

Imperial College London
National Heart and Lung Institute

**Developing a model of mitochondrial
heteroplasmy in human pluripotent stem cell-
derived cardiomyocytes**

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December 2017

*Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy of
Imperial College London*

Abstract

Hypertrophic cardiomyopathy and arrhythmias are common in patients with heteroplasmic mtDNA mutations but their pathogenesis and how the levels of mutation change in patients over time remain poorly understood. Therefore, a robust *in vitro* model of heteroplasmy in cardiomyocytes (CMs) is currently needed. This thesis addressed the hypothesis that human induced pluripotent stem cell-cardiomyocytes (hiPSCs-CMs) with heteroplasmic mitochondrial dysfunction recapitulated cardiac features associated with mtDNA disease. We have applied maturation strategies to identify culture conditions where the maximal metabolic potential of healthy hiPSC-CMs could be achieved to later determine if that was compromised in models of heteroplasmy. Time in culture and T₃ did not improve mitochondrial function in hiPSC-CMs and consequently were not further applied. Antimycin A and menadione impair the electron transport chain and increase the production of reactive oxygen species and were used to generate a drug-induced model of heteroplasmy using healthy hiPSC-CMs. We found that the cells' mitochondrial function recovered from antimycin A and menadione damage, possibly due to a quick mitochondrial turnover and so this approach could not be pursued. Instead, CMs were generated by differentiating hiPSCs reprogrammed from somatic cells of a patient with the A3243G mtDNA mutation. The levels of heteroplasmy increased with hiPSC passaging and we obtained pure populations of A3243G hiPSC-CMs with heteroplasmy levels ranging from 23 to 45% and preserved structural properties. Most functional properties were also maintained but mutant hiPSC-CMs showed a delay in the initiation of the calcium transient, which could explain the episodes of bradycardias that occur in patients with mtDNA diseases. Here, we have demonstrated that hiPSC-CMs can be used to investigate the cardiac abnormalities associated with mtDNA disease but concluded that higher levels of heteroplasmy would be required to detect a disease phenotype in these cells. Future work on heteroplasmic drifts in hiPSC culture will provide crucial information about the pathogenesis of mtDNA disease and into the drift that might happen with age in children resulting from mitochondrial replacement therapy, where a small amount of mutant mtDNA is carried over.

Declaration of originality

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Conference Proceedings

“Electron transport chain inhibition for the generation of human stem cell-derived cardiomyocyte cultures with various levels of impaired mitochondrial activity aimed at studying arrhythmias”

Ricardo CP, Hellen N, Kodagoda T, Mitalipov S, Terracciano C, Harding S E

Poster presentation at Joint British Heart Foundation Regenerative Medicine Centres Meeting 2016, University of Cambridge / Cambridge, U.K.; 23 June 2016.

“Low levels of the A3243G mtDNA mutation in human induced pluripotent stem cell-cardiomyocytes do not cause functional or metabolic disturbances but increase with further passaging”

Ricardo CP, Hellen N, Foldes G, Kodagoda T, Terracciano CM, Duchon MR, Harding SE

Poster presentation at Joint British Heart Foundation Regenerative Medicine Centres Meeting 2017, University of Nottingham / Nottingham, U.K.; 12 June 2017.

“Low levels of the A3243G mtDNA mutation in human induced pluripotent stem cell-cardiomyocytes do not cause functional or metabolic disturbances but increase with further passaging”

Ricardo CP, Hellen N, Foldes G, Kodagoda T, Terracciano CM, Duchon MR, Harding SE

Oral presentation at Mitochondria: Form and Function Meeting 2017, University College London / London, U.K.; 14-15 September 2017

Acknowledgements

Firstly, I would like to thank my supervisor Professor Sian Harding for challenging me to embark in such an exciting project, so unique compared to the research done at NHLI Myocardial Function Section. I am very grateful for all the opportunities I have been afforded during my time in this lab. Thank you for helping me to grow as a scientist, not only through constant supervision but also by giving me complete freedom to find my own direction. To my supervisor Professor Cesare Terracciano I must thank you for the support, guidance and especially for always casting a critical eye over my work.

A big thank you to the members of the Harding group who directly contributed to this project, Dr. Nicola Hellen, Dr. Gabor Foldes, Thusharika Kodagoda and Magdalena Kloc. A special acknowledgement to Dr. Maeve Elder and Micaela Vidal Jenkins, the two other “powerhouses of the cell”, with whom I was lucky enough to discuss the complex nature of mitochondria and of the Seahorse Analyser... I cannot forget all the other colleagues on the floor who helped me with this thesis through advice and discussion, moral support and friendship but would like to thank in particular Dr. Matthew Turley, Peter O’Gara, Dr. Chris Kane, Liam Couch, Dr. Filippo Perbellini and the “Pineapple Team”.

This work would not be complete without the collaboration with Professor Michael Duchon and the members of his lab, Dr. Will Kotiadis and Dr. Shona Joy, at University College London. Thank you Michael for the enthusiastic discussions about this project and for sharing with us with such a precious cell line, which would not have been possible without Will’s hard work. I would also like to thank Dr. Shoukhrat Mitalipov’s lab in Oregon University, in particular Dr. Hong Ma, who shared the protocol and cell line used for heteroplasmy quantification. Again, this was an integral part of my thesis. Finally, I must thank Dr. Tristan Rodriguez at Imperial College London for establishing contact with the University of Edinburgh to provide the GFP

cell line and for guiding me through the cell competition studies; thank you to Thomas Adejumo at the MRC London Institute of Medical Sciences Flow Cytometry Facility for helping me to execute them.

A special mention goes to the NHLI Foundation, who awarded me with the PhD scholarship, and the British Heart Foundation, who funded most of the experimental work presented here.

Last but not least I would like to thank my family and friends for their unconditional support and love during these three years, in particular my parents for being my role models as scientists; my grandfather for teaching me to work hard and who I very much wished to be here to see this work completed; and Sam for always being present at work and at home and for never letting me down when the tough times came.

“If you think you are too small to make a difference, try sleeping with a mosquito.”

Tenzin Gyatso, 14th Dalai Lama

Abbreviations

$\Delta\mu_{\text{H}^+}$	electrochemical proton gradient
Δp	proton motive force
ΔpH_m	mitochondrial pH gradient
$\Delta\psi_\text{m}$	mitochondrial membrane potential
ANOVA	analysis of variance
AP	action potential
ARMS qPCR	amplification-refractory mutation system real-time PCR
ARVC	arrhythmogenic right ventricular cardiomyopathy
b.p.m.	beats per minute
Ca^{2+}	calcium ion
CASQ2	calsequestrin-2 gene
CDI	Cellular Dynamics International
CDS	cell dissociation solution
CM	cardiomyocyte
CPVT	catecholaminergic polymorphic ventricular tachycardia
CTRL	control
D3	deiodinase 3
DAD	delayed afterdepolarizations
DCM	dilated cardiomyopathy
dNTPs	deoxyribonucleoside triphosphates

E8	essential 8
EAD	early afterdepolarization
EB	embryoid body
EC	excitation contraction
ECAR	extracellular acidification rate
ESC	embryonic stem cell
ETC	electron transport chain
F0	baseline fluorescence
F	peak fluorescence
FCCP	carbonyl cyanide p-trifluoromethoxyphenyl-hydrazine
H ⁺	hydrogen ion
H ₂ O ₂	hydrogen peroxide
HCM	hypertrophic cardiomyopathy
hESC	human embryonic stem cell
hiPSC	human induced pluripotent stem cell
hMSC	human mesenchymal stem cell
hPSC	human pluripotent stem cell
I _{CaL}	L-type calcium current
I _f	funny/pacemaker current
I _{K1}	inwardly rectifying potassium current
I _{Kr}	rapid delayed potassium current

I_{Ks}	slow activating delayed potassium current
IM	inner membrane
IMS	intermembrane space
I_{Na}	sodium current
IOP	iopanoic acid
iPSC	induced pluripotent stem cell
I_{to}	transient outward potassium current
KH	Krebs–Henseleit
LQT	long QT
LS	Leigh Syndrome
MCU	mitochondrial Ca^{2+} uniporter
MEF	mouse embryonic fibroblast
MELAS	myopathy, encephalopathy, lactic acidosis, stroke-like episodes
MERRF	myoclonic epilepsy and ragged red fibres
mESC	mouse embryonic stem cell
MIDD	maternally inherited diabetes and deafness
mMSC	mouse mesenchymal stem cell
MPTP	mitochondrial permeability transition pore
mtDNA	mitochondrial DNA
MUT	mutant
Na^+	sodium ion

NADH	nicotinamide adenine dinucleotide (NAD) + hydrogen (H)
NARP	neuropathy, ataxia, retinitis pigmentosa
NCX	sodium-calcium exchanger
NO	nitric oxide
O_2^-	superoxide
OCR	oxygen consumption rate
OM	outer membrane
OXPHOS	oxidative phosphorylation
Q	ubiquinone
qPCR	real-time PCR
RB+	RPMI 1640 + 1x B27 with insulin
RB-	RPMI 1640 + 1x B27 without insulin
RMP	resting membrane potential
ROS	reactive oxygen species
rRNA	ribosomal RNA
rT ₃	reverse T ₃
RyR2	ryanodine receptor type 2 gene
s.e.m.	standard error of the mean
SERCA	sarcoplasmic reticulum calcium ATPase
SOD2	superoxide dismutase
SR	sarcoplasmic reticulum

T ₃	triiodothyronine
T50	time to 50% decay
T50 Relax	time to 50% relaxation
TALEN	transcription activator-like effector nucleases
TNT	tunnelling nanotube
TPeak	time to peak
tRNA	transfer RNA
ZFP	zinc finger peptide

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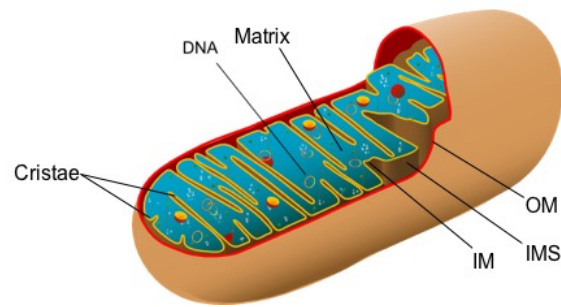
Chapter 1 : Introduction

1.1 Mitochondria – form and function

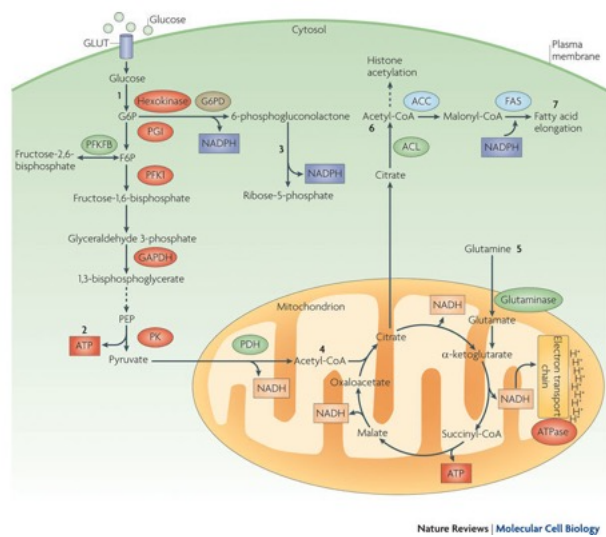
1.1.1 Basic structure

Mitochondria arose approximately two billion years ago from the engulfment of an α -proteobacterium by a precursor of the eukaryotic cell (Lane and Martin, 2010). Although their main function is the production of ATP by oxidative phosphorylation (OXPHOS), these organelles share other important functions such as the generation and control of reactive oxygen species (ROS), regulation of programmed cell death (apoptosis) and calcium homeostasis. Like their ancestor, mitochondria are still comprised of two separate membranes, the inner (IM) and outer (OM) membranes, which encapsulate an intermembrane space (IMS) and of a matrix compartment. The IM folds to form cristae, increasing the IM surface area. Mitochondria contain their own circular genome, mitochondrial DNA (mtDNA), which is present in the matrix region. See Figure 1.1a for a schematic diagram of a mitochondrion. An electron transport chain (ETC) comprising of a series of complexes is located in the IM cristae and is responsible for the transfer of electrons and for the transport of protons (H^+) from the matrix to the IMS, across the IM. This creates an electrochemical proton gradient ($\Delta\mu_{H^+}$) across the IMS that drives ATP production during OXPHOS. See Figure 1.1b/c for a schematic overview of cellular metabolism and of the ETC.

a



b



c

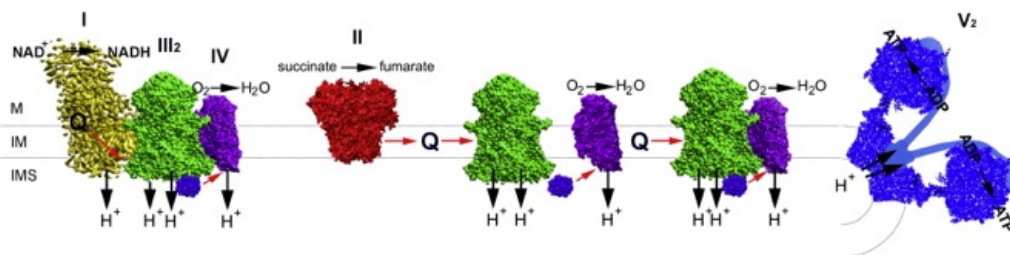


Figure 1.1: Schematic diagrams of the mitochondrial ultrastructure and of the electron transport chain.

a) Each mitochondrion is comprised of an outer membrane (OM) and an inner membrane (IM), which encapsulate an intermembrane space (IMS), and of a matrix compartment. The IM folds to form cristae. Molecules of mitochondrial DNA are located in the matrix. b) Glycolysis occurs in the cell's cytoplasm and generates pyruvate which enters the mitochondrial Krebs cycle to generate NADH and succinate, the fuel of the electron transport chain (ETC), generating ATP during mitochondrial oxidative phosphorylation (OXPHOS). From (Buchakjian and Kornbluth, 2010). c) The ETC chain is located in the IM and is comprised of complexes I (yellow), II (red), III (green), IV (purple) and V (ATP synthase, blue). It is responsible for the transfer of electrons (electron pathways represented by red arrows) and the accumulation of protons (H^+) in the

IMS, which are transferred back to the matrix (M) by complex V. Q, ubiquinone. From (Chaban, Boekema and Dudkina, 2014)

1.1.2 Mitochondrial dynamics

Previously thought to keep a rod-shaped structure, mitochondria are now known to be highly dynamic. Their shape can vary between small spheres or short rods to long tubules, depending on the cell type. Mitochondria within a single cell also constantly undergo structural changes known as fusion and fission. During fusion, the double membranes of individual mitochondria merge and their matrix contents are mixed to form a bigger single organelle. On the other hand, during fission, a single mitochondrion can divide to generate two or more smaller organelles(Chaban, Boekema and Dudkina, 2014). Recently, it has also been shown that mitochondria can also travel between cells in a process known as mitochondrial transfer(Detmer and Chan, 2007). These dynamic processes will be discussed in more detail below.

The main mediators of fusion and fission are proteins members of the Dynamin family. In mammals, mitofusins (MFN1 and MFN2) mediate OM fusion and the protein OPA1 mediates IM fusion. During fusion, mitofusins of neighbouring mitochondria bind, allowing the OM to merge. This is followed by merging of the IM through association of OPA1 proteins of adjacent mitochondria. During fission DRP1, another protein of the Dynamin family, is recruited from the cytosol to the OM. When localised to the OM, DRP1 oligomerizes into a spiral structure that constricts and separates the mitochondrion(Rustom *et al.*, 2004). Mitochondrial dynamics have numerous biological roles in health and disease. For maintenance of a healthy mitochondrial population, a deficient mitochondrion (e.g. lacking mtDNA) can regain function by fusing with a healthy organelle, sharing its contents and subsequently undergoing fission(van der Bliek, Shen and Kawajiri, 2013). Increased fission leading to fragmentation of mitochondria is involved in apoptosis. This enables the release of cytochrome c from the IMS which activates a cascade of caspases involved in the apoptotic programme(Koshiba *et al.*, 2004). Fusion and fission are also involved in cell growth, division, differentiation and calcium

homeostasis and perturbations in any of these aspects can cause disease(Youle and Karbowski, 2005).

A study published over a decade ago by the Gerbes group was the first to describe intercellular organelle transport between rat cells, followed by multiple studies showing evidence of mitochondrial transfer between other mammalian cell types in culture(Detmer and Chan, 2007). Transport of mitochondria between cells *in vitro* seems to occur via tunnelling nanotubes (TNTs), which are protrusions that extend from the plasma membrane, usually containing cytoskeletal elements such as actin/myosin filaments and microtubules(Rustom *et al.*, 2004). As examples of mitochondrial transfer, human lung carcinoma cells depleted of mitochondria (rho zero) regained mitochondria and aerobic respiration was restored when co-cultured with human mesenchymal stem cells (hMSCs)(Berridge *et al.*, 2016); repair of rotenone injured mouse tracheal epithelial cells was promoted by transfer of mitochondria from mouse MSCs (mMSCs)(Spees *et al.*, 2006); mitochondrial transfer from mice astrocytes to neurons after stroke contributed to endogenous neuroprotective and neurorecovery mechanisms(Ahmad *et al.*, 2014). Overall these studies highlighted the importance of mitochondrial transfer to tackle mitochondrial dysfunction. It is also possible that mitochondrial transfer is present under physiological conditions to maintain the healthy status of the cell.

1.1.3 Mitochondrial DNA

Mitochondrial DNA (mtDNA) contains 37 genes which encode for 13 proteins involved in the ETC, 22 tRNAs and 2 rRNAs. The remaining proteins involved in OXPHOS, totalling 80 in humans, are encoded by the nuclear DNA. mtDNA is packaged into protein-DNA complexes, known as nucleoids, and exists in 2 to 10 copies per organelle(Hayakawa *et al.*, 2016). On average there are between 10000 and 100000 copies of mtDNA in human cells(Wiesner,

Rüegg and Morano, 1992). Mutations can either affect all copies of mtDNA (homoplasmic) or just a certain number (heteroplasmic). By definition, heteroplasmy is the presence of more than one type of mtDNA within a cell and we will base ourselves on this theory for the purpose of this thesis(Wallace and Chalkia, 2013). However, it is important to mention that since there are multiple mtDNA copies inside a single mitochondrion, it is possible that heteroplasmy levels vary between mitochondria of the same cell. Similarly, heteroplasmy levels can vary in different cell populations, tissues and organs. To date, quantification of heteroplasmy levels is possible in DNA extracted from a population of cells and we should be aware of this limitation. Mutations can be either transmitted maternally or acquired in somatic cells. High levels of heteroplasmy of deleterious mutations can cause mitochondrial disorders, but this will be further discussed later. First it is important to describe the pattern of mtDNA inheritance.

1.1.3.1 Mitochondrial DNA inheritance

In contrast to nuclear DNA, mtDNA is inherited maternally exclusively. The exact reason for this remains unclear but is it possible that paternal mtDNA is eliminated to avoid greater levels of heteroplasmy to be passed on to offspring. The mechanism of paternal mtDNA elimination has been widely studied using *D. melanogaster* and *C. elegans*. In *D. melanogaster* paternal mtDNA is degraded by mitochondrial Endonuclease G during sperm elongation pre-fertilization. In contrast, in *C.elegans*, mtDNA is eliminated by mitochondrial autophagy (mitophagy) shortly post-fertilization(Rooney *et al.*, 2015). Although it has been established that paternal mtDNA elimination is conserved in mammals, the exact mechanisms are still unknown. It is thought that it might involve elimination both pre and post-fertilization and that mitophagy might also play a role(Mishra and Chan, 2014).

Even though paternal mtDNA is eliminated, it has been shown that levels of heteroplasmy can change dramatically during germ line transmission. For instance, a mother with no apparent mitochondrial disorder can give birth to a child with a mitochondrial condition and *vice versa*, as mature oocytes from the same mother show differences in the mtDNA genome(Mishra and Chan, 2014). This led to the mtDNA genetic bottleneck theory, which states that only a small proportion of the maternal mtDNA is passed on to offspring(Tuppen *et al.*, 2010). The mechanisms behind the mtDNA genetic bottleneck are not fully understood yet but studies have shown that it could be explained by three different models: the reduction in mtDNA copy number in primordial germ cells; the unequal partitioning of homoplasmic segregating units in primordial germ cells or the replication of a subgroup of genomes during oocyte maturation(Stewart and Chinnery, 2015). A schematic diagram showing the proposed theories of mtDNA segregation is shown in Figure 1.2. Heteroplasmy can vary between oocytes from the same mother but also from cell to cell and between tissues in the same individual. At present, the pattern of mtDNA segregation is still thought to be random(Carling, Cree and Chinnery, 2011).

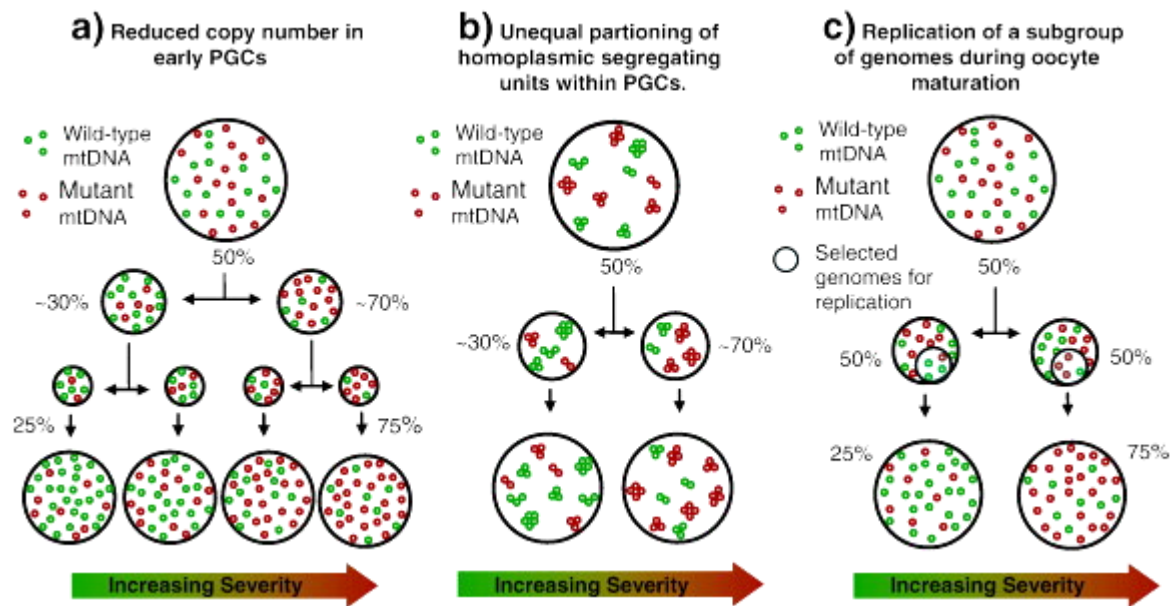


Figure 1.2: Proposed models of mtDNA segregation in embryogenesis.

Diagram shows the three proposed theories for the mtDNA genetic bottleneck in the generation of mature oocytes from primordial germ cells (PGCs): a) reduced copy number in PGCs, b) unequal partitioning of homoplasmic segregating units within in PGCs and c) replication of a subgroup of genomes during oocyte maturation can all give rise to mature oocytes with varying levels of heteroplasmy. Heteroplasmy is represented as a % of mutant to wild-type mtDNA. From (Stewart and Chinnery, 2015).

1.1.4 The electron transport chain

Probably the most important role of mitochondria is the production of ATP during OXPHOS. The IM accommodates multiple copies of the respiratory complexes (I-V) that form the ETC. The main function of ETC is the transfer of electrons and the transport of protons (H^+) across the IM to the IMS to generate an $\Delta\mu_H^+$ across the IMS that is used by the last complex (V, ATP Synthase) to produce ATP.

The ETC is initiated by NADH and succinate, by-products of glycolysis, fatty acid oxidation and of the Krebs cycle. See Figure 1.1b/c for schematic diagrams of cellular metabolism and of the ETC. Complex I accepts electrons from NADH and transfers them to ubiquinone. Protons are also pumped across the IM at this stage. Complex II separately accepts electrons from succinate and also transfers them to ubiquinone. Complex II is not involved in H^+ transport. Ubiquinone transfers electrons to complex III which in turn transfers them to cytochrome c and additionally pumps H^+ across the IM. Complex IV accepts electrons from cytochrome c and delivers them to the last electron acceptor, an oxygen molecule, to convert it to two water molecules. Protons are also pumped at this stage. Leakage of electrons to oxygen at earlier stages generates the formation of the ROS superoxide (O_2^-). Superoxide formation is common in complexes I and III. At the end of the ETC, an electrochemical gradient across the IMS has been generated. Protons are then pumped back into the mitochondrial matrix by the F_0 component of the ATP synthase, causing a conformational change in the enzyme which releases free energy catalysing the generation of ATP from ADP + Pi at site F_1 (Carling, Cree and Chinnery, 2011). At this stage it is important to mention that the proton motive force (Δp) determines H^+ transport back to the mitochondrial matrix and hence the rate of ATP synthesis. Δp has two components: the mitochondrial membrane potential ($\Delta\psi_m$, a charge or electrical gradient) and the mitochondrial pH gradient (ΔpH_m , an H^+ chemical or

concentration gradient). For this reason fluorescent probes to measure $\Delta\psi_m$ are often used to assess mitochondrial function(Chaban, Boekema and Dudkina, 2014).

1.1.4.1 Inhibitors and uncouplers of the electron transport chain

Agents that inhibit or dissipate the $\Delta\mu_{H^+}$ across the IM are classified as inhibitors or uncouplers of mitochondrial respiration. Some of these compounds also increase the generation of superoxide in mitochondria. They can be useful to induce mitochondrial dysfunction but also to investigate specific properties of mitochondrial respiration in cells. For the purpose of this thesis we will focus on the agents rotenone, antimycin A, oligomycin, FCCP and menadione.

If mitochondria require to increase oxygen consumption rate (OCR) for ATP production then the $\Delta\mu_{H^+}$ decreases causing an increase in the rate of respiration (or activity of the ETC). On the other hand, if the ATP requirements are lower and mitochondria consume less oxygen, then the $\Delta\mu_{H^+}$ increases and the rate of respiration decreases. Rotenone is a member of the rotenoid family of isoflavonoids produced by Leguminosae plants and is a specific inhibitor of complex I. The antibiotic antimycin A is an inhibitor of complex III and works by inhibiting electron transfer from b heme bH to ubiquinone at center Qi. Electron leakage to oxygen and superoxide formation at complex III consists mainly of one-electron reduction of oxygen at centre Qo and this is normally rapidly reduced by centre Qi. Consequently, antimycin A also increases the rate of superoxide formation at centre Qo. Blockage of complex I and III by rotenone and antimycin A decreases mitochondrial respiration and OCR, so the drop in OCR caused by their application allows measurement of non-mitochondrial respiration in cells. Oligomycin is an antibiotic that inhibits the ATP synthase enzyme by preventing H^+ transport across the F_0 subunit and hence stops ATP production. Consequently, OCR also drops and so the drop in OCR caused by oligomycin can be used to quantify the levels of ATP production in cells. The uncoupler carbonyl cyanide p-trifluoromethoxyphenyl-hydrazine (FCCP) is a

lipophilic weak acid that moves H^+ across the IM (protonophore). It travels to the IMS, combines with H^+ , travels back to the mitochondrial matrix across the IM to release H^+ , thereby reducing the $\Delta\mu_{H^+}$. The decrease in $\Delta\mu_{H^+}$ causes an increase in the rate of respiration and in OCR and hence the rise in OCR caused by FCCP can be used to measure the maximal respiration in cells. Efficient H^+ transport across the IM for the generation of a $\Delta\mu_{H^+}$ requires that electron transfer occurs through the ordered sequence of electron acceptors in the ETC. Menadione is an alternate electron acceptor, another type of uncoupler, which accepts electrons from complex I and feeds them directly to complex IV. This bypasses H^+ being pumped from complex III. Additionally, menadione also increases the rate of superoxide formation by redox cycling and generation of oxygen free radicals(Perry *et al.*, 2011).

1.1.5 Mitochondrial function in the heart

As mentioned before, the main function of mitochondria is the production of ATP via OXPHOS but they have other important functions such as calcium homeostasis, the generation and control of ROS and regulation of programmed cell death. Here, the relevance of these mitochondrial roles to the heart will be evaluated.

1.1.5.1 Involvement in ATP utilisation, calcium homeostasis and ROS

ATP is required for cardiac excitation-contraction (EC) coupling and it is expected that cells in the human heart daily synthesize 30kg of ATP(Wallace and Starkov, 2000). In the failing human heart, a low phosphocreatine-to-ATP ratio has been described which indicates a cardiac energy deficit(Lemieux and Hoppel, 2009). During the cardiac action potential, the sarcolemma is depolarized by activation of voltage-gated Na^+ channels. Calcium enters the cell via L-type Ca^{2+} channels, triggering opening of RyR2 subtype of ryanodine receptors and Ca^{2+} release from the sarcoplasmic reticulum (Ca^{2+} -induced Ca^{2+} -release). The cytosolic free

Ca^{2+} concentration rises and activates contractile proteins, causing cell contraction. During relaxation, Ca^{2+} is re-uptaken mainly by the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) at the expense of ATP hydrolysis and by the sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). There is also some small contribution by the sarcolemmal Ca^{2+} -ATPase, again at the expense of ATP hydrolysis, and by the mitochondrial Ca^{2+} uniporter (MCU)(Beer *et al.*, 2002). There is some controversy as to whether mitochondria of cardiomyocytes (CMs) uptake Ca^{2+} in a beat-to-beat fashion(Bers, 2002). It is more likely that Ca^{2+} uptake is slow, causing a slow accumulation of Ca^{2+} in the mitochondrial matrix. Nevertheless, it is known that this stimulates dehydrogenase activity, increasing mitochondrial respiration and ATP production. Additionally, mitochondrial Ca^{2+} uptake stimulates mitochondrial nitric oxide synthase (mtNOS), causing NO production which in turn is involved in mitochondrial Ca^{2+} homeostasis(Dedkova and Blatter, 2008).

Alterations in mitochondrial Ca^{2+} are central in ischemia-reperfusion injury. Ischemia-reperfusion causes a drop in ATP production by CMs which results in a reduction in Na^+/K^+ ATPase function. This increases intracellular Na^+ and Ca^{2+} and hence mitochondrial Ca^{2+} . Excessive mitochondrial Ca^{2+} causes the mitochondrial permeability transition pore (MPTP) to open which in turn enables antioxidant enzymes to escape mitochondria and increases ROS. Increased mitochondrial permeability also abolishes the $\Delta\psi_m$ and therefore ATP production decreases. Together, these factors cause cell death(Dedkova and Blatter, 2008).

1.1.5.2 Involvement in apoptosis

Cardiomyocytes undergo apoptosis in response to a number of stimuli including ischemia-reperfusion, heart failure, acidosis, oxidative stress and stretch(Griffiths, 2012). The evidence for ischemia-reperfusion-induced apoptosis is particularly strong, in cells(Crow *et al.*, 2004), animal models(Bialik *et al.*, 1999) and humans(Fliss and Gattinger, 1996). Inhibition of cardiomyocyte apoptosis by overexpression of anti-apoptotic protein Bcl-2 in mice subject to ischemia-reperfusion reduces infarct size up to 70% and ameliorates cardiac dysfunction(Saraste *et al.*, 1997).

It was discussed before that cytochrome c release from the IMS, which increases during mitochondrial fission, activates a cascade of caspases involved in the apoptotic programme. This process also occurs in response to ischemia-reperfusion. Apoptosis is mediated by two central pathways, the extrinsic (or death receptor) and the intrinsic (or mitochondrial) pathways, both with the end goal of procaspase activation, mitochondrial function abolishment and proteolytic cell destruction. In the intrinsic pathway, BH3-only proteins and Bax transmit intracellular and extracellular death signals to mitochondria. Bax translocates to the OM and together with Bak induces OM membrane permeabilisation, causing release of cytochrome c and of other apoptogens from the IMS. This step is opposed by Bcl-2 and Bcl-xL. Cytochrome c then assembles into the apoptosome, triggering procaspase-9 activation, subsequent procaspase-3 activation and cell death(Brocheriou *et al.*, 2000; Chen *et al.*, 2001). It is possible that OM permeabilisation is further increased by the opening of MPTP and that this abolishes the $\Delta\psi_m$, but this link is still unclear(Crow *et al.*, 2004).

1.2 Mitochondrial DNA disease

1.2.1 Mutation rate in mitochondrial DNA

The spontaneous mutation rate in mtDNA has been estimated to be 100 times higher than in nuclear DNA and several factors could be responsible for this (Green and Kroemer, 2004). Superoxide production by the ETC can cause excessive damage to mtDNA and in addition mtDNA lacks histones which are thought to serve as a barrier against ROS damage (Marcelino and Thilly, 1999). Although mitochondria possess a base excision repair system, other DNA repair systems found in the nucleus have not been found in mammalian mitochondria (Alexeyev, 2009). Previously it was thought that another factor contributing to the high mutation rate were mtDNA replication errors, but the mtDNA polymerase can replicate mtDNA very precisely due to a high nucleotide selectivity and the ability to excise nucleotide misinsertions. Instead, asymmetric pools of deoxyribonucleoside triphosphates (dNTPs), with predominance of dGTPs, have been described in rat mitochondria and it is thought that this imbalance is contributing towards the high mutation rate of mtDNA (Bogenhagen, 1999).

1.2.2 Specific characteristics of mtDNA mutations

Several factors are responsible for the onset of clinical symptoms and the phenotypic variability typical between patients with mtDNA mutations. The mtDNA genetic bottleneck contributing to variable levels of heteroplasmy in offspring from the same mother was already described above (see 1.1.3.1). In addition, three more features are specific to mitochondrial disease: the threshold effect, mitotic segregation and clonal expansion, all of which will be discussed below.

In the case of heteroplasmic mtDNA mutations, the ratio of mutant to wild-type mtDNA determines the clinical phenotype. A certain threshold of mutant mtDNA must be reached for

tissue dysfunction to occur. The threshold depends on the type of mutation and on the tissue affected. Generally, the threshold lies between 60 and 90%(Song *et al.*, 2005). In mitotic tissues, mitochondria are randomly segregated by induction of mitochondrial fission during phases G2 and mitosis of the cell cycle(Rossignol *et al.*, 2003). Therefore, the mutant load can drift in cells with heteroplasmic mtDNA mutations. In fast-dividing tissues such as the blood, an annual 1% reduction in the A3243G mutation has been described(Mishra and Chan, 2014). Particularly in post-mitotic tissues like the heart, clonal expansion of mtDNA mutations is common, due to preferential amplification of mutant mtDNA during mitochondrial turnover(Rahman *et al.*, 2001). However, the reasons for this amplification are still unclear. These three features apply to both inherited and acquired mtDNA mutations, which will be covered next.

1.2.3 Inherited mtDNA mutations

Homoplasmic DNA mutations are transmitted maternally to all offspring. Transmission of heteroplasmic mtDNA mutations is more complex, as described before. In the mutator mouse, deleterious mutations, usually involving amino acid replacement, are often eliminated during germ cell development. On the other hand, point mutations in tRNA or rRNA genes are better tolerated(Tuppen *et al.*, 2010). Although the tRNA genes only occupy approximately 10% of the mtDNA genome, multiple pathogenic mutations affecting the human mtDNA lie in specific tRNA genes(Stewart *et al.*, 2008). The majority of inherited pathogenic mtDNA mutations are heteroplasmic and within the most common are: A3243G in the L1 gene, involved in Myopathy, Encephalopathy Lactic Acidosis, Stroke-like episodes (MELAS) and Maternally Inherited Diabetes and Deafness (MIDD); G13513A in the ND5 gene, involved in MELAS and Leigh Syndrome (LS); T8993G in the ATP6 gene, involved in Neuropathy, Ataxia, Retinitis Pigmentosa (NARP) and LS(Park and Larsson, 2011). Figure 1.3 shows the location and syndromes associated with the most common inherited mtDNA mutations.

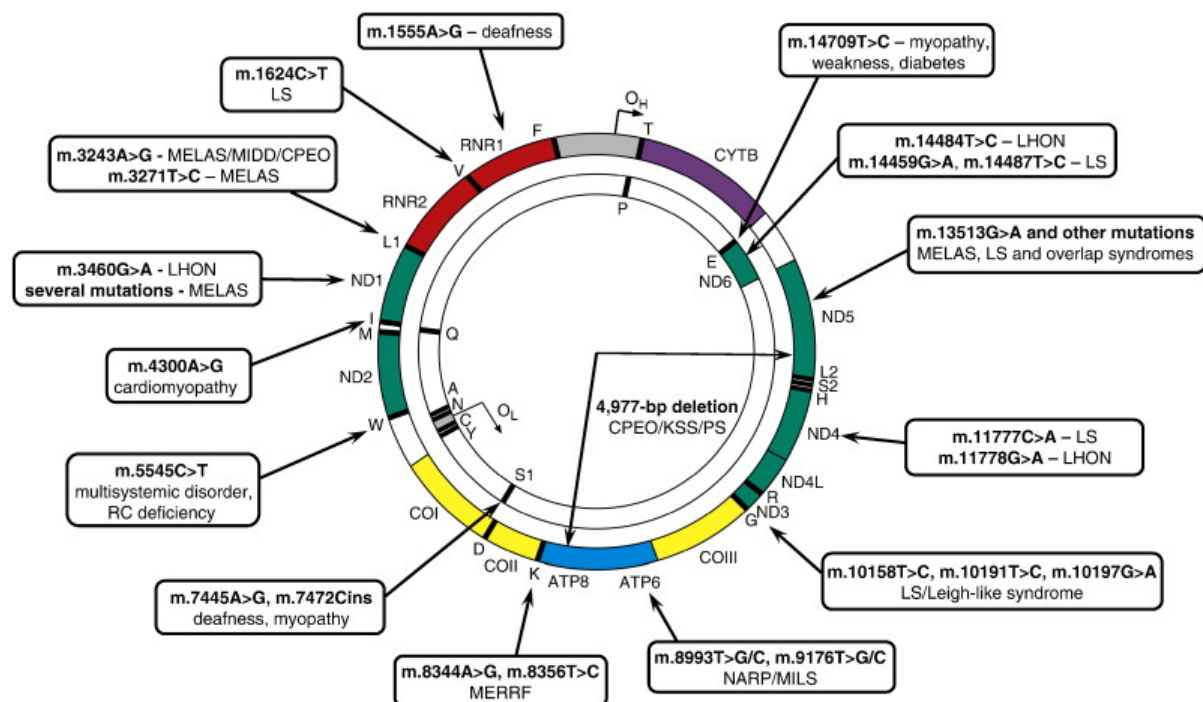


Figure 1.3: Structure of mtDNA and syndromes associated with the most common mtDNA mutations.

Diagram shows the circular double stranded mtDNA genome and genes encoding for mt-rRNAs (red), mt-tRNAs (black bars) and complex I (green), III (purple), IV (yellow) and V (blue) polypeptides. Non-coding regions are represented in grey. Arrows point to the location of the most common mtDNA mutations and associated syndromes. From (Tuppen *et al.*, 2010).

1.2.4 Acquired mtDNA mutations

As described above (see 1.2.2), high levels of a specific maternally inherited mtDNA point mutation can result in mitochondrial disease. On the other hand, accumulation of multiple types of mtDNA mutations in somatic cells are thought to be involved in ageing and cancer. The mitochondrial theory of ageing states that production of superoxide by mitochondria leads to progressive accumulation of somatic mtDNA mutations during a lifetime, causing mitochondrial dysfunction. This results in greater superoxide production and hence a ROS vicious cycle(Tuppen *et al.*, 2010). To test the mitochondrial theory of ageing, knock-in mice expressing a proofreading-deficient version of mtDNA polymerase were developed. This causes accumulation of mtDNA mutations over time and mutator mice show many features of ageing(Harman, 1992). In humans, cytochrome c oxidase-deficient CMs detected in the ageing heart showed a similar phenotype to patients with inherited mitochondrial disease(Trifunovic *et al.*, 2004). Although these studies provide evidence that somatic mtDNA mutations are present in ageing, there is some controversy as to whether these mutations reach a level that causes the ageing process.

High levels of somatic mtDNA mutations have also been reported in multiple studies of solid tumours and leukaemias in humans(Müller-Höcker, 1989). It is still unclear how these mutations accumulate and although it seems that they do not contribute to tumorigenesis directly, they may serve as a useful biomarker.

1.2.5 Pathogenesis and clinical manifestations of mitochondrial DNA disease

Since mitochondria are vital constituents of all nucleated cells, it is not surprising that mtDNA diseases affect multiple tissues. Due to the complexity of mtDNA mutations, the clinical features of mtDNA diseases are also highly variable. There are classic syndromes associated

with particular mtDNA mutations and mtDNA mutations are also involved in common disease phenotypes, such as diabetes and cardiomyopathies. Finally, mtDNA mutations are also implicated in ageing and cancer, as discussed above (see 1.2.4). Typically, features of mtDNA disease in adults involve: neurological (e.g. strokes; epilepsy; myopathy), cardiac (e.g. cardiomyopathy; arrhythmias; heart failure), ophthalmological (e.g. optic atrophy; ophthalmoplegia), endocrinal (e.g. diabetes; thyroid disease), respiratory (e.g. respiratory failure) and gastrointestinal (e.g. irritable bowel) symptoms (Kim, 2014). For the purpose of this thesis we will focus on the pathogenesis and clinical features associated with the mtDNA mutation A3243G and the cardiac involvement in mtDNA disease.

MELAS is one of the most common maternally inherited mtDNA disorders and the A3243G mutation is the most common mtDNA mutation involved in MELAS, present in over 80% of patients with the disease. The mutation is also specific to MELAS and is not found in patients with other mitochondrial syndromes (Taylor and Turnbull, 2005). It is a point mutation involving the substitution of one adenine for a guanine at nucleotide pair 3243, in the D (dihydrouridine)-loop encoding the tRNA Leu (UUR), which possibly reduces the translation efficiency and alters the ratio of mitochondrial rRNA and mRNA transcripts (Goto, Nonaka and Horai, 1990). The pathogenesis of the A3243G mutation involves decreased mitochondrial protein synthesis leading to impaired mitochondrial energy production, which in turn causes oxidative stress, NO deficiency and mitochondrial proliferation. All of these factors contribute to multi-organ dysfunction typical in MELAS. Generally, symptoms manifest before the age of 20 and patients tend to present multiple symptoms. Neurological symptoms such as stroke-like episodes, epilepsy, encephalopathy and dementia are very common, occurring in more than 90% of the patients. These are usually accompanied by other symptoms like cortical vision loss, severe headaches and muscle weakness. Cardiac problems including cardiomyopathies and cardiac conduction abnormalities have also been reported, but in less than 25% of patients (Wallace,

1992). Cardiac involvement in patients with mtDNA mutations will be discussed in more detail further down.

1.2.6 Cardiac involvement in mtDNA mutations

The pathogenesis of cardiac dysfunction caused by mtDNA mutations is poorly understood given the limited access to human cardiac tissue and the absence of reliable animal models. It is known that cardiomyopathies are more common in patients with mutations in mitochondrial tRNAs, such as the A3243G mutation(El-Hattab *et al.*, 2015). It is also understood that increased biogenesis of mutated mitochondria is a maladaptive mechanism that causes a pronounced increase in mitochondrial-derived ROS and is involved in cardiomyopathies(Majamaa-Voltti *et al.*, 2008). Generation of CMs from induced pluripotent stem cells (hiPSC-CMs) derived from patients with mtDNA mutations will be an important step forward in this area.

Together with neurological disease, cardiac disease appears to be the most common cause of early death in patients with MELAS caused by the A3243G mutation(Sebastiani *et al.*, 2007). There is no cardiac phenotype unique to patients with mtDNA mutations so, unless the patients or family members show other symptoms of mitochondrial disorders or unless a cause of cardiac dysfunction cannot be determined, the mutations can remain unidentified. The cardiac pathologies mostly involved in patients with mtDNA mutations are cardiomyopathies (hypertrophic, dilated, or restrictive) and arrhythmias (bradyarrhythmias or tachyarrhythmias). Hypertrophic remodelling, which mimics hypertrophic cardiomyopathy (HCM), is more frequent over other types of cardiomyopathies in patients with mtDNA mutations. Bradyarrhythmias are the most common type of arrhythmias in patients with mtDNA disorders(Majamaa-Voltti *et al.*, 2008).

