Organs-on-chip: towards therapies for cardiovascular disease using human stem cells

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CHAPTER 1

Towards therapies for mitochondrial cardiomyopathies using advanced human stem cell models

INTRODUCTION

The heart has been central to much symbolism throughout the history of human culture, sometimes being seen as the location of the soul and the origin of the emotional and intellectual ratio (Figueredo, 2021). This metaphorical role of the heart is reflected in its vital anatomical role, connecting tissues and organs throughout the body and supplying them constantly with oxygen and nutrients while shuttling away carbon dioxide and waste products. The heart's vital role is demonstrated by the fact that it is the first organ to form during organogenesis during embryological development. Cardiomyocytes in the linear heart tube derived from the first heart field (FHF) initiate circulation during the fourth week of gestation (Buijtendijk *et al.,* 2020; Schoenwolf *et al.,* 2014). While these foetal cardiomyocytes are capable of contraction, propagating action potentials and contributing to the foetal circulation, they are just a prelude to what they will become postnatally. Throughout the foetal stage of development, cardiomyocytes remain smaller than their adult counterparts and do not show the same hallmark intracellular striations of sarcomeres and mitochondria (Ulmer & Eschenhagen, 2020). The morphology rapidly changes postnatally due many anatomical, structural and metabolic changes in the heart as a whole. The postnatal anatomical changes of the heart, the increased workload on the left ventricle compared to the right, combined with new environmental conditions and the increasing circulatory demand of the growing body, necessitate rapid adaptation of the cardiomyocytes around birth, a process commonly referred to as maturation.

For a short period, the initial increase in cardiac output is facilitated by hyperplasia, similar to what occurs during foetal development. However, postnatal cardiomyocytes quickly lose the ability to proliferate, and the influx of newly differentiated cardiomyocytes ceases. Subsequent cardiac mass and output increases are achieved through cardiomyocyte hypertrophy (enlargement of individual cells) and the influx and proliferation of non-myocytes. In the first year, postnatally, the heart triples in weight. However, this growth is not proportional to the rest of the body, which grows more rapidly (Hew & Keller, 2003). Due to the heart becoming relatively smaller compared to the rest of the body, an increase in cardiac efficiency is required to maintain sufficient output. This increase in contractile efficiency of the cardiomyocytes is achieved through a rapid rise in the organisation of the sub-cellular architecture, resulting in more efficient excitation-contraction coupling and energy metabolism. The regulation of energy metabolism and the efficiency of the contractile apparatus are inherently linked, and dysregulation of either process results in the adaptation of the other. How cardiomyocytes regulate this energy metabolism, how we can manipulate this in cultured cardiomyocytes and what happens to the heart when genetic or other disease disrupt normal energy availability and use, is the subject of this thesis.

METABOLIC AND MITOCHONDRIAL DEVELOPMENT OF THE HEART

The increased sub-cellular organisation in adult cardiomyocytes compared to foetal cardiomyocytes is evident at the macro cellular level, with the shape of cardiomyocytes changing from polygonal towards elongated rod- or "brick-"like. The myofibres are organised along the long axis of the cardiomyocytes, creating a force of contraction in the same direction. Additionally, after birth, when hyperplasia is no longer the driving force underlying increased cardiac output, hypertrophic growth increases the size of cardiomyocytes by more than 15 times as they move towards their adult maturation state (Fig. 1A). Together, this results in an enormous increase in the force of contraction exerted by each individual cell.

During the hypertrophic phase, there are major changes in the organisation of the myofibres, sarcoplasmic reticulum (SR) and mitochondria at the subcellular level. Most of the intracellular space in adult cardiomyocytes is occupied, with very little free cytoplasm. The nucleus, the aligned myofibres and SR, and the large mitochondria directly connected to the sarcomeres and SR occupy the most space, leaving less than 2% of the cytoplasm free (Ventura-Clapier *et al.,* 1998).

These changes greatly impact what drives the cardiomyocyte towards being such an efficient generator of force but also greatly impact how cardiomyocytes supply their subcellular organelles with sufficient ATP to ensure proper function.

