesis, and future work could continue following the same course as *MINCR*. Using an hiPSC-CM model designed to recapitulate HCM phenotypes caused by the R453C-*MYH7* SNP, CRISPR/Cas9 and overexpression tools of the lncRNA targets were created and applied to the cardiomyocytes, followed by phenotyping assays to elucidate their phenotypic effects.

6.1 Use of hiPSC-CMs as a model of human CVD

The acquisition of hiPSC-CMs from hiPSCs grants researchers an unlimited source of material, with the possibility to create isogenic lines using gene-editing methods, such as CRISPR/Cas9. As a model, hiPSC-CMs, in addition to gene editing, denote greater experimental prospects and the opportunity to create isogenic control lines maintaining patient background genetics. Other human models that have greater complexity such as cardiac muscle strips and myofibrils from heart tissue have limitations in availability and practicalities. Those of lower

complexity, which include isolated cardiomyocytes and purified actin/myosin, cannot recapitulate an accurate representation of the heart and are a limited source.

Small animal models do not demonstrate physiologically relevant characteristics to those of the human heart. Differences in isoform expression of key sarcomeric components and beat rate mean that any experimental outcomes need to be investigated in a human model to confirm mechanisms/functions exist in the same format and have the potential to lead to improved therapies. This is of even greater importance when investigating the role of lncRNAs in human disease. The poor conservation of lncRNAs between species requires the use of a human model, as some lncRNAs discovered in animal models do not exist within the human genome.

Although hiPSC-CMs are regarded as one of the best models of human cardiac disease, there are still advancements required until their full potential is reached. Current issues revolve around maturity and composition not reflecting that of the human adult heart. The maturity of hiPSC-CMs can partially be overcome by using 3D EHTs which present greater similarity to human hearts in terms of contractility. Still, cardiomyocytes are just one cell type present in the multicellular heart. Although ventricular cardiomyocytes are predominantly affected by mutations in *MYH7*, using hiPSC-CMs to model the disease indicates only some phenotypes that might occur. A comprehensive model would encompass all cell types present in the heart in a multi-culture format; cardiomyocytes, fibroblasts, mural cells and endothelial cells [341]. Realistically, there are challenges to this due to the different culture/differentiation requirements of each cell type. Co-culture was first achieved by Mummery and colleagues with two cell types (CMs and endothelial) [342], followed by three types (CMs, endothelial and fibroblasts) [343]. For the purposes of lncRNA investigation, a comprehensive multi-culture model would be ideal to fully understand the phenotypic effects. Until this exists, hiPSC-CMs are a sufficient model and the work in this thesis sets a precedent on why further investigations should be carried out in hiPSC-CMs, as opposed to animal models that are still widely used.

6.2 lncRNAs acting as gene modifiers in HCM

Integrating hiPSCs with gene editing and modulating technologies, the work in this thesis revealed novel lncRNAs involved in HCM acting as gene modifiers. While these data are still preliminary, the hypertrophy data (BNP assay) strongly suggests that there are lncRNA mechanisms underlying hypertrophy in HCM. One method to illuminate these mechanisms is through the prediction of interacting proteins of the lncRNA targets. catRAPID is a server that has been used to predict such interactions, identifying proteins of high probability to directly interact with the RNA in question [344]. In addition, determining the location of the lncRNAs would be beneficial as nuclear and cytoplasmic lncRNAs perform different functions – the same location of the lncRNA and potential interacting protein may add strength to the catRAPID predictions. To confirm the predictive results, RNA immunoprecipitation (RIP) of the proteins will quantify the enrichment – high enrichment is suggestive of interactions between the two molecules.

6.3 *MINCR* expression relates to cell cycle progression in hiPSCs

In Chapter 4, the arguments for whether cardiomyocytes are dividing cells were reviewed. While the debate continues as to whether cardiomyocytes are proliferative or not, a 2020 study by Wu and colleagues demonstrated that WNT activation and reduced cell to cell contact enabled the proliferation of cardiomyocytes [345]. In chapter 5, the knockout of *MINCR* in hiPSCs revealed an unanticipated result which led to the hypothesis that *MINCR* is involved in cell cycle progression and/or apoptosis in hiPSCs. Whether this extends to hiPSC-CMs is a cause for future experiments, as asked by question 2 in chapter 5.6.1. If, however, this were the case, whether the overexpression of *MINCR* promoted cardiomyocyte differentiation would be of significant interest to the field. Identification of interacting proteins and other molecules which can be initially predicted by *in silico* modelling may enable the understanding of the role of *MINCR* in proliferation, in addition to the results of differential expression of cell cycle genes.