A study of adult patients with a range of mitochondrial disorders revealed that minor electrocardiogram abnormalities were apparent in 78% of the patients and cardiomyopathy was present in 25%(Bates *et al.*, 2012). In a study of MELAS patients and carriers of the A3243G mutation, most patients (mean heteroplasmy in skeletal muscle samples was 60%) had left ventricular dysfunction and hypertrophy. Carriers (mean heteroplasmy in skeletal muscle samples was 30%) did not show cardiac symptoms(Limongelli *et al.*, 2010). This study highlighted a correlation between heteroplasmy levels and cardiac function. Bradyarrhythmias are thought to occur in 5-10% of patients with A3243G and are possibly caused by atrio-ventricular block although this is still unclear(Vydt *et al.*, 2007).

1.2.7 Management of mtDNA disease

1.2.7.1 Treatment options

Due to the complexity of mtDNA mutations, effective treatments for mtDNA diseases are still not available. The two main treatment approaches that have been explored are exercise therapy and gene therapy but the best strategy is to prevent transmission of mtDNA mutations in the first place.

Exercise therapy is an option for patients presenting myopathy with very high levels of heteroplasmic mtDNA mutations in mature muscle fibres but low levels in undifferentiated satellite cells. Resistance training should cause necrosis of mutant mature muscle fibres and incorporation of wild-type satellite cells into existing muscle fibres, thereby reducing the proportion of mutant to wild-type cells(Bates *et al.*, 2012). Twelve-week resistance training studies in patients with large-scale mtDNA deletions caused a decrease in cytochrome c oxidase muscle fibres and an increase in satellite cells, together with improvements in oxidative capacity and muscular strength(Taivassalo *et al.*, 1999). Unfortunately, exercise

therapy is limited to myopathies and there is always the risk of amplification of the mtDNA mutation.

Mitochondrial genome editing by CRISPR/Cas9 has been reported a few years ago but there is some scepticism(Murphy *et al.*, 2008). Scientists were not able to reproduce this and correcting or introducing specific pathogenic mtDNA mutations has not been achieved yet. However, other types of gene therapy have been recognised as a treatment option for several mtDNA diseases. Allotropic expression of the protein of interest has been attempted to treat mitochondrial tRNA diseases. For instance, in the case of the A3243G mtDNA mutation where the levels of tRNA^{Leu}(UUR) are reduced and mitochondrial translation is defective, overexpression of human mitochondrial leucyl-tRNA synthase in human cybrid cells (see section 1.2.8.2 for a definition) with the A3243G mutation caused increased levels of tRNA^{Leu}(UUR), of mitochondrial translation products and of oxygen consumption(Jo *et al.*, 2015). Other similar strategies have been attempted, including xenotropic expression of wild-type genes of other species that encode proteins with analogous function. Overexpression of the ATPase 6 (CrATP6) gene from the alga *Chlamydomonas reinhardtii* in human cybrid cells with the T8993G mtDNA mutation resulted in cell viability recovery and improvements in ATP production(Park, Davidson and King, 2008).

Finally, zinc finger peptides (ZFPs) and more recently Transcription Activator-Like Effector Nucleases (TALENs), that can bind DNA specifically and can include nucleases and targeting sequences, have been shown to bind and cleave mtDNA point mutations and deletions in human cybrid cells but efficient delivery to target tissues is still a big challenge(Ojaimi *et al.*, 2002).

1.2.7.2 Prevention of transmission

Prenatal genetic diagnosis from amniocentesis is an option to prevent transmission of homoplasmic mutations, but is not as predictive for heteroplasmic phenotypes because of the variability of mutant load between tissues and with time. For similar reasons, preimplantation genetic diagnosis from early human embryos has been effective but is not routinely performed (Minczuk *et al.*, 2006; Bacman *et al.*, 2013). Mitochondrial replacement therapy, which was recently approved in the UK, offers a great potential to prevent maternal mtDNA disease transmission and it can be accomplished by two main procedures: pronuclear transfer and spindle transfer. In pronuclear transfer, pronuclei are removed from the maternal zygote post-fertilization and inserted into an enucleated fertilised donor zygote. In spindle transfer, the nuclear genetic material is removed from a maternal pre-fertilization oocyte at metaphase II and inserted into an enucleated donor oocyte which is subsequently fertilized (Steffann *et al.*, 2005). To protect nuclear integrity, some cytoplasmic material containing mutant mtDNA is carried over, particularly in the case of spindle transfer due to the nebulous nature of the spindle complex. Pre-clinical experiments using human zygotes reported that mtDNA carryover was <2% in the majority (79%) of pronuclear transfer blastocysts (Fogleman *et al.*, 2016). Although these experiments have shown successful replacement of mtDNA, the effect of small amounts of mutant mtDNA carryover is not completely understood. Embryonic stem cell (hESCs) lines have been generated from human blastocysts following pronuclear transfer (Hyslop *et al.*, 2016). This study showed that heteroplasmy remained low and stable in most lines but that in some the mutant load increased with time to >90%. If this was recapitulated in children born after mitochondrial replacement therapy the consequences could be very serious.

1.2.8 Experimental models of mitochondrial DNA disease

1.2.8.1 Mice models

Mainly due to similar and well described genetics, mice have become an essential model system to study mtDNA diseases, particularly in respect to patterns of segregation and tissue specificity. It is still not possible to directly manipulate the mitochondrial genome so scientists have applied indirect methods to engineer mouse mtDNA. The first heteroplasmic mice were generated by introducing cytoplasm of an embryo from a mouse strain with one mtDNA genotype into the cytoplasm of another embryo from a different mouse strain with a second mtDNA genotype (Yamada *et al.*, 2016). The generated mice, which had two mtDNA genotypes and harboured polymorphic mtDNA mutations, were used to examine the segregation of the different mtDNA genotypes in different tissues. In most tissues the pattern of mtDNA segregation was explained by random genetic drift that occurred during early oogenesis but in others directional selection for one mtDNA genotype was repeatedly observed.

Transmitochondrial mice have more recently been generated by microinjection of mtDNA originated from somatic cells of patients with specific mtDNA mutations into mouse zygotes. Heteroplasmic tissues of transmitochondrial mice with the A3243G mutation showed impaired OXPHOS with reduced activity of complexes I/IV of the ETC and increased ROS generation and activity of the antioxidant protein superoxide dismutase (SOD2) (Jenuth *et al.*, 1996). Mice generated from another transmitochondrial model with a heteroplasmic mtDNA deletion showed symptoms similar to Pearson's syndrome, including anaemia, myopathy, and pancreatic and renal insufficiency (Li *et al.*, 2008).

Other researchers have instead focused on manipulating nuclear genes which encode for mitochondrial proteins. For example, the mutator mice expresses a proof-reading-deficient version of the nucleus-encoded catalytic subunit of mtDNA polymerase and shows accumulation of mtDNA mutations and signs of ageing(Nakada *et al.*, 2004). Although mice models provide important clues in terms of patterns of mtDNA segregation and tissue specificity, genetics and molecular mechanisms of mtDNA disease are better studied in cybrid cells, which will be covered below (see 1.2.8.2).

1.2.8.2 Cybrid cells

Cybrid cells are normally generated by fusion of enucleated patient cells (cytoplasts) with immortalised human cell lines lacking mtDNA (rho zero) and have been extensively used to study mtDNA diseases. Rho zero cells were first discovered in yeast, where conditions which favoured glycolysis over OXPHOS caused depletion of mtDNA(Trifunovic *et al.*, 2004). Immortalised human cell lines can now be artificially generated by depleting mtDNA using ethidium bromide(Wilkins, Carl and Swerdlow, 2014). Platelets, which do not contain a nucleus, from a patient with the mtDNA disease Myoclonic-Epilepsy-and-Ragged-Red-Fibers (MERRF) encephalomyopathy have also been used to generate cybrid cells by fusion with a rho zero cell line(King and Attardi, 1989).

The applications of cybrid cells to investigate mtDNA mutations include testing heteroplasmy thresholds, assessing mtDNA/nuclear DNA interactions and evaluating differences in the functional properties of cell lines generated from different patients with the same mitochondrial disorder. Cybrid cells have been generated from somatic cells of patients with a wide range of mtDNA diseases but we will focus on the A3243G mutation for the purpose of this thesis. Cybrids have been made by fusing enucleated cells of MELAS patients with multiple immortalised human rho zero cell lines. In osteosarcoma models, at least 95% heteroplasmy was necessary to observe phenotypic changes(Chomyn *et al.*, 1994). One of the studies

reported a 30% decrease in cytochrome c oxidase activity in cybrids with 90% heteroplasmy(King *et al.*, 1992). The same study also described that in lung carcinoma cybrids produced using the same patient's cells, 55% heteroplasmy resulted in 50% reduction in cytochrome oxidase activity, highlighting the importance of nuclear DNA/mitochondrial DNA interactions. Nuclear differences have also been shown responsible for mutation drifts occurring in culture over time. Different cybrids both with the A3243G mutation have shown drifts away and towards the mutant mtDNA over time in culture(Dunbar *et al.*, 1995).

Cybrids do not share the same nuclear and mitochondrial DNA and it has been shown that the cybridisation process interferes with nuclear transcripts involved in OXPHOS(Yoneda *et al.*, 1992; Turner *et al.*, 2005). Cybrid cells produced using tumour cells are also mainly dependent on glycolysis and in contrast, mtDNA mutations mainly affect cells or tissues with high OXPHOS utilisation(Tuppen *et al.*, 2010). The discovery of hiPSCs gave a new direction to mtDNA disease research and their applications will be discussed below (see 1.2.8.3).

1.2.8.3 Human induced pluripotent stem cells

Embryonic stem cells (ESCs) can be derived from the inner cell mass of the blastocyst embryo and are distinguished by their self-renewal and pluripotency (differentiation into the three primary germ cell layers endoderm, ectoderm and mesoderm) potential. In 2006, Yamanaka and colleagues discovered that mouse somatic cells, such as fibroblasts, could be reprogrammed to an ESC-like state by using a cocktail of the four pluripotency factors OCT4, SOX2, KLF4 and MYC(Wilkins, Carl and Swerdlow, 2014). These cells were termed induced pluripotent stem cells (iPSCs) and one year later the same group reported generation of iPSCs from human fibroblasts (hiPSCs)(Takahashi and Yamanaka, 2006). Since then, hiPSCs have been derived from multiple sources of somatic cells, including blood and urine, and using

different reprogramming techniques, including nonintegrating viruses (e.g. sendai virus) and nonviral methods (e.g. episomal plasmids)(Takahashi *et al.*, 2007). Human iPSCs, which can be derived from patients with all sorts of diseases, are particularly relevant for drug discovery and disease modelling. Besides, recent advances in the CRISPR-Cas9 gene editing technology now enables scientists to eliminate disease-causing mutations in patient-derived hiPSCs to create isogenic controls and to introduce mutations in wild-type hiPSCs(Malik and Rao, 2013; Raab *et al.*, 2014). Unfortunately CRISPR-Cas9 has not been achieved in mtDNA but several studies have applied the iPSC technology to generate models of mtDNA diseases, which will be discussed below.

In contrast with other models of mtDNA disease, hiPSCs from all types of mtDNA mutations can be obtained, as long as patient-derived somatic cells are available. In addition, these cells share the same nuclear DNA and mtDNA and can be differentiated into multiple cell types. Human iPSCs have been generated from somatic cells of patients with the following mutations: A3243G (from MELAS(Shi *et al.*, 2016) and diabetic(Hamalainen *et al.*, 2013; Ma *et al.*, 2015; Perales-Clemente *et al.*, 2016) patients); G13513A (from MELAS(Fujikura *et al.*, 2012) and Leigh Syndrome(Folmes *et al.*, 2013) patients); C5541T (from MELAS patients(Ma *et al.*, 2015)); T8993G (from Leigh Syndrome patients(Kodaira *et al.*, 2015)) and mtDNA deletions (from Pearson marrow pancreas syndrome patients(Ma *et al.*, 2015)). Four important findings originated from these studies: 1-the mitochondrial genetic bottleneck that happens in embryogenesis happens when somatic cells are reprogrammed to hiPSCs, which has been demonstrated by clones of hiPSCs with different levels of heteroplasmy originating from the same somatic cells; 2-levels of heteroplasmy drift in culture; 3-mtDNA mutations do not seem to impede hiPSC differentiation to different lineages and 4-disease phenotypes are recapitulated in hiPSCs. Results from studies using hiPSCs with the A3243G mutation will be described in more detail below.

Hämäläinen *et al.* reprogrammed fibroblast lines from three MELAS patients with the A3243G mtDNA mutation to hiPSCs (Cherry *et al.*, 2013). The levels of heteroplasmy in the patients' skeletal muscle were 73, 50, and 90%, and in the fibroblasts 22, 21, and 35%, respectively. After reprogramming, hiPSC clones with high (>80%) and undetectable heteroplasmy were obtained from the same patient. Both MELAS high and MELAS low hiPSCs could be differentiated to neurons but only MELAS high cells showed mitochondrial dysfunction, specifically down-regulation of mitochondrial respiratory chain complex I and enhanced mitophagy induction via the PARKIN-PINK1 pathway.

Ma *et al.* also obtained fibroblasts from a MELAS patient with the A3243G mtDNA mutation for reprogramming to hiPSCs (Hämäläinen and Suomalainen, 2015). The original fibroblasts had 29% heteroplasmy and, once again, the generated hiPSCs had levels of heteroplasmy ranging from undetectable to 100%. Human iPSCs with 100% heteroplasmy could be differentiated back to fibroblasts and both hiPSCs and generated fibroblasts with high mutant loads showed impaired mitochondrial respiration in terms of basal respiration, ATP production, maximal respiration and spare respiratory capacity.

Perales-Clemente *et al.* generated hiPSCs from fibroblasts of three MELAS patients with the A3243G mutation (Ma *et al.*, 2015). The levels of heteroplasmy in the fibroblasts was around 40% for two of the patients and 20% for the third. Generated hiPSC clones had levels of heteroplasmy ranging from undetectable to 100%. Authors also observed drifts in hiPSC heteroplasmy over 6 passages in culture. Out of the 25 hiPSC clones analysed, heteroplasmy decreased in 18, did not change in 5 and increased in 2. Cardiomyocytes with mutation levels of 83 and 83% were successfully differentiated from two clones of hiPSCs with 73 and 83% heteroplasmy, respectively, and both showed impaired mitochondrial respiration in terms of basal respiration and uncoupled respiration.

Finally, hiPSCs have also been generated from fibroblasts of two diabetic patients with the A3243G mtDNA mutation, one of them also showing sensorineural hearing loss, epilepsy and cardiomyopathy(Perales-Clemente *et al.*, 2016). Fujikura *et al.* reported that heteroplasmy levels in original fibroblasts were 18 and 24% for patients 1 and 2 but levels for respective hiPSCs varied between undetectable and 87% for patient 1 and undetectable and 79% for patient 2. The mutant hiPSCs had potential to differentiate into the three primary germ cell layers *in vitro* and formed teratomas when injected into SCID mice.

No studies have addressed the potential of hiPSC-CMs from patients with the A3243G mutation to investigate the mechanisms behind the cardiac abnormalities associated with this mutation and other mtDNA diseases. The generation, applications and properties of hiPSC-CMs will be covered in the next section.

1.3 Human induced pluripotent stem cell-cardiomyocytes

1.3.1 Cardiac differentiation protocols

Two years after the discovery of iPSCs by Yamanaka and colleagues, generation of CMs from mice(Fujikura *et al.*, 2012) and human iPSCs was described(Mauritz *et al.*, 2008; Narazaki *et al.*, 2008). Initial protocols were very inefficient and very variable between batches and cell lines. Over the years differentiation efficiency has improved tremendously and recent methods generate virtually pure cultures of hiPSC-CMs and are highly reproducible.

1.3.1.1 Embryonic body formation

The initial strategy employed to differentiate hESCs(Zhang *et al.*, 2009; Zwi *et al.*, 2009) to CMs was embryoid body (EB) formation and this was then adapted to hiPSCs(Itskovitz-Eldor *et al.*, 2000; Kehat *et al.*, 2001). When colonies of hiPSCs are cultured in suspension for up to 10 days they aggregate to form EBs and the fraction of the EBs containing hiPSC-CMs spontaneously beats and can be dissected. The molecular, structural and functional properties of these beating hiPSC-CMs shared similarities with those of adult CMs. The low yield of hiPSC-CMs (as low as 1%) and lack of reproducibility are the main limitations of this technique. These are caused by several reasons, including the presence of undefined components such as serum and the heterogenous EB size which is difficult to control(Zhang *et al.*, 2009).

1.3.1.2 Small molecule-based

As an attempt to improve cardiac differentiation, monolayer-based protocols using chemically defined media composed of small molecules have been developed. These methods involve the manipulation of pathways central to cardiac development. Treatment of H7 hESC monolayers sequentially with the members of the TGF β family activin A and BMP4 resulted in >30% CMs but this has not been successfully applied to other hESC or hiPSC lines(Mummery *et al.*, 2012). In addition to members of TGF β family, temporal manipulation of the Wnt/ β -catenin signaling pathway has also been employed for cardiac differentiation of hiPSCs. This pathway has vital biphasic roles in cardiac development, with early Wnt signaling contributing to mesoderm induction and later Wnt inhibition being crucial for cardiac specification(Laflamme *et al.*, 2007). CHIR99021 is a GSK-3 inhibitor which inhibits β -catenin proteolysis thereby causing β -catenin nuclear translocation, activation of specific transcription factors and consequently activated Wnt signaling. Wnt-C59 is a Wnt antagonist and causes Wnt receptor saturation and Wnt signaling inactivation(Ueno *et al.*, 2007). See Figure 1.4 for

a schematic diagram of Wnt/ β -catenin signaling pathway manipulation with CHIR99021 and Wnt-C59. The studies published by Lian in 2013 and Burrridge in 2015 considerably improved the generation of CMs from hESCs and hiPSCs(Burrridge *et al.*, 2014). The authors have separately shown that Wnt activation with CHIR99021 followed by Wnt inhibition with Wnt-C59 (or analogous IWP2 and IWP4) of multiple hESC and hiPSC lines, in the presence of the defined RPMI/B-27 medium, enables the generation of virtually pure (up to 95% troponin-T positive) cultures of functional CMs in two weeks. For further purification of the generated cells, metabolic selection can be performed by glucose starvation, as CMs can use lactate as an energy source when glucose is lacking(Lian *et al.*, 2013; Burrridge *et al.*, 2014). This protocol has been shown to be very efficient and highly reproducible by several groups and has been applied to multiple healthy and diseased hESC and hiPSC lines(Tohyama *et al.*, 2013; Sharma *et al.*, 2015).

The Wnt/ β -catenin signalling pathway

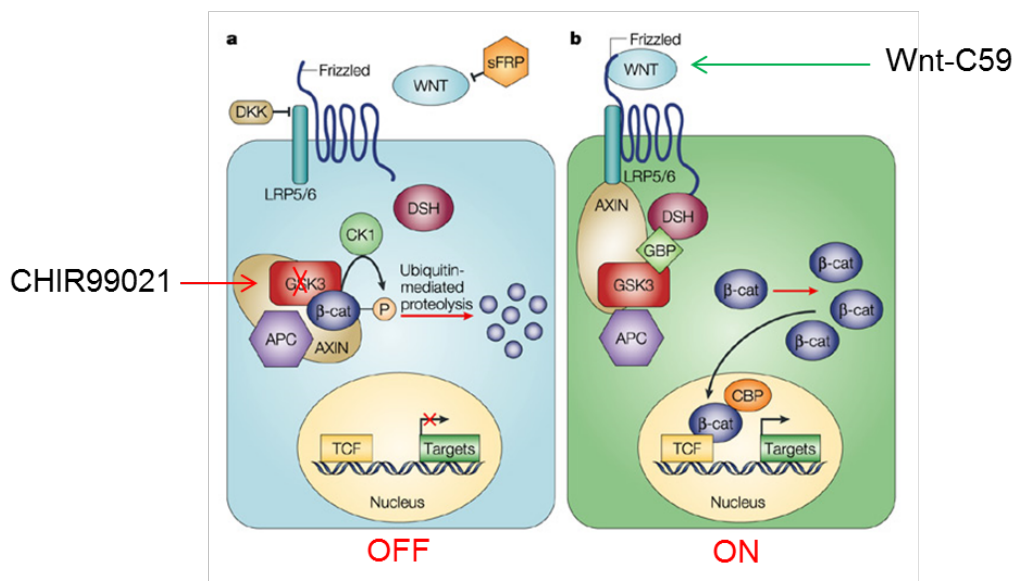


Figure 1.4: The Wnt/ β -catenin signaling pathway.

When Wnt is unbound to its receptor, GSK3, APC and AXIN form a destruction complex causing β -catenin proteolysis. Consequently, the pathway is inactivated (left, OFF). On the other hand, binding of Wnt to its receptor causes incorporation of the GSK3 complex and so β -catenin is able to translocate to the nucleus without being destroyed, binding to specific transcription factors and causing transcription of certain proteins. The pathway is activated (right, ON). CHIR99021 is a GSK3 inhibitor, thereby inhibiting β -catenin proteolysis and causing pathway activation. Wnt-C59 is a Wnt antagonist, causing β -catenin proteolysis and therefore pathway inactivation. Adapted from (Denning *et al.*, 2016).

1.3.2 Applications of human induced pluripotent stem cell-cardiomyocytes

As discussed before in section 1.2.6, mtDNA mutations are associated with cardiac abnormalities, including arrhythmias and cardiomyopathies, in particular hypertrophic cardiomyopathy. As covered in section 1.2.8.3, although hiPSCs have been applied to model a number of mtDNA diseases, no studies have addressed their potential to investigate arrhythmias and cardiomyopathies caused by mtDNA mutations. On another note, other groups have used hiPSC-CMs to model arrhythmogenic disease and cardiomyopathies caused by other underlying mechanisms and these studies will be described below (see 1.3.2.1).

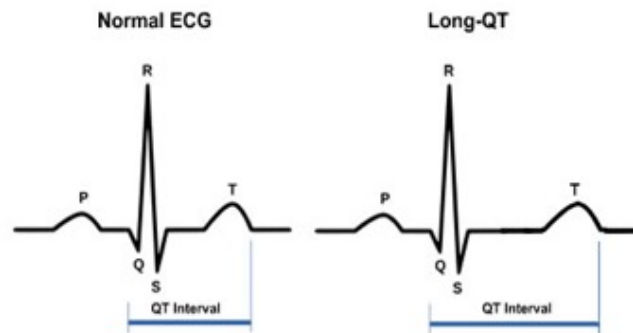
1.3.2.1 Modelling arrhythmias

Early afterdepolarizations (EADs) are an important cause of lethal arrhythmias in patients with prolongation of the QT interval on the ECG, also known as long QT (LQT) syndrome. Inherited mutations in cardiac ion channel proteins can result in prolongation of the action potential which in turn can trigger EADs and even more widespread arrhythmias (Moon *et al.*, 2004). See Figure 1.5 a/b for a schematic diagram illustrating LQT syndrome and the occurrence of EADs. At least 500 different mutations in more than 13 genes encoding cardiac ion channel proteins are involved in LQT syndrome. However, LQT1 and LQT2 are the most prevalent, accounting for >50% and 30-40% of the cases, respectively (Weiss *et al.*, 2010). Both LQT1 and LQT2 are caused by mutations in potassium channels. Human iPSC-CMs were firstly generated from patients with LQT1 but this has now been extended to other types of LQT syndrome (Dell'Era *et al.*, 2015). Studies using LQT1 hiPSC-CMs reported a higher susceptibility to tachyarrhythmias after the application of the catecholamine isoprenaline, but this could be mitigated by the non-selective beta-blocker propranolol (Dell'Era *et al.*, 2015). Most studies with LQT2 hiPSC-CMs also described arrhythmia propensity, demonstrated by

EADs either during spontaneous recordings or after application of isoprenaline(Moretti *et al.*, 2010; Egashira *et al.*, 2012).

Human iPSC-CMs have also been used to model the inherited catecholaminergic polymorphic ventricular tachycardia (CPVT) which is characterized by ventricular tachycardia caused by physical or emotional exertion. The two types of CPVT, CPVT1 and CPVT2, are linked to mutations in the cardiac ryanodine receptor type 2 gene (RYR2) and in the calsequestrin-2 gene (CASQ2), respectively(Itzhaki, Maizels, *et al.*, 2011; Matsa *et al.*, 2011; Lahti *et al.*, 2012; Mehta *et al.*, 2014). Mutations in the RYR2 gene, which encodes for the major mediator of Ca^{2+} -induced Ca^{2+} -release from the sarcoplasmic reticulum (SR), and in the CASQ2 gene, encoding for the SR calcium binding protein involved in EC coupling, are thought to cause Ca^{2+} leakage from the SR during catecholaminergic stimulation, increased intracellular free Ca^{2+} , activation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and ultimately delayed afterdepolarizations (DADs)(Priori and Chen, 2011). Multiple studies using hiPSC-CMs derived from CPVT1 and CPVT2 patients showed recapitulation of the disease phenotype, including arrhythmias and DADs during spontaneous beating and in response to isoprenaline(Iyer and Armondas, 2006). See Figure 1.5b for a schematic diagram illustrating the occurrence of DADs.

a



b

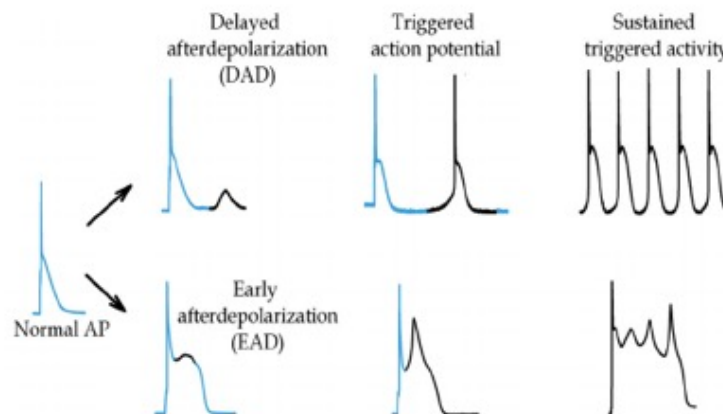


Figure 1.5: Long QT syndrome, delayed afterdepolarizations and early afterdepolarizations.

a) Long QT (LQT) syndrome is characterized by a prolongation of the QT interval of the electrocardiogram (ECG). b) Both delayed and early afterdepolarizations (DADs and EADs) of the action potential (AP) can cause sustained triggered activity. In particular, LQT syndrome can cause EADs which in turn cause arrhythmias. From (Sedj and Pieske, 2012).

1.3.2.2 Modelling cardiomyopathies

Cardiomyopathies are a class of inherited cardiac diseases more common and complex than arrhythmias and can be classified as hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC). At least 1400 mutations in more than 11 genes encoding thin and thick myofilaments of the sarcomere or the adjacent Z-disc are involved in HCM (Fatima *et al.*, 2011; Jung *et al.*, 2012; Kujala *et al.*, 2012; Novak *et al.*, 2012). HCM consists of left-ventricular hypertrophy and is usually asymptomatic but can cause progressive heart failure, arrhythmias and sudden cardiac death (Maron and Maron, 2013). Human iPSC-CMs have been a useful tool to explain the underlying mechanisms of HCM, particularly in terms of the genetic-environmental interactions. Lan and colleagues obtained hiPSC-CMs from families with a mutation in MYH7 causing HCM and showed that irregular Ca^{2+} transients and increased intracellular Ca^{2+} were implicated in the pathogenesis of HCM (Dell'Era *et al.*, 2015). Pharmacological restoration of Ca^{2+} handling prevented HCM progression. Another study found that the hypertrophy-inducing factor endothelin-1 promoted increased cardiomyocyte area and intracellular myofibrillar disarray in HCM but not in control hiPSC-CMs (Lan *et al.*, 2013). Both DCM (Tanaka *et al.*, 2014) and ARVC (Siu *et al.*, 2012; Sun *et al.*, 2012; Tse *et al.*, 2013) have also been modelled using hiPSC-CMs and this is covered in more detail in a review by Eschenhagen and colleagues (Caspi *et al.*, 2013; Kim *et al.*, 2013; Ma *et al.*, 2013).

This was just an overview of the application of hiPSC-CMs for modelling arrhythmias and cardiomyopathies. These cells have been applied to model other diseases and for drug discovery and regenerative medicine purposes. A review by Yoshida and Yamanaka published a few months ago covers the cardiac applications of hiPSC-CMs in more depth (Eschenhagen, Mummery and Knollmann, 2015).

1.3.3 Properties of human induced pluripotent stem cell-cardiomyocytes

Although hiPSC-CMs recapitulate the phenotype of a variety of disease or drug-induced states, it is well established that hiPSC-CMs generated from current differentiation protocols have an immature phenotype that resembles mid-gestation of human foetal heart development (Yoshida and Yamanaka, 2017). It is also known that their structural and functional properties are very heterogeneous limiting their characterization into specific chambers of the heart (Rajamohan *et al.*, 2013).

1.3.3.1 Gene expression features

Like adult CMs, hiPSC-CMs express genes encoding for sarcomeric proteins, including MYL2, MYL7, MYH6, MYH7, TNNT2, TNNI1 and TNNI3 (Kane and Terracciano, 2017). However, in contrast to adult human ventricular CMs, MYH6 (α MHC) predominates over MYH7 (β MHC) and TNNI1 (foetal ssTnI) predominates over TNNI3 (cTnI) in hiPSC-CMs (Burridge *et al.*, 2014). Major cardiac ion channel genes found in the human adult left ventricle are also expressed in hiPSC-CMs: L-type Ca^{2+} channels (CACNA1C and CACNA1D), Na^{+} channel (SCN5A) and K^{+} channels (KCNH2 and KCNQ1) (Denning *et al.*, 2016). Even though their levels of expression are lower than those found in human adult left ventricular tissue, hiPSC-CMs also express genes encoding for proteins involved in EC coupling such SERCA, RYR2, CASQ2 and NCX (Liang *et al.*, 2013). In terms of metabolic genes, hiPSC-CMs express genes encoding for proteins involved in the ETC (e.g. ATP5b) and in protection against oxidative stress (e.g. SOD1, SOD2) (Itzhaki, Rapoport, *et al.*, 2011; Denning *et al.*, 2016).

1.3.3.2 Ultrastructural features

Adult CMs are large rod-shaped poly-nuclear cells with organised sarcomeres of 2.2 μm with longitudinal alignment. Adult CMs express T-tubules and show Z-discs and I-, H-, A- and M-bands(Rana *et al.*, 2012). In contrast, hiPSC-CMs are small round/angular-shaped mono-nuclear cells with disorganized sarcomeres of 1.6 μm with no alignment. The cells lack T-tubules and usually only show Z-discs and I-bands(Severs *et al.*, 1985; Slade *et al.*, 1985). Mitochondria in hiPSC-CMs are fewer than in adult CMs, where they can occupy up to 25% of the cytoplasmic volume(Zhang *et al.*, 2009; Zwi *et al.*, 2009; Gherghiceanu *et al.*, 2011). Finally, in hiPSC-CMs mitochondria tend to localize around the nucleus in a disorganized fashion but are aligned with myofibrillar proteins or in the subsarcolemmal compartment in adult CMs(Barth *et al.*, 1992; Hattori *et al.*, 2010).

1.3.3.3 Electrophysiological properties

Human iPSC-CMs exhibit robust and reproducible electrophysiological properties and currents of several voltage-gated ion channels appear to be comparable to adult CMs. This includes the Na^+ (I_{Na}), the L-type Ca^{2+} (I_{CaL}), the outward K^+ (I_{to}) and the rapid and slow activating components of the delayed rectifier K^+ (I_{Kr} and I_{Ks} , respectively)(St. John *et al.*, 2005; García-Pérez, Hajnóczky and Csordás, 2008); although the presence of an I_{Ks} current in hiPSC-CMs has been recently called into question(Denning *et al.*, 2016). A unique characteristic of hiPSC-CMs is their spontaneous beating, in contrast to adult ventricular CMs which are quiescent. This is partially caused by high expression of the funny/pacemaker current, I_{f} , and by low expression of the inwardly rectifying potassium current, I_{K1} . Consequently, the resting membrane potential (RMP) in hiPSC-CMs is -20 to -60 mV and in adult CMs is -80 to -90 mV(Christ, Horvath and Eschenhagen, 2015). Two other characteristics specific to hiPSC-CMs are a low action potential (AP) upstroke velocity of 10-

50 V/s, versus 150-350 V/s in adult CMs, and a slower conductance velocity of 10–20 cm/s, versus 60 cm/s in adult CMs. The latter appears to be caused by gap junctions located around the cell circumference and not at the intercalated discs (Ma *et al.*, 2011; Denning *et al.*, 2016).

1.3.3.4 Excitation-contraction coupling

Human iPSC-CMs show robust L-type Ca^{2+} currents, twitch Ca^{2+} transients and caffeine-releasable SR Ca^{2+} stores, comparable to adult CMs, suggesting a functional EC coupling (Denning *et al.*, 2016). However, transient kinetics are significantly different compared to hESCs and adult rabbit and mouse CMs; the rate of transient rise and decline is significantly slower in hiPSC-CMs (Itzhaki, Rapoport, *et al.*, 2011; Hwang *et al.*, 2015). Transport of Ca^{2+} by SERCA and NCX is slower compared to adult rabbit and mouse CMs but the relative contribution of SERCA and NCX to Ca^{2+} transport is comparable (Lee *et al.*, 2011; Hwang *et al.*, 2015). Together, data indicate that EC coupling in hiPSC-CMs is functional but under developed (Hwang *et al.*, 2015).

1.3.3.5 Metabolic properties

It has been shown by several different studies that the ETC complexes I, III and ATP synthase are fully functional in hiPSC-CMs as demonstrated by the drop in oxygen consumption after application of rotenone, antimycin A and oligomycin (Kane, Couch and Terracciano, 2015). Like adult CMs, hiPSC-CMs generated by recent methods produce ATP primarily through OXPHOS (Rana *et al.*, 2012; Wang *et al.*, 2014; BurrIDGE *et al.*, 2016; Perales-Clemente *et al.*, 2016). However, while 90% of acetyl-CoA produced by adult CMs derives from oxidation of fatty acids, hiPSC-CMs mostly utilize glucose and lactate (Rana *et al.*, 2012). Nevertheless, hiPSC-CMs are capable of performing fatty acid oxidation and in fact their levels of ATP production and the ratio of OXPHOS/glycolysis increase when glucose is substituted by

galactose (which blocks ATP production by glycolysis) or fatty acids(Harris and Das, 1991; Rana *et al.*, 2012).

1.3.4 Promoting *in vitro* maturation of human induced pluripotent stem cell-cardiomyocytes

Given the immature phenotype of hiPSC-CMs *in vitro*, many attempts have been made to promote their maturation to a more adult-like phenotype. Strategies that have been applied will be discussed below, with a particular focus on time in culture and chemically induced maturation using triiodothyronine (T_3) hormone. Evidence suggests that although an adult CM phenotype is never acquired, several structural and functional properties of hiPSC-CMs can be improved. Refer to Figure 1.6 for an illustrated summary of strategies applied to induce hiPSC-CM maturation.

1.3.4.1 Time in culture

Intuitively, increasing time in culture would be expected to promote the maturation of hiPSC-CMs. Morphological maturation has been reported when hiPSC-CMs were kept in culture up to 100 days versus up to 40 days, with increased cell size, elongated shape, multinucleation, sarcomere length and MYH7 and MYH6 gene expression(Rana *et al.*, 2012). In another study, hiPSC-CMs kept up to 1 year in culture showed gradual changes in sarcomere organisation: 30 days-old hiPSC-CMs only showed Z- and I-bands whereas 90 days-old cells also developed A-bands and 360 days-old hiPSC-CMs had M-bands in addition(Lundy *et al.*, 2013). Maturation of ion channel densities and kinetics has also been shown: increased densities of I_{Na} and I_{CaL} in 231 days-old hiPSC-CMs versus 28 days-old(Kamakura *et al.*, 2013). In terms of function, in contrast to 40 days-old hiPSC-CMs, 100 days-old hiPSC-CMs have been shown to have a higher AP upstroke velocity and amplitude, a more negative RMP and quicker Ca^{2+} transient rise and decline rates(Ivashchenko *et al.*, 2013).

1.3.4.2 Chemically induced maturation

One of the most promising chemicals promoting hiPSC-CM maturation is T_3 , which is known to have essential roles in foetal maturation(Lundy *et al.*, 2013). In studies using foetal sheep, T_3 has been shown to increase CM size and the number of terminally differentiated binucleated cells(Forhead and Fowden, 2014). On the other hand, thyroidectomy of the sheep foetus has been shown to reduce the number of binucleated CMs and the relative heart weight(Chattergoon, Giraud and Thornburg, 2007). In CMs, T_3 also has an important role in the perinatal switch from β - to α -myosin heavy chains in the sarcomeres and in the prepartum upregulation of the β -adrenergic receptors and response to β -agonists(Chattergoon *et al.*, 2012). Functionally, studies in the ovine foetal heart have shown that T_3 is essential for growth adaptation to pressure overload that occurs during late gestation(Birk *et al.*, 1992; Edwards *et al.*, 1994).

Addition of T_3 to culture media for the duration of 1 week increased hiPSC-CM size, sarcomere length and elongation(Segar *et al.*, 2013). T_3 treated hiPSC-CMs also showed improvements in force generation and in rates of Ca^{2+} transient rise and decline. The levels of SERCA gene expression also increased. Finally, the authors also reported increases in mitochondrial maximal and spare respiratory capacity after T_3 treatment but no changes in mtDNA or ATP production. T_3 is now a component of most commercially available media to promote *in vitro* hiPSC-CM maturation. Treatment of hiPSC-CMs with ascorbic acid, which promotes cardiac differentiation(Yang *et al.*, 2014), also caused improvements in force of contraction, sarcomeric organization, intercalated disc formation and metabolic activity(Cao *et al.*, 2012).

1.3.4.3 Other strategies

Several other strategies have been employed to promote maturation of hiPSC-CMs. Culturing hiPSC-CMs on micro-grooved patterns increased cell alignment, sarcomere organisation and improved Ca^{2+} kinetics(Kensah *et al.*, 2013). In a separate study, culture on polyacrylamide hydrogels to resemble the extracellular matrix of the heart increased force of contraction of hiPSC-CMs(Rao *et al.*, 2013). Three-dimensional culture in a microfabricated system resulted in aligned 'biowires' of hiPSC-CMs that, when submitted to electrical stimulation, displayed increased myofibril ultrastructural organization and improvements in several Ca^{2+} handling and electrophysiological properties(Hazeltine *et al.*, 2012). Mechanical strain, co-culture with non-CMs and overexpression of specific genes have also been employed as an attempt to mature hiPSC-CMs. A recent review by Veerman and colleagues covers all these aspects in more detail(Nunes *et al.*, 2013).

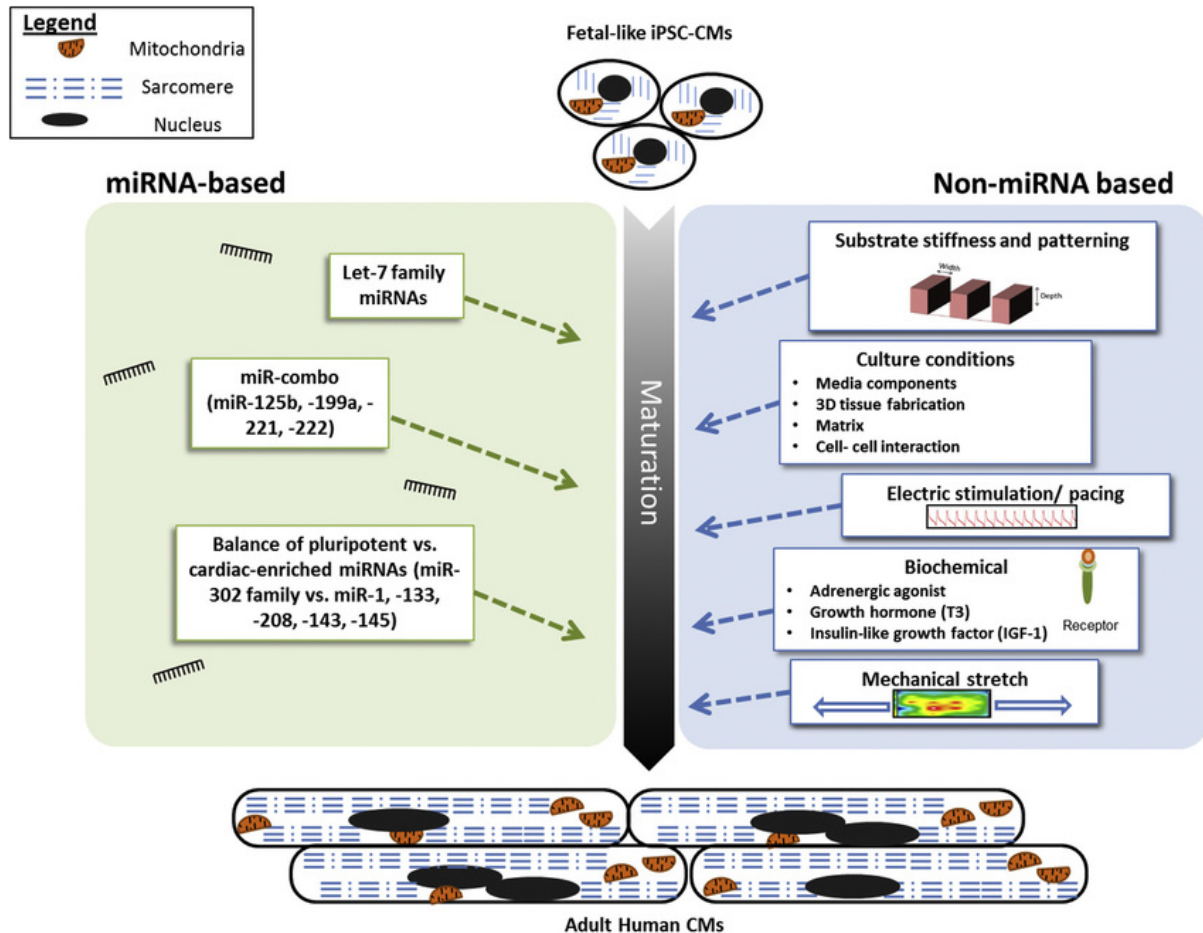


Figure 1.6: Strategies to promote the maturation of human pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs).

Human iPSC-CMs show foetal-like properties in terms of density and positioning of mitochondria, sarcomeric alignment and number of nuclei, compared to adult CMs. Strategies such as miRNAs, substrate stiffness and patterning, culture conditions, electric stimulation/pacing, biochemical or mechanical stretch can be applied to direct foetal-like hiPSC-CMs towards an adult-like phenotype. From (White, Pang and Yang, 2016).

1.4 Aims and Hypotheses

Cardiac abnormalities, including hypertrophic cardiomyopathy and arrhythmias, are common in patients with heteroplasmic mtDNA mutations but the pathogenesis remains poorly understood. Due to lack of biopsies from organs such as the heart, there is also limited understanding on how the levels of mutation change in these patients over time. A robust *in vitro* model of heteroplasmy in cardiomyocytes is needed as currently available cybrids and mice models are inappropriate. Human induced pluripotent stem cells (hiPSCs) can be generated from somatic cells of patients with all types of mtDNA mutations, recapitulate features of corresponding mtDNA disorders and have been used to understand their pathogenesis. Despite showing an immature phenotype, cardiomyocytes can be differentiated from hiPSCs at high efficiencies using recent methods. However, no studies have addressed their potential to investigate the pathogenesis of cardiac abnormalities in mtDNA disease and the dynamics of mtDNA mutations over time.

Therefore this thesis addresses the hypothesis that:

Human induced pluripotent stem cell-cardiomyocytes with heteroplasmic mitochondrial dysfunction recapitulate cardiac features associated with mtDNA disease.

The following specific hypotheses were addressed:

1. Maturation strategies induce metabolic maturation of hPSC-CMs

Given the immature phenotype of human pluripotent stem cell cardiomyocytes (hPSC-CMs), this hypothesis was tested to identify culture conditions where maximal metabolic potential of healthy hPSC-CMs (hESC-CMs and hiPSC-CMs) can be achieved to later determine if that is

compromised in hiPSC-CMs with mitochondrial dysfunction. This was addressed by applying T_3 / extending time in culture in hPSC-CMs and measuring mitochondrial function (Chapter 3).