In foetal cardiomyocytes, ATP is mostly generated by glycolysis or lactate oxidation in the mitochondria, and the subcellular organelles receive this ATP primarily via passive diffusion (Anmann *et al.,* 2014; Lopaschuk & Jaswal, 2010). After birth, oxygen saturation increases, and the substrate availability for the heart changes, with glucose and lactate availability decreasing while the concentration of free fatty acids (FFAs) increases. These changes drive the heart to shift rapidly towards an increased reliance on oxidative phosphorylation (OXPHOS), specifically, the oxidation of FFAs (beta-oxidation), caused by changes in several metabolic pathway regulators. After birth, the HIF-1α pathway, important in regulating the maintenance of anaerobic glycolysis and active during the foetal development of the heart, is inhibited, while simultaneously, the PGC-1α/PPARα and PGC-1α/PPAR-ß/δ pathways increase in activity. These pathways are the master regulators of fatty-acid beta-oxidation; they act by increasing the translation of fatty-acyl dehydrogenases and carnitine palmitoyl transferases (Lopaschuk & Jaswal, 2010). Beta-oxidation occurs within the mitochondria, with each cycle shortening the fatty acids by two carbons, yielding one $FADH_2$, one NADH and one acetyl-CoA. The oxidation of fatty acids allows the heart to use an energy source which carries more potential energy, with long-chain fatty acids potentially generating 0.5 moles of ATP per gram. In comparison, glucose only generates 0.21 moles of ATP per gram. However, the efficacy of this process relies on a constant supply of oxygen, consuming more oxygen in the generation of ATP compared to the oxidation of carbohydrates.

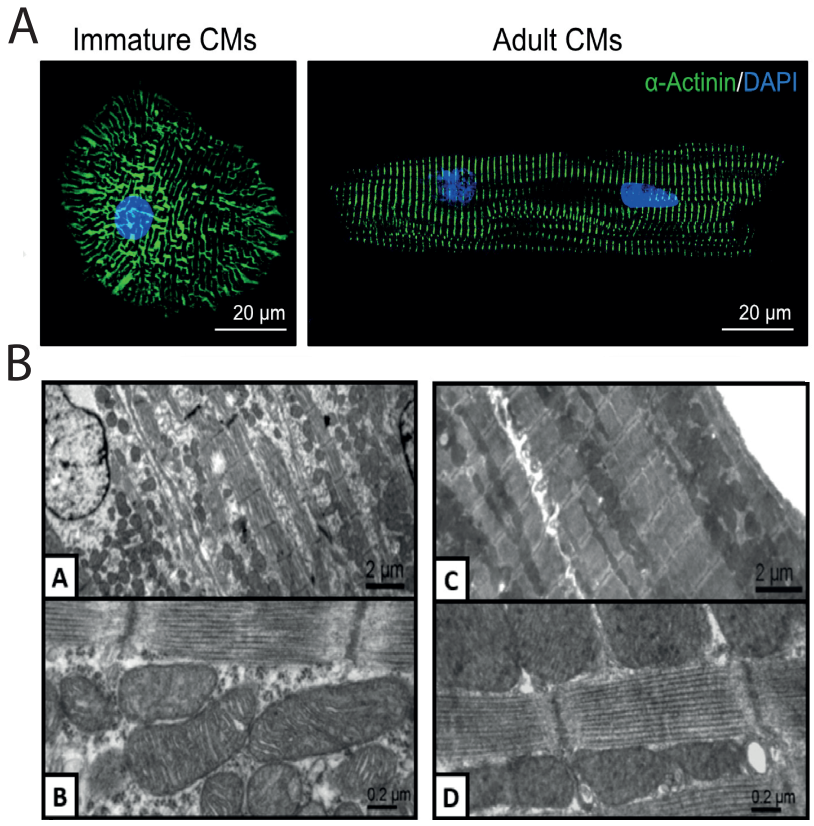

Figure 1: Microscopic and subcellular changes organisation changes drastically during cardiomyocyte maturation. A) The left panel shows immature cardiomyocytes derived from human induced pluripotent stem cells, which have a polygonal shape and unaligned sarcomere. In contrast, the right-panel shows a mature cardiomyocyte derived from the adult mouse heart. Upon maturation the shape of the cardiomyocytes increase in size, elongate and show typical striation of the sarcomeres. The image was adapted from Ahmed et al. 2020. B) Electron microscopy images of mouse papillary muscle. I-II were obtained from 3-day old mice and III-IV from 63-day old mice. The density of the intracellular organelles increases during maturation, leaving less free cytoplasm. The sarcomeres align themselves and alternate with larger mitochondria that closely align with the sarcomeres, enabling for efficient exchange of ATP via direct nucleotide shuttling or the creatine-kinase cycle. The image was adapted from Piquereau et al. 2010

