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

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ORGANS-ON-CHIP:
TOWARDS THERAPIES FOR CARDIOVASCULAR
DISEASE USING HUMAN STEM CELLS

Ruben W.J. van Helden

Colofon

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

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

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CHAPTER

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-1 α pathway, important in regulating the maintenance of anaerobic glycolysis and active during the foetal development of the heart, is inhibited, while simultaneously, the PGC-1 α /PPAR α and PGC-1 α /PPAR- β/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 FADH₂, 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.

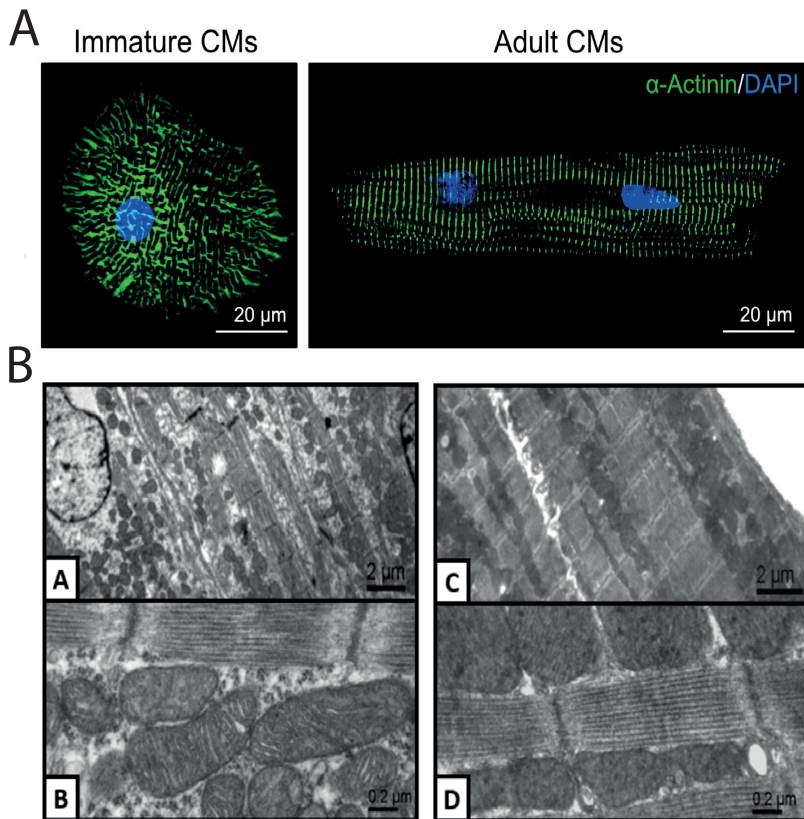


Figure 1: Microscopic and subcellular changes organisation changes drastically during cardiomyocyte maturation. A) The left panel shows immature cardiomyocytes derived from human induced pluripotent stem cells, which have a polygonal shape and unaligned sarcomere. In contrast, the right-panel shows a 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.

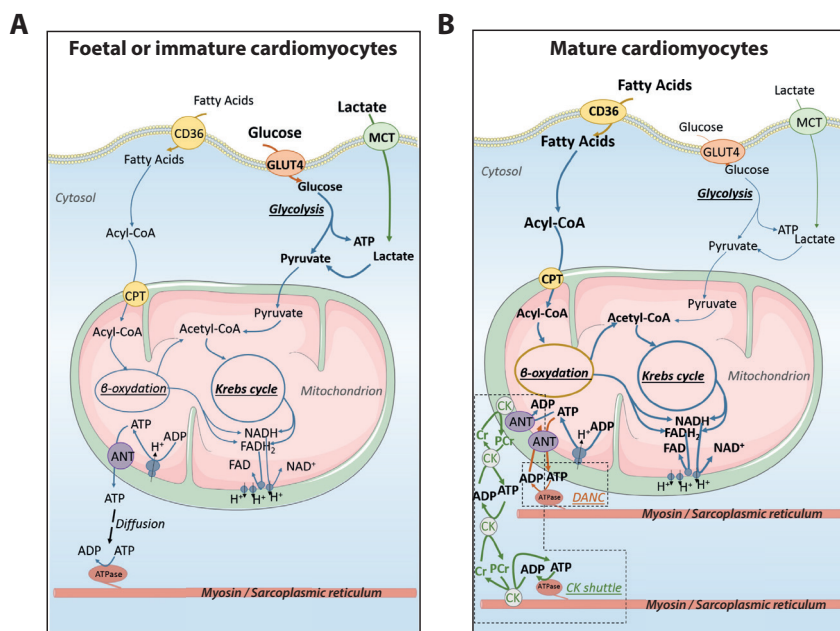


Figure 2: the development of energy-producing system in foetal and adult cardiomyocytes. A) Foetal cardiomyocytes rely heavily on carbohydrates in the form of glucose and lactate. ATP produced either by glycolysis in the cytosol or via OXPHOS in the mitochondria reaches the ATPases on the myofibres and 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 nucleoside 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 nucleoside 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 ($\text{ATP} + \text{Cr} \leftrightarrow \text{ADP} + \text{pCr}$). 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 heteroplasmic 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 heteroplasmic 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- κ B 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 4th 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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CHAPTER

Generation of three human induced pluripotent stem cell lines, LUMCi024-A, LUMCi025-A, and LUMCi026-A, from two patients with Combined Oxidative Phosphorylation Deficiency 8 and a related control

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ABSTRACT

Combined Oxidative Phosphorylation Deficiency 8 (COXPD8) is an autosomal recessive disorder causing lethal childhood-onset hypertrophic cardiomyopathy. Homozygous or compound heterozygous mutations in the nuclear-encoded mitochondrial alanyl-tRNA synthetase 2 (*AARS2*) gene underly the pathology. We generated induced pluripotent stem cells (hiPSCs) from two patients carrying the heterozygous compound c.1774C>T, c.2188G>A and c.2872C>T *AARS2* mutations, as well as a related healthy control carrying the c.2872C>T *AARS2* mutation. All hiPSC-lines expressed pluripotency markers, maintained a normal karyotype, and differentiated towards the three germ layer derivatives *in vitro*. These lines can be used to model COXPD8 in humans or mitochondrial dysfunction.

RESOURCE UTILITY

These hiPSC lines are a valuable resource for studying COXPD8 and primary mitochondrial cardiomyopathies. Additionally, they can be used to study the cellular metabolism of human cardiomyocytes. These lines provide a unique tool to uncover the role of mitochondrial function in lethal infantile cardiomyopathy and to search for potential treatments.

RESOURCE TABLE

Unique stem cell lines identifier	LUMCi024-A (https://hpscereg.eu/cell-line/LUMCi024-A) LUMCi025-A (https://hpscereg.eu/cell-line/LUMCi025-A) LUMCi026-A (https://hpscereg.eu/cell-line/LUMCi026-A)
Alternative names of stem cell lines	LUMC0057iAARS03 LUMC0058iAARS01 LUMC0059iCTRL03
Institution	Leiden University Medical Center, LUMC
Contact information of distributor	Milena Bellin (m.bellin@lumc.nl) Ruben van Helden (r.w.j.van_helden@lumc.nl) Christian Freund (c.m.a.h.freund@lumc.nl)
Type of cell lines	iPSCs
Origin	Human
Cell Source	Skin Fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai virus, <i>OCT3/4</i> , <i>SOX2</i> , <i>KLF4</i> and <i>MYC</i>
Multiline rationale	Non-isogenic cell lines obtained from patients with three heterozygous compound mutations in the same gene (<i>AARS2</i>) and a related control with a single heterozygous mutation in the <i>AARS2</i> .
Gene modification	YES
Type of modification	De novo
Associated disease	Combined Oxidative Phosphorylation Deficiency 8 (OMIM: 614096)
Gene/locus	<i>AARS2</i> 6p21.1, NM_020745.4 Heterozygous <i>AARS2</i> c.1774C>T and c.2188G>A (LUMCi024-A, LUMCi025-A) Heterozygous <i>AARS2</i> c.2872C>T (LUMCi024-A, LUMCi025-A, LUMCi026-A)
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	August 2014 (LUMCi024-A & LUMCi025-A) September 2014 (LUMCi026-A)
Cell line repository/bank	N/A
Ethical approval	Skin fibroblasts were collected after the patient (and children's parent) signed informed consent and approved by the ethical committee of the Erasmus MC University Medical Centre. The generation of the lines was approved by the Leiden University ethics committee under the P 13.080 "Parapluprotocol: hiPSC".

RESOURCE DETAILS

Combined Oxidative Phosphorylation Deficiency 8 (COXPD8) is a rare recessive familial disease characterized by mitochondrial dysfunction in the heart leading to fatal infantile hypertrophic cardiomyopathy (Götz *et al.*, 2011). Part of a broader group of pathologies known as primary mitochondrial cardiomyopathies, COXPD8 is caused by mutations in the nuclear-encoded mitochondrial gene Alanine tRNA-synthetase 2 (*AARS2*). The leading hypothesis is that mutations in *AARS2* cause instability in alanine-synthetase 2, leading to faulty loading of tRNAs in the mitochondria, defective assembly of mitochondrial-encoded oxidative phosphorylation chain proteins and impaired respiratory capacity (Euro *et al.*, 2015; Sommerville *et al.*, 2019). Due to the rare and lethal nature of this disease, primary tissue for research is rare. Human-induced pluripotent stem cells (hiPSCs) can be differentiated into patient-specific cardiomyocytes to uncover the mechanisms underlying this poorly understood pathology. Two patients with COXPD8 were identified just a few days after birth (Fig. 1A). Sequencing of both the patients and their mother revealed the presence of three mutations in *AARS2* (Fig. 1B): the novel c.2872C>T (p.958 R/*, rs779332260) mutation was present on the maternal allele; while in the patients the paternal allele contained two mutations: the pathogenic c.1774C>T (p.592 R/W, rs138119149) and the non-pathogenic c.2188G>A (p.730 V/M, rs35623954) mutations. Dermal fibroblasts from all three donors were reprogramed using a replication-defective Sendai virus (SeV) containing the *OCT3/4*, *SOX2*, *KLF4* and *MYC* reprogramming factors (Nishimura *et al.*, 2011). Three clones were isolated and expanded for each individual and cryopreserved at passage 5. One selected clone per line, hereafter named LUMCi024-A, LUMCi025-A, and LUMCi026-A, was assessed for *in vitro* pluripotency characteristics as reported in Table 2. All hiPSC lines showed characteristic stem cell colony-forming growth with a high nucleus/cytoplasm ratio (Fig. 1C). Expression and localization of pluripotency markers SSEA4, *OCT3/4*, and *NANOG* were verified using immunofluorescence (IF) analysis (Fig. 1D); fluorescence-activated cell sorting (FACS) confirmed expression of these proteins in the undifferentiated hiPSCs (Fig. 1E). Additionally, the absence of SeV was confirmed using qPCR for the SeV-NP DNA-vector in all lines (supplementary Fig. 1A). Sanger sequencing showed all three patient mutations in LUMCi024-A and LUMCi025-A, and the presence of the c.2872C>T mutation in LUMCi026-A (Fig. 1F). Karyotype G-banding with a resolution of 5-10Mb was used to confirm the genomic integrity of the hiPSC-lines, showing a normal karyotype and confirming gender (Fig. 1H). Differentiation potential of the lines was confirmed using an *in vitro* spontaneous differentiation assay with subsequent IF analysis for markers of the three germ layer derivatives, AFP (endoderm), PECAM1 (mesoderm), and *TUBB3* (ectoderm) (Figure 1G). To facilitate genotyping of all the lines and discrimination of the patient lines LUMCi024-A and LUMCi025-A, two primers were designed for an intronic region in the gene *MIF-AS1* (ENSG00000218537) which has a heterozygous SNP (g.117 T>G, rs2000466) in LUMCi025-A (supplementary Fig. 1B). Finally, clones tested negative for the presence of mycoplasma (Supplementary Fig. 1C).

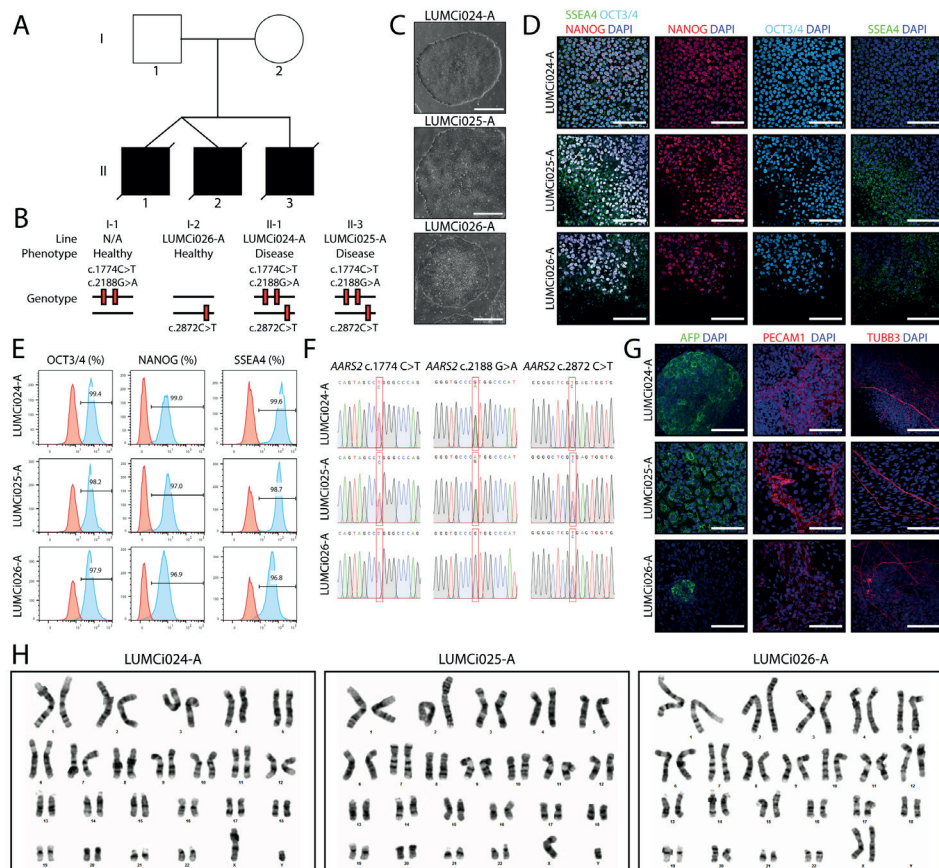


Figure 1. Generation and characterization of hiPSC lines LUMCi024-A, LUMCi025-A and LUMCi026-A from patients affected by infantile cardiomyopathy due to mutations in the AARS2 gene.

(A) Family tree. (B) Schematic showing hiPSC lines with the corresponding genotypes. (C) Representative brightfield images showing hiPSC colony morphology. Scale bar: 600 μ m (D) Immunofluorescence staining of iPSC colonies showing expression of the pluripotency markers NANOG (red), OCT3/4 (cyan) and SSEA4 (green) in all lines; nuclei stained with DAPI (blue). Scale bar: 100 μ m. (E) FACS analysis of undifferentiated hiPSCs stained for OCT3/4, NANOG and SSEA4. All lines show >95% marker expression versus unstained hiPSCs. (F) Sanger sequencing of genomic DNA isolated from hiPSC lines showing the presence of all three mutations (LUMCi024-A, LUMCi025-A) or only one mutation (LUMCi026-A). (G) Immunofluorescence staining of hiPSCs after spontaneous differentiation protocol for markers of the three germ-layers, AFP (endoderm, green), PECAM1 (mesoderm, red), and TUBB3 (ectoderm, red). (H) Karyotyping of the three hiPSC lines showing no abnormalities.

MATERIALS AND METHODS

Ethical statement

This study was approved by the Medical Ethical Committees at the Leiden University Medical Center (P13.080), the Erasmus Medical Center and the Maastricht University Medical Center. Written informed consent was obtained for all patients.

Cell culture and reprogramming

Skin fibroblasts were isolated from a skin biopsy and cultured in DMEM/F12 Glutamax supplemented with 10% FBS, 1% penicillin-streptomycin, 1% non-essential amino acids, 9 μ M 2-mercaptoethanol and 1 μ g/ml vitamin-C (all from ThermoFisher Scientific) at 37°C, 5% CO₂ and 21% O₂. hiPSCs from skin fibroblasts were generated using Sendai virus with 1x10⁵ cells transduced once with 3-10 MOI SeVdp (KOSM302L). The day after transduction, cells were dissociated with TrypLE select and replated onto irradiated MEFs. The following day culture was continued in DMEM/F12 Glutamax supplemented with 20% KOSR, 10 ng/ml human FGF2, 1% non-essential amino acids, 0.5% penicillin-streptomycin and 0.1 μ M 2-mercaptoethanol until the appearance of hiPSC clusters. Subsequently, cells were passaged once onto MEFs, and siRNA treatment for removal of SeV was initiated (Nishimura *et al.*, 2011). Finally, colonies were picked and transferred onto Matrigel/mTESR at 37°C, 5% CO₂ and 21% O₂ for expansion and stored at passage 5. Passaging was performed using Gentle Cell Dissociation Buffer (Stem Cell Technologies) by dissociating the cells into small clumps.

Immunofluorescence staining

hiPSCs at passage 10 were fixed in 2% PFA for 30 min, permeabilized with 0.1% Triton X-100, and blocked with 4% normal swine serum (NSS, DAKO) at room temperature (RT) for 1 h. Primary antibodies were added and incubated overnight at 4°C. After washing, secondary antibodies were added for 1 h at RT (Table 3). Nuclei were stained with DAPI, and coverslips were mounted using Mowiol (Merck Millipore). Images were acquired on a Leica TCS SP8 microscope.

FACS staining

hiPSCs were dissociated at passage 15 with TrypLE for 5 min at 37°C. Samples were fixed for 15 min in 200 μ l of Reagent A of the fix & perm kit (Thermo Fisher Scientific). The cells were washed once in FACS buffer (PBS without Ca²⁺ and Mg²⁺ with 0.5% BSA and 2 mM EDTA), resuspended in 80 μ l of Reagent B with the conjugated antibodies (table 3) and incubated in the dark for 60 min

at RT. Subsequently, the samples were washed once and resuspended in FACS buffer before being measured on the MACSQuant VYB flow cytometer (Miltenyi Biotec). Analysis was performed using the FlowJo™ v.10.6.1.

***In vitro* spontaneous trilineage differentiation**

The ability of hiPSCs to differentiate into the three germ layers (ectoderm, mesoderm, and endoderm) was assessed using a spontaneous differentiation protocol. The differentiated areas of the hiPSC colonies were picked and plated onto vitronectin-coated coverslips in TESR-E8 (Stem Cell). The next day the medium was refreshed with DMEM/F12 (Gibco) with 20% FBS (Gibco) and consecutively refreshed every second day. After three weeks of culture, the samples were fixed and stained as described.

qPCR

Total RNA from hiPSCs at passage 10 was extracted using the NucleoSpin® RNA kit (Macherey-Nagel). Following the manufacturer's instruction, reverse transcription was done on 1 µg RNA using iScript™ cDNA Synthesis Kit. qPCR was performed with primers, as shown in Table 3, using IQ SYBR Green Supermix and CFX96 Real-Time PCR Detection System (all from BioRad).

Karyotype analysis

hiPSCs at passage 9 were karyotyped by Cell Guidance systems (UK) and a total of 20 metaphases were counted.

Sequencing

Genomic DNA was isolated from hiPSCs using Quick Extract (Lucigen) according to the manufacturer's instructions. The *AARS2* gene was amplified at the three sites of the mutations using Platinum™ Taq DNA Polymerase High Fidelity (Invitrogen) for mutation analysis using the primers listed in Table 3. The PCR reaction was performed, and samples were submitted for Sanger Sequencing to confirm the presence of the *AARS2* mutations.

STR analysis

Cell identity was assessed on genomic DNA from both hiPSCs and corresponding skin fibroblasts by Promega PowerPlex Fusion System 5C autosomal STR kit (Westen *et al.*, 2014).

Mycoplasma test

All lines were tested for the presence of mycoplasma and other human pathogens using the IDEXX BioAnalytics PCR analysis. (Supplementary File 1).

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COMPETING INTERESTS

Christine L. Mummery is co-founder of Pluriomics bv (now Ncardia bv).

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Table 1: Summary of lines

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
LUMCi024-A	LUMCi024-A	Male	Four days postnatal	Caucasian	Compound <i>AARS2</i> c.1774C>T, c. 2188G>A, c. 2872C>T	COXPD8
LUMCi025-A	LUMCi025-A	Male	One day postnatal	Caucasian	Compound <i>AARS2</i> c.1774C>T, c. 2188G>A, c. 2872C>T	COXPD8
LUMCi026-A	LUMCi026-A	Female	37	Caucasian	Heterozygous c.2872C>T	Healthy

Table 2: Characterization and validation

Classification	Test	Result	Data
Morphology	Brightfield microscopy	Normal morpholog	Figure 1 panel C
Phenotype	Qualitative analysis of IF staining	Positive immunostaining of pluripotency markers: SSEA4, OCT3/4 & NANOG	Figure 1 panel D
	Quantitative analysis by FACS	Increased fluorescent signal over negative control of pluripotency markers: SSEA4, OCT3/4 & NANOG	Figure 1 panel E
Genotype	G-band Karyotyping 5-10Mb	Normal karyotype: 46, XY for LUMCi024-A and LUMCi025-A and XX for LUMCi026-A.	Supplementary Fig. 1B
Identity	Microsatellite PCR (mPCR) OR STR analysis	N/A	N/A
		24 markers tested with 100% match	Available with the authors
Mutation analysis	Sequencing	<i>AARS2</i> c.1774C>T, c. 2188G>A, c. 2872C>T	Figure 1 panel F
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Sendai virus	qPCR: Negative	Supplementary Fig. 1A
	Mycoplasma	PCR analysis: Negative	Supplementary File 1
Differentiation potential	Spontaneous differentiation <i>in vitro</i> by IF analysis	Positive immunostaining of the three germ layer markers: ectodermal (TUBB3), mesodermal (PECAM-1) and endodermal (AFP)	Figure 1 panel G
Donor screening (OPTIONAL)	N/A	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 3: Reagents details

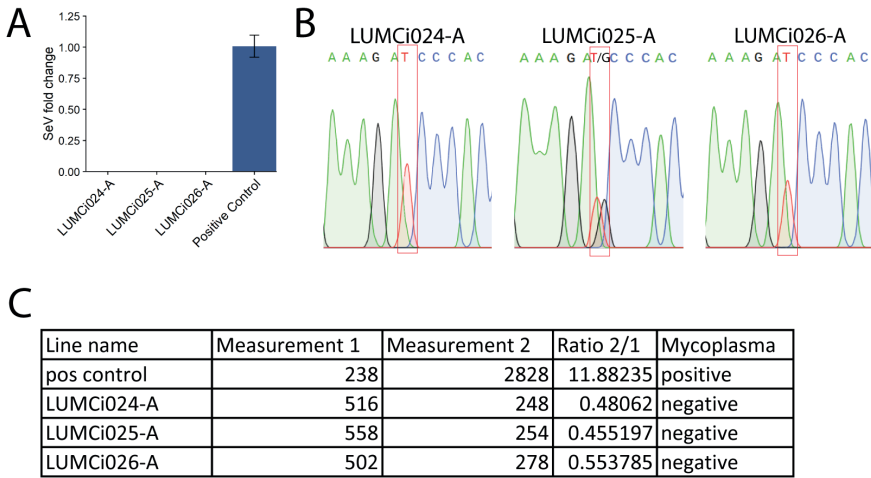
Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers (FACS)	BV421 Mouse Anti-OCT3/4	1:25 (100k cells in 80 ul)	BD Biosciences Cat# 565644, RRID:AB_2739320
Pluripotency Markers (FACS)	Mouse Anti-Human NANOG Monoclonal Antibody, PE Conjugated	1:5 (100k cells in 80 ul)	BD Biosciences Cat# 560483, RRID:AB_1645522
Pluripotency Markers (FACS)	Anti-SSEA-4-FITC antibody	1:25 (100k cells in 80 ul)	Miltenyi Biotec Cat# 130-098-371, RRID:AB_2653517
Pluripotency markers (Immunocytochemistry)	Mouse IgG2b anti-OCT3/4	1:100	Santa Cruz Biotechnology Cat# sc-5279 RRID: AB_628051
Pluripotency markers (Immunocytochemistry)	Mouse IgG1 anti-NANOG	1:150	Santa Cruz Biotechnology Cat# sc-293121 RRID: AB_2665475
Pluripotency markers (Immunocytochemistry)	Mouse IgG3 anti-SSEA4	1:30	BioLegend Cat# 330402, RRID:AB_1089208
Differentiation markers (Immunocytochemistry)	Mouse IgG2a anti-TUBB3	1:4000	Covance Cat# MMS-435P, RRID:AB_2313773
Differentiation markers (Immunocytochemistry)	Mouse IgG1 anti-CD31 (PECAM1)	1:100	Agilent Cat# M0823, RRID:AB_2114471
Differentiation markers (Immunocytochemistry)	Rabbit IgG anti-AFP	1:25	Quartett Cat# 2011200530, RRID:AB_2716839
Secondary antibodies (Immunocytochemistry)	Donkey anti-Mouse IgG (H+L) Highly cross-adsorbed Secondary, Alexa Fluor 488	1:500	Molecular Probes Cat# A-21206, RRID:AB_2535792
Secondary antibodies (Immunocytochemistry)	Donkey anti-Goat Mouse IgG (H+L) Highly cross-adsorbed Secondary, Alexa Fluor 647	1:500	Thermo Fisher Scientific Cat# A-11031, RRID:AB_144696
Secondary antibodies (Immunocytochemistry)	Goat anti-Mouse IgG3 Cross-adsorbed Secondary Antibody, Alexa Fluor 488	1:250	Thermo Fisher Scientific Cat# A-21151, RRID:AB_2535784
Secondary antibodies (Immunocytochemistry)	Goat anti-Mouse IgG1 Cross-adsorbed Secondary Antibody, Alexa Fluor 568	1:500	Thermo Fisher Scientific Cat# A-21124, RRID:AB_2535766
Secondary antibodies (Immunocytochemistry)	Goat anti-Mouse IgG2b Cross-adsorbed Secondary Antibody, Alexa Fluor 647	1:250	Thermo Fisher Scientific Cat# A-21242, RRID:AB_2535811

Antibodies used for immunocytochemistry/flow-cytometry

Antibody	Dilution	Company Cat # and RRID
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Primers

	Target	Forward/Reverse primer (5'-3')
Mutation analysis (PCR/ sequencing)	<i>AARS2</i> (c.1774C>T)	GCCCACCACTAATAACCATTC/ TTCAGGATCCCACATCAGAG
Mutation analysis (PCR/ sequencing)	<i>AARS2</i> (c.2188G>A)	GACTGCATGTTGGTCTTTCC/ ATAACCAGGTCCCCTACAGC
Mutation analysis (PCR/ sequencing)	<i>AARS2</i> (c.2872C>T)	TTGAGGGTACCTTGAGTGGA/ TAGCCCATGTCTCCTTGTGT
Genotyping (PCR/sequencing)	<i>MIF-AS1</i> (Intron 2 g.166G>T)	TAGGCCTAGACCTTTCTCTC/ TCTTCCTAATCTTCACCCCA
Sendai virus vector (RT-qPCR)	<i>SeV</i>	GCAGCTCTAACGTTGTCAAA/ CCTGGAGCAAATTCACCATGA
Housekeeping gene (RT- qPCR)	<i>GAPDH</i>	TCCTCTGACTTCAACAGCGA/ GGGTCTTACTCCTTGGAGGC



Supplemental fig 1. Further characterisation of the three hiPSC lines.

(A) Quantification by RT-PCR of SeV-NP DNA-vector RNA isolated from the three hiPSC-lines. (B) PCR amplification of the intronic region in the gene *MIF-AS1* and the detection of a unique SNP present in line LUMCi025-A. (C) Detection of the presence of Mycoplasma in the tissue culture.



CHAPTER

Disrupted energetics and contraction in cardiomyocytes with infantile mitochondrial cardiomyopathy

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ABSTRACT

Infantile mitochondrial cardiomyopathy causes death shortly after birth. We used human-induced pluripotent stem cells derived cardiomyocytes (hiPSC-CMs) from siblings carrying two compound mutations in the nuclear-encoded mitochondrial-Alanine-tRNA synthetase 2 gene (*AARS2*) to investigate the cardiac pathology. We analysed the structural, metabolic, and functional properties of patient hiPSC-CMs and compared them with familial and gene-corrected controls. The patient's hiPSC-CMs displayed reduced energetic capacity in both oxidative phosphorylation and glycolysis, resulting in reduced ATP production. However, an increase in mitochondrial copy number was identified as a possible compensatory mechanism. Quantitative metabolomics and RNA-sequencing supported metabolic dysregulation, showing less intracellular NAD⁺ and failure to produce NAD⁺ from pyruvate. Additionally, we found alternative use of lipids and lipid precursors and a dependency on extracellular aspartate. Finally, *AARS2* mutations caused dysregulation in calcium handling and cardiac ion channel balance, causing arrhythmia and contractile defects. Surprisingly, the patient hiPSC-CMs showed reduced sensitivity to pro-arrhythmic drugs. In conclusion, we captured the pathological features related to infantile mitochondrial cardiomyopathy showing a valuable resource for studying the disease pathology and ways to rescue the phenotype.

Keywords: hiPSC-derived cardiomyocytes/Infantile cardiomyopathy/metabolomics/mitochondrial cardiomyopathy/mitochondrial disease model

INTRODUCTION

Mitochondrial diseases can lead to a variety of pathologies in different human organs (Gorman *et al.*, 2016; Vafai & Mootha, 2012; Koopman *et al.*, 2012). Since mitochondria crucially regulate cell metabolism, it is not surprising that the heart, which has a high energetic demand due to its contractile work, is among the most affected by mitochondrial dysfunction and disease. Mitochondrial diseases are often caused by genetic mutations in either the mitochondrial or nuclear DNA (mtDNA and nDNA, respectively), affecting proteins involved in mitochondrial function (Finsterer & Kothari, 2014). One particular mitochondrial disease is the rare, autosomal recessive combined oxidative phosphorylation deficiency 8 (COXPD8, OMIM: 614096), which leads to lethal infantile cardiomyopathy shortly after birth (Götz *et al.*, 2011). The disease is caused by mutations in the nuclear gene alanyl-tRNA-synthetase 2 (*AARS2*) that encodes the mitochondrial protein alanine-tRNA synthetase (mt-AlaRS). This enzyme plays a crucial role in the protein translation machinery of mitochondria, charging the naïve mitochondrial transfer-RNA^{Ala} with alanine to create alanyl-tRNA while also playing an essential role in verifying the correct charge on other aminoacyl-tRNAs (Euro *et al.*, 2015; Aibara *et al.*, 2020; Sokabe *et al.*, 2009; Guo *et al.*, 2009; Hilander *et al.*, 2018). The role of mitochondrial translation is relatively small, with the mtDNA only encoding 22 tRNAs, 2 ribosomal-RNAs and 13 protein-coding genes (Vafai & Mootha, 2012; Koopman *et al.*, 2012; Anderson *et al.*, 1981). However, these 13 proteins form essential parts of the respiratory chain complexes I, III, IV and V, while complex II is composed solely of subunits encoded in the nDNA. The respiratory chain complexes I-IV are the central drivers of oxidative phosphorylation (OXPHOS), performing the reduction and oxidative (redox) reactions of Nicotinamide-adenine-dinucleotide (NADH) and Flavine-adenine-dinucleotide (FADH₂). These reactions create and maintain the proton gradient essential in driving the F₁F₀-ATPase complex V, the final OXPHOS step, and charge ADP with an additional phosphate group to ATP. Mutations in *AARS2*, therefore, contribute to translational defects in these 13 subunits, with the misincorporation of amino acids leading to decreased stability of the OXPHOS complexes I, III, IV and V and a lower abundance of fully assembled complexes (Watanabe, 2010; Hilander *et al.*, 2018).

Previous studies have shown that mutations in *AARS2* can lead to either neurological pathologies, known as (ovario)leukodystrophy, or to early-onset lethal infantile cardiomyopathy, depending on the mutational burden (Dallabona *et al.*, 2014; Euro *et al.*, 2015; Taylor *et al.*, 2014; Tang *et al.*, 2019; Sommerville *et al.*, 2019; Götz *et al.*, 2011; Zhang *et al.*, 2022). Similar to other mt-Aminoacyl-tRNA-related pathologies (Watanabe, 2010; Yao & Fox, 2013; Nguyen *et al.*, 2021) and mitochondrial defects in general, the tissue or organ affected depends on which gene is mutated or even the specific genetic mutation (Koopman *et al.*, 2012; Nguyen *et al.*, 2021; Yapliito-Lee *et al.*, 2007; Nielsen *et al.*, 2020). In COXPD8, the *AARS2* c.1774C>T mutation is recognised as a central mutation leading to infantile cardiomyopathy, either as a homozygous mutation or accompanied by an additional defective

allele with another compound mutations (Götz *et al.*, 2011; Sommerville *et al.*, 2019; Mazurova *et al.*, 2016). While the disease is very rare, the pathology observed in patients is well described. Pathological features common to all COXP8 patients include (hypertrophic) cardiomyopathy, cytochrome oxidase (COX) negative muscle fibres, reduced expression of the OXPHOS complexes I, III, IV and V in both heart and muscle fibres, and lactic acidosis, in addition to a varying degree of neurological pathology. However, few studies to date have examined the characteristics of the mutations in detail and compared this directly with functional features of affected cells such as cardiomyocytes.

Human-induced pluripotent stem cells (hiPSCs) offer an opportunity to model mitochondrial cardiomyopathies, explore the underlying pathological molecular mechanisms and test therapeutic options (Pavez-Giani & Cyganek, 2022). Indeed, mitochondrial cardiomyopathies due to nDNA mutations, mainly with hypertrophic and dilated phenotypes, have previously been modelled using cardiomyocytes differentiated from hiPSCs (hiPSC-CMs). These studies include research into pathologies such as Leigh syndrome (Hallas *et al.*, 2018), Friedreich's ataxia (Hick *et al.*, 2013; Wong *et al.*, 2019; Lee *et al.*, 2016; Li *et al.*, 2019), Barth syndrome (Wang *et al.*, 2014; Jin *et al.*, 2016; Fatica *et al.*, 2019; Dudek *et al.*, 2016; Chowdhury *et al.*, 2018), dilated cardiomyopathy with ataxia (Rohani *et al.*, 2020), and very long-chain acyl-coenzyme A dehydrogenase deficiency (Knottnerus *et al.*, 2020). These studies using hiPSC-CMs, either in 2-dimensional culture or engineered heart constructs, have revealed ultrastructural abnormalities of the mitochondria, reduced oxidative capacity, aberrant calcium handling and electrophysiological properties, and impaired force of contraction. However, so far, no hiPSC-based cardiac models have been reported for *AARS2* mutations with prominent infantile cardiomyopathy phenotype, possibly due to the fatal and early onset (perinatal to early post-natal) disease phenotype leading to difficulties in obtaining primary material (Mazurova *et al.*, 2016).

Here, we studied cardiomyocytes differentiated from hiPSCs generated from newborn-sibling patients carrying compound mutations in *AARS2* and their apparently healthy mother who carried one heterozygous *AARS2* mutation (van Helden *et al.*, 2021). We also created genetically corrected hiPSC controls matching the unaffected father's genotype. In the patient hiPSC-CMs, mitochondrial dynamics were disrupted, and metabolic homeostasis was altered, reducing ATP and altering the usage of amino acids and lipids. These changes led to dysregulation in calcium handling and electrophysiological properties, resulting in arrhythmic behaviour and altered contractility kinetics. The results in this study provide insights into *AARS2* mutation effects on hiPSC-CM function and could ultimately help identify targets for therapy.