2. Populations of hiPSC-CMs with varying degrees of mitochondrial dysfunction and the same nuclear background can be generated by addition of mitochondrial drugs to healthy hiPSC-CMs

This would circumvent the lack of isogenic controls for hiPSCs with mtDNA mutations and the issues involved with differentiating hiPSCs with high mtDNA mutation loads. This hypothesis was tested by applying antimycin A and menadione to a set of healthy hiPSC-CMs, mixing with untreated healthy hiPSC-CMs at different ratios and measuring mitochondrial function (Chapter 4).

3. Human iPSCs from a patient with the heteroplasmic mtDNA A3243G mutation can be differentiated to cardiomyocytes. Mutant hiPSC-CMs recapitulate cardiac abnormalities associated with mtDNA disease, show mitochondrial dysfunction and can be used to investigate how levels of heteroplasmy change over time

This hypothesis was tested by performing cardiac differentiation of mtDNA mutant hiPSCs, measuring differentiation efficiency, cell size and beating rate and Ca^{2+} handling properties (Chapter 5). Mitochondrial properties and mutant load was also assessed (Chapter 6).

Chapter 2: General Methods

2.1 Human hiPSC culture

2.1.1 Cell lines

Control and 20% A3243G mutant hiPSCs lines were obtained from Michael Duchen's Lab (UCL). Fibroblasts of a patient containing 20% heteroplasmy for the transposition mutation A3243G in the mtDNA were reprogrammed to hiPSCs using an episomal-based approach. The Control hiPSC line originated from fibroblasts of a family member who did not carry the mutation (same generation method as above).

A hiPSC line with the homoplasmic (100%) A3243G mtDNA mutation was obtained from Shoukhrat Mitalipov's Lab (Oregon University). Dermal fibroblasts from a patient with MELAS syndrome were reprogrammed to hiPSCs using a Sendai virus-based method.

The commercial control lines IMR90-4 (hiPSCs derived from lung fibroblasts via lentiviral reprogramming) and H7 (hESCs) were supplied by WiCell Research Institute. The control CAG-ZsGreen1 hiPSC line, targeted to the endogenous AAVS1 locus, was obtained from Richard Axton (University of Edinburgh).

2.1.2 Thawing of hiPSCs

Cryovials were stored in liquid nitrogen. For thawing, a cryovial was immersed in a water bath (37°C) and the cell suspension diluted 1 in 10 with Essential 8 (E8) medium (Thermo Fisher Scientific) supplemented with 10µM ROCK Inhibitor Y-27632 2HCl (Selleckchem), slowly and

drop-wise to prevent osmotic shock to the cells. Human iPSC suspensions were always handled with wide orifice 1000 μ L tips (Starlab group) to avoid breaking the colonies into single cells. The suspension was centrifuged at 200g for 4 minutes and the pellet resuspended in E8 + 10 μ M ROCK inhibitor. ROCK inhibitor was only supplemented when cells were thawed or passaged. The contents of 1 vial were plated on 2 wells of a 6 well-plate (Becton Dickinson), which had been previously coated with growth factor-reduced Matrigel (Corning) at 37°C for 30 minutes. The Matrigel used consisted of a 1:50 dilution of the stock with KnockOut DMEM (Thermo Fisher Scientific). Cultures were maintained at 37 °C in a 170-300 Galaxy R CO₂ humidified incubator (RS Biotech), with 5% CO₂ and 21% O₂.

2.1.3 Maintenance and passaging of hiPSCs

Medium was replaced with fresh E8 (Thermo Fisher Scientific) every day and the hiPSCs were passaged every 4 days. Cells were lifted off by incubating with the EDTA solution Versene (Thermo Fisher Scientific) at 37°C for 5 minutes and resuspended in E8 + 10 μ M ROCK inhibitor (Selleckchem). The cell suspension was added at a 1:16 split ratio to new plates previously coated with Matrigel (see 2.1.2).

2.1.4 Cryopreservation of hiPSCs

For cryopreservation, each well of a 6 well-plate of hiPSCs was lifted off as described in 2.1.3 and was resuspended in 2mL of mFreSR Cryopreservation Medium (Stem Cell Technologies). The suspension was quickly added at 1mL per cryovial (Corning), the vials were placed at - 80°C in an isopropanol (VWR) containing Mr Frosty Freezing Container (Thermo Fisher Scientific) overnight, and transferred to liquid nitrogen the following day.

2.1.5 Homoplasmic A3243G hiPSCs

All the hiPSC lines in 2.1.1, except for the homoplasmic A3243G hiPSCs, were handled as described in 2.1.2-2.1.4. The following methods apply to the homoplasmic A3243G hiPSC line only.

2.1.5.1 Preparation of MEF feeders

Human iPSCs were expanded on mitomycin C inactivated CF-1 mouse embryonic fibroblast (MEF) feeders (Amsbio). One day before thawing hiPSCs, MEFs were thawed and plated at 0.5 million fibroblasts per well of a 6 well-plate according to the manufacturer's instructions. The following day, and at least 4 hours before thawing hiPSCs, MEF medium was replaced with hiPSC medium consisting of knockout DMEM medium (Thermo Fisher Scientific) supplemented with 20% of knockout serum replacement (Thermo Fisher Scientific), 0.1 mM nonessential amino acids (Thermo Fisher Scientific), 1 mM L-glutamine (Thermo Fisher Scientific), 0.1 mM beta-mercaptoethanol (Thermo Fisher Scientific), 1× penicillin–streptomycin (Thermo Fisher Scientific) and 4 ng/ml basic fibroblast growth factor (R&D Systems) for conditioning.

2.1.5.2 Thawing, maintenance and passaging of homoplasmic A3243G hiPSCs

Cryovials were stored and thawed as described in 2.1.2, except medium used was hiPSC medium outlined in 2.1.5.1. After centrifugation, colonies were resuspended in MEF-conditioned hiPSC medium (see 2.1.5.1) and added to MEF feeders. Medium was replaced every day and when large enough, colonies with typical undifferentiated hiPSC morphology were manually picked and propagated as described before (Veerman *et al.*, 2015). Cultures were maintained at 37 °C in a NuAire hypoxic incubator (Triple Red), with 3% CO₂ and 5%

O₂, controlled by the injection of carbon dioxide and nitrogen, to prevent the occurrence of spontaneous hiPSC differentiation (Tachibana *et al.*, 2013).

2.1.5.3 Cryopreservation of homoplasmic A3243G hiPSCs

For cryopreservation, colonies were manually picked as described in 2.1.5.2, centrifuged at 200g for 4 minutes and resuspended in freezing solution composed of 65% knockout DMEM medium, 25% FBS and 10% DMSO (all Thermo Fisher Scientific). The suspension was quickly added at 1mL per cryovial (Corning), each containing approximately 15 colonies. The vials were placed at -80°C in an isopropanol (VWR) containing Mr Frosty Freezing Container (Thermo Fisher Scientific) overnight and transferred to liquid nitrogen the following day.

2.2 Cell competition assays

Embryonic stem cells exhibit a competitive behaviour which allows cell fitness regulation by recognition and elimination of mutant cells and survival of wild-type cells, so we aimed to investigate the occurrence of cell competition in hiPSCs with the A3243G mtDNA mutation (Yoshida *et al.*, 2009). At day 0, human iPSCs were incubated with Trypsin-EDTA 0.05% (Thermo Fisher Scientific) for 1 minute at 37°C and dissociated into single cells by pipetting several times using a normal 1000µL tip (Starlab group). Trypsin-EDTA was inactivated with twice the volume of E8 medium supplemented with 10% FBS (both Thermo Fisher Scientific) and the cells were centrifuged at 200g for 4 minutes. At this stage, cells were either resuspended in E8 medium supplemented with 10µM ROCK Inhibitor Y-27632 2HCl (Selleckchem) for plating, or in 1% FBS in DPBS for flow cytometry analysis. The number of viable cells was determined as described in 2.3.3. 100000 hiPSCs were plated into Matrigel-coated (see 2.1.2) 24-well plates (Becton Dickinson). Cells were plated separately or as a mixture of control green hiPSCs and A3243G mutant hiPSCs at a 50:50 ratio. Medium was

replaced to E8 after 24 hours and every day thereafter. Flow cytometry analysis was performed at days 0, 1, 2 and 4 after plating. For maximum accuracy, for the separate cultures, cells were trypsinized, mixed in equal volume and analysed by flow cytometry. Cell suspensions were ran through a BD LSR2 cytometer equipped with a 525/50nm filter, and data was analysed using the FACSDiva software (both BD Biosciences). A primary gate was manually set to exclude debris and doublets based on forward (FSC) and side scatter (SSC). For each sample, 10000 events were recorded inside the population of interest, and the proportion of control green hiPSCs (525/50nm positive) and A3243G mutant hiPSCs (525/50nm negative) was determined.

2.3 Cardiac differentiation of hiPSCs

Human iPSCs were differentiated to cardiomyocytes (hiPSC-CMs) by small molecule modulation of the Wnt/ β -catenin signalling pathway, based on the protocols published by Lian *et al.* in 2013 and Burrridge *et al.* in 2014 (Sancho *et al.*, 2013).

2.3.1 Differentiation protocol

At day 4 after passaging (day 0), medium was changed to RPMI 1640 + 1x B27 without insulin (RB-, both Thermo Fisher Scientific) supplemented with 6 μ M GSK-3 inhibitor CHIR99021 (Tocris, stock made up to 12mM with DMSO) and left for 48 hours. On day 2, CHIR-containing medium was replaced with just RB- and left for 24 hours, and on day 3 medium was changed to RB-, supplemented with 2.5 μ M Wnt inhibitor Wnt-C59 (Tocris, stock made up to 20mM with DMSO). Thereafter, medium was changed every other day with RB- and contracting hiPSC-CMs were usually visible from day 8. At day 11 of differentiation, cells were subjected to glucose starvation to obtain a pure population of hiPSC-CMs, as shown before (Lian *et al.*, 2013; Burrridge *et al.*, 2014). This was performed by changing to RPMI 1640 without glucose

+ 1x B27 with insulin (metabolic selection, both Thermo Fisher Scientific) for 4 days and then returning to normal RPMI 1640 + 1x B27 with insulin (RB+, both Thermo Fisher Scientific). From this point, hiPSC-CMs were dissociated and either re-plated as a monolayer and matured, or cryopreserved, for future experiments. All hiPSC-CMs were 30-40 days old when experiments were conducted. Cultures were maintained at 37 °C in a 170-300 Galaxy R CO₂ humidified incubator (RS Biotech), with 5% CO₂ and 21% O₂.

2.3.2 Dissociation of purified hiPSC-CMs

Purified hiPSC-CM clusters were dissociated by treatment with cell dissociation solution (CDS), which consisted of 40% RPMI 1640, 40% enzyme free cell dissociation buffer and 20% Trypsin-EDTA 0.05% (all Thermo Fisher Scientific), for 8 min at 37°C. Cells were triturated with a 1000µL tip and each 1 mL of CDS was inactivated with 4 mL of RPMI 1640 (same as above) supplemented with 10% FBS (Thermo Fisher Scientific). Suspensions were centrifuged at 200g for 4 minutes.

2.3.3 Determination of cell viability

An Automated LUNA-FL Dual Fluorescence Cell Counter (Logos Biosystems) was used to detect green (live) and red (dead) fluorescing cells after staining with Acridine Orange/Propidium Iodide Stain (Logos Biosystems). The cells were prepared according to the manufacturer's instructions and the counter automatically determined the total number of viable cells. Briefly, 9 parts of cell suspension were mixed with 1 part of the stain.

2.3.4 Replating and maintenance of purified hiPSC-CMs

Stock fibronectin (bovine, Sigma-Aldrich) was diluted 1:100 with DPBS. Fibronectin-coated plates were incubated for 2 hours at 37°C. Human iPSC-CMs pellets were resuspended in RB+, supplemented with 10% FBS (all Thermo Fisher Scientific) and 10µM Rock Inhibitor (Selleckchem), and the cell suspension was added to pre-coated plates at 1 million hiPSC-CMs per well of a 6 well-plate. The medium was changed after 24 hours to just RB+ and every 2 days thereafter, until the cells reached the desired age and were replated for experiments.

2.3.5 Cryopreservation of purified hiPSC-CMs

For cryopreservation, hiPSC-CM pellets were resuspended in freezing solution composed of FBS with 10% DMSO (both Thermo Fisher Scientific). At least 2 million hiPSC-CMs were added per cryovial (Corning) in 1mL of freezing solution. The vials were quickly placed at -80°C in an isopropanol (VWR) containing Mr Frosty Freezing Container (Thermo Fisher Scientific) overnight and transferred to liquid nitrogen the following day.

2.3.6 Thawing of purified hiPSC-CMs

Fibronectin coated plates (see 2.3.4) were prepared and hiPSC-CMs were thawed as described in 2.1.2, except medium used for thawing was RPMI 1640 supplemented with 10% FBS (both Thermo Fisher Scientific). After centrifugation, pellets were resuspended in RB+, supplemented with 10% FBS (all Thermo Fisher Scientific) and 10µM ROCK Inhibitor (Selleckchem), and the cell suspension was added to pre-coated plates at 2 million cardiomyocytes per well of a 6 well-plate. The medium was changed after 24 hours to just RB+ and every 2 days thereafter until the cells reached the desired age and were replated for experiments.

2.4 iCell cardiomyocytes

Commercially available hiPSC-CMs (iCell Cardiomyocytes) were purchased from Cellular Dynamics International (CDI). These cells are purified (>90% cardiomyocytes) and matured (30 days old) before shipping.

2.4.1 Thawing and maintenance of CDI hiPSC-CMs

The cells were thawed according to the manufacturer's instructions using commercially available plating medium (CDI), and cellular content was calculated on the basis of provided plating efficiency and viability measurements by the manufacturer. Human iPSC-CMs were added to plates pre-coated with 0.1% gelatin (Sigma-Aldrich) in DPBS for 1 hour at 37°C. Cells were allowed to adhere for two days, medium was then changed over to maintenance medium (CDI), and replaced every two days thereafter until the hiPSCs were used for experiments. Cultures were maintained at 37 °C in a 170-300 Galaxy R CO₂ humidified incubator (RS Biotech), with 5% CO₂ and 21% O₂.

2.5 Measurement of contraction in hiPSC-CMs

2.5.1 Cell preparation for contraction studies

At the end of the differentiation protocol, hiPSC-CMs were dissociated as described in 2.3.2 and left to mature until about 30 days old. At this stage, they were replated again onto 35mm dishes with a 7mm glass bottomed well (MatTek Corporation), which had been previously coated with fibronectin as described in 2.3.4. 30,000 cells were added to obtain a confluent monolayer and were left to attach for around 5 days before the experiment was conducted.

2.5.2 Preparation of Krebs–Henseleit solution and drugs

Human iPSC-CMs were superfused with Krebs–Henseleit (KH) solution composed of 119mM NaCl, 4.7mM KCl, 0.94mM MgSO₄, 1.2mM KH₂PO₄, 25mM NaHCO₃, 11.5mM glucose, 2mM CaCl₂ and 100 µM L-ascorbic acid (all Sigma-Aldrich), freshly prepared in distilled water. For catecholamine and cholinomimetic response studies, KH was supplemented with 0.01µM isoprenaline hydrochloride (Sigma, made up to 10mM stock with MQ H₂O) or 0.01µM isoprenaline + 1µM carbachol (Sigma, made up to 10mM stock with MQ H₂O), respectively.

2.5.3 Experimental set-up for contraction studies

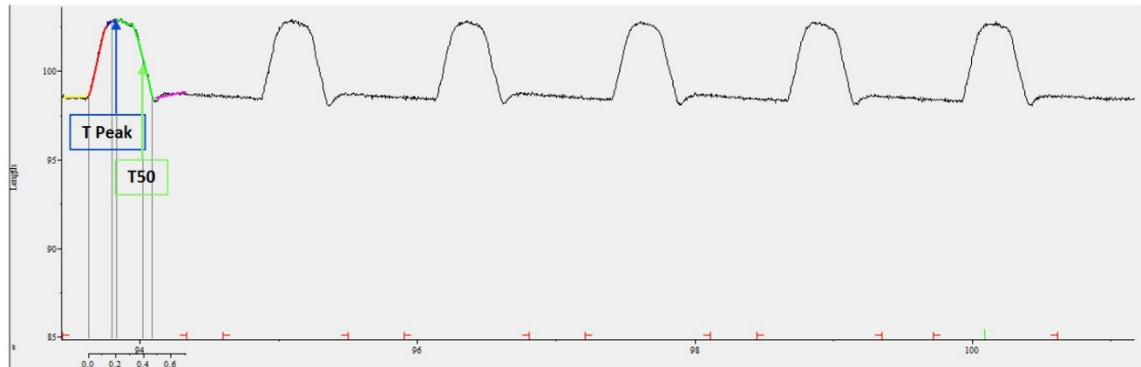
Dishes were transferred to the stage of a Nikon TE200 inverted microscope, which was connected with an IonOptix MyoCam on the side-port (IonOptix Corporation). This was then linked to a PC and data was acquired using the IonWizard software (IonOptix Corporation). Briefly, human iPSC-CMs were superfused with KH, continuously bubbled with 5% CO₂/ 95% O₂ and warmed to 37°C by a heater placed next to the chamber inlet, at a rate of 2 mL/min. Beating hiPSC-CM monolayers were tracked using a video edge-detection system (IonWizard software, Ionoptix Corporation). Cells were left to equilibrate for 15 minutes in standard KH and then were superfused for 15 minutes with β-adrenoceptor agonist isoprenaline, 15 minutes with isoprenaline + muscarinic agonist carbachol, and were finally washed with standard KH for an additional 15 minutes.

2.5.4 Analysis of contraction

Acquired data was opened and analysed using the IonWizard software (IonOptix Corporation). Beating rate was calculated by manually counting the number of contractions over a period of 60 seconds. Measurements were made at basal, every 5 minutes during drug superfusion,

and after washing. Absolute beats per minute (bpm) and % change from bpm were plotted. Time-to-peak shortening (T Peak) and time from peak to 50% relaxation (T50 Relax) were calculated by the IonWizard software for 6 contractions, at basal and after 5 minutes of isoprenaline superfusion (see Figure 2.1 for representative traces).

a



b

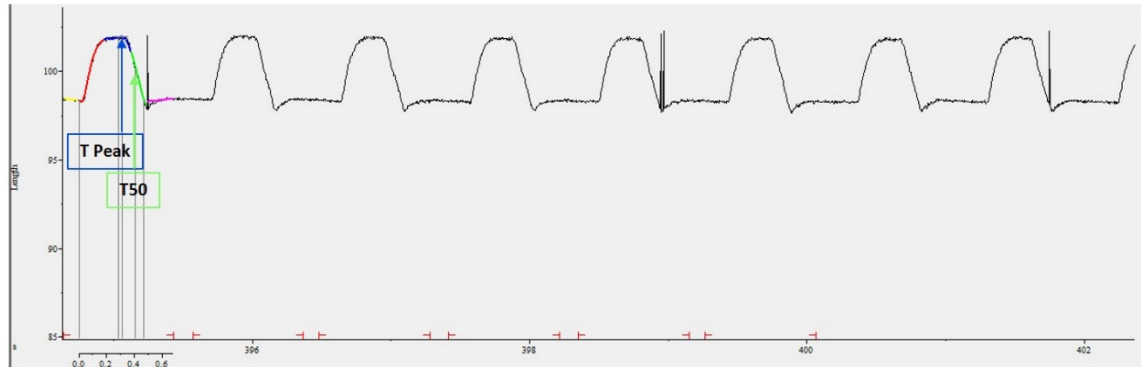


Figure 2.1: Analysis of contraction in hiPSC-CMs.

Representative traces show how time-to-peak shortening (T Peak, blue) and time from peak to 50% relaxation (T50, green) were calculated by the IonWizard software for 6 contractions, a) at basal and b) after 5 minutes of isoprenaline superfusion.

2.6 Measurement of calcium transients in hiPSC-CMs

Fluo-4 AM (Thermo Fisher Scientific) is a cell-permeable molecule that exhibits an increase in fluorescence upon binding intracellular Ca^{2+} , and was used for the visualisation of Ca^{2+} transients.

2.6.1 Cell preparation for calcium transient studies

Human iPSC-CMs were prepared as described in 2.5.1.

2.6.2 Preparation of Tyrode's solution

During optical mapping recordings, medium was replaced by normal Tyrode's Solution composed of 140 mM NaCl, 4.5 mM KCl, 10 mM glucose, 10 mM HEPES, 1 mM MgCl_2 and 1 mM CaCl_2 , freshly prepared in distilled water and pH adjusted to 7.4.

2.6.3 Optical mapping of intracellular calcium

Human iPS-CMs were loaded with 5 μM fluo-4 AM (stock prepared from 50 μg fluo-4 in 50 μl 20% pluronic/DMSO, both Thermo Fisher Scientific) in 1 mL RB+ (both Thermo Fisher Scientific) for 20 minutes at 37°C. Medium was then replaced with fresh RB+ and incubated for a further 20 minutes to allow for dye de-esterification. Dishes were mounted on a Nikon Eclipse TE2000-U confocal microscope (Nikon) using a 40x oil-immersion objective, and were continuously superfused with normal Tyrode's solution warmed to 37°C. Fluo-4 was excited using a 470nm wavelength LED light, and emitted fluorescence collected through a 530 $\pm$ 35 nm long-pass filter. The cells were field stimulated at 1Hz using a 20 $\pm$ 10V pulse of 5ms

duration, and recordings were captured using a Neuro CMOS camera (Redshirt Imaging) at 0.5 kHz with a temporal bin of 2 (final frame rate 250/s). Recordings were made from 3 different areas for each dish (see Figure 2.2a).

2.6.4 Analysis of calcium transients

The first four transients of each recording were combined using signal averaging, and individual parameters of the calcium transient analysed using a custom in-house code for MATLAB R2016b (MathWorks). The following parameters were calculated: normalised fluorescent amplitude (F/F_0 , where F is peak fluorescence intensity and F_0 is baseline fluorescence), time to peak (T Peak, calculated as time from stimulation to peak fluorescence) and time to 50, 80 and 90% decay (T50, T80, T90, calculated as time from peak fluorescence to 50, 80 and 90% reduction in amplitude). Transients were also visualized using pCLAMP 10 software (Molecular Devices), and excitability manually calculated for the first four transients of each recording (defined as the time in ms from stimulation to beginning of the calcium transient). Parameters from the 3 different regions imaged in each dish were averaged. See Figure 2.2.

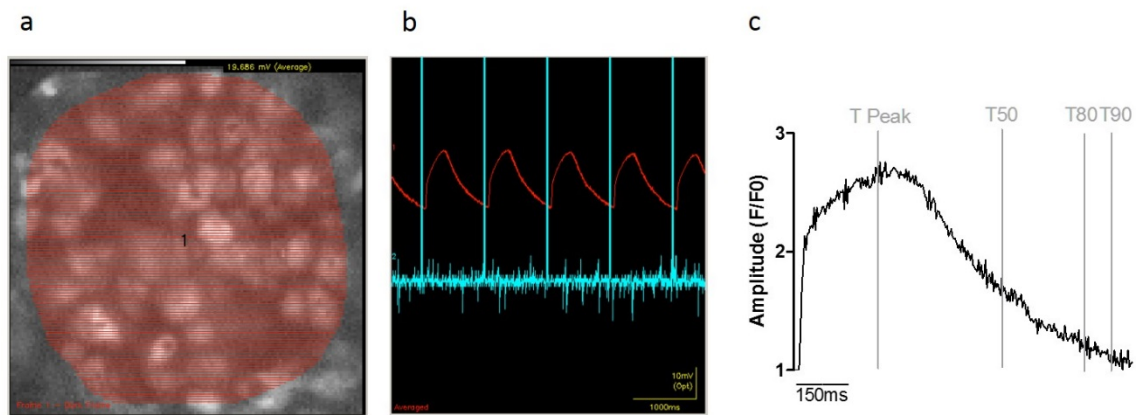


Figure 2.2: Calcium transient analysis in hiPSC-CMs.

a) Selection of region 1 with fluorescing hiPSC-CMs in the back can be seen (transients were recorded from 3 different regions per dish). b) Representative averaged transients recorded from region 1. Cells were stimulated at 1Hz (stimulus shown in blue) and recordings were made for 5 minutes. c) The first 4 transients of each recording were combined using signal averaging, and individual parameters of the calcium transient analysed using a custom in-house code for MATLAB.

2.7 Measurement of mitochondrial respiration in hiPSC-CMs

The Seahorse extracellular flux analysers XF24 and XFp (Agilent) were used to assess mitochondrial substrate utilization in hiPSC-CMs. With optical fluorescent sensors for O_2 and H^+ embedded in each well probe, Seahorse Analysers measure OCR and extracellular acidification rate (ECAR) in live cells to account for mitochondrial respiration and glycolysis, respectively. The Mitochondrial Stress Test (Agilent) involves the application of mitochondrial drugs for the measurement of specific parameters of mitochondrial respiration. Oligomycin (an ATP synthase blocker), FCCP (a mitochondrial membrane uncoupler) and antimycin A + rotenone (electron transport chain complex III and I inhibitor, specifically) are sequentially injected, and their effect on OCR and ECAR is recorded. This enables the determination of key mitochondrial parameters such as Basal Respiration, ATP Production, Maximal Respiration and Spare Respiratory Capacity. See Figure 2.3 for a better understanding of the test and how these parameters are calculated.

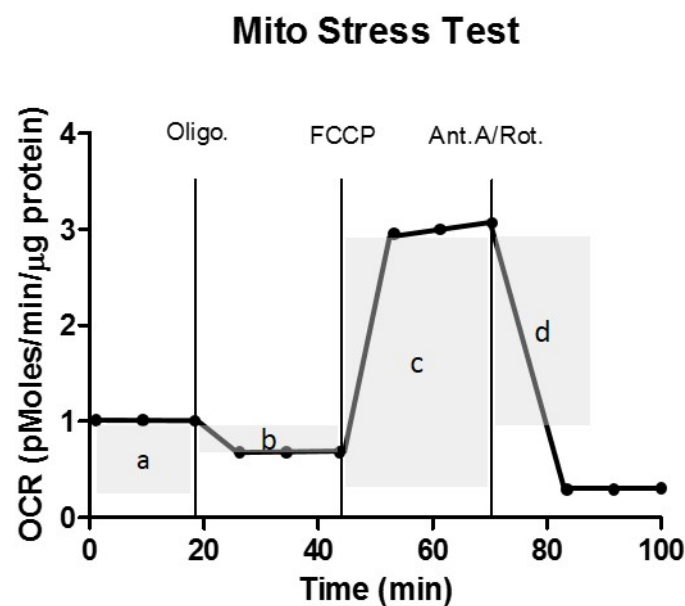


Figure 2.3: A representation of the Mitochondrial Stress test.

Oxygen consumption rate (OCR) is measured in live cells. 3 measurements are taken for each treatment period. Basal OCR is recorded, followed by the injection of: oligomycin (blocks the ATP synthase and hence causes a drop in OCR), FCCP (mitochondrial membrane uncoupler, triggering a maximal rise in OCR) and lastly antimycin A + rotenone (electron transport chain inhibitors, which completely abolish OCR). The four key parameters of mitochondrial function basal respiration (a), ATP production (b), maximal respiration (c) and spare respiratory capacity (d) can then be determined.

2.7.1 Cell preparation for the Mitochondrial Stress Test

At the end of the differentiation protocol, hiPSC-CMs were dissociated as described in 2.3.2 and left to mature until about 30 days old. At this stage, they were replated again onto XFp plates (Agilent), which had been previously coated with fibronectin as described in 2.3.4. 21,000 cells were added to obtain a confluent monolayer and were left to attach for around 5 days before the experiment was conducted. For CDI hiPSC-CMs, 20,000 and 14,000 cells were added to XF24 and XFp well plates, respectively, to achieve a confluent monolayer (see 2.4.1).

2.7.2 Preparation of Seahorse assay medium and drugs

For the Mitochondrial Stress Test, medium was replaced with bicarbonate-free Seahorse assay medium which was made up to 1L with 8.3g DMEM Base (Sigma-Aldrich), 10mM glucose (Sigma-Aldrich), 2mM Glutamax-1 (Thermo Fisher Scientific) and 1mM sodium pyruvate (Thermo Fisher Scientific), sterilized, and pH adjusted to 7.4 on the day of the experiment. The following drugs were applied during the test: oligomycin (Abcam, stock prepared to 10mM in DMSO), FCCP (Abcam, stock prepared to 10mM in DMSO) and antimycin A (Sigma-Aldrich, stock prepared to 10mM in DMSO) + rotenone (Abcam, stock prepared to 10mM in DMSO).

2.7.3 The Mitochondrial Stress Test

The experiment was conducted according to the manufacturer's instructions (Agilent). Briefly, medium was exchanged for Seahorse assay medium and hiPSC-CMs were left to equilibrate for one and a half hours at 37°C in a non-CO₂ incubator (INCU-Line®, IL 10 Digital incubators, VWR). The Seahorse Analyser XF24 or XFp (Agilent) was calibrated, as instructed by the

manufacturer, and the drug cartridge was loaded in ports A (oligomycin), B (FCCP) and C (antimycin A + rotenone). Final concentration in well after injection 1.5 μ M oligomycin, 0.5 μ M FCCP and 1 μ M antimycin A + rotenone. Drugs were previously diluted in Seahorse assay medium and these concentrations were recommended by the company (Agilent). Each assay cycle comprised of probe mixing, waiting and measuring, as suggested by the manufacturer, and three cycle measurements were taken for each treatment period. OCR and ECAR were reported in pmol/min and mpH/min, respectively, and were normalised to total protein levels in each well, determined according to the manufacturer's instructions using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Absorbance was measured at 562nm on a LT-4500 plate reader (Labtech).

2.7.4 Analysis of mitochondrial respiration

For each experiment, results were visualized using Wave 2.3 Software (Agilent) and average OCR and ECAR values for each experimental group were exported. Basal respiration, ATP production, maximal respiration and spare respiratory capacity were defined by the differences between average measurements 3 and 12, 3 and 6, 7 and 12, 7 and 3, respectively (See Figure 2.3). The ratio between OCR and ECAR was determined by dividing the last basal OCR measurement by the last basal ECAR measurement. Data are represented as absolute values or as a percentage change from first basal measurement.

2.8 Measurement of ATP production in hiPSC-CMs

At the end of the differentiation protocol, hiPSC-CMs were dissociated as described in 2.3.2 and left to mature until about 30 days old. At this stage, they were replated again on 96-well plates (Becton Dickinson), which had been previously coated with fibronectin as described in 2.3.4. 20,000 cells were added to obtain a confluent monolayer and were left to attach for

around 5 days before the experiment was conducted. ATP production was quantified using a Luminescent ATP Detection Assay Kit (Abcam) according to the manufacturer's instructions, and the luminescent signal was measured using a Paradigm Microplate Detection Platform (Beckman Coulter). ATP levels relative to control were determined as a percentage.

2.9 Immunostaining

Cells were washed with DPBS, fixed with 4% paraformaldehyde (Electron Microscopy Services) in DPBS for 15 min at room temperature and washed 3 times with DPS. At this stage, they were either kept at 4°C in DPBS until needed or permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in DPBS for 10 min at room temperature, followed by three 5 minute washes with DPBS. Cells were then incubated with blocking solution composed of 4% FBS (Thermo Fisher Scientific) in DPBS for 1 hour at room temperature.

See Table 2.1 for details on the antibodies used. Primary antibodies were diluted in 3% BSA (Sigma-Aldrich) in DPBS to the desired concentration, cells were incubated in primary antibody for 1 hour at room temperature, and at the end of the incubation period washed with DPBS as above. Appropriate secondary antibodies were diluted in 3% BSA in DPBS to the desired concentration, and added to the preparation for a 45 minute incubation period. Cells were washed again as above and nuclei were stained for 10 minutes at room temperature with Hoechst 33342 (Thermo Fisher Scientific) in DPBS.

Target	Description	Dilution	Reference
Oct-4	Rabbit polyclonal	1:100	ab19857 (Abcam)
Troponin-T	Rabbit monoclonal	1:200	ab45932 (Abcam)
Alexa Fluor 488	Goat anti-rabbit	1:400	A-11008 (Thermo Fisher Scientific)
Hoechst 33342	-	1:500	H3570 (Thermo Fisher Scientific)

Table 2.1: Primary and secondary antibodies used in immunostaining.

2.10 Imaging

2.10.1 High content imaging

Images were acquired on ArrayScan™ VTI automated microscopy and image analysis system (Thermo Fisher Scientific), with the ×10 objective. All the cells were identified with Hoechst fluorescence that defined the nuclear area (channel 2) and hiPSC-CMs were distinguished by troponin-T fluorescence (channel 1). The fluorescence intensity threshold discriminating Troponin-T positive and Troponin-T negative cells was set manually. Morphology Explorer BioApplication was used for the measurement of hiPSC-CM size (area of detected object in μm^2) and shape (based on ratio of length μm to width μm of detected object). Compartmental Analysis BioApplication was used for the calculation of troponin-T positive cells (average channel 1 fluorescent intensity detected in a defined annular region of the cytoplasm outside the nucleus), and the obtained results were expressed as a percentage calculated as (total number troponin-T positive cells x 100)/total number Hoechst detected cells, following threshold correction and normalization for secondary antibody control. See Figure 2.4.

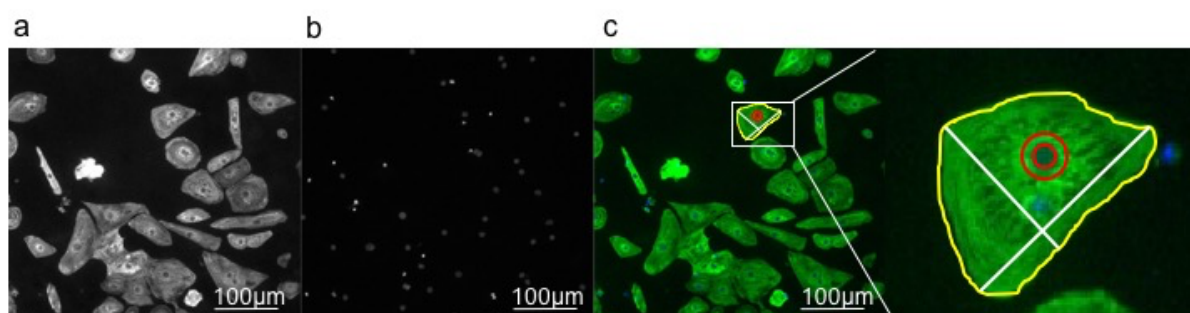


Figure 2.4: Calculation of troponin-T positive cells and cell size and shape.

Cells were stained for troponin-T and images were acquired using an automated microscopy and image analysis system. a) Human iPSC-CMs were distinguished by troponin-T fluorescence (channel 1). b) All the cells were identified with Hoechst fluorescence that defined the nuclear area (channel 2). c) Average channel 1 fluorescent intensity detected in a defined annular region of the cytoplasm outside the nucleus (red) was used for the calculation of troponin-T positive cells. Cell size (area of detected object in μm^2) and shape (based on ratio of length μm to width μm of detected object) were also calculated. Scale bar 100 μm .

2.10.2 Brightfield and confocal microscopy

Brightfield images were acquired using a Nikon eclipse TE200 microscope with NIS-elements BR software. Confocal images were acquired using an inverted Zeiss LSM-780 confocal microscope with Zen Zeiss software.

2.11 Gene expression

2.11.1 RNA extraction and cDNA synthesis

Cells were washed with DPBS and lysed using TRI Reagent (Sigma-Aldrich), according to the manufacturer's instructions. Lysates were stored at -80°C until needed. RNA was isolated using an RNeasy Mini kit (Qiagen), according to the kit's instructions, and quantified using a NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). RNA was converted to single-stranded cDNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), as instructed by the manufacturer. Reverse transcription was performed in a GeneAmp PCR Sytem 2700 thermal cycler (Applied Biosystems).

2.11.2 Genomic DNA extraction

For genomic DNA extraction, cells were dissociated accordingly, centrifuged at 200g for 4 minutes and pellets were washed once in DPBS and stored at -80°C until needed. DNA was extracted using the DNeasy Blood And Tissue Kit (Qiagen), according to the manufacturer's instructions, and quantified using a NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Fisher Scientific).

2.11.3 Real-time PCR (qPCR)

Real-time PCR (qPCR) was performed for RNA quantification, mitochondrial DNA copy number quantification and amplification-refractory mutation system (ARMS) quantification of A3243G heteroplasmy. Primers used are listed in Table 2.2.

2.11.3.1 mRNA expression and mitochondrial DNA copy number quantification

The reaction mix was prepared in accordance with the protocol provided for the TaqMan Gene Expression Assays (Thermo Fisher Scientific). Each reaction contained 50ng cDNA template or 10ng genomic DNA, for mRNA expression and mitochondrial DNA copy number quantification, respectively. All reactions were performed in triplicate. The reaction mix was transferred to a 384-well plate (Thermo Fisher Scientific), centrifuged at full speed for 1 minute, and loaded into a 7900HT Fast Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were denaturation at 95°C for 10 min, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

For mRNA quantification, fold changes in gene expression were determined using the comparative CT method ($\Delta\Delta C_t$) with normalization to the housekeeping gene B2M (Thermo Fisher Scientific). Mitochondrial DNA copy number quantification was determined according to the following equations, previously described by others (Sharma *et al.*, 2015): $\Delta C_T = (\text{nucDNA } C_T - \text{mtDNA } C_T)$ and relative mitochondrial DNA copy number = $2 \times 2^{\Delta C_T}$. NucDNA C_T was determined by amplification of nuclear gene SDHA and mtDNA C_T was determined by amplification of the mitochondrial gene MTND1.

2.11.3.2 Amplification-refractory mutation system real-time PCR

Amplification-refractory mutation system real-time PCR (ARMS qPCR) is a sensitive method used to detect and quantify the amount of heteroplasmic mtDNA point mutations. The technique uses two sets of primers, one amplifies total mtDNA and the other amplifies the mutant mtDNA sequence. By generating a standard curve (based on four 8-fold serial dilutions plus last 4-fold dilution) using a positive DNA control which is homoplasmic (100% mutant) for the mutation of interest, it is possible to quantify the amount of mutant mtDNA relative to total mtDNA in the samples of interest (Rooney *et al.*, 2015). Briefly, genomic DNA was extracted from samples of interest and from the homoplasmic A3243G hiPSC line, as described in 2.11.2. Each reaction contained 10ng of genomic DNA. For amplification of total mtDNA, the MTND1 primer (Thermo Fisher Scientific) was used and the reaction mix was prepared in accordance with the protocol provided for the TaqMan Gene Expression Assays (Thermo Fisher Scientific). For amplification of the A3243G mtDNA mutant sequence, in-house designed primers and probes (Sigma-Aldrich, see Table 2.2) and the HOT FIREPol DNA polymerase chemistry (Solis BioDyne) were used as described before (Venegas and Halberg, 2012). All reactions were performed in duplicate. The reaction mix was transferred to a 384-well plate (Thermo Fisher Scientific), centrifuged at full speed for 1 minute and loaded into a 7900HT Fast Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were denaturation at 95°C for 15 min, and 45 cycles of 95°C for 15 seconds and 62°C for 1 minute. Quantity of total and mutant mtDNA was automatically calculated by the SDS 2.3 software based on the generated standard curve. The percentage of mutant mtDNA in relation to the total mtDNA content was determined by the equation $100 \times (\text{quantity A3243G sequence} / \text{quantity total mtDNA})$ (Lee *et al.*, 2012).

Target	Sequence	Description	Reference	Supplier
SOD2	-	TaqMan Assay	Hs00167309_m1	Thermo Fisher Scientific
B2M	-	TaqMan Assay	Hs00187842_m1	Thermo Fisher Scientific
SDHA	-	TaqMan Assay	Hs03560884_cn	Thermo Fisher Scientific
MTND1	-	TaqMan Assay	Hs02596873_s1	Thermo Fisher Scientific
A3243G	GGGTTTGTTAAGATGGCTGG	Forward Primer	MelasAS(3)-F	Sigma-Aldrich
A3243G	TAGGAATGCCATTGCGATT	Reverse Primer	MelasAS-R	Sigma-Aldrich
A3243G	FAM-CCCATGGCCAACCTCCTACTCCTCA-BHQ1	Probe	MelasCR-FAM	Sigma-Aldrich

Table 2.2: Primers and probes used in real-time PCR.

2.12 Statistical analysis

Unless specified otherwise, the following parametric tests were performed for normally distributed data: unpaired or paired student's t-tests were used for comparisons between two groups, and one-way or two-way analysis of variance (ANOVA) followed by a Bonferroni's Post-Hoc test were used for comparisons between more than two groups. Data are presented as mean $\pm$ standard error of the mean (s.e.m.) and $p < 0.05$ was considered significant. The analysis was carried out with the Prism6 software (GraphPad Software).

Chapter 3: The involvement of maturation strategies on human pluripotent stem cell derived cardiomyocytes mitochondrial respiration

3.1 Introduction

As explored in section 1.3.3 of the Introduction, the differentiation methods developed to date still result in rather immature, or foetal-like hPSC-CMs(Lee *et al.*, 2012). This is true in terms of morphology(Veerman *et al.*, 2015), sarcomere organisation(Snir *et al.*, 2003), electrophysiology(Snir *et al.*, 2003) and, of particular interest to us, metabolism(Kane and Terracciano, 2017). Human PSC-CMs have fewer yet more disorganised mitochondria, and rely on glucose and lactate primarily, as opposed to fatty acids in the adult heart(Denning *et al.*, 2016). As discussed in section 1.3.4, several strategies have been employed to drive maturation of hPSC-CMs, including prolonging the time in culture and application of chemical molecules(Denning *et al.*, 2016). The thyroid hormone T_3 lies amongst the most promising substances identified to date as promoting hPSC-CM maturation(Veerman *et al.*, 2015). Together, these strategies lead to some improvements in maturation, but hPSC-CMs that are functionally identical to adult CMs have not yet been described, under any condition(Yang *et al.*, 2014). Some issues remain unsolved though, for instance the metabolic state of hPSC-CMs is rarely a focus of maturation studies.

Over the last few years, our lab has been investigating the role of time in culture and T_3 in promoting the maturation of CMs derived from several hPSC lines (hiPSCs and hESCs). Initial experiments were performed using the commercially available line CDI hiPSC-CMs (from Cellular Dynamics International) but the protocols separately published by Lian(Veerman *et al.*, 2015) and Burridge(Lian *et al.*, 2013) enabled us to use CMs from several hPSC lines (hiPSCs and hESCs) generated at high efficiencies in-house. Using CDI hiPSC-CMs, we have performed a study looking at the proteomic changes that occurred when the cells were

kept in culture for 2 weeks after thawing (45 days old) versus 1 month after thawing (60 days old). After 1 month in culture, proteins involved in OXPHOS were upregulated and proteins involved in glycolysis were downregulated compared to 2 weeks (in submission). Functionally, AP properties were improved at 1 month compared to 2 weeks but beating rate and Ca^{2+} handling properties were unchanged. In a separate study, we have shown that, compared to 48 hours, 1 week and 2 weeks of T_3 treatment, 1 month treatment increased the ratio of α/β MHC and β_1 adrenergic receptor expression in CDI hiPSC-CMs (unpublished data). Next, we established that treatment of CDI hiPSC-CMs with T_3 for 1 month improved beating rate, Ca^{2+} handling and AP properties of the cells and resulted in upregulation of proteins involved with OXPHOS and mitochondrial dysfunction (unpublished data). In this chapter we aimed to determine the effects of 2 weeks versus 4 weeks in culture and of 1 month T_3 treatment on CDI hiPSC-CM mitochondrial respiration. We were also interested in looking at the effects of T_3 treatment on mitochondrial respiration of CMs generated in house from other hiPSCs and hESC lines. Unpublished data from our lab has also shown that H7 hESC-CMs express high levels of the deiodinase 3 (D3) enzyme, which inactivates the active T_3 to inactive reverse T_3 (rT_3) and T_2 and is upregulated in foetal tissues for protection against maternal high levels of T_3 (Burridge *et al.*, 2014). In contrast, levels of D3 in CDI hiPSC-CMs are very low. Therefore, we also wanted to determine the effects of iopanoic acid (IOP), which blocks D3 activity, or GC-1, which is a thyroid hormone receptor β -specific agonist not broken down by D3, on mitochondrial respiration of hESC-CMs (Larsen and Zavacki, 2012).