This shift towards fatty acid oxidation promotes cardiomyocyte maturation by increasing the ATP turnover rate. The main energy consumers within the heart are the sarcoplasmic reticulum ATPase (SERCA), responsible for the uptake of Ca^{2+} into the SR, and the ATPases of the myosin myofilaments. In the immature heart, the mitochondria are mostly located peri-nuclear, and the myocytes rely on the passive diffusion of ATP through the cytoplasm (Ventura-Clapier *et al.,* 1998). In the first days after birth, the mitochondria migrate and remodel in proximity to the myofibres. This alignment

facilitates greater availability of ATP at the organelles yet is still limited by the resulting ATP/ADP concentrations at the energy-utilising and energy-generating organelles (Anmann *et al.,* 2014; Wilding *et al.,* 2006). This limitation is, in effect, caused by energy transfer via diffusion through the cytoplasm, as it requires a significant concentration gradient or it will be inhibited by kinetic and thermodynamical limitations (Dzeja & Terzic, 2003; Saks *et al.,* 2008). These same restrictions are mirrored in ATPase activity restrictions, which benefit from very low concentrations of ADP and high concentrations of ATP (Dzeja & Terzic, 2003). Thus, an alternative system is required to provide the subcellular organelles efficiently with a high ATP/ADP ratio while maintaining a low ATP/ADP ratio in the mitochondria. Within the heart and striated muscles, cells have found ingenious methods to maintain high ATP/ADP ratios close to the energy-demanding organelles while keeping a low ATP/ADP ratio in and near the mitochondria to ensure the highest degree of OXPHOS.

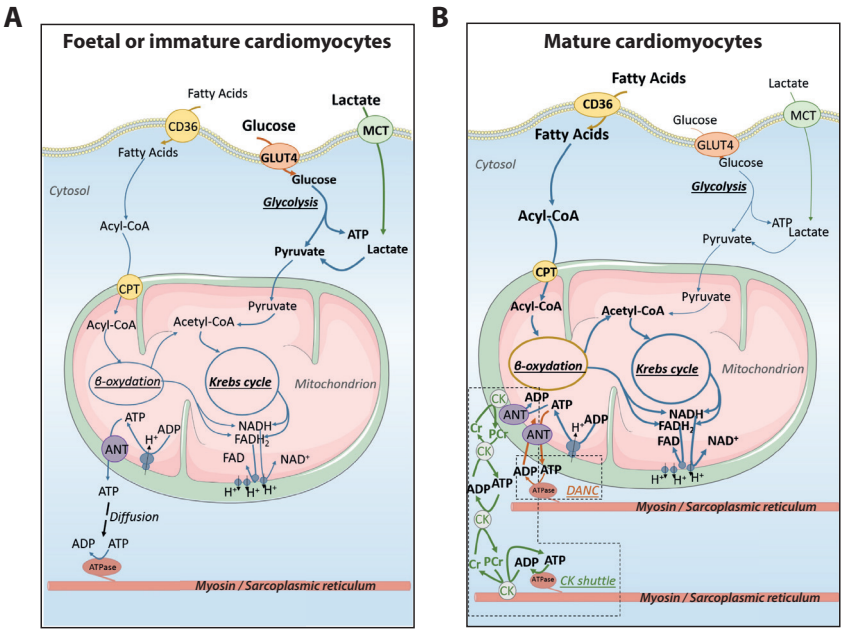

Figure 2: the development of energy-producing system in foetal and adult cardiomyocytes. A) Foetal cardiomyocytes rely heavily on carbohydrates in the form of glucose and lactate. ATP produced either by glycolysis in the cytosol or via OXPHOS in the mitochondria reaches the ATPases on the myofibres and SR through passive diffusion. This process results in a detrimental concentration ratio of ATP and ADP, with high concentrations of ATP in and near the mitochondria and low concentrations of ATP near the ATPases of the Myosin and SR. B) Mature cardiomyocytes primarily generate ATP via beta-oxidation of fatty acids in the mitochondria. The cardiomyocytes employ DANC and the CK-shuttle to ensure high ratios of ATP/ADP near the ATPases and low ratios near the mitochondria.
Adenine nucleotide translocase (ANT), creatine kinase (CK), Carnitine palmitoyltransferase I (CPT), direct adenosine nuclease channelling (DANC), glucose transporter type 4 (GLUT4), Monocarboxylate transporter (MCT). Figure adapted from Piquereau & Ventura-Clapier et al. 2018.