RESULTS

Generation of isogenic pairs of hiPSC lines from patients with COXPD8 and their differentiation into cardiomyocytes

Three siblings, male twins and another brother, presented with circulatory and respiratory insufficiency shortly after birth (Kamps *et al.*, 2018). Fig. 1A shows the family tree, where patient 1 (II-1) showed hypertrophic cardiomyopathy and additional signs of hypotonia and convulsions with persistent lactic acidosis. The patient died five days postpartum. Patient 2 (II-3) was diagnosed with hypertrophic cardiomyopathy shortly after birth, presented with lactic acidosis and died several days postpartum. Genetic testing by exome sequencing revealed two compound mutations in the *AARS2* gene, c.1774C>T inherited via the father's allele and c.2872C>T present on the mother's allele (Fig. 1A, B) (Kamps *et al.*, 2018). The c.1774C>T missense mutation is associated with the COXPD8 pathology when present homozygously or in conjunction with a pathological compound mutation on the other allele (Taylor *et al.*, 2014; Sommerville *et al.*, 2019). The c.2872C>T nonsense mutation, which has not been described in other patients before, is present in the gene's last exon, resulting in protein truncation. For our study, hiPSCs from the two patients carrying the compound mutations, brothers both from different pregnancies (II-1 and II-3), and their mother, carrying the heterozygous truncation (I-2) were available (Fig. 1A, LUMCi024-A, LUMCi025-A and LUMCi026-A, respectively and as described previously (van Helden *et al.*, 2021), hereafter referred to as Pt 1, Pt 2 and Ctrl).

We designed a gRNA to correct the c.2872C>T mutation using CRISPR/Cas9 technology to create isogenic control hiPSC lines from Pt 1 and Pt 2 that matched the father's genotype (Fig. S1A and table S1). By using ddPCR for screening, we simultaneously detected the wild-type- (WT), mutant- and corrected alleles (Fig. S1A, B). Three clones from Pt 1 and two from Pt 2 were isolated (3/82 and 2/89), with a correction efficiency of 2.8% (Fig. S1C). Further characterisation of the clones confirmed correction of the mutation and the introduction of the three silent mutations (Fig. 1C, Fig. S1F). All corrected clones showed normal karyotypes by G-banding and expressed the pluripotency markers OCT3/4, SSEA4, and NANOG (Fig. S1D, E), similar to the respective parental lines (van Helden *et al.*, 2021). We selected one corrected clone per line, hereafter referred to as Iso 1 and Iso 2 (LUMCi024-A-corrected and LUMCi025-A-corrected on hPSCreg) for further characterisation and used the following nomenclature throughout the manuscript: LUMCi024-A and LUMCi025-A are patient 1 (Pt 1) and patient 2 (Pt 2), respectively, LUMCi024-A-corrected and LUMCi025-A-corrected are Iso 1 and Iso 2, respectively; LUMCi026-A is the control (Ctrl).

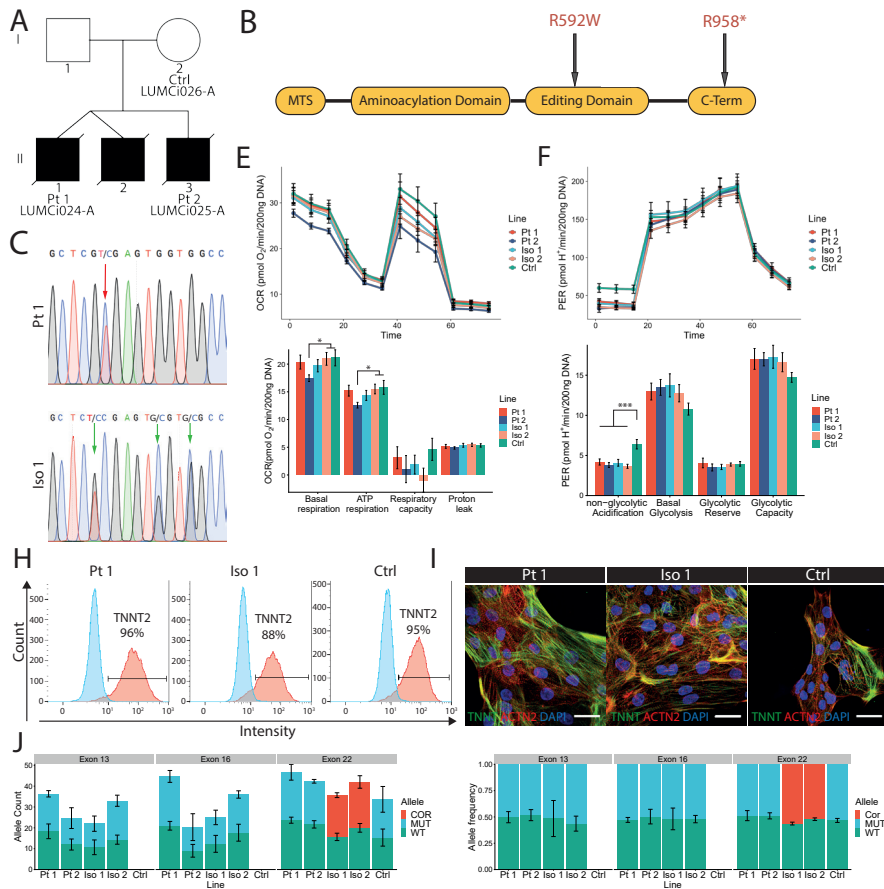


Figure 1: genealogy, genetic correction in hiPSCs, metabolic characterisation of hiPSCs and differentiation into cardiomyocytes.

A) Genealogy showing unaffected (white) and affected individuals (black). Circle: female; square: male. The hiPSC lines were generated from the unaffected mother (I-2, LUMCi026-A or Ctrl), and two affected children (II-1 and II-3, LUMCi024-A or Pt 1, and LUMCi025-A or Pt 2, respectively). B) Graphical representation of the enzyme Mt-AlaRS with the different protein domains. The locations of the two pathological amino acid mutations, R592W and R958*, are shown in red, and the additional polymorphism V730M is indicated in a lighter shade. C) Sanger sequencing chromatogram of DNA isolated from Pt 1 and Iso 1 hiPSCs showing the corrected c.2872C>T mutation in Iso 1. D and E) Traces of extra-cellular flux assays in undifferentiated hiPSCs by Seahorse measuring Oxygen consumption rate (OCR) (D) and proton extrusion rate (PER) (E). Below is the quantification of the different processes (basal respiration, ATP respiration, respiratory capacity, proton leak, (E, bottom) and non-glycolytic acidification, basal glycolysis, glycolytic reserve, and glycolytic capacity (F, bottom)) is shown as a bar graph. N ≥ 18, n = 3 biological repeats, data represented as mean and standard error. F) Cardiomyocyte differentiation protocol. Day 0 (D 0) was defined as the first induction of the mesoderm. BMP 4: Bone morphogenetic protein 4; CHIR = CHIR99021; XAV: XAV-939. Lactate treatment was used as metabolic selection for enriching the cardiomyocyte population. G) Representative FACS measurement (histograms) for cardiac troponin T (TNNT) (red) in Pt 1, Iso 1, and Ctrl hiPSC-CMs after lactate selection. Cells stained with isotype-matched antibodies were used as a negative control in blue. H) Representative immunofluorescence images of cardiac sarcomeric proteins TNNT (green) and α-actinin 2 (ACTN2, red) in Pt 1, Iso 1, and Ctrl hiPSC-CMs. Nuclei are stained in blue with DAPI. Scale bar = 25 μm. I) AARS2 allelic expression quantification from RNA-sequencing showing the copy number for WT (green),

mutated (blue), and corrected (red) alleles. Left panel: absolute count; right panel: allele frequency. N = 3 biological repeats. Error bar: standard error. Statistics are indicated as follows unless otherwise indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with student's t-test.

An unregular cellular phenotype was observed during the culture of the patient and isogenic hiPSC lines. Massive cell death would occur when these lines were allowed to grow at confluencies higher than 80%. This behaviour, however, was not apparent in the Ctrl line. Higher basal rates of glycolysis and lower rates of OXPHOS could be depleting glucose more rapidly in the medium, resulting in this observed phenotype. We performed Seahorse extra-cellular flux measurements to uncover whether metabolic differences were already present in the undifferentiated hiPSCs. These measurements revealed that Pt 2 had lower basal respiration and ATP-coupled respiration compared to Iso 2 and the Ctrl (fig. 1E.), but the other lines did not show any differences in respiration. Measuring glycolysis, we observed that both the Pt and Iso lines had a lower non-glycolytic acidification rate compared to Ctrl, suggesting a lower total contribution to glycolysis (Fig. 1F), however, this did not result in a significantly lower basal glycolytic rate.

We modified the differentiation protocol by shortening the mesoderm induction period from 3 to 2 days and adjusted the concentration of the growth factors Activin A and BMP4 per line (Fig. 1G). This led to >85% hiPSC-CMs in differentiated populations after lactate selection as determined by flow cytometric analysis for cardiac troponin T (TNNT2) for all five lines (Fig. 1H, Fig. S1J). The hiPSC-CMs showed typical sarcomere organisation, independent of whether they were Pt, Iso, or Ctrl (Fig. 1I, Fig. S1K). Finally, whole-genome gene expression by RNA sequencing (RNA-seq) revealed similar *AARS2* allele frequencies in all the lines, suggesting that the *AARS2* mutations and correction of c.2872C>T did not affect either allele expression or mRNA stability (Fig. 1J).

Patient hiPSC-CMs have altered mitochondrial function

The *AARS2* mutations lead to decreased mitochondrial OXPHOS complexes I, III, IV and V in cardiac biopsies from patients, as well as lactic acidosis in their blood plasma (Kamps *et al.*, 2018). To assess the mitochondrial properties of our hiPSC-CMs, mitochondrial OXPHOS complexes and the protein mt-AlaRS were quantified by Western blot. We found reduced mt-AlaRS expression compared to both the corresponding Iso and Ctrl hiPSC-CMs, and reduced complex IV mitochondrially encoded cytochrome c oxidase I (MTCO1) in Pt 1 when compared to Iso 1 (Fig. 2A,B, Fig. S2A,B, Fig. S3A,B). Additionally, the mitochondrial household gene and loading control, Voltage-dependent anion-selective channel 1 (VDAC1), was unchanged (Fig. S2C, Fig. S3C) when normalised to the whole cell lysate, indicating no differences in mitochondrial biogenesis. Strikingly, we found that the copy number of mtDNA per cell was increased in the patient hiPSC-CMs versus the isogenic

controls, with the maternal control showing even lower numbers than the corrected control (Fig. 2C, Fig S3D). TEM was used to examine mitochondrial ultrastructure (Fig. 2D, Fig. S3E), which did not differ between hiPSC-CMs from all lines. Finally, mitochondrial membrane potential (MMP) and network organisation were assessed using TMRM (Fig. S2D). Both MMP, expressed here as mean mitochondrial intensity, and the mean mitochondrial volume was increased in the patients compared to their isogenic controls and Ctrl (Fig. 2E, Fig. S3F).

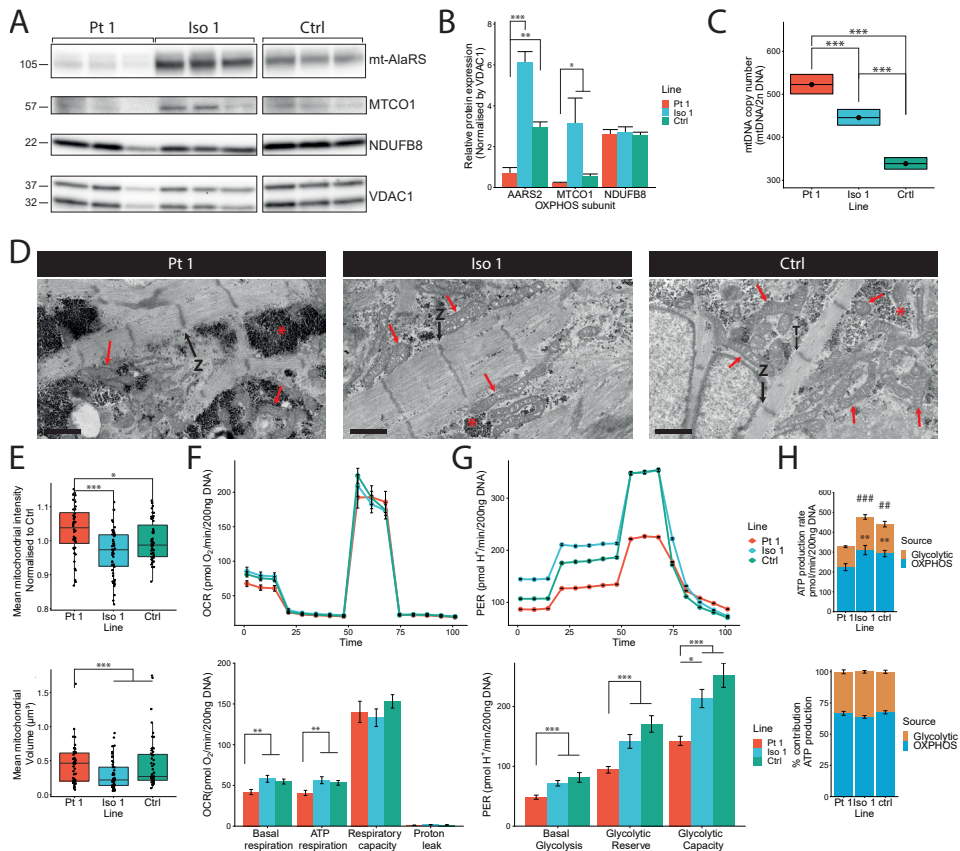


Figure 2: Altered mitochondrial protein expression, mitochondrial organisation and metabolic flux in Pt 1 hiPSC-CMs.

A) Western blot showing expression of mitochondrial proteins mt-AlaRS, MTCO1, NDUFB8, and VDAC1 in Pt 1, Iso 1 and Ctrl hiPSC-CMs. Each column represents an independent differentiation (biological replicate). **B)** Quantification of mitochondrial protein expression, normalised by VDAC1 expression as a marker for total mitochondrial biomass. **C)** mtDNA copy number per cell, quantified by ddPCR. The plot represents the Poisson minimum, mean, and maximum number of mtDNA per 2N nDNA copy number. Calculations based on Poisson distribution. N = 22-26, n = 3-4 biological repeat, ** P < 0.01, *** P < 0.001, Poisson statistics. **D)** Representative transmission electron microscopy (TEM) images from Pt 1, Iso 1, and Ctrl hiPSC-CMs. The red arrow indicates mitochondria, the red star indicates glycogen deposits and black Z the Z-disk. Scale bar = 1 μ m. **E)** Quantification

of the intensity of the TMRM dye in mitochondria, normalised to the mean of the Ctrl and mean volume of each identified mitochondria. Each dot represents the mean value obtained per microscope field. $N = 12$, $n = 1$ biological repeat. F and G) Traces of extra-cellular flux assays in hiPSC-CMs by Seahorse measuring Oxygen consumption rate (OCR) (F) and proton extrusion rate (PER) (G). At the bottom of each panel, quantification of the different processes (basal respiration, ATP respiration, Respiratory capacity, proton leak, (F, bottom) and basal glycolysis, glycolytic reserve, and glycolytic capacity (G, bottom)) is shown as a bar graph. $N \geq 18$, $n = 3-4$ biological repeats. H) Total ATP production (top) and contribution of OXPHOS and glycolysis to total ATP production (bottom), calculated based on extra-cellular flux measurements. Statistical differences are denoted in comparison to Pt 1. # marks statistical differences when comparing Glycolytic ATP production rate: ## $P < 0.01$, ### $P < 0.001$, and * for the OXPHOS ATP production rate ** $P < 0.01$. $N \geq 18$, $n = 3-4$ biological repeats. All data shown are mean with standard error and are $n = 3$ biological repeats unless otherwise stated. Statistics are indicated as follows unless otherwise indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with student's t-test.

In contrast to the increase in MMP and mtDNA copy number, mitochondrial respiration was reduced in the patient hiPSC-CMs compared to the Iso and Ctrl hiPSC-CMs, with lower OCR during basal ATP generation, and additionally, a lower spare respiratory capacity in Pt 2 compared to Iso 2 (Fig. 2E, Fig. S3G). Glycolysis was also reduced in the patient hiPSC-CMs, with lower glycolytic capacity and glycolytic reserve, and an additional reduced basal glycolytic activity in Pt 1 compared to Iso 1 (Fig. 2G, Fig. S3H). Together, this resulted in an overall reduction of 30% in oxidative and glycolytic ATP production rates in both the patient hiPSC-CMs compared with the controls, while the relative contribution of both pathways remained the same between Pt 1 and Iso 1 and had a higher reliance on glycolysis in Pt 2 compared to Iso 2 (Fig. 2H, Fig. S3I).

Altogether, these results showed that patient hiPSC-CM mitochondria had altered structure and function compared with controls.

Altered central carbon and lipid metabolism, as well as gene expression in patient hiPSC-CMs

Quantitative metabolomics of hiPSC-CMs and the exchange of metabolites with the culture medium revealed reduced intracellular concentrations of several amino acids and energy-carrying substrates such as ATP, NAD^+ and creatine phosphate in Pt 1 when compared to Iso 1 or Ctrl, as well as reduced concentrations of key lipid precursors acetate, choline, glycerol, and sn-Glycero-3-phosphocholine and increased concentration of O-phosphocholine in Pt 1 compared to Iso 1 hiPSC-CMs, with the Ctrl showing the highest levels among the samples (Fig. 3A). Pt 2 showed a similar profile, with reduced energy-carrying substrates, notably ATP, GTP, NAD^+ and lactate, with the exception of creatine. The lipid precursor profile was less clear, with decreased concentrations of acetate and sn-Glycero-3-phosphocholine, but with increased choline, o-phosphocholine and glycerol (fig. S4A). Quantitative lipidomics revealed higher lipid concentrations in Ctrl compared with both Iso 1 and Pt 1 hiPSC-CMs (Fig. 3B, Table S3).

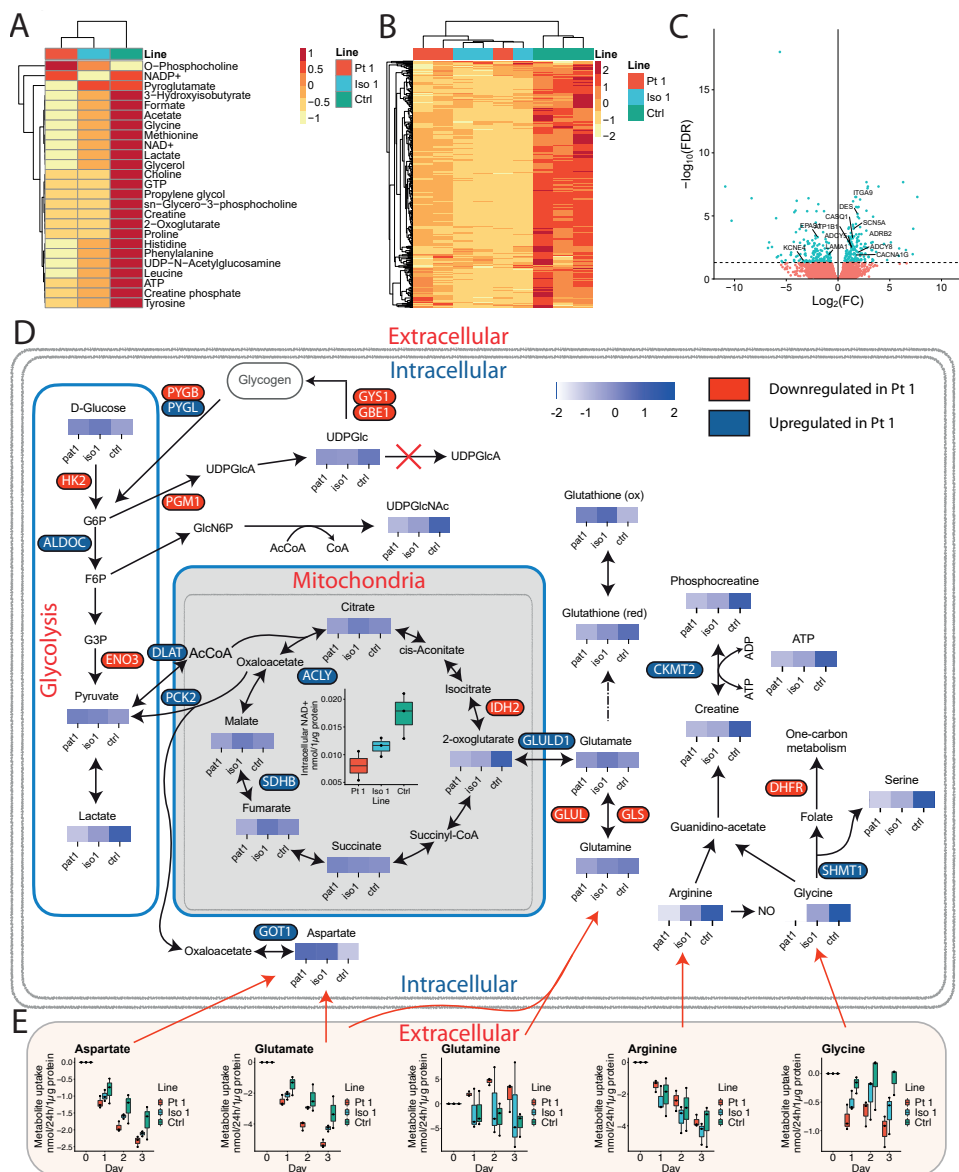


Figure 3. Metabolomic and transcriptomic analysis shows altered metabolic pathways utilisation in Pt 1 hiPSC-CMs.

A) Hierarchical clustering based on intracellular metabolites quantified by NMR (nuclear magnetic resonance); $n = 2-3$ biological repeats. The scale represents Z-score. B) Hierarchical clustering based on all detected lipids on the Lipidzyer platform; $n = 3$. The scale represents Z-score. C) Volcano plot showing the distribution of all genes detected by RNA-sequencing, in blue genes equal to or lower than the 5% false-discovery rate. The marked genes are subselections of genes related to hypertrophy and cardiac development. D) Combined pathway analysis of intracellular metabolites; upregulated genes (blue) and downregulated (red) genes in Pt 1 versus Iso 1 and Ctrl hiPSC-CMs are visualised (p.value < 0.15 and 20% altered fold-change). E) Metabolite exchange from the medium.

Transcriptomic analysis indicated that 44% of the variance in the data could be explained by principal components 1 and 2, resulting in the separation of Pt 1 from Iso 1 and Ctrl but closer alignment between Pt 2 and Iso 2 (Fig. S4B, C). Analysis of differentially expressed genes (DEGs) identified a total of 689 DEGs across three sets of analyses, with 368 DEGs detected when comparing Pt 1 hiPSC-CMs with Iso 1 hiPSC-CMs, 335 DEGs between Pt 1 and Ctrl, and 361 DEGs when comparing Pt 1 with the “healthy” lines (Pt 1 vs Iso 1 and Ctrl, with correction of genetic background, Fig. S4D). Hierarchical clustering based on the 361 DEGs from Pt 1 vs healthy lines showed that the Pt 1 hiPSC-CMs clustered separately from both the Iso 1 and Ctrl CMs, driven mainly by differences between Pt 1 and Iso 1 (Fig. S4E). Finally, hierarchical clustering on the 42 DEGs generated by comparing the patients to both isogenic lines showed different gene expression clusters between the patient lines versus isogenic and control lines (Fig. S4F), indicating the presence of the *AARS2* mutations does influence general gene expression in hiPSC-CMs.

Pathway analysis combining intracellular metabolite concentrations, their exchange with the growth medium and gene expression indicated key differences between Pt 1 and the Iso 1 and Ctrl hiPSC-CMs (Fig. 3D and 3E). In particular, and in agreement with the Seahorse assay, Pt 1 and Pt 2 cells produced less lactate. However, a more detailed analysis of the data pointed to differences in mitochondrial metabolism, redox balance, substrates for energy generation and links to lipogenesis/lipolysis. The data also highlighted the following: i) the upregulation of dihydroliipoamide S-acetyltransferase (*DLAT*) in Pt 1 combined with reduced lactate production suggests that most of the intracellular pyruvate is transferred into the mitochondria and converted to AcCoA and subsequently to citrate, ii) decreased 2-oxoglutarate in Pt 1 combined with the downregulation of Isocitrate dehydrogenase (*IDH2*), indicates the reduced oxidative conversion of citrate to 2-oxoglutarate. iii) lower NAD⁺ in Pt 1 and Pt 2 as a result of impaired respiration (mitochondrial NAD⁺) and reduced conversion of pyruvate to lactate (production of cytosolic NAD⁺), further verified by additional independent measurements of intracellular NAD⁺ in both patients (Fig. S4G). iv) higher intracellular aspartate in Pt 1 and Iso 1, and a significantly higher influx of extra-cellular aspartate in Pt 1 combined with upregulation of cytosolic glutamic-oxaloacetic transaminase 1 (*GOT1*) to facilitate aspartate *de novo* synthesis from oxalacetate. v) a higher influx of extra-cellular glutamate in Pt 1 combined with upregulated glutamate dehydrogenase 1 (*GLUD1*) and with downregulation of glutamate-ammonia ligase (*GLUL*) and glutaminase (*GLS*), additionally identified as altered regulation in the mitochondrial pathway analysis in both Pt 1 and Pt 2. These data suggest that Pt 1 cells preferentially utilise extra-cellular glutamate rather than from glutaminolysis, which in turn can be converted to 2-oxoglutarate in an NAD⁺-dependent step. vi) the upregulation of ATP citrate lyase (*ACLY*) in Pt 1, suggesting conversion of cytosolic citrate, which can be generated from either reductive carboxylation of glutamine and/or from pyruvate entering the TCA via AcCoA to cytosolic oxalacetate. vii) Pt 1

cells exhibited more oxidised and less reduced glutathione suggesting differences in ROS production. viii) Pt 1 and Pt 2 had lowered intra-cellular glycine, and Pt 1 also showed increased consumption of extra-cellular glycine. This altered glycine concentrations, combined with the upregulation of serine hydroxymethyltransferase 1 (*SHMT1*) and downregulation of dihydrofolate reductase 2 (*DHFR*), might also contribute to higher serine biosynthesis and utilisation in Pt 1 rather than contributing one-carbon units to the folate pathway. Interestingly, these pathways were also found to be altered in regulation according to the MitoCarta3 dataset (Fig. S4H). ix) While overall, Pt 1 exhibited lower pools of high energy phosphates (ATP, phosphocreatine), the upregulation of mitochondrial creatine kinase 2 (*CKMT2*) could indicate consumption of extra-cellular glycine by Pt 1 to produce phosphocreatine. x) Choline and ethanolamine pathways were also different in Pt 1, including the phosphorylation to phosphocholine (PCho) and phosphoethanolamine (PEtn), as well as the levels of formed *sn*-glycerophosphocholine (GPC). Interestingly, the expression of enzymes degrading PC into GPC, as well as those that convert PEtn to acetate and AcCoA, were all increased in Pt 1 (Fig. S4I). xi) In addition to the overall lower levels of all lipids classes in Pt 1 (Fig. 3B), we also observed lower intracellular free glycerol – a source of the three-carbon backbone of TAG and other lipids. This lower free glycerol combined with increased uptake of glycerol from the medium in Pt 1 and Iso 1, lower lipoprotein lipase (*LPL*) (degradation of lipids to free glycerol) and higher glycerol kinase (*GK5*) (conversion of glycerol to glycerol-3-phosphate to facilitate the production of pyruvate), matched by lower expression of glycerol-3-phosphate dehydrogenase 1 like enzyme (*GPDI1*) (conversion of dihydroxyacetone phosphate to glycerol-3-phosphate, away from glycolysis) in Pt 1 (Fig. S4I). Combining all glycerol-related and PE/PC-related data suggests different capacities of Pt 1 for lipid synthesis and utilisation of lipolysis products, albeit these links need further investigation.

Mitochondrial pathways were analysed using MitoCarta3 by performing four comparisons; Pt 1 vs Iso 1, Pt 2 vs Iso 2, both patients vs both isogenic, correcting for genetic background and finally, both patients vs the isogenic and the Ctrl line, again correcting for genetic background. This pathway analysis revealed some consensus (Fig. S4H). Specifically, genes related to mtRNA regulation and ribosome seemed to be affected in both Pt 1 and Pt 2. Furthermore, glutamate, glycine and 1-carbon metabolism were found to be altered in regulation, compounding the observations made with the metabolomics analysis.

Further analysis of the RNA-seq using KEGG also revealed several altered pathways related to metabolic homeostasis, contractile apparatus or adhesion molecules, calcium handling, and translation and transcription (Fig. 4A). Additionally, GO-term analysis revealed altered transport and phosphorus metabolism pathways (Fig. 4B).

Calcium transients in patient hiPSC-CMs showed altered dynamics and arrhythmic behaviour

We were particularly interested in investigating whether the observed transcriptional differences in calcium handling and contraction pathways were paralleled by functional consequences, as calcium dynamics play an essential role in the excitation-contraction coupling of cardiomyocytes. Therefore, we measured the calcium transients and contraction profiles of the patient and control hiPSC-CMs. The cardiomyocytes from each of the hiPSC lines displayed a range of different calcium transient profiles, with some wells showing typical, rhythmic calcium traces and other wells presenting arrhythmic calcium transients (Fig. 4C, D). The arrhythmic behaviour was analysed by plotting the data in Poincaré plots. In Pt 1 hiPSC-CMs, the Poincaré profile showed a greater spread and quantification showed a larger standard deviation along the intersecting line, indicating a higher incidence of arrhythmic transients than the Iso 1 and Ctrl hiPSC-CMs (Fig. 4E, F).

Quantifying the calcium transient characteristics during non-arrhythmic transients revealed prolonged peak-to-peak-intervals (PtP-intervals) under spontaneous beating, with subsequent increases in the calcium-transient duration at 90% decay and calcium amplitude in Pt 1 compared to Iso 1 and Ctrl (Fig. S5A). Additionally, both the rising- and decay-slope were decreased, in line with an increase in rising and decay time in Pt 1 compared to Iso 1 and Ctrl CMs (Fig. S5A). We used caffeine, a Ryanodine Receptor 2 (RyR2) channel agonist, to induce calcium release from the sarcoplasmic reticulum (SR) and measure total cytosolic calcium. The addition of caffeine prolonged peak duration and increased amplitude in the Ctrl compared to both Pt 1 and Iso 1 hiPSC-CMs, while the peak area did not differ between the lines (Fig. S5B, C). These results suggest that the observed calcium changes between Pt 1 and Iso 1 were not due to differences in total cytosolic calcium.

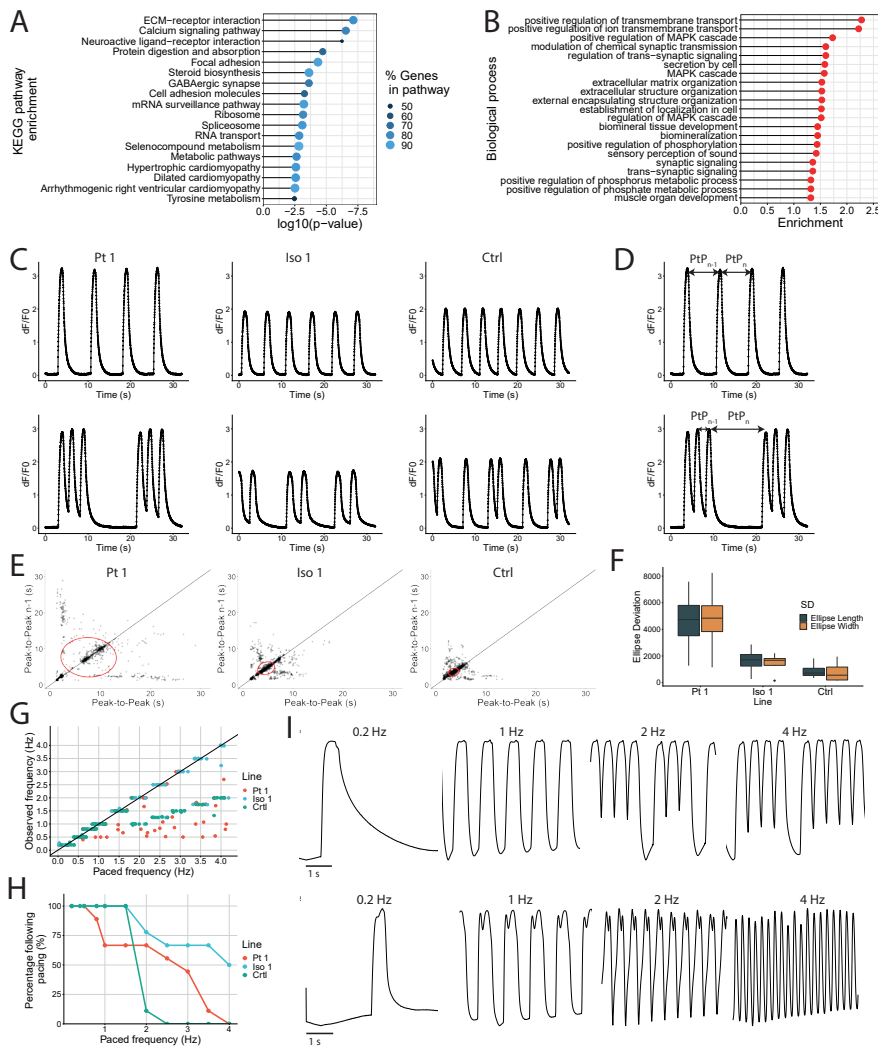


Figure 4. Altered calcium homeostasis and arrhythmic behaviour Pt 1 in hiPSC-CMs.

A) KEGG pathways were found altered in their expression. (FDR adjusted p-value < 0.05). B) Gene-ontology (GO) biological processes analysis of enriched pathways (Kruskal-Wallis p-value < 0.05). C) Representative calcium transient traces showing rhythmic calcium profiles (top) and arrhythmic calcium profiles (bottom) in spontaneously contracting hiPSC-CMs. D) Graphs showing the method for calculating the poincaré plots from rhythmic (top) and arrhythmic (bottom) calcium traces; each peak-to-peak (PtP) time as compared to the previous peak. E) Poincaré plots showing arrhythmia, marked as a red ellipse drawn based on calculating standard deviation along and perpendicular to the dissecting line ($N = 60$, $n = 4$ biological repeats). F) Quantification of the ellipse statistics. G) Ability of hiPSC-CMs to follow increasing pacing frequencies. The x-axis shows paced frequency. The y-axis shows the observed beating rate of the hiPSC-CMs. All data points underneath the intersecting line reflect hiPSC-CMs not contracting at the paced frequency. ($N = 8-9$, $n = 3$ biological repeats). H) Representative traces show different contractile characteristics during ramped pacing. Pt 1 hiPSC-CMs (top) show arrhythmic contractile properties at higher frequencies, while Iso 1 hiPSC-CMs (bottom) retain regular beating intervals. I) Quantification of the percentage of samples able to follow the paced frequency up to 4 Hz.

We then measured the contraction characteristics of the hiPSC-CMs. In agreement with the calcium transient analysis, Pt 1 hiPSC-CMs showed a higher spontaneous Beat-to-Beat (BtB) rate, higher contraction duration and increased relaxation time compared to Iso 1 and Ctrl, while time-to-peak was unchanged (Fig. S5D). To mimic physiologically relevant beat-rates and the cellular stress induced by high beat rates, we paced the hiPSC-CMs at increasing frequencies, starting from 0.2 Hz and ramping up to 4 Hz (Fig. 4G). From 0.8 Hz onwards, both the patient hiPSC-CMs showed diminished capability to follow the increased pacing frequencies compared to their corresponding isogenic CMs (Fig. 4G, H). Additionally, when the patient hiPSC-CMs failed to follow the paced frequency, both lines showed an arrhythmic behaviour (Fig. 4I). Conversely and, different to the isogenic CMs that were able to respond to all the pacing frequencies, the Ctrl hiPSC-CMs could not be paced above 2 Hz. Instead, they reliably contracted at half of the pacing frequency, resulting in beat rates of 1, 1.5 and 2 Hz while paced at 2, 3 and 4 Hz, respectively. Although Pt 2-hiPSC-CMs did not differ from Iso 2 in calcium handling (Fig. S6A-E), the contraction duration and time-to-peak were both increased in Pt 2 (Fig. S6F). Additionally, Pt 2 showed the same high prevalence of arrhythmias as Iso 2 (Fig. S6G-H). These results indicated that the patient-derived CMs, harbouring the compound *AARS2* mutations, CMs were more prone to arrhythmic events than their respective isogenic controls and the independent control.