Altogether, this knowledge would allow us to understand the conditions necessary to achieve a maximal/mature OXPHOS capacity in hPSC-CMs. This could then be applied to determine whether this metabolic state is compromised in the *in vitro* models of heteroplasmic mitochondrial dysfunction to be developed in hiPSC-CMs.

3.2 Methods

3.2.1 Cell preparation

CDI hiPSC-CMs were handled as in section 2.4 of General Methods. 2 days after plating, medium was changed to Maintenance Medium (CDI) and studies were conducted after 2 weeks (for optimisation experiments or for 2 weeks experiments) or 1 month (for drug treatment experiments). IMR90-4 hiPSCs and H7 hESCs were maintained and differentiated as described in sections 2.1 and 2.3. At day 30 post-beginning of differentiation, cells were either used for optimisation experiments or treated with drugs for 1 week or 1 month, accordingly.

3.2.2 Drug treatments

The following drugs were diluted to the desired concentration in culture medium:

- 3, 3', 5 Triiodo-L—thyronine sodium salt (T_3) – Sigma-Aldrich, stock prepared to 30 μ M in 1M NaOH
- Iopanoic acid (IOP) - Tokyo Chemical Industry, stock prepared to 65mM in ethanol absolute
- GC-1 – Tocris, stock prepared to 10mM in DMSO

3.2.3 Mito Stress Test experiments

All Mito Stress test experiments were performed as stated in section 2.7. Seahorse Assay Medium was prepared as described in section 2.7.2, but supplemented with either 10mM glucose or 10mM galactose (both Sigma-Aldrich), accordingly. Mitochondrial drugs were prepared as in section 2.7.2, and diluted in Seahorse Assay Medium to the required

concentration. The % change in OCR, worked out for drug optimisation, represents the change between last basal measurement and first measurement after drug injection. Determination of the mitochondrial respiration parameters is defined in section 2.7.4.

3.2.4 Quantification of mtDNA copy number

Refer to section 2.11.3.1 for information.

3.2.5 Statistical Analysis

Details are described in section 2.12.

3.3 Results

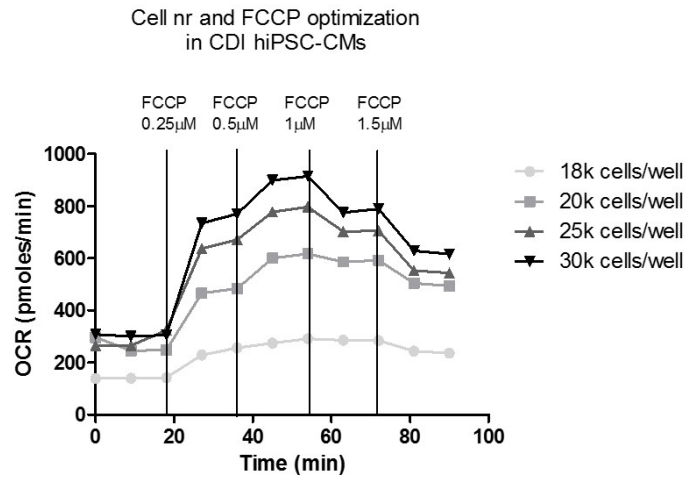
3.3.1 The Mito Stress Test was optimised for CDI hiPSC-CMs

Cell density is a main aspect that requires optimisation in the Mito Stress Test. It is crucial that the cells form a confluent monolayer, in particular in the centre of the well, where the probe takes measurements from. Differences in cell density between assays will cause high variability in the results. For optimisation of cell density, 18,000, 20,000, 25,000 and 30,000 CDI hiPSC-CMs were plated in each well of an XF24 plate (numbers are reduced by 30% for XFp plates). By observing under an inverted brightfield microscope, it was decided that 20,000 cells were needed to obtain a confluent monolayer across the whole well.

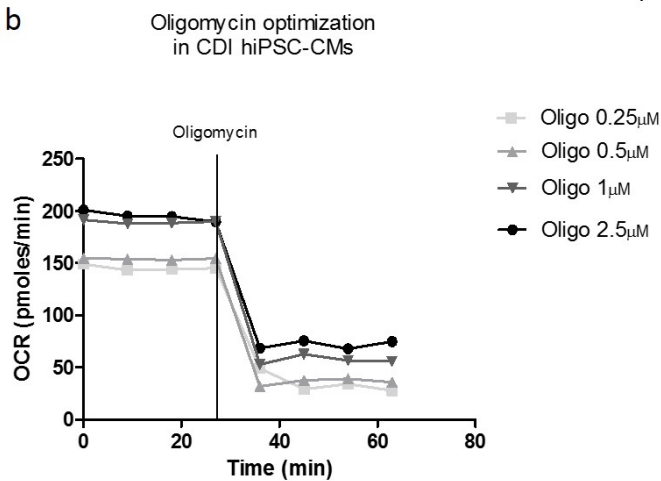
The test is performed using a Seahorse Analyser, which accounts for changes in OCR, at basal and after the addition of several mitochondrial drugs. This enables the measurement of

the mitochondrial parameters basal respiration, ATP production, maximal respiration and spare respiratory capacity (see section 2.7.4 for details on how the calculations are performed). Due to the narrow range from maximal inhibition to associated cell death, FCCP and oligomycin must also be titrated carefully to minimize toxicity. The OCR response of CDI hiPSC-CMs to increasing concentrations of FCCP (0.25 μ M, 0.5 μ M, 1 μ M and 1.5 μ M) can be seen in Figure 3.1a. FCCP 0.5 μ M resulted in the highest rise in OCR. Higher concentrations generally resulted in a drop of OCR, suggesting toxicity. Oligomycin was similarly titrated in CDI hiPSC-CMs, with injections of 0.25 μ M, 0.5 μ M, 1 μ M and 2.5 μ M, this case in separate wells to avoid preconditioning. This is shown in Figure 3.1b. The percentage drop in OCR for every concentration was calculated (Figure 3.1c), and the highest drop in OCR occurred upon injection of 0.5 μ M oligomycin to hiPSC-CMs (reduction of 79.4%). Again, higher concentrations resulted in a drop of OCR, suggesting toxicity. Based on these data, it was decided that 20,000 CDI hiPSC-CMs, 0.5 μ M oligomycin and 0.5 μ M FCCP would be used for future experiments.

a



b



c

Oligomycin (μ M)	Drop in OCR (%)
0.25	66.0
0.5	79.4
1	72.3
2.5	63.9

Figure 3.1: Mito Stress Test optimisation in CDI hiPSC-CMs.

a) Cell density and FCCP were optimised by plating 18,000, 20,000, 25,000 and 30000 cells per well of a XF24 plate, and measuring the changes in oxygen consumption rate (OCR, in pmoles/min) upon sequentially increasing the concentration of FCCP injected. b) Oligomycin concentration was optimised by observing the drop in OCR at the different concentrations injected. c) The percentage (%) drop in OCR was calculated for the different concentrations used.

3.3.2 One month in culture did not result in improved mitochondrial respiration in CDI hiPSC-CMs compared to 2 weeks

We wanted to establish whether extending the time in culture of CDI hiPSC-CMs was linked to improved mitochondrial respiration, as previous results from our lab showed upregulation of OXPHOS proteins and downregulation of glycolysis proteins when CDI hiPSC-CMs were kept in culture for 1 month (60 days old) compared to 2 weeks (45 days old) after thawing (see Figure A2 of the Appendix – data kindly provided by Nicola Hellen). Functionally, AP properties were improved at 1 month compared to 2 weeks but beating rate and Ca^{2+} handling properties were unchanged (see Figure A1 of the Appendix– data kindly provided by Nicola Hellen). For this, the Mito Stress Test was performed using 2 weeks and 1 month old CDI hiPSC-CMs (Figure 3.2a). There were no significant differences between 2 weeks and 1 month in culture in terms of the mitochondrial respiration parameters analysed: basal respiration, ATP production, maximal respiration and spare respiratory capacity (Figure 3.2b). The ratio between OCR (mitochondrial respiration) and ECAR (glycolysis), an indication of the energetic phenotype, was also calculated and, similarly, there were no differences after prolonged time in culture (Figure 3.2c). It is also worth noting that, in both groups, OXPHOS outweighed glycolysis (Figure 3.2c, OCR/ECAR ratios were greater than 1).

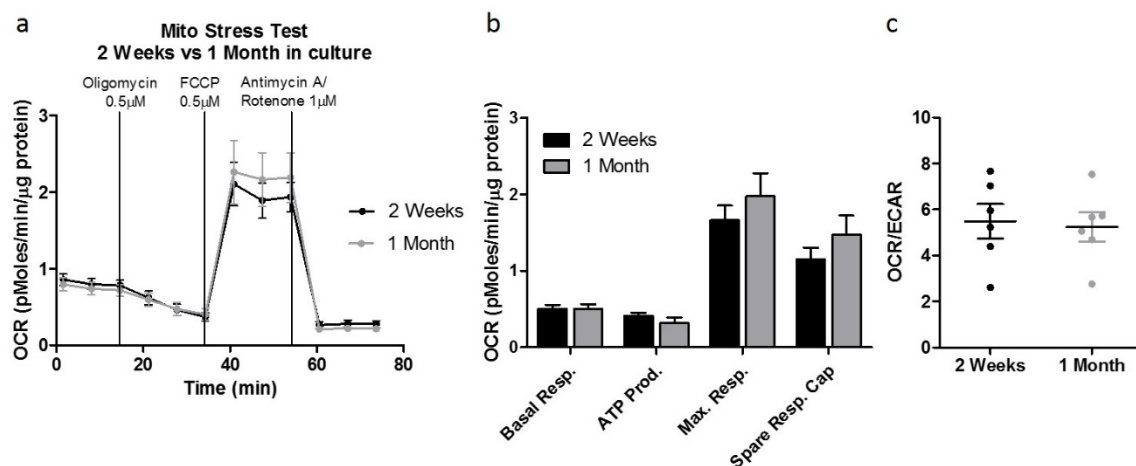


Figure 3.2: The effect of prolonged time on culture in CDI hiPSC-CM mitochondrial respiration.

a) The Mito Stress Test was performed at 2 weeks and 1 month in culture, and oxygen consumption rate (OCR, in pMoles/min/ μ g protein) was measured at basal, and following the injection of 0.5 μ M oligomycin, 0.5 μ M FCCP and 1 μ M antimycin A/rotenone. b) Basal respiration, ATP production, maximal respiration and spare respiratory capacity. c) The ratio between OCR and ECAR (extracellular acidification rate, in mpH/min/ μ g protein). n=6 independent batches. All the graphs represent mean $\pm$ s.e.m., Unpaired Student's t-test.

3.3.3 Treatment with T₃ for 1 month did not result in improved mitochondrial respiration in CDI hiPSC-CMs

The positive effects of T₃ on hPSC-CM maturation have been established by our lab. Improvements in functional and molecular properties of hiPSC-CMs were observed after 1 month treatment with T₃. Functionally, T₃ improved beating rate, Ca²⁺ handling and AP properties of hiPSC-CMs (see Figure A3 of the Appendix– data kindly provided by Nicola Hellen). Of particular relevance to this project were the results obtained from the proteomic studies, which revealed upregulation of several proteins involved in OXPHOS but also upregulation of several proteins involved in mitochondrial dysfunction, including some responsible for apoptosis and mitochondrial fission (see Figure A4 of the Appendix– data kindly provided by Nicola Hellen). We wanted to determine the effects of T₃ treatment on mitochondrial respiration of CDI hiPSC-CMs.

The Mito Stress Test was performed in CDI hiPSC-CMs after 1 month in culture with 30nM T₃ (Figure 3.3a), using the standard Seahorse Assay Medium which contains 10mM glucose. Basal respiration and ATP production decreased after T₃ treatment (Figure 3.3b; basal respiration: control OCR 0.61 ± 0.07 , T₃ OCR 0.42 ± 0.04 , $p = 0.0270$; ATP production: control OCR 0.55 ± 0.06 , T₃ OCR 0.37 ± 0.04 , $p = 0.0368$). Maximal respiration and spare respiratory capacity were unchanged (Figure 3.3b). The energetic phenotype, in terms of OCR/ECAR ratio, was also unchanged and both groups primarily relied on OXPHOS (Figure 3.3c).

The full list of components present in CDI hiPSC-CM Maintenance Medium was not disclosed by the company. However, we were informed that it did not contain glucose and that galactose was present. Cells are relatively plastic in terms of their metabolism, and galactose as a substrate in cell culture shifts the ATP production from glycolysis to OXPHOS (Kraiem, 2005). Therefore, we questioned whether the presence of glucose in Seahorse Assay medium could

have hampered the improvements in mitochondrial respiration by T_3 . Similarly, we wondered if the effects of T_3 had worn off by the time of measurements, since medium was replaced by Seahorse Assay Medium without T_3 before the assay. Thus, the Mito Stress Test was repeated using Seahorse Assay Medium, instead supplemented with 10mM galactose and 30nM T_3 (Figure 3.3d). In the presence of galactose, T_3 treatment did not cause significant changes in any of the parameters of mitochondrial respiration analysed, basal respiration, ATP production, maximal respiration and spare respiratory capacity. There were no differences between hiPSC-CMs just treated with T_3 during 1 month of culture and those that also had T_3 throughout the assay (Figure 3.3e). It is worth noting that we noticed a reduction in spare respiratory capacity when using galactose, compared to glucose, but this was not significant (Figure 3.3b and e, for control group $p=0.0669$; Unpaired Student's t-test). In addition, there were no differences in the OCR/ECAR ratio. Again, both control and T_3 -treated cells used primarily OXPHOS (Figure 3.3f).

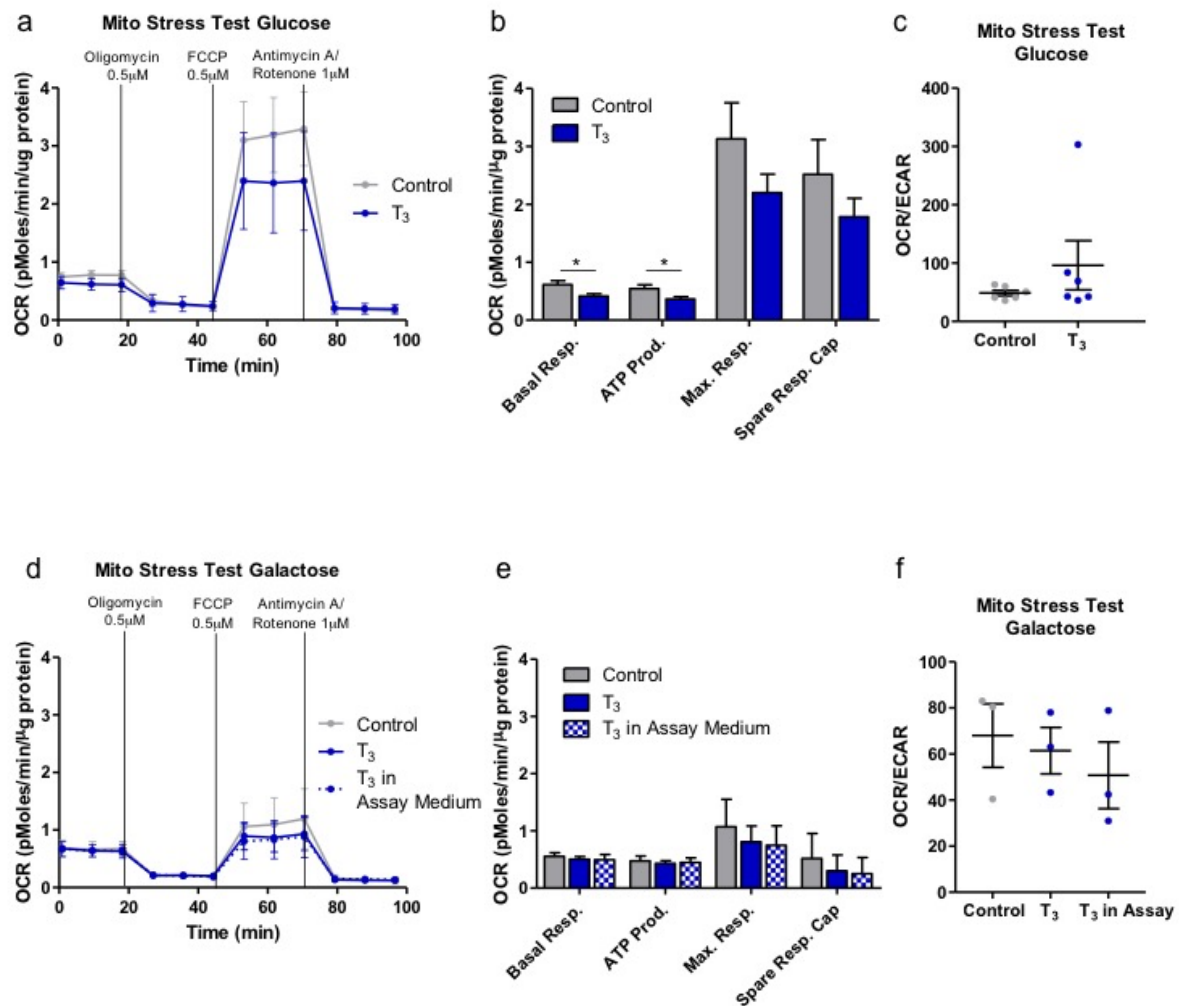


Figure 3.3: The effect of T₃ treatment on CDI hiPSC-CM mitochondrial respiration.

a) The Mito Stress Test was performed after 1 month treatment with 30nM T₃ using the standard glucose containing Seahorse Assay Medium, and oxygen consumption rate (OCR, in pMoles/min/ μ g protein) was measured at basal, and following the injection of 0.5 μ M oligomycin, 0.5 μ M FCCP and 1 μ M antimycin A/rotenone. b) Basal respiration, ATP production, maximal respiration and spare respiratory capacity. c) The ratio between OCR and ECAR (extracellular acidification rate, in mP_H/min/ μ g protein). n=6 independent batches. The graphs represent mean $\pm$ s.e.m., Unpaired Student's t-test. *p<0.05. d) The Mito Stress Test performed after 1 month treatment with 30nM T₃, using galactose containing Seahorse Assay Medium with or without T₃. e) The parameters of mitochondrial respiration and f) the OCR/ECAR ratio were also calculated. n=3 independent batches. The graphs represent mean $\pm$ s.e.m. One-way ANOVA followed by Bonferroni's Post-Hoc.

3.3.4 The Mito Stress test was optimised for hiPSC-CMs differentiated in-house

In recent years, the efficiency and reproducibility of cardiomyocyte differentiation from hPSCs has highly improved (Rossignol *et al.*, 2004). This enabled us to differentiate multiple hPSC lines in-house and to reduce the experiments performed with commercial lines, such as CDI hiPSC-CMs. One of the main advantages of this is being aware of the exact culture conditions used, in particular the energy substrate provided.

The Mito Stress Test was optimised for in-house produced CMs, using cells generated from the hiPSC IMR90-4 line. 30,000 IMR90-4 hiPSC-CMs per well were needed to produce a confluent monolayer in XF24 plates (number reduced by 30% for XFp plates), as observed under a brightfield inverted microscope. Oligomycin concentration was optimised by injecting 0.5µM, 1µM and 1.5µM into separate wells and measuring the reduction in OCR (Figure 3.4a). Oligomycin 1.5µM resulted in the biggest drop in OCR (Figure 3.4b, reduction of 45.2%). FCCP concentration was then optimised by injections of 0.25µM, 0.5µM and 0.75µM into separate wells, after the cells had already been exposed to 1.5µM oligomycin (Figure 3.4c). The largest rise in OCR was observed at 0.5µM FCCP (Figure 3.4d, 45.2% rise). Based on these results, it was decided that 30,000 cells, 1.5µM oligomycin and 0.5µM FCCP would be used for future experiments using in-house generated hPSC-CMs.

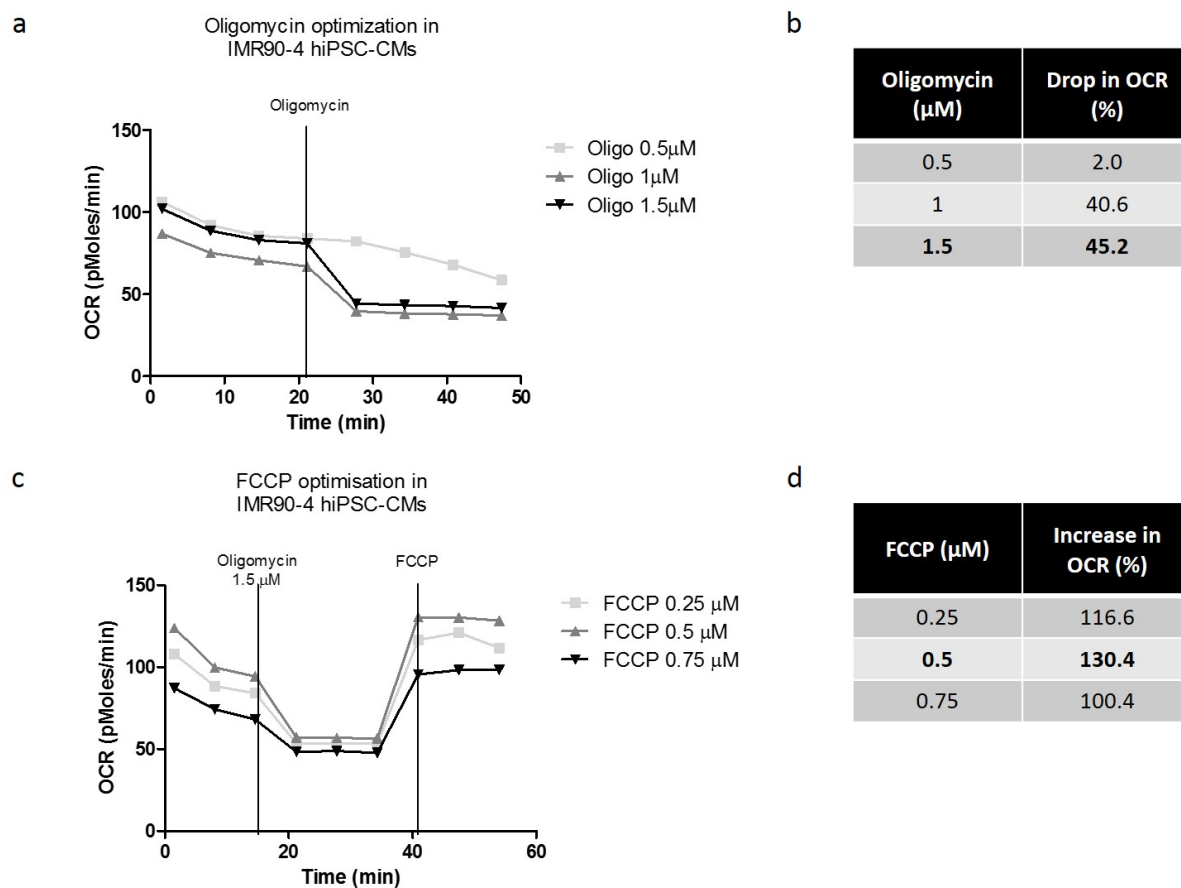


Figure 3.4: Mito Stress Test optimisation in IMR90-4 hiPSC-CMs.

a) Oligomycin and c) FCCP were injected at different concentrations, and their effect on oxygen consumption rate (OCR, in pMoles/min) was measured. The concentration responsible for the biggest b) drop and d) rise in OCR was determined.

3.3.5 Treatment with T₃ for 1 week did not result in improved mitochondrial respiration in IMR90-4 hiPSC-CMs

No improvements in mitochondrial respiration were observed in CDI hiPSC-CMs after 1 month T₃ treatment. In fact, a reduction in basal respiration and ATP production was observed when the Mito Stress Test was performed using 10mM glucose (Figure 3.3b). A study published by Yang *et al.* in 2014 determined that treatment of IMR90-4 hiPSC-CMs generated in-house with T₃ for 1 week resulted in improvements in mitochondrial respiration, in terms of basal respiration, maximal respiration and spare respiratory capacity (Lian *et al.*, 2013; BurrIDGE *et al.*, 2014). To investigate whether these results were reproduced using IMR90-4 hiPSC-CMs generated in-house by us, we repeated the Mito Stress Test using the standard glucose containing Seahorse Assay Medium (Figure 3.5a). Mitochondrial respiration, in terms of all the parameters analysed, was not significantly different in T₃-treated compared to untreated cells (Figure 3.5b, for basal respiration, ATP production, maximal respiration and spare respiratory capacity). No differences were detected in terms of OCR/ECAR ratio. Once again, both groups performed OXPHOS primarily (Figure 3.5c).

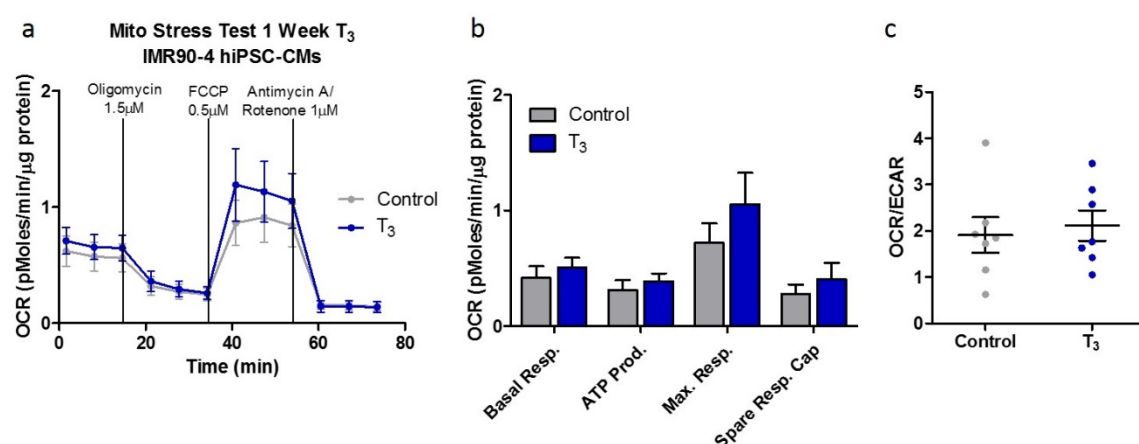


Figure 3.5: The effect of T_3 treatment on IMR90-4 hiPSC-CM mitochondrial respiration.a) The Mito Stress Test was performed after 1 week treatment with 30nM T_3 using the standard glucose containing Seahorse Assay Medium, and oxygen consumption rate (OCR, in pMoles/min/ μ g protein) was measured at basal, and following the injection of 1.5 μ M oligomycin, 0.5 μ M FCCP and 1 μ M antimycin A/rotenone. b) Basal respiration, ATP production, maximal respiration and spare respiratory capacity. c) The ratio between OCR and ECAR (extracellular acidification rate, in mpH/min/ μ g protein). n=7 independent differentiations. The graphs represent mean $\pm$ s.e.m., Unpaired Student's t-test.

3.3.6 T₃ treatment for 1 month increased mitochondrial respiration in H7 hESC-CMs but bypassing the D3 pathway did not improve mitochondrial respiration further than treatment with T₃

Results from the Mito Stress test performed using the standard glucose containing Seahorse Assay Medium showed that, in contrast with what was observed with hiPSC-CMs, 30nM T₃ treatment for 1 month increased all the parameters of mitochondrial respiration in H7 hESC-CMs compared to control, including basal respiration (Figure 3.6b, $p<0.001$), ATP production (Figure 3.6c, $p<0.05$), maximal respiration (Figure 3.6d, $p<0.01$) and spare respiratory capacity (Figure 3.6e, $p<0.01$).

To test the hypothesis that high levels of D3 in H7 hESC-CMs could be preventing T₃ from further improving mitochondrial respiration, the cells were also treated for 1 month with the drugs 5 μ M IOP and 300nM GC-1 (Figure 3.6a). GC-1 increased basal respiration (Figure 3.6b, $p<0.05$) and ATP production (Figure 3.6c, $p<0.05$) compared to control, but its effects were not significantly different from those of T₃. IOP+T₃ did not affect any parameter of mitochondrial respiration, compared to control (Figure 3.6b-e). Interestingly, basal respiration and ATP production were significantly lower in IOP+T₃ treated cells compared to just T₃ (Figure 3.6b and c, basal respiration $p<0.05$; ATP production $p<0.05$). All groups relied on OXPHOS primarily, but only IOP+T₃ resulted in a significantly higher OCR/ECAR ratio (Figure 3.6f, $p<0.05$).

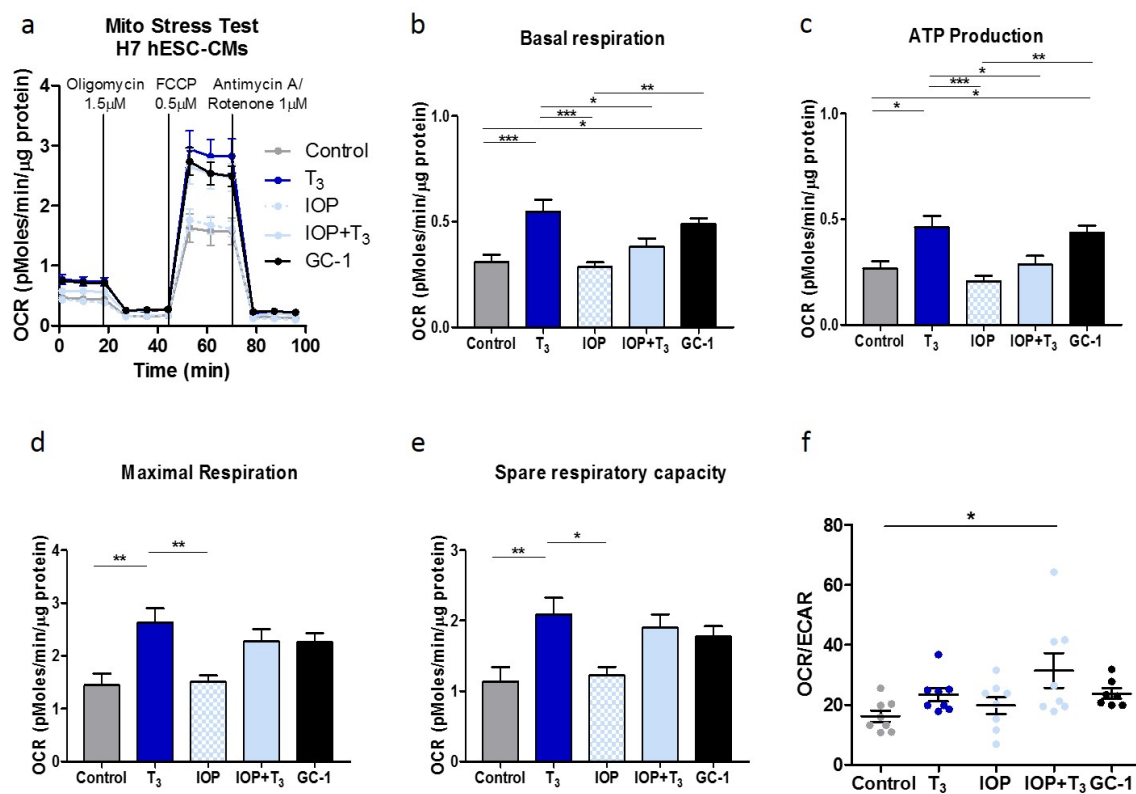


Figure 3.6: The effect of 1 month T₃ treatment and of bypassing the D3 pathway on H7 hESC-CM mitochondrial respiration.

a) The Mito Stress Test was performed after 1 month treatment with 30nM T₃, 5 μ M iopanoic acid (IOP), 5 μ M IOP+ 30nM T₃ or 300nM GC-1, and oxygen consumption rate (OCR, in pMoles/min/ μ g protein) was measured at basal, and following the injection of 1.5 μ M oligomycin, 0.5 μ M FCCP and 1 μ M antimycin A/rotenone. b) Basal respiration, ATP production, maximal respiration and spare respiratory capacity. c) The ratio between OCR and ECAR (extracellular acidification rate, in mpH/min/ μ g protein). n=8 independent differentiations (n=7 for GC-1). The graphs represent mean $\pm$ s.e.m. One-way ANOVA followed by Bonferroni's Post-Hoc. *p<0.05, **p<0.01, ***p<0.001.

3.3.7 Mitochondrial copy number was significantly higher in H7 hESC-CMs compared to CDI hiPSC-CMs

MtDNA is the template for transcription of essential electron transport chain proteins. Therefore, it can serve as a marker of the OXPHOS capacity of hPSC-CMs (Yang *et al.*, 2014). MtDNA copy number was measured as an attempt to explain the different responses of H7 hESC-CMs and CDI hiPSC-CMs to T_3 pathway modulation. Results show that mtDNA copy number was significantly higher in H7 hESC-CMs than in CDI hiPSC-CMs. This was apparent for all hESC-CM conditions comparing to both control and T_3 hiPSC-CMs (Figure 3.7; hESC-CMs versus hiPSC-CMs: control vs control or vs T_3 $p < 0.05$, T_3 vs control or vs T_3 $p < 0.01$; IOP vs control or vs T_3 $p < 0.01$; IOP+ T_3 vs control or vs T_3 $p < 0.001$; GC-1 vs control $p < 0.01$ and GC-1 vs T_3 $p < 0.05$). There were no significant differences in mtDNA copy number within hESC-CMs and hiPSC-CMs in any condition.

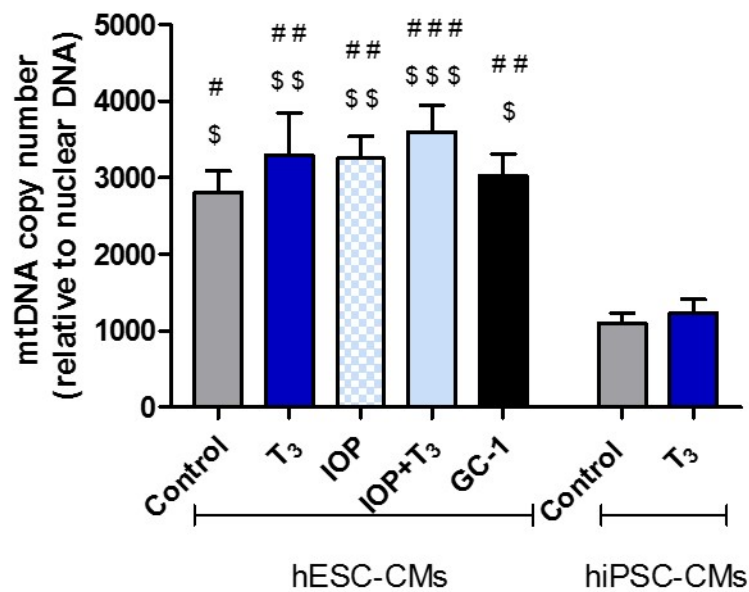


Figure 3.7: Mitochondrial DNA copy number in hESC-CMs and hiPSC-CMs.

Mitochondrial DNA (mtDNA) copy number was determined by real-time qPCR by measuring the ratio of mtDNA (MTND1 gene) relative to nuclear DNA (SDHA gene). Graph shows mtDNA levels in H7 hESC-CMs and CDI hiPSC-CMs after 1 month treatment with 30nM T₃, 5μM IOP, 5μM IOP + 30nM T₃ or 300nM GC-1. n=4 independent differentiations. The graphs represent mean ± s.e.m. One-way ANOVA followed by Bonferroni's Post-Hoc. # and \$ represent relationship to control and T₃ treated hiPSC-CMs, respectively. 1, 2 or 3 symbols represent p<0.05, p<0.01 or p<0.001, respectively.

3.4 Discussion

The results presented here demonstrate that the Mito Stress Test can give detailed information about the mitochondrial respiration status of hPSC-CMs. We showed that the effects of the maturation strategies implemented on mitochondrial respiration differed between the lines CDI and IMR90-4 hiPSC-CMs and H7 hESC-CMs. Time in culture and T_3 treatment did not improve mitochondrial respiration in CDI and IMR90-4 hiPSC-CMs, whereas T_3 treatment enhanced mitochondrial respiration in H7 hESC-CMs. At the molecular level, the levels of mtDNA were unchanged after T_3 modulation in CDI hiPSC-CMs and H7 hESC-CMs, but were significantly higher in H7 hESC-CMs when compared to CDI hiPSC-CMs.

3.4.1 Selection of maturation strategies and of the method to assess mitochondrial function

As discussed in the Introduction (section 1.3.4), hPSC-CMs present structural and functional immature features that can be tackled using several maturation strategies (Burridge *et al.*, 2016). We thought it would be important to establish whether, once implemented, these strategies could help revealing the maximal OXPHOS capacity in hiPSC-CMs, with the final aim of determining if this metabolic state was compromised in the *in vitro* models of heteroplasmic mitochondrial dysfunction to be developed in hiPSC-CMs

We have selected time in culture as it is probably the most studied, cost-effective and straightforward maturation strategy. Improvements in morphology, ion channel current densities and kinetics, and calcium handling and action potential upstroke velocities over time in culture have been extensively described in hPSC-CMs (Veerman *et al.*, 2015). However, only recently has a group described the link between time in culture and mitochondria. Dai and colleagues reported an increase in mitochondrial mass and mitochondrial membrane potential but with no improvement in the activity of mitochondrial respiratory enzyme

complexes over time in culture(Veerman *et al.*, 2015). Here, we have compared the mitochondrial respiration of 45 days-old against 60 days-old CDI hiPSC-CMs.

Thyroid hormone T_3 is one of the most encouraging reagents to date identified as promoting hPSC-CM maturation, causing improvements in shape, sarcomere organisation, calcium handling, contractility and gene expression(Dai *et al.*, 2017). Of particular interest to us, Yang *et al.* found improvements in mitochondrial function, in terms of basal respiration, maximal respiration and spare respiratory capacity, without reporting ATP production, following 1 week treatment of IMR90-4 hiPSC-CMs with 30nM T_3 . The authors did not see any changes in mitochondrial mass after the treatment(Veerman *et al.*, 2015). We have treated IMR90-4 hiPSC-CMs for 1 week, and CDI hiPSC-CMs and H7 hPSC-CMs for 1 month, with 30nM T_3 .

The predominant function of mitochondria is to generate ATP through OXPHOS, and as such measuring mitochondrial respiration is the most useful tool to determine mitochondrial dysfunction in the case of mtDNA disorders(Yang *et al.*, 2014). The status of the mitochondrial membrane potential, which directly correlates with mitochondrial function, is also a useful tool to detect mitochondrial dysfunction(Brand and Nicholls, 2011). That being said, the additional roles of mitochondria should not be overlooked, especially if specific disturbances have been associated with the disease of interest. By using intact cells to investigate mitochondrial function, we missed the complexity of cell-cell interactions *in vivo*, but cells still provided considerable more information than using intact mitochondria alone. The measurement of cell respiratory control, which can be accomplished by the Mito Stress Test using a Seahorse Analyser, provides the most information about mitochondrial function in intact cells using a single test. The principles behind the test are described in the General Methods section 2.7. We have successfully optimised the Mito Stress Test for commercially available and in-house generated hiPSC-CMs, carefully titrating oligomycin and FCCP, and have managed to record the maximal response to these drugs, and hence obtained accurate measurements of ATP production and maximal respiration (Figure 3.1 and 3.4).

3.4.2 Effect of time in culture on CDI hiPSC-CM mitochondrial respiration

Prolonging the time in culture from 2 weeks to 1 month did not enhance mitochondrial respiration in CDI hiPSC-CMs (Figure 3.2). CDI hiPSC-CMs are shipped at 30 days-old and the assays were conducted 2 weeks (45 days-old) and 1 month (60 days-old) after plating. Studies which reported maturation of hPSC-CMs over time in culture generally compared 30 days-old cells versus at least 80 days-old, but up to a year-old cells (Brand and Nicholls, 2011). Therefore, it is possible that a gap of 15 days is not enough to detect differences in mitochondrial respiration between the two groups. Similarly, it is also likely that 60-days are not sufficient to completely achieve functional maturation. Results from our lab show some improvements in gene expression, proteomics and AP properties at 1 month versus 2 weeks, but no changes in beating rate or Ca^{2+} handling properties in CDI hiPSC-CMs (unpublished data).

Dai and colleagues did not see increased activity of mitochondrial respiratory enzyme complexes when comparing 30 day-old to >100 day-old hPSC-CMs (Ivashchenko *et al.*, 2013; Kamakura *et al.*, 2013; Lundy *et al.*, 2013), so another possibility is that it is simply not conceivable for hPSC-CMs to achieve the bioenergetics of the postnatal heart *in vitro*. A recent study found that the transcriptome of hPSC-CMs is comparable to that of the first trimester foetal heart, and that after maturation strategies are implemented it clusters closer to the second trimester foetal heart (Dai *et al.*, 2017). In humans, the electron transport chain seems to be active by late embryonic period, and mitochondria are actively engaged in OXPHOS in the foetal heart. Fatty acid oxidation has also begun to occur. However, lactate and glucose still account for a significant proportion of energy production. It is not until the first week of life that the final energetic transition occurs, with glycolysis and lactate metabolism decreasing and fatty acid oxidation increasing to adult levels (van den Berg *et al.*, 2015). Strategies fail to

mature hPSC-CMs past the foetal stage, so this most likely applies to bioenergetics as well, unless glucose is completely removed from the system, which will be discussed further down.

3.4.3 Effect of T_3 on CDI and IMR90-4 hiPSC-CM mitochondrial respiration

The conditions used in the differentiation and maintenance of CDI hiPSC-CMs were not transparent, but it is very likely that T_3 is present. Being one of the most promising factors promoting maturation of hPSC-CMs, T_3 is now present in most commercial available differentiation and maturation media (Stem Cell Technologies; Thermo Fisher Scientific; R&D Systems; Pluriomics). The physiological circulating levels of total T_3 and free T_3 in humans are in the range of 1-3nM and 3-8 pM, respectively (Porter *et al.*, 2011). We treated CDI hiPSC-CMs for 1 month with 30nM T_3 (based on what was done by others (Hulbert, 2000)), and observed a reduction in mitochondrial respiration, in particular in basal respiration and ATP production. Consequently, there is an indication that the total amount of T_3 received by CDI hiPSC-CMs was excessively high, probably at the level of hyperthyroidism. It has been well established that T_3 treatment increases OXPHOS in mitochondria, and that increased OXPHOS leads to increased production of ROS and ROS-related damage. This is further enhanced in the case of hyperthyroidism, hence the antioxidative capacity of cells becomes limited (for example superoxide dismutase and catalase activities) (Yang *et al.*, 2014). We hypothesize that ROS-damage is impairing mitochondrial respiration in T_3 treated CDI hiPSC-CMs. In line with this hypothesis, proteomic analysis of CDI hiPSC-CMs under the same conditions revealed upregulation of several proteins involved in mitochondrial dysfunction, including some responsible for apoptosis (unpublished data, shown in Appendix Figure A4).

Treating in-house differentiated IMR90-4 hiPSC-CMs with T_3 30nM for a shorter period of time (1 week), had no significant effect in any parameter of mitochondrial function. Yang *et al.* in

2014 claimed that treatment of IMR90-4 hiPSC-CMs with T_3 for 1 week resulted in improvements in mitochondrial function, increasing basal respiration, maximal respiration and spare respiratory capacity, without reporting ATP production (Harper and Seifert, 2008). The experiments were also performed using the Mito Stress Test in a Seahorse Analyser, so it is unclear why the authors do not report the data obtained for ATP production, as it probably provides the most information about mitochondrial function. None of the other parameters validate that T_3 has improved mitochondrial respiration. An increase in basal respiration can be explained by either higher ATP production or higher proton leak. Elevated proton leak is an indication of mitochondrial membrane uncoupling or dysfunction. Maximal respiration is determined by basal respiration and spare respiratory capacity, and elevated spare respiratory capacity indicates the cells are operating further from their bioenergetic limit (Yang *et al.*, 2014).

3.4.4 Effect of T_3 on H7 hESC-CM mitochondrial respiration

In contrast to CDI hiPSC-CMs, treatment of H7 hESC-CMs with 30nM T_3 for 1 month resulted in improvements in mitochondrial function. The maximal respiration potential increased due to an increase in ATP production. However, spare respiratory capacity also increased, meaning the cells did not operate at the maximal capacity even in the presence of T_3 ; we believe this comes down to the substrate provided, which will be discussed in the next section. Bypassing the D3 activity, by using the T_3 agonist GC-1 also increased mitochondrial respiration but not further than T_3 . It is likely that the maximal OXPHOS potential of hESC-CMs has been reached and cannot be enhanced in current culture conditions.