One such method arises during the remodelling phase of cardiomyocytes when mitochondria start to align with the SR and myofibres. The mitochondria directly couple with these organelles, directly exchanging ATP and ADP between the mitochondria and the ATPases. This process is called direct adenosine nuclease channelling (DANC). It relies on the adenine nucleotide translocase (ANT), anchored in the inner membrane of the mitochondria, to actively transport ADP generated by the ATPases into the mitochondria while shuttling ATP out of the mitochondria (Fig. 2B). This process does not rely on the diffusion rates of ATP or ADP as the ANTs are situated in direct conjecture with the ATPases and thereby kinetically favour the ATPases (Piquereau & Ventura-Clapier, 2018). The other energy transfer system relies on the creatine kinase enzyme (CK), which provides energy transfer from ATP onto creatine (Cr) or from phosphorylated Cr (pCr) onto ADP (ATP + Cr ↔ ADP + pCr). Cytosolic CK (MM-CK) can be found within the cytosol of cardiomyocytes or near the ATPases on the SR and myofibres, while mitochondrial CK (MT-CK) is anchored within the inner membrane of the mitochondria, near the ANT translocase (Fig. 2B). The highly energetic pCr leaving the mitochondria is shuttled by the MM-CK in the cytosol to the bound MM-CK at the ATPases. Here, they maintain a high ATP/ADP ratio by constantly transferring the phosphate group onto ADP, relieving the ATPases of constant inhibition by preventing increased ADP concentrations and providing constant ATP. The inverse happens within the mitochondria. In close proximity to ANT, the MT-CK maintains high ADP/ATP ratios, favouring increased oxidative phosphorylation. In this manner, the CK-shuttle provides an increased output of the ATPases on the SR and myofibres and the OXPHOS capacity of the mitochondria (Piquereau & Ventura-Clapier, 2018). In the heart, these two systems are constantly in competition, with the CK system meeting most of the energy demand in the cardiomyocytes, with around 90% of the energy leaving the mitochondria in the form of pCR. These systems are also in flux, and both the pCR and DANC systems can compensate for each other (Piquereau *et al.,* 2020). The reliance of the adult heart on mitochondrial respiration and the precarious balance in which this system resides means that any dysfunction of the mitochondria can cause severe heart pathologies.

MITOCHONDRIAL PATHOLOGIES OF THE HEART

The detection and definition of mitochondrial diseases started in 1988 when two papers first reported detecting pathogenic variants in mitochondrial DNA (mtDNA) (Holt *et al.,* 1988; Wallace *et al.,* 1988). After these seminal papers, many more groups discovered novel mtDNA mutations, broadening the scope of mitochondrial diseases. These mutations in the mtDNA were detected relatively easily due to the limited size of mtDNA versus nuclear DNA (nDNA). The first mitochondrially linked nDNA mutation was discovered in 1995 in a patient with Leigh syndrome (Bourgeron *et al.,* 1995). With the number of mutations linked to mitochondrial diseases still continuing to grow, the estimates of the

prevalence of mitochondrial diseases are still uncertain. This uncertainty is largely due to vast differences in population frequencies in different global regions and the difficulty in defining mitochondrial diseases, which can present at any age, affect a single organ or multiple tissues simultaneously and are clinically heterogeneous. Generally, a frequency of 1:5,000 cases is estimated, but this frequency can be much greater in consanguineous family populations affected by founder mutations (Vafai & Mootha, 2012). Broadly speaking, a distinction can be made between childhood- (< 16 years of age) and adult-onset mitochondrial diseases due to differences in the pathological and genetic presentation. For adult-onset mitochondrial diseases, ~80% of the pathologies can be explained by heteroplastic mutations in the mtDNA, while in childhood-onset, these mtDNA mutations only contribute 20-25%, the majority caused by autosomal recessive mutations in the nDNA (Gorman *et al.,* 2016). Childhood-onset mitochondrial disease is associated with greater severity and higher mortality rates, although the diseases are not always fatal. The highest mortality risk is in children presenting with mitochondrial cardiomyopathies, estimated to be 20-40% of all childhood mitochondrial diseases (Brunel-Guitton *et al.,* 2015). These patients show an increased risk of mortality of 71% compared to 26% mortality in children presenting with mitochondrial diseases without cardiac involvement (Holmgren *et al.,* 2003). The mutations causing these cardiomyopathies can be subdivided into 5 broad categories: 1) isolated complex deficiencies caused by mutations in genes encoding for the electron chain subunits (ETCs), 2) mitochondrial translation defects, mutations in proteins involved in protein synthesis within the mitochondria, 3) mtDNA depletion syndrome, mutations affecting mtDNA maintenance resulting in a marked depletion of mtDNA, 4) 3-Methylglutaconic acidurias, defined by markedly increased excretion of 3-methylglutaconic acid (3MGC) in diseases such as Barth-syndrome, and 5) secondary mitochondrial involvements, pathologies with mutations in genes closely linked to ETC function, but not directly by defects in any ETC gene (extensively reviewed in Duran et al., 2019; El-Hattab & Scaglia, 2016; Enns, 2017). The heteroplastic nature of these mutations leads to heterogeneous clinical and biochemical presentation, making proper diagnosis difficult (Byers, 2014).