Lower calcium and NaV1.5 ion-channel sensitivity revealed by flecainide treatment in MEA

To further exacerbate the phenotype in Pt 1, we performed a small drug screen in hiPSC-CMs of Pt 1, Iso 1 and Ctrl, pacing at 1 Hz. In the presence of both 5 μ M Thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump, and 15 μ M flecainide, a potent anti-arrhythmic drug blocking the Ryanodine receptor 2 (RYR2), the cardiac sodium channel NaV1.5, and the human Ether-à-go-go-Related Gene (hERG), none of the hiPSC-CMs be paced at 1 Hz. The calcium transients changed to show a typical “shoulder” during the downslope of the calcium transient that is thought to be a precursor for early afterdepolarisations (EAD) (Blinova *et al.*, 2017). This was more apparent in the Ctrl and Iso 1 lines, and subsequently led to actual EAD-induced sustained triggered activity in the Ctrl line (Fig. 5A). Quantification confirmed that Pt 1 hiPSC-CMs had a weaker drug response compared to both Iso 1 and Ctrl, with the changes in beat rate, decay slope, transient area, PWD50 and PWD90 all being less pronounced (Fig. 5B).

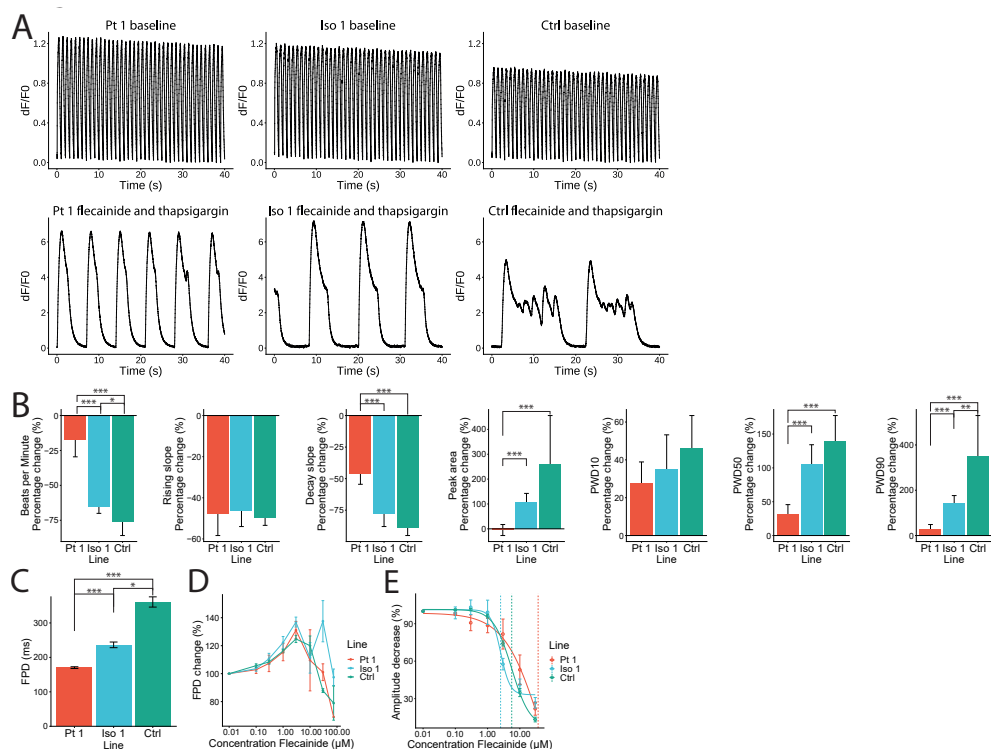


Figure 5: Calcium, contraction and electrophysiological properties of hiPSC-CMs.

A) Representative calcium transients from cardiomyocytes paced at 1 Hz (top) at baseline without drug intervention and at 1 Hz (bottom), including 15 μ M Flecainide and 5 μ M thapsigargin. B) Quantification of calcium transients parameters after the addition of flecainide and thapsigargin. DMSO treatment was used as a reference control. $N = 8$ and $n = 2$ biological repeats. Statistics indicated as follows, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with Wilcoxon signed-rank test. C) Field potential duration (FPD) as assessed by multi-electrode array measurements. $N \geq 18$, $n = 2$ biological repeats. Statistics indicated as follows, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with student's t-test. D-E) FPD change (D) sodium spike amplitude change (E) upon Flecainide treatment. $N \geq 6$ and $n = 2$ biological repeats. For amplitude decrease, the $EC_{50} = 36.8$, 2.52 and 5.59 μ M for Pt 1, Iso 1 and Ctrl, respectively. All data shown are mean with standard error.

Because EADs are an arrhythmic response of cardiomyocytes to hERG-channel blockers, we investigated whether the arrhythmic behaviour we observed was due to hERG inhibition. Treatment with either thapsigargin or flecainide alone showed that the effect was solely due to flecainide (Fig. S7A), which is known to block hERG with an EC_{50} of 1.5 μ M (Melgari *et al.*, 2014). Indeed, the transcriptomic analysis revealed that key genes related to excitation-contraction coupling had an altered expression profile in Pt 1, possibly impacting action-potential generation (Fig. S7B); however, well-known $NaV1.5$ modulator *SIRT1* and was not altered (Fig. S7C).

To verify whether the arrhythmic behaviour was caused by hERG blocking, we measured the hiPSC-CM field potential duration (FPD) using MEAs. Interestingly, FPDs of Pt 1 hiPSC-CMs were shorter than Iso 1 or Ctrl hiPSC-CMs (Fig. 5D). When treating the hiPSC-CMs with flecainide, the change in FPD between the lines was similar, suggesting that the hERG-blockage effect of flecainide did not drive the mechanism of action behind the observed differences in the calcium transients. (Fig. 5E). Instead, EADs observed in the calcium measurements were likely driven due to sodium-channel blockage by flecainide, where Pt 1 showed a lower sensitivity to the drug with a smaller reduction in spike amplitude (Fig. 5F).

Altogether, these results indicated a difference in ion-channel and calcium handling in Pt 1 hiPSC-CMs, resulting in altered drug response and decreased FPD *in vitro*.

DISCUSSION

Infantile cardiomyopathy is a devastating and fatal disorder that presents shortly after birth and is often caused by mitochondrial defects. Currently, no effective treatment exists for these young patients, and it is unclear how mitochondrial defects lead to the development of cardiomyopathies.

In this study, we used previously described patient hiPSC lines (van Helden *et al.*, 2021). We generated isogenic control lines by repairing the maternally inherited mutation to investigate how the *AARS2* mutations impacted essential pathways for cardiomyocyte function.

The undifferentiated hiPSCs from the patients were not able to grow at higher confluencies, resulting in massive cell death and complete colony collapse within 24 hours. While this behaviour is not evident in the Ctrl line or any other of our regular in-house lines, it was not fixed by correcting the maternal allele. Further metabolic characterisation of the hiPSC-lines could also not explain the observed phenotype, with an increase in non-glycolytic acidification in the Ctrl-line, but these differences were not sufficient to show a lower glycolytic activity,

It is therefor tempting to speculate that the *AARS2* c.1774C>T mutation, even in heterozygosity, might result in impaired mitochondrial respiratory function since all the hiPSC lines, patients' (*AARS2*^{c.1774C>T/c.2872C>T}) and isogenic controls (*AARS2*^{c.1774C>T/WT}), showed similar behaviour. However, a mutation-cause effect could not be ruled out due to potential line-to-line and sex differences. In addition, the undifferentiated patient and isogenic hiPSC lines showed differences in the acidification rates compared to the control, possibly resulting in more rapid acidification of the medium during normal culture conditions. Only Pt 2 showed decreased OXPHOS potential compared to Iso 2 and Ctrl, while Pt 1 remained unchanged compared to Iso 1 and Ctrl. It is possible that the *AARS2* c.1774C>T mutation on the paternal allele can impair cellular function in hiPSCs. However, based

on our data, we cannot exclude the possibility of other factors playing a role, such as sex and epigenetic status of the primary samples from which the hiPSCs were derived.

The patients in this study and others diagnosed with COXPD8 showed a substantial reduction in mitochondrial complexes I, III and IV and consequent loss of COX activity in the heart (Kamps *et al.*, 2018). Here, we found reduced respiratory capacity and reduced abundance of complex IV in Pt 1 hiPSC-CMs, suggesting that our *in vitro* COXPD8 infantile cardiomyopathy hiPSC model recapitulates these important aspects of the disease. However, we did not observe complete ablation of the OXPHOS complexes I, III and IV. In an earlier study in patients diagnosed with the same pathology, a greatly reduced abundance of these OXPHOS protein complexes was described in both heart and skeletal muscle biopsies (Sommerville *et al.*, 2019). However, skin fibroblasts from these same patients remained capable of assembling these complexes, while mt-AlaRS abundance was still markedly reduced, similar to the patient hiPSC-CMs presented in this study. This illustrates the tissue specificity of mitochondrial diseases, where cells or tissues from different organs can be differently affected. The immature state of hiPSC-CMs, could explain the presence of complex I and III in our patient. Our hiPSC-CMs are more resemblant of fetal-like cardiomyocytes, while it is known that the mitochondria develop rapidly in the first weeks post-natal (Piquereau & Ventura-Clapier, 2018; Tylková, 2009; Anmann *et al.*, 2014). Indeed, most patients make it to birth, often without pre-natal signs of cardiac impairment (Euro *et al.*, 2015; Mazurova *et al.*, 2016). The metabolic switch towards predominant fatty-acid utilisation in the neo-natal heart is a major factor that drives cardiomyocyte maturation, including mitochondrial development (Ulmer & Eschenhagen, 2020; Lopaschuk & Jaswal, 2010; Piquereau & Ventura-Clapier, 2018). We speculate that these metabolic changes, along with increased mechanical load upon birth, are a deadly trigger in the heart of these young patients. Our study, based on relatively immature hiPSC-CMs that have not undergone the metabolic switch towards fatty-acid metabolism, may not reveal the entire phenotype. Nevertheless, we did find early signs of pathology, leading us to hypothesise that the patient's heart must already be predisposed to developing the disease, but that the cells have enough compensatory mechanisms in place to buffer for the loss in function.

In addition to changes in mitochondrial protein expression, the patients also showed abnormal mitochondrial morphology in the heart (Kamps *et al.*, 2018). Mitochondria are highly malleable organelles changing shape and size by fusion and fission, as well as changing their cristae ultrastructure and mitochondrial membrane potential depending on their cellular context (Vafai & Mootha, 2012). Our study found that these mitochondrial dynamics were also affected in the *AARS2* patient hiPSC-CMs. We observed an increase in both the mtDNA copy number, MMP and mean mitochondrial volume but no differences in the mitochondrial cristae ultrastructure. During fetal heart development, the absolute mtDNA copy number increases, although it remains the same

in relation to the absolute mass of the heart (Pohjoismäki *et al.*, 2013). Increases in mtDNA copy number are frequently observed during mitochondrial dysfunction and metabolic diseases, and it has been hypothesised that this is a cellular compensatory mechanism to increase mitochondrial biomass and energetic capacity (McManus *et al.*, 2019; Li *et al.*, 2018). In this regard, the increase in MMP in Pt 1 and Pt 2 was striking, as mitochondrial dysfunction is often associated with a decreased MMP. However, several other published studies have shown similar increases in the MMP in relation to mitochondrial dysfunction (McManus *et al.*, 2019; Otten *et al.*, 2018). A possible explanation is a reduction in the availability of NAD⁺ in the patient hiPSCs-CMs. NAD⁺ regeneration can be achieved in the mitochondria through the ETC-mediated NADH oxidation produced in the TCA cycle. Alternatively, in cells with ETC dysfunction, NAD⁺ regeneration may also occur from pyruvate in the cytosol, through the reduction step to lactate, by lactate dehydrogenase (LDH). While intracellular pyruvate levels between all hiPSCs-CMs do not differ, we found less lactate production in the patient cells. Thus, in both the mitochondria and the cytosol, NAD⁺ regeneration is limited in the patient hiPSCs-CMs. The role of NAD⁺ concentration and NAD⁺/NADH ratio has been well described in the literature in the context of cardiovascular disease, and recent investigation has solidified the relationship between reduced NAD⁺ and metabolic, cardiac diseases, including bradycardia, to altered regulation of the NaV1.5 channel (Yoon *et al.*, 2022). Here the NADH-dependent acetylation activity of SIRT1 is the key modulator of NaV1.5, altering the excitability of the hiPSC-CMs. In our study, we observed arrhythmic behaviour, reduced NaV1.5 expression and blockage sensitivity (induced by flecainide) in our patient hiPSC-CMs, and reduced intracellular NAD⁺ concentrations. The RNA-seq data indicated only slightly increased NAD-dependent deacetylase sirtuin 2 (*SIRT2*) expression, and *SIRT1* expression remained unchanged (Kane & Sinclair, 2018). We have not been able to uncover the exact nature of what causes the increased incidence of arrhythmia in the patient hiPSC-CMs, warranting further investigation. However, it seems likely that the combination of reduced NAD⁺ availability and reduced expression of NaV1.5 lie at the origin.

Our metabolomics analysis also showed further evidence of altered NAD⁺-dependent processes. Aspartate was the only amino acid increased in patient and isogenic hiPSCs-CMs, while Pt 1 also consumed significantly more aspartate from the culture medium. Aspartate *de novo* synthesis usually takes place in the mitochondria via malate dehydrogenase 2 (MDH2) and Glutamic-Oxalacetic Transaminase 2 (GOT2) and is then transported to the cytosol, where the cytosolic aspartate aminotransferase GOT1 consumes aspartate to transfer electrons into the mitochondria. However, aspartate synthesis via the NAD⁺-dependent MDH2 is limited in cells with ETC dysfunction. This induces a shift in bidirectional GOT1 to generate aspartate in the cytosol instead of consuming it by utilising oxalacetate (Birsoy *et al.*, 2015). In agreement with this, we found an upregulation of *GOT1* in Pt 1. The necessary source of oxalacetate for GOT1 is further supported by i) the upregulation of

DLAT in Pt1, which catalysis the conversion of pyruvate to AcCoA in the mitochondria, which in turn can enter the TCA cycle to form citrate, ii), the upregulation of *ACLY* in Pt 1 which converts glutamine-derived citrate in the cytosol to oxalacetate and iii), the increased uptake of glutamate by the patient hiPSCs-CMs which can further contribute to the synthesis of oxaloacetate. The fact that glutamine- or glutamate- derived reductive synthesis of aspartate also requires NAD^+ (Sullivan *et al.*, 2015), which is limited in Pt 1 cells, might also explain the significantly higher uptake of aspartate from the culture medium by the patient hiPSCs-CMs. It is well known that increased aspartate biosynthesis or its exogenous addition rescues cell proliferation in cells with impaired ETC, regardless of which ETC-complex shows dysfunction (Birsoy *et al.*, 2015; Sullivan *et al.*, 2015). In the same studies, it was also shown that adding aspartate does not restore NAD^+/NADH ratio in cells with reduced respiration but rather supported proliferation via the biosynthesis of nucleotides.

Many previous studies have shown the essential role that mitochondria play in excitation-contraction coupling and calcium handling. It is, therefore, not surprising that calcium handling abnormalities have been previously identified in hiPSC-CM models for mitochondrial cardiomyopathy (Knottnerus *et al.*, 2020; Liu *et al.*, 2021; Li *et al.*, 2018; Lee *et al.*, 2016; Hallas *et al.*, 2018). Here we show that moderate changes in mitochondrial function and ATP availability are linked with disrupted calcium handling and contraction in hiPSC-CMs and, in addition, electrophysiological changes. The evident arrhythmia observed in the calcium transient measurements in our hiPSC-CMs, their inability to follow higher pacing frequencies and their altered sensitivity to flecainide illustrate the interplay between mitochondria and the calcium handling in the heart.

Patients with *AARS2* mutations frequently present with an HCM phenotype, including reduced cardiac contractile capacity and arrhythmic events. It is interesting to note that mitochondrial and calcium dysfunction underlies the decrease in heart function in HCM, like acquired HCM (caused by increased strain on the heart) or familial HCM (mainly due to mutations in sarcomeric proteins) (Lan *et al.*, 2013; Mosqueira *et al.*, 2018, 2019; Smith *et al.*, 2018; Zhou *et al.*, 2019). Mutations in myosin-binding proteins have also been shown to disrupt mitochondrial, and calcium handling in both humans and mice, and the mitochondrial disruptions are thought to be caused by an increased ATP demand by the sarcomeres, leading to increased oxidative stress (Wijnker *et al.*, 2019, 2016; McNamara *et al.*, 2017; Parbhudayal *et al.*, 2020; Güçlü *et al.*, 2017). While we do find transcriptomic evidence of HCM in our patient hiPSC-CMs, 2D hiPSC-CM models are not ideally suited to model the more severe outcomes of HCM, such as the increase in cell-size and the reduction in contractile strength. Regardless, the underlying mechanisms leading to the progressive nature of HCM are evident in our data, the early incidences of arrhythmia triggered by decreased mitochondrial function and aberrant calcium dynamics.

How increased mitochondrial stress leads to further developing calcium and contractile defects is currently not fully understood. These changes were observed in our model, which represents a fetal-like stage of cardiomyocyte development, thereby revealing early phenotype development not described before in patients with COXPD8. Our model could be used in future studies to understand how early mitochondrial dysfunction interacts with excitation-contraction coupling, uncover the cascade of events that lead to COXPD8 cardiac failure, and possibly test potential pharmacological or metabolic interventions.

In summary, we have shown that compound mutations in *AARS2* lead to insufficiencies in OXPHOS and glycolysis and can disrupt excitation-contraction coupling resulting in defective calcium homeostasis and arrhythmic behaviour in the patient hiPSC-CMs and revealing dysfunction in the entire cascade central to cardiomyocyte function. It is possible that by carefully controlling the energy source supplied to the neonate at birth, the development of life-threatening cardiomyopathy could be slowed down or even prevented.

MATERIAL AND METHODS

hiPSC culture and differentiation into cardiomyocytes

LUMC0024i-A, LUMC0025i-A and LUMC0026i-A hiPSC lines, previously generated as described (van Helden *et al.*, 2021) and their isogenic corrected hiPSCs were maintained on recombinant human vitronectin in E8 medium (both Thermo Fisher Scientific). The hiPSCs were passaged as small clumps twice a week using 0.5 mM Ethylenediaminetetraacetic acid (EDTA, Thermo Fisher Scientific) in phosphate-buffered saline without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (PBS⁻, Thermo Fisher Scientific), and plated in E8 medium with addition of Revitacell (1:200, Thermo Fisher Scientific).

The hiPSCs were differentiated into cardiomyocytes as described previously (Campostrini *et al.*, 2021), with the following modifications: on day -1, $1.5\text{--}3.5 \times 10^4$ cells per cm^2 cells were plated on growth factor-reduced Matrigel-coated plates (150 $\mu\text{g}/\text{ml}$) and cardiac mesoderm was induced on day 0 using LI-BPEL medium with the concentrations of the following cytokines differing per line: Activin A (15, 30, 30 ng/ml, Pt 1/Iso 1, Pt 2/Iso 2, and Ctrl, respectively, Miltenyi Biotec), BMP4 (20, 10, 15 ng/ml, Pt 1/Iso 1, Pt 2/Iso 2, and Ctrl, respectively, R&D Systems) and CHIR99021 (1.5 μM , Axon Medchem). After 48 h, the medium was replaced with LI-BPEL containing XAV939 (5 μM , Tocris), then refreshed every 2-3 days thereafter. Enrichment for the cardiomyocyte population was performed by metabolic treatment from day 12-16 in DMEM without glucose (Thermo Fisher Scientific) with 5mM sodium L-lactate (Sigma-Aldrich). All the differentiations resulted in 85-95% of TNNT2+ cells as measured by flow cytometry (Fig. 1E, Fig. S1G). The hiPSC-CMs were dissociated

using the Multi-tissue dissociation kit 3 (Miltenyi Biotec) for 10 min at 37°C and cryopreserved in KnockOut Serum Replacement (KOSR, Thermo Fisher Scientific) with 10% DMSO (van den Brink *et al.*, 2020).

Correction of *AARS2* c.2872C>T using CRISPR/Cas9

Genetic correction of the *AARS2* c.2872C>T mutation was performed by transfecting the hiPSCs with a gRNA recognising the mutated allele, high-fidelity Cas9 Ribonucleoprotein (RNP, IDT), and a single-stranded oligonucleotide (ssODN) comprising the corrected sequence and several silent mutations to avoid re-cutting of the Cas9 after homologous recombination. Briefly, the hiPSCs were dissociated into single cells using Accutase (Accutase) and a total of 1×10^5 cells were electroporated using the NEON electroporation kit and the NEON Transfection system (Thermo Fisher Scientific) with 240 ng gRNA, 1 µg Cas9 RNP and 13.3 µM ssODN according to the manufacturer's recommendation. The cells were plated into a 48-well plate pre-coated with Synthemax-II substrate (6µg/ml, Corning) and containing CloneR E8 (Stemcell Technologies). After 24 h, the cells were refreshed with TesR E8 (Stemcell Technologies) and cultured until near confluence. These bulk transfected cells were dissociated with Accutase and plated at clonal density (1500 cells) in CloneR E8 on a 10 cm dish pre-coated with Synthemax-II. The remainder of the cells were used for DNA isolation, followed by ddPCR screening for identifying successful correction. The cells plated at clonal density were picked, expanded, and frozen, and DNA was isolated for a final ddPCR screen to determine which clones had successfully undergone homologous recombination. Sequences for the gRNA and ssODN can be found in Table S1.

ddPCR for CRISPR/Cas9 correction screening and for estimating mitochondrial copy number

Genomic DNA was isolated using the high pure PCR template preparation kit (Roche) as described by the manufacturer. The ddPCR screen for identifying corrected clones was based on a double probe approach (Miyaoaka *et al.*, 2014) that allowed simultaneous detection of WT, mutant and corrected alleles. The FAM probe targeting the WT allele was designed to allow binding of the mutant allele as well, while the HEX probe was designed only to bind the corrected allele (Table S1). For each sample, no more than 60 ng gDNA was mixed with both probes, ddPCR supermix for probes (Biorad) and 50 units of restriction enzymes CviQI and HindIII-HF (both from NEB), and each sample was placed in a 96-well twin-tech semi skirted PCR plate (Eppendorf). Droplet generation, PCR-amplification and droplet reading were performed using an AutoDG droplet generator, T100 Thermocycler and QX200 Droplet Digital PCR System (all from Biorad). Analysis was performed using QuantaSoft™ Analysis Pro V.1.0.596 (Biorad).

Mitochondrial copy number was determined using a HEX probe targeting the nuclear *RPP30* gene and a FAM probe targeting the mitochondrially encoded *ND4* gene (BioRad). DNA was isolated from hiPSC-CMs using a chloride-based DNA lysis buffer consisting of 10 mM Tris-HCl (Merck) pH 8.0, 1 mM EDTA pH 8.0, 50 mM KCl and 2 mM MgCl₂ (Sigma-Aldrich) with the addition of Proteinase K (Thermo Fisher Scientific) (200 µg/ml). The cells were lysed overnight at 60°C. The ddPCR procedure was adjusted to use 4 units of HaeIII per reaction and 2.5 ng of DNA. Due to the greater abundance of mtDNA compared to nDNA, less DNA was loaded to ensure empty droplets allowing proper quantification of DNA copies. Mitochondrial copy number estimation was calculated in R (v. 4.0.3) based on the mathematical model described previously (Dube *et al.*, 2008). Sequences of the ddPCR probes and primers can be found in Table S1.

Mutation screening

Genomic DNA was amplified by polymerase chain reaction using primers surrounding the three *AARS2* mutations using the Platinum Taq HiFi (Invitrogen). The PCR products were purified (QIAquick PCR Purification Kit, Qiagen) and sent for Sanger sequencing. The sequences for the genotyping primers are listed in Table S1.

Flow cytometry

Cardiac differentiation efficiency was assessed by flow cytometry analysis between days 17-21 of differentiation using cardiac troponin T (TNNT2) staining on fixed cells. Cardiomyocytes were dissociated as described above and subsequently fixed and permeabilised with Fix and Perm reagent A and B (Thermo Fisher Scientific). Cells were then stained in permeabilisation reagent B with Cardiac Troponin T-VioBlue (1:10 or 1:50, Miltenyi Biotec) for 15 min at RT and subsequently analysed in FACS buffer (PBS⁻ with 2% FCS and 2mM EDTA) using a MACSQuant VYB flow cytometer (Miltenyi Biotec). Data were analysed using FlowJo (Tree star).

Pluripotency of the isogenic corrected hiPSC lines was assessed using the same method, using the following antibodies: OCT3/4-BV421, NANOG-PE (BD Biosciences) and SSEA-4-FITC (Miltenyi Biotec). Catalogue numbers and used dilutions for the antibodies are listed in Table S2.

Immunocytochemistry analysis

The hiPSC-CMs were fixed in 4% paraformaldehyde, permeabilised with 0.1% Triton-X (Sigma-Aldrich) in PBS with Ca²⁺/Mg²⁺ (PBS⁺) and blocked with 10% fetal bovine serum (Thermo Fisher Scientific). Samples were stained overnight in blocking buffer at 4°C degrees with primary antibodies

against TNNT2 (rabbit polyclonal 1:1000, Abcam) and ACTN2 (mouse monoclonal 1:800, Sigma-Aldrich). Primary antibodies were counter stained using Alexa-Fluor 488 (Goat- α -rabbit, 1:200, Thermo Fisher Scientific) and Alexa-Fluor 594 (Donkey- α -mouse 1:200, Thermo Fisher Scientific), and nuclei were stained with DAPI (Thermo Fisher Scientific). Images were acquired on a Leica SP8 WLL confocal laser-scanning microscope. Catalogue numbers and used dilutions for the antibodies are listed in Table S2.

Western blot

The hiPSC-CMs were lysed on ice in a RIPA lysis and protein extraction buffer (Thermo Fisher Scientific) with an additional 2 μ l/ml protease inhibitor mix (Sigma Aldrich). Protein content was quantified using a BCA protein kit (Thermo Fisher Scientific). 30 μ g proteins per sample were used for Western blot experiments. Proteins were resolved by SDS-PAGE gel (4-15% Criterion Precast Gel, Bio-Rad) and subsequently transferred to a Polyvinylidene fluoride (PVDF) membrane (Bio-rad). Total protein was assessed by Ponceau staining before being blocked by incubation with TBS with 0.1% tween 20 with 5% non-fat dry milk for 1 h at RT. Primary antibodies against mt-AlaRS, NDUFB8, SDHA, UQCRC2, MTCO1, ATPB and VDAC1 (all Abcam) were incubated overnight at 4°C in milk on the membrane. The membrane was subsequently washed in TBS with 0.1% tween-20 and incubated with the isotype appropriate HRP-conjugated antibody (Cell Signalling) for 1 h at RT. Images were acquired with ChemiDoc MP Imaging System (Biorad) and quantified with ImageJ 1.8.0_66. Catalogue numbers and used dilutions for the antibodies are listed in Table S2.

Transmission electron microscopy (TEM)

hiPSC-CMs were fixed for 1 h by adding to the culture medium an equal volume of fixative (3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4). After washing the monolayers three times with 0.1 M cacodylate buffer, the cells were postfixed for 1 h on ice with 1% OsO₄, 1.5% potassium ferricyanide in 0.1M cacodylate buffer, followed by dehydration in a series of washing steps consisting of ethanol and ethanol mixed with EPON (LX112, Ladd research industries). After the final step with EPON only, BEEM capsules were filled with EPON and positioned on the fixed cells. After polymerisation of the EPON, the BEEM capsules were snapped off the surface of the culture dishes. Ultrathin sections (90 nm) parallel to the surface was made on a Reichert Ultracut S (Leica Microsystems). After the post staining of the sections with uranyl acetate and lead citrate, the electron microscopy images were taken with a twin transmission electron microscope (Thermo Fisher Scientific (formerly Fei) operating at 120 kV, using a Gatan Oneview camera (Gatan, Pleasanton) on binning 2. Overlapping images were collected and stitched together into a large image as described (Faas *et al.*, 2012).

Live imaging of mitochondrial membrane potential and mitochondrial network

hiPSC-CMs were thawed and allowed to recover for 4 days in LI-BPEL medium. After recovery, the cells were dissociated and plated onto a glass 18-chamber slide (Ibidi) coated with 75 µg/ml Matrigel. Four days post-seeding, the hiPSC-CMs were treated and measured as previously described (Rech *et al.*, 2019). Briefly, the mitochondria were stained for 30 min with 10 nM of TMRM (Thermo Fisher) in a medium without phenol red at 37°C and 5% CO₂. Images were acquired using a spinning disk CorrSight fluorescent microscope equipped with a Zeiss 63x numerical aperture (NA) 1.4 objective and a Hamamatsu ORCA-Flash 4.0 V2 camera (FEI). The fluorescent signal was acquired using a 561 nm laser coupled to a 600 nm emission filter. The cells were maintained within a bioculture chamber at 37°C and 5% CO₂. The images consisted of 10 fields of view per well, and the z stacks contained 100 images at a Nyquist Z-distance of 170 nm. The z stacks underwent deconvolution using the Constrained Inference for Linear Mixed-Effects Models (CLME) algorithm of Huygens Professional (SVI Hilversum). Image quantification was performed using MATLAB 2017 (Mathworks) in 3D. Further data analysis was performed in R (4.0.3), and the data was normalised to the average values of the Ctrl line for each replicate.

Measurement of oxidative and glycolytic metabolic capacity

Respiration and acidification rates of hiPSCs and hiPSC-derived cardiomyocytes were assessed using a Seahorse XF-96 Analyzer (Agilent). For undifferentiated hiPSCs, the cells were plated the day before the assay in E8 medium in an assay plate coated with vitronectin. On the day of the assay, the cells were washed and kept for 1 h in the assay medium consisting of Dulbecco's Modified Eagle Medium (DMEM) without bicarbonate (Sigma-Aldrich), 5 mM glucose (Sigma-Aldrich), 2 mM L-glutamine (Thermo Fisher Scientific), 2 mM pyruvate (Thermo Fisher Scientific) and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma-Aldrich) for performing the mitochondrial stress test and the same medium without glucose for assessing the glycolytic capacity. For assessing OXPHOS, 1 µM Oligomycin, 0.5 µM FCCP and 1 µM Antimycin A and Rotenone were injected, while for measuring glycolysis, 5 mM glucose, 1 µM Oligomycin and 10 mM 2-DG were injected (Sigma-Aldrich). Measurements of the metabolic capacity of the hiPSC-CMs were performed as follows. Thawed cardiomyocytes were plated on 75 µg/ml Matrigel in LI-BPEL four days before the assay. The medium consisted of bicarbonate DMEM, 5 mM glucose, 2 mM L-glutamine, 5 mM HEPES and 0.22% fatty acid-free BSA (Bovogen) for assessing OXPHOS and without glucose for assessing glycolytic capacity. The drug injection concentrations were adjusted to 2.5 µM Oligomycin, 2.5 µM FCCP, 2 µM Antimycin A and Rotenone, 5 mM Glucose and 100 mM 2-DG. After the assay, normalisation was performed using a Pico-green assay (Thermo Fisher Scientific). Quantification of

ATP production rates was calculated according to the Real-time ATP production kit. All analysis was performed using R (version 4.0.3).

Metabolomics

Four days after hiPSC-CMs were thawed, the medium was replaced with HEPES-free LI-BPEL, made using Iscove's Modified Dulbecco's Medium (IMDM) without HEPES (Biowest). Additionally, the culture medium was added to an empty well serving as a background control. On days 0-3, 200 μL of the medium was taken from each well with hiPSC-CMs or the control well. Each medium sample was then mixed with 400 μL of ice cold (-20°C) ultra-pure methanol (MeOH; Sigma-Aldrich), vortexed for 10 s and stored at -80°C until further processing. On day 3 after the last medium sample collection, the remaining medium was aspirated, each well was quickly washed with cold PBS⁺ (Thermo Fisher Scientific) and liquid N₂ was added to each well in order to snap freeze the hiPSC-CMs.

Metabolite extraction from the medium was performed by centrifuging the mixed medium – MeOH samples at 18,000 χg for 20 min at 0°C . The supernatant containing the metabolites in the medium was collected and dried under nitrogen gas.

Extraction of both polar and nonpolar fractions from the cells was performed as follows. Keeping the plate on ice, 600 μL of 0°C 60% (v/v) MeOH was added to each well, and cells were lysed using a scraper. The lysate was transferred to pre-cooled Eppendorf tubes and vortexed for 10 min at 4°C . Subsequently, 500 μL of 0°C chloroform (Sigma-Aldrich) was added, and the tubes were vortexed for an additional 10 min at 4°C and then left at -40°C for 30 min to allow separation of the polar and nonpolar phases. Samples were then centrifuged at 4,000 χg for 10 min at 4°C . The polar (upper) and nonpolar (bottom) phases were collected and transferred to glass vials. All extracts were dried under nitrogen gas and stored at -80°C until further processing. The middle protein-containing layer from each sample was dissolved in a buffer consisting of 3M Urea (Sigma-Aldrich), 0.25M TRIS-HCl (Merck) at pH 8.0 and 1% SDS (Sigma-Aldrich) and was subsequently quantified by Pierce BCA protein assay (Thermo Fisher Scientific) for normalisation of the metabolomics data.

Polar cell extracts and media extracts were dissolved in 0.22mL 50mM K₂HPO₄/KH₂PO₄ (Sigma-Aldrich) solution (pH 7.4) in 99.9% deuterated water (D₂O; CortecNet) containing 0.2 mM NaN₃ (Sigma-Aldrich) and 0.05 mM of trimethylsilylpropionic-*d*₄ acid sodium salt (TSP-*d*₄; Sigma-Aldrich). From each sample, 185 μL were transferred to 3 mm Nuclear Magnetic Resonance (NMR) tubes (Bruker), and quantitative metabolomics was performed by NMR spectroscopy as described in detail previously (Kostidis *et al.*, 2017). Briefly, NMR spectra were recorded using a 14.1T NMR spectrometer (600MHz for ¹H; Bruker Avance Neo) under standardised instrumental settings for all

samples. For each sample, a one-dimensional ^1H spectrum was collected using the *noesygppr1d* pulse sequence. All spectra data were phased, baseline corrected and referenced to TSP-d4 methyl protons at δ 0.00 ppm and subsequently imported into Chenomx NMR suit 8.5 (Chenomx Edmonton, Canada) for the quantification of metabolites.

Dried nonpolar cell extracts were used for lipidomic analysis by the commercial Lipidyzer platform (The Lipidyzer, Sciex). Lipid extracts were dissolved in a 250 μL buffer solution of 10 mM ammonium acetate in 50:50 (*v/v*) dichloromethane/methanol (Lipidyzer running buffer), including the Lipidyzer internal standard kit with >50 labelled internal standards for 13 lipid classes. The Lipidyzer platform is a flow-injection-based ion-mobility triple quadrupole system consisting of a Sciex 5500 QTrap equipped with SelexIon technology coupled to a Shimadzu Nexera series UHPLC system used for injection and delivering running buffer at 7 $\mu\text{L}/\text{min}$. A total of 50 μL of the resuspended samples were injected using 2 dedicated methods. Method #1 operated with active differential mobility spectrometry (DMS) separation under the following conditions: DMS temperature low, modifier (propanol) composition low, separation voltage 3500 V, and DMS resolution enhancement low. For method #2, the DMS cell was not activated. The MS operated under the following conditions: curtain gas 17, CAD gas medium, ion spray voltage 4100 V in ESI + mode and -2500 V in ESI- mode, temperature 200 $^{\circ}\text{C}$, nebulising gas 17, and heater gas 25. First, phosphatidyl choline (PC), phosphatidylethanolamine (PE), lysophosphatidyl choline (LPC), lysophosphatidylethanolamine (LPE), and sphingomyelin (SM) lipid classes were analysed with method #1. Next, free fatty acid (FFA), triacylglyceride (TAG), diacylglyceride (DAG), ceramide (CER), dihydroceramide (DCER), lactosylceramide (LCER), hexosylceramide (HCER), and cholesterol ester (CE) lipids were analysed applying method #2. Further technical detail, including a list of all monitored transitions and detailed experimental settings can be found elsewhere. All concentrations from polar metabolites and lipids ($\mu\text{mol}/\text{L}$) were normalised to the protein mass of each cell extract.