Several aspects could explain the discrepancy in the response to prolonged T_3 exposure between H7 hESC-CMs and CDI and hiPSC-CMs. A very likely explanation for the observed differences could simply be the inherent heterogeneity between different hPSC lines. Several studies have now established that gene expression and epigenetic differences between hPSC

lines resulted in functional differences (Brand and Nicholls, 2011). A recent study using 711 hiPSCs cell lines derived from 301 healthy individuals found that the heterogeneity of hiPSCs arises from genetic differences between donors (Bock *et al.*, 2011).

Another possibility is that high D3 levels in H7 hPSC-CMs inactivate excess T_3 , protecting the cells against ROS-related damage. The D3 enzyme inactivates T_3 and T_4 , with major roles in the placenta, uterus, and the developing embryo/foetus. The foetus is protected from maternal T_3 and T_4 adult levels, allowing its own autonomous developmental program to control the differentiation process (Kilpinen *et al.*, 2017). Results from our lab show high levels of D3 activity in hESC-CMs and absence in hiPSC-CMs (unpublished data). Further validating this theory, it was shown here that that D3 inhibition (IOP) + T_3 treatment of hESC-CMs caused a significant decrease in basal respiration and ATP production, compared to T_3 treatment without D3 inhibition.

Finally, T_3 demand for ATP production and thus for OXPHOS is known to induce mitochondriogenesis (Larsen and Zavacki, 2012). However, increased ATP production in response to T_3 did not affect mitochondrial copy number in H7 hESC-CMs. It indicates that mitochondria were able to increase their ATP production without having to increase in numbers. It was also seen that mitochondrial copy number was significantly lower in CDI hiPSC-CMs compared to H7 hESC-CMs, which once again can be attributed to genetic and epigenetic differences between the two cell lines.

3.4.5 Impact of substrate provided on hPSC-CM mitochondrial respiration

The OCR/ECAR ratio is an indication of the proportion of OXPHOS/glycolysis performed by the cells. Here it was seen that hPSC-CMs, differentiated from both H7 hESCs and CDI and

IMR90-4 hiPSCs, primarily relied on OXPHOS for ATP production. The OCR/ECAR ratio did not change after implementation of any maturation strategy. These results were not surprising as it is established that in hPSC-CMs, similarly to adult cardiomyocytes, OXPHOS is the main energy production system operating (Harper and Seifert, 2008), although glycolysis still accounts for a significant proportion of ATP production (Denning *et al.*, 2016). Human PSC-CMs mainly metabolise glucose and lactate (Rana *et al.*, 2012) like the embryonic and foetal heart, where fatty acid oxidation accounts for less than 15% of ATP production via OXPHOS (Denning *et al.*, 2016). In contrast, the adult heart, fatty acid oxidation accounts for 90% of ATP production via OXPHOS.

Metabolic plasticity enables cells to shift their metabolism according to the bioenergetic demands of different cell states (Robertson, Tran and George, 2013). Human iPSC-CMs have been previously maintained for short periods of time in a cocktail of fatty acids but with no indication that under these conditions their ATP production via OXPHOS becomes primarily dependent on fatty acid oxidation (Folmes *et al.*, 2012). Surprisingly, the most novel methods of hPSC cardiac differentiation still utilise glucose during CM generation and maintenance (Rana *et al.*, 2012; Drawnel *et al.*, 2014). Here, we have observed that in the presence of glucose, CDI hiPSC-CMs did not operate to maximal OXPHOS potential, noticed by the high levels of spare respiratory capacity. When glucose was substituted by galactose, spare respiratory capacity decreased, but did not reach significance. A reduction in spare respiratory capacity indicates that cells are operating closer to their maximal OXPHOS potential (Lian *et al.*, 2013; Burrige *et al.*, 2014). Galactose is oxidised to pyruvate via glycolysis but this yields no net ATP, forcing cells to use OXPHOS to generate sufficient ATP for survival (Brand and Nicholls, 2011). However, this still does not recapitulate fatty acid oxidation that occurs in the adult heart. Most likely, *in vitro* recapitulation of the metabolic status of the *in vivo* heart is not possible.

3.5 Summary

In summary, we have presented in this chapter a detailed assessment of the effects of different maturation strategies on mitochondrial respiration of hPSC-CMs (CDI and IMR90-4 hiPSC-CMs and H7 hESC-CMs) and concluded that their application does direct hiPSC-CMs towards a maximal OXPHOS potential. We have also proposed that using glucose as a substrate impedes hPSC-CM metabolic maturation, but this requires further investigation. Prolonged time in culture did not improve mitochondrial respiration of CDI hiPSC-CMs. We suggested a longer period might be necessary for changes, but it is possible that the bioenergetics of the adult heart will not be achieved *in vitro*. Prolonged treatment with T_3 improved mitochondrial respiration in H7 hESC-CMs, but not in CDI hiPSC-CMs. We attributed these changes primarily to genetic and epigenetic differences between the two cell lines. Additionally, bypassing the D3 pathway does not further improve mitochondrial respiration in H7 hESC-CMs which indicates that the OXPHOS capacity of these cells *in vitro* reached its limit. It is uncertain whether complete maturity of hiPSC-CMs will ever be achieved, but it is clear that these cells provide useful insights into the pathogenesis of cardiac abnormalities associated with heteroplasmic mtDNA diseases. Aware of these limitations, in the following chapters we will attempt to develop *in vitro* models of heteroplasmic mitochondrial dysfunction using hiPSC-CMs and will not apply maturation strategies.

Chapter 4: Mitochondrial drugs to model heteroplasmy in human induced pluripotent stem cell derived cardiomyocytes

4.1 Introduction

As discussed in section 1.1.4.1 of the Introduction, agents that affect mitochondrial bioenergetics mostly do so by either inhibiting or dissipating the proton gradient across the IM of mitochondria. These compounds are classified as inhibitors or uncouplers of mitochondrial respiration. The second complex of the ETC, complex III, can be blocked by the antibiotic antimycin A, which is the most frequently used agent to block complex III as it has the highest affinity. This drug inhibits the transfer of electrons at the Q_i center of complex III, increasing the probability of ROS production at center Q_o . Consequently, together with blocking complex III, antimycin A also causes toxicity by increasing ROS generation (Rossignol *et al.*, 2004). Low concentrations of antimycin A have been extensively used to block complex III activity in hiPSCs (Wallace and Starkov, 2000) and hPSC-CMs (Cherry *et al.*, 2013; Folmes *et al.*, 2013; Ma *et al.*, 2015) for Mito Stress Test experiments. A higher concentration of antimycin A has also been used as a positive control for the generation of ROS in hiPSC-CMs (Wang *et al.*, 2014; Yang *et al.*, 2014; Burridge *et al.*, 2016). In these studies, effects of the drug were monitored immediately after administration. It is not known whether the effect of antimycin A on hiPSC-CMs subsides and if it is reversible. Studies with other preparations were performed in the 1960s but were contradictory, showing some weak evidence of reversibility of antimycin A by uncouplers (Burridge *et al.*, 2016).

Alternate electron acceptors are examples of uncouplers of OXPHOS. These drugs dissipate the proton gradient across the IM of mitochondria by diverting the transfer of electron away from the electron acceptors in the respiratory chain, thereby decreasing the efficiency of energy production and speeding up the oxidation of substrates. Menadione is an alternate

electron acceptor that is toxic by two major mechanisms: oxidative stress by generation of ROS due to enzymatic redox cycling and loss of protein thiols due to sulfhydryl arylation(Rieske, 1967). Menadione has been used as a positive control for the generation of ROS in hiPSC-CMs(Wallace and Starkov, 2000). Beat frequency, cAMP formation, ROS formation and mitochondrial integrity after short and long term exposures to menadione have also been investigated in hiPSC-CMs(Burridge *et al.*, 2016). In our lab, menadione has been shown to decrease mitochondrial respiration in 2 lines of hiPSC-CMs (unpublished data). Again, it is not known whether the effect of toxic levels of menadione on hiPSC-CMs vanishes and if it is reversible.

Studies using hiPSCs reprogrammed from patients with heteroplasmic mtDNA mutations showed that the level of heteroplasmy correlated with mitochondrial function assessed using the Mito Stress Test. A decrease in OCR has been reported with heteroplasmy levels higher than 60%(Grimm *et al.*, 2015). However, the availability of patient samples is limited and attempts to expand and differentiate hiPSCs with high mutation loads by our lab have been challenging. Production of ROS by the ETC is thought to be involved in the accumulation of mtDNA mutations, which in turn cause mitochondrial dysfunction and ageing(Cherry *et al.*, 2013; Ma *et al.*, 2015).

Therefore, in this chapter, we were particularly interested in applying the ROS generators complex III inhibitor antimycin A and uncoupler menadione to generate mitochondrial damage in healthy hiPSC-CMs. It was hypothesized that antimycin A and menadione would cause mitochondrial dysfunction in the treated cells. Treated and untreated hiPSC-CMs would be mixed and based on the data from the literature discussed above, cultures with more than 60% antimycin or menadione-treated hiPSC-CMs were expected to display mitochondrial dysfunction, in particular lower OCR. It was also hypothesized that increasing the number of

treated cells would cause a progressive decrease in OCR and that these cultures could then be used as future *in vitro* models to determine how mitochondrial heteroplasmy might affect cardiac function.

4.2 Methods

4.2.1 Cell preparation and drug treatments

IMR90-4 hiPSCs were maintained and differentiated as described in sections 2.1 and 2.3. All hiPSC-CMs were 30-40 days old when experiments were conducted. The following drugs were diluted to the desired concentration in RB+ culture medium:

- Antimycin A – Sigma-Aldrich, stock prepared to 10mM in absolute ethanol
- Menadione - Sigma-Aldrich, stock prepared to 100mM in DMSO

Two (for antimycin A experiments) or 5 (for menadione experiments) days before the experiments were conducted, hiPSC-CMs were treated with the drugs at the desired concentration and incubated for 2 hours at 37°C. The cells were then washed with DPBS, dissociated (see 2.3.2), counted (see 2.3.3), mixed accordingly and replated (see 2.3.4) for experiments. For the galactose experiments, glucose was substituted by galactose in RB+ medium at this stage. For antimycin A experiments, hiPSC-CMs were mixed at: 30% antimycin A-treated + 70% untreated cells and 60% antimycin A-treated + 40% untreated cells. For menadione experiments, hiPSC-CMs were mixed at 50% menadione-treated + 50% untreated cells.

4.2.2 Quantification of CellTracker Red-positive cells

For live cell tracking, hiPSC-CMs were treated with 1µM CellTracker™ Red CMTPX Dye (Thermo Fisher Scientific, stock prepared to 1mM in DMSO) in RB+ medium, or with RB+

medium, for 15 minutes at 37°C, according to the manufacturer's instructions. Cells were then dissociated (see 2.3.2), counted (see 2.3.3) and mixed at: 30% CellTracker Red-treated + 70% untreated cells and 60% CellTracker Red-treated + 40% untreated cells. hiPSC-CMs were plated at 30,000 cells onto 35mm dishes with a 7mm glass bottomed well (MatTek Corporation), which had been previously coated with fibronectin as described in 2.3.4. The cells were left to attach for 2 days, after which nuclei were labelled with Hoechst (Thermo Fisher Scientific, 1:500 dilution in RB+ medium) by incubating for 15 minutes at 37°C, according to the manufacturer's instructions, and imaged using an inverted Zeiss LSM-780 confocal microscope with Zen Zeiss software. Images were acquired from three different regions for each dish, using the red (excitation / emission 577 / 602nm) and blue (excitation / emission 350 / 461nm) channels. Images were opened using the Fiji (Image J) software and the number of red cells out of the total number of blue nuclei was manually counted.

4.2.3 Mito Stress Test experiments

All Mito Stress test experiments were performed as stated in section 2.7. Seahorse Assay Medium was prepared as described in section 2.7.2, but supplemented with either 10mM glucose or 10mM galactose (both Sigma-Aldrich), accordingly. Mitochondrial drugs were prepared as in section 2.7.2, and diluted in Seahorse Assay Medium to the required concentration. Determination of the mitochondrial respiration parameters is defined in section 2.7.4.

4.2.4 ATP production quantification

ATP production was quantified using a Luminescent ATP Detection Assay Kit (Abcam), as described in section 2.8.

4.2.5 Quantification of SOD2 mRNA levels by qPCR

Following the Mito Stress Test, RNA was extracted and cDNA synthesized as described in section 2.11.1. RNA from foetal and adult heart tissue was purchased (Agilent). qPCR was performed as described in 2.11.3.1 and results are shown relative to Control hiPSC-CMs.

4.2.6 Statistical Analysis

Details are described in section 2.12.

4.3 Results

4.3.1 The antimycin A heteroplasmy model was developed for IMR90-4 hiPSC-CMs

Antimycin A blocks complex III of the electron transport chain and was used to generate an *in vitro* model of mitochondrial heteroplasmy in IMR90-4 hiPSC-CMs (Lagouge and Larsson, 2013). Two separate cultures of hiPSC-CMs, a control group and a group presenting ETC impairment by treatment with antimycin A, were mixed accordingly to generate groups of 0, 30, 60 and 100% impaired cells. A diagram of the model is shown in Figure 4.1.

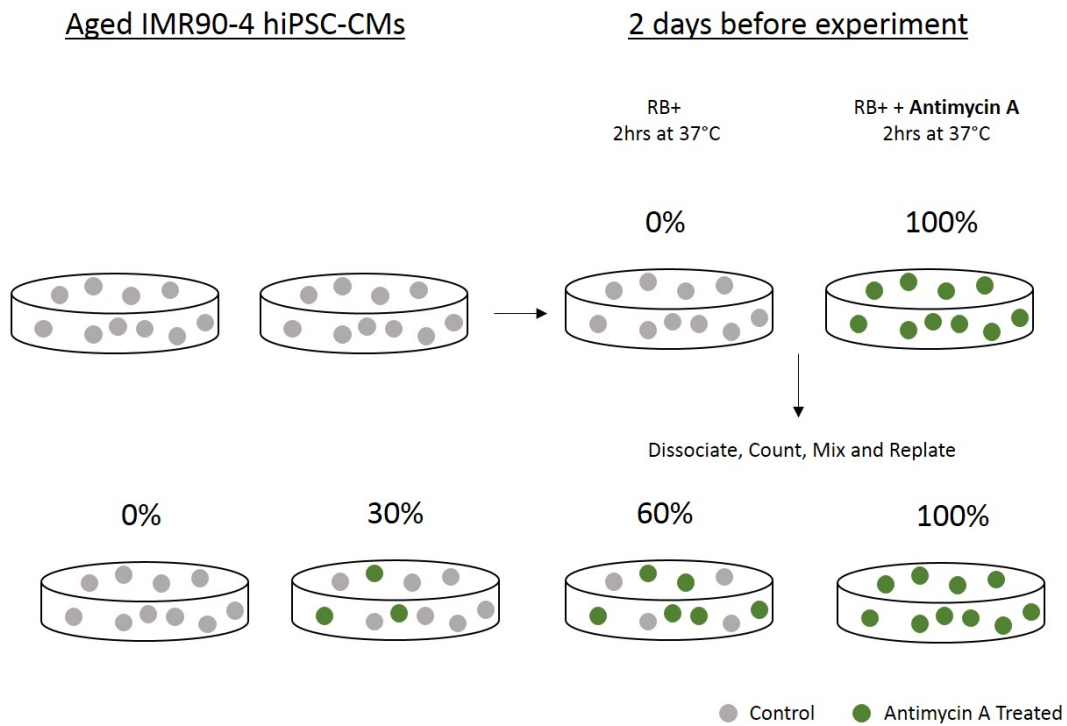


Figure 4.1: Diagram of the antimycin A heteroplasmy model.

Two days before the experiment was conducted aged IMR90-4 hiPSC-CMs were incubated for 2 hours with antimycin A (green) or just RB+ medium (grey). The cells were then washed, dissociated and counted. Control and antimycin A-treated hiPSC-CMs were mixed and replated for the generation of 0, 30, 60 and 100% cultures.

To determine the accuracy of the dissociation, mixing and replating process, a preliminary experiment was performed by labelling a group of hiPSC-CMs with CellTracker Red and mixing these with unlabelled cells to generate cultures of 0, 30, 60 and 100% CellTracker Red-positive cells. The cells were left to attach for two days, when the cultures were imaged using a confocal microscope. The representative images in Figure 4.2a show that the generation of cultures with varying levels of CellTracker Red-positive cells was successfully achieved. The percentage of red cells out of the total number of cells was determined and can be seen in Figure 4.2b. In the untreated group, due to auto fluorescence, an average of 7.72% (s.e.m. $\pm$ 0.93%) of the detected cells were red. In the 30% and 60% groups an average of 36.01% (s.e.m. $\pm$ 2.46%) and 72.32% (s.e.m. $\pm$ 2.13%) red-positive cells were detected, respectively. In the 100% group an average of 91.56% (s.e.m. $\pm$ 1.41%) of cells were red. All groups were significantly different to control and between each other ($p < 0.001$ for all comparisons).

After determining that the mixing process was effective, antimycin A was titrated by incubating hiPSC-CMs with 0.3, 1, 3, 10, 30 and 100 μ M for 2 hours (Figure 4.2c). The cells were then replated and left to attach for 2 days before a Mito Stress Test was conducted. When compared to control, hiPSC-CMs incubated with antimycin A up to 30 μ M had similar basal respiration rates and responded equally to oligomycin, indicating that ATP production had not been impaired. Therefore, 30 μ M antimycin A, the lowest concentration resulting in a drop in basal respiration and in the response to oligomycin, was chosen for future experiments.

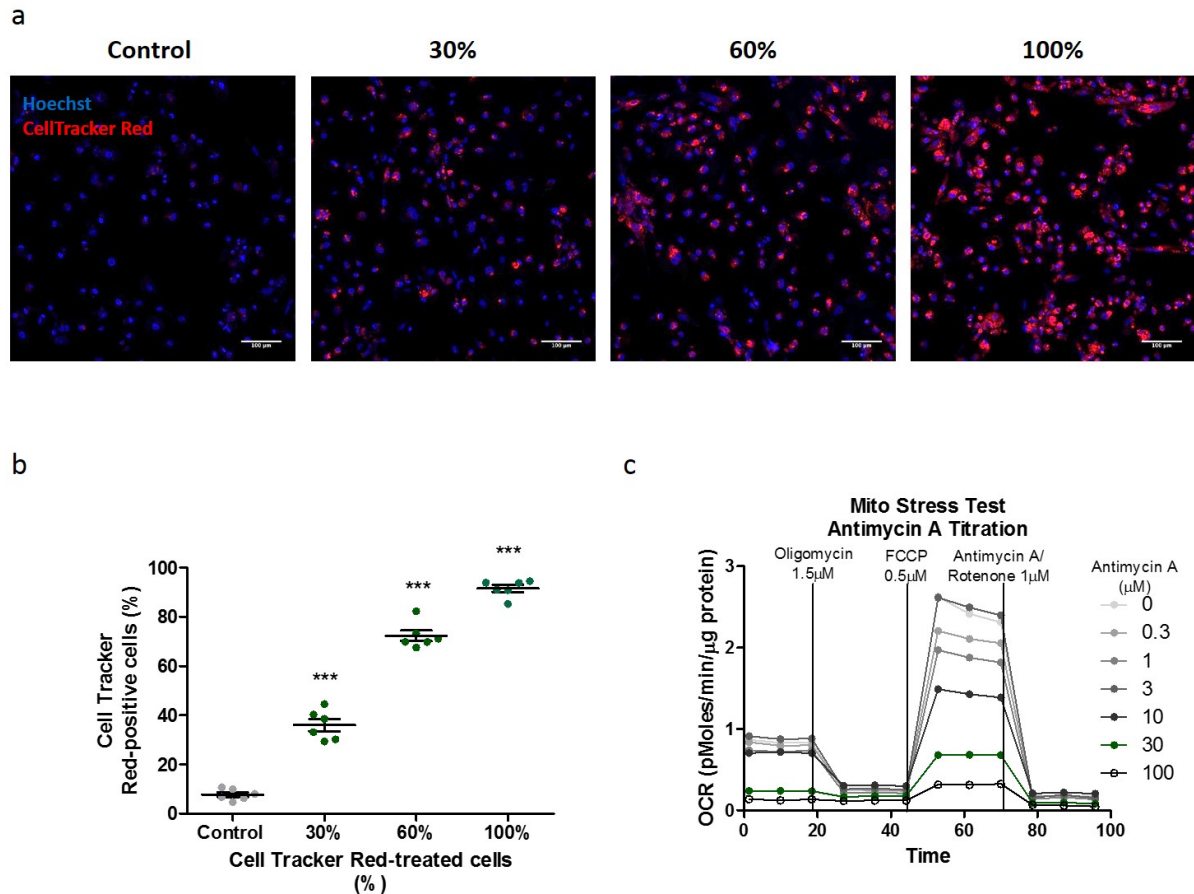


Figure 4.2: Development of the antimycin A heteroplasmy model.

a) Human iPSC-CMs were labelled with CellTracker Red and mixed with unlabelled cells at 0, 30, 60 and 100% labelled cells. Two days after, the cultures were labelled with the nuclear dye Hoechst (blue) and imaged using a confocal microscope. Images shown are representative. Scale bar 100μm. b) the number of red/total blue hiPSC-CMs was manually counted and the percentage of CellTracker Red-positive cells was calculated for each group. n=6 images acquired from 2 dishes for each group. Graph represents mean ± s.e.m., One-way ANOVA followed by Bonferroni's Post-Hoc. ***p<0.001 for all comparisons. c) The Mito Stress test was performed to titrate antimycin A, 2 days after the cells were incubated with the drug and replated. Oxygen consumption rate (OCR, in pMoles/min/μg protein) was measured at basal, and following the injection of 1.5μM oligomycin, 0.5μM FCCP and 1μM antimycin A/rotenone. n=1 differentiation.

4.3.2 IMR90-4 hiPSC-CMs recovered from antimycin A treatment

In the previous chapter it was concluded that prolonging time in culture or supplementing culture medium with the maturation agent T_3 does not improve mitochondrial respiration in hiPSC-CMs, so the standard conditions were used for subsequent experiments. Control and 30 μ M antimycin-treated IMR90-4 hiPSC-CMs were mixed as described above and the Mito Stress Test was conducted 2 days after replating, using the standard glucose buffer (Figure 4.3a). No significant difference was observed in any of the mitochondrial respiration parameters between the control group and any of the antimycin A treated groups (Figure 4.3b). Similarly, the ratio of OXPHOS/glycolysis (OCR/ECAR) was unchanged (Figure 4.3c).

To rule out the possibility of glucose supplementation enabling hiPSC-CMs to switch to glycolysis to protect against further ROS production, glucose was removed from the culture medium on the day of mixing and until the Mito Stress test was repeated (Figure 4.3c)(Wallace and Starkov, 2000). Glucose was replaced by galactose, which forces the cells to utilise OXPHOS to produce ATP. Again, data from the Mito Stress in the presence of galactose revealed no differences in any of the mitochondrial respiration parameters between the control group and any of the antimycin A treated groups (Figure 4.3d). The ratio of OCR/ECAR of the 60% group was surprisingly significantly higher than the control, but no other significant changes were observed (Figure 4.3f).

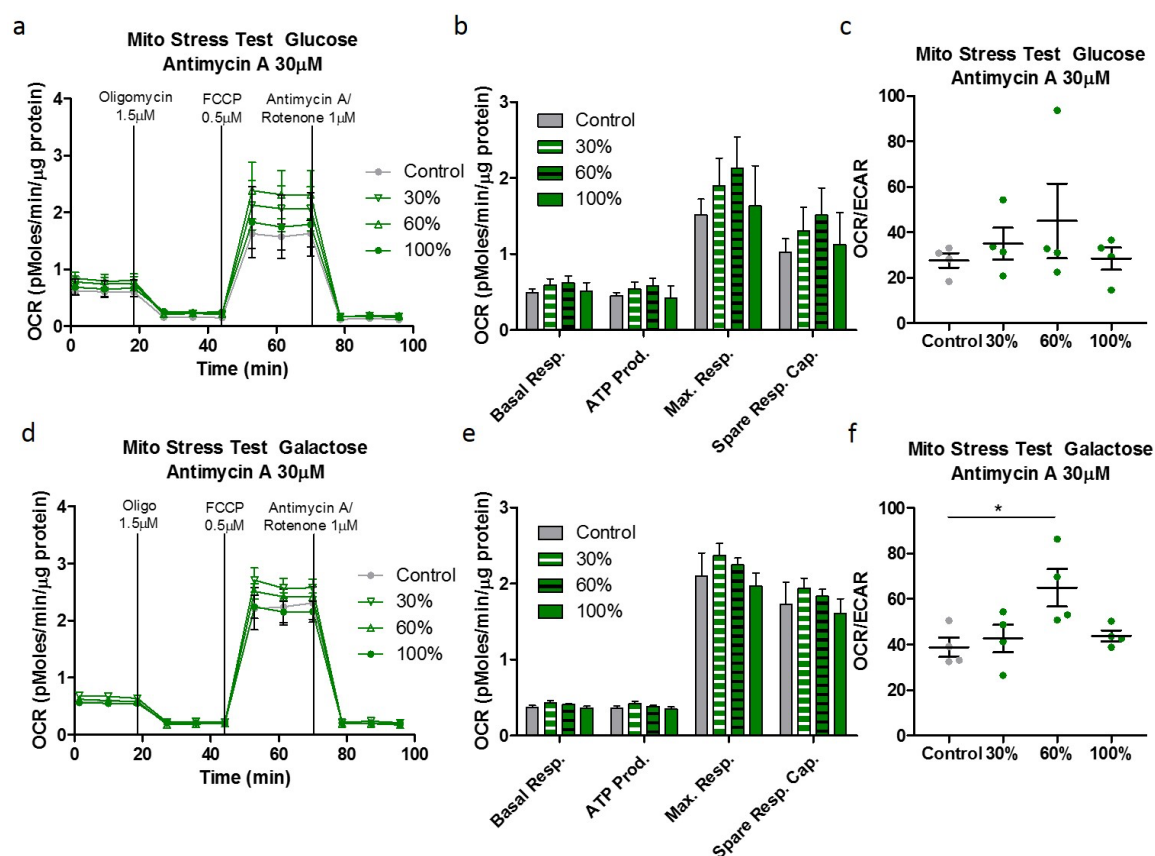
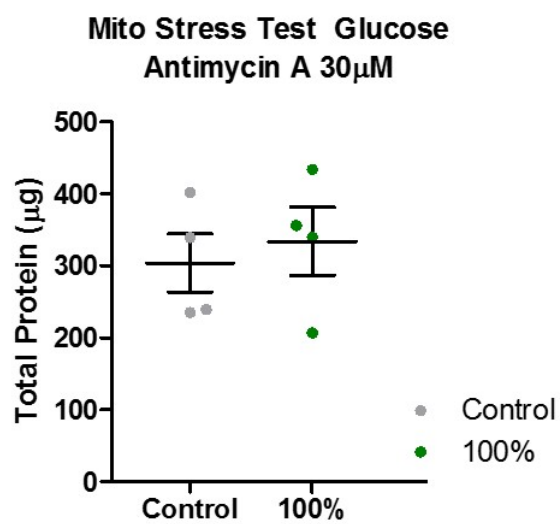


Figure 4.3: The effect of antimycin A treatment on hiPSC-CM mitochondrial respiration.

The Mito Stress Test was performed using a) glucose or c) galactose medium 2 days after cells were treated with 30μM antimycin A, mixed and replated. Each group is represented by the percentage of antimycin A-treated hiPSC-CMs. Oxygen consumption rate (OCR, in pMoles/min/μg protein) was measured at basal, and following the injection of 1.5μM oligomycin, 0.5μM FCCP and 1μM antimycin A/rotenone. Basal respiration, ATP production, maximal respiration and spare respiratory capacity for b) glucose and e) galactose experiments. The ratio between OCR and ECAR (extracellular acidification rate, in mpH/min/μg protein) for c) glucose and f) galactose experiments. n=4 independent differentiations. The graphs represent mean ± s.e.m. One-way ANOVA followed by Bonferroni's Post-Hoc. *p<0.05.

Antimycin A treatment could have altered hiPSC-CM survival and/or attachment, so it was fundamental to confirm that there were no differences in cell number between the control and antimycin A-treated groups, as this would have changed the mixing ratios. For this, the cells were lysed and the total protein was measured after each Mito Stress Test experiment. There were no significant differences in the total protein levels between the control and the 100% group, in both glucose and galactose experiments (Figure 4.4a and b). This confirms that the observed OCR readouts originated from the same number of cells, suggesting that hiPSC-CMs might have recovered from antimycin A damage in the 2-day gap between replating and experiments.

a



b

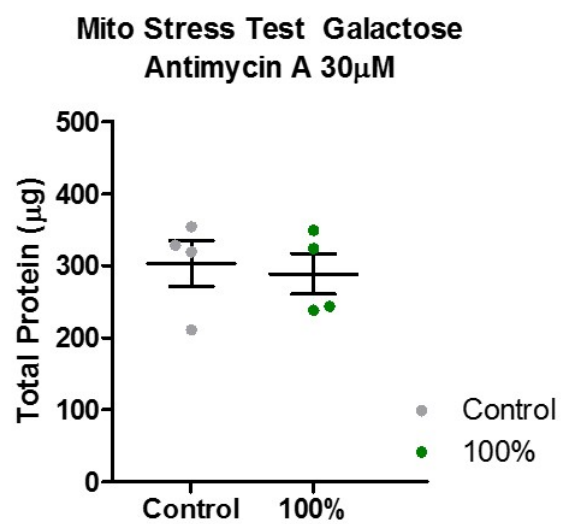


Figure 4.4: Total protein levels in hiPSC-CMs treated with antimycin A.

At the end of each a) glucose or b) galactose Mito Stress Test experiment the cells were lysed and the total protein (μ g) was quantified for the control (grey) and treated (green) groups. $n=4$ independent differentiations. The graphs represent mean $\pm$ s.e.m. Unpaired Student's t-test.

4.3.3 The menadione heteroplasmy model was developed for IMR90-4 hiPSC-CMs

Menadione is a potent generator of ROS and so was chosen as the second drug to generate an *in vitro* model of mitochondrial heteroplasmy in hiPSC-CMs(Chen, Qian and Wu, 2015). Furthermore, menadione had been previously successfully used by our lab to impair mitochondrial respiration in two lines of hiPSC-CMs.

An outline of the model developed can be seen in Figure 4.5. It was decided that a 5-day gap would be necessary after mixing, if the model was to be extended to other types of investigations. For instance, optical imaging of calcium requires that connections between adjacent cells are fully established, which takes longer than 2 days. Additionally, the new generated groups were control, 50% and 100% menadione-treated IMR90-4 hiPSC-CMs, for simplification.

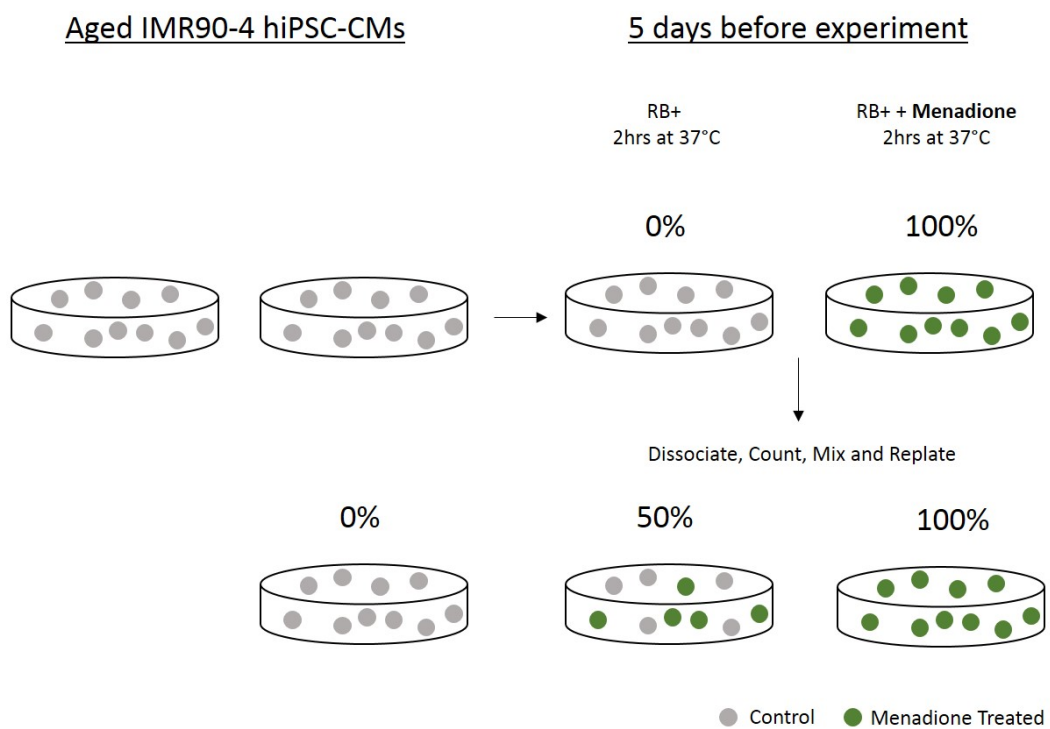


Figure 4.5: Diagram of the menadione heteroplasmy model.

Five days before the experiment was conducted aged IMR90-4 hiPSC-CMs were incubated for 2 hours with menadione (green) or just RB+ medium (grey). The cells were then washed, dissociated and counted. Control and menadione-treated hiPSC-CMs were mixed and replated for the generation of 0, 50 and 100% cultures.

To titrate menadione, hiPSC-CMs were incubated with 3,10,15,20 and 25 μ M for 2 hours (Figure 4.6a). After the 2 hours incubation period, hiPSC-CMs treated with 3 and 10 μ M remained attached to the plate and beating. Cells treated with any concentration higher than 10 μ M completely lifted off the plate and stopped beating by the end of the incubation period. Therefore, 10 μ M menadione, the highest concentration not causing hiPSC-CMs to stop beating and lift off, was chosen for future experiments. Another member of the lab had also previously reported that the range of concentrations of menadione that caused a drop in mitochondrial respiration without associated cell death was very narrow.

4.3.4 IMR90-4 hiPSC-CMs recovered from menadione treatment

The Mito Stress Test experiments were conducted 5 days after menadione-treated and untreated cells were mixed and replated (Figure 4.6b). Comparable to what was observed before with antimycin A, mitochondrial respiration of hiPSC-CMs treated with 10 μ M menadione was not significantly different from that of control hiPSC-CMs. None of the parameters of mitochondrial respiration were changed in the 50% and 100% groups (Figure 4.6c). The ratio of OCR/ECAR was also unchanged (Figure 4.6d). For a second readout of mitochondrial respiration, ATP production was also measured using a Luminescent ATP Detection Assay Kit and it was unchanged (Figure 4.6e). Previous results from our lab showed that menadione treatment for 2 hours at concentrations of 4 μ M and 15 μ M immediately followed by the Mito Stress Test resulted in significantly lower OCR in hiPSC-CMs obtained from CDI and Axiogenesis, respectively (see Figures A5 and A6 of the Appendix – data kindly provided by Micaela Jenkins).

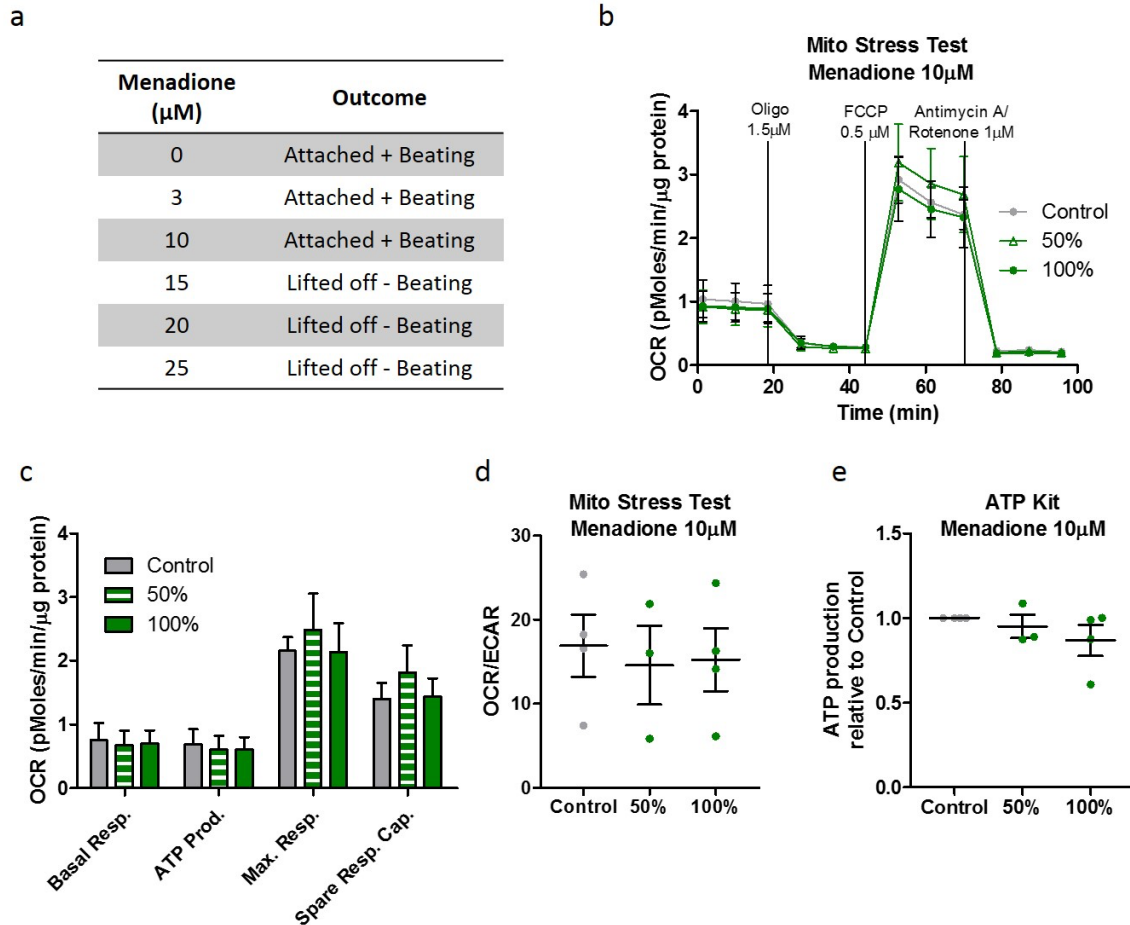


Figure 4.6: The effect of menadione treatment on hiPSC-CM mitochondrial respiration.

a) Menadione was titrated in hiPSC-CMs. + (cells were beating) – (cells stopped beating). b) The Mito Stress Test was performed 5 days after cells were treated with 10μM menadione, mixed and replated. Each group is represented by the percentage of menadione-treated hiPSC-CMs. Oxygen consumption rate (OCR, in pMoles/min/μg protein) was measured at basal, and following the injection of 1.5μM oligomycin, 0.5μM FCCP and 1μM antimycin A/rotenone. c) Basal respiration, ATP production, maximal respiration and spare respiratory capacity. d) The ratio between OCR and ECAR (extracellular acidification rate, in mpH/min/μg protein) was determined. e) ATP production was measured using an ATP kit and is represented relative to Control. n=4 (3 for 50% group) independent differentiations. The graphs represent mean ± s.e.m. One-way ANOVA followed by Bonferroni's Post-Hoc.

To confirm that similar numbers of menadione-treated and control hiPSC-CMs survived and/or attached and that the mixing ratio was correct, the total protein content was measured at the end of the Mito Stress Test experiments. There were no differences in the total protein levels between the two groups (Figure 4.7a).

The enzyme SOD2 is responsible for the conversion of superoxide (O_2^-) into hydrogen peroxide (H_2O_2). SOD2 is crucial in the protection against ROS and it is upregulated in periods of oxidative stress (Wallace and Starkov, 2000). To understand whether hiPSC-CMs were still under oxidative stress 5 days after menadione treatment, the levels of SOD2 mRNA were measured by qPCR. SOD2 mRNA expression was also compared to that of foetal and adult heart tissue. The results are shown in Figure 4.7b. No significant differences were found in SOD2 mRNA levels between control, 50% and 100% menadione-treated hiPSC-CMs. It was also observed that SOD2 mRNA levels of hiPSC-CMs were comparable to foetal heart tissue, and approximately 20 times lower than in adult heart tissue. Together, these observations indicate that hiPSC-CMs recovered from menadione-induced oxidative stress during the 5-day gap between treatment and the experiments.

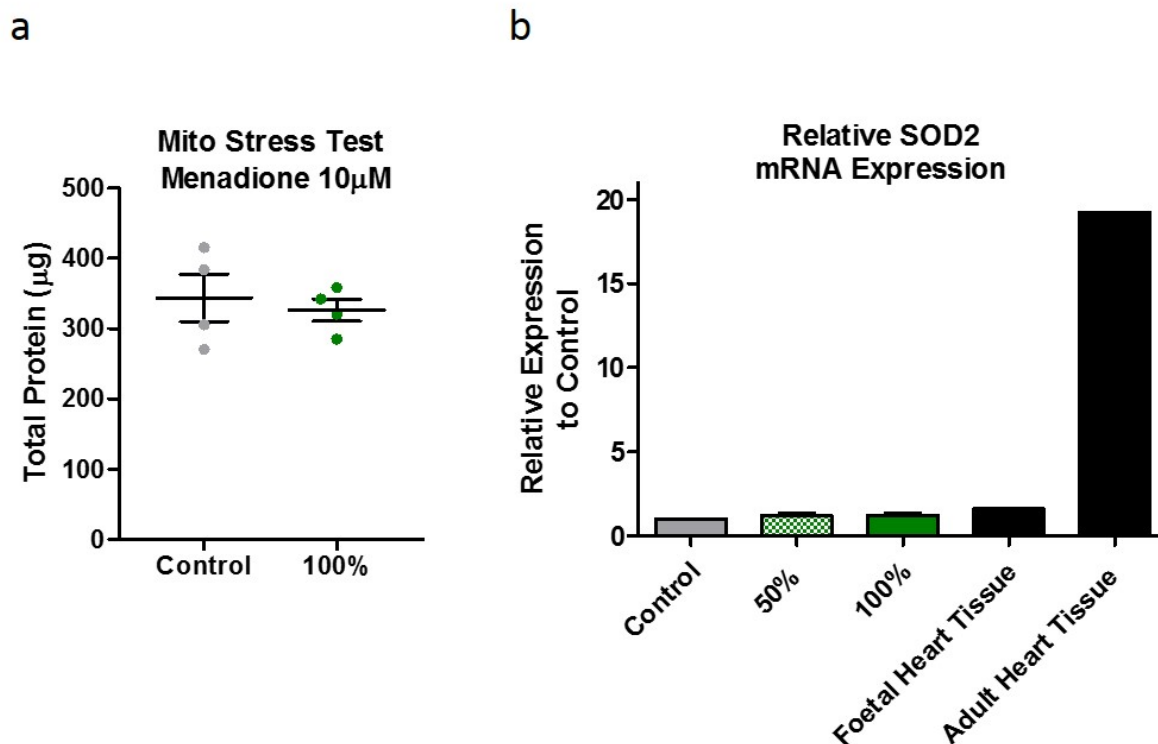


Figure 4.7: Total protein and SOD2 mRNA levels in menadione-treated hiPSC-CMs.

a) At the end of each Mito Stress Test experiment the cells were lysed and the total protein (µg) was quantified for the control (grey) and treated (green) groups. Each group is represented by the percentage of menadione-treated hiPSC-CMs. n=4 independent differentiations. The graphs represent mean $\pm$ s.e.m. Unpaired Student's t-test. b) The SOD2 mRNA levels of menadione-treated hiPSC-CMs and of foetal and adult heart tissue were measured using qPCR. Data were normalised to the housekeeping gene B2M and are represented relative to Control hiPSC-CMs. n=3 independent differentiations for hiPSC-CMs; n=1 for foetal and adult heart tissue. The graph represents mean $\pm$ s.e.m for hiPSC-CMs and mean for foetal and adult heart tissue. One-way ANOVA followed by Bonferroni's Post-Hoc between hiPSC-CMs.