Quite often, these diseases develop into multi-organ pathologies, either due to the direct effect of the mutation on other organs, such as the brain, liver and kidneys, or due to cardiac insufficiency indirectly affecting other organs, in the case of pulmonary hypertension. The most common cardiac manifestations are in the myocardium and are detected shortly postnatally. Hypertrophic cardiomyopathy (HCM), the pathological increase in the size of the ventricle walls, resulting in a loss in pumping efficiency, is the most frequent pathology in 40% of patients with mitochondrial cardiomyopathies. Dilated cardiomyopathy, an increased ventricle volume with decreased contractile force, can either present as a primary pathology or be due to pre-existing hypertrophic cardiomyopathy. Additionally, more rare myopathies might present as left-ventricular non-compaction, histiocytoid cardiomyopathies, or restrictive cardiomyopathies, all leading to decreased cardiac output (Finsterer

& Kothari, 2014). Due to the high metabolic demand, it is no surprise that the heart is commonly affected by mitochondrial disease and that the onset of these pathologies shortly after birth coincides with the metabolic remodelling of the heart. However, it is unclear how the mutations contribute to the pathologies of many of these mitochondrial diseases. A notable instance of this ambiguity is the discrepancies in disease manifestation caused by mutations in nuclear-encoded mitochondrial tRNA synthetases. Despite their shared role in mitochondrial protein translation, these enzymes lead to clinically distinct pathologies. Moreover, the phenotypes do not correspond to mutations in the matching tRNAs encoded within the mitochondrial DNA, further highlighting the complexity of mitochondrial disease pathogenesis (Vafai & Mootha, 2012).

CURRENT (HIPSC) MODELS FOR MITOCHONDRIAL AND METABOLIC HEART DISEASE

The discovery of human induced pluripotent stem cells (hiPSC) holds tremendous promise for revolutionising human disease modelling and drug discovery. The ability to generate many different organ-specific cells from hiPSCs and derive them from any patient or (apparently) healthy population makes these cells particularly useful for unravelling the complex pathophysiology of mitochondrial cardiomyopathies (Caudal *et al.,* 2022; de Korte *et al.,* 2020; Giacomelli *et al.,* 2017). The majority of the >1500 proteins involved in mitochondrial function and homeostasis are encoded in the nDNA. Mutations occurring in these genes can be studied according to the principles of Mendelian genetics, with most mitochondrial cardiomyopathies requiring either homozygosity or compound mutations for their pathogenicity. These principles can provide abundant opportunities for disease modelling using hiPSCs *in vitro*. Many hiPSC lines can be derived from large patient families with a varying mutational burden, allowing the *in vitro* characterisation of these pathologies within a restricted and controlled genetic background. However, more commonly, genetic correction of the pathogenic mutation, using CRISPR/cas9, can be utilised to generate isogenic clones, elucidating mutation-specific mechanisms of action and progression towards cellular pathology.

Several studies have studied mitochondrial cardiomyopathies using hiPSC in the last ten years, focusing on the same type of mitochondrial pathologies, chiefly in 3-Methylglutaconic acidurias. The most studied disease is Barth syndrome, a myopathy caused by a mutation in the X-chromosomal gene *Tafazzin* (*TAZ)*. This protein is responsible for the acetylation of cardiolipin, an essential phospholipid of the inner mitochondrial membrane. The first study employing hiPSC-derived cardiomyocytes (hiPSC-CMs) to investigate this disease was also the most extensive: hiPSC-CMs from two different patients, unrelated controls, and isogenic lines with cas9-induced mutations in *TAZ* were investigated. The study found reduced cardiolipin biogenesis and OXPHOS capacity and utilised an Organ-on-