RNA-sequencing analysis

RNA was isolated and purified from the hiPSC-CMs using the NucleoSpin RNA-kit (Macherey-Nagel). RNA-sequencing and whole-genome transcriptome data were generated by Novogene (United Kingdom) on the Illumina NovaSeq6000 platform, paired-end sequencing and read length of 150bp with a sequencing depth of 20M raw reads per sample.

Raw RNA-seq reads were processed using the open-source BOWDL RNAseq pipeline v3.0.0 (<https://zenodo.org/record/3713261#.YoQ9mxxBxhE>) developed at the LUMC. This pipeline performs FASTQ preprocessing (including quality control, quality trimming, and adapter clipping), RNA-Seq alignment, read quantification, and optionally transcript assembly. FastQC was used for

checking raw read QC. Adapter clipping was performed using Cutadapt (v2.4) with default settings. RNA-Seq reads' alignment was performed using STAR (v2.7.3a) on GRCh38 human reference genome. The gene read quantification was performed using HTSeq-count (v0.11.2) with the setting “--stranded=no”. The gene annotation used for quantification was Ensembl version 99.

Count tables were normalised against gene length and GC content using the R-package *cqn* v1.4 (Hansen *et al.*, 2012), and genes were filtered based on their expression across all the samples according to (Chen *et al.*, 2016), resulting in a final dataset of 19120 genes and 15 samples. Differentially expressed genes (DEG) were identified using a general linearised model based on the package EdgeR (McCarthy *et al.*, 2012), correcting for cellular origin. Benjamini and Hochberg FDR were computed to adjust p-values obtained for each differentially expressed gene. Using a cutoff of 0.05 at the adjusted p-values, we identified all up and down-regulated genes. Heatmaps were produced using the pheatmap package (Kolde, 2019). KEGG-pathway enrichment was performed based on the sum of rank test, with adjusted p-values < 0.05 considered to be significant according to the Benjamini-Hochberg procedure (Irizarry *et al.*, 2009), gene-ontology (GO) enrichment, using the Kolmogorov-Smirnov Goodness of Fit Test with a cutoff p-value of < 0.05, were performed using the topGO package (2.44 (Alexa & Rahnenfuhrer, 2021)), and mitochondrial pathway analysis enrichment was performed using the sum of rank tests, a less stringent use of un-adjusted p-values of < 0.05 considered significant. Both the tested genes and the involvement in mitochondrial processes was based on the curated MitoCarta 3.0 dataset (Rath *et al.*, 2021).

NAD⁺ measurements

Intracellular NAD⁺ concentrations were Dissociated hiPSC-CMs were plated on a µclear white transparent 384 well plate (Greiner) precoated with 75 µg/ml Matrigel (Corning). After a four-day recovery period, the cells were lysed using the NAD/NADH-Glo assay (Promega) according to manufacturer's protocol, and total NAD⁺ was measured on a SpectraMax iD3 plate reader. Data was analysed using R (4.1.1), and the data were normalised to the average values of the Ctrl line for each replicate.

Calcium transient measurements

Calcium transients were assessed on hiPSC-CMs thawed and seeded in a black, flat-bottomed 96-well plate coated with 75 µg/ml Matrigel (Corning) and cultured for four days before the assay. The calcium-6 dye (Molecular Devices) was dissolved in 10 mL HBSS buffer B and subsequently diluted 3-fold in Buffer B (Molecular Devices). The diluted dye solution was added 1:1 to the wells containing hiPSC-CMs in a bicarbonate-free medium consisting of DMEM, 5 mM Glucose, 2 mM

L-glutamine, 1 mM pyruvate, 5 mM HEPES and 0.2% BSA. Cardiomyocytes were incubated for 1 hr at 37°C and 0% CO₂ before being measured on the FDSS/μcell (Hamamatsu Photonics) at 37°C and 0% CO₂ with an exposure time of 0.017s. Caffeine injection was performed by preparing a “compound plate” including 100 μM caffeine (Sigma Aldrich) in the assay medium, which was then automatically injected into the assay plate, reaching a final concentration of 25 μM (Liu *et al.*, 2007). Analysis was performed using R (4.0.3). Poincaré graphs were generated by plotting the peak-to-peak (PtP) interval of one cycle against the PtP-interval of the previous cycle (PtP_{n-1}). Ellipse statistics were calculated by taking the SD-length (deviation along the intersecting line) and SD-width (deviation perpendicular to the intersecting line), according to (Tulppo *et al.*, 1996; Fishman *et al.*, 2012) and using additional R package ggforce (Pedersen, 2021).

The pacing was performed using the EFS pacing head add-on (Hamamatsu Photonics). Cells were paced at frequencies of 0.5 or 1 Hz, with a pulse of 7.5 V, 10 ms width and the probe at the height of 0.5-1 mm. The addition of flecainide and thapsigargin was performed manually by adding a 10x concentrated stock of 150 μM and 50 μM for each, respectively. Analysis was performed in R (4.0.3), and induced change was calculated against baseline and corrected for DMSO controls.

Contraction measurements

Thawed hiPSC-CMs were seeded in a black, flat-bottomed 96-well plate coated with 75 μg/ml Matrigel (Corning) and cultured for four days to recover. Videos of cardiomyocyte contraction were recorded on a microscope using a 40x objective (Leica Inverted microscope IBDE) with a Thorlabs DCC3240M camera at 100 frames per second. The cells were kept in a culture chamber at 37°C and 5% CO₂. When capturing spontaneous contraction, videos were recorded for 15-30 seconds. The pacing of the cardiomyocytes was performed with a pulse of 21V/cm for 12.5 ms. The duration of each recording was adjusted to allow for at least 4 contractions to be captured. Videos were analysed using MUSCLEMOTION (Sala *et al.*, 2018) ImageJ macro (ImageJ 1.8.0_66), and analysis was performed using R (4.0.3).

Field potential measurements

Seven days after recovery from thaw, hiPSC-CMs were dissociated and plated onto a Matrigel-coated 48-well multi-electrode array (MEA) plate (Axion Biosystems), by adding 8 μl droplets of cell suspension containing 70k hiPSC-CMs to the middle of each well, taking care to cover the measuring electrodes and not the reference electrode. After initial attachment for 2 h in the incubator, an additional 300 μl of LI-BPEL was gently added to the wells. The cells were allowed to recover for 7 days before evaluating drug responses using the Maestro Pro MEA system (Axion Biosystems). E4031

(Torcis) and flecainide (Torcis) were dissolved in DMSO at stock concentrations of 50mM. From this stock, further dilutions were made in LI-BPEL medium with a final DMSO concentration of 0.1%. Data were analysed using R (4.0.3), and dose-response curve statistics were calculated using (Ritz *et al.*, 2015).

Data and statistical analysis

All data analysis was performed using R (4.0.3), relying on additional packages (Wickham *et al.*, 2019; Wilke, 2020; Xie, 2021; Xiao, 2018). Data is represented as mean with standard error, and statistical analysis was performed using student's t-test or Mann-Whitney U test. P-values < 0.05 were considered statistically significant.

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COMPETING INTERESTS

C.L.M. is co-founder of Ncardia bv. The authors have no additional financial interests.

AUTHOR CONTRIBUTIONS

Conceptualisation: R.W.J.v.H, C.L.M., M.B. Formal Analysis: R.W.J.v.H, T.K., S.K., H.M., R.C.S., K.K.,

Funding Acquisition: C.L.M, M.B, M.G., R.P.D. Investigation: R.W.J.v.H, T.K., S.K., K.K., A.A.M.

Methodology: R.W.J.v.H, T.K., S.K., Project administration: R.W.J.v.H, C.L.M., M.B. Resources: R.d.C

Software: R.C.S., H.M. Supervision: M.B, C.L.M., M.M. Visualisation: R.W.J.v.H. Writing – original draft: R.W.J.v.H, C.L.M., M.B. Writing - review & editing: R.W.J.v.H, T.K., S.K., C.L.M., M.B.

DATA AVAILABILITY

The datasets and computer code produced in this study are available in the following databases:

- **RNA-Seq data: Gene Expression Omnibus GSE46843 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE208272>)**
- Datasets of intracellular lipidomics and metabolomics as well as metabolomic measurements on medium are attached as supplemental Tables 3, 4 and 5. Data are represented as nmol normalised to the total amount (µg) of protein

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EXPANDED VIEW FIGURE LEGENDS

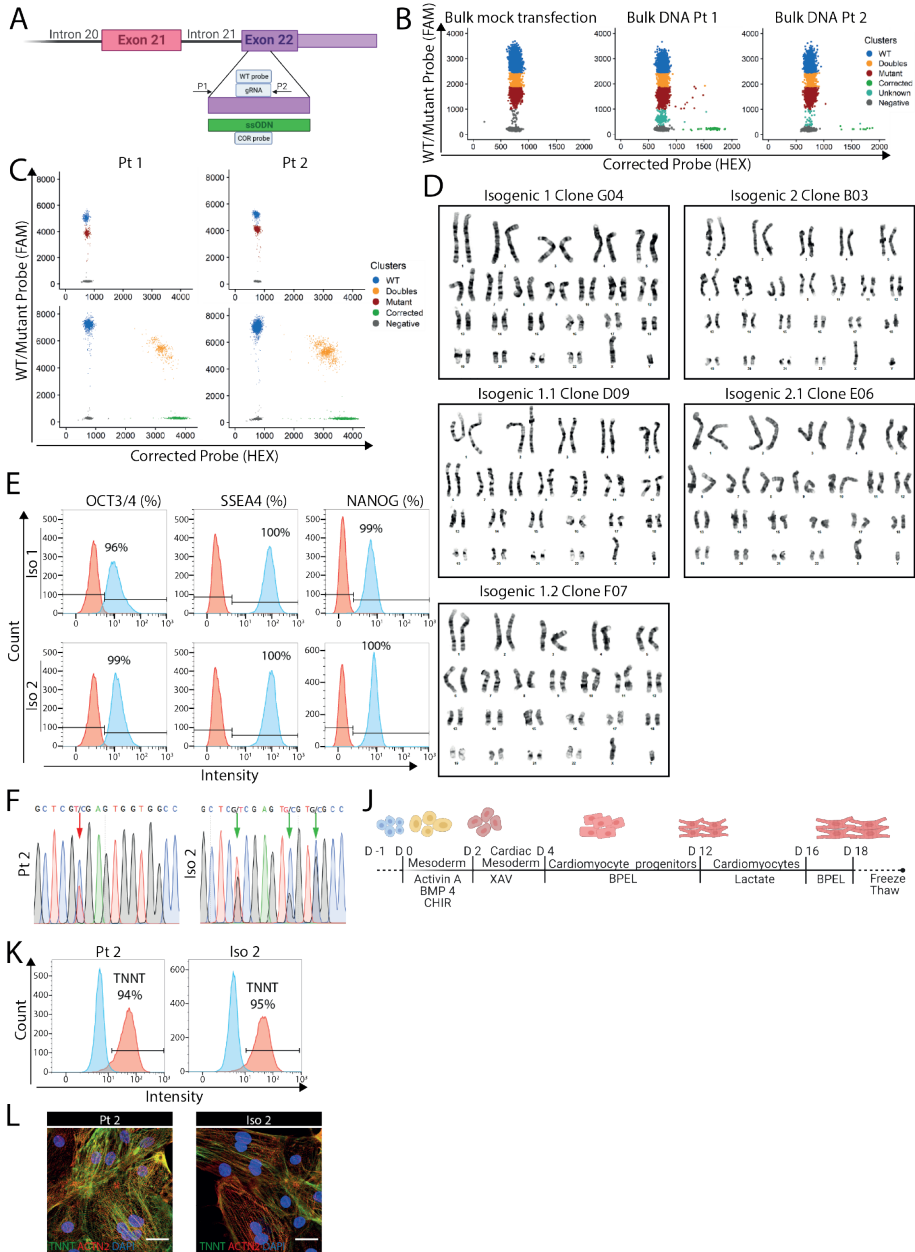


Figure S1. Genetic correction in hiPSCs and differentiation into cardiomyocytes.

A) Schematic representation for the CRISPR/Cas9 correction of the AARS2 c.2872C>T mutation and the screening

approach. The gRNA is uniquely complementary to the mutated allele. The WT probe binds to the WT allele with higher affinity than to the mutant allele. The COR probe binds uniquely to DNA matching the donor ssODN. P1 = forward primer 1, P2 = reverse primer 2, used for the screening. B) Screening of the transfected bulk hiPSC population. Mock transfection was performed without Cas9 protein but with the gRNA. WT droplets (blue) contain only the WT allele(s). Mutant droplets (red) contain only mutant allele(s). Mixed droplets (orange) contain one or multiple copies of both WT and mutant alleles. Corrected droplets (green) contain corrected alleles by homologous directed repair. Unknown droplets (turquoise) contain either unsuccessful PCR reactions or mutated alleles that underwent non-homologous end joining and therefore contained indels. Negative droplets (grey) do not contain any DNA. C) Screening of the hiPSC-clones grown from single-cell deposition from the bulk populations. The absence of the mutant cluster and the presence of the corrected cluster indicate the successful correction of the c.2872C>T mutation. D) Karyotypes from the targeted and corrected clone; Isogenic 1 clone G04 and Isogenic 2 clone B03 are Iso 1 and Iso 2, respectively. E) FACS analysis (histogram) of pluripotency markers OCT3/4, SSEA4 and NANOG (orange). Cells stained with isotype-matched antibodies were used as a negative control (blue). F) Sanger sequencing chromatogram of DNA isolated from Pt 2 and Iso 2 hiPSCs showing that the c.2872C>T mutation was corrected in Iso 2. G) Representative FACS analysis (histograms) of cardiac troponin (red) expression in Pt 2 and Iso 2 hiPSC-CMs after lactate selection. In blue, as a control the same cells were stained with isotype-matched antibodies. H) Immunofluorescence images from Pt 2 and Iso 2 hiPSC-CMs for TNNT2 (green), α -actinin-2 (ACTN2, red). Nuclei were stained in blue with DAPI, scale bar = 25 μ m.

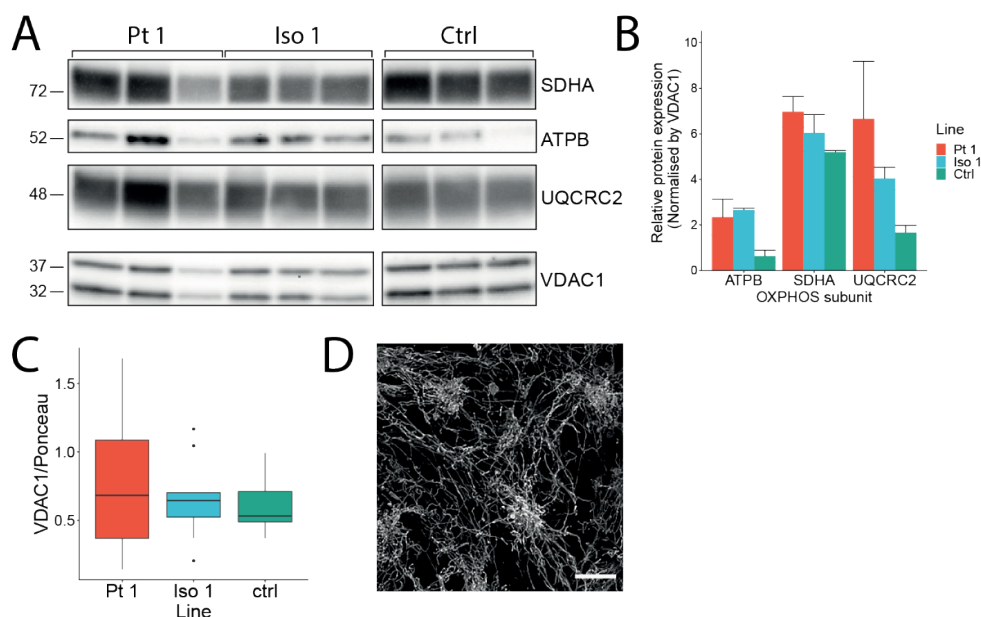


Figure S2. Mitochondrial proteins and mitochondria network analysis.

A) Western blot showing expression of the mitochondrial protein subunits SDHA, ATPB, UQCRC2 and VDAC1 in Pt 1, Iso 1 and Ctrl hiPSC-CMs. Each column represents an independent differentiation (biological replicate, n = 3). B) Densitometric quantification (ADU: arbitrary units) of mitochondrial protein expression normalised to VDAC1 as a marker for total mitochondrial biomass. Data shown as mean with standard error. C) Expression of mitochondrial membrane porin VDAC1 normalised against total lysate protein assessed using Ponceau staining. D) Representative image of hiPSC-CMs where mitochondria were stained with the TMRM dye. Scale bar = 10 μ m.

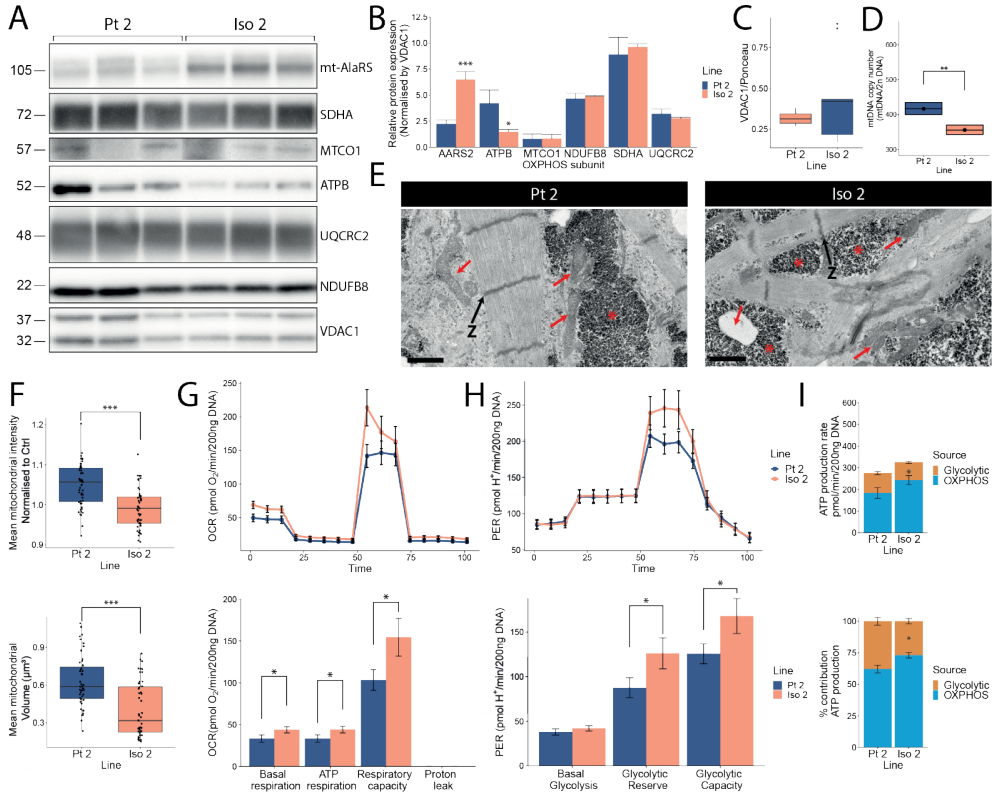


Figure S3. Mitochondrial protein expression, mitochondrial organisation and metabolic flux in Pt 2 hiPSC-CMs.

A) Western blot showing expression of the mitochondrial proteins mt-AlaRS, SDHA, MTCO1, ATPB, UQCRC2, NDUFB8 and VDAC1 in Pt 2 and Iso 2 hiPSC-CMs. Each column represents a single differentiation (biological replicate, $n = 3$). B) Densitometric quantification of mitochondrial protein expression normalised to VDAC1 expression as a marker for total mitochondrial biomass. C) Expression of mitochondrial membrane porin VDAC1 normalised against total lysate protein assessed using Ponceau staining as a quantification for mitochondrial biomass corrected for total biomass. D) mtDNA copy number per cell, quantified by ddPCR. The plot represents the Poisson minimum, mean, and maximum number of mtDNA per 2N nDNA copy number. Calculations based on Poisson distribution. $N = 26$, $n = 4$ biological repeat, ** $P < 0.01$, *** $P < 0.001$, Poisson statistics. E) transmission electron microscopy (TEM) images from Pt 2 and Iso 2 hiPSC-CMs, scale bar = $1 \mu\text{m}$. F) Quantification of the intensity of the TMRM dye in mitochondria, normalised to the mean of the Ctrl and mean volume of each identified mitochondria. Each dot represents the mean value obtained per microscope field. $N = 51$, $n = 3$ biological repeat. G-H) Traces of extra-cellular flux assays in hiPSC-CMs by Seahorse measuring Oxygen consumption rate (OCR) (G) and proton extrusion rate (PER) (H). At the bottom of each panel, quantification of the different processes (basal respiration, ATP respiration, respiratory capacity, proton leak, (G, bottom) and basal glycolysis, glycolytic reserve, and glycolytic capacity (H, bottom)) is shown as a bar graph. $N \geq 18$, $n = 3-4$ biological repeats. I) Total ATP production (top) and contribution of OXPHOS and glycolysis to total ATP production (bottom), calculated based on extra-cellular flux measurements. Statistical differences are denoted in comparison to Pt 2. $n = 3-4$ biological repeats. All data shown are mean with standard error. Statistics were indicated as follows unless otherwise indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with student's t-test.



A) Heatmap Hierarchical clustering of intra-cellular metabolites quantified by NMR (n = 3 biological repeats). The scale represents Z-score. B) Proportion of variance of all samples per principal component (PC) from bulk transcriptomics analysis. C) Scatter plot of PC1 and PC2 performed on hiPSC-CMs derived from all 5 lines. n = 3 biological repeats. D) Venn diagram portraying the total number (and percentages) of differentially expressed genes (DEGs) found by comparing Pt 1 with either Iso 1 (368 DEGs) or Ctrl (335 DEGs) or as a combined analysis of Pt 1 vs healthy (representing Iso 1 and Ctrl) (361 DEGs). E) Hierarchical clustering of the 361 DEGs from the Pt 1 vs healthy comparison. The scale represents Z-score. F) Hierarchical clustering of the 42 DEGs identified when comparing both patients against both isogenic lines. The scale represents Z-score. G) Pathway analysis on genes related to mitochondrial function from the MitoCata 3.0 atlas (p-value < 0.05). Each H) Combined pathway analysis of intracellular metabolites; upregulated genes (blue) and downregulated (red) genes in Pt 1 versus Iso 1 and Ctrl hiPSC-CMs are visualised (p-value < 0.15 and 20% altered fold-change).

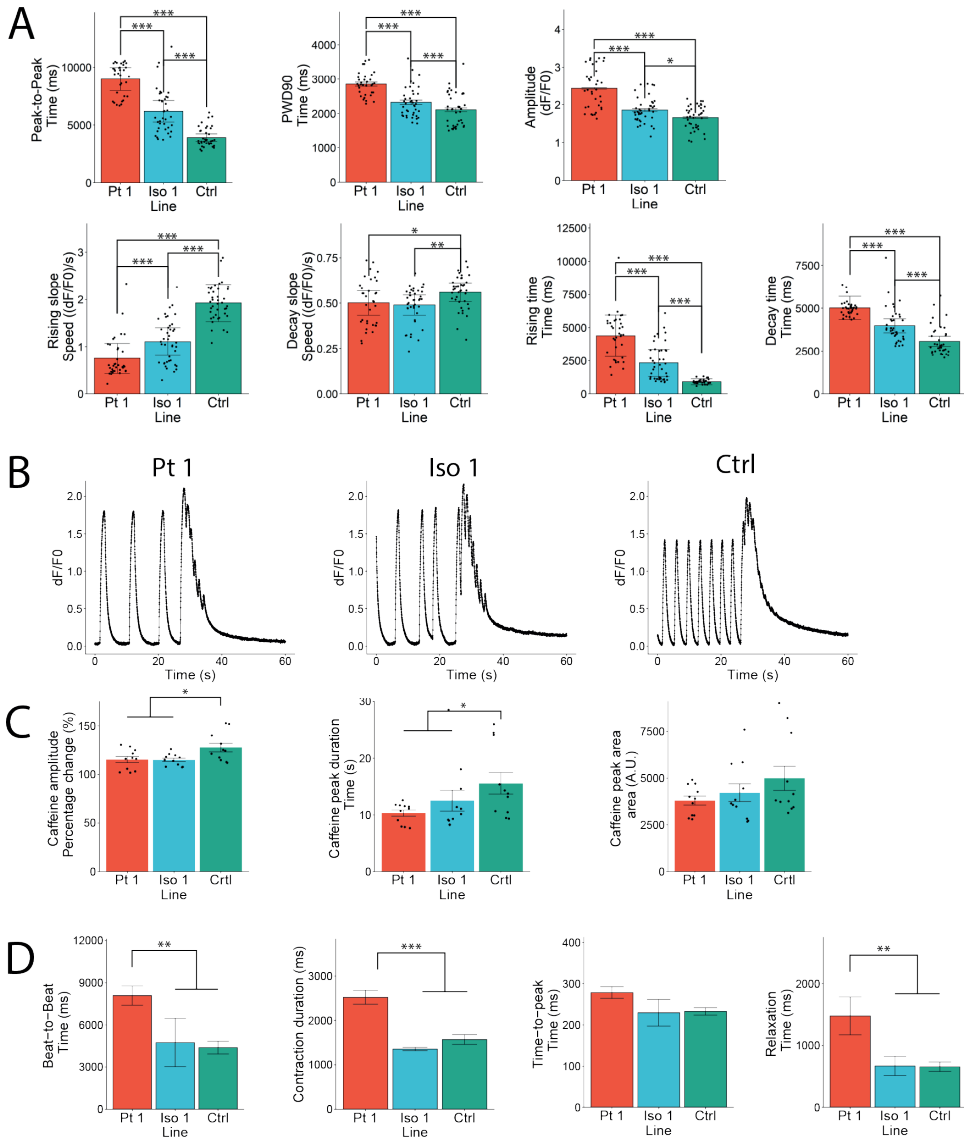


Figure S5. Quantitative analysis of calcium transients and contraction properties.

A) Analysis of rhythmic calcium transients showing peak-to-peak time, calcium transient width duration at 90% decay (PWD90), amplitude, rising slope, decay slope, rising time and decay time. Fluorescent amplitude was normalised using average background signal. Each dot represents the average of all calcium traces from a single well. $N \geq 35$, $n = 3$ biological repeats. B) Representative traces showing calcium release from the sarcoplasmic reticulum upon the addition of 50 mM caffeine. C) Quantification of the caffeine-induced calcium release transient, amplitude change calculated over normal average amplitude, caffeine peak duration as PWD90, and area under the curve for the PWD90 duration. Each dot represents one well and analysed peak, $N = 11$, $n = 3$ biological repeats. D) Contraction analysis. All data shown are mean with standard error $N \geq 6$ and $n = 3$ biological repeats.

All data shown are mean with standard error. Statistics indicated as follows, unless otherwise indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with student's t-test.

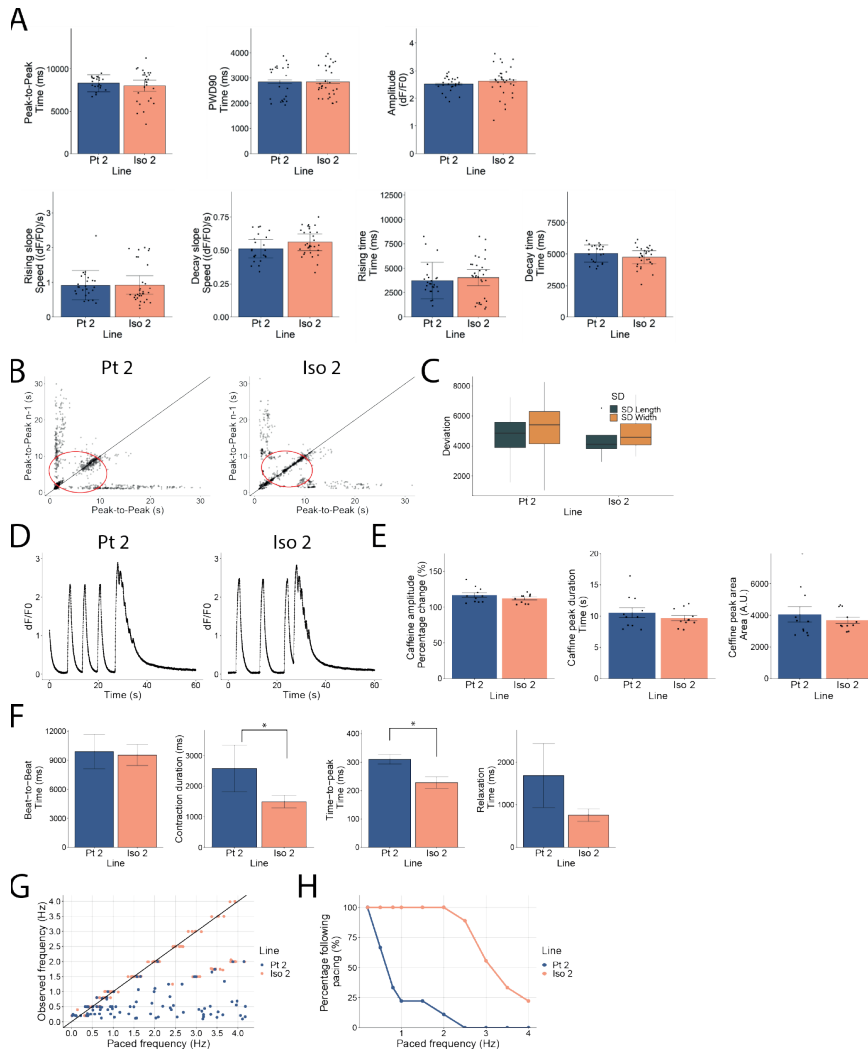


Figure S6. Calcium and contractile analysis of Pt 2 and Iso 2.

A) Analysis of rhythmic calcium transients in spontaneous contracting hiPSC-CMs showing peak-to-peak time, calcium transient width duration at 90% decay (PWD90), amplitude, rising slope, decay slope, rising time and decay time. Fluorescent amplitude normalised using average background signal. Each dot represents the average of all calcium traces from a single well. $N \geq 25$, $n = 4$ biological repeats. B) Poincaré plots showing arrhythmia, marked as a red ellipse that was drawn based on calculating standard deviation along and perpendicular to the dissecting line. $N = 60$, $n = 4$ biological repeats. C) Quantification of the standard-deviation statistics used to generate the ellipse. D) Representative traces showing calcium release from the SR upon addition of 50 mM caffeine. E) Quantification of the caffeine-induced calcium release transient, induced amplitude change calculated based on average calcium transient amplitude, caffeine peak duration as PWD90 and area under the curve for the PWD90 duration. Each dot represents one well and analysed peak, $N = 11$, $n = 3$ biological repeats. F) Contraction analysis showing beat-to-beat time, total contraction duration, time-to-peak and relaxation time. $N = 9$, $n = 3$ biological repeats. G) Ability of hiPSC-CMs to follow increasing pacing frequencies. The x-axis shows paced frequency, the y-axis shows the observed beating rate of

the hiPSC-CMs. All dots underneath the intersecting line reflect hiPSC-CMs not contracting at the paced frequency. (N = 9, n = 3 biological repeats). H) Quantification of the percentage of samples able to follow the paced frequency up to 4 Hz. All data shown are mean with standard error and n = 3 biological repeats. Statistics indicated as follows, * P < 0.05, ** P < 0.01, *** P < 0.001 with student's t-test.

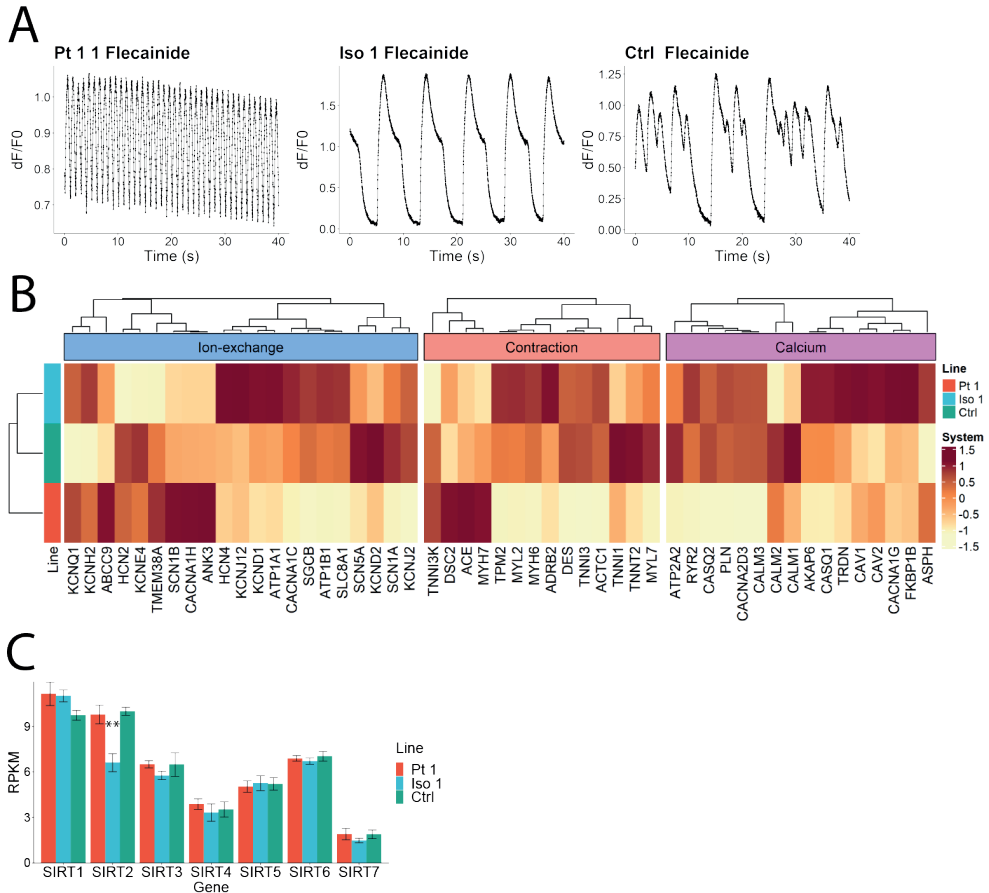


Figure S7. Decreased sensitivity to Flecainide in Pt1, with altered expression profiles in ion-exchange, contraction and calcium related genes, but not in Sirtuins.

A) Representative traces showing calcium transients of hiPSC-CMs being paced at 1 Hz and exposed to 15 μ M flecainide. B) Heatmap showing a curated list of genes based on KEGG and GO pathways related to ion exchange (blue), cardiomyocyte contraction (pink) and calcium homeostasis (purple). Final selection based on either p-value < 0.05 altered regulation versus Pt 1 or manually curated key genes. C) Expression of all sirtuin genes from transcriptomic data-analysis. Expression indicated as RPKM, n = 3 biological repeats.

All data shown are mean with standard error. Statistics indicated as follows, unless otherwise indicated. * P < 0.05, ** P < 0.01, *** P < 0.001 with student's t-test.



CHAPTER

Generation, functional analysis and applications of isogenic three- dimensional self-aggregating cardiac microtissues from human pluripotent stem cells

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ABSTRACT

Tissue-like structures from human pluripotent stem cells containing multiple cell types are transforming our ability to model and understand human development and disease. Here we describe a protocol to generate cardiomyocytes (CMs), cardiac fibroblasts and cardiac endothelial cells, the three principal cell types in the heart, from human induced pluripotent stem cells (hiPSCs) and combine these in three-dimensional cardiac microtissues (MTs). We include details of how to differentiate, isolate, cryopreserve and thaw the component cells and how to construct and analyze the MTs. The protocol supports hiPSC-CM maturation and allows replacement of one or more of the three heart cell types in the MTs with isogenic variants bearing disease mutations. Differentiation of each cell type takes approximately 30 days, while MT formation and maturation requires another 20 days. No specialist equipment is needed and the method is low cost, requiring just 5,000 cells per MT.