4.4 Discussion

The results presented here demonstrate that two populations of hiPSC-CMs can be mixed at different ratios and that the ratios are maintained up to 5 days after mixing. Antimycin A and menadione were used to generate a drug-induced model of heteroplasmy in hiPSC-CMs. In contrast to the effect directly after treatment, it was shown after 2 days that the mitochondrial respiration of 30, 60 and 100% antimycin A-treated hiPSC-CMs was not significantly different from that of control. This was identical when the substrate was replaced by galactose. Similarly, mitochondrial respiration of 50 and 100% menadione-treated hiPSC-CMs was not significantly different from control, 5 days after treatment. The mRNA levels of the antioxidant mitochondrial enzyme SOD2 were not altered after menadione treatment. SOD2 mRNA levels in hiPSC-CMs were comparable to foetal heart tissue and approximately 20 times lower than adult heart tissue.

4.4.1 Heteroplasmy model development

Generating a drug-induced model of heteroplasmy for the study of mitochondrial disorders in hiPSC-CMs would circumvent the limited availability of patient-derived hiPSCs and the difficulties of expanding and differentiating hiPSCs with very high levels of mtDNA mutations. The mitochondrial free radical theory of ageing, which was initially proposed by Harman in 1956(Miao and St Clair, 2009), suggests that the production of ROS by the ETC is thought to be involved in the accumulation of mtDNA mutations, which in turn cause mitochondrial dysfunction(Harman, 1956). The complex III inhibitor antimycin A and the uncoupler menadione were picked for the generation of a heteroplasmy model in hiPSC-CMs. These agents were chosen due to their ability of both impairing the ETC and increasing the generation of ROS. Confocal imaging of hiPSC-CMs cultures with 0, 30, 60 and 100% CellTracker Red labelled cells revealed that the ratio was accurate and was maintained 2 days

after replating. Additionally, total protein levels of drug-treated and untreated hiPSC-CMs were similar at the end of the Mito Stress Test experiments, indicating that the ratios were also maintained in both antimycin A and menadione models.

4.4.2 Effects of antimycin A and menadione on hiPSC-CMs

The effect of antimycin A on hiPSC-CMs OCR was titrated using a Mito Stress Test. In the titration experiment, a drop in hiPSC-CM basal respiration and ATP production was detected 2 days after treatment with 30 μ M antimycin A. On the other hand, in subsequent experiments, there was no difference in any mitochondrial respiration parameters between control and all antimycin A-treated groups. Similarly, incubation with menadione concentrations above 10 μ M caused hiPSC-CMs to lift off the plate and stop beating. However, in the following experiments, hiPSC-CMs incubated with 10 μ M menadione did not show any differences in mitochondrial respiration compared to control. Unfortunately, only one batch of hiPSC-CMs was used for titration of antimycin A and menadione. Several reagents used for hiPSC maintenance and differentiation show considerable batch-to-batch variability (e.g. matrigel, fibronectin, growth factors) and can result in generated hiPSC-CMs with different phenotypes (Lagouge and Larsson, 2013). It is possible that the batch of hiPSC-CMs used for each titration experiment was particularly sensitive to mitochondrial drugs and titrations should have been repeated using numerous differentiation batches. We are aware of this limitation.

Nonetheless, antimycin A (20 μ M) and menadione ($\geq$ 10 μ M) have been shown to increase ROS production and decrease mitochondrial integrity, beating rate and cAMP formation in hiPSC-CMs (Rajamohan *et al.*, 2013). BurrIDGE and colleagues reported an increase in ROS production after the application of 20 μ M antimycin A or menadione to hiPSC-CMs (Grimm *et al.*, 2015; BurrIDGE *et al.*, 2016). In another study, Grimm and colleagues investigated the

effect of 30 minutes or 24h hours of menadione exposure on ROS production, mitochondrial integrity, beating rate and cAMP formation of hiPSC-CMs (Burridge *et al.*, 2016). Dose-response curves show an increase in ROS and a drop in beating rate and cAMP formation after 30 minutes of 10 μ M exposure and a decrease in mitochondrial integrity after 30 minutes of 100 μ M menadione exposure. In our lab, mitochondrial respiration was impaired when the Mito Stress Test was performed on CDI and Axiogenesis hiPSC-CMs that had just been treated for 2 hours with 4 μ M and 15 μ M menadione, respectively.

After treatment with both antimycin A and menadione, mitochondrial respiration was similar between all three groups: control, mixed and 100% treated hiPSC-CMs. Unchanged mitochondrial respiration in the mixed group only could have been explained by transfer of mitochondria from the untreated cells to the treated. However, this cannot explain results obtained from the 100% treated group. Therefore, it seems clear at this point that the reason for not detecting a change in mitochondrial respiration was the gap between drug treatments and the time of the experiments. There could have been three main reasons for this, which will be covered below: glycolysis supported hiPSC-CM respiration to protect them from oxidative stress; mitochondrial turn over (mitophagy of damaged mitochondria and mitochondrial biogenesis) in hiPSC-CMs enabled recovery from oxidative stress; foetal phenotype of hiPSC-CMs protected them from oxidative stress.

Finally, we are aware of the limitations of looking at time zero for only one batch of hiPSC-CMs and also of not investigating responses at intervening points from 0 to 5 days. This could have revealed whether or not the drugs did induce mitochondrial damage on the cells. At these time points, it would have been particularly relevant to investigate if the drugs instigated oxidative stress, which could have been achieved using the dye MitoSox, or whether they abolished mitochondrial membrane potential, which can be measured using the dye JC-1.

4.4.3 Glycolysis as a protection against oxidative stress

Reliance on glycolysis seems to protect against oxidative stress damage and this is particularly well described in the case of cancer cells(Grimm *et al.*, 2015). It was hypothesized that hiPSC-CMs relied on glycolysis after antimycin A treatment to protect against ROS damage. Human iPSC-CMs largely depend on OXPHOS but glycolysis still plays a part in their energy production(Chen, Qian and Wu, 2015). Depending on the substrate provided hiPSC-CMs can shift between glycolysis and OXPHOS, galactose completely inhibiting ATP generation via glycolysis(Denning *et al.*, 2016). Therefore, glucose in RB+ medium was replaced by galactose and the Mito Stress Test experiment was repeated. However, there were no differences in any parameter of mitochondrial respiration between the antimycin A-treated groups and control. Surprisingly, the OCR/ECAR ratio (OXPHOS/glycolysis) of the 60% group was significantly higher than control, which could be indicative of mitochondrial turn over of stressed cells, which will be discussed further down. These observations indicate that reliance on glycolysis was not responsible for protection of hiPSC-CMs against oxidative stress,

4.4.4 Mitochondrial turnover as a protection against oxidative stress

Mitochondrial turnover (mitophagy and biogenesis) must be tightly regulated to prevent mitochondrial dysfunction than can originate from oxidative stress. In the young rat heart mitochondria turn over with a half-life of 16 days and this is known to decline with age(Rana *et al.*, 2012). Assuming turn over occurs at a constant rate and considering that rat cardiomyocytes have approximately 10,000 mitochondria, one mitochondrion per cell is replaced every 4 minutes. This accounts for 3–4 % of the mitochondrial mass being replaced each day at basal conditions and increases in periods of stress (Menzies and Gold, 1971). Selective fission followed by fusion of dysfunctional mitochondria seems to contribute to their

segregation and removal by mitophagy(Gottlieb and Stotland, 2015). Mitophagy is balanced by mitochondrial biogenesis which enables replenishment of the pool of healthy mitochondria. It is likely that over the period of 2 or 5 days after treatment with antimycin A or menadione, respectively, at least part of the affected mitochondria in hiPSC-CMs were eliminated and substituted by healthy ones. A quick mitochondrial turnover is particularly expected in the case of hiPSC-CMs, which have an embryonic-like phenotype. This could be the reason why changes in mitochondrial function could not be detected several days after treatment with concentrations of antimycin A and menadione known to result in ROS-induced damage of hiPSC-CMs. Additionally, antimycin A-induced oxidative damage has been previously shown to lead to selective elimination of defective mitochondria by mitophagy in mouse cardiomyocytes(Twig *et al.*, 2008). The fact that the OCR/ECAR ratio of the 60% antimycin A-treated group was significantly higher than control could be explained by the elimination of dysfunctional mitochondria and the biogenesis of healthy mitochondria, more efficient in OXPHOS generation. As a future investigation, blocking mitochondrial biogenesis could help elucidate whether mitochondrial turnover was the responsible factor(Dutta *et al.*, 2013).

4.4.5 Levels of SOD2 expression in hiPSC-CMs

Oxidative stress in cells occurs when there is an imbalance between ROS generation and detoxification. Superoxide dismutases (SODs), including SOD2, are the main ROS scavenger enzymes and are upregulated during times of oxidative stress. SOD2 localizes in the mitochondrial matrix and prevents ROS from escaping mitochondria by converting O_2^- into H_2O_2 , which is then transformed into H_2O and O_2 by catalase or glutathione peroxidase(De Luca *et al.*, 2015). SOD2 mRNA levels were measured in hiPSC-CMs to test if the cells were still under oxidative stress 5 days after treatment with menadione. Results showed that SOD2 levels were not significantly different to control hiPSC-CMs. This suggests that excess amounts of superoxide were eliminated before experiments were conducted, removing the

stimulus for SOD2 expression to be raised. Nevertheless, there is no evidence in the literature that damage from oxidative stress caused by antimycin A and menadione is reversible(Miao and St Clair, 2009).

SOD2 levels in hiPSC-CMs were also compared to foetal and adult heart tissue. SOD2 levels in hiPSC-CMs were similar to those in foetal heart tissue and approximately 20 times lower than in adult heart tissue. SOD2 expression is known to increase during development of the rat heart(Rieske, 1967), possibly to protect against the superoxide generated from increased mitochondrial respiration(Yu *et al.*, 2014). The results here presented demonstrate once more the immature phenotype of hiPSC-CMs, which should be taken into consideration. But importantly, hiPSC-CMs might be more resistant to oxidative stress due to their embryonic features. In contrast with adult cardiomyocytes which are highly susceptible to stress, it seems that embryonic cardiomyocytes orchestrate various protective mechanisms to survive mitochondrial stress(Porter *et al.*, 2011). Furthermore, it is well known that the failing adult heart reverts to a foetal metabolic gene profile for stress protection(Magarin *et al.*, 2016).

4.5 Summary

In this chapter we investigated the potential uses of the mitochondrial drugs antimycin A and menadione for the development of a heteroplasmy model in hiPSC-CMs and it can be concluded here that this strategy is not suitable. Although the mixing ratios of control versus treated hiPSC-CMs were maintained, the effect of the two drugs on mitochondrial function could not be detected days after treatment. It was hypothesized that glycolysis could have been protecting hiPSC-CMs against antimycin A oxidative damage but results from experiments where glycolysis was blocked by galactose supplementation were not indicative of this. Instead, it is likely that mitochondrial turnover occurs relatively fast in hiPSC-CMs and

was responsible for the elimination of dysfunctional mitochondria that were subject to antimycin A and menadione-induced oxidative damage. In the future it would be interesting to block mitochondrial biogenesis to confirm this. Given that SOD2 mRNA levels were comparable to foetal heart tissue, it was finally suggested that hiPSC-CM immature phenotype also equips them with protective mechanisms against oxidative stress. Further studies are needed for validation.

To conclude, we have established here that applying drugs for the development of a heteroplasmy model in hiPSC-CMs is limited and dependent on a drug which causes permanent mitochondrial damage. Therefore, cardiac differentiation of hiPSCs derived from patients with heteroplasmic mtDNA mutations still appears to be the best strategy to investigate heteroplasmy in hiPSC-CMs and we decided to carry on with this approach.

Chapter 5: Investigation of cardiac-specific properties of A3243G mutant human induced pluripotent-stem cell cardiomyocytes

5.1 Introduction

As discussed in section 1.2.8.3 of the Introduction, hiPSCs have been previously generated by reprogramming somatic cells of patients with various mtDNA mutations, including: A3243G (from MELAS(Taegtmeyer, Sen and Vela, 2010)and diabetic(Hamalainen *et al.*, 2013; Ma *et al.*, 2015; Perales-Clemente *et al.*, 2016) patients); G13513A (from MELAS(Fujikura *et al.*, 2012) and Leigh Syndrome(Folmes *et al.*, 2013) patients); C5541T (from MELAS patients(Ma *et al.*, 2015)); T8993G (from Leigh Syndrome patients(Kodaira *et al.*, 2015)) and mtDNA deletions (from Pearson marrow pancreas syndrome patients(Ma *et al.*, 2015)). Authors described generation of hiPSC clones with different mutation loads and recapitulation of disease-causing phenotypes. Most studies have focused on investigating the metabolic properties of mutant hiPSCs, with only one study reporting the generation of CMs from hiPSCs of MELAS patients with the mtDNA A3243G mutation(Cherry *et al.*, 2013). Perales-Clemente and colleagues obtained pure populations of CMs positive for troponin T from hiPSCs with approximately 80% heteroplasmy for the A3243G mutation. Generated CMs showed impaired mitochondrial respiration but investigation of cardiac-specific properties was not performed. A line of hiPSCs with 100% A3243G mutant load derived from a patient with MELAS has been previously obtained by our lab. Unfortunately, the cells easily lost their pluripotency in culture and as such cardiac differentiation attempts have failed. We subsequently obtained hiPSCs with 20% heteroplasmy for the A3243G mutation to start, derived from a patient presenting diabetes but no other marked symptoms of MELAS.

Tissues with high energy requirements, including the heart, are most frequently involved in mitochondrial disorders(Perales-Clemente *et al.*, 2016). Dependant on the patient's age and

mutant load, multiple cardiac phenotypes are associated with pathogenic mtDNA mutations, the most prevalent being cardiomyopathies (hypertrophic, dilated, or restrictive), arrhythmias (bradyarrhythmias or tachyarrhythmias) and sudden death (Taylor and Turnbull, 2005). Hypertrophic remodelling, which mimics HCM, occurs in up to 20-60% of all patients presenting mitochondrial conditions and it explains the diagnosis of some HCM patients where sarcomeric protein mutations are not identified (Bates *et al.*, 2012). Bradyarrhythmias are thought to occur in 5-10% of patients with A3243G and in up to 84% of patients harbouring an mtDNA deletion causing the mitochondrial condition Kearns-Sayre Syndrome. Finally, tachyarrhythmias are thought to be less common but were still identified in 3-27% of patients with the A3243G mutation (Daehmlow *et al.*, 2002; Scaglia *et al.*, 2004; Limongelli *et al.*, 2010).

Unless the patients present unexplained HCM and clinical features of mitochondrial disorders, mtDNA mutations are usually late diagnosed or not even identified. Therefore, little is still known about cardiac involvement in most patients with mtDNA mutations. In the previous chapter, it was determined that application of mitochondrial drugs for the generation of a heteroplasmy model using hiPSC-CMs was limited. As such, the aim of this chapter was to characterize the cardiac-specific properties of CMs differentiated from hiPSCs originating from a patient harbouring 20% heteroplasmy for the common heteroplasmic mtDNA mutation A3243G. It was hypothesized that the A3243G mutation caused abnormalities in the cardiac-specific properties of mutant hiPSC-CMs. Increased cell size and differences in beating and calcium handling properties were expected to occur in mutant hiPSC-CMs.

5.2 Methods

5.2.1 Human iPSC maintenance and cardiac differentiation

Control and 20% A3243G mutant hiPSCs lines were maintained and differentiated as described in sections 2.1 and 2.3. All hiPSC-CMs were 30-40 days old when experiments were conducted.

5.2.2 Assessment of pluripotency

Human iPSCs were prepared for immunostaining as described in section 2.9. Antibodies used were rabbit anti-Oct4 (ab19857, Abcam) followed by Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (A11008, Thermo Fisher Scientific). Hoechst 333 (H3570, Thermo Fisher Scientific) was used for nuclear labelling. Representative confocal images were acquired as described in 2.10.2.

5.2.3 Image acquisition during hiPSC cardiac differentiation

On differentiation days -2,-1, 0, 2 (after CHIR99021), 5 (after Wnt-C59) and 13 (during metabolic selection) representative brightfield images were acquired as described in 2.10.2.

5.2.4 High content imaging of Troponin T

Human iPSC-CMs were prepared for immunostaining as described in section 2.9. 10,000 cells were plated per well of a 96 well-plate and in triplicate. Antibodies used were rabbit anti-cardiac troponin T (ab45932, Abcam) followed by Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (A11008, Thermo Fisher Scientific). Hoechst 333 (H3570, Thermo Fisher Scientific) was used for nuclear labelling. Plates were run and analysed as described in section

2.10.1 for determination of the percentage of troponin T-positive cells, along with cell area and shape.

5.2.5 Ionoptix measurement of contraction

Basal beating rate, response to isoprenaline and carbachol and time to peak contraction and to 50% relaxation were determined as described in section 2.5.

5.2.6 Optical mapping measurement of Ca^{2+} transients

Human iPSC-CMs were loaded with the Ca^{2+} indicator Fluo-4 AM and Ca^{2+} handling was assessed as described in 2.6.

5.2.7 Statistical Analysis

Details are described in section 2.12.

5.3 Results

5.3.1 A3243G mutant hiPSCs expressed the pluripotency marker Oct-4 and could be differentiated to beating CMs

To assess the differentiation potential of A3243G mutant hiPSCs, the cells were stained for the pluripotency marker Oct-4. As can be seen in Figure 5.1a, mutant hiPSCs expressed the nuclear pluripotency protein at high levels. Figure 5.1b shows phase contrast images taken throughout the differentiation of mutant hiPSCs to CMs. Differentiation was started 4 days after splitting and when the cells reached approximately 90% confluency (d 0). Minimal cell

death was observed during differentiation. Two days after the application of the first inhibitor CHIR99021 (d 2) cell morphology began to change and two days after the application of the second inhibitor Wnt-C59 (d 5) networks of CMs-to-be started to be visible. Beating was generally observed around day 8 and metabolic selection was performed on day 11 to eliminate non-CMs using glucose starvation. Cultures of mutant hiPSC-CMs were successfully purified during metabolic selection (d 13).

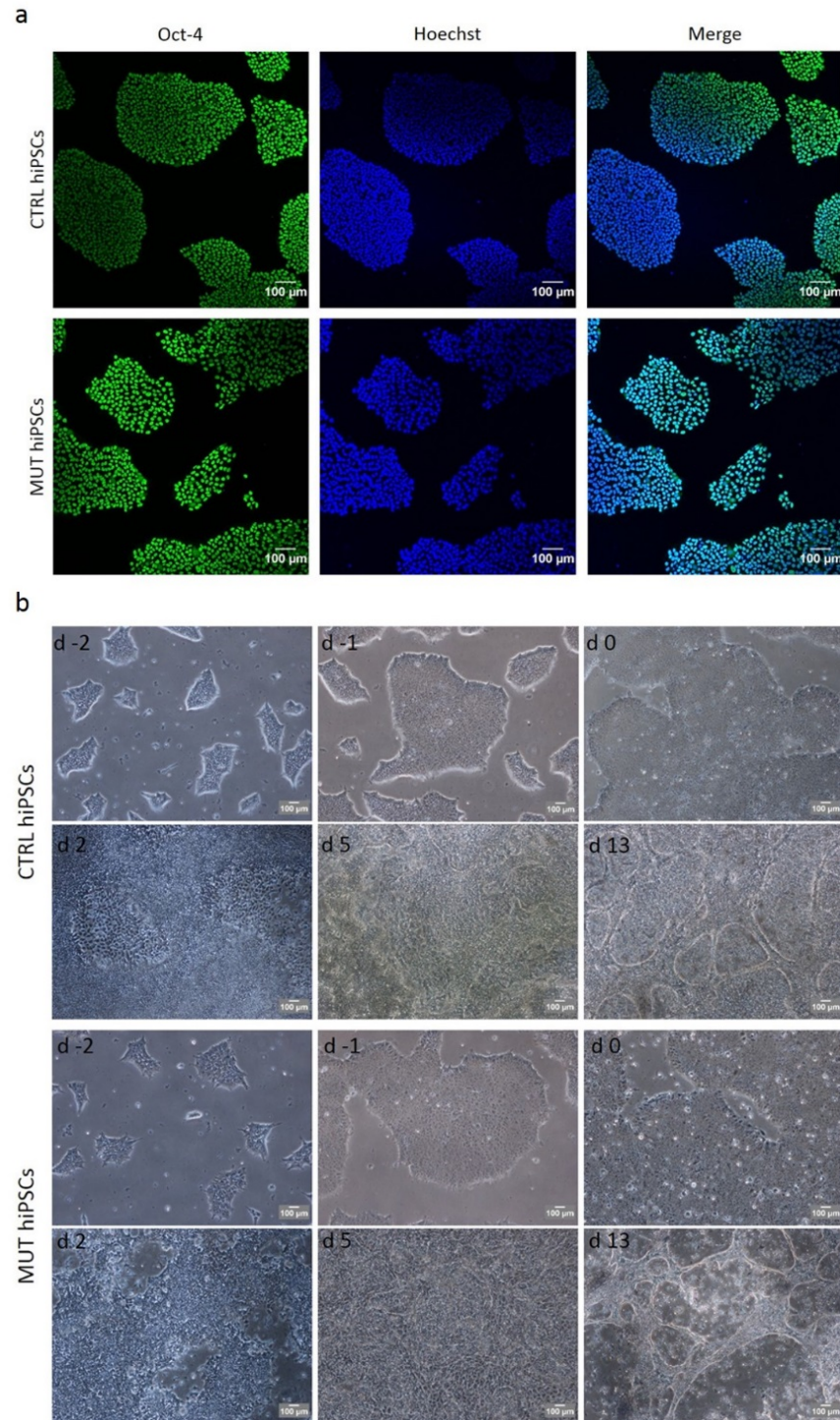


Figure 5.1: Oct-4 expression and cardiac differentiation of A3243G mutant hiPSCs.a) Representative confocal images of immunofluorescence staining of control (CTRL) and mutant (MUT) hiPSC for pluripotency marker Oct-4. Samples stained at day 2 after passaging. Scale bars, 100µm. b) Phase contrast images of CTRL and MUT hiPSCs during chemically defined differentiation. Days relative to beginning of differentiation (d 0). Scale bars, 100µm.

5.3.2 A3243G mutant hiPSC-CMs expressed the cardiac marker Troponin T and did not show differences in cell morphology

The cardiac protein troponin T is usually the marker of choice to determine differentiation efficiencies (Bates *et al.*, 2012). A3243G mutant hiPSC-CMs were stained for troponin T 30-40 days post beginning of differentiation and were screened using a high-content fluorescent microscope (Figure 5.2a). As determined by the analysis software, over 90% of the cells were positive for troponin T and this was not statistically different to control (Figure 5.2b). Mutant hiPSC-CM morphology was heterogeneous, the cells presenting a wide range of sizes and shapes. Area and shape was analysed for all control and mutant hiPSC-CMs that were screened (10,000 cells plated in triplicate per differentiation for a total of 6 differentiations) and, as can be seen in Figure 5.2c, there was an overlay between the area and shape of the two populations. Results indicated no changes in mutant hiPSC-CM morphology.

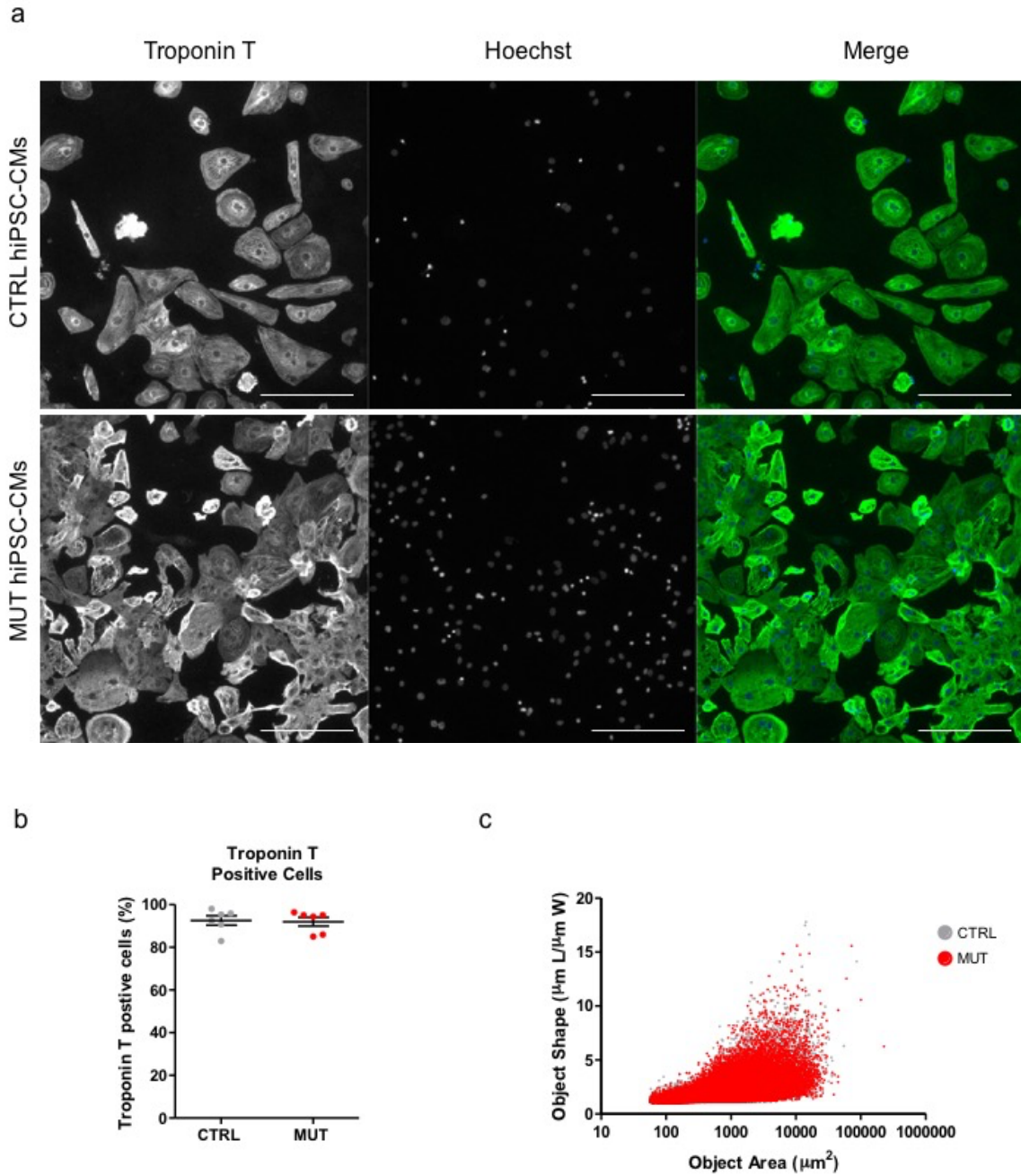


Figure 5.2: Troponin T expression and morphological analysis of A3243G mutant hiPSC-CMs. a) Representative high-content microscopy images of immunofluorescence staining of control (CTRL) and mutant (MUT) hiPSC-CMs for cardiac marker troponin T. Samples stained at day 35 post start of differentiation. Scale bars, 100μm. b) Percentage of troponin T-positive cells in control (CTRL) and MUT hiPSC-CM cultures was calculated using the Cellomics ArrayScan software. n=6 independent differentiations; Graph represents mean $\pm$ s.e.m; Unpaired Student's t-test. c) Cellomics ArrayScan software was also used to analyse shape (length μm / width μm) and area (μm²) for each of CTRL and MUT hiPSC-CMs screened. n=10,000 cells plated on triplicate for a total of 6 independent differentiations.

5.3.3 A3243G mutant hiPSC-CMs did not present beating disturbances and responded to isoprenaline and carbachol

For characterization of A3243G mutant hiPSC-CMs in terms of their cardiac-specific properties, we started by performing beating rate assessment. The average basal beating rate of mutant hiPSC-CMs was 39 b.p.m. and was not significantly different to control (Figure 5.3a). Isoprenaline is a non-selective β -adrenergic receptor agonist and was applied to investigate β -adrenergic signalling in mutant hiPSC-CMs (Figure 5.3b). In response to 0.01 μ M isoprenaline, mutant hiPSC-CMs increased the beating rate by an average 58% and this was similar to control. After 15 minutes of perfusion with 0.01 μ M isoprenaline, control hiPSC-CMs showed signs of desensitisation. At 15 minutes, beating rate of control hiPSC-CMs had dropped and was significantly lower than mutant hiPSC-CMs (138.20 ± 5.66 and 154.30 ± 5.32 % for control and mutant hiPSC-CMs, respectively; $p=0.0470$). The muscarinic agonist carbachol was subsequently applied in the presence of isoprenaline to investigate muscarinic signalling in mutant hiPSC-CMs (Figure 5.3b). Application of 1 μ M carbachol decreased the beating rate of mutant hiPSC-CMs to an average of 112% basal b.p.m., and was not significantly different to control. Results demonstrated functional β -adrenergic and muscarinic signalling in A3243G mutant hiPSC-CMs.

To determine if contractile kinetics were affected in mutant hiPSC-CMs, time to peak contraction and to 50% relaxation were additionally measured. Time to peak of mutant hiPSC-CMs was similar to control, at basal and after 5 minutes with isoprenaline. Additionally, within each group, time to peak did not change from baseline to after 5 minutes with isoprenaline (Figure 5.3c). Similarly, time to 50% relaxation was unchanged between mutant and control hiPSC-CMs. Within each group, time to 50% relaxation got shorter from baseline to after 5 minutes of isoprenaline application (Figure 5.3d; $p=0.0420$ and $p=0.0005$ for control and mutant hiPSC-CMs, respectively). These results indicated that the effect of isoprenaline on increasing hiPSC-CM beating rate involved a shorter time to 50% relaxation.

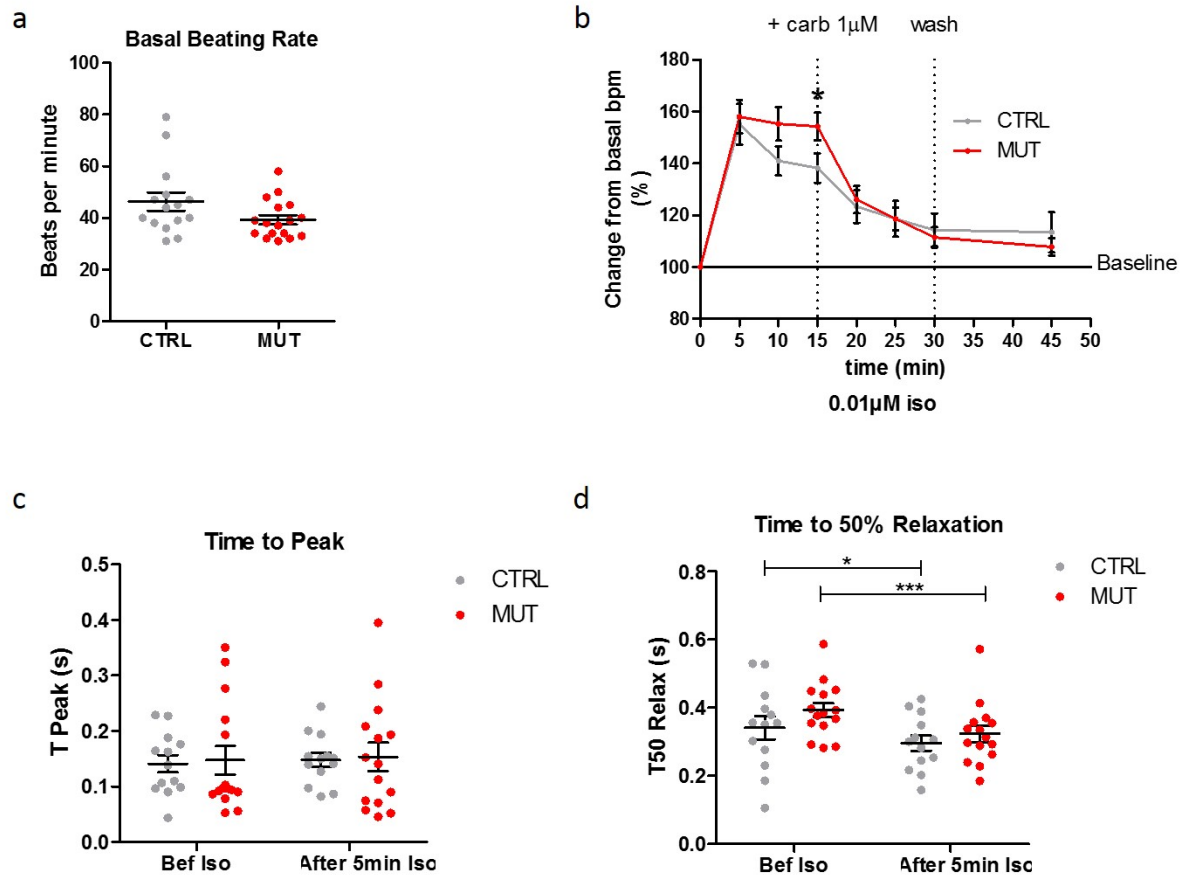


Figure 5.3: Contraction assessment of A3243G mutant hiPSC-CMs.

a) Basal beating rate (in beats per minute, b.p.m.) of control (CTRL) and mutant (MUT) hiPSC-CMs. $n=15$ and 17 dishes from 6 independent differentiations for CTRL and MUT hiPSC-CMs, respectively; Unpaired Student's t -test. b) The time course of beating rate (% change from basal b.p.m.) in CTRL and mutant MUT hiPSC-CMs upon application of $0.01\mu\text{M}$ isoprenaline (iso) followed by $1\mu\text{M}$ carbachol in the presence of isoprenaline (+ carb). $n=15$ and 17 dishes from 6 independent differentiations for CTRL and MUT hiPSC-CMs, respectively; Unpaired Student's t -test for CTRL vs MUT, $*p<0.05$. c) Time to peak contraction and d) to 50% relaxation at basal and after 5 minutes of isoprenaline (iso) application in CTRL and MUT hiPSC-CMs. $n=13$ dishes from 6 independent differentiations. Unpaired Student's t -test CTRL vs MUT; Paired Student's t -test before vs after iso, $*p<0.05$, $**p<0.01$. All graphs represent mean $\pm$ s.e.m.

5.3.4 Response to electrical field stimulation was slower in A3243G mutant hiPSC-CMs but Ca^{2+} signalling kinetics were maintained

To investigate Ca^{2+} signalling in A3243G mutant hiPSC-CMs, cells were loaded with the Ca^{2+} indicator Fluo-4 AM, field stimulated at 1Hz and Ca^{2+} transients were recorded using a confocal microscope. Field stimulation was performed to account for any differences in basal beating rate. Representative Ca^{2+} transients can be seen in Figure 5.4a. Visually, the initiation of the Ca^{2+} transient in mutant hiPSC-CMs was delayed in comparison to control hiPSC-CMs. For this reason, the time from stimulation to the beginning of the transient was measured for the two groups and was approximately 2-fold higher in mutant hiPSC-CMs and significantly greater than control (Figure 5.4b 18.85 ± 3.05 and 38.08 ± 6.13 ms for control and mutant hiPSC-CMs, respectively; $p = 0.0084$). To confirm that this delay was exclusive to A3243G mutant hiPSC-CMs, it was also measured in hiPSC-CMs generated from the control line IMR90-4 (Figure 5.4c). There was no significant difference in the delay from stimulation between control hiPSC-CMs and IMR90-4 hiPSC-CMs.

Mutant hiPSC-CMs did not show differences in the transient amplitude (Figure 5.4d). Ca^{2+} transient kinetics were also determined and in terms of Ca^{2+} release: time to transient peak was significantly higher in mutant hiPSC-CMs (Figure 5.4e; 303.50 ± 26.90 and 367.40 ± 12.95 ms for control and mutant hiPSC-CMs, respectively; $p = 0.0402$). For clarification, these parameters were measured from beginning of stimulation and no differences in time to peak were detected when the delay from stimulation was subtracted from the total time to peak (Figure 5.4f). These data suggested that the significantly higher time to peak of mutant hiPSC-CMs was a result of a greater delay from stimulation and not of any other disturbances in Ca^{2+} signalling. Finally, the duration of Ca^{2+} uptake, in terms of time to 50, 80 and 90% transient decay, was unchanged in mutant hiPSC-CMs (Figure 5.4g-i).

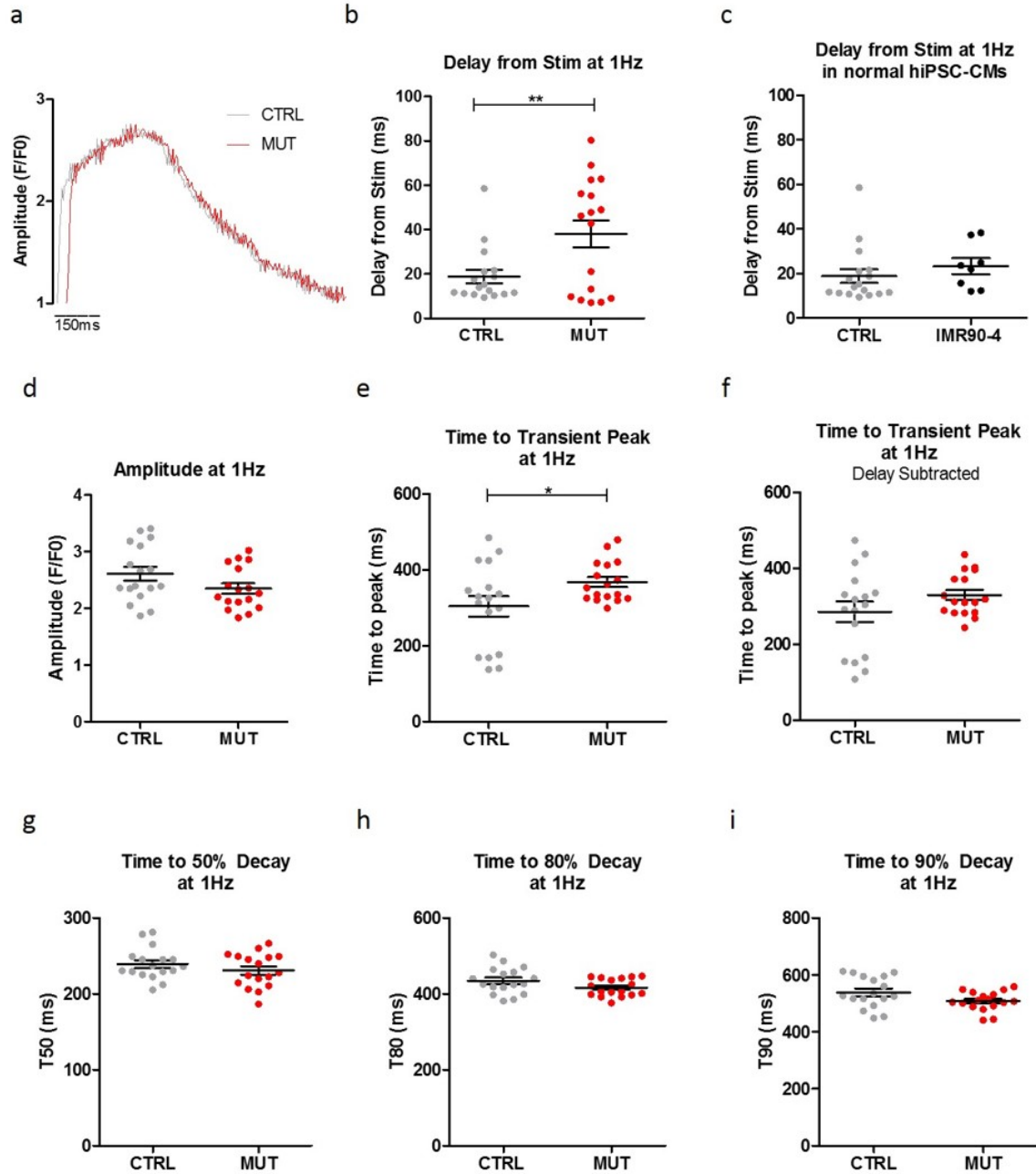


Figure 5.4: Ca²⁺ handling assessment of A3243G mutant hiPSC-CMs.

a) Representative Ca²⁺ transients for control (CTRL) and mutant (MUT) hiPSC-CMs field stimulated at 1 Hz. Delay from stimulation to beginning of the Ca²⁺ transient in b) MUT hiPSC-CMs and c) normal hiPSC-CMs. d) Amplitude of the Ca²⁺ transient represented as F/F₀, where F is the peak fluorescence value and F₀ is the baseline fluorescence value. e) - i) Ca²⁺ transient kinetics. n=17 dishes from 6 independent differentiations, 8 dishes from 2 independent differentiations for IMR90-4. Unpaired Student's t-test; **p<0.01. All graphs represent mean ± s.e.m.

5.4 Discussion

In this chapter we characterized the cardiac-specific properties of CMs produced from hiPSCs with 20% heteroplasmy for the mtDNA mutation A3243G. Beating CMs were successfully generated at high efficiencies and the cells did not present any changes in size or shape. Basal beating rate of mutant hiPSC-CMs was comparable to control and the cells had functional β -adrenergic and muscarinic signalling. No differences were observed in time to peak contraction and to 50% relaxation. Initiation of the Ca^{2+} transient in response to electrical field stimulation was delayed in mutant hiPSC-CMs. This resulted in a longer time to transient peak and suggested an impairment in the cells' excitability. However, no changes were observed in the other Ca^{2+} signalling parameters.

5.4.1 Cardiac differentiation of A3243G mutant hiPSCs

In the previous chapter it was shown that applying mitochondrial drugs to create a model of heteroplasmy in hiPSC-CMs was limited, since the drugs used did not permanently impair mitochondrial function. As such, it was concluded that the best way to investigate the effects of heteroplasmy on CM function would be to differentiate hiPSCs originating from patients with heteroplasmic mtDNA mutations. Previously, cardiac differentiation of a line of hiPSCs with 100% A3243G mutant load derived from a patient with MELAS was unsuccessful. Therefore, a hiPSC line with 20% heteroplasmy for the A3243G mtDNA mutation to start and a control line were obtained through a collaboration with UCL. The control line used originated from a family member who did not have the mutation as, unfortunately, it is still not possible to correct mtDNA mutations for the generation of isogenic controls. Both lines were positive for the pluripotency marker Oct-4, which is required for lineage specification (Burridge *et al.*, 2014).

In recent years there has been a significant improvement in the protocols for cardiac differentiation of hPSCs. Lian and Burridge have developed monolayer-based approaches

based on the temporal modulation of Wnt/ β -catenin signalling pathway and found that sequential activation of canonical Wnt signaling by treatment with GSK3 inhibitor CHIR99021 and inhibition of Wnt signalling by treatment with Wnt inhibitor C-59 was sufficient to drive several hPSC lines to CMs(Wang *et al.*, 2012). Since then many groups have been able to generate functional CMs at very high purities (>80%) from multiple healthy and diseased hiPSCs lines(Lian *et al.*, 2013; BurrIDGE *et al.*, 2014). Here, it was shown that very pure populations of CMs (>90% positive for troponin T) could also be produced by differentiation of A3243G mtDNA mutant hiPSCs with 20% heteroplasmy to start. It was also observed that mutant hiPSC-CMs survived the glucose starvation step to specifically eliminate non-CMs based on the metabolic differences between CMs and non-CMs(Sharma, Wu and Wu, 2013). Another group has also applied small molecule modulation of Wnt/ β -catenin signalling pathway to produce CMs from hiPSCs with approximately 80% A3243G heteroplasmy. Perales-Clemente and colleagues reported generation of CM cultures positive for the cardiac markers troponin T (>80%), troponin I (>70%) and myosin II heavy chain (>70%)(Sharma *et al.*, 2015). The authors also withdrew glucose from the culture medium to perform metabolic selection and did not report any associated cell death. Therefore, it seems that mitochondrial respiration can still support the metabolism of hiPSC-CMs with up to 80% A3243G heteroplasmy. In accordance with this, phenotypic manifestation of the A3243G mutation has only been reported in patients with mutant loads exceeding 73%(Perales-Clemente *et al.*, 2016). Additionally, the onset of MELAS syndrome, which is mainly caused by the A3243G mutation, commonly occurs in late childhood or adulthood so there is no indication that CM development would be impaired(Rossignol *et al.*, 2003).

At this stage, it is important to say that, although the level of heteroplasmy was 20% in the reprogrammed fibroblasts and the initial hiPSC colonies generated, it was unknown whether this remained constant during hiPSC culture and differentiation to CMs. Therefore, an important part of this work was to quantify the levels of mutation in the hiPSCs and in the CMs

generated. Investigation of this and other mitochondrial properties will be described in the next chapter.