Chip model employing PDMS muscular thin films to demonstrate reduced sarcomeric assembly and contractile force (Wang *et al.,* 2014). Two subsequent studies relied on the same control and induced mutant hiPSC lines to perform further metabolic characterisation. These studies showed that *TAZ* mutated lines have an accumulation of cellular long-chain acylcarnitines, increased reliance on glycolysis and decreased beta-oxidation, demonstrating the incapability of the mitochondria in using oxygen-reliant energy pathways properly for the generation of ATP (Fatica *et al.,* 2019). The second study focused more on cardiomyocyte-specific phenotypes. Here, aberrant Ca^{2+} handling was found to lead to contractile defects, implicating the relationship between increased mitochondrial ROS and activation of the CaMKII pathway as a root cause of the Ca^{2+} defects (Liu *et al.,* 2021). Two additional studies relied mostly on data generated from a mouse model of Barth disease and appended their paper by using a single hiPSC patient line and one unrelated control to study the effect of mutated *TAZ* in hiPSC-CMs. In the mutated hiPSC-CMs, a reduction in sarcomere organisation was found, as well as less reliance on OXPHOS and reduced assembly of heavy OXPHOS supercomplexes in the mitochondria (Dudek *et al.,* 2016). In addition, a prominent role for NF-κB signalling and *HIF-1a* activation in the context of increased ROS production was uncovered (Chowdhury *et al.,* 2018).

Another study of mitochondrial cardiomyopathy classified as a 3-Methylglutaconic acidurias was performed on two patients presenting with dilated cardiomyopathy due to homozygous intronic mutations in the *DNAJC19* gene. This protein is located in the inner membrane of the mitochondria, where its function has not been completely elucidated. Although the pathology and the function of the gene share similarities with Barth syndrome, the cellular phenotypes were distinct from mutations in the TAZ protein. Small dysregulations in cardiolipin were observed compared to unrelated control hiPSC-CMs, but the study's main finding was mitochondrial fragmentation and a shift in the OPA1 isoform, a key regulator in mitochondrial fusion and fission. The authors reversed these phenotypes by treating cells with the small molecule SS-31, which is thought to play a role in ROS reduction and stabilising the mitochondrial membrane (Chavez *et al.,* 2020), uncovering a possible avenue for treatment (Rohani *et al.,* 2020).

The second most studied disease is Friedreich's ataxia, a pathology characterised by secondary mitochondrial involvement and caused by an expanded GAA repeat in the first intron of the *Frataxin* gene. The protein plays a role in synthesising iron-sulphur complexes, essential in several iron-dependent proteins within the mitochondria. The first study generated hiPSC-lines from two patients and two age-matched controls and demonstrated mitochondrial dysfunction, with reduced mitochondrial membrane potential and abnormalities in the mitochondrial ultrastructure (Hick *et al.,* 2013). Another study expanded on these observations using the same hiPSC lines, showing increased ROS and disrupted Ca^{2+} homeostasis (Lee *et al.,* 2016). However, studies relying on isogenic lines are rare due to the difficulty in gene editing repeat sequences. Two groups have successfully corrected

the GAA repeats and published studies on the isogenic hiPSC pairs. One report focused on the transcriptomic analysis of hiPSC-CMs, revealing a predisposition towards an HCM phenotype (Li *et al.,* 2019), while the other utilised engineered heart tissues (EHTs) to demonstrate deficiencies in excitation-contraction coupling and reduced force generation (Wong *et al.,* 2019).

A different type of mitochondrial heart disease involving beta-oxidation of very long long-chain fatty acids has also been modelled. Here, hiPSC-CMs derived from two patients presented with mutations in the gene *ACADVL.* These hiPSC-CMs showed lipid accumulation and disruption in electrical action-potential generation, Ca^{2+} homeostasis and delayed after depolarisations (DADs), reflecting the clinical presentation of the patients (Knottnerus *et al.,* 2020).

Finally, a single study examined primary oxidative phosphorylation deficiency in Leigh's disease. Here, the authors generated hiPSCs from two patients with homozygous or compound mutations in the gene *SCO2*. This protein is part of the COX complex and facilitates copper delivery, which is necessary for proper ETC function. Mutations in the *COX* gene are the most common cause of complex IV deficiency, resulting in reduced activity in the 4^{th} OXPHOS complex, primarily leading to HCM in affected patients. Here, in the hiPSC-CMs, the authors showed aberrant mitochondrial ultrastructure, loss of positive inotropic effect as induced by isoproterenol, and an increased incidence of DADs accompanied by an increased propensity towards arrhythmic behaviour (Hallas *et al.,* 2018).