KEYWORDS: human induced pluripotent stem cell-derived cardiomyocytes; human induced pluripotent stem cell-derived cardiac endothelial cells; human induced pluripotent stem cell-derived cardiac fibroblasts; cardiac microtissue; cardiomyocyte maturation; multicellular cell diseases and drug efficacy platform.

Key papers:

1. Giacomelli, E., Meraviglia V., Campostrini G. *et al.* Human-iPSC-Derived Cardiac Stromal Cells Enhance Maturation in 3D Cardiac Microtissues and Reveal Non-cardiomyocyte Contributions to Heart Disease. *Cell Stem Cell* **26**, 862-879 e811, doi:10.1016/j.stem.2020.05.004 (2020).
2. Giacomelli E. *et al.* Co-Differentiation of Human Pluripotent Stem Cells-Derived Cardiomyocytes and Endothelial Cells from Cardiac Mesoderm Provides a Three-Dimensional Model of Cardiac Microtissue. *Curr Protoc Hum Genet.* 2017 Oct 18;95:21.9.1-21.9.22. doi: 10.1002/cphg.46. PMID: 29044469.
3. Giacomelli, E. *et al.* Three-dimensional cardiac microtissues composed of cardiomyocytes and endothelial cells co-differentiated from human pluripotent stem cells. *Development* **144**, 1008-1017, doi:10.1242/dev.143438 (2017).

INTRODUCTION

The dialogue between cells in developing or damaged tissues critically determines the functional phenotype and (patho)physiological properties of the tissue. Most major organs (for example the intestine, liver and pancreas) can now be mimicked in vitro as organoid-like structures¹⁻³. These organoid-like structures have been established from either adult stem cells or hiPSCs and contain various cell types.

The heart contains three major cell types: cardiomyocytes (CMs), cardiac endothelial cells (ECs) and cardiac fibroblasts (CFs). CMs make up approximately 80% of the adult human heart by volume but only 30% of the total cell number^{4,7}. The developing heart, by contrast, contains 60-70% CMs⁸. The majority of the other cells are ECs, CFs and immune cells, although the estimation of their exact proportion varies between studies⁵⁻⁷. All the cells in the heart communicate via physical interactions and paracrine factors⁴, thus regulating heart development and function^{9,10}.

Here we describe a protocol to construct three-dimensional (3D) structures here termed “cardiac microtissues” (MTs) composed of 70% CMs, 15% ECs, and 15% CFs derived from human induced pluripotent stem cells (hiPSCs). We used this protocol to generate cardiac MTs from multiple hiPSC lines¹¹. We also describe how to evaluate some standard functional parameters on whole MTs or single CMs dissociated from them. These procedures allow assessment of electrical, structural, mechanical and metabolic maturation of hiPSC-CMs.

Development of the Protocol

During organogenesis, CMs, ECs and CFs differentiate from common precursors in the cardiac mesoderm¹²⁻¹⁴. We previously described how to co-differentiate CMs and ECs simultaneously from hiPSC-derived cardiac mesoderm progenitors by combining Wnt-inhibition with vascular endothelial growth factor (VEGF) addition¹⁵. From the combined culture, ECs and CMs were isolated using the surface proteins CD34 and VCAM1, respectively. In a more recent study¹¹, we further optimized the previous procedures for EC and CM differentiation by driving the CM and EC differentiation pathways independently after cardiac mesoderm induction. Independent culture resulted in production of monotypic cultures, circumventing the need to isolate ECs and CMs. This avoided the need to select CMs with VCAM1, which is also expressed by CFs¹⁶. We also described previously how to differentiate hiPSCs to epicardial cells (EPI) that normally cover the surface of the heart¹⁷, and which are the major source of CFs developmentally¹⁸. We recently extended this differentiation procedure to generate CFs from hiPSC-EPI¹¹. Specifically, we adapted a monolayer differentiation protocol described previously¹⁹, based on low insulin BPEL (LI-BPEL) medium for cardiac mesoderm induction and for the initial induction of CFs, then used a commercially available medium (FGM3)

for subsequent stages, as this supported CF expansion and increased reproducibility between batches.

Cell-cell communication in 3D spatial organization is essential for organ formation and to physiologically mimick their multicellularity^{20,21}. Adding hiPSC-ECs to 3D aggregates containing hiPSC-CMs enhanced some aspects of maturation of the hiPSC-CMs¹⁵. We recently improved the maturation of the hiPSC-CMs further by also including CFs¹¹. The triple cell type combination supported self-assembly of MTs *in vitro*. The features exhibited by the hiPSC-CMs in the three-cell type cardiac MTs that indicated enhanced maturation were: sarcomere length and organization, contraction duration and amplitude, T-tubule-like structures (albeit sparse), action potential profiles characterized by more hyperpolarized resting membrane potential, the presence of an action potential “notch” and expression of postnatal sarcomere isoforms¹¹. We also demonstrated that the features indicating enhanced electrophysiological maturity of the hiPSC-CMs were retained after their dissociation from the MTs¹¹. The dialogue among all three cell types is essential to ensure full maturation of the CMs. Coupling between CMs and CFs through connexin 43 (CX43) gap junctions and cAMP-pathway activation were found to be two key mechanisms contributing to hiPSC-CM maturation¹¹.

Comparison with other methods

Table 1 compares the cellular composition and substrate/scaffold of the cardiac MTs that we describe in this protocol with those used in various other systems using human pluripotent stem cells (hiPSCs) or embryonic stem cells, hESCs, collectively called hPSCs).

Table 1. Cellular composition and scaffold/substrate of published engineered heart tissue models.

Study	Number of cells per cardiac tissue	CM source	EC source	Stromal cell source	Substrate/scaffold
(Stevens et al. 2009) ⁵²	3 x 10 ⁶ cells	hESCs	hESCs or HUVEC	Neonatal human dermal fibroblasts (NHDFs)	Scaffold-free
(Tulloch et al. 2011) ³¹	2 x 10 ⁶ CMs or 2 x 10 ⁶ CMs + 1 x 10 ⁶ HUVEC or 2 x 10 ⁶ CMs + 1 x 10 ⁶ HUVEC + 1 x 10 ⁶ MSCs or MEFs	hESCs or hiPSCs	HUVEC	Human marrow stromal cells (MSCs)	Type I Collagen; 11% Geltrex
(Schaaf et al. 2011) ³²	0.5 x 10 ⁶ cells	hESCs			Fibrinogen/Matrigel plus thrombin; rectangular casting molds with two flexible silicone posts

Study	Number of cells per cardiac tissue	CM source	EC source	Stromal cell source	Substrate/scaffold
(Nunes et al. 2013) ²³	0.5×10^6 cells	hESCs and hiPSCs	NA	NA	Type I Collagen; two layers masks made with SU-8 photolithography, propylene glycol monomethyl ether acetate and PDMS
(Wang et al. 2014) ²⁷	1.5×10^6 cells/cm ²	hiPSCs	NA	NA	Elastomers micropatterned with fibronectin lines
(BurrIDGE et al. 2014) ³³	2.5×10^5 CMs + 5×10^4 ECs + 5×10^4 MSCs	hESCs	hiPSCs	Human amniotic mesenchymal stem cells (hAMSCs)	3D Hydrogel platform (Matrigel-based)
(Hinson et al. 2015) ³⁴	1.1×10^6 cells per mold ($\sim 1 \times 10^3$ cells per tissue)	hiPSCS	NA	Human mesenchymal stem cells (hMSCs)	PDMS, type I collagen and human fibrinogen
(Ravenscroft et al. 2016) ⁴⁹	5×10^3 cells at ratio CMs:ECs:CFs (or DFs) of 4:1:2	hESCs or hiPSCs	Human cardiac microvascular ECs (hCMECs) or human coronary artery ECs (hCAECs) or human dermal microvascular ECs (hDMECs)	Primary human cardiac fibroblasts (hCFs) or normal human dermal fibroblasts (NhDFs)	Scaffold-free
(Masumoto et al. 2016) ⁴⁶	3×10^6 cells	hiPSCs*	hiPSCs*	Mural cells (MCs) from hiPSCs*	Acid-soluble rat-tail type I collagen neutralized with alkaline buffer; Matrigel
(Mannhardt et al. 2016) ³⁵	1×10^6 cells	hiPSCs	NA	NA	Agarose casting molds with solid silicon racks; Fibrin matrix (Matrigel, fibrinogen, thrombin)
(Huebsch et al. 2016) ⁴⁷	2×10^3 CMs + 2×10^3 stromal cells	hiPSCs*	NA	Fibroblast-like stromal cells from hiPSCs*	PDMS stencils coated with fibronectin
(Keung et al. 2016) ³⁶	1×10^6 cells per mold ($\sim 1 \times 10^3$ cells per tissue)	hESCs	NA	NA	PDMS, type I collagen and human fibrinogen
(Tiburcy et al. 2017) ³⁷	1.5×10^6 cells	hESCs or hiPSCs	NA	Human fibroblasts	Circular casting molds; Matrigel/rat collagen matrix; bovine collagen (Matrigel-free method)
(Gao et al. 2017) ²⁴	25×10^3 CMs + 12.5×10^3 ECs + 12.5×10^3 SMCs	hiPSCs*	hiPSCs*	Smooth muscle cells from hiPSCs*	3D-MPE printed scaffold; methacrylated gelatin

Study	Number of cells per cardiac tissue	CM source	EC source	Stromal cell source	Substrate/scaffold
(Lee et al. 2017) ³⁸	1×10^5 CMs + 1×10^5 FBs	hESCs	NA	Human foreskin fibroblasts	PDMS elastomer molds; Matrigel/ type I collagen matrix
(Giacomelli et al. 2017) ¹⁵	5×10^3 cells at ratio CMs:ECs of 85:15	hESCs or hiPSCs*	hESCs or hiPSCs*	NA	Scaffold-free
(Polonchuk et al. 2017) ⁵³	12×10^3 cells at ratio CMs:ECs:CFs of 2:1:1	iCMs from Axiogenesis	hCAECs	iCFs from Axiogenesis	Scaffold-free
(Mills et al. 2017) ²²	5×10^4 cells CMs:stromal cells of 70:30	hESCs and hiPSCs*	NA	CD90 ⁺ stromal cells from hESCs and hiPSCs*	SU-8 photolithography and PDMS molds; Matrigel/ type I collagen matrix
(Pointon et al. 2017) ⁵⁰	5×10^3 cells at ratio CMs:ECs:CFs of 4:1:2	hiPSCs	hCMECs	hCFs	Scaffold-free
(Ong et al. 2017) ³⁸	33×10^3 cells at ratio CMs:ECs:CFs of 70:15:15	hiPSCs	HUVEC	Human adult ventricular cardiac fibroblasts	Biomaterial free, 3D bioprinted
(Shadrin et al. 2017) ³⁹	$0.5\text{--}1 \times 10^6$ cells	hESCs and hiPSCs	NA	NA	PDMS square molds; Hydrogel matrix (fibrinogen, Matrigel, thrombin)
(Ronaldson-Bouchard et al. 2018) ⁴⁰	2×10^6 cells at ratio CMs:FBs of 75:15	hiPSCs	NA	Skin fibroblasts	PDMS elastomeric pillars with polycarbonate support structures; fibrin hydrogel (fibrinogen, thrombin)
(Ma et al. 2018) ²⁸	3×10^6 cells	hiPSCs	NA	NA	Cell-encapsulation gel-free filamentous matrix (OrmoClear*polymer)
(Forsythe et al. 2018) ⁵¹	1.5×10^3 cells at ratio CMs:CFs of 90:10	iCMs from Axiogenesis	NA	hCFs	Scaffold-free
(Tsuruyama et al. 2019) ⁴¹	6×10^6 CMs/sheet + 6×10^6 FBs/sheet	hiPSCs	NA	hDFBs	Cell sheets wrapped around a hollow octagonal tubular column; fibrin and collagen gels to seal the extremities
(Zhao et al. 2019) ³⁰	7.47×10^4 CMs + CFs 3.5×10^4	hESCs and hiPSCs	NA	hCFs	Flexible poly (octa-methylene maleate (anhydride) citrate) (POMaC) wires; hydrogel matrix (collagen, Matrigel)

Study	Number of cells per cardiac tissue	CM source	EC source	Stromal cell source	Substrate/scaffold
(Mills et al. 2019) ⁴²	5 × 10 ⁴ cells at ratio CMs:stromal cells of 70:30	hESCs*		CD90 ⁺ stromal cells from hESCs*	PDMS molds; Matrigel/ type I collagen matrix
(Goldfracht et al. 2019) ²⁶	2 × 10 ⁶ CMs	hiPSCs	NA	NA	Chitosan-enhanced ECM from decellularized pig heart
(Wanjare et al. 2020) ²⁹	1 × 10 ⁶ CMs + 4 × 10 ⁴ ECs	hiPSCs*	hiPSCs*	NA	Circular microfibrous polycaprolactone sheets; Geltrex
(Goldfracht et al. 2020) ⁴³	2 × 10 ⁶ CMs	hESC- (atrial and ventricular differentiation)	NA	NA	Ring-shaped casting molds; collagen-based hydrogel matrix
(Jang et al. 2020) ⁴⁴	1 × 10 ⁶ CMs + 2 × 10 ⁵ CFs	hESCs and hiPSCs	NA	Human adult ventricular cardiac fibroblasts	PDMS support; collagen I mixture (layer-by-layer deposition)
(Sebastiao et al. 2020) ⁵⁴	1.5 × 10 ³ CMs	hiPSCs	NA	NA	Scaffold-free
(Richards et al. 2020) ⁵⁵	1.5 × 10 ⁵ cells 50% CMs and 50% non-myocyte (at a 4:2:1 ratio of FBs:HUVECs:hADSCs)	hiPSCs	HUVEC	hCFs Human adipose stem cells from hiPSCs (hADSCs)	Scaffold-free
(Dostanic et al. 2020) ⁴⁵	4.7 × 10 ⁴ cells, 3.1 × 10 ⁴ cells or 1.6 × 10 ⁴ cells (at 4:1 ratio of CMs:CFs)	hiPSCs*	NA	hiPSCs*	PDMS, acid solubilized collagen I and Matrigel
(Giacomelli et al. 2020) ¹¹	5 × 10 ³ cells at ratio CMs:ECs:CFs of 70:15:15	hiPSCs*	Cardiac specific ECs from hiPSCs*	Cardiac Fibroblasts from hiPSC-epicardium*	Scaffold free

*Indicates hiPSC from the same donor were used to derive the different cell types.

Most of the designs are based on specific tissue-engineered formats using methods such as SU-8 photolithography^{22,23}, bioprinting^{24,25}, decellularization of whole hearts²⁶, surface micropatterning²⁷⁻²⁹, biowires^{23,30}, or casting molds as hydrogel (collagen, Matrigel or fibrinogens) scaffolds^{22,31-47}. Engineered heart tissues (EHTs) are grown on mechanical supports or pillars providing mechanical load and have been described to induce hiPSC-CM maturation^{35,37}. One system uses forced supraphysiological pacing up to 6 Hz to facilitate this⁴⁰. The initial designs⁴⁸ were low-throughput,

time-consuming and costly for both academic laboratories and pharma but miniaturized versions have now been made^{22,30,34,36,42,45,47}. However, EHTs still require specialized technical skills and often costly equipment, thus fewer independent constructs can be made from a single hiPSC-CM batch. Moreover, the thickness of the resulting tissue might limit diffusion of, for example, drugs and nutrients. The cell distribution within the EHTs is not often examined, so it can be unclear whether this is homogenous (as *in vivo*) or heterogeneous. Other groups have addressed the shortcomings of engineered systems by including hiPSC-CMs in 3D self-assembled (scaffold-free) structures generally using fewer cells and straightforward techniques^{15,49-55}. For both engineered and scaffold-free systems, some use (only) non-purified CM populations which contain non-CM cells in undefined numbers^{22,23,27,28,32,35,39,43,54}. Others have developed multi-cell type systems that included purified CM population with specific non-CM cardiac cells but most required primary human cardiac microvascular ECs and/or primary human fibroblasts^{25,30,31,33,34,37,38,40,41,44,49-53,55}. Such cells are by definition limited in supply and can be particularly difficult to obtain from patients. Furthermore we have found that dermal fibroblasts express little or no CX43, which can limit their ability to couple with hiPSC-CMs and hence induce CM maturation¹¹. Thus, whilst skin fibroblasts are readily obtainable and expandable they are not able to support hiPSC-CM maturation in the same way as CFs.

Advantages and applications of our protocol

The self-organizing cardiac MT platform we describe here is scaffold-free, hence is easy to fabricate and does not require advanced tissue engineering or specialized apparatus. It requires only 5,000 input cells per MT. A single operator can generate and process up to 1,500-2,000 MTs in a single run, and the 96-well format is compatible with multiple semi-automatic analysis systems for two-dimensional (2D) screening of CMs (e.g. fluorescent plate readers, FLIPR calcium assays, the Hamamatsu FDSS system). Additionally, cells can be cryopreserved at intermediate differentiation stages, so that they are readily available for MT construction from banked stocks. All the cell types necessary for building MTs can be derived from a single hiPSC source, allowing isogenic disease modelling. By systematically interchanging each of the cell types in the cardiac MTs with an isogenic or mutant variant, it is possible to identify the cell type(s) responsible for a particular disease phenotype. We have also demonstrated that the procedure is highly reproducible across multiple hiPSC lines and differentiated cell batches¹¹ (Table 2). All three cell types survive equally well in basal medium and do not require complex media formulations. Because the system does not require specialized equipment and the differentiated cellular components of the cardiac MTs can be cryopreserved, both the procedure and the cells can be easily transferred and distributed to other laboratories in both academia and pharma, enabling multidisciplinary collaborations. Using a 96-well format, production of thousands of MTs is low cost ($\pm$ 0.22€ per MT; Table 3). In addition, cardiac MTs as described here are entirely

hiPSC-derived allowing generation of models for investigating patient-specific multicellular responses in diseases and to pharmacological compounds. For this reason, they may have greater utility than unicellular monolayer cultures of hiPSC-CMs in screening drugs that affect the heart⁵⁶. Moreover, because enhanced CM maturation takes place in MTs, they can facilitate hiPSC-CM based modelling of cardiac diseases that manifest in adults (or postnally)⁵⁷.

Table 2. Variability table of morphological, contraction and single-cell electrophysiological parameters.

The table reports quantitative parameters showing the expected sample-sample, batch-batch and line-line reproducibility. Size: diameter of MTs mounted on coverslips; IBI: inter-beat interval; RMP: resting membrane potential; APA: action potential amplitude; Vmax: maximal upstroke velocity; APD₉₀: action potential duration at 90% repolarization; SD: standard deviation; CV: coefficient of variation (calculated as SD/mean*100). These data have been previously shown in ¹¹. Raw data are reported in Supplementary Table 1.

		Morphology			Contraction		Single-cell electrophysiology				
		Size (μm)	Sarcomere length (μm)	Sarcomere alignment index	IBI (ms)	Contraction duration (normalized)	RMP (mV)	APA (mV)	Vmax (dV/dT)	APD90 (ms)	
Sample to sample variability	mean	482.4	1.78	0.027	3631.4	393.9	-71.0	102.4	52.8	254.9	
	SD	38.3	0.06	0.004	819.3	118.1	6.7	10.7	32.3	89.2	
	CV	7.8	3.1	13.4	22.1	31.8	9.4	10.5	61.5	33.8	
Batch to batch variability	mean	471.4	1.78	0.027	3334.2	386.6	-71.2	102.9	49.4	277.1	
	SD	31.0	0.02	0.002	1347.9	140.7	2.3	4.2	9.3	31.8	
	CV	6.8	1.3	6.6	35.9	35.4	3.2	41	16.3	10.6	
Line to line variability	mean	471.4	1.78	0.026	3331.2	393.5	-71.5	102.9	49.9	272.0	
	SD	46.7	0.004	0.000	584.2	24.4	1.1	0.8	11.8	68.6	
	CV	9.9	0.2	0.02	17.5	6.2	1.5	0.8	23.7	25.2	

Table 3. Estimated costs to generate hiPSC cardiac MTs based on 2019 quotations including discounts for bulk orders of the suppliers indicated in the Materials.

The costs can vary per supplier, applied discounts or change with inflation.

Material/Reagent/Procedure	Cost (€)
Cost to generate ~ 4,000,000 hiPSC-CMs (starting from to one well of a 6-well plate of undifferentiated hiPSCs)	
Matrigel	0,4
Growth factors	1,13
Culture medium	0,9
Dissociation	4,32
Extra medium for dissociation and re-suspension	0,6
Extra material: tips, tubes, plates	2,17
Total	9,52
Total for 180 microtissues	1,5
Total for 1 microtissue	< 0,01

Material/Reagent/Procedure	Cost (€)
Cost to generate ~ 800,000 hiPSC-ECs (starting from to one well of a 6-well plate of undifferentiated hiPSCs)	
Matrigel	0,4
Growth factors	1,53
Culture medium	0,36
Kit for enrichment	26,87
Extra medium for isolation, dissociation, re-suspension, freezing and thawing	2
Fibronectin for coating	1,2
CryoStor CS10 medium for freezing	2,08
TrypLE 1X for dissociation	0,85
Extra material: tips, tubes, plates	3,29
Total	38,58
Total for 180 microtissues	6,5
Total for 1 microtissue	< 0,04
Cost to generate ~1,200,000 hiPSC-CFs (starting from to one well of a 6-well plate of undifferentiated hiPSCs)	
Matrigel	0,4
Growth factors	3,95
Culture medium	1,54
Fibronectin for coating	2,7
TrypLE 1X for dissociation	1,7
FGM3 medium	11,1
CryoStor CS10 medium for freezing	2,08
Extra FGM3 medium for dissociation, freezing and thawing	5,55
Extra material: tips, tubes, plates	4,96
Total	33,98
Total for 180 microtissues	3,82
Total for 1 microtissue	< 0,02
Costs to generate and maintain 180 microtissues (corresponding to 3 V-shaped 96-well plates)	
Cells	11,82
Growth factors	9,44
Culture medium	2,16
Material: reservoirs, tips, tubes, plates	15,4
Total (excluding material)	23,42
Total (including material)	38,82
Total (excluding material) for 1 microtissue	~ 0,13
Total (including material) for 1 microtissue	~ 0,22

Immediate applications for the cardiac MT protocol described include modelling multicellular cardiac diseases, drug screening and studies to understand signalling pathways controlling human heart physiology. The multi-cell type model of the heart we describe allows in-depth mechanistic insight into cardiac diseases which may be poorly characterized because the non-myocyte cells in the heart play active roles in the pathogenesis but cannot be distinguished in end-stage outcomes in patients such as heart failure. Contributions of the non-myocyte cell population to (both genetic and environmental) cardiac diseases is becoming increasingly evident, with fibrosis, arrhythmias and electrical conduction defects all thought to have a non-myocyte contribution. To model these kind of diseases, the versatility of the cardiac MT model described allows modification of non-cardiomyocyte cell ratios during generation, that can be defined depending on the specific application. Moreover, MTs with pre-defined ratios of non-cardiomyocytes enable cellular “culprits” and “victims” in cardiac disease to be distinguished. For example, we demonstrated that modelling Arrhythmogenic Cardiomyopathy (ACM) caused by a mutation in plakophilin 2, a protein of the desmosome, only required mutated CFs for arrhythmias to appear in paced cardiac MTs, indicating CFs and not just the CMs contribute to the disease¹¹. Individual CMs and CFs are known to couple and communicate through homo- and heterocellular gap junctions to control electrical activity⁵⁸ and conduction in the heart⁵⁹. This is also evident in the cardiac MT model. In addition, CFs appear to play a specific role in CM maturation during development. This is independent of or additional to the maturation that results from the metabolic switch in energy source, from glucose prenatally to fatty-acids postnatally. Together, these cardiac cell combinations and variations in energy sources can provide opportunities to understand heart failure occurring just after birth in patients with inborn errors of metabolism or mitochondrial disease⁶⁰. Automated handling systems could further increase throughput and thus facilitate testing of thousands of compounds in parallel in cardiac drug discovery and in assessment of cardiac toxicity. We recently demonstrated the utility of MTs to assess cardiotoxic drug effects in a study designed to identify doxorubicin analogues that do not induce late heart failure, in contrast to doxorubicin itself and other anthracycline cancer chemotherapeutics. Human cardiac MTs were able to distinguish the “safe” variants and gave similar outcomes to 3-month studies in mice⁵⁶. Cardiac MTs enable elucidation of cell-cell interactions and could thus show how individual as well as combinations of genetic variants identified from large-scale genetic studies contribute to the pathogenicity of cardiac diseases in which multicellularity plays a role.

Limitations of the protocol

Mechanical load is a physiological stress that helps heart development and maturation. In our system we are able to achieve a certain degree of cardiomyocyte maturation even without mechanical loading; however, adding load to our system could potentially increase maturation even further. Many key

functional parameters, such as electrophysiology, calcium handling, contraction amplitude and dynamics, mitochondrial respiration and glycolytic activity can be measured in cardiac MTs. However, the contraction force determined in EHTs cannot be measured in MTs as it requires contraction against a load. Nevertheless, comparison of contraction amplitude measurements using MUSCLEMOTION software⁶¹ with the absolute force measurements assessed by pole deflection in EHTs³⁵ previously showed highly significant correlation ($R^2=0.879$) between force of contraction and cell displacement during contraction⁶¹. However, this correlation might be influenced by the viscoelasticity of the MTs, which may change substantially with the state of the cardiomyocytes and microenvironment. Measurements of conduction velocity and propagation via optical mapping are also challenging in MTs, due to their small size. However, appropriate camera setups with high sensitivity or light sheet microscopy⁶² might overcome this issue.

We demonstrated that in cardiac MTs, key ultrastructural features of hiPSC-CMs improve (mitochondria with well-defined cristae, M-bands and T-tubule-like structures) and that maturity was retained upon isolation of the CMs from the MTs with most CMs displaying typical adult action potential “notches”, hyperpolarized resting membrane potential, and increased upstroke velocity¹¹. Nevertheless, the maturation level achieved was not identical in all cells and did not reach that of CMs in the adult heart. Whether contraction against resistance, fluid flow in organ-on-chip formats allowing perfusion and delivery of nutrients, micropatterned surfaces to induce sarcomere alignment or forced and prolonged pacing would promote further maturation or even aging remains to be investigated. As for all hPSC-derivatives described to date, hiPSC-ECs and hiPSC-CFs are also probably immature: RNA-seq data obtained before incorporation in the 3D MTs showed that hiPSC-CFs clustered closely to both human primary fetal- and adult CFs, and that hiPSC-ECs clustered closely to human primary fetal ECs¹¹. We have not investigated whether the MT environment promotes their maturation, and this warrants further analysis. For this reason, we cannot exclude that possible immaturity of hiPSC-ECs and hiPSC-CFs might affect some aspects of disease modelling, for example cardiac fibrosis, which is not driven only by increased number of fibroblasts but also by a their activation into myofibroblasts.

In the heart other cell types contribute to tissue homeostasis and play a role in disease pathology. Further development of this tricelltype microtissue system will undoubtedly benefit from the addition of other cardiac cells, such as mural cells to promote formation and maintenance of microcapillary structures and macrophages, dendritic cells and neurons, which are relevant for disease modelling.

Experimental design

The procedure describes the steps required to produce scaffold-free 3D cardiac MTs composed of hiPSC-CMs, hiPSC-ECs and hiPSC-CFs and how to analyse their structural, functional (electrical and contraction) and metabolic properties.

hiPSC expansion and initial differentiation

The first part of the procedure describes hiPSC expansion and differentiation into the three hiPSC-derived cardiac cell types. Provided pluripotency of the starting population is high, the hiPSCs can be cultured using various maintenance protocols without affecting differentiation efficiency outcome. The expected results described here were obtained using two independent control hiPSCs lines but we have also successfully applied the protocol to various hiPSC lines for modeling of cardiac diseases (e.g. ACM, long QT syndrome 1)¹¹. The differentiation of CMs, ECs, and CFs starts with induction of cardiac mesoderm, as outlined schematically in Fig. 1. Some optimization of the differentiation protocol may be required for different hiPSC lines, most specifically the starting seeding density. If the optimal seeding density is not pre-determined, variable differentiation efficiencies can result, particularly for CMs. The initial steps of differentiation for the three cell types is carried out in low insulin, BSA-based medium containing polyvinyl alcohol and essential lipids (LI-BPEL medium, see MATERIALS), which was initially optimized for CM differentiation^{63,64} and subsequently proved efficient for CM and EC co-differentiation^{15,65}. The cytokines, Activin-A and BMP4, together with the Wnt-signalling pathway activator CHIR99021 are added to LI-BPEL for 72 h to direct the differentiation to cardiac mesoderm. Alternatively, CM differentiation can be induced using a modified medium that lacks several of the components present in LI-BPEL, but includes various trace elements⁶⁶ (mBEL, see Box 1). This circumvents the need to use cytokines as the first induction step requires only CHIR99021 for 48 h.

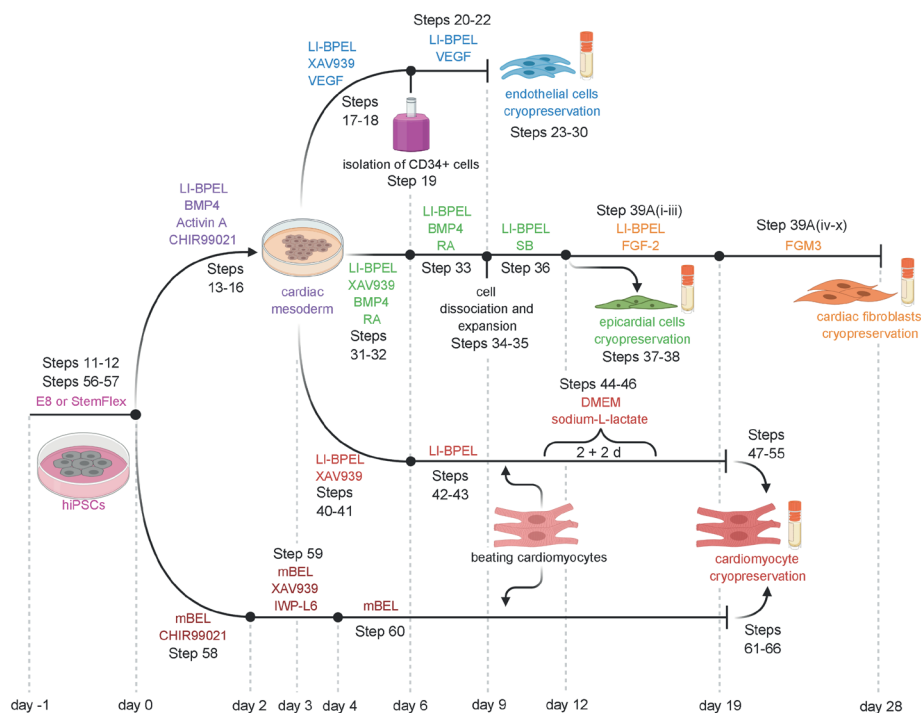


Fig. 1: Outline of *in vitro* differentiation protocol from hiPSCs to ECs, CFs and CMs.

Schematic of the protocol for differentiation of the three cell types used for MT production with indication of the media and supplements used.

Specific differentiation to EC, CF and CM

The next part of the procedure comprises separate differentiations of EC, CF and CM.

EC differentiation is based on the addition of VEGF and inhibition of the Wnt-pathway with XAV 939 for 72 h. ECs can be cryopreserved once they reach confluence in the plate without affecting their properties. Although ECs do not undergo significant cell death in the freezing/thawing process, they are sensitive to cell contact and will undergo cell cycle withdrawal and death if they become over-confluent in culture. Therefore the density of cell seeding after thawing should take into consideration the time in culture and proliferative capacity of the cells.

CFs are differentiated from EPI cells that are generated using a protocol we published previously with some modifications¹⁷. EPI specification is based on Wnt-inhibition with XAV 939 and addition of BMP4 and retinoic acid (RA) to the basal medium. The differentiation to EPI cells takes 9 d, after which the cells can be expanded and cryopreserved. This creates stocks from which CF differentiation

can be initiated and allows large numbers of CFs to be derived from the same EPI batch. CF differentiation is induced by adding basic fibroblast growth factor (FGF-2) to LI-BPEL medium for 5 d. A commercially available medium optimized for fibroblast culture (FGM3 medium, see MATERIALS) is then used. During this step, the proliferation of CFs is slower than, for example, dermal fibroblasts. CFs can be expanded by passaging the culture twice before adapting the cells to grow on uncoated tissue culture plates. CFs can be cryopreserved between passages 2-6. After 7 or more passages, they show reduced proliferation and signs of senescence and should not be used to form MTs.

CMs can be differentiated from cytokine-induced cardiac mesoderm with addition of XAV 939⁶³. Alternatively, the CMs can be differentiated using mBEL medium supplemented with small molecules⁶⁶ (Box 1), similar to protocols described previously^{67,68}. For the specification to CMs by Wnt-signalling inhibition, the differentiated cells are treated with both XAV 939 and IWP-L6 for 48 h. Small molecules can be useful in improving standardization since there is likely less lot-to-lot variability than with cytokines. Moreover, the use of small molecules is cost-effective compared to cytokines. The small molecule protocol has however only been optimized for CM differentiation and the cytokine protocol should be used if a common cardiac mesoderm for the differentiation of other cell types is required. In general, both CM differentiation protocols are highly efficient and we have seen no differences in CM behaviour in MTs using either method. For hiPSC lines where differentiation efficiencies are poor (< 70% cTnT+ cells), purification based on metabolic selection with lactate can be used to enrich for CMs (> 85-90% cTnT+ cells)^{67,69}. Basic DMEM medium with no glucose or pyruvate but supplemented with lactate supports CM survival only and not that of other glucose-dependent cells⁶⁹. The CMs can also be cryopreserved without affecting their functional properties⁶⁶, thereby allowing large cell numbers from the same differentiation batch to be available to form MTs. However, the freeze/thawing procedure does reduce CM recovery to ~30-50%, so this needs to be taken into account for MT formation.

Quality checks of the hiPSC-derivatives are recommended before use in MT formation, as described in the protocol. The methods we describe are based on flow cytometry (Supplementary Figures 1 and 2) and immunofluorescence analyses.

MT generation

MTs are generated as embryoid body-like structures by self-aggregation of in total 5,000 of the three cell types in controlled ratios that remain constant over time in culture¹¹. The process is outlined schematically in Fig. 2. MTs can be generated from freshly differentiated cells or cells derived from frozen stocks. Differences in the differentiation protocol for each of the cell types dictates the overall

timing of the procedure. Using frozen stocks avoids the need to synchronize differentiation of the three cell types, thereby reducing simultaneous workload and enabling the production of more MTs from the same batches of cell types.

To form MTs, the different hiPSC-cardiac cell types are dissociated, counted and combined in fixed ratios (70:15:15, CMs:ECs:CFs) in homogeneous cell suspension, and a total of 5,000 cells are seeded in each well of V-shaped 96-well plates. Centrifugation is recommended to improve cell self-aggregation and tissue compaction, which is usually completed within 24-48 h. Cells are aggregated in 50 μ L LI-BPEL containing VEGF and FGF-2 to support ECs and CFs survival. The MTs start beating spontaneously within 3-4 d of seeding and this is maintained during the entire culture period of 21 d. To refresh the medium, half of the volume is replaced with fresh LI-BPEL containing VEGF and FGF-2 every 3 days.

MT analysis

Analysis of MTs after 21 d in culture showed that maturation of hiPSC-CMs was significantly improved at the structural (immunostaining to analyse sarcomere length and organization) and functional level (video analysis of contraction and calcium transients, metabolic function using Seahorse assays, electrophysiology using sharp electrode recordings). For some assays requiring single-cell analysis, we developed a procedure to dissociate MTs, based on cardiac embryoid body dissociation using collagenase II⁷⁰. This is also described here. Dissociated cells can be used for example, for single-cell RNA-sequencing (scRNA-seq) or plated for single-cell electrophysiology or immunostaining. These assays allow examination of CM maturation and cell type-specific features like gene expression, structure, functional activity. The process is optimized for the recovery of hiPSC-CMs but CFs are also obtained although in a smaller numbers. The survival of ECs after the dissociation and replating is poor, thus alternative procedures are necessary to recover ECs in sufficient numbers for the majority of downstream analyses. Nonetheless, sufficient numbers of each cell type can be recovered after dissociation from MTs for scRNA-seq analysis¹¹.