5.4.2 Morphological analysis of mutant hiPSC-CMs

The link between cardiac abnormalities and mitochondrial mutations is well-known (Taylor and Turnbull, 2005). In particular, presentation of cardiomyopathies has been extensively described in patients with the A3243G mutation (Bates *et al.*, 2012). Follow up studies suggested that patients start by presenting HCM which later develops to dilated cardiomyopathy and heart failure (Sato *et al.*, 1994; Majamaa-Voltti *et al.*, 2002; Vydut *et al.*, 2007). Hoping to investigate whether hypertrophy could be detected in hiPSC-CMs with the A3243G mutation, the cells were screened using a Cellomics ArrayScan for determination of cell size (area in μm^2) and shape (length μm / width μm). A protocol for the characterization of the hypertrophic profile of hiPSC-CMs using the automated fluorescence imaging platform and high-content analysis Cellomics ArrayScan that had been previously developed by our group was applied (Sato *et al.*, 1994). No differences in morphology were detected in mutant hiPSC-CMs. As described above, higher levels of heteroplasmy are usually present in symptomatic patients, so these results could suggest that mutant load of 20% is not sufficient to induce hypertrophy in hiPSC-CMs.

5.4.3 Contraction studies of A3243G mutant hiPSC-CMs

The main functional abnormalities that are observed in failing CMs are reduced contraction, reduced β -adrenergic response, increased duration of contraction and relaxation and greater likelihood of arrhythmias (Földes and Mioulane, 2013). Therefore, characterization of cardiac-specific properties of A3243G mutant hiPSC-CMs was performed to establish whether features of heart failure were detected. Using the IonoOptix system it is not possible to

measure force of contraction in hPSC-CMs. However, basal beating rate and time to peak contraction and relaxation can be determined. Additionally, healthy hPSC-CMs respond to isoprenaline and carbachol and so it is possible to measure β -adrenergic and muscarinic signalling using the IonOptix system(Harding, 2005). In terms of contraction, it is well-known that there is a reduction of contraction in the failing CM and that this is more pronounced at higher stimulation frequencies. Failing cells cannot increase force of contraction in response to increased beating rate(Brito-Martins, Harding and Ali, 2008). Loss of responsiveness to endogenous or exogenous catecholamines (such as isoprenaline) is also a hallmark of heart failure(Harding, 2005). Isolated atrial or ventricular CMs from the failing heart show functional desensitisation to isoprenaline(Harding, 2005). Here, mutant hiPSC-CMs did not show differences in basal beating rate. It was also shown that mutant hPSC-CMs were responsive to isoprenaline and carbachol, indicating functional β -adrenergic and muscarinic signalling, and the maximal response of mutant hiPSC-CMs to isoprenaline was not different to control. Finally, isolated ventricular CMs from patients with heart failure show increased duration of contraction, specifically longer time to peak and to 50 and 90% relaxation(Harding *et al.*, 1990, 1992). Results from A3243G mutant hiPSC-CMs indicated that time to peak and to relaxation were unaffected. Therefore, overall, it seems that the beating properties of hiPSC-CMs with 20% heteroplasmy for the mtDNA mutation A3243G were unaffected and no features of heart failure were identified. Again, it is likely that 20% mutant load is not sufficient to induce beating disturbances in hiPSC-CMs.

5.4.4 Ca^{2+} handling in A3243G mutant hiPSC-CMs

Previous studies have established that disturbances in contraction and in catecholamine response of failing CMs seem to be linked to abnormalities in Ca^{2+} handling(Davies *et al.*, 1995). Although there are contradictory opinions on whether Ca^{2+} entry is impaired, it seems clear that the amount of Ca^{2+} supply to the myofilaments is reduced in patients with heart

failure, causing depression and prolongation of the Ca^{2+} transient (Harding, 2005)(Gwathmey *et al.*, 1987). This was not observed in A3243G mutant hiPSC-CMs, which suggests normal Ca^{2+} binding to myofilaments. Although most parameters of Ca^{2+} signalling were unchanged in mutant hiPSC-CMs, the cells had a longer time to transient peak, which was caused by a delayed response to electrical field stimulation. The beginning of the transient, and hence rise in intracellular Ca^{2+} , took approximately double the time in mutant hiPSC-CMs when compared to two control hiPSC-CMs lines, following electrical field stimulation. These results can be indicative of reduced or delayed opening of voltage operated L-type Ca^{2+} channels; or perhaps predominance of T-type Ca^{2+} channels which play a crucial role in hypertrophy, heart failure and arrhythmias and are less effective in Ca^{2+} entry but this requires further investigations(Beuckelmann, Näbauer and Erdmann, 1992). This delay in the beginning of the Ca^{2+} transient could explain the bradyarrhythmias that are common in patients with mtDNA disorders(Richard *et al.*, 1998; Zhou and January, 1998).

5.5 Summary

In summary, we have performed in this chapter a detailed assessment of cardiac-specific properties of A3243G mtDNA mutant hiPSC-CMs and demonstrated that pure populations can be generated to model what happens to CM function in the case of mitochondrial mutations. We concluded that 20% mutant load was not sufficient to induce a hypertrophic phenotype. Similarly, mutant hiPSC-CMs showed maintained contraction and response to isoprenaline and carbachol which was again proposed to be due to a low mutant load. Most Ca^{2+} handling parameters were normal in mutant hPSC-CMs but the cells displayed a delay in the beginning of the Ca^{2+} transient which was proposed to be caused by a disturbance in Ca^{2+} channel opening and Ca^{2+} entry. In the future it would be interesting to extend these evaluations to hiPSC-CMs with higher mutant loads to establish the threshold at which CM disturbances can be detected.

All the results presented in this chapter were obtained from bulk cultures of A3243G hiPSC-CMs. Given the nature of mtDNA mutations, it is possible that heteroplasmy was not homogenous within a population of cells and that some cells expressed more than 20% heteroplasmy and had abnormal cardiac properties. To understand this, we would have to perform single cell analyses and we are aware of this being a limitation of this project.

Finally, as stated before, it is essential to measure the mutant load of the hiPSCs and of the CMs used for these experiments. It is likely that the lack of a disease phenotype was caused by a low mutant load but it is also possible that hiPSC-CMs were able to adapt to a certain level of mitochondrial dysfunction. Therefore, it is also important to evaluate if the mitochondria of these mutant cells were dysfunctional. Investigation of mitochondrial properties of the A3243G mutant hiPSC-CMs will be discussed in the next chapter.

Chapter 6: Investigation of mitochondrial properties of A3243G mutant human induced pluripotent-stem cell cardiomyocytes

6.1 Introduction

In the previous chapter it was shown that most cardiac properties of hiPSC-CMs generated from patient-derived hiPSCs originally with 20% heteroplasmy for the A3243G mtDNA mutation were unaffected. The absence of a disease phenotype could be attributed to a capacity of hiPSC-CMs to adapt to a certain level of mitochondrial dysfunction or to low mutant load. Therefore, in this chapter, we set out to assess mitochondrial properties and to quantify mutant load in the different batches of mutant hiPSC-CMs used for experiments.

As described in section 1.2.8.3 of the Introduction, most studies which reprogrammed somatic cells of patients with mtDNA mutations reported generation of clones of hiPSCs with different levels of heteroplasmy originating from the same somatic cells; drifting of heteroplasmy levels in culture; successful differentiation of mutant hiPSCs into different lineages and recapitulation of disease phenotypes (Bates *et al.*, 2012). Mitochondrial properties were evaluated in hiPSCs (Fujikura *et al.*, 2012; Cherry *et al.*, 2013; Folmes *et al.*, 2013; Hamalainen *et al.*, 2013; Kodaira *et al.*, 2015; Ma *et al.*, 2015; Perales-Clemente *et al.*, 2016) and hiPSC-CMs (Hämäläinen and Suomalainen, 2015; Ma *et al.*, 2015) with the mtDNA mutation A3243G. Investigation of mitochondrial respiration using the Mito Stress Test performed in a Seahorse Analyser provides the most information about mitochondrial function in cells and impaired mitochondrial respiration was reported in hiPSCs homoplasmic for the A3243G mutation (Perales-Clemente *et al.*, 2016) and in hiPSC-CMs with 83% heteroplasmy for the A3243G mutation (Ma *et al.*, 2015). Other mitochondrial disturbances such as down-regulation of mitochondrial respiratory chain complex I and enhanced mitophagy induction have also been reported in hiPSCs with high (>80%) levels of the A3243G mutation (Perales-Clemente *et al.*, 2016).

Here, the level of A3243G heteroplasmy in the patient's somatic cells and in the first generated hiPSC clones was 20%. However it was important to determine if heteroplasmy levels changed during hiPSC culture and after cardiac differentiation. Amplification Refractory Mutation System-quantitative polymerase chain reaction (ARMS-qPCR) is a sensitive (detection threshold of 0.5%) single-step method to detect and quantify heteroplasmic mtDNA point mutations(Hämäläinen and Suomalainen, 2015). The reaction uses two sets of primers to simultaneously amplify the wild-type and mutant mtDNA sequences, enabling the quantification of mutant mtDNA levels in a group of cells. ARMS qPCR has been previously used to quantify levels of A3243G heteroplasmy in patient-derived hiPSCs(Venegas and Halberg, 2012).

In the next pages we will present an evaluation of the mitochondrial properties and heteroplasmy levels of A3243G mutant hiPSC-CMs.

6.2 Methods

6.2.1 Cell culture

Human iPSCs were maintained and differentiated as described in sections 2.1 and 2.3. All hiPSC-CMs were 30-40 days old when experiments were conducted.

6.2.2 Mito Stress Test experiments

All Mito Stress test experiments were performed as stated in section 2.7. Determination of the mitochondrial respiration parameters is defined in section 2.7.4, except OCR data are represented as a percentage change from the first basal measurement for each cell type (control and mutant). Basal glycolysis corresponds to the third ECAR measurement.

6.2.3 ATP production quantification

ATP production was quantified using a Luminescent ATP Detection Assay Kit (Abcam), as described in section 2.8.

6.2.4 Quantification of mtDNA copy number by qPCR

The levels of mtDNA relative to nuclear DNA were assessed by qPCR as described in section 2.11.3.1.

6.2.5 Quantification of A3243G mutation levels by ARMS qPCR

ARMS qPCR was used to quantify the levels of A3243G mutation in mutant hiPSCs and hiPSC-CMs as described in section 2.11.3.2.

6.2.6 Investigation of cell competition by flow cytometry

Cell competition assays between A3243G mutant hiPSCs and GFP⁺ control hiPSCs were performed as described in section 2.2.

6.2.7 Statistical Analysis

Details are described in section 2.12

6.3 Results

6.3.1 Mitochondrial function and mtDNA copy number were unchanged in A3243G mutant hiPSC-CMs

In this chapter, a detailed assessment of mitochondrial properties of A3243G mutant hiPSC-CMs was performed. Firstly, mitochondrial function was measured using the Mito Stress Test (Figure 6.1a). Mutant hiPSC-CMs did not show differences in any mitochondrial parameter (basal respiration, ATP production, maximal respiration and spare respiratory capacity, Figure 6.1b). Basal glycolysis rate, along with the ratio of OXPHOS/glycolysis (OCR/ECAR, Figures 6.1c and 6.1d, respectively), were also measured and were unchanged. Both mutant and control hiPSC-CMs relied primarily on OXPHOS (OCR/ECAR >1) for energy production. Finally, ATP levels were also measured using a luminescence kit for validation and were not statistically different relative to control (Figure 6.1e).

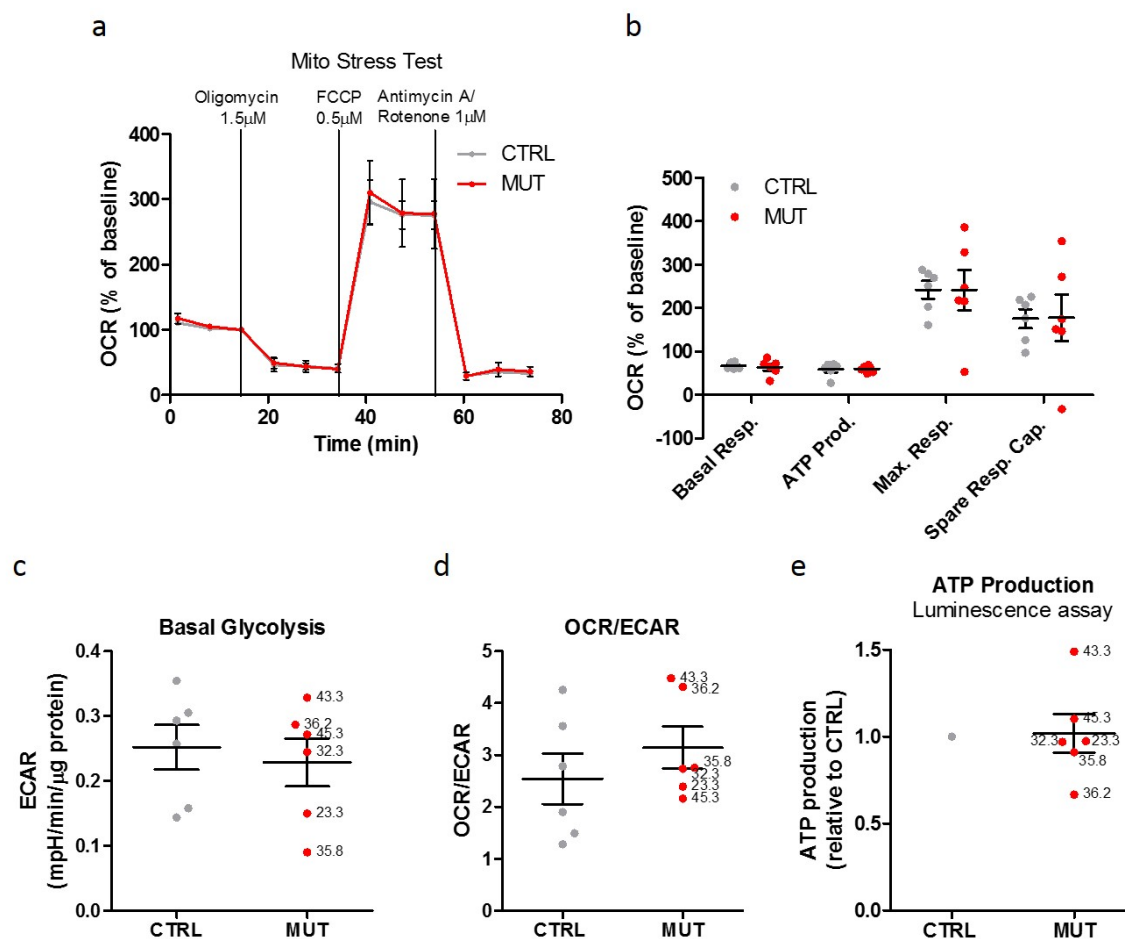


Figure 6.1: Mitochondrial respiration in A4354G mutant hiPSC-CMs.

a) The Mito Stress Test. Oxygen consumption rate (OCR, in pMoles/min/μg protein) was measured at basal, and following the injection of 1.5μM oligomycin, 0.5μM FCCP and 1μM antimycin A/rotenone. Data represented as a percentage change from first OCR measurement for mutant (MUT) and control (CTRL) hiPSC-CMs. b) Basal respiration, ATP production, maximal respiration and spare respiratory capacity were calculated. c) Basal glycolysis represented as extracellular acidification rate (ECAR, in mpH/min/ μg protein). d) The ratio between OCR and ECAR. e) ATP production was measured using an ATP kit and is represented relative to Control. n=6 independent differentiations. The graphs represent mean ± s.e.m. Unpaired Student's t-test. Numbers next to the data points correspond to the % of A3243G heteroplasmy in each mutant hiPSC-CM batch.

To investigate whether the number of copies of mtDNA was altered in A3243G mutant cells, the ratio of mtDNA to nuclear DNA was evaluated in hiPSCs and differentiated hiPSC-CMs (Figure 6.2). mtDNA copy number in mutant hiPSCs was not significantly different to control (Figure 6.2a). Similarly, mutant hiPSC-CMs did not show different levels of mtDNA when compared to control (Figure 6.2b).

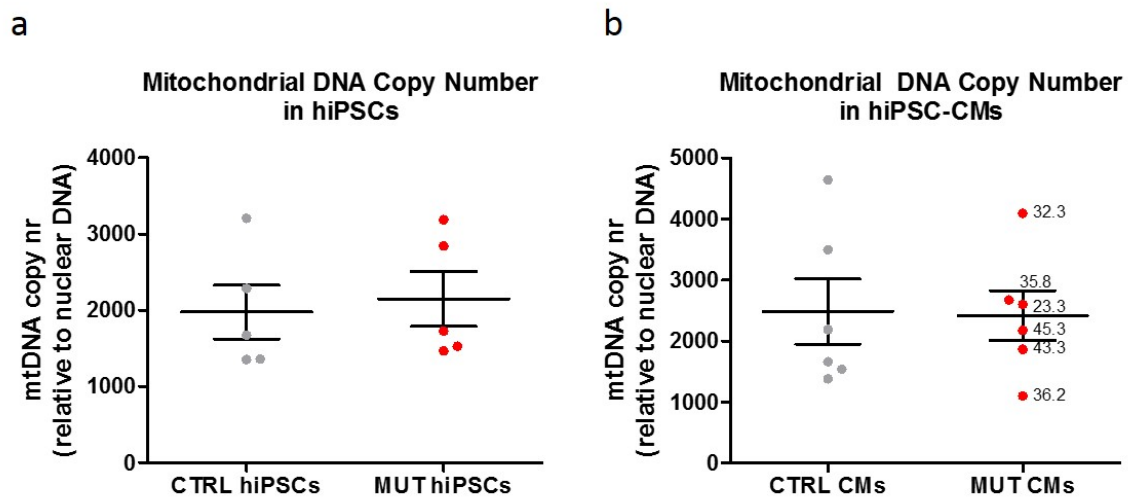


Figure 6.2: Mitochondrial DNA copy number in A3243G in mutant hiPSCs and hiPSC-CMs.

Mitochondrial DNA (mtDNA) copy number was measured by qPCR in a) mutant (MUT) and control (CTRL) hiPSCs and b) hiPSC-CMs. Data represented relative to levels of nuclear DNA. n=6 independent differentiations. The graphs represent mean $\pm$ s.e.m. Unpaired Student's t-test. Numbers next to the data points correspond to the % of A3243G heteroplasmy in each mutant hiPSC-CM batch.

6.3.2 Levels of A3243G mutation were similar between hiPSCs and related hiPSC-CMs but seemed to increase with hiPSC passaging

It was known that the level of A3243G heteroplasmy in the patient's somatic cells and resulting hiPSCs colonies ranged 20%, but it was also critical to know the mutant load of the hiPSC-CMs used for the present experiments. The same 6 batches of cells were used for all mitochondrial and cardiac-specific studies in this and the previous chapter (differentiation numbers 2, 3, 5, 6, 8 and 10, see Figure 6.3a). The level of A3243G heteroplasmy of every batch was determined using ARMS qPCR and is shown in Figure 6.3a. Figure 6.3a also shows the passage number of the hiPSCs used to generate hiPSC-CMs. It can be seen that the A3243G mutation is dynamic, with levels ranging from 23 to 45% in different batches of hiPSC-CMs. Glycolysis, OCR/ECAR, ATP production and mtDNA copy number data points were labelled with mutant load corresponding to each hiPSC-CM batch used (Figures 6.1c-e and Figure 6.2b). It can be observed that higher levels of heteroplasmy were not associated with higher rates of glycolysis, lower ATP production and lower mtDNA copy number.

Samples of hiPSCs were not available for every batch, but it can be seen in Figure 6.3a that levels of heteroplasmy remained fairly constant after differentiation (see differentiation numbers 5, 6, 8 and 10). But at this stage it was apparent that hiPSC heteroplasmy levels were raised at higher passage numbers (26.4%, 31.3%, 37.2%, 40.5% and 38.7% for passages 17,18,19,21 and 23, respectively). Therefore, in order to determine if the level of mutation was increasing with passaging, a fresh vial of hiPSCs at passage 15 was thawed and samples were taken up to passage 30. Sampling was made at every passage from passage 15 to 25 and every 5 passages afterwards. Results are shown in Figure 6.3b. Once more, the dynamics of the A3243G mutation are very clear, with heteroplasmy levels drifting with passaging. Mutation levels appear to increase with passaging over time, which is clearer when looking at the data every 5 passages (see bars with pointing arrows; 24.1%, 30.7%, 34% and 43.4% for passages 15, 20, 25 and 30, respectively).

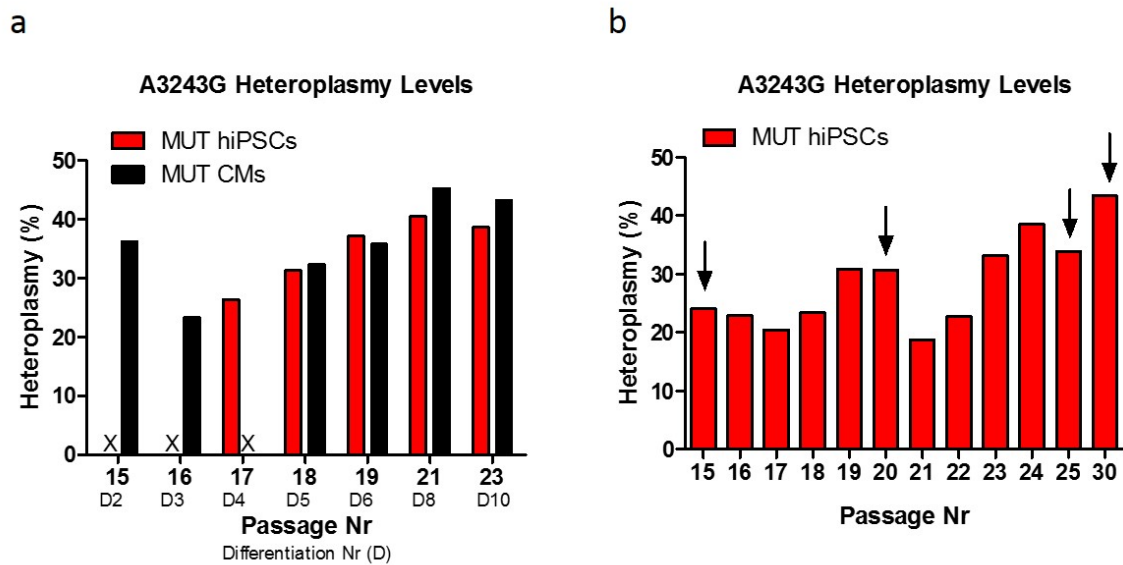


Figure 6.3: Assessment of A3243G heteroplasmy levels.

a) Levels of heteroplasmic mutation A3243G in the different differentiation batches of mutant hiPSCs (MUT hiPSCs) and hiPSC-CMs (MUT CMs) used for experiments were determined by ARMS qPCR. The corresponding hiPSC passage number for each differentiation is shown. X, Sample not available. b) A3243G heteroplasmy levels of a fresh vial of MUT hiPSCs thawed and passaged from P15 to P30. Arrows were used to highlight data from P15, 20, 25 and 30. Data represented as a percentage of mutant DNA relative to total DNA.

6.3.3 Possible transfer of cytoplasmic material from GFP+ control hiPSCs to A3243G mutant hiPSCs

Mutant mouse ESCs (mESCs) have been shown to be eliminated in the presence of wild-type mESCs by competitive interactions. For example, mESCs with defective BMP signalling cultured on their own proliferate normally. However, when cultured together with wild-type mESCs, mutant mESCs are eliminated and wild-type mESCs take over (Ma *et al.*, 2015). This can be measured by flow cytometry by sorting cultures of GFP+ (wild-type) and GFP negative (mutant) cells over time (see Figure A7 of the Appendix – from (Sancho *et al.*, 2013)). It was particularly interesting to investigate cell competition in the case of the A3243G mutation since mutation levels prevailed, and increased, during mutant hiPSC passaging. For cell competition assays, mutant hiPSCs were mixed at a 50:50 ratio with GFP+ control hiPSCs at day 0 and the two populations were sorted by flow cytometry for green fluorescence at days 0, 1, 2 and 4 after mixing. The hiPSC GFP+ line used was a CAG-ZiessGreen stably transfected line, targeted to the AAVS locus. Obtained results are shown in Figure 6.4. As a control, the two cell types were also grown separately and mixed at a 50:50 ratio on the day of the experiment before sorting, which can be seen in the Appendix Figures A12-A14. The full set of data, including gating selection, can also be found in the Appendix. Results confirmed that at day 0 after mixing and plating, the ratio of the two cell types was accurate (Figure 6.4.a and Figure 6.4.e; 52.0% mutant to 47.9% control). When grown separately from mutant hiPSCs, proliferation of GFP+ control hiPSCs originated daughter cells with similar levels of GFP expression over the course of the 4 days; similarly, mutant hiPSCs maintained their lack of GFP expression (Appendix). However, when the two cell types were co-cultured, a new population of cells with intermediate levels of GFP expression originated over time. As can be seen in Figures 6.4a – d, it seems that mutant hiPSCs might have acquired GFP expression by retaining cytoplasmic material transferred from GFP+ control hiPSCs. Whether cytoplasmic transfer was unidirectional remained undetermined. Only comprising 0.1% of the total gated cells at day 0, at day 4 the population of hiPSCs with intermediate GFP expression levels had

increased to 25.4% (Figure 6.4e). Consequently, it was not possible to assess cell competition, as mutant and control hiPSCs could not be accurately distinguished.

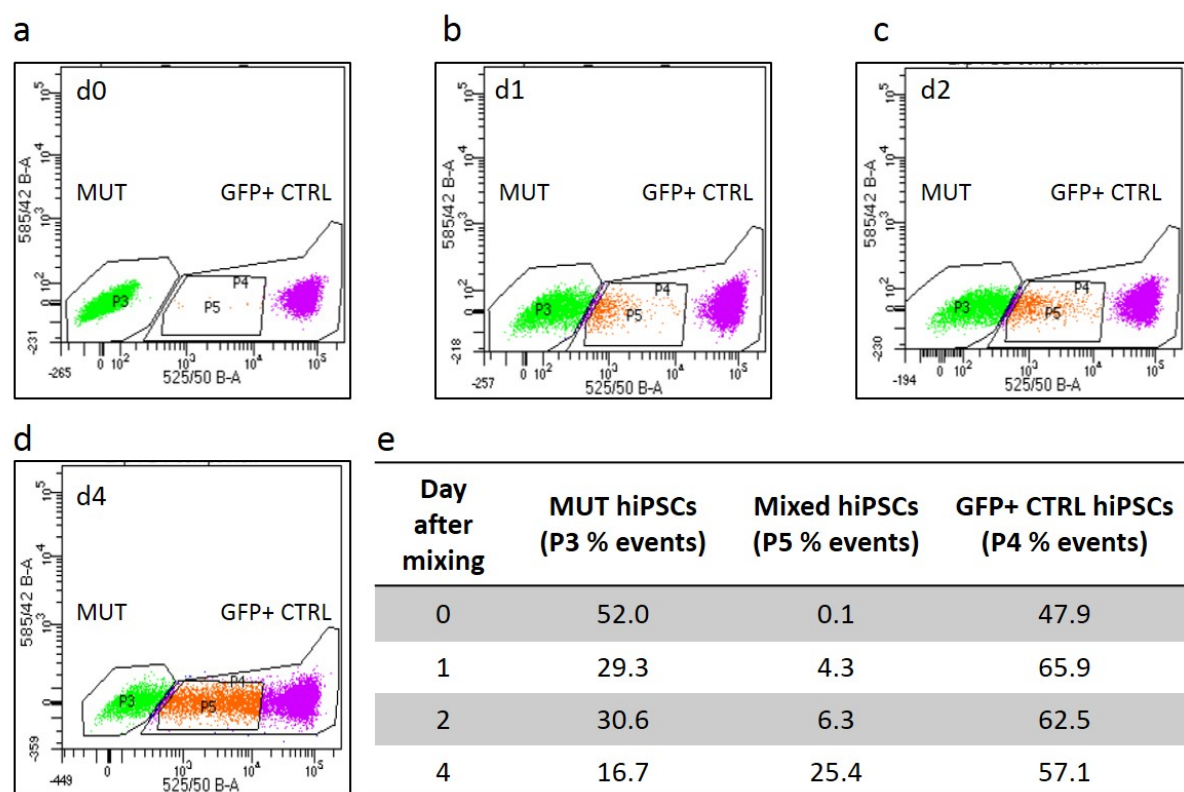


Figure 6.4: Cell competition assay between A3243G mutant and GFP+ control hiPSCs.

Mutant (MUT) and GFP+ control hiPSCs (GFP+ CTRL) were mixed at a 50:50 ratio on day 0 and co-cultured for 4 days. Cells were sorted for GFP expression by flow cytometry on days a) 0, b) 1, c) 2 and d) 4 after plating. a-d) Flow cytometry dot plots show gates created to identify hiPSCs according to their GFP expression profile (525/50 fluorescence; P3 GFP negative, P5 GFP intermediate and P4 GFP positive). e) Percentage of events for each gate during the co-culture period, out of total events.

6.4 Discussion

In this chapter we characterized the mitochondrial properties of CMs produced from hiPSCs originally with 20% heteroplasmy for the mtDNA mutation A3243G. All parameters of mitochondrial respiration were unchanged in mutant hiPSC-CMs. Mutant hiPSC-CMs also had comparable rates of glycolysis and similar energy phenotypes in terms of the OCR/ECAR ratio. The number of mtDNA copies was equally unaffected in mutant hiPSC-CMs. The level of A3243G heteroplasmy was also measured for all differentiation batches of mutant hiPSC-CMs used for experiments and was reported to vary between 23 and 45%. This variation was believed to be caused by changes in mutant load of hiPSCs occurring over time in culture. Long term culture of mutant hiPSCs caused heteroplasmy to almost double over 15 passages. To investigate whether mutant cells would be eliminated in the presence of non-mutant, cell competition assays were established. However, cell competition between mutant and GFP+ control hiPSCs could not be assessed due to possible transfer of cytoplasmic GFP+ particles to mutant hiPSCs.

6.4.1 Assessment of mitochondrial respiration and mtDNA copy number in A3243G mutant hiPSC-CMs

In the last chapter it was shown that most cardiac properties of hiPSC-CMs with the A3243G mutation were unchanged. Given the plasticity of CMs, it was hypothesised that hiPSC-CMs were able to adapt to a certain level of mitochondrial dysfunction. In this chapter we aimed to determine whether mitochondria in mutant hiPSC-CMs were actually dysfunctional. Results showed that all parameters of mitochondrial respiration, including ATP production, were not changed in hiPSC-CMs with the A3243G mutation. In previous studies using patient-derived hiPSCs with several mtDNA mutations, impaired mitochondrial respiration, including decreased ATP production, was reported only in clones with heteroplasmy levels greater than

60%(Sancho *et al.*, 2013). Human iPSC-CMs with 80% heteroplasmy for the A3243G mutation also exhibited lower mitochondrial respiration in a separate study.

Similarly to control hiPSC-CMs, mutant cells relied primarily on OXPHOS for energy production and had comparable basal glycolysis rates. A study using cybrid cells harbouring the A3243G mutation described that increased glycolysis (demonstrated by increased levels of extracellular lactate and pyruvate) and defective OXPHOS (demonstrated by decreased rates of ATP synthesis and O₂ consumption) contributed to the pathogenesis of the mutation (Cherry *et al.*, 2013; Folmes *et al.*, 2013; Ma *et al.*, 2015). Together, these results indicate that impaired mitochondrial respiration was not detected in mutant hiPSC-CMs due to low mutant load.

Mitochondrial DNA encodes 13 polypeptides involved in the ETC and so reduced mtDNA copy number can be used as a marker of mitochondrial dysfunction(Pallotti *et al.*, 2004). MtDNA copy number was unchanged in both A3243G mutant hiPSCs and in hiPSC-CMs. Studies using patient derived hiPSCs with the A3243G and G13513A mutations also reported that the mutations did not cause significant alterations in mtDNA copy number(Picard *et al.*, 2014). Although mtDNA copy number was normal, mtDNA transcription was found to be downregulated and instead contributing to mitochondrial dysfunction(Fujikura *et al.*, 2012; Folmes *et al.*, 2013; Hamalainen *et al.*, 2013). As such, mtDNA transcription is probably a better marker to assess mitochondrial dysfunction in the future. Using other methods of assessing mitochondrial function, for example fluorescent probes for monitoring mitochondrial membrane potential, would also be encouraged in the future.

6.4.2 Assessment of A3243G heteroplasmy levels

Initial experiments with the patient's somatic cells and with the first generated hiPSC clones indicated that the level of A3243G heteroplasmy was 20%. Quantification of heteroplasmy in the differentiated mutant hiPSC-CMs used for all studies presented in this and the previous chapter actually revealed that the levels fluctuated between 23 and 45% throughout different batches. However, higher levels of heteroplasmy were not associated with higher rates of glycolysis, lower ATP production and lower mtDNA copy number. Heteroplasmy levels remained constant between hiPSCs and related hiPSC-CMs but fluctuated over time during hiPSC culture. Results from other studies also reported minor variations in heteroplasmy levels over time in hiPSCs with the A3243G mutation(Hamalainen *et al.*, 2013). It is believed that the genetic bottleneck which happens during embryogenesis and leads to mtDNA diversity could be provoked during reprogramming or subsequent asymmetrical hiPSC divisions to generate a range of healthy and diseased clones and a shift in heteroplasmy(Fujikura *et al.*, 2012; Kodaira *et al.*, 2015; Perales-Clemente *et al.*, 2016). In the future it could be interesting to perform selection and expansion of hiPSC clones with higher mutant loads and to differentiate these cells to obtain CMs with impaired cardiac and mitochondrial properties for arrhythmia studies. In fact, some published studies reported the generation of hiPSC clones with a wide range of heteroplasmy levels after somatic cells with a specific mutant load were reprogrammed(Folmes *et al.*, 2013). For instance, Ma and colleagues have reprogrammed patient fibroblasts with 29% heteroplasmy for the A3243G mutation and reported generation of 10 hiPSC clones with the following heteroplasmy levels: 5 at 0%, 33%, 78%, 88 and 2 at 100%(Cherry *et al.*, 2013; Folmes *et al.*, 2013; Hamalainen *et al.*, 2013; Kodaira *et al.*, 2015; Ma *et al.*, 2015).

To date, segregation of mtDNA during embryogenesis is mostly believed to occur in a random fashion, although there is no clear understanding of the underlying process(Ma *et al.*, 2015).

Here, it was shown that over the course of 15 passages, levels of the A3243G mutation almost doubled in hiPSCs. The results suggest that perhaps segregation of mtDNA is not random and could have implications in mitochondrial replacement therapy where, although most of the mtDNA is left behind, pre-clinical experiments show that to maintain nuclear integrity a small percentage (2-4%) is carried over(Stewart and Chinnery, 2015).

The observed increase in A3243G mtDNA mutation over time in culture could be explained by the fact that mtDNA mutations confer mutant cells with a certain advantage over non-mutant, which will be discussed below. On another note, very recent preliminary experiments from our collaborators at UCL seem to indicate that levels of A3243G mutation in the parental fibroblasts also increase with passaging. Importantly, they report involvement of the AKT signalling pathway and elimination of the mutation when the pathway is inhibited. Interestingly, the AKT pathway regulates CM size, survival, angiogenesis and inflammation in cardiac hypertrophy(Hyslop *et al.*, 2016) and could well explain the hypertrophic phenotype of patients with the A3253G mutation and other mtDNA diseases. It would be interesting to investigate AKT pathway inhibition in our A3243G mutant hiPSCs.

6.4.3 Investigating cell competition between A3243G mutant hiPSCs and GFP+ control hiPSCs

The progressive increase in A3243G mutation observed over time in hiPSC culture could be attributed to some sort of selective advantage of mtDNA mutant hiPSCs. This is because mutant, or unfit, cells are usually eliminated by fitter cells in the first stages of development in a process known as cell competition(Aoyagi and Matsui, 2011). A study using cell lines containing the heteroplasmic A3243G mutation constructed by introducing mitochondria from MELAS patients into mtDNA depleted human cells (Rho zero) reported shifts towards the pure mutant genotype and pointed this to a replicative advantage of mutant mtDNA(Di Gregorio,

Bowling and Rodriguez, 2016). Perhaps prevalence of mtDNA mutations is important for maintenance of genetic diversity. Cell competition was first discovered in *Drosophila* and has been shown to be conserved in mammals (Yoneda *et al.*, 1992). A study found that mouse ESCs displaying defective bone morphogenetic protein signalling, defective autophagy or that were tetraploid were eliminated by wild-type cells (Di Gregorio, Bowling and Rodriguez, 2016). Here, GFP⁺ control hiPSCs were co-cultured with A3243 mutant hiPSCs to assess cell competition by measuring the proportion of each cell type over time by flow cytometry. Unfortunately, cell competition could not be assessed since mutant hiPSCs might have acquired cytoplasmic GFP particles transferred from control hiPSCs, becoming impossible to distinguish the two populations by flow cytometry. Besides the transfer of cytoplasmic particles, we must consider that GFP acquisition by mutant hiPSCs could have been due to fusion and/or clumping of GFP⁺ control with GFP⁻ mutant hiPSCs. However, we do think of this possibility as highly unlikely since the cells were filtered prior to flow cytometry analysis, which would have resulted in any clumps or fused cells to be excluded from the analysis. In the future, and to avoid the spread of green fluorescence, experiments should be repeated with a control GFP⁺ cell line where GFP has not been tagged to proteins expressed in the cytoplasm. It is peculiar though that authors of the mouse ESC competition study did not experience such a phenomenon. There is no indication that the transfer of cytoplasmic material is unidirectional (just control to mutant hiPSCs) but it could suggest that mitochondria are being transferred from control hiPSCs to mutant hiPSCs to support mitochondrial function of the less fit cells.

As described in section 1.2 of the Introduction, transport of mitochondria between cells *in vitro* is thought to be mediated by TNTs and to have an important role in preventing mitochondrial dysfunction (Sancho *et al.*, 2013). Transfer of mitochondria from mice astrocytes to neighbouring neurons after stroke has been shown to improve neuron's viability, mitochondrial membrane potential, oxygen consumption and ATP production (Berridge *et al.*, 2016). In

addition, rho zero human lung carcinoma cells regained mitochondria and aerobic respiration was restored when co-cultured with hMSCs (Berridge, Schneider and McConnell, 2016) and repair of rotenone injured mouse tracheal epithelial cells was promoted by transfer of mitochondria from mouse mMSCs (Spees *et al.*, 2006). In the future, further investigations could be performed by GFP labelling of mitochondria or the use of fluorescent mitochondrial probes.

6.5 Summary

In this chapter the mitochondrial properties of A3243G mutant hiPSC-CMs were evaluated. All parameters of mitochondrial respiration, including ATP production, and mitochondrial copy number were unchanged in mutant hiPSC-CMs, which was linked to low mutant load. Levels of heteroplasmy in different batches of mutant hiPSC-CM varied between 23 and 45%. Variation was attributed to mtDNA segregation occurring during asymmetrical hiPSC divisions which generates hiPSC clones with a wide range heteroplasmy levels. In the future, it would be interesting to attempt to isolate hiPSC clones with higher mutant loads for arrhythmia studies. Although mtDNA segregation is believed to be random, it was seen here that the A3243G mutation almost doubled in hiPSCs over the course of 15 passages. It was suggested that mtDNA mutant hiPSCs could have some sort of selective advantage that prevents their elimination and that this permits a higher genetic diversity. These findings could impact on mitochondrial replacement therapy, where small amounts of mutant mtDNA are carried over. Very recent preliminary data from our collaborators indicated that the AKT pathway could be responsible for sustained mtDNA mutant load so it would be interesting to investigate whether blocking this pathway eliminates the A3254G mutation in hiPSCs in culture. Finally, we observed a possible transfer of cytoplasmic material and/or mitochondria, which occurred from control to mutant hiPSCs, could indicate that mitochondria are transferred via TNTs to support

the function of less fit cells with mtDNA mutations. GFP labelling of mitochondria or the use of fluorescent mitochondrial probes would facilitate future investigations.

Chapter 7: General Discussion

7.1 Overview of key findings

The principal aim of this thesis was to develop an *in vitro* model of heteroplasmy using hiPSC-CMs, to understand the mechanisms behind the cardiac abnormalities present in patients with mtDNA diseases.

Given the immature phenotype of hiPSC-CMs, including metabolism, in Chapter 3 we applied maturation strategies with the aim of achieving a maximal/mature OXPHOS capacity in hPSC-CMs. This would allow us to determine whether this metabolic state was compromised in the *in vitro* models of heteroplasmic mitochondrial dysfunction to be developed in hiPSC-CMs. Prolonged time in culture did not improve mitochondrial respiration in CDI hiPSC-CMs. Treatment of CDI hiPSC-CMs and IMR90-4 hiPSC-CMs with T_3 did not improve mitochondrial respiration but treatment of H7 hESC-CMs with T_3 for 1 month caused increases in all parameters of mitochondrial respiration. Since H7 hESC-CMs express high levels of D3, which inactivates T_3 , we thought that bypassing the D3 pathway with the molecules IOP and GC-1 would cause a higher increase in mitochondrial respiration but it was not different to T_3 alone. Treatment of T_3 to both CDI hiPSC-CMs and H7 hESC-CMs did not cause a change in mitochondrial copy number but this was significantly lower in CDI hiPSC-CMs compared to H7 hESC-CMs. It was concluded that maturation strategies would not be employed in future models of mitochondrial heteroplasmy using hiPSC-CMs.

There is low availability of samples from patients with mtDNA diseases and there are challenges associated with differentiating hiPSCs with high mtDNA mutant loads to CMs. In Chapter 4 we aimed to apply the drugs antimycin A and rotenone, which impair the ETC and

increase the production of ROS, to generate a model of heteroplasmy using CMs from the control line IMR90-4 hiPSCs. Heteroplasmy models were generated by mixing hiPSC-CMs previously treated with either drug with non-treated hiPSC-CMs. Mitochondrial respiration of untreated, mixed and 100% treated hiPSC-CMs was assessed. The two drugs impair mitochondrial respiration when the Mito Stress Test is conducted straight after treatment. However, although the mixing ratios were maintained, when the test was conducted 2 (antimycin A) or 5 (menadione) days after treatment and mixing, mitochondrial respiration was similar between untreated, mixed and 100% treated hiPSC-CMs. After 5 days of treatment with menadione, the levels of the antioxidant enzyme SOD2 were also similar between the three groups. SOD2 levels were also comparable to foetal heart tissue and lower than in adult heart tissue. It was concluded that antimycin A and menadione could not be applied to generate a model of heteroplasmy and that the best strategy would be to differentiate hiPSCs generated from reprogramming somatic cells of patients with mtDNA mutations.

Fibroblasts of a diabetic patient with the A3243G mtDNA mutation were reprogrammed to hiPSCs and these were obtained through a collaboration. Heteroplasmy levels in the fibroblasts and in the first generated hiPSC colonies were 20%. In Chapter 5 we showed that cardiac differentiation of mutant hiPSCs generated pure populations of troponin T-positive beating CMs and performed their characterisation. Cell size and shape were unchanged in mutant hiPSC-CMs, along with beating rate, response to a β -adrenergic and to a muscarinic agonist, time to peak contraction and time to 50% relaxation. Most Ca^{2+} handling properties were maintained in mutant hiPSC-CMs but the cells showed a marked delay in the initiation of the Ca^{2+} transient. Lack of a disease phenotype could have been caused by the low mutant load or by an adaptation of the CMs to a certain level of mitochondrial dysfunction. Therefore, mitochondrial properties of mutant hiPSC-CMs were assessed in Chapter 6.

Mutant hiPSC-CMs primarily relied on OXPHOS. All parameters of mitochondrial respiration, basal glycolysis and mitochondrial copy number were unaffected in mutant hiPSC-CMs. The levels of the A3243G mutation ranged between 23 and 45% in the batches of mutant hiPSC-CMs used for experiments in Chapters 5 and 6. However, higher mutant loads were not associated with higher rates of glycolysis, lower ATP production and lower mtDNA copy number. It was also observed that passaging of hiPSCs caused heteroplasmy to almost double, over the course of 15 passages. It was not possible to determine whether the mutation accumulated due to a selective advantage by these cells since there was transfer of GFP+ cytoplasmic material, and possible mitochondria, to mutant hiPSCs from the GFP+ control hiPSCs used for cell competition studies.