Most of these studies failed to include data on isogenic corrected lines compared to the patient lines. While many studies employ multiple patient lines and sometimes multiple clones of each line, using a single hiPSC or hESC control line is a potential concern: hiPSC-CMs are well-known for a tendency to show line- and batch-dependent variability in phenotype, potentially masking mutation-linked pathology or revealing differences that are not due to the disease-causing mutation (Sala *et al.,* 2017). Additionally, only a single study of actual oxidative phosphorylation defects has been performed on lines with nDNA mutations, and none have studied the role of mutations in the mitochondrial protein translation machinery. Studies on these primary mitochondrial defects have been performed much more extensively in the neuronal field but for hiPSC-CMs, studies are still lacking.

Modelling mitochondrial cardiomyopathies due to mtDNA mutations

It is worth noting that not all cardiomyopathies can be studied by relying on the generation of isogenic lines using CRISPR/cas9. Multiple mitochondrial cardiomyopathies are caused by mutations in the mtDNA, such as Kearns-Sayre syndrome, Leigh syndrome, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS). These diseases do not follow Mendelian inheritance patterns and depend on the mutated mtDNA amount established at the mitochondrial bottleneck

transfer during oogenesis (Gorman *et al.,* 2016). This process results in oocytes carrying multiple variants of mtDNA, leading to a state of heteroplasmy, where the presence of pathology is determined by the contribution of mutant mtDNA towards the whole pool of mtDNA. In these cases, generating isogenic clones by altering the mtDNA with CRISPR/cas9 is not feasible due to the high mtDNA copy number per cell and the ineffective mtDNA recombination machinery. Historically, this has meant that generating models for mitochondrial diseases was challenging, with a considerable need for alternative experimental approaches (Caudal *et al.,* 2022; Silva-Pinheiro & Minczuk, 2022). Recent breakthroughs have shown that hiPSC-clones derived from single patients have varying degrees of heteroplasmy. The mechanism underlying the heteroplasmy observed between hiPSC-clones seems driven by mtDNA segregation during the reprogramming of somatic cells. During this process, segregation of the mitochondria occurs unevenly and is apparently random. This results in hiPSC-clones with large differences in mtDNA heteroplasmy from mutation-rich with 80-90% mtDNA penetration to completely mutation-free cells (Fujikura *et al.,* 2012; Hsu *et al.,* 2016). The heteroplasmy within these hiPSC clones was maintained through prolonged culture and upon differentiation. This unique ability to derive multiple heteroplasmic clones from a single patient allows the investigation of the impact of mutational burden upon the cellular phenotype (Inak *et al.,* 2017). This feature is useful for in vitro determination of the mutational effect on cellular phenotype and provides insight into heteroplasmy within the human body and into variability in clinical presentation in patients (Hatakeyama & Goto, 2016). Only a limited number of studies have looked into mitochondrial cardiomyopathies using this approach (extensively reviewed by Caudal *et al.,* 2022; Pavez-Giani & Cyganek, 2022).

The first was performed in hiPSC-CMs derived from two patients with Myoclonic Epilepsy with ragged red fibre syndrome (MERRF). These patients carried a heteroplasmic mutation in the mitochondrial transfer RNA MT-TK at m.8344A>G. At a mutational burden of around 50%, these hiPSC-CMs started manifesting altered phenotypes, with impaired OXPHOS, increased ROS and abnormal mitochondrial ultrastructure (Chou *et al.,* 2016). Another study investigated heteroplasmy in patients with MELAS. Here, they derived multiple hiPSC clones, two clones carrying only WT mtDNA, two clones with an 80% heteroplasmic mutation in MT-TL1 at m.3243A>G, two clones with a mutation in MT-CO3 at m.9547G>T at either 40 and 70% and two clones with a mutation at m.19318T>C in the gene MT-ND5 at a mutation load of 83%. In this impressive study, the authors showed that hiPSC-CMs with mutations in MT-TL1 and the clone carrying a 70% mutational load in MT-CO3 had reduced OXPHOS potential, while all the other clones showed similar energetics compared to the WT clones, indicating the pathogenic mutational burden necessary to cause a phenotype for multiple mutations (Perales-Clemente *et al.,* 2016).

Two additional studies reported a loss in cardiogenic differentiation potential in hiPSCs from two patients affected by MELAS. hiPSCs from one patient carrying the mutation m.13513G>A showed a loss of cardiogenic potential at mutational burdens of >40%. Furthermore, hiPSC-CMs below a threshold of <30% still showed disrupted contractile properties and increased apoptosis rates compared to WT mtDNA clones (Galera-Monge *et al.,* 2019). In the other study, hiPSC clones that carried a mutation in the MT-TL1 gene at m.3243A>G were derived. Here, the authors reported a loss of hiPSC differentiation potential towards cardiomyocytes at a mutational burden of >90%, while clones with homoplasmic WT or 40% mutational burden were unaffected, further elucidating the phenotypes observed by Perales-Clemente *et al.* Furthermore, differentiation efficiency towards neuronal lineages was unaffected (Yokota *et al.,* 2017).