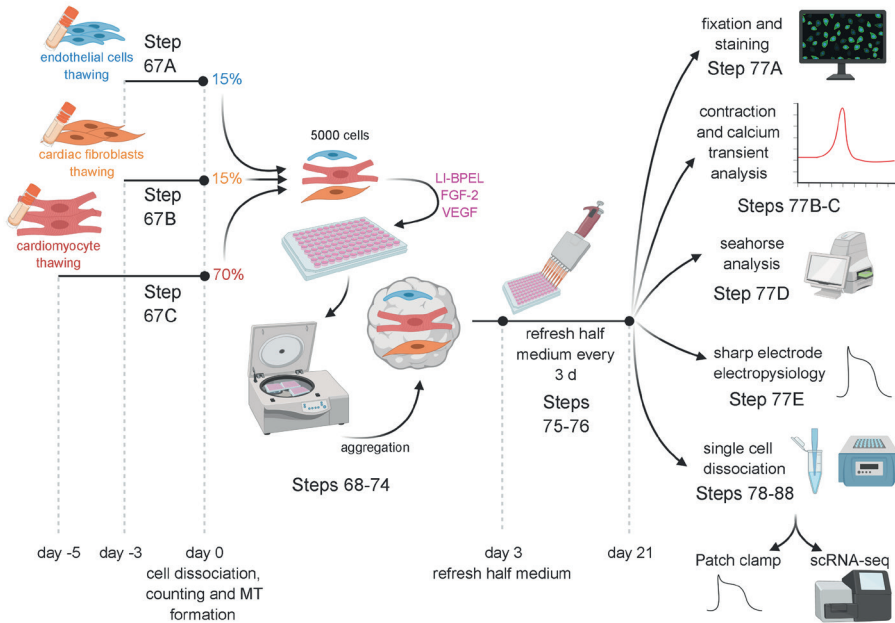


Fig. 2: Outline of *in vitro* 3D cardiac microtissues (MTs) formation and analysis.

Graphical illustration describing MT generation from frozen stocks of ECs, CFs and CMs and characterization of structural and functional properties using: immunofluorescence, contraction and calcium transient analysis, Seahorse analysis, sharp electrode electrophysiology and single-cell dissociation.

MATERIALS

Biological materials

hiPSC. The results we show here used hiPSC line LUMC0020iCTRL-06 (<https://hpscreg.eu/cell-line/LUMCi028-A>; RRID:CVCL_ZA25; https://scicrunch.org/resolver/RRID:CVCL_ZA25)^{11,15,71}. This protocol has been used successfully on six hiPSC lines to date. These are: LUMC0020iCTRL06 (female), LUMC0099iCTRL04 (<https://hpscreg.eu/cell-line/LUMCi004-A>; RRID:CVCL_UK77; https://scicrunch.org/resolver/RRID:CVCL_UK77)¹¹ (female), LUMC0059iCTRL03¹¹ (female), LUMC0021iKCNQ-30¹¹ (female), LUMC0153iPKP03^{11,72} (female), which were reprogrammed from primary skin fibroblasts using Sendai virus by the LUMC hiPSC core facility, and NCRM1¹¹ (RRID:CVCL_1E71; https://scicrunch.org/resolver/RRID:CVCL_1E71) (male), which was obtained from RUDCR Infinite Biologicals at Rutgers University.

! CAUTION The stem cell research protocols described here were approved by the medical ethical committee at Leiden University Medical Center.

! CAUTION All hiPSC lines should be routinely tested to ensure pluripotency and genomic integrity by karyotyping (approximately after 15-20 passages)^{73,74}, and to exclude mycoplasma infection.

! CAUTION Sex, defined as being XX or XY, is an important biologic variable in preclinical research⁷⁵. As such, the sex of the donors of the hiPSC lines used should be considered in research areas where sex differences play a role.

! CAUTION hiPSC lines need to be handled following minimum biosafety 1 level precautions, always wearing personal protection equipment and working in a class 1-2 biosafety cabinet.

Reagents

General reagents

! CAUTION Each reagent should be handled based on information reported in reagent specific material safety data sheets and always using proper personal protective equipment.

! CAUTION Room temperature is considered as 20-25 °C.

- Essential 8™ (E8™) Medium (Thermo Fisher Scientific, cat. no. A1517001) or StemFlex™ Medium (Thermo Fisher Scientific, cat. no. A3349401)

CRITICAL The medium used to maintain the hiPSC lines is dependent on how the user prefers to passage the cells. If the user prefers to passage the hiPSCs as cell clusters using EDTA, then E8 medium should be used for maintenance. If the user prefers to enzymatically passage as single cells (e.g. with 1x TrypLE Select), then the hiPSCs should be cultured with StemFlex Medium.

- Matrigel™ hESC-Qualified Matrix (Corning, cat.no. 354277)
- Vitronectin, truncated recombinant human (VTN-N) (Thermo Fisher Scientific, cat.no. A14700)
- Fibronectin bovine plasma (Sigma-Aldrich, cat.no. F1141)
- Laminin (BioLamina, cat. no. LN521)
- RevitaCell™ Supplement (100X) (Thermo Fisher Scientific, cat.no. A2644501)
- UltraPure™ 0.5 M EDTA, pH 8.0 (Thermo Fisher Scientific, cat.no. 15575020)
- TrypLE Select, 10X (Thermo Fisher Scientific, cat.no. A1217701)
- Phosphate-buffered Saline with calcium, magnesium (PBS^{+/+}) (Thermo Fisher Scientific, cat. no. 14040-091)
- Phosphate-Buffered Saline without calcium and magnesium (PBS^{-/-}) (Thermo Fisher Scientific, cat. no. 14190-169)
- Hanks' Balanced Salt Solution (HBSS) no calcium, no magnesium, no phenol red (Thermo Fisher

Scientific, cat. no. 14175-053)

- DMEM/F-12, Glutamax supplement (Thermo Fisher Scientific, cat. no. 31331-028)
- DMEM, no glucose, no pyruvate (Thermo Fisher Scientific, cat. no. 11966-025)
- DMEM/F-12, HEPES (Thermo Fisher Scientific, cat. no. 31330-038)
- Distilled water (Thermo Fisher Scientific, cat. no. 15230-089)
- Fibroblast Growth Medium 3 (FGM3) (PromoCell, cat. no. C-23025)
- CryoStor CS10 medium (Stem Cell Technologies, cat. no. 07930)
- Fluo-4, AM, cell permeant (Thermo Fisher Scientific, cat. no. F14201)
- Powdered DMEM, without glucose, L-glutamine, phenol red, sodium pyruvate and sodium bicarbonate (Sigma-Aldrich, cat. no. D5030)

CRITICAL Phenol red has steroid activity and can affect oestrogen receptors; its inclusion could modify results in a sex-specific way.

- D-Glucose (Sigma-Aldrich, cat. No. G8270)
- Sodium Pyruvate 100mM (Thermo Fisher Scientific, cat. no. 11360070)
- L-Glutamine 200mM (Thermo Fisher Scientific, cat. no. 25030024)
- Oligomycin (Sigma-Aldrich, cat. no. O4876)
- Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (Sigma-Aldrich, cat. no. C2920)
- Antimycin A (Sigma-Aldrich, cat. no. A8674)
- Rotenone (Sigma-Aldrich, cat. no. R8875)
- 2-Deoxy-D-glucose (2-DG) (Sigma-Aldrich, cat. no. D8375)
- EasySep™ Human CD34 Positive Selection Kit II (Stem Cell Technologies, cat. no. 17856)
- Multi Tissue Dissociation Kit 3 (Miltenyi Biotec, cat. no. 130-110-204)

CRITICAL The dissociation of hiPSC-CMs using Miltenyi Biotec Multi Tissue Dissociation Kit 3 is crucial for MT generation protocol to ensure reproducible single cell suspensions of CMs without affecting their viability.

- DNase Vial (D2) (Worthington-Biochem, cat. no. LK003170)
- Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific, cat. no. P7589)
- Paraformaldehyde (PFA) (Merck, cat. no. 104005)

! CAUTION Paraformaldehyde is a flammable solid, carcinogen, irritant (skin, eye, and respiratory tract), toxic (by skin contact and inhalation). It can be stored in a flammable storage cabinet. During work, avoid contact and inhalation. Wear protective gloves/protective clothing/eye protection/face protection.

- Triton X-100 (Sigma-Aldrich, cat. no. T8787)

! CAUTION Triton X-100 can cause acute oral toxicity and serious eye damage/eye irritation. For prevention, wash face, hands and any exposed skin thoroughly after handling. Do not eat, drink or smoke when using this product. Wear protective gloves/protective clothing/eye protection/face

protection.

- Bovine Serum Albumin BSA (Sigma-Aldrich, cat. no. A3311)
- Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, cat. no. 10270-106)
- Fixation Medium (Reagent A) (Thermo Fisher Scientific, cat. no. GAS001S5)
- Permeabilization Medium (Reagent B) (Thermo Fisher Scientific, cat. no. GAS002S5)
- Sodium-L-Lactate (Sigma-Aldrich cat. no. 71718)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat. no. D2650)
- **! CAUTION** DMSO is flammable. Keep away from heat/sparks/open flames/hot surfaces. During work, wear protective gloves/protective clothes/eye protection/face protection.
- KnockOut™ Serum Replacement (KOSR) (Thermo Fisher Scientific, cat. no. 10828028)
- Trypan Blue Stain solution (0.4%) (Thermo Fisher Scientific, cat. no. T10282)
- 4',6-Diamidino-2-Phenylindole, Dilactate (DAPI nucleic acid stain) (Thermo Fisher Scientific, cat. no. D3571)
- ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific, cat. no. P36930)
- Iscove's Modified Dulbecco's Medium (IMDM) (Thermo Fisher Scientific, cat. no. 21056-023)
- F12 nutrient mixture (Ham) (Thermo Fisher Scientific, cat. no. 31765-027)
- Glutamax (Thermo Fisher Scientific, cat. no. 35050-038)
- Penicillin/Streptomycin (Thermo Fisher Scientific, cat. no. 15070-063)
- Phenol Red (Sigma-Aldrich, cat. no. P3532)
- Protein-free Hybridoma Medium-II (PFHMI) (Thermo Fisher Scientific, cat. no. 12040-077)
- Bovine Serum Albumin (BSA) (Bovogen Biologicals Australia, cat. no. BSAS05)
- Poly(Vinyl Alcohol) (PVA) (Sigma-Aldrich, cat. no. P8136)
- Chemically-defined Lipid Concentrate (CDLC) (Thermo Fisher Scientific, cat. no. 11905-031)
- Insulin-Transferrin-Selenium Ethanolamine (ITS-X) 100X (Thermo Fisher Scientific, cat. no. 51599-056)
- L-ascorbic acid 2-phosphate (Sigma-Aldrich, cat. no. A8960)
- α -Monothioglycerol (Sigma-Aldrich, cat. no. M6145)

Recombinant proteins and small molecules

- Collagenase type II (Worthington, cat. no. LS004176)
- Recombinant Human BMP-4 Protein (R&D Systems, cat. no. 314-BP)
- Human Activin A, premium grade (Miltenyi Biotec, cat. no. 130-115-010)
- CHIR 99021 (Axon Medchem, cat. no. Axon1386)
- XAV 939 (Tocris, cat. no. 3748/10)
- Human VEGF, premium grade (Miltenyi Biotec, cat. no. 130-109-386)

- Retinoic Acid (Sigma-Aldrich, cat. no. R2625)
- TGF β inhibitor, SB431542 (Tocris, cat. no. 1614/10)
- Human FGF-2, premium grade (Miltenyi Biotec, cat. no. 130-093-842)

Primary antibodies

- Anti-Troponin I antibody (H-170) (TNNI) (1:500 dilution; Santa Cruz Biotechnology, cat. no. sc-15368; RRID: AB_793465; https://scicrunch.org/resolver/RRID:AB_793465) used for immunofluorescence
- Anti-alpha-Actinin (Sarcomeric) antibody (ACTN2) (1:800 dilution; Sigma-Aldrich, cat. no. A7811; RRID: AB_476766; https://scicrunch.org/resolver/RRID:AB_476766) used for immunofluorescence
- Anti-CD31/PECAM-1 antibody (1:200 dilution; R&D Systems, cat. no. AF806; RRID: AB_355617; https://scicrunch.org/resolver/RRID:AB_355617) used for immunofluorescence
- Anti-Collagen Type I, clone 5D8-G9 antibody (COL1A1) (1:200 dilution; Millipore, cat. no. MAB3391; RRID: AB_94839; https://scicrunch.org/resolver/RRID:AB_94839) used for immunofluorescence
- Anti-Wilm's Tumor Protein antibody (WT1) (1:200 dilution; Millipore, cat. no. CA1026; RRID: AB_437848; https://scicrunch.org/resolver/RRID:AB_437848) used for immunofluorescence
- Anti-TBX18 antibody (TBX18) (1:200 dilution; Sigma-Aldrich, cat. no. HPA029014; RRID: AB_10601597; https://scicrunch.org/resolver/RRID:AB_10601597) used for immunofluorescence
- Anti-ZO-1 antibody (ZO-1) (1:200 dilution; Thermo Fisher Scientific, cat. no. 33-9100; RRID: AB_87181; https://scicrunch.org/resolver/RRID:AB_87181) used for immunofluorescence
- Anti-Connexin 43/GJA1 antibody (CX43) (1:200 dilution; Abcam, cat. no. ab11370; RRID: AB_297976; https://scicrunch.org/resolver/RRID:AB_297976) used for immunofluorescence
- Anti-Vimentin, clone V9 antibody (VIM) (1:100 dilution, Sigma-Aldrich, cat. no. V6630; RRID: AB_477627; https://scicrunch.org/resolver/RRID:AB_477627) used for immunofluorescence
- Anti-GATA4 (1:100 dilution, Santa Cruz Biotechnology, cat. no. sc-25310; RRID: AB_627667; https://scicrunch.org/resolver/RRID:AB_627667)
- Anti-cardiac Troponin T (cTnT) antibody (clone REA400)-VioBlue(1:50 dilution; Miltenyi Biotec, cat. no. 130-120-402; RRID: AB_2783891; https://scicrunch.org/resolver/RRID:AB_2783891) used for flow cytometry
- REA Control Antibody (I), human IgG1 (clone REA293)-VioBlue (1:50 dilution; Miltenyi Biotec, 130-108-346, RRID: AB_2661680; https://scicrunch.org/resolver/RRID:AB_2661680) used for flow cytometry
- CD31 (PECAM-1) antibody (clone WM59)-APC (1:100 dilution, eBioscience, cat. no. 17-0319-

42, RRID: AB_10852842; https://scicrunch.org/resolver/RRID:AB_10852842) used for flow cytometry

- IgG1 kappa Isotype Control (clone P3.6.2.8.1)-APC (1:100 dilution, eBioscience, cat. no. 17-4714-42, RRID: AB_1603315; https://scicrunch.org/resolver/RRID:AB_1603315) used for flow cytometry

Secondary antibodies

- Cy3-AffiniPure Donkey Anti-Mouse IgG (H+L) antibody (1:100 dilution; Jackson ImmunoResearch Labs, cat. no. 715-165-150; RRID: :AB_2340813; https://scicrunch.org/resolver/RRID:AB_2340813) used for immunofluorescence
- Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:200 dilution; Thermo Fisher Scientific, cat. no. A-21206; RRID: AB_2535792; https://scicrunch.org/resolver/RRID:AB_2535792) used for immunofluorescence
- Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (1:200 dilution; Thermo Fisher Scientific, cat. no. A-21203; RRID: AB_2535789; https://scicrunch.org/resolver/RRID:AB_2535789) used for immunofluorescence
- Donkey Anti-Sheep IgG (H+L) Antibody, Alexa Fluor 647 (1:200 dilution; Thermo Fisher Scientific, cat. no. A21448; RRID: AB_10374882; https://scicrunch.org/resolver/RRID:AB_10374882) used for immunofluorescence

Equipment

- 6-well cell culture plates (Greiner Bio-one, cat. no. 657 160)
- 12-well cell culture plates (Greiner Bio-one, cat. no. 665 180)
- 24-well cell culture plates (Greiner Bio-one, cat. no. 662 160)
- 96-well black microplates (Corning Falcon 96-Well, cat. no. 353219)
- 96-well V-bottom microplates (Greiner Bio-one, cat. no. 651161)
- 5-, 10-, and 25-mL sterile plastic serological pipettes (Greiner Bio-One, cat. no. 606180, 607180, and 760180, respectively)
- 15- and 50-mL polystyrene conical tubes (Corning Falcon, cat. no. 352097 and 352098, respectively)
- 1.5-mL microcentrifuge tubes (Eppendorf, cat. no. 0030 120.086)
- Flow cytometry tubes with cell strainer snap cap (Corning Falcon, cat. no. 352235)
- 10-, 200-, and 1000- μ L filter tips (Corning, cat. no. 4807, 4810, and 4809, respectively)
- 12-mm, round, glass coverslips (Menzel-Glaser, cat. no. CB00140RA1)

- Vacuum filter/storage bottle system (0.22 µm pore size) (Millipore, cat. no. S2GVU05RE)
- Multichannel pipetting reservoirs (VWR, cat. no. 613-1175)
- Cryovials (Greiner Bio-One, cat. no. 123263)
- Fuchs-Rosenthal counting chamber (VWR, cat. no. 15170-230)
- Parafilm (Sigma-Aldrich, cat. no. P7793)
- Handheld pipetman (P-10 (10 µL), P-200 (200 µL), P-1000 (1,000 µL) (Gilson international, cat. no. 4807, 4810, and 4809, respectively)
- Multichannel, 12-channel 5-50 ul (Finnpipette, cat. no. 4610130)
- Nalgene® Mr. Frosty freezing container (Sigma-Aldrich, cat. no. C1562-1EA)
- Water bath (Julabo TW20; MCO-18AIC, cat. no. 08010017)
- Milli-Q® Water Purification System (Millipore, cat. no. C79625)
- Biological safety cabinet/laminar flow-hood (CleanAir)
- CO₂ cell-culture incubator (Sanyo, cat. no. MCO-15A)
- Centrifuge (Eppendorf, cat. no. 5810R)
- Seahorse XFe96 Analyzer (Agilent)
- Seahorse XFe96 FluxPak (Agilent cat. no. 102601-100)
- WTW Lab pH meter inoLab pH 7110 (Xylem analytics cat. No. 1AA110)
- Leica MZ6 stereomicroscope (Leica Microsystems)
- Glass pasteur capillary pipettes 150mm (Wilhelm Ulbrich cat. No. 10150)
- 250 µL pipette tips ultra-low attachment (Mettler Toledo cat. no. 17007958)
- MACSQuant®VYB flow cytometry (Miltenyi Biotech, cat. no. 130-096-116)
- Nikon Eclipse Ti inverted microscope (or equivalent commercially available device, preferably for phase contrast microscopy) connected to a Nikon DS- 2MBW camera (Nikon) or connected to ThorLabsDCC3240M camera
- EVOS M7000 Imaging System (Thermo Fisher Scientific)
- LAS AF6000 fluorescent microscope (Leica Microsystems)
- SP8WLL fluorescent confocal laser-scanning microscope equipped with a violet (405-nm), blue (488-nm), and orange (532-nm) laser (Leica Microsystems)
- Thin walled, glass capillaries with filament (World Precision Instruments, cat. no. TW150F-3)
- Micropipette puller P-97 (Sutter Instrument)

Software

- Fiji-ImageJ (Schindelin, J. et al., 2012 PMID: 22743772) (ImageJ: <https://imagej.net/Fiji/Downloads>)
- FlowJo (FlowJo: <https://www.flowjo.com/>)
- GraphPad Prism 8.2.0 (GraphPad: <https://www.graphpad.com/scientific-software/prism/>)

- MUSCLEMOTION (Sala, L. et al., 2018 PMID: 29282212) (<https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.117.312067>). MUSCLEMOTION is freely accessible on the web at <https://gitlab.com/bjvanmeer/MUSCLEMOTION> or <https://github.com/l-sala/MUSCLEMOTION/>. After downloading the macro, users can access the code through a text editor or directly through FIJI. For more details, see CODE AVAILABILITY STATEMENT.
- Rstudio (RStudio: <https://rstudio.com/products/rstudio>)
- Biorender (<https://biorender.com/>)

Reagent setup

Undifferentiated hiPSC culture media

Either E8™ or StemFlex™ medium can be used.

E8™ medium reconstitution: mix E8™ basal medium together with 50X E8™ supplement and 0.5% penicillin-streptomycin.

StemFlex™ reconstitution: mix StemFlex™ basal medium together with 10X StemFlex™ supplement and 0.5% penicillin-streptomycin.

Filter-sterilize the media by passing it through a 0.22-μm filter. Store the media at 4 °C for up to 2 weeks. Following reconstitution, 40-mL aliquots can be prepared and stored at -20 °C for longer period (up to 6 months).

CRITICAL Thaw E8™ and StemFlex™ supplements for 1-2 h at room temperature or overnight at 4 °C. Do not thaw both frozen supplements at 37° C.

CRITICAL Before use, warm the media at room temperature. Do not warm the medium in the water bath at 37° C.

HiPSC dilution medium preparation

HiPSC dilution medium consists of DMEM-F-12, HEPES containing 0.5% BSA. The hiPSC dilution medium can be stored at 4 °C for up to 1 month.

Freezing medium for hiPSC-derived cardiomyocytes (hiPSC-CMs)

1X freezing medium for hiPSC-CMs is composed of 90% (vol/vol) KOSR and 10% (vol/vol) DMSO. The freezing medium can be stored at 4 °C for up to 1 week.

0.5 mM EDTA solution

Dilute 0.5 M EDTA solution in PBS^{-/-} to make 0.5 mM solution (dilution 1:1,000). Store the diluted solution at room temperature for up to 6 months.

1X TrypLE Select solution

Dilute TrypLE Select 10X solution in PBS^{-/-} to make 1X (dilution 1:10) diluted TrypLE Select solution. Store the diluted solution at room temperature for up to 6 months.

Vitronectin (VTN) stock

Thaw the vial of VTN at room temperature. Prepare 60-μL aliquots of VTN and store them at -80 °C.

Laminin stock

Thaw laminin overnight at 4° C. Prepare 1-mL aliquots of undiluted Laminin (100 μg/mL) and store them at 4° C for up to 3 months under aseptic conditions. Laminin aliquots can be stored at -80 °C for longer period.

CRITICAL Repeated freeze-thaw cycles should be avoided.

Matrigel™ hESC-Qualified Matrix stock

Thaw the bottle of Matrigel overnight at 4° C. Prepare 50- and 100-μL aliquots (containing 0.5 mg and 1 mg of protein, respectively) and store them at -20 °C.

CRITICAL During the entire procedure, it is necessary to always work on ice and use cold tips since Matrigel quickly polymerizes at room temperature. Repeated freeze-thaw cycles should be avoided.

Cardiac fibroblast culture medium (FGM3)

Reconstitute the Fibroblast Growth Medium 3 by mixing the FGM3 basal medium together with the 10X Supplement Mix and 0.5% penicillin-streptomycin. Store the complete medium at 4 °C for up to 6 weeks or prepare 40-mL aliquots and store them at -20 °C for longer period.

CRITICAL Thaw Supplement mix for 1-2 h at room temperature or overnight at 4 °C. Do not thaw supplements at 37 °C.

Seahorse metabolic flux stock medium

Reconstitute 1 bottle of DMEM no glucose powder (sufficient for 1 litre DMEM) in 800 mL Milli-Q water and rinse the stock bottle. Place a large stirrer and mix until all the powder is dissolved. Measure the pH of the reconstituted medium and titrate with 1 M NaOH until a pH of 7.4 is reached. Add Milli-Q water up to 1 L and sterile filtrate using a 0.22 µm filter. This stock medium can be stored at 4 °C for 1 year.

Seahorse metabolic flux assay medium

Two different media are used during the assay, one including glucose to measure the mitochondrial oxidative potential, called SH+ medium, and one without glucose to measure the glycolytic capacity of the MTs, called SH- medium. For SH- medium, take 49 mL of the Seahorse stock medium and add 500 µL of both 200mM L-Glutamine and 100mM sodium pyruvate. For SH+, medium take 48.25 mL of seahorse stock medium and add 750 µL of 1 M D-glucose and 500 µL of 200 mM L-Glutamine and 500 µL 100 mM sodium pyruvate. This will result in media containing 1 mM pyruvate, 2 mM L-glutamine and in the SH+ medium also 15 mM D-Glucose. Warm the medium to 37 °C in a water bath and subsequently adjust the pH to 7.4 with 1 M NaOH. Filter-sterilize the medium through a 0.22 µm filter. Take 35 mL of both media and add to their own tube. To this add 875 µL 8% (wt/vol) BSA, this will yield the media used for washing the MTs prior to plating them, called Wash+ and Wash-. Prepare Seahorse media fresh on day of experiment or the day before the assay.

Fixative solution

Dilute 8% (wt/vol) PFA in 0.2 M phosphate buffer, pH 7.4 in order to make 4% (vol/vol) PFA solution. Store the solution at 4 °C up to 1 month.

! CAUTION PFA is carcinogen and it causes skin, eye and respiratory tract irritation. Always wear the appropriate personal protection equipment and work under a fume hood.

Permeabilization solution for immunostaining on 2D cells

Add 50 µL of Triton X-100 to 50 mL of PBS^{+/+} in order to make 0.1% (vol/vol) Triton X-100 solution. Store the solution up to 3 months at 4 °C.

Permeabilization solution for immunostaining on 3D cardiac MTs

Add 100 µL of Triton X-100 to 50 mL of PBS^{+/+} in order to make 0.2% (vol/vol) Triton X-100 solution. Store the solution up to 3 months at 4 °C.

Blocking solution for immunostaining on 2D cells and 3D cardiac MTs

Prepare blocking solution for 3D MTs with PBS^{+/+} containing 10% (vol/vol) FBS. Store the solution up to 1 week at 4 °C.

CM dissociation enzyme mix (Multi Tissue Dissociation Kit 3)

To prepare CM dissociation enzyme mix, add Enzyme T to Buffer X of the Multi Tissue Dissociation Kit 3 in a ratio of 1:10. Prepare fresh each time and use immediately.

CRITICAL Upon receipt of the kit, immediately prepare 500-μL and 1-mL aliquots and store them at -20 °C up to 6 months. Avoid repeated freeze-thaw cycles of the aliquots.

DNAse solution

Dissolve in PBS to a final concentration of 1000 units/mL. Add to CM dissociation enzyme mix to a final concentration of 100 units/mL. Store solution at 4°C for maximum 1 week before use, or prepare fresh.

Collagenase type II enzymatic solution for cardiac microtissue dissociation

Dissolve Collagenase type II in HBSS solution in order to make 290U/mg solution. Filter-sterilize the medium by passing through a 0.22-μm filter. Prepare fresh each time, pre-warm the enzymatic solution at 37 °C and use it immediately.

BMP4 stock solution

Reconstitute BMP4 powder at 100 μg/mL in sterile 4 mM HCl 0.1% BSA solution and then dilute in sterile 0.1% BSA in PBS^{-/-} to make BMP4 stock at 25 μg/mL. Prepare 30-μL aliquots of BMP4 and store them at -80 °C.

CRITICAL Thaw BMP4 aliquot on ice and spin down before using. Store the residual aliquot up to 7 d at 4 °C. Avoid freeze-thaw cycles.

Activin A stock solution

Reconstitute Activin A powder at 100 μg/mL in distilled sterile-filtered water and then proceed with further a dilution at 25 μg/mL in sterile 0.1% BSA in PBS^{-/-}. Prepare 30-μL aliquots of Activin A and store them at -80 °C.

CRITICAL Thaw Activin A aliquot on ice and spin down before using. Store the residual aliquot up to 7 d at 4 °C. Avoid freeze-thaw cycles.

CHIR99021 stock solution

Dissolve CHIR99021 at 4mM stock solution in DMSO. Prepare 50- and 100- μ L aliquots and store them at -20 °C.

CRITICAL Thaw CHIR99021 aliquot at room temperature, protecting the aliquot from light. Before using, spin it down. After thawing, store the aliquot up to 10 d at 4 °C.

XAV 939 stock solution

Dissolve XAV 939 at 5mM stock solution in DMSO. Prepare 100- μ L aliquots and store them at -20 °C.

CRITICAL Thaw XAV 939 aliquot at room temperature and spin it down, before using. After thawing, store the aliquot up to 10 d at 4 °C.

VEGF stock solution

Reconstitute VEGF powder at 100 μ g/mL in distilled sterile-filtered water and then proceed with further a dilution at 50 μ g/mL sterile in 0.1% BSA in PBS^{-/-}. Prepare 50- μ L and 100- μ L aliquots of VEGF and store them at -80 °C.

CRITICAL Thaw VEGF aliquot on ice and spin down before using. Store the residual aliquot up to 7 d at 4 °C. Avoid freeze-thaw cycles.

FGF-2 stock solution

Reconstitute FGF-2 powder at 100 μ g/mL in distilled sterile-filtered water and then proceed with further a dilution at 10 μ g/mL sterile in 0.1% BSA in PBS^{-/-}. Prepare 50- μ L aliquots of FGF-2 and store them at -80 °C.

CRITICAL Thaw FGF-2 aliquot on ice and spin down before using. Store the residual aliquot up to 7 d at 4 °C. Avoid freeze-thaw cycles.

SB431542 (TGF β inhibitor) stock solution

Dissolve SB431542 at 20 mM stock solution in DMSO. Prepare 50- μ L aliquots and store them at -20 °C.

CRITICAL Thaw SB431542 at room temperature and spin it down, before using. After thawing, store the aliquot up to 10 d at 4 °C.

RA stock solution

Dissolve RA at 100 μ M stock solution in DMSO. Prepare 50- μ L and 100- μ L aliquots and store them at -20 °C.

CRITICAL Thaw RA at room temperature and spin it down, before using. After thawing, discard the leftover.

CRITICAL RA is sensitive to air and light. Always protect it from light, switching off the light of the safety cabinet.

Sodium-L-Lactate stock solution

Dissolve Sodium-L-Lactate at 100 mM in DMEM no glucose, no pyruvate. Prepare 500- μ L and 1-mL aliquots and store them at -20 °C.

Phenol Red solution for LI-BPEL medium

Dissolve 1 mg of Phenol Red in 25 mL of IMDM medium, vortex to properly mix and incubate at 37 °C in a water bath for 10 min. Then, use it immediately for LI-BPEL medium preparation.

Bovine Serum Albumin (BSA) stock solution for LI-BPEL medium

Dissolve 10% (wt/vol) BSA (Bovogen Biologicals Australia, cat. no. BSAS05) in IMDM medium. Place the tube(s) on a rollerbank at 4 °C for 2 h to properly ensure BSA is totally dissolved. Filter-sterilize BSA stock solution by passing it through a 0.22- μ m filter and store it at 4 °C up to 1 month.

Polyvinyl Alcohol (PVA) stock solution for LI-BPEL medium

Dissolve 5% (wt/vol) PVA in distilled sterile-filtered water and place the tube(s) on a rollerbank at least overnight (up to 2 d) at 4 °C. Incubate PVA solution at 75 °C for 10-15 min, vortexing in between. Once PVA solution is completely dissolved, store it at 4 °C up to 1 month.

CRITICAL PVA is hard to dissolve. Following these instructions is essential to ensure proper PVA dissolution.

L-ascorbic acid 2-phosphate (AA2P) stock solution for LI-BPEL medium

Dissolve AA2P at 5 mg/mL stock solution in distilled sterile-filtered water. Prepare 1-mL aliquots and store them at -20 °C.

Low insulin BSA-based, Polyvinyl Alcohol, Essential Lipids (LI-BPEL) medium preparation

To prepare 100 mL of LI-BPEL medium, combine all the components/supplements as listed in the table below. Filter the medium using a 0.22- μ m filter system and store the medium at 4 °C for up to 2 weeks.

Component	Quantity for 100 mL
IMDM	43 mL
F12	43 mL
Protein Free Hybridoma Medium-II (PFHMII)	5 mL
BSA (10% wt/vol in IMDM)	2.5 mL
PVA (5% wt/vol in distilled water)	2.5 mL
Chemically Defined Lipid Concentrate (CDLC) 100X	1 mL
Insulin-Transferrin-Selenium-Ethanolamine (ITS-X) 1000X	0.1 mL
α -Monothioglycerol (13 μ L in 1 mL IMDM)	0.3 mL
L-ascorbic acid 2-phosphate (5 mg/mL in distilled water)	1 mL
Glutamax	1 mL
Penicillin-streptomycin	0.5 mL
Phenol red	1mg

FACS buffer

FACS buffer consists of PBS^{-/-} containing 2% (vol/vol) FBS and 2 mM EDTA. Store at 4 °C for up to 3 months.

Bovine Serum Albumin (BSA) stock solution for Seahorse medium

Dissolve 8% (wt/vol) BSA (Bovogen Biologicals Australia, cat. no. BSAS05) in Seahorse stock medium. Place the tube(s) on a roller bank at 4 °C for 2 hours to properly ensure BSA is dissolved. Filter-sterilize BSA stock solution by passing it through a 0.22-μm filter and store it at 4 °C up to 1 month or in smaller stocks at -20 °C for up to a year.

Oligomycin stock solution

Dissolve the entire 5 mg content of the Oligomycin bottle in 2.52 mL of DMSO. This will result in a 2.5 mM stock which can be frozen in 50 μL stocks at -20 °C.

FCCP stock solution

Add 1.3 mL DMSO to 10 mg of FCCP to achieve a concentrated superstock solution of 30 mM. This can be stored at -20 °C. Regular stocks are made by further dilution of the superstock by 10X. These stocks can be frozen in 40 μL aliquots at -20 °C.

Antimycin-A stock solution

Weigh 25 mg of Antimycin-A and add 4.5 mL of Ethanol (DMSO can also be used if preferred) to make a 10 mM superstock which can be stored at -20 °C. Regular stocks are made by further dilution of the superstock by 10X. These stocks can be frozen in 60 μL aliquots at -20 °C.

Rotenone stock solution

Weigh 10 mg of Rotenone and add 2.5 mL of DMSO to make a 10 mM superstock which can be stored at -20 °C. Regular stocks are made by further dilution of the superstock by 10X. These stocks can be frozen in 60 μL aliquots at -20 °C.

2-DG stock solution

Dissolve 5 g of 2-DG powder in 15.15 mL of seahorse stock medium. This will make a 2 M solution at close to max solubility. If small clumps remain a short period of heating at 37 °C is sufficient to

dissolve all the powder. Subsequently, freeze in small aliquots of 1 mL at -20 °C.

D-Glucose stock solution

Weigh 1.8 g of D-glucose powder and add it to 10 mL of seahorse stock medium. Freeze in aliquots of 1 mL at -20 °C.

Fluo-4 AM stock solution

Reconstitute 50 µg of Fluo-4 AM in 10 µL of DMSO to make a stock solution of 5 mM, mixing thoroughly to ensure it is completely dissolved. Store Fluo-4 AM stock solution up to 1 week at 4 °C.

CRITICAL Protect the solution from light to avoid bleaching of the fluorescent dye.

Fluo-4 AM loading solution

Dilute 5 mM Fluo-4 AM stock solution in LI-BPEL medium to make a 5 µM Fluo-4 AM loading solution (dilution 1:1,000). Vortex briefly the diluted solution to ensure homogenous distribution of the fluorescent dye. Prepare fresh each time and use immediately.

CRITICAL Protect the solution from light to avoid bleaching of the fluorescent dye.

Equipment setup

VTN-coated plates

Remove an aliquot of VTN from -80 °C and thaw it at room temperature. Dilute VTN in sterile PBS^{-/-} (dilution 1:100) at room temperature, gently pipetting the VTN dilution up and down. Coat the growth surface of the plates, using 1 mL per 10-cm², in order to obtain a final concentration of 0.5 µg/cm². Incubate the plates at room temperature for 1 h and use them immediately or store at 4 °C for up to 2 weeks. Before using the coated plates, pre-warm them at room temperature for at least 30 min.