7.2 Implications

The work presented here has several implications on the stem cell, cardiac and mitochondrial fields.

Human PSC-CMs hold great promise in the areas of disease modelling, drug discovery and regenerative medicine. However, two limitations of this technology are the high variability between different hPSC lines and the immature phenotype of hPSC-CMs. Opposing responses of different hPSC-CM lines to maturation strategies highlighted the importance of addressing variability when conducting studies with hPSCs. Data validation using hPSCs generated from different donors is crucial to ensure that studies can be reproduced. Maturation strategies, including prolonging time in culture and application of T_3 , can improve certain features of hPSC-CMs. Though, it was demonstrated that these strategies did not alter the energy phenotype or mitochondrial copy number in several hPSC-CMs lines and it was discussed that metabolic maturation of hPSC-CMs would require a shift from glucose/lactate

metabolism to fatty acid oxidation. This could be attempted by substrate modulation but, due to the dependence of hPSC-CMs on glucose, it seems unlikely that the metabolic phenotype of the adult heart can be replicated *in vitro*.

Models of heteroplasmy to study pathogenesis of mtDNA diseases in CMs are lacking. The mitochondrial drugs antimycin A and menadione have been shown to impair mitochondrial respiration in hiPSC-CMs, when this is assessed straight after their application. Here, antimycin A and menadione could not be used to generate a model of heteroplasmy in hiPSC-CMs as within a few days mitochondrial function had recovered from the drug treatments. Mitophagy and mitochondrial biogenesis replace 3-4% of the mitochondrial population at basal conditions in rat CMs and this is further increased following stress. Therefore, it was hypothesised that mitochondrial turnover was responsible for the elimination of damaged mitochondria in hiPSC-CMs. If this proves to be true in the future, it will provide important information about the response of CMs to oxidative stress and to mitochondrial dysfunction in the regulation of cell fitness.

The pathogenesis of the cardiac abnormalities associated with patients with mtDNA mutations, including the A3243G, is unknown. It was concluded here that the best strategy to investigate this would be to generate CMs from hiPSCs reprogrammed from somatic cells of patients with mtDNA mutations. Results indicate that heteroplasmy levels higher than 23-45% will be required for an impairment of most cardiac and mitochondrial properties of A3243G mutant hiPSC-CMs. Still, the delay in the initiation of the Ca^{2+} transient observed in mutant hiPSC-CMs has important implications as could be the underlying mechanism to the bradyarrhythmias that are observed in some patients with mtDNA mutations. The increase in the A3243G mutation that occurred during hiPSC passaging is implicated on mitochondrial replacement therapy, where a small amount of mutant mtDNA is carried over. It is possible

that the mutant load increased due to a selective advantage of mutant mtDNA or high activity of the AKT pathway. If this proves to be true in the future, it could contradict the model of random mtDNA segregation.

7.3 Strengths and Limitations

A major limitation of this study is the fact that only one patient-derived mtDNA mutant hiPSC line was available. Our lab had access to two other mtDNA mutant hPSC lines (A3243G and T8993G, both 100% mutant) but their maintenance was a big challenge and their cardiac differentiation was not achievable. A group generated cardiomyocytes from A3243G hiPSCs with 83% heteroplasmy but no other studies have reported successful generation of cardiomyocytes from cells with higher mutant loads. We are aware that the low mutant load of the line used was most likely responsible for the absence of a disease phenotype in culture. In addition, the variability between different hPSC lines could also impact on the reproducibility of our findings with other lines.

Small molecule modulation of the Wnt/ β -catenin signalling pathway for cardiac differentiation of hPSCs was a big advantage. This method enabled the generation of mutant hiPSC-CMs at high efficiencies. The use of hPSC-CMs also presents strengths and limitations. The immature phenotype of these cells was discussed in depth before and logically restricts the translation of our findings to adult human physiology. However, the differences between hPSC-CMs and adult CMs in culture were also beneficial. In contrast to adult CMs, hPSC-CMs can be maintained in culture for long periods of time and can form a confluent, electrically coupled monolayer which facilitates electrophysiological and arrhythmia studies.

A great advantage of having mostly used the Mito Stress Test, performed using a Seahorse Analyser, to assess mitochondrial function in hPSC-CMs is that it provides the most information about mitochondrial function in live cells. However, we are aware that this technique only takes O₂ consumption into account, which is not truly representative of all aspects of mitochondrial function. In addition, the technique is very variable and so makes it very difficult to account for subtle changes in mitochondrial function.

Patient-derived hiPSCs can be obtained from patients with all types of mutations and share the same nuclear and mitochondrial genome. This is not possible with other mtDNA disease models. In addition, the cells can be differentiated to other cell types, including CMs which are not easily available. As a final limitation, it must be said that the available methods of quantifying mtDNA mutant load, including ARMS qPCR used here, are not very precise. To date, only the average level of heteroplasmy in a lysate of cells can be measured. It is still not possible to detect mtDNA mutations at the mitochondrion level and being able to do this would be very important for modelling arrhythmias. Heterogeneity between cardiomyocytes has been shown to be involved in the occurrence of arrhythmias, hence it would be crucial to understand if heterogeneity in mitochondrial function caused by differences in heteroplasmy between adjacent cells has an impact in the arrhythmogenic potential of cardiac cells.

7.4 Future directions

The work presented here opened up several new lines of investigation. Manipulation of the energy substrate provided during generation and maintenance of hPSC-CMs will be very important to understand if fatty acid oxidation as the main source of OXPHOS production is achievable in these cells. This can be attempted by gradual elimination of glucose together

with fatty acid supplementation. It will also be interesting to determine whether mtDNA mutant hiPSC-CMs can utilise fatty acids instead of glucose.

To understand the mechanisms behind the recovery of hiPSC-CMs to oxidative stress, it would be interesting to block one or more components of mitochondrial dynamics. Mitochondrial dynamics is comprised of biogenesis, fission, fusion and mitophagy, which work together to ensure mitochondrial quality control. For instance, blocking the PGC family of transcriptional coactivators (regulators of biogenesis) would be a possibility. This knowledge would be critical for the development of new models of heteroplasmy based on healthy hiPSC-CMs.

For the development of new models of heteroplasmy based on patient-derived hiPSC-CMs, it is critical that hiPSCs with higher mutant loads are obtained for CM generation, characterization and arrhythmia studies. This can be achieved by reprogramming patient-derived somatic cells with high mutant loads, if these are available. Instead, clonal selection of the available A3243G hiPSC line could be attempted. Segregation of mtDNA and shifts in heteroplasmy are thought to occur during hiPSC divisions. Individual colonies can be separately expanded and cardiac differentiation performed on those with high mutant loads, as assessed by ARMS qPCR. As an increase in heteroplasmy was observed at higher passages, further passaging of A3243G mutant hiPSCs is another option.

Understanding the mechanisms behind the increase in the A3243G mtDNA mutation with passaging is a critical future investigation. Since preliminary data from our collaborators suggest that the AKT pathway is responsible for maintenance of high mutant loads, it would be very relevant to block the AKT signalling pathway in hiPSCs and assess whether the mutation is eliminated. Equally, competition studies of mtDNA mutant hiPSCs with a new GFP⁺ control line would help elucidate whether the mutation is maintained due to a selective

advantage by the mutant mtDNA. Together, these investigations could provide important insights into the pattern of mutant mtDNA segregation.

7.5 Conclusion

In this thesis we have demonstrated the ability of using hiPSC-CMs to investigate the cardiac abnormalities associated with mtDNA disease. Our work highlighted the importance of addressing the variability between different hiPSC lines for disease modelling applications. It was also shown here that available maturation strategies are not sufficient to generate adult-like hiPSC-CMs, also in terms of metabolic activity. However, we illustrated that immature hiPSC-CMs can provide important information about the plasticity of cardiomyocytes, in particular in response to oxidative stress. It can also be concluded that high levels of heteroplasmy are necessary to detect a phenotype in mtDNA mutant hiPSC-CMs and that in contrast to what is currently known, segregation of mtDNA might not occur in a random fashion. Future work will help to elucidate this and will provide crucial information about the pathogenesis of mtDNA disease.

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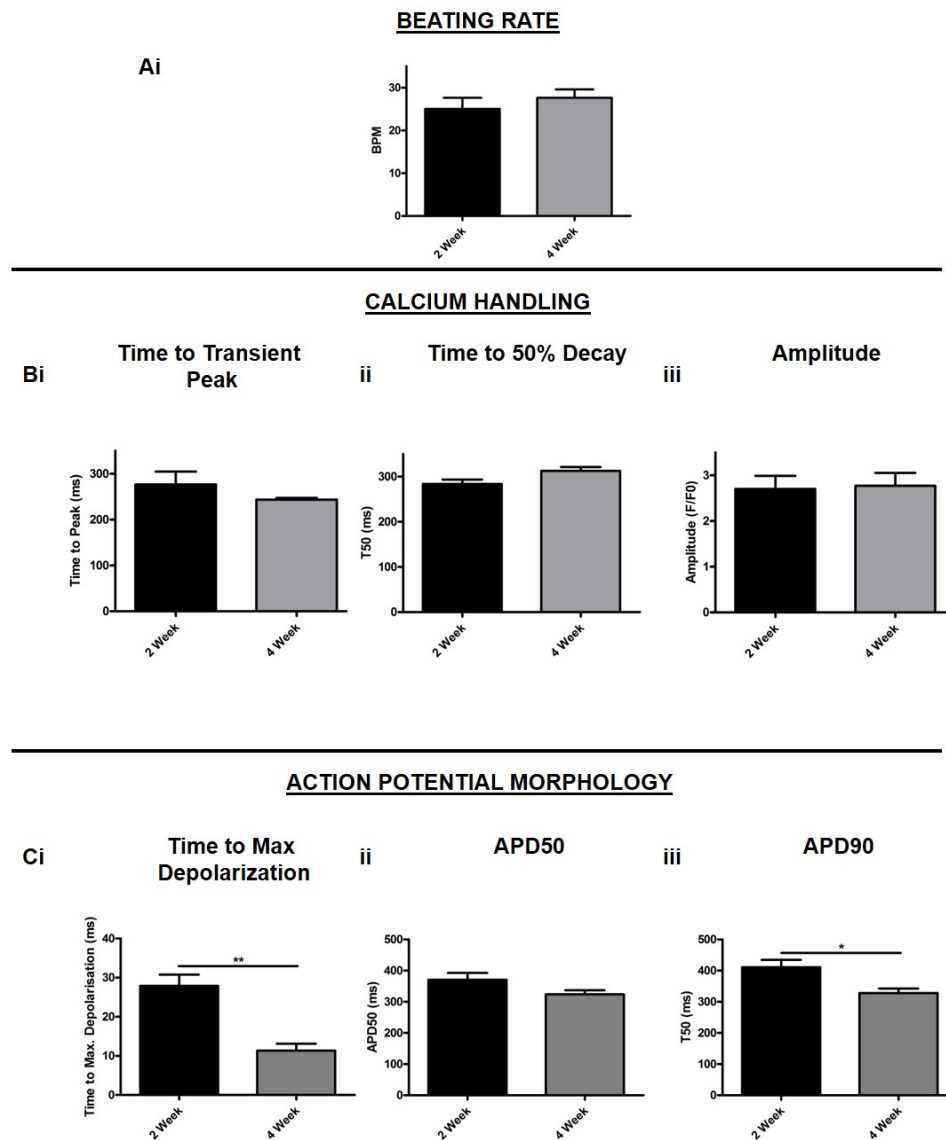
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Appendix

Figure A1: Functional properties of CDI hiPSC-CMs kept in culture for 2 weeks and 1 month.



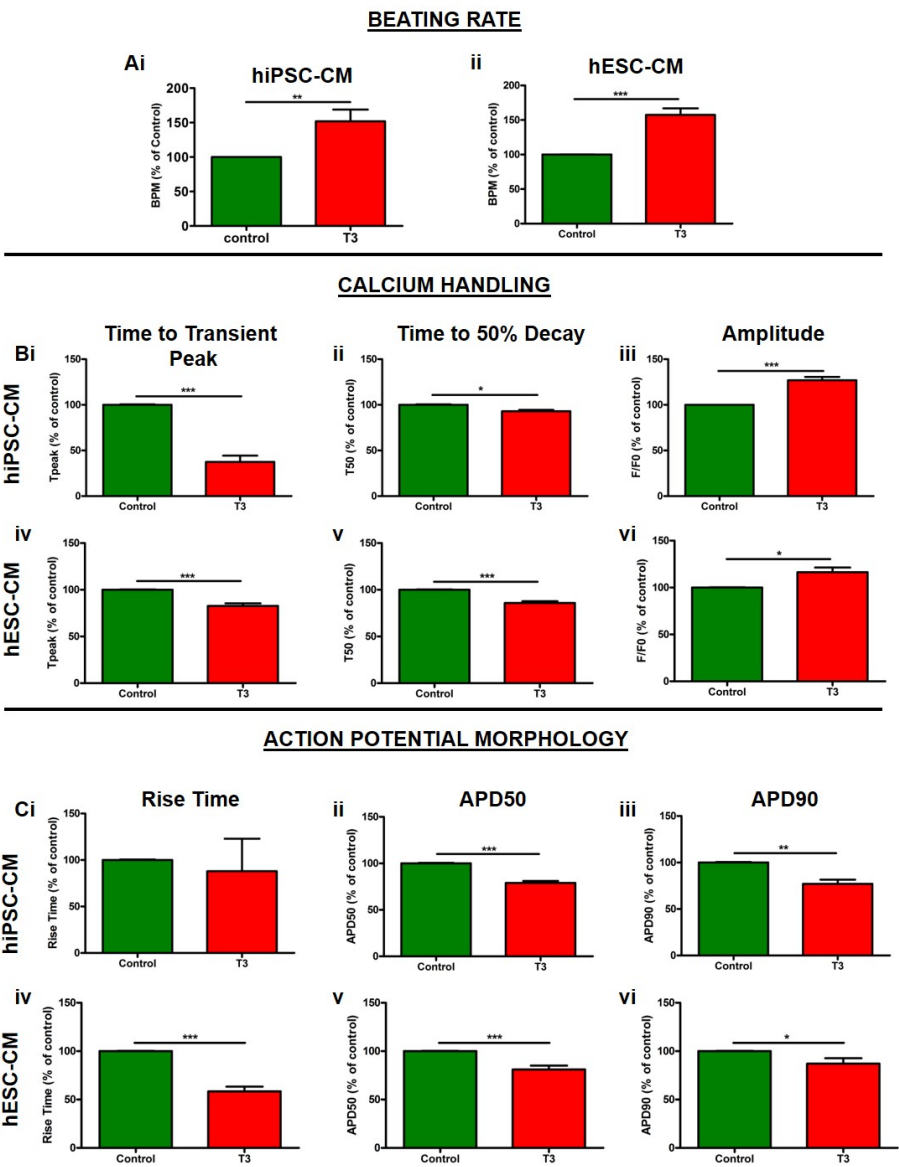
Data by Nicola Hellen.

Figure A2: Top pathways of proteins involved when CDI hiPSC-CMs were kept in culture for 1 month, compared to 2 weeks.

Pathway	P value	No. Proteins ↑	No. Proteins ↓
Oxidative phosphorylation	2.75E-18	21	0
Glycolysis1	3.00E-11	0	9
Granzyme A signalling	6.40E-9	1	6
Protein kinase A signalling	8.05E-9	5	18
tRNA charging	5.56E-8	0	8

Data by Nicola Hellen.

Figure A3: Functional properties of CDI hiPSC-CMs and H7 hESC-CMs following 1 month treatment with T₃.



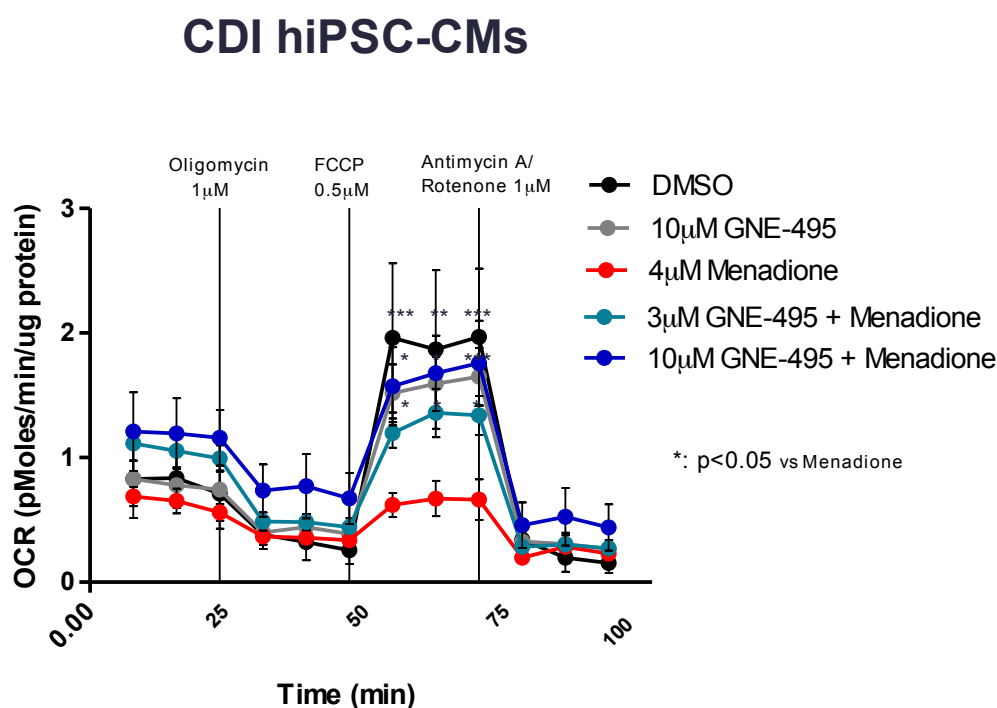
Data by Nicola Hellen.

Figure A4: Top pathways of proteins involved following 1 month treatment of CDI hiPSC-CMs with T₃.

Pathway	P value	Proteins ↑	Proteins ↓
Mitochondrial dysfunction	2.52E-33	44	2
Oxidative phosphorylation	1.88E-32	37	1
Signalling by Rho family GTPases	2.04E-11	2	27
Epithelial adherens junction signalling	4.32E-11	1	21
Rho A signalling	6.95E-11	1	19

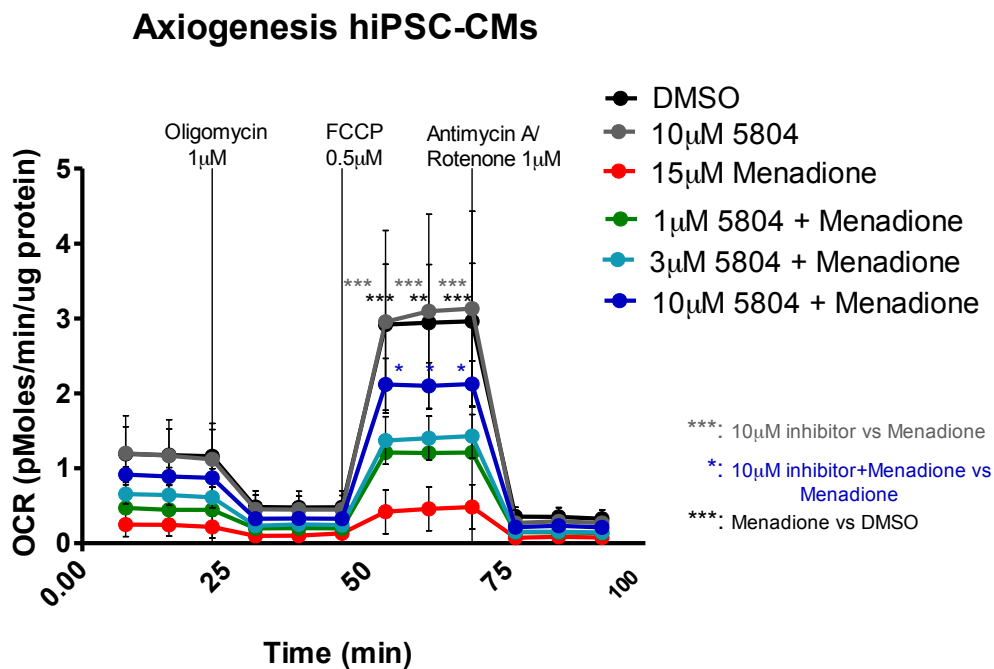
Data by Nicola Hellen.

Figure A5: Mito Stress Test of CDI hiPSC-CMs treated for 2 hours with 4μM menadione.



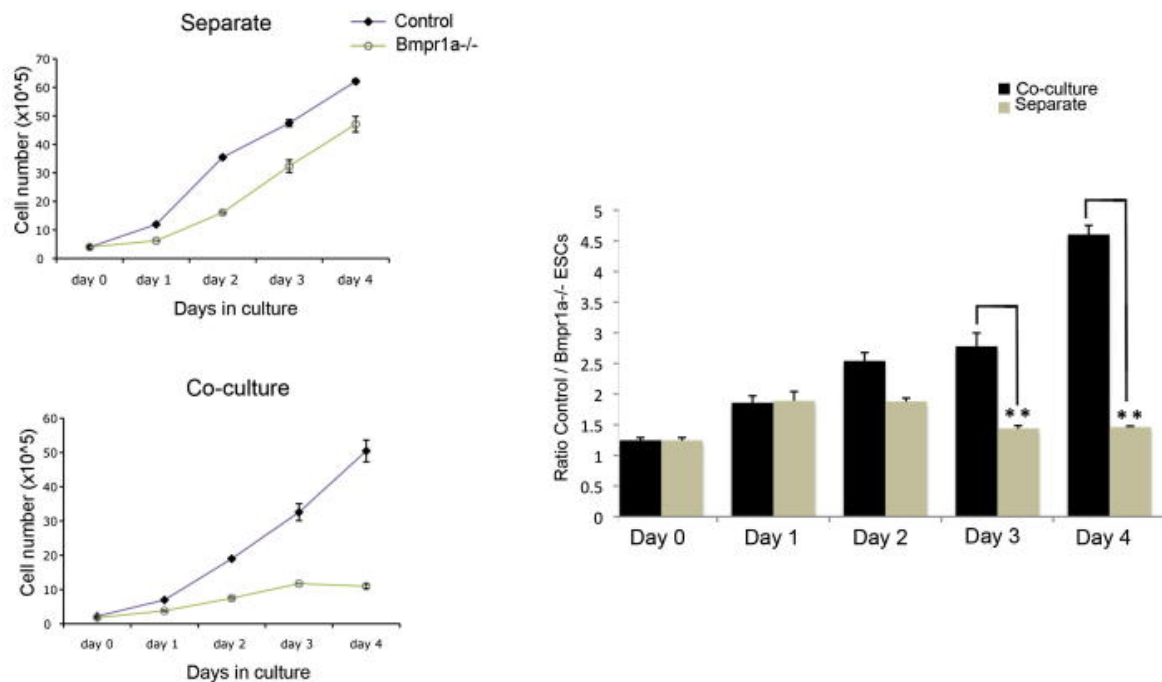
Data by Micaela Jenkins.

Figure A6: Mito Stress Test of Axiogenesis hiPSC-CMs treated for 2 hours with 15 μ M menadione.



Data by Micaela Jenkins.

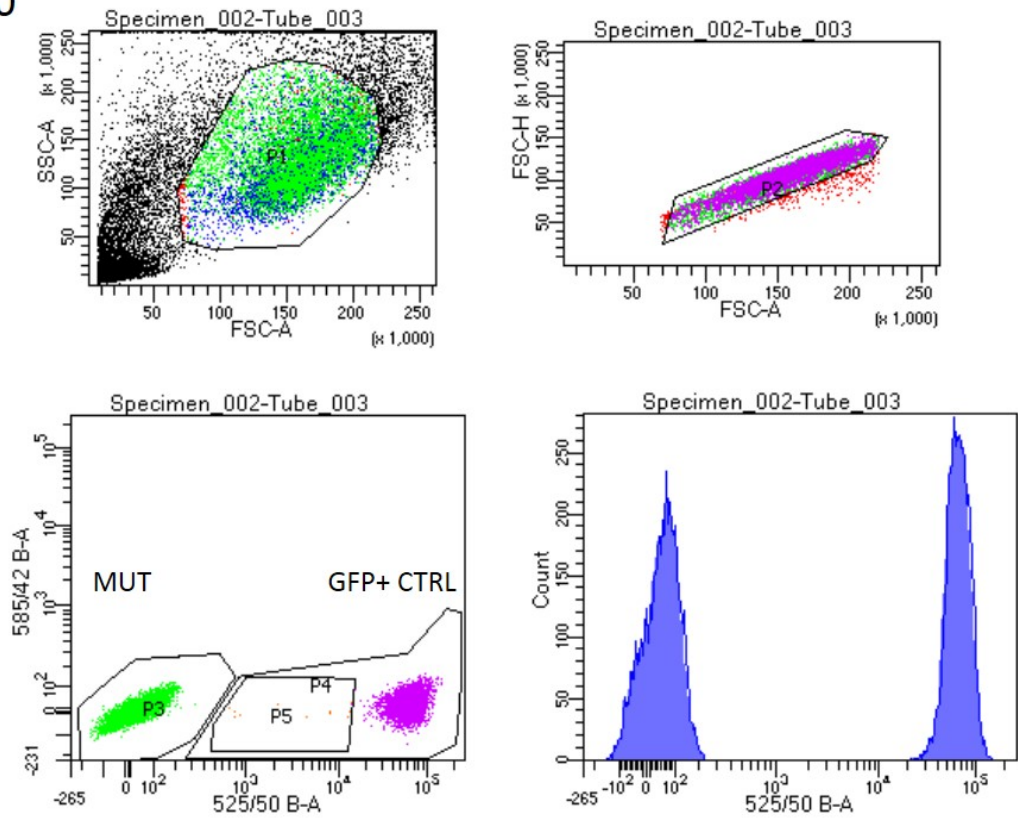
Figure A7: Elimination of mouse ESCs with defective BMP signalling in the presence of wild-type mouse ESCs.



From (Ahmad *et al.*, 2014).

Figure A8: Cell competition assay - Co-culture day 0.

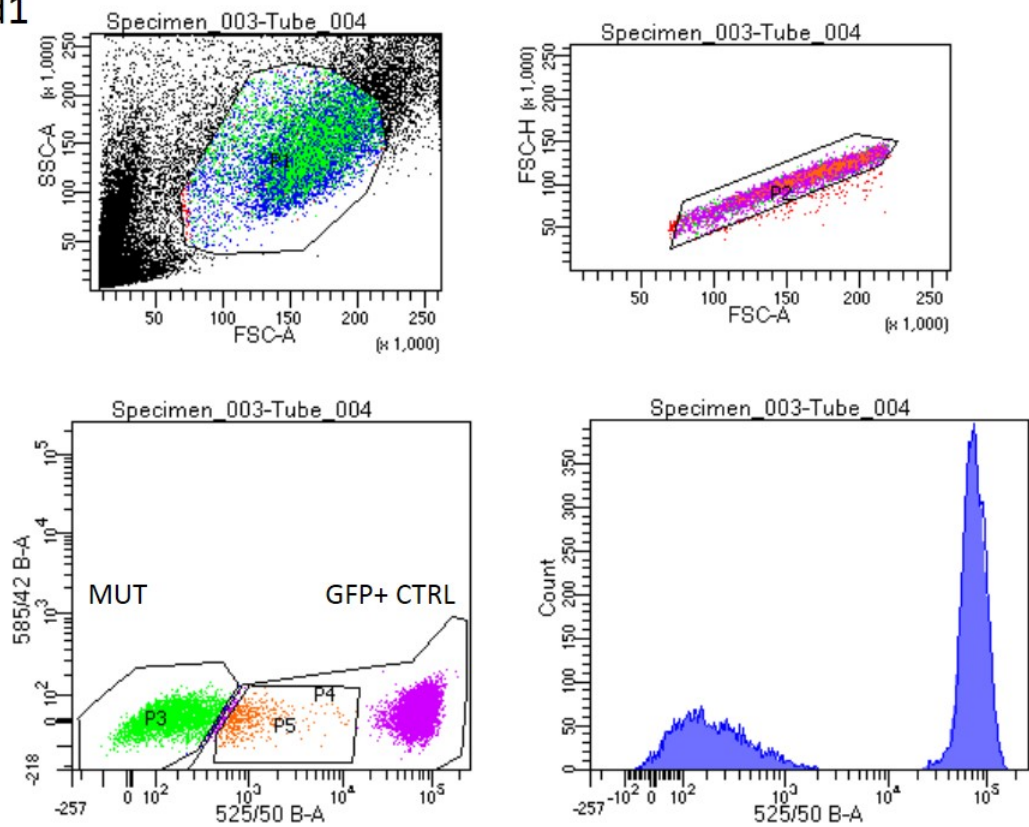
d0



Tube: Tube_003			
Population	#Events	%Parent	%Total
■ All Events	23,501	####	100.0
■ P1	10,369	44.1	44.1
■ P2	10,000	96.4	42.6
■ P3	5,200	52.0	22.1
■ P4	4,799	48.0	20.4
■ P5	11	0.1	0.0

Figure A9: Cell competition assay - Co-culture day 1.

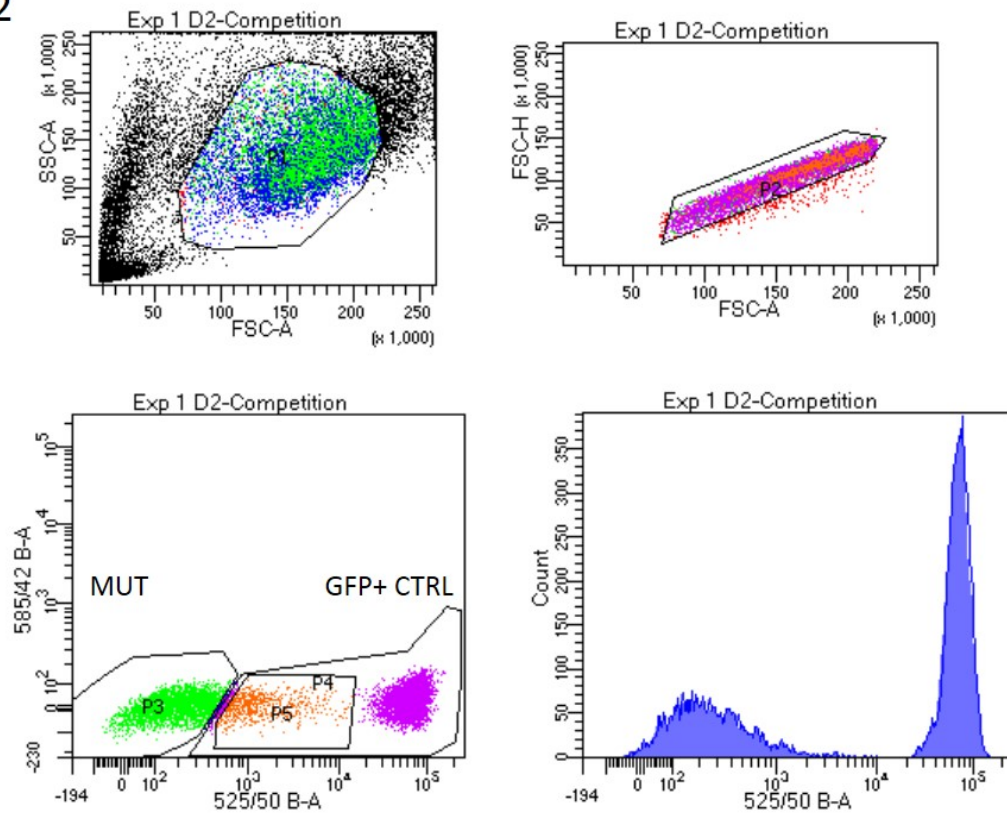
d1



Tube: Tube_004			
Population	#Events	%Parent	%Total
All Events	37,075	####	100.0
P1	10,186	27.5	27.5
P2	10,000	98.2	27.0
P3	2,928	29.3	7.9
P4	7,022	70.2	18.9
P5	429	4.3	1.2

Figure A10: Cell competition assay - Co-culture day 2.

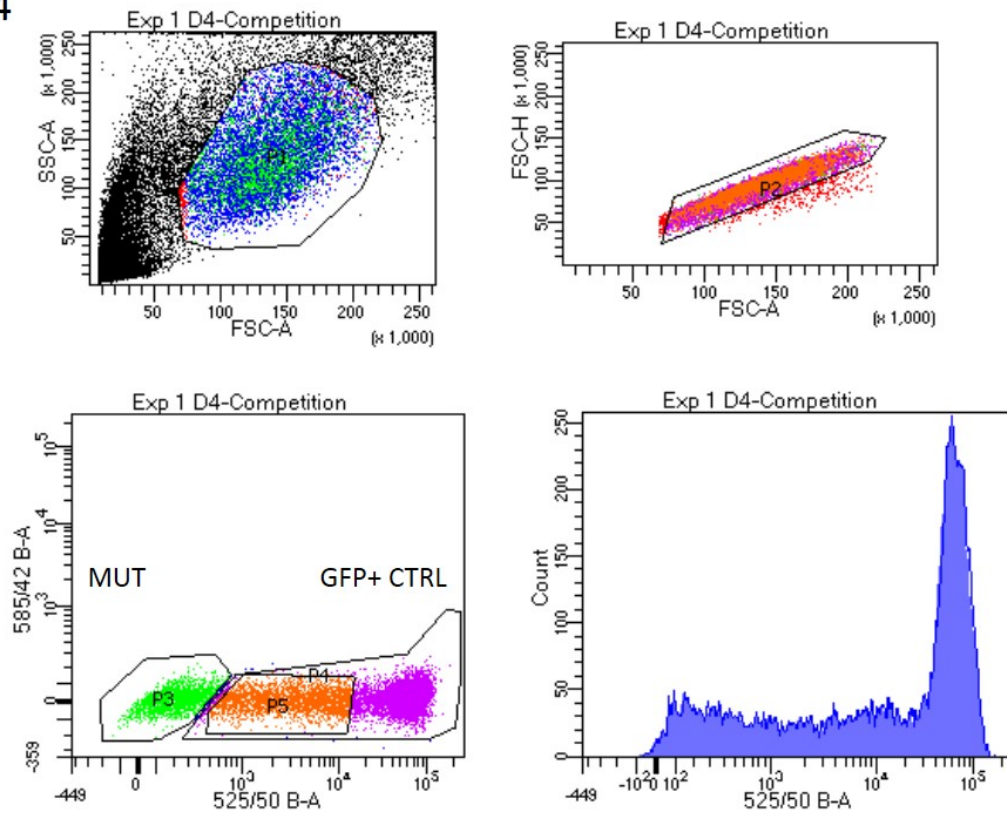
d2



Tube: Competition			
Population	#Events	%Parent	%Total
■ All Events	21,093	####	100.0
■ P1	10,338	49.0	49.0
■ P2	10,000	96.7	47.4
■ P3	3,058	30.6	14.5
■ P4	6,879	68.8	32.6
■ P5	634	6.3	3.0

Figure A11: Cell competition assay - Co-culture day 4.

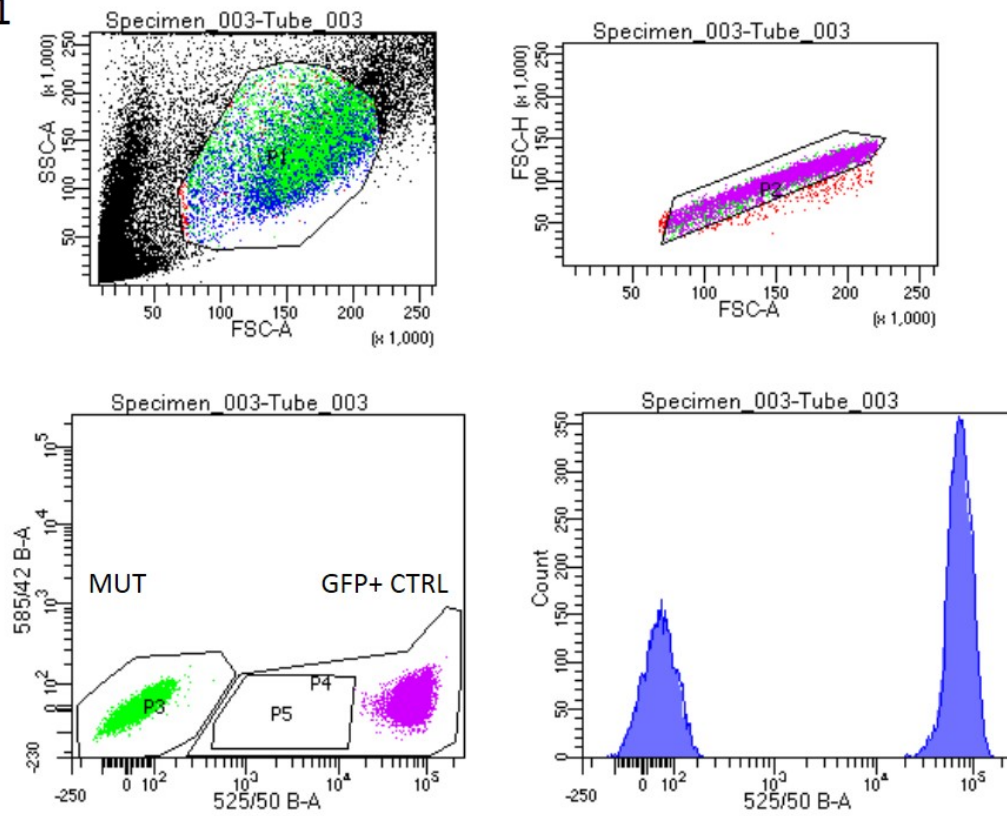
d4



Tube: Competition			
Population	#Events	%Parent	%Total
■ All Events	29,013	####	100.0
■ P1	10,497	36.2	36.2
■ P2	10,000	95.3	34.5
■ P3	1,668	16.7	5.7
■ P4	8,254	82.5	28.4
■ P5	2,542	25.4	8.8

Figure A12: Cell competition assay - Separate culture day 1.

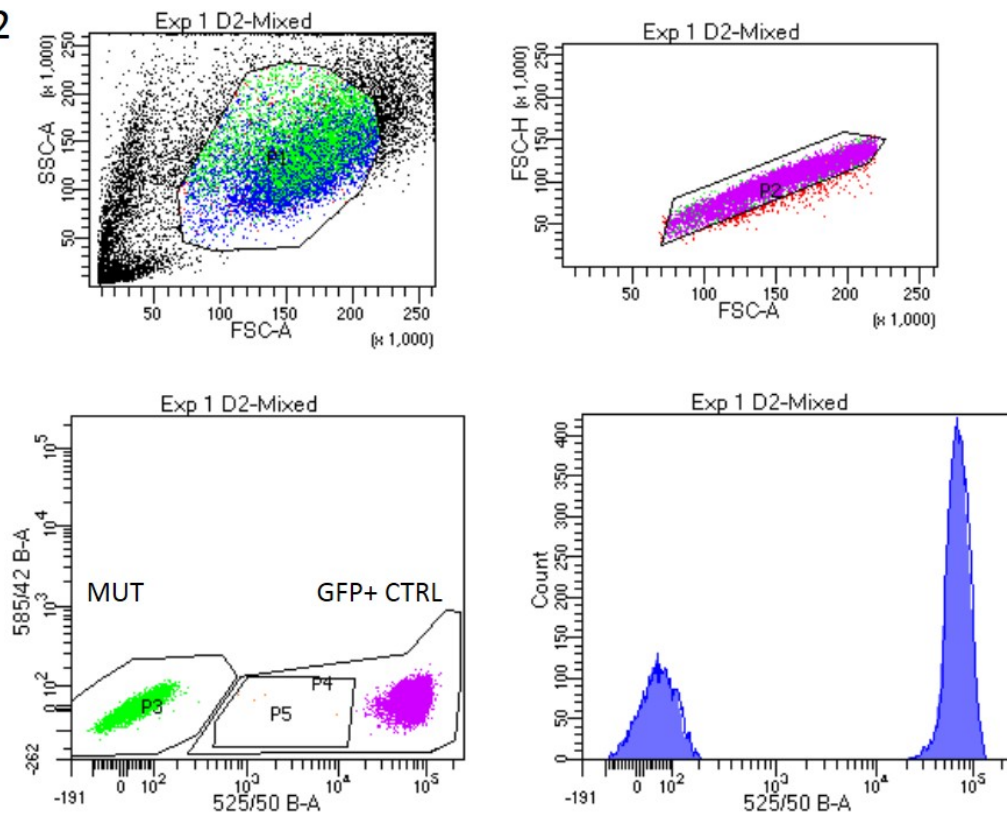
d1



Tube: Tube_003			
Population	#Events	%Parent	%Total
■ All Events	45,091	####	100.0
■ P1	10,323	22.9	22.9
■ P2	10,000	96.9	22.2
■ P3	3,742	37.4	8.3
■ P4	6,258	62.6	13.9
■ P5	0	0.0	0.0

Figure A13: Cell competition assay - Separate culture day 2.

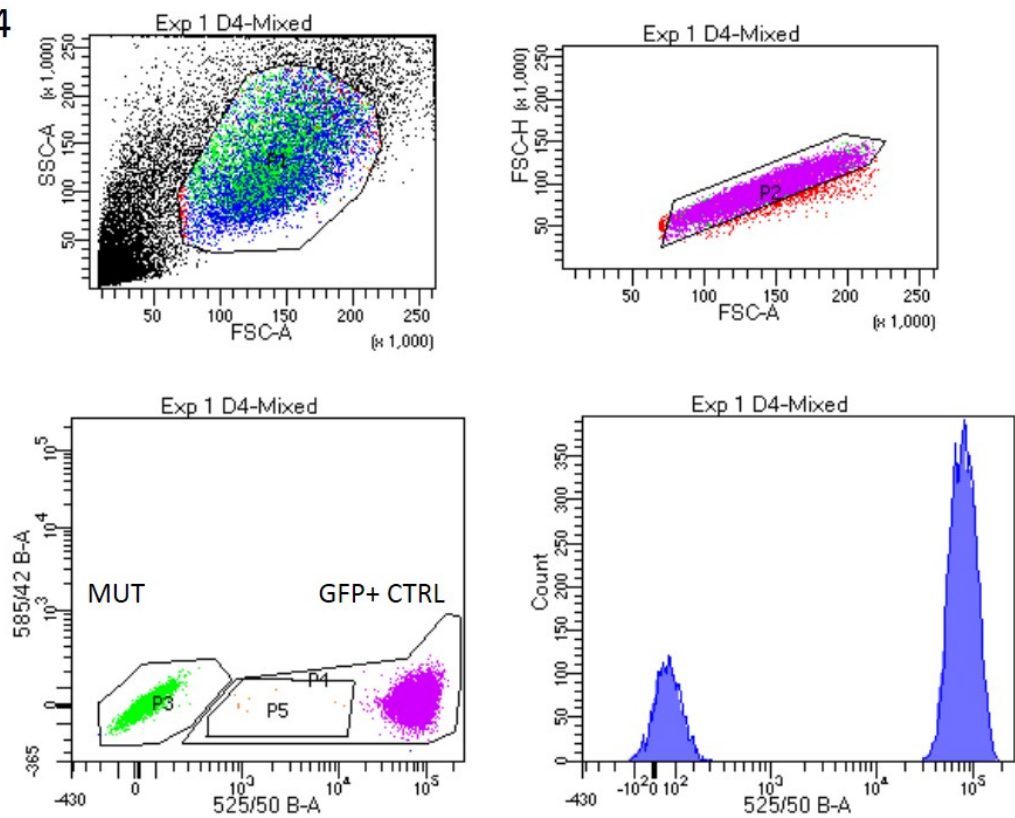
d2



Tube: Mixed			
Population	#Events	%Parent	%Total
■ All Events	18,778	####	100.0
■ P1	10,364	55.2	55.2
■ P2	10,000	96.5	53.3
■ P3	3,204	32.0	17.1
■ P4	6,796	68.0	36.2
■ P5	4	0.0	0.0

Figure A14: Cell competition assay - Separate culture day 4.

d4



Tube: Mixed			
Population	#Events	%Parent	%Total
■ All Events	23,596	####	100.0
■ P1	10,629	45.0	45.0
■ P2	10,000	94.1	42.4
■ P3	2,360	23.6	10.0
■ P4	7,638	76.4	32.4
■ P5	10	0.1	0.0