6.4 Study limitations

6.4.1 Assessing transfection efficiency

Transfection efficiency was assessed in hiPSC-CMs not transfected with lncRNA-CAG vectors, but cells separately transfected with pmaxGFP. Therefore, the efficiency of transfection was not assessed in each well but assumed based on the pmaxGFP wells. Another approach would have been to incorporate GFP and a promoter into the lncRNA-CAG vectors or perform dual transfection, but it is unknown whether this expression would impact lncRNA overexpression, hence transfection was assessed in separate wells. In addition, transfection with multiple plasmids can cause toxicity and affect cell viability.

6.4.2 Phenotypic assays

High content imaging assays can provide high throughput interpretations of HCM phenotypes. There are a few issues with the automated analysis of these assays, however. The scripts used to analyse the images are not optimised therefore the distinction between cardiomyocytes is not perfect. Additionally, due to the multinucleation of cardiomyocytes, the software tended to identify each of the cells separately. In an attempt to combat this, a building block was inserted into the algorithm with a value intended to cluster nuclei that likely belonged to one cell together within a certain distance. While the algorithm performs the analysis, it does so based on the conditions fed to it by the user, therefore between each biological repeat, there can be some variation in the analysis applied.

Mitochondrial respiration profiles can be obtained in a high throughput manner using the Seahorse XF analyser. Issues using this platform relate to the fact that the data is obtained from monolayer cultures, which do not represent normal cardiac physiology and respiration. There are further issues with normalisation based on cell counts due to multinuclear tendencies of cardiomyocytes. These issues are discussed above. Other methods of normalisation that could have been used are DNA or protein quantification using Picogreen fluorescence labelling or Bradford assays, respectively. DAPI staining and high content imaging were opted for due to the potential disproportionate effects that multinucleated cells may

have on DNA quantification and the possible higher protein content in diseased lines in terms of protein quantification.

6.5 Future work

Although this work developed novel gene-editing tools and an optimised transfection protocol specifically for hiPSC-CMs, additional experiments should be performed to improve the targeting strategy and elucidate the more HCM hallmark phenotypes. Due to the impact of COVID-19 on this project, some of the suggested phenotyping analyses could not be performed and therefore are pivotal to furthering the understanding of the lncRNA targets.

1. FISH analysis of the localisation of the lncRNAs in the HCM model. For those lncRNA targets of shorter length (target 2 and *MINCR*), prediction models could be used.
2. Functional analysis of the contractility of lncRNA target knockouts in hiPSC-CMs could be analysed using EHTs. In addition, the enhanced maturation state associated with EHTs would provide greater physiological relevance. This technique enables consistent data collection and can be used for pharmacological and histological analysis.
3. Analysis of the electrophysiology of lncRNA expression modulated hiPSC-CMs using multi-electrode arrays (MEA) or patch-clamp technologies.
4. Using CelloPTIQ for the analysis of calcium transients in lncRNA modulated hiPSC-CMs.
5. *MINCR* knockdown in hiPSC and hiPSC-CMs to perform proliferation/apoptotic assays to determine if the results obtained in this study were reproducible. Further analysis of the overexpression of *MINCR* in hiPSC-CMs to determine if it is possible to harness *MINCR* to promote the proliferation of hiPSC-CMs by assessing proliferation rates.
6. *In silico* modelling using the catRAPID program to identify lncRNA to protein interactions. This could elucidate the mechanisms of action of lncRNAs and allow precise pharmacological targeting.

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Appendix - Publications

1. **Bodbin S. E**, Denning C, Mosqueira D. Transfection of hPSC-Cardiomyocytes Using Viafect™ Transfection Reagent. *Methods Protocols*. 2020 Aug 9;3(3):57
2. Bhagwan JR, Mosqueira D, Chairez-Cantu K, Mannhardt I, **Bodbin S. E**, Bakar M, Smith JGW, Denning C. Isogenic models of hypertrophic cardiomyopathy unveil differential phenotypes and mechanism-driven therapeutics. *J Mol Cell Cardiology*. 2020 Aug;145:43-53
3. Kargaran, P. K., Evans, J. M., **Bodbin, S. E.**, Smith, J. G. W., Nelson, T. J., Denning, C., & Mosqueira, D. (2020). Mitochondrial DNA: Hotspot for potential gene modifiers regulating hypertrophic cardiomyopathy. *Journal of Clinical Medicine*, 9(8), [2349]