CRITICAL Do not allow the coated surface to dehydrate as this will not support cell growth. Prior to storing at 4 °C, top up the plates with additional PBS^{-/-} and seal with parafilm (to prevent evaporation and contamination).

Laminin-coated plates

Remove an aliquot of laminin from -80 °C and thaw slowly at 4 °C. Dilute the laminin stock (100 µg/

mL) to a final concentration of 5 µg/mL in sterile PBS^{+/+} (dilution 1:20) at room temperature. Coat the growth surface of the plates (1 mL per 10-cm²) and incubate the coated plates for a minimum of 2 h at 37 °C or overnight at 4 °C for a more reliable coating. Store the plates for up to 4 weeks at 4 °C, adding extra PBS^{+/+} and sealing with parafilm to prevent the plates from drying out.

CRITICAL The use of PBS^{+/+} is crucial for proper coating since divalent cations are important for the protein structure and function.

Matrigel-coated plates

Thaw Matrigel aliquot on ice and dilute it in ice-cold DMEM/F12 (dilution 1:120). Coat the growth surface of the plates (1 mL per 10-cm²) and incubate them at room temperature for 1 h. Use them immediately or top up with extra DMEM/F12 and store them at 4 °C up to 2 weeks.

CRITICAL During the entire procedure, keep Matrigel and DMEM/F12 on ice and use prechilled conical tubes and pipette tips, in order to prevent Matrigel polymerization.

Fibronectin-coated plates

Dilute fibronectin in sterile PBS^{-/-} at final concentration of 5 µg/mL (dilution 1:200) at room temperature and coat the growth surface of the plates (1 mL per 10-cm²). Incubate the plates at room temperature for 1 h, then use them immediately or add extra PBS without calcium and magnesium, seal with parafilm and store at 4 °C up to 2 weeks.

Cell culture incubators for Seahorse experiments

Set cell culture incubators to 37 °C and 0% CO₂ for incubation of the cartridge and cardiac microtissues prior Seahorse experiments.

Seahorse assay Matrigel-coated plates

Thaw Matrigel aliquot on ice and dilute it in ice-cold DMEM/F12 (dilution 1:60). Add 80 µL to each well of the seahorse tissue culture plate and incubate at room temperature for 1 h. The plate can be used immediately or stored at 4 °C for up to 2 weeks.

CRITICAL During the entire procedure, keep Matrigel and DMEM/F12 on ice and use prechilled conical tubes and pipette tips, to prevent Matrigel polymerization.

PROCEDURE

Thawing and expansion of undifferentiated hiPSCs

TIMING 10 min plus 3–4 d for expansion before passaging hiPSCs

1. Prepare a 15-mL conical tube filled with 5 mL of hiPSC medium (E8 or StemFlex), previously equilibrated at room temperature.
2. Remove the cryovial of hiPSCs from liquid nitrogen storage tank and transfer it on dry ice or in a portable liquid nitrogen container to the cell culture room.
3. Immerse the cryovial in a 37 °C water bath or incubate in a 37 °C, 5% CO₂ cell culture incubator and swirl it gently, until only an ~2 mm³ ice crystal remains.

CRITICAL STEP To prevent contamination, do not submerge the cap of the cryovial.

4. Remove the cryovial from the water bath or from the 37 °C, 5% CO₂ cell culture incubator, spray with 70% ethanol and place it into the biological safety cabinet.
5. Gently transfer the content of the cryovial into the 15-mL conical tube containing the hiPSC medium (E8 or StemFlex). Rinse the vial with additional 1 mL of hiPSC medium to recover any residual cells and add it into the same 15-mL conical tube.

CRITICAL STEP Transfer the content of the cryovial drop-wise to minimize osmotic shock

6. Centrifuge the cells at 300g for 3 min at room temperature, aspirate and discard the supernatant. Resuspend the cell pellet in the desired volume of hiPSC medium supplemented with RevitaCell (1:200), by gently pipetting the cells up and down a few times.
7. Dispense the cell suspension onto VTN or laminin-coated plate (for cells to be incubated with E8 or StemFlex, respectively) and place it gently into a 37 °C, 5% CO₂ cell culture incubator, moving the plate side-to-side and back-and-forth to ensure a homogenous distribution of the cells.
8. Refresh the medium (2 mL per 10-cm² growth surface) after 24 h with fresh hiPSC medium (without RevitaCell).
9. Refresh the hiPSC medium daily or every-other-day (for E8 or StemFlex, respectively), until the cells reach approximately 70% or 80% (for E8 or StemFlex respectively) of confluency. This generally takes 3–4 days.

CRITICAL STEP From initial thawing, hiPSCs require 1 week (2 passages) to adapt to culture conditions before starting any differentiation protocol.

? TROUBLESHOOTING

Passaging of undifferentiated hiPSCs

10. Passage hiPSCs cultured using E8 medium/VTN-coated plate as described in option A and hiPSCs using StemFlex medium/laminin-coated plate as described in option B.

(A) Passaging undifferentiated hiPSCs cultured using E8 medium/VTN-coated plate
TIMING 10 min

- (i) When the cells are 70% confluent, aspirate the culture medium and wash with room temperature PBS^{-/-}.
- (ii) Incubate hiPSCs at room temperature with 0.5 mM EDTA for 3 min in 37 °C, 5% CO₂ cell culture incubator (1 mL of EDTA per 10-cm² growth surface).

CRITICAL STEP Monitor the cells under a microscope. Different cell lines may require different incubation times in EDTA. EDTA should be removed when cells start to round up and the colonies appear to have holes in them.

- (iii) Aspirate and discard EDTA solution and add E8 medium (1 mL of E8 per 10-cm² growth surface).
- (iv) Detach the cells from the surface of the well(s) by gently pipetting the medium up and down until all cells are in suspension. Collect the cells in a 15-mL conical tube.

CRITICAL STEP Do not pipette the cells too vigorously, cell clusters are required rather than a single cell suspension.

CRITICAL STEP Some hiPSC lines re-adhere very rapidly after the addition of hiPSC medium to neutralize EDTA. For this reason, it is advisable to work with no more than 1 to 3 wells at a time.

CRITICAL STEP To avoid cross contamination between cell lines, it is advisable to work with 1 cell line at a time.

- (v) Rinse the well surface with E8 medium (1 mL per 10 cm² growth surface) and put this medium in the 15-mL conical tube containing the cell suspension.
- (vi) Add an appropriate volume of hiPSC medium supplemented with Revita-Cell, 1:200 to each well of a VTN-coated 6-well plate so that each well contains a total volume of 2 mL after addition of the cell suspension.
- (vii) Plate the cells at a density such that they reach 80% confluency and are ready to be passaged after 3 or 4 days, respectively.

CRITICAL STEP The appropriate plating ratio must be determined empirically and may vary among different cell lines.

- (viii) Incubate the plate in the 37 °C, 5% CO₂ incubator and change the medium (without RevitaCell) every 24 h.

? TROUBLESHOOTING

- (B) Passaging undifferentiated hiPSCs cultured using StemFlex medium/laminin-coated plate

TIMING 10 min

- (i) When the cells are 80% confluent, aspirate the culture medium and wash with room temperature PBS^{-/-}.
- (ii) Incubate hiPSCs with room temperature 1X TrypLE Select for 5 min in a 37 °C, 5% CO₂ cell culture incubator (1 mL of 1X TrypLE Select per 10-cm² growth surface).

CRITICAL STEP Monitor the cells under a microscope. The cells need to be harvested, when they round up and start to detach.

- (iii) Detach the cells from the surface of the well(s) by gently pipetting the dissociation reagent up and down using a P-1000 pipette.
- (iv) Collect the cells in a 15-mL conical tube containing 3x volume of hiPSC dilution medium to dilute the dissociation reagent.
- (v) Wash the well with an additional 1 mL of hiPSC dilution medium to collect any remaining cells and add to the conical tube.
- (vi) Centrifuge the cells at 300g for 3 min.
- (vii) Aspirate the supernatant and resuspend the cells in 1 mL of StemFlex medium.
- (viii) Aspirate the laminin solution from the coated surface of a new plate and add an appropriate volume of StemFlex medium so that each well will contain a total of 2 mL of medium per 10-cm² after addition of the cell suspension.
- (ix) Plate the cells at a density such that they reach 80% confluency and are ready to be passaged after 3 or 4 days.

CRITICAL STEP The appropriate plating ratio must be determined empirically and may vary among different cell lines.

- (x) Incubate the plate in a humidified incubator at 37 °C and 5% CO₂. Change the medium 24 h later, followed by every 48 h thereafter until the cells are

confluent. Alternatively, 24 h after passaging the cells can be fed with double the volume of StemFlex medium, which means that they do not require feeding for a further 72 h.

? TROUBLESHOOTING

Cardiac mesoderm induction with cytokines

TIMING 5 d

CRITICAL STEP To ensure optimal differentiation success rate, use high quality hiPSC cultures at passages lower than 40-50.

CRITICAL STEP The cardiac mesoderm induction with cytokines has been optimized for hiPSCs cultured in E8 medium.

11. Repeat step 10 option A i-v. Take a 20- μ L aliquot of cell suspension and count the cell number using a hemocytometer and microscope.
12. Seed 1.5×10^4 (for differentiation of cardiac endothelial cells) and 2.5×10^4 cells per cm^2 (for differentiation of epicardial cells and cardiomyocytes) on Matrigel-coated plate in E8 medium supplemented with RevitaCell (1:200). Incubate the plate overnight into the 37°C , 5% CO_2 incubator. This is day -1 of differentiation.

CRITICAL STEP The differentiation efficiency is profoundly affected by the initial seeding density of undifferentiated hiPSCs (Fig.3) . When starting differentiation of new hiPSC lines, it is crucial to identify the optimal seeding density to get highly efficient differentiation, thus we recommend seeding plates at 2 different densities.

13. After 24 h, prepare the appropriate volume of cardiac mesoderm induction medium composed of LI-BPEL medium supplemented with 20 ng/mL BMP4, 20 ng/mL Activin A, and 1.5 μM CHIR 99021.

CRITICAL STEP Warm LI-BPEL medium at room temperature. Add the cytokines fresh each time and use the medium immediately.

14. Aspirate E8 medium and wash the cells once with PBS^{-/-}, followed by an additional washing step with LI-BPEL medium.
15. Add cardiac mesoderm induction medium to the well(s) (3 mL per 10-cm^2 growth surface). This is day 0 of differentiation. Incubate cells. Do not change the medium until day 3, to avoid disturbing cardiac mesoderm differentiation.

CRITICAL STEP To improve differentiation efficiency and reproducibility, cardiac mesoderm

induction should be started exactly 24 h after the seeding of hiPSCs.

CRITICAL STEP To guarantee the success of subsequent differentiation, the next step should be started exactly 96 h after the seeding of hiPSCs.

? TROUBLESHOOTING

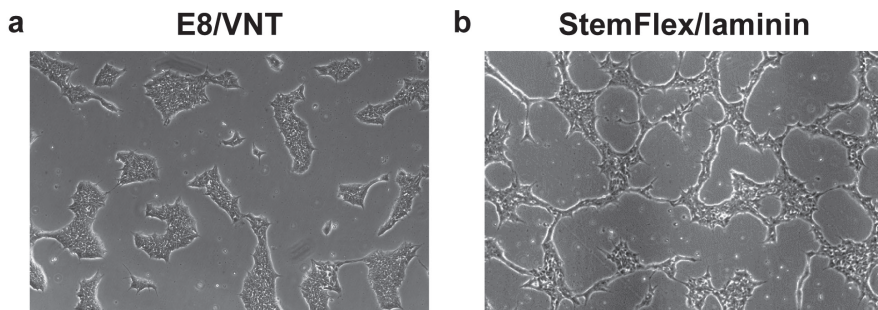


Fig. 3: Morphology of undifferentiated hiPSCs.

Representative brightfield images of hiPSCs cultured in E8/VTN (a) or StemFlex/Laminin (b). Scale bars, 200 μm .

Differentiation into cardiac endothelial cells, epicardial cells and cardiomyocytes.

16. On day 3 of differentiation, start the process of differentiating the common mesodermal precursors into cardiac endothelial cells and epicardial cells by following A and B. Also, either differentiate common mesodermal precursors to cardiomyocytes by following C or differentiate from hiPSCs without requiring growth factors (BMP4 and Activin-A) as described in Box 1. All three cell types can be cryopreserved once generated so differentiations can be carried out in parallel or sequentially.

- (A) Cardiac endothelial cell differentiation, isolation, culture and cryopreservation

TIMING 6 d

- (i) On day 3 of differentiation, prepare endothelial cell specification medium composed of LI-BPEL medium supplemented with 5 μM XAV 939 and 50 ng/mL VEGF.

CRITICAL STEP Warm LI-BPEL medium at room temperature. Add the cytokines fresh each time and use the medium immediately.

- (ii) Aspirate and discard the cardiac mesoderm induction medium from the well(s) seeded with 1.5×10^4 cells per cm^2 and add endothelial cell specification medium (3 mL per 10-cm^2 growth surface).

CRITICAL STEP To guarantee the success of the differentiation, cardiac mesoderm induction should be started exactly 96 h after the seeding of hiPSCs. Different cell lines may require different seeding density thus this might require optimization.

- (iii) On day 6 of differentiation, isolate endothelial cells (ECs) by immunomagnetic selection of CD34⁺ cells by using EasySep Human CD34 Positive Selection Kit II as described in the manufacturer's instructions (also described previously¹⁵). After immunomagnetic isolation, collect a 20- μL aliquot of freshly isolated CD34⁺ ECs for cell count.
- (iv) Dilute ECs in endothelial cell medium composed of LI-BPEL medium supplemented with 50 ng/mL VEGF and plate the cells on fibronectin (FB-N)-coated plate at 2.0×10^4 per cm^2 seeding density.

CRITICAL STEP Different cell lines may require different seeding density thus seeding density might require optimization.

- (v) Incubate the plate overnight into the 37°C , 5% CO_2 incubator and then replace medium with LI-BPEL medium supplemented with 50 ng/ml VEGF after 24 h.
- (vi) Check cells regularly. On day 9-10 of differentiation, or when cells reach 90-95% confluency (Fig. 4a, b), move to the next step to dissociate EC cells.
- (vii) Aspirate cell culture medium and wash with room temperature PBS^{-/-}.
- (viii) Incubate the cells with 1X TrypLE Select for 5 min in 37°C , 5% CO_2 cell culture incubator (1 mL of 1X TrypLE Select per 10-cm^2 growth surface).
- (ix) Neutralize 1X TrypLE Select by diluting with the same amount of LI-BPEL medium per well.
- (x) Detach the cells from the surface of the well(s) by pipetting the medium up and down and collect the cells in a 15-mL conical tube.
- (xi) Wash each well with 1 mL of LI-BPEL per 10-cm^2 growth surface, in order to collect residual cells and transfer them into the same 15-mL tube.

CRITICAL STEP Sample an aliquot of cell suspension (approximately 250,000-300,000 cells) to run flow cytometry for endothelial marker CD31 as quality control to evaluate the purity of EC population (CD31⁺ cells > 90%) (see Box 2 and Fig. 4c).

- (xii) Centrifuge the cells at 300g for 3 min at room temperature, aspirate and

discard the supernatant. Proceed to next step to cryopreserve cells or start to expand cells for MT production as described in step 18Biii.

- (xiii) Resuspend the cell pellet in appropriate volumes of CryoStor CS10 freezing medium to transfer 3.5, 7.0, or 10×10^4 cells/500 μ L into a cryovial. Transfer appropriate volumes of cells to cryovials.

CRITICAL STEP Cryovials are prepared to contain different numbers of frozen cells so that only the right amounts (without waste) are available for further experiments.

- (xiv) Transfer the cryovial(s) into a Mr. Frosty freezing container and place it at -80°C . Approximately 24–48 h later, transfer the cryovials in liquid nitrogen tank for longer storage.

? TROUBLESHOOTING

PAUSEPOINT Cells can be stored in liquid nitrogen indefinitely.

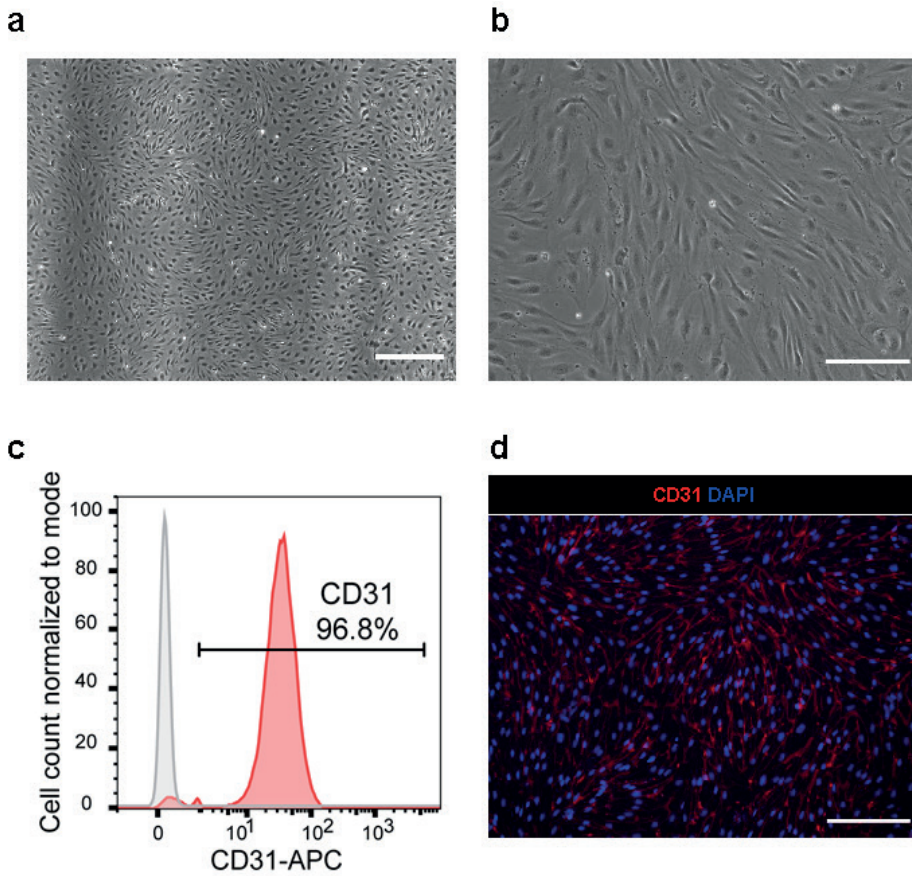


Fig. 4: hiPSC-EC characterization.

(a) Representative brightfield image of CD34⁺ hiPSC-ECs 6 d post-isolation and before freezing. Scale bar, 200 μ m. (b) Representative brightfield image of CD34⁺ hiPSC-ECs after thawing at higher magnification. Scale bar, 200 μ m. (c) Flow cytometry analysis of CD34⁺ hiPSC-ECs showing a representative histogram for the expression of the EC marker CD31. Isotype negative control (grey) and hiPSC-ECs are stained with CD31 (red). (d) Representative immunofluorescence images of the endothelial marker CD31 in hiPSC-ECs (day 12). Nuclei are stained with DAPI (blue). Scale bar, 50 μ m.

(B) Epicardial cell differentiation, culture and cryopreservation

TIMING 9 d

- (i) On day 3 of differentiation, prepare epicardial cell induction medium I composed of LI-BPEL medium supplemented with 5 μ M XAV 939, 30 ng/mL BMP4, and 1 μ M RA.

CRITICAL STEP Warm LI-BPEL medium at room temperature. Add the cytokines fresh each time and use the medium immediately. RA is light sensitive, thus use in the dark.

- (ii) Aspirate the cardiac mesoderm induction medium from the well(s) seeded with 2.5×10^4 cells per cm^2 and add epicardial cell induction medium (3 mL per 10-cm^2 growth surface). Incubate cells.

CRITICAL STEP To guarantee the success of the differentiation, cardiac mesoderm induction should be started exactly 72 h after the seeding of hiPSCs. Different cell lines may require different seeding density thus seeding density might require optimization.

- (iii) On day 6 of differentiation, prepare the appropriate volume of epicardial cell specification medium II composed of LI-BPEL medium supplemented with 30 ng/mL BMP4, 1 μM RA. Replace medium on the cells with this medium (3 mL per 10-cm^2 growth surface) and incubate cells.

CRITICAL STEP Warm LI-BPEL medium at room temperature. Add the cytokines fresh each time and use the medium immediately. RA is light sensitive, thus carry out this step in the dark.

- (iv) On day 9 of differentiation, follow step 16 A vii-Xii to dissociate the newly formed epicardial cells (EPI).
- (v) Resuspend EPI cells in epicardial cell maintenance medium composed of LI-BPEL medium supplemented with 10 μM SB431542 and plate the cells on FBN-coated plate at 1.5×10^4 per cm^2 seeding density.

CRITICAL STEP Different cell lines may require different seeding densities, thus seeding density might require further optimization.

- (vi) Check cells regularly. On day 12 of differentiation, EPI cells should be 90–95% confluent. Either cryopreserve (as described in step 16A vii-Xiv) or proceed with cardiac fibroblast differentiation as described in the next step (Fig. 5a).

CRITICAL STEP To evaluate efficiency of epicardial differentiation, perform immunofluorescence for WT1, CX43, TBX18, and ZO-1 epicardial markers (see Box 3 and Fig. 5b).

? TROUBLESHOOTING

PAUSEPOINT Cells can be stored in liquid nitrogen indefinitely.

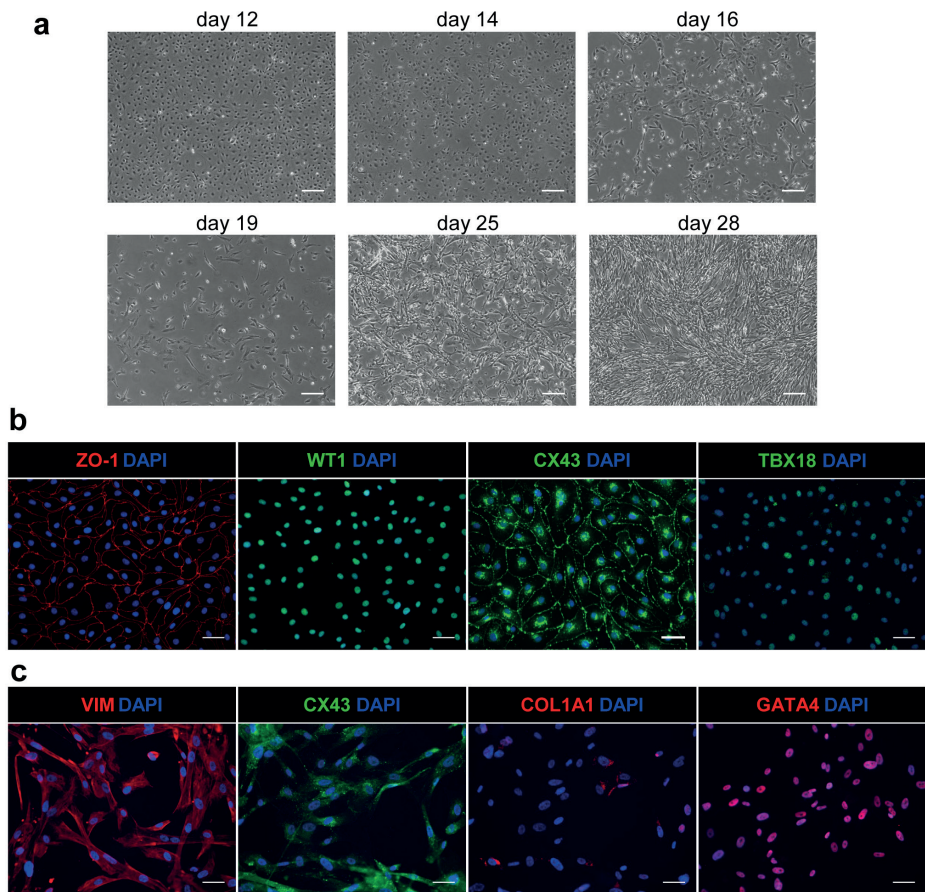


Fig. 5: hiPSC-EPI and hiPSC-CF characterization.
(a) Brightfield images at different time points showing the epithelial mesenchymal transition of hiPSC-EPI (day 12) toward hiPSC-CFs (day 28) during cardiac fibroblast differentiation. Scale bar, 200 μ m. (b) Representative immunofluorescence images of the epicardial markers WT1, TBX18, CX43 (green) and ZO-1 (red) in hiPSC-EPI (day 12). Nuclei are stained with DAPI (blue). Scale bar, 50 μ m. (c) Representative immunofluorescence images of the fibroblast markers VIM, COL1A1, GATA4 (red) and CX43 (green) in hiPSC-CFs (day 28). Nuclei are stained with DAPI (blue). Scale bar, 50 μ m.

(C) Cardiomyocyte differentiation, lactate purification, culture and cryopreservation

TIMING 15-17 d

(i) On day 3 of differentiation, prepare cardiomyocyte specification medium

composed of LI-BPEL medium supplemented with 5 μM XAV 939.

CRITICAL STEP At this stage the cells should have proliferated, formed a confluent monolayer and medium should be yellow. If the monolayer is not confluent, this will negatively affect the efficiency of cardiomyocyte differentiation, resulting in a low percentage of cTnT positive cells in the populations and the formation of non homogenous beating monolayer.

CRITICAL STEP Warm LI-BPEL medium at room temperature. Add the cytokines fresh each time and use the medium immediately.

- (ii) Aspirate the cardiac mesoderm induction medium from the well(s) seeded with 2.5×10^4 cells per cm^2 and add cardiomyocyte specification medium (3 mL per 10-cm^2 growth surface). Incubate cells.

CRITICAL STEP To improve differentiation efficiency and reproducibility, perform this step 72 h after cardiac mesoderm induction. Different cell lines may require different seeding density thus seeding density might require optimization.

- (iii) On day 6 of differentiation, replace the medium with LI-BPEL without supplements (3 mL per 10-cm^2 growth surface).
- (iv) On day 9 of differentiation, replace the medium with fresh LI-BPEL without supplements. Beating CMs should start to appear at day 9-10. Replace medium with fresh LI-BPEL without supplements every 2-3 d until the beating cells appear.
- (v) When the wells show a homogenous beating monolayer (usually at day 10-12 of differentiation), aspirate LI-BPEL medium and replace with lactate purification medium composed of DMEM (no glucose, no pyruvate) supplemented with 4 mM Sodium-L-Lactate. Further incubate cells.
- (vi) Replace medium on beating CMs with fresh lactate purification medium after 48 h (day 12-14 of differentiation).

CRITICAL STEP Metabolic selection of CMs with lactate treatment maximizes CM enrichment (> 90% cTnT positive cells)

- (vii) After an additional 48 h of lactate purification treatment (day 14-16 of differentiation), replace medium with LI-BPEL medium.

CRITICAL STEP Before proceeding to CM dissociation for cryopreservation, it is crucial to let CMs recover from metabolic selection by culturing and re-adapting the CMs to LI-BPEL culture condition for at least 24 h.

- (viii) Check cells. On day 18-20 of differentiation, beating CMs should be ready to be dissociated as detailed in the following steps (Fig. 6a and Supplementary Video 1).
- (ix) Aspirate and discard cell culture medium and wash cells with room temperature PBS^{-/-}.
- (x) Incubate the cells with CM dissociation enzyme mix for 10 min in 37 °C, 5% CO₂ cell culture incubator (1 mL of enzyme mix per 10-cm² growth surface).
- (xi) Neutralize the enzyme mix by diluting with double the amount of LI-BPEL medium per well (2 mL of LI-BPEL per 10-cm² growth surface).
- (xii) Dislodge the cells from the surface of the well(s) and dissociate the CMs by gently pipetting the medium up and down several times using a P-1000 pipette (orifice 1.5 mm). Collect the cell suspension in a 15-mL conical tube.

CRITICAL STEP To avoid too much mechanical stress and damage of the CM cell membranes, stop pipetting as soon as big clusters are no longer visible.

? TROUBLESHOOTING

- (xiii) Rinse each well with an additional 1 mL of LI-BPEL per 10-cm² growth surface to collect residual cells and transfer into the 15-mL tube used in the previous step. Collect a 20-μL aliquot of cell suspension and count cell number.

CRITICAL STEP To check for the extent of the differentiation, also remove an aliquot of cell suspension (approximately 250,000-300,000 cells) and run flow cytometry, checking for the presence of the cardiomyocyte marker cTnT (see Box 4 and Fig. 6b).

- (xiv) Centrifuge the cells at 300g for 3 min at room temperature, aspirate and discard the supernatant. Proceed to the next step for cryopreservation or use freshly dissociated CMs to proceed directly to MT generation (step 20).
- (xv) Resuspend the cell pellet in appropriate volume of CM freezing medium (90% KOSR with 10% DMSO) and transfer 2.5-3.0 x 10⁶ cells/300 μL into a cryovial.
- (xvi) Transfer the cryovial(s) into a Mr. Frosty freezing container and place it at -80 °C. Approximately 24-48 h later, transfer the cryovials in liquid nitrogen tank for longer storage.

? TROUBLESHOOTING

PAUSEPOINT Cells can be stored in liquid nitrogen indefinitely.

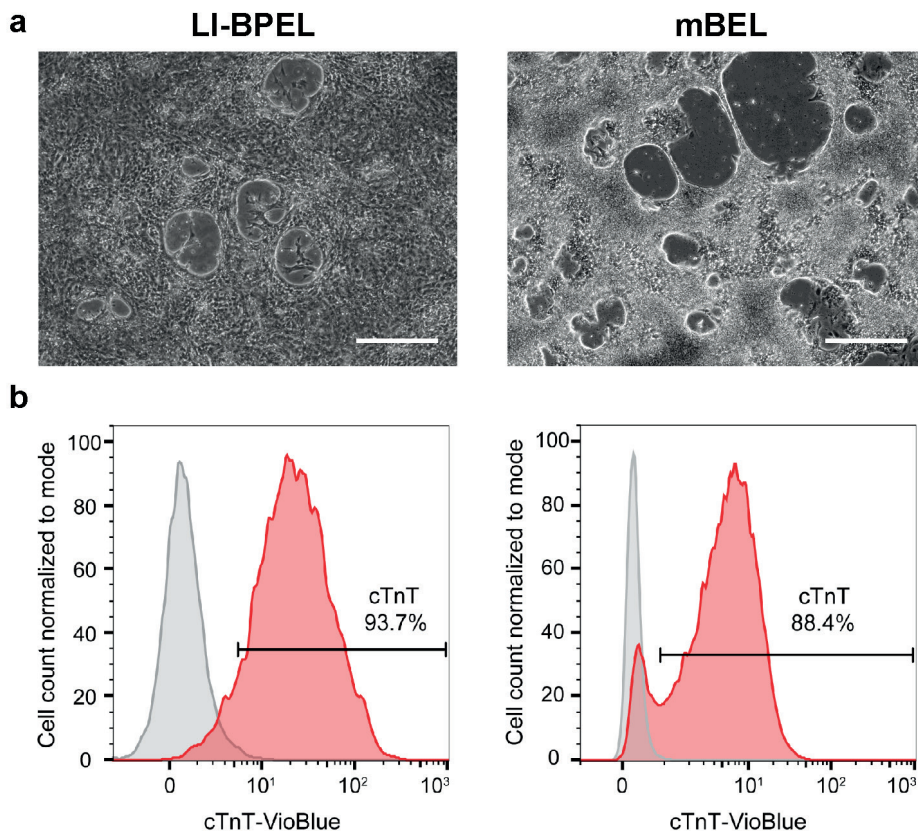


Fig. 6: hiPSC-CM characterization.

(a) Brightfield images of hiPSC-CMs after 21 d of differentiation using LI-BPEL protocol with lactate purification (left panel) or mBEL protocol (right panel). Scale bars, 200 μm . (b) Expression of cardiac Troponin T (cTnT) by hiPSC-CMs. Representative flow cytometry analysis of hiPSC-CMs generated using either the LI-BPEL with lactate purification (left panel) or mBEL (right panel). Histograms show hiPSC-CMs stained with isotype control (LI-BPEL with lactate purification) or unstained (mBEL) (grey), and stained with cTnT (red).

Cardiac fibroblasts differentiation, culture and cryopreservation

17. If differentiating cardiac fibroblasts (CFs) from freshly dissociated EPI cells, follow option A. To differentiate CFs from cryopreserved EPI cells, follow option B.

(A) Cardiac fibroblast differentiation from freshly dissociated EPI cells

TIMING 16 d

- (i) On day 12 of differentiation, dissociate the EPI cells as described in step 16 A vii-Xii . Then resuspend EPI cells in CF induction medium composed of LI-BPEL medium supplemented with 10 ng/mL FGF-2 and plate the cells on a VTN-coated plate at 2.5×10^4 per cm^2 seeding density.

CRITICAL STEP Different cell lines may require different seeding density thus seeding density might require optimization.

- (ii) Incubate the plate overnight in the 37 °C, 5% CO₂ incubator and replace the medium with fresh LI-BPEL supplemented with 10 ng/mL FGF-2 after 24 h.
- (iii) Replace CF induction medium every 2 d for the next 6 d (i.e. until day 19 of differentiation).
- (iv) On day 19 of differentiation, start culturing the newly formed CFs by replacing the medium with Fibroblast Growth Medium 3 (FGM3), to support the expansion of CFs.
- (v) Replace the medium with FGM3 every 2 d for approximately 9-10 d.
- (vi) On day 28-29 of differentiation, CFs should be 90-95% confluent (Fig. 5a). Repeat step 16 A vii-Xii to dissociate CFs.
- (vii) Resuspend CFs in FGM3 medium and plate the cells on VTN-coated plate at a split ratio of 1:2.

CRITICAL STEP The first amplification step should be carried out on a VTN-coated plate. Note that different cell lines may require different seeding ratios.

- (viii) After 3-4 d, CFs should be confluent and can be amplified and adapted to grow on (uncoated) tissue culture plates by repeating step 16 A vii-Xii and plating in FGM3 medium at a split ratio of 1:2.

CRITICAL STEP After this passage (passage 2), CFs can be further amplified, or cryopreserved (as described in step 16A vii-Xiv) or immediately expanded for MT production (by proceeding with step 18Cii).

CRITICAL STEP To evaluate efficiency of cardiac fibroblast differentiation, perform IF for COL1A1, Vimentin, GATA4, and CX43 (see Box 3 and Fig. 5c).

? TROUBLESHOOTING

- (B) Cardiac fibroblast differentiation from cryopreserved EPI cells

TIMING 10 min for thawing plus 3-4 d for expansion of EPI cells, and 16 d for CF differentiation

- (i) To thaw EPI cells, prepare a 15-mL conical tube filled with 5 mL of LI-BPEL medium, previously equilibrated at room temperature.
- (ii) Remove the cryovial of frozen EPI cells from liquid nitrogen storage tank and transfer it on dry ice or in a portable liquid nitrogen container to the cell culture room.
- (iii) Immerse the cryovial in a 37 °C water bath and swirl it gently, until only an ice crystal remains.

CRITICAL STEP To prevent contamination, do not submerge the cap of the cryovial.

- (iv) Remove the cryovial from the water bath, spray with 70% ethanol and place it into the biological safety cabinet.
- (v) Gently transfer the content of the cryovial into the 15-mL conical tube containing the LI-BPEL medium. Rinse the vial with additional 1 mL of LI-BPEL medium to recover any residual cells and add it into the 15-mL conical tube.
- (vi) Centrifuge the cells at 300g for 3 min at RT, aspirate and discard the supernatant.
- (vii) Resuspend the cell pellet in EPI medium composed of LI-BPEL medium supplemented with 10 μ M SB431542 and re-plate $2.5\text{--}3.0 \times 10^4$ cells per cm^2 on a FBN-coated plate.
- (viii) Place the cells gently into a 37 °C, 5% CO_2 cell culture incubator and replace the medium (2 mL per 10- cm^2 growth surface) after 24 h with fresh LI-BPEL medium supplemented with 10 μ M SB431542. After 3 d, the cells should have reached approximately 85-90% of confluency. Proceed with step 17Ai-viii to dissociate and differentiate to CF.