Finally, a recent study was the first to report mutations in a gene affecting mitochondrial translation. Two patients presented with a common mutation present in the Finnish population. The mutation m.3243A>G in the *tRNA-Leu(UUR)* gene was studied. Interestingly, only one of the two patients included had developed cardiomyopathy. The two mutant hiPSC lines were derived from each patient with a mutation load of 60-85%, and two control lines were generated from the same patients with a mutational load of <3%. No changes in differentiation capacity were observed, and only minor mitochondrial changes were seen, with reduced expression in Complex I. OXPHOS capacity was reduced in both patient lines. However, glycolysis, ROS production and calcium handling (see fig. 2) were all changed only in the hiPSC-CMs derived from the patient with cardiomyopathy (Ryytty *et al.,* 2022). This was the first study in hiPSC-CMs of the effect of defects in the mitochondrial protein translation and also the first to uncover differences between patients affected by the same heteroplasmic mutation but presenting with a different pathology. These results suggest that additional SNPs could influence the pathology within mitochondrial diseases, possibly explaining some of the clinical heterogeneity observed in patients.

AIM AND SCOPE OF THIS THESIS

Studying mitochondrial disease, particularly mitochondrial cardiomyopathies, remains challenging due to the broad spectrum of clinical features and the complex relationship between mutation effects and the presentation of disease pathology. Moreover, the lack of reliable models that sufficiently replicate the complexity of the adult human heart hinders the accurate assessment of the phenotypes associated with mitochondrial diseases. This thesis aims to address some of these challenges by improving the utilisation of hiPSC models in modelling mitochondrial cardiomyopathies. To achieve this, hiPSC-CMs were used to investigate one specific mitochondrial cardiomyopathy; further hiPSC-CMs models and measurement techniques were explored to improve the accuracy of readouts in these models.

While many mitochondrial cardiomyopathies have been studied, this field is far from complete. The **second chapter** contributes to this field by describing a family in which three sons were affected by infantile cardiomyopathy caused by a mitochondrial disease called combined oxidative phosphorylation deficiency 8 (COXPD8). HiPSCs were derived from two of these patients and their mother, showing that functional hiPSCs can be obtained from the skin fibroblasts of these diseased patients. **Chapter 3** explores how these hiPSCs can be used to create novel *in vitro* models for this condition by generating isogenic clones from the patient lines that were genetically corrected and studying the effects of these mutations on the phenotype of the cardiomyocytes *in vitro.* Taking advantage of the immature fetal phenotype of 2D differentiated hiPSC-CMs shortly after differentiation, the cardiomyocytes could be observed at a developmental stage where there is no overt pathology, and the condition normally remains undetected in patients. Mitochondrial function, transcriptomics, metabolomics, and excitation-contraction coupling behaviour were assessed to investigate the effects of the mutations on cellular phenotype.

Chapter 4 presents an extensive protocol for generating more mature hiPSC-CMs in the form of small cardiac microtissues, which can be used for disease modelling and drug discovery. The chapter describes the derivation, culture and preservation of hiPSCs towards cardiomyocytes, cardiac endothelial cells and cardiac fibroblasts, as well as the methodology for incorporating these three cell types into a single 5000-cell microtissue. Protocols are described for immunofluorescence, video analysis of contraction and calcium, metabolic functionality in the whole microtissue and electrophysiology using sharp-electrode recordings.

Further recent advances in cellular models have been made in the Organ-on-Chip field, where cells are placed in an environment better resembling the physiological state of the specific organ. Many of these models use microfluidic channels, microfluidic flow and novel measuring and sensing methods. **Chapter 5** describes incorporating pH and oxygen sensors within a microfluidic channel, allowing live monitoring of cellular states and optimising tissue-culture conditions by tracking extracellular conditions.

Finally, **chapter 6** summarises and discusses the implications of the collective work presented in this thesis and offers a framework for future investigation into mitochondrial cardiomyopathies. Overall, the thesis aims to address the challenges associated with studying mitochondrial cardiomyopathies by improving the utilisation of hiPSC models and provides insights into disease modelling, measurement techniques, and organ-on-chip approaches with mitochondrial diseases being used as an exemplar.

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