? TROUBLESHOOTING

Box 1: Alternative protocol for cardiomyocyte differentiation using small molecules and cryopreservation **TIMING 21 d**

This procedure differentiates cardiomyocytes from hiPSCs without requiring growth factors (BMP4 and Activin-A) or a lactate purification step, thereby reducing the cost of generating the hiPSC-CMs. It can thus be used in place of steps 11-15 and 16C, however note that steps 11-15 are still required as part of the process to differentiate cells to CFs and ECs.

ADDITIONAL MATERIALS REQUIRED

- Trace elements B (Corning, cat. no. 25022CI)
- Trace elements C (Corning, cat. no. 25023CI)
- IWP-L6 (AbMole, cat. no. M2781)

ADDITIONAL REAGENT SETUP

IWP-L6 stock solution

Dissolve IWP-L6 at 25 mM stock solution in DMSO. From this stock solution, dilute further to 0.25 mM in DMSO. Prepare 10-μL aliquots and store at -20 °C.

CRITICAL Thaw IWP-L6 aliquots at room temperature. Before use, briefly centrifuge to collect solution at the bottom of tube. After thawing, store the aliquot up to 10 d at 4 °C.

3-step Modified LI-BPEL (mBEL) medium preparation

To prepare 100 mL of each of the 3-step basal mBEL medium, combine all the components/supplements listed in the tables below. Filter the medium using a 0.22-μm filter system and store the medium at 4 °C for up to 1 month.

Cardiomyocyte Induction Medium (Day 0-4) – mBEL minus insulin and essential lipids:

Component	Quantity for 100 mL
IMDM	47.25 mL
F12	47.25 mL
BSA (10% wt/vol in IMDM)	2.5 mL
α-Monothioglycerol (13 μL in 1 mL IMDM)	0.3 mL
L-ascorbic acid 2-phosphate (5 mg/mL in distilled water)	1 mL
Glutamax	1 mL
Trace elements B	0.01 mL
Trace elements C	0.1 mL
Penicillin-streptomycin	0.5 mL

Cardiomyocyte Specification Medium (Day 4-onwards) – mBEL minus essential lipids:

Component	Quantity for 100 mL
IMDM	47.25 mL
F12	47.25 mL
BSA (10% wt/vol in IMDM)	2.5 mL
Insulin-Transferrin-Selenium-Ethanolamine (ITS-X) 100X	0.1 mL
α -Monothioglycerol (13 μ L in 1 mL IMDM)	0.3mL
L-ascorbic acid 2-phosphate (5 mg/mL in distilled water)	1 mL
Glutamax	1 mL
Trace elements B	0.01 mL
Trace elements C	0.1 mL
Penicillin-streptomycin	0.5 mL

Cardiomyocyte Maintenance Medium (after thawing and replating) – mBEL:

Component	Quantity for 100 mL
IMDM	47.25 mL
F12	47.25 mL
BSA (10% wt/vol in IMDM)	2.5 mL
Insulin-Transferrin-Selenium-Ethanolamine (ITS-X) 100X	0.1 mL
Chemically Defined Lipid Concentrate (CDLC)	1 mL
α -Monothioglycerol (13 μ L in 1 mL IMDM)	0.3mL
L-ascorbic acid 2-phosphate (5 mg/mL in distilled water)	1 mL
Glutamax	1 mL
Trace elements B	0.01 mL
Trace elements C	0.1 mL
Penicillin-streptomycin	0.5 mL

PROCEDURE

- Seed $4.3\text{--}5.7 \times 10^4$ hiPS cells per cm^2 on Matrigel-coated plates (i.e. 150,000-200,000 cells per well of a 12-well plate) in StemFlex or E8 medium supplemented with RevitaCell (1:200). Incubate the plate overnight in a humidified incubator at 37 °C and 5% CO_2 . This is day -1 of differentiation

CRITICAL STEP The differentiation efficiency is affected by the confluency of the undifferentiated hiPSCs when the differentiation is initiated. It is crucial to identify the optimal seeding density for individual hiPSC lines to maximise the purity and total number of CMs acquired.

- After 24 h (day 0 of differentiation), remove the StemFlex or E8 medium and add cardiomyocyte induction medium supplemented with 5 μM CHIR 99021 (1 mL per well of a 12-well plate).

CRITICAL STEP To improve differentiation efficiency and reproducibility, the differentiation should be started exactly 24 h after seeding the hiPSCs. Do not change the medium until day 2 of differentiation.

3. On day 2 of differentiation, replace the medium with cardiomyocyte induction medium supplemented with 5 μ M XAV 939 and 0.25 μ M IWP-L6 (1 mL per well of a 12-well plate).

CRITICAL STEP To improve differentiation efficiency and reproducibility, perform this step exactly 48 h after the last medium replacement.

4. On day 4 of differentiation, replace the medium with cardiomyocyte specification medium (1 mL per well of a 12-well plate).
5. Completely replace the medium 2 d later, and then every 2-3 d thereafter. Spontaneously beating CMs should be apparent between day 8-10 of differentiation.
6. On day 21 of differentiation, the cells can be dissociated (Fig. 6a and Supplementary Video 2). First, aspirate the culture medium and wash the cells with room temperature PBS^{-/-}.
7. Incubate the cells with 5X TrypLE Select for 10 min in a humidified incubator at 37°C and 5% CO₂ (0.5 mL of 5X TrypLE Select per well of a 12-well plate).
8. Detach the cells from the tissue culture plate by gently pipetting the medium up and down using a P-1000 pipette (orifice 1.5 mm). Collect the cells in a 15-mL conical tube containing 3X volume of cardiomyocyte specification medium.

CRITICAL STEP Generate a single cell suspension of CMs by very gentle pipetting to avoid damaging the CMs.

9. Wash the tissue culture plate with cardiomyocyte specification medium (1 mL per well of a 12 well plate) to collect residual cells and transfer to the same 15-mL tube. Collect an aliquot to count the cells.

CRITICAL STEP Remove an aliquot of the cell suspension (approximately 200,000-300,000 cells) to evaluate by flow cytometry the purity of CM population based on cTnT expression (see Box 4 and Fig. 6b). The differentiated cells should be >80% cTnT⁺.

10. If you wish to cryopreserve cells, follow steps described in step 16C Xiv-Xv. Otherwise cells are ready to be used in step 20.

? TROUBLESHOOTING

Thawing and re-plating the three cardiac cell types (ECs, CFs and CMs) for cardiac microtissue generation

18. Thaw (if necessary) CMs (cytokine/growth factor and small molecule protocols), thaw (if necessary) and expand ECs and CFs as described in options A, B and C, respectively. Option A needs

to be started 5 days before step 19, while options B and C need to be started 3 days before step 19. If cells are already thawed, start at steps Avii, Biii and Cii respectively.

(A) Thawing and re-plating of cardiomyocytes (CMs)

TIMING 10 min plus 5 d for cell recovery before cardiac microtissue generation

- (i) 5 d before MT production, remove a cryovial of CMs from the liquid nitrogen storage tank and transfer to a biological safety cabinet on dry ice or in a portable liquid nitrogen container.
- (ii) Immerse the cryovial in a 37 °C water bath or into a 37°C, 5% CO₂ cell culture incubator, holding the tube stationary, without swirling it, until only a 2 mm³ ice crystal remains.

CRITICAL STEP To prevent contamination, do not submerge the cap of the cryovial. It is recommended to use a floating microcentrifuge tube rack.

- (iii) Immediately remove the cryovial from the water bath, spray with 70% ethanol and place it into the biological safety cabinet.
- (iv) Gently transfer the contents of the cryovial (2.5-3.0 x 10⁶ cells in 300 µL of CM freezing medium) to a 15-mL conical tube using a P1000 pipette (orifice 1.5 mm). Avoid pipetting the cell suspension up and down.
- (v) Rinse the empty cryovial with 1 mL of LI-BPEL medium or cardiomyocyte maintenance mBEL medium, previously equilibrated at RT. Using a P1000 pipette, add the medium drop-wise to the 15-ml tube containing the cells while gently swirling the tube for 1-5 seconds after the addition of each drop.

CRITICAL STEP The drop-wise addition of the medium facilitates suitable mixing of the cells thus minimizing osmotic shock and increasing CM recovery and viability.

- (vi) Slowly add an additional 4.7 mL of medium (LI-BPEL or mBEL medium) to the 15-ml tube containing the cell suspension using a 5-ml pipette. Collect an aliquot of cell suspension for cell count.
- (vii) Centrifuge the cells at 300g for 3 min at room temperature and discard the supernatant.
- (viii) Gently resuspend the cell pellet from the previous step, Box 1 or step 16Cxiv in the appropriate volume of the original culture medium (LI-BPEL or mBEL medium) and plate 1.0-4.0 x 10⁵ cells per cm² seeding density on Matrigel-coated plate.

- (ix) Place the cells gently into a humidified incubator at 37°C, and 5% CO₂. Refresh the medium after 24 h. The CMs should start spontaneously beating 24-48 h post thawing.
- (x) Refresh with the appropriate medium every 2-3 d. After 5 d, CMs should be ready for use in step 19.

CRITICAL STEP Expected survival after thawing is approximately 30-50% of the initial number of cells cryopreserved. Before MT production, it is crucial to wait at least 5 d to ensure complete recovery of the CMs from the thawing procedure.

? TROUBLESHOOTING

(B) Thawing and expansion of endothelial cells (ECs)

TIMING 10 min plus 3 d for cell expansion before cardiac microtissue generation

- (i) 3 d before you plan to produce cardiac microtissues (MTs), prepare a 15-mL conical tube filled with 5 mL of LI-BPEL medium, previously equilibrated at room temperature.
- (ii) Thaw ECs (from step 16Axiiv) as described in step 17B(ii-vi).
- (iii) Resuspend the cell pellet (from previous step or step 16Axiiv) in EC medium composed of LI-BPEL medium supplemented with 50 ng/ml VEGF and re-plate $2.5\text{--}3.0 \times 10^4$ cells per cm² on FBN-coated plate.
- (iv) Place the cells gently into a 37 °C, 5% CO₂ cell culture incubator and replace the medium (2 mL per 10-cm² growth surface) after 24 h with fresh LI-BPEL medium supplemented with 50 ng/mL VEGF.
- (v) After 3 d, the cells should reach approximately 85-90% of confluency and are ready for use in step 19.

CRITICAL STEP Expected survival after thawing should be > 80%. For MT production, it is crucial to let ECs recover from thawing for at least 3 d, being careful that cells do not become over confluent and detach.

(C) Thawing and expansion of cardiac fibroblasts (CFs)

TIMING 10 min plus 3 d for cell expansion before cardiac microtissue generation

- (i) Thaw CFs by following steps 17B(ii-vi)..
- (ii) Resuspend the cell pellet from previous step or step 17Aviii in FGM3 medium and re-plate $25\text{--}30 \times 10^3$ cells per cm² on (uncoated) plastic plates tissue

culture treated.

- (iii) Place the cells gently into a 37°C, 5% CO₂ cell culture incubator and replace the medium (2 ml per 10-cm² growth surface) after 24 h with fresh FGM3. After 3 d, the cells should reach approximately 85-90% of confluency and are ready for use in step 19.

CRITICAL STEP Expected survival after thawing should be > 80%. Before MT production, it is crucial to let CFs recover from thawing for at least 2 days, being careful that cells do not become over confluent and detach.

Generation and culture of 3D cardiac microtissues (MTs) composed of hiPSC-CMs, -ECs and -CFs

TIMING 2h for cardiac microtissue generation plus 21 d for cardiac microtissue culture

19. Dissociate ECs and CFs as described in step 16Avii-xii and dissociate CMs as described in step 16Cix-xiv.

CRITICAL STEP Monitor the results of each cell type dissociation under the microscope to ensure single-cell detachment. Single-cell suspension and absence of cell clumps are crucial for precise cell counting and proper MT production.

20. Resuspend all three cell types in 1 mL of LI-BPEL medium and collect a 20-μL aliquot of each cell type suspension. Count cells using a hemocytometer and microscope.
21. Prepare a 15-mL conical tube and dilute ECs, CFs and CMs in LI-BPEL medium supplemented with 50 ng/mL VEGF and 5ng/mL FGF-2 combining 5,000 cells in the defined ratio of 70% CMs, 15% ECs and 15% CFs (3,500 CMs, 750 ECs and 750 CFs for each MT) per 50 μL of medium.

CRITICAL STEP 50 μL cell suspension will be required per well (cells are later plated in a 96-well V-bottom microplate).

22. Gently resuspend the cell suspension using a 5- or 10-mL sterile plastic serological pipette and transfer to a sterile plastic reservoir.
23. Fill each external well with 100 μL of PBS. Seed 50 μL of cell suspension per internal well (for each MT) of a 96-well V-bottom microplate using a multichannel pipette.

CRITICAL STEP Do not seed MTs in the outermost wells of the 96-well V-bottom microplate to avoid possible medium evaporation. Each 96-well microplate will contain a maximum of 60 MTs.

CRITICAL STEP For initial optimization of the protocol it is recommended to prepare 120

MTs corresponding to two 96-well plates.

CRITICAL STEP Move the plastic reservoir side-to-side after plating each 3 rows of the 96-well microplate to assure maintenance of a homogeneous cell suspension. This is critical to obtain MTs which are similar in size and cellular composition.

24. Centrifuge the microplate(s) at 300g for 10 min at room temperature to facilitate the aggregation of the cell suspension on the bottom of the well(s). This is day 0 of MT formation.
25. Place the microplates gently into a 37°C, 5% CO₂ cell culture incubator. To allow cellular aggregation and proper MT formation, do not move the plates for 3 d.

CRITICAL STEP Proper cell aggregation and subsequent tissue formation is evident as compact and round-shaped MTs observed after 24h (Fig. 7).

26. Every 3 d, refeed MTs by removing 25 µL of old medium and replacing it with 25 µL of fresh LI-BPEL supplemented with 50 ng/ml VEGF and 5ng/ml FGF-2. After 21 d, MTs should be ready for the next step (further characterization and analyses).

? TROUBLESHOOTING

CRITICAL STEP Note that for subsequent video analysis of calcium transients (step 27 option C) cells need to be transferred to a black plate 2 days prior to analysis, i.e. on day 19.

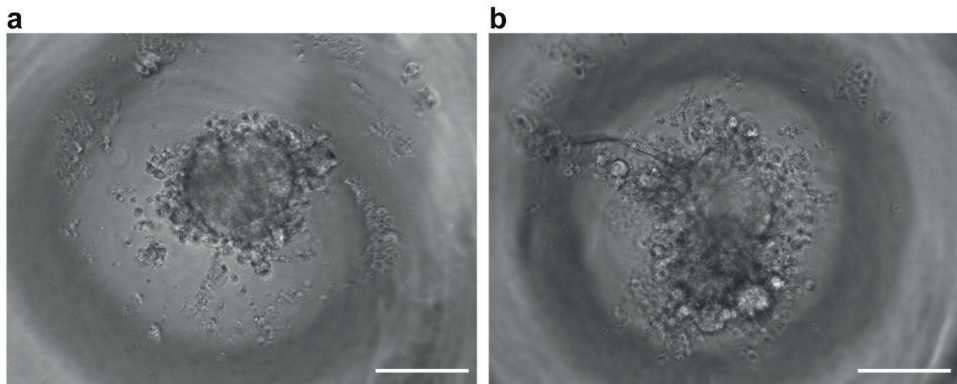


Fig. 7: Examples of optimal and sub-optimal cardiac MTs after 24h after formation.

Representative images of compact and round-shaped MT (a) and not properly formed MT (b). Scale bar 200 µm.

Assays that can be undertaken on 3D cardiac microtissues

27. After 21 d in culture from initial aggregation, 3D whole MTs can be used for various assays. Option A describes how to undertake structural analysis by immunofluorescence staining. Option B describes video analysis to assess contraction. Option C describes video analysis of intracellular calcium transients. Option D describes how to use the Seahorse XFe96 assay to assess metabolic functionality. Option E describes how to assess electrical properties by sharp electrode electrophysiology. Option F describes how to dissociate MTs into single cells and then undertake scRNAseq or electrophysiology or immunostaining of single cells.

(A) Immunofluorescence staining

TIMING 3 d

- (i) Collect at least 30 MTs and transfer them to a 15-mL conical tube using a P-1000 pipet. Wait for 3-5 min until all MTs sediment at the bottom of the tube.
- (ii) Carefully aspirate the supernatant using a 1-mL pipet and wash MTs with 1 mL of PBS^{+/+} to completely remove residual culture medium. Transfer PBS solution containing MTs to a 1.5-mL tube and wait for 3-5 min until all MTs sediment at the bottom of the tube.

CRITICAL STEP Avoid pipetting MTs up and down in order to preserve the integrity of microtissues.

- (iii) Carefully aspirate the supernatant using a 1-mL pipet and fix MTs with 100 μ L of 4% (vol/vol) PFA solution for 1 h at 4 °C.
- (iv) Aspirate and discard the fixative solution without disturbing MTs on the bottom of the tube and wash three times (5 min each) with 100 μ L of PBS^{+/+} to completely remove residual fixative solution.

CRITICAL STEP Avoid pipetting MTs up and down in order to preserve the integrity of microtissues.

PAUSEPOINT Fixed MTs can be stored in 1 mL of PBS^{+/+} at 4 °C up to 2-3 weeks before proceeding with immunostaining protocol.

- (v) Carefully aspirate the supernatant using a 1-mL pipet and permeabilize MTs with 100 μ L of permeabilization solution 0.2% (vol/vol) Triton X-100 for 30 min at room temperature.

CRITICAL STEP This and the following steps that describe how to permeabilize and immunostain with primary and secondary antibodies are the same as those previously described^{15,65}.

- (vi) Aspirate and discard the permeabilization solution without disturbing MTs on the bottom of the tube and wash three times (5 min each) with 100 μ L of PBS^{+/+} to completely remove residual permeabilization solution.
- (vii) Block to minimize non-specific binding of the primary antibodies by adding 100 μ L of blocking solution composed of PBS^{+/+} containing 10% (vol/vol) FBS for 2-3 h at room temperature.
- (viii) Stain MTs with 60 μ L of blocking solution containing primary antibodies: α -Sarcomeric Actinin (ACTN2), Troponin I (TNNI) for CMs; CD31 for ECs; Collagen Type I (COL1A1) for CFs (see recommended dilution in 'Materials' section) and incubate them overnight at 4 °C.
- (ix) The following day, wash three times with 100 μ L of PBS^{+/+}, each time for 20 min, waiting for microtissues to sediment on the bottom of the tube.
- (x) Stain MTs with 60 μ L of blocking solution containing secondary antibodies (see dilution in "Materials" section) and incubate them overnight at 4 °C in the dark.
- (xi) The following day, wash three times with 100 μ L of PBS^{+/+} in the dark, each time for 20 min, waiting for microtissues to sediment on the bottom of the tube.
- (xii) Stain with 60 μ L of DAPI at 1:500 dilution in PBS^{+/+} for 30 min at room temperature in the dark.
- (xiii) At the end of staining procedure, carefully aspirate the supernatant using a 200- μ L pipet to completely remove residual DAPI solution.
- (xiv) Resuspend no more than 25-30 MTs in 10 μ L of liquid mountant, transfer MTs on microscope slides and mount them on top of 12-mm round, glass coverslips, avoiding bubbles and making sure MTs are well distributed throughout the coverslip to avoid MT overlap. After completion of the immunostaining protocol, proceed with the image acquisition of whole 3D MTs mounted on microscope slides on the top of glass coverslips using a confocal laser-scanning microscope.

CRITICAL STEP Before starting with image acquisition, allow the slides to dry at least 24 h at room temperature in the dark.

- (xv) In order to evaluate the architecture and cell composition of whole 3D MTs, acquire images using either a 20× or 25× objective magnification and Z-stack acquisition. MTs should stain positive for cardiac marker TNNI, endothelial marker CD31 and fibroblast marker COL1A1.
- (xvi) In order to perform structural analysis of sarcomeres (length and organization), acquire images of 3D whole 3D MTs using 100× objective magnification and Z-stack acquisition. MTs stain positive for cardiac markers TNNI and ACTN2.
- (xvii) Determine sarcomere length by measuring the average distance between Z-bands (stained with ACTN2) using standard analysis plugin in open-source Fiji Software, ImageJ.
- (xviii) Evaluate sarcomere organization as sarcomere alignment index based on the regularity and periodicity of ACTN2 positive Z-bands (stained with ACTN2) using a standard fast Fourier transform (FFT) analysis (Fiji Software, ImageJ) as previously described^{76,77}.

? TROUBLESHOOTING

(B) Video analysis of contraction

TIMING 2-3 min per cardiac microtissue

CRITICAL It is advisable to avoid re-plating of MTs to reduce possible variation of contraction parameters due to potential attachment of MTs on flat bottom plates, thus analyse MTs in the V-shaped culture microplate in which they were generated.

- (i) Record movies of MTs spontaneously beating or paced at 1, 2 or 3 Hz (20 V, 3 ms) using custom-made electrodes fitting the V-shaped 96-wells (Supplementary Figure 3) for at least 20 s at video acquisition speed of 100 frames per s (f.p.s.), using 10× objective phase containing the entire tissue within the field of view.

CRITICAL STEP To adequately perform contraction video analysis, use a high-speed recording camera for the movie acquisition. Avoid variations of the lighting conditions during measurements.

CRITICAL STEP Record videos for at least 20 s in order to capture multiple contraction cycles within each video and possibly identify beating abnormalities.

- (ii) Perform contraction video analysis using the MUSCLEMOTION ImageJ macro as described previously⁶¹, including in a detailed protocol^{61,78}.

(C) Video analysis of calcium transients

TIMING 1 h for dye incubation and 2-3 min for analysis of single cardiac microtissue

CRITICAL For analysis on day 21, MTs need to be picked 18-19d following initial MT formation (by aspirating the content of each well with a P1000 pipette) and seeded on Matrigel-coated 96-well black plates. It is important to seed no more than 3-4 MTs per well to ensure homogeneous distribution within the surface of the well, avoiding direct contact between different MTs.

- (i) On day 21 (48-72 h after re-plating) aspirate LI-BPEL medium from the well(s) and add 50 μ L of 5 μ M Fluo-4 AM loading solution to each well containing MTs. Place in a 37 °C, 5% CO₂ cell culture incubator for 30 min.
- (ii) Remove Fluo-4 AM loading solution using P200 pipet and replace with LI-BPEL medium. Incubate loaded MTs for an additional 30 min into a 37 °C, 5% CO₂ cell culture incubator, before proceeding to the next step for recordings.

CRITICAL STEP Carefully aspirate Fluo-4 AM loading solution to remove any dye nonspecifically associated with the surface of MTs. A further 30 min incubation before recordings is crucial to allow complete de-esterification of intracellular AM esters.

- (iii) Transfer the plate with loaded MTs under a fluorescence microscope (with proper filters for excitation at 494 nm and emission at 516 nm) equipped with an environmental chamber to perform measurements at 37°C and 5% CO₂.
- (iv) Acquire calcium transients of MT paced at 1.5 Hz, 20 V, 3 ms by recording videos for at least 20 s at video acquisition speed of at least 56 f.p.s., using 10x objective phase containing the entire tissue within the field of view.

CRITICAL STEP Record videos for at least 20 s in order to capture multiple calcium transient cycles within each video and possibly identify calcium abnormalities.

CRITICAL STEP The use of opaque black-walled 96-well plates with clear bottom is crucial for assays using fluorescent dye as Fluo-4 AM to reduce fluorescent signal crosstalk and background.

? TROUBLESHOOTING

(D) Metabolic functionality of whole MTs using Seahorse XFe96

TIMING 6 h for plating MTs plus 3 h for measurements

CRITICAL The Seahorse XFe96 sensor cartridge must be hydrated one day prior to the assay. To do this, open the Seahorse Flux assay kit in a sterile biosafety cabinet. Take the sensor cartridge off the utility plate and place it upside down, sensors facing up. Add 200 μL of ddH₂O to all the wells of the utility plate and place the sensor cartridge back into the utility plate. Additionally, take 25 mL of seahorse calibration medium and place in a 50 mL falcon tube. Place both the plate and the tube in a 0% CO₂ incubator overnight.

CRITICAL STEP Make sure the incubator is set to 0% CO₂ and that it is properly humidified.

CRITICAL STEP Make sure the Probes of the sensor cartridge do not touch any surface as they are sensitive to damage

CRITICAL Two separate assays are described: a Mitochondrial stress test and measurement of glycolytic capacity. Both can be performed on the same plate (if preferred) by running different assays in different wells because they have the same injection timing scheme (3 injections total at the same time points).

- (i) On the day of the assay, take both the Seahorse calibration medium and the Seahorse XFe96 sensor cartridge out of the incubator. Again, take off the sensor cartridge, place it upside down, and aspirate the ddH₂O from all wells. Subsequently, add 200 μL of Seahorse Calibration medium to all the wells. Leave for at least one hour.

CRITICAL STEP Ensure that all the wells are filled, including those that are not used during the assay.

CRITICAL STEP Allow at least 1 h of incubation in the calibration fluid. The cartridge should be used on the day it is placed in the calibration fluid.

- (ii) Prepare the Seahorse assay media. (This can also be prepared the day before the assay).
- (iii) Using a P1000 carefully take 20 MTs for the mitochondrial stress test plus 20 additional MTs for the glycolytic capacity test and place them in separate 15 mL Falcon tubes.

CRITICAL STEP Each MT measurement condition should consist of 4 technical repeats each containing a total of 4 MTs. Therefore, a total of 16 MTs per measurement condition is required.

However, MTs can be lost or damaged during transfer and plating. We therefore recommend taking 20 MTs per condition.

- (iv) Let the MTs sediment for 30 s and aspirate the supernatant without disturbing the MT pellet
- (v) Wash the pellets intended for the mitochondrial stress test with Wash+ medium and the glycolytic capacity pellets with Wash- medium 3 times, allowing at least 30 s after medium addition for the MTs to settle.

CRITICAL STEP BSA must be included in the wash medium, in its absence, the MTs will stick to both the surface of the Falcon tube and the pipette tips.

- (vi) Place 2 ml of wash media in each well that will be required of a 6-well ultra-low attachment plate (1 well is required per condition being tested).
- (vii) After the last wash, resuspend the MTs in 750 μ L of their wash medium and take up all the MTs with a P1000 and transfer them to a pre-filled well in the ultra-low attachment plate.

CRITICAL STEP Ensure that MTs are added underneath the surface of the liquid as they tend to float on top of the medium.

CRITICAL STEP The medium in the wells must contain BSA otherwise the MTs will stick to the surface of the well and pipette tips.

- (viii) Take the coated Seahorse tissue culture plate and aspirate the Matrigel. Add 160 μ L of preheated SH+ or SH- medium to all wells that will be used for the assay and keep the plate in the biosafety cabinet.

CRITICAL STEP Bear in mind that at least 4 control wells are required. These must be filled with the medium but do not have MTs added in the next step. These wells serve as blank measurements for correction of the data.

CRITICAL STEP Although all wells can be used in the assay, using the centre 60 wells is recommended as these provide the most reliable data due to there being less evaporation from these wells compared to wells at the edge.

- (ix) Place the plate in a stereomicroscope and using a P20 pipette pick 4 MTs in a single 20 μ L volume and place them in the centre of a Seahorse tissue culture well. Repeat until all desired wells are filled.
- (x) Take a Glass Pasteur pipette and pull the tip by holding it in a gas flame to create a sealed blunt tip. Subsequently, bend the thin part of the pipette in a 90° angle in the gas flame (Fig. 8a).

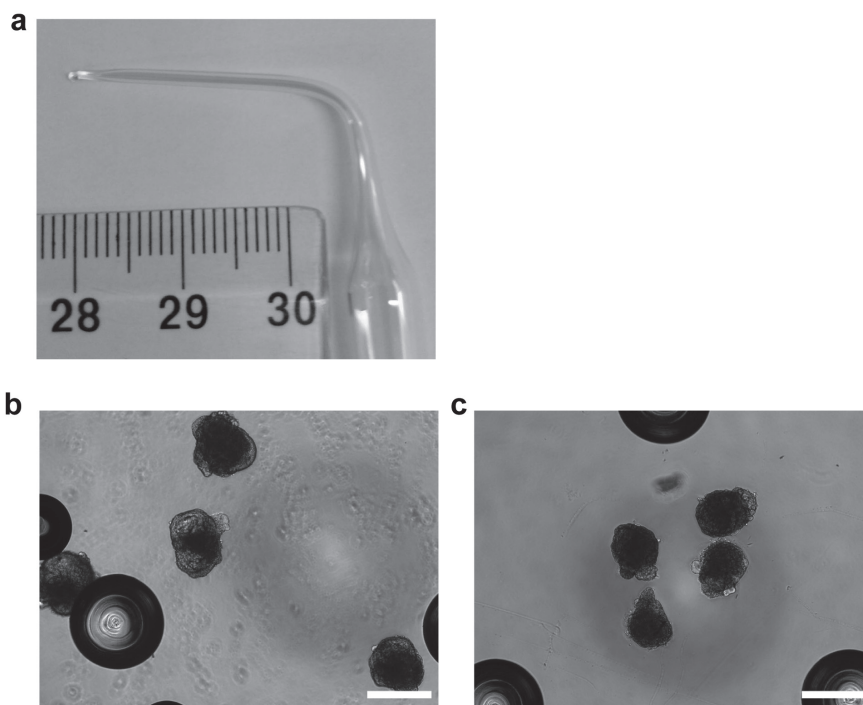


Fig. 8: Overview of correct Seahorse plating conditions.

(a) Bent and stubbed Pasteur pipette used for MT positioning. Scale in centimetres. (b) MTs directly after plating at an incorrect position in the plate. Scale bar, 400 μm . (c) Correct positioning of MTs in the middle of the Seahorse well. Scale bar, 400 μm .

- (xi) Under a stereomicroscope, use the blunt Pasteur pipette to position the four MTs carefully in the centre of the well without the MTs touching each other (Fig. 8b, c).
- (xii) Carefully place the Seahorse tissue culture plate in a 0% CO_2 incubator for 4 h to allow the MTs to lightly attach to the plate

CRITICAL STEP Do not allow the MTs to attach overnight. Leaving them longer results in the CFs migrating out of the MT and attaching to the bottom of the plate, changing the MT composition.

- (xiii) Thaw all the prepared drug stocks (oligomycin, antimycin A, rotenone) at room temperature. Fill all ports that will not be used for drug injection in the next steps with equal amounts of liquid to ensure proper

ejection of the compounds during the assay.

CRITICAL STEP Drug concentrations might require optimisation for use with different cell lines. Due to the 3D nature of the tissues, high concentrations are usually required compared to those used for cells growing in 2D culture.

- (xiv) For the mitochondrial stress test, load the desired cartridge drug injection ports using a multichannel pipette and Mettler Toledo pipette tips as follows: add 41 μL of the oligomycin stock to 3 mL of SH+ medium yielding a final concentration of 35 μM and load 20 μL into port A. Put 20 μL FCCP in 1.5 mL of SH+ medium at a final concentration of 40 μM and load 22 μL in port B. Finally add 30 μL of both antimycin A and rotenone to 1.5 mL of SH+ medium, final concentration 2 μM , and load 25 μL into port C.
- (xv) To test glycolytic capacity, load the cartridge drug injection ports using a multichannel pipette and Mettler Toledo pipette tips as follows: dilute 225 μL of the glucose stock in 1275 μL of SH- medium and load 20 μL in port A. Port B will be loaded with 22 μL of oligomycin, which was already prepared in the previous step. Finally, dilute 750 μL of 2-DG stock in 750 μL of SH- medium and put 25 μL in port C.
- (xvi) Run the Seahorse according to Seahorse manual and using a 3-injection scheme. Set 4 measurements in the base measurements and 6 measurements for all subsequent injections.

CRITICAL STEP Longer measurement periods are necessary after each injection than those used for 2D cell experiments to allow for proper diffusion of the drugs into the 3D tissues. The above recommendations are based on oligomycin addition. We recommended measuring until the curve has flattened and the OCR no longer decreases compared to the previous measuring point.

- (xvii) Normalize the data per well based on either protein measurement, using Bradford or BCA, or DNA content, using Picogreen, or any other preferred normalisation method.

PAUSEPOINT After the addition of the lysis buffer the plate can be kept at -20°C for several weeks before performing the normalisation assay.

? TROUBLESHOOTING

(E) Sharp electrode recordings**TIMING** 10-15 min for recording of single cardiac microtissue

- (i) On day 21 after the initial MT formation, collect MTs as in option A (i) and transfer them to Matrigel-coated plastic coverslips.
- (ii) 48-72 h after re-plating MTs, check they are attached to the plate and ready to be recorded.
- (iii) Immediately before recording, cut MTs into 2-3 smaller pieces using a syringe needle.
- (iv) Place the coverslip onto the patch clamp setup and perfuse with Tyrode solution at a controlled temperature of 37 °C.
- (v) Prepare sharp electrodes with a final resistance of 80-100 MOhM from glass capillaries using a micropipette puller.
- (vi) Gently impale a single MT piece using sharp electrode and penetrate it using the zap function of the amplifier.
- (vii) Start recording action potentials of MT spontaneous activity.

(F) Dissociation of cardiac microtissues into single-cells**TIMING** 2-3 h

CRITICAL The efficient dissociation of MTs at single-cell level is crucial for several downstream characterizations such as single-cell RNA sequencing, single-cell electrophysiology analysis, or single-cell immunostaining.

- (i) Collect MTs and transfer them to a 15-mL conical tube using a P-1000 pipet. Wait for 3-5 min until all MTs sediment at the bottom of the tube.
CRITICAL STEP The number of MTs to use for dissociation is strongly dependent on downstream application and needs to be optimized.
- (ii) Carefully aspirate and discard the supernatant using a P-1000 pipet (orifice 1.5 mm) and wash MTs with 0.5 mL of HBSS buffer to completely remove residual culture medium.
- (iii) Transfer HBSS containing MTs to a 1.5-mL tube. Wash the original 15-mL tube with 0.5 mL HBSS and add it to the same 1.5-mL tube. Wait until all MTs sediment at the bottom of the tube.
- (iv) Carefully aspirate and discard the HBSS solution using a P-1000 pipet and add 300 µL of collagenase II enzymatic solution (290U/mg) pre-warmed at 37 °C.

- (v) Incubate the 1.5-mL tube for 20 min at 37 °C, under constant shaking at 450 rpm in a heating block.
- (vi) Let MTs sediment on the bottom of the tube, transfer the supernatant to a 15-mL tube containing 4 mL of LI-BPEL medium supplemented with 10% FBS.
- (vii) Add 300 μ L fresh collagenase II enzymatic solution pre-warmed at 37 °C to MTs in the 1.5-mL tube and incubate for 10 min at 37 °C, under constant shaking at 450 rpm in a heating block.
- (viii) Gently pipette the dissociated cells 3 times up and down using P-1000 pipet and let undissociated MTs sediment, before transferring the supernatant into the same 15-mL tube as that used in step ii.
- (ix) Repeat steps vii and viii an additional 3-8 times, until MTs are completely dissociated.

CRITICAL STEP The number of dissociation cycles required for single cell dissociation may vary for each experiment. Check if aggregates are still visible before starting a new dissociation cycle. Avoid over-digestion of MTs to recover as many single cells as possible.

- (x) Collect a 20- μ L aliquot of cell suspension and count. Centrifuge the remaining cells at 300g for 3 min at room temperature, aspirate and discard the supernatant.
- (xi) Depending on the downstream application, proceed as follows:
 - For single-cell RNA-sequencing:
 - Prepare a single-cell suspension in LI-BPEL medium following the manufacturer's instructions. For 10x Genomics®, for example, if the total number of input cells required is 1700, then the optimal concentration is in the range of ~200 to 700 cells/ μ L.
 - For single-cell electrophysiology using patch clamp:
 - Resuspend the cell pellet in the appropriate volume of LI-BPEL medium supplemented with 50 ng/mL VEGF and 5ng/mL FGF-2 and plate 20-100 $\times$ 10³ cells on Matrigel-coated glass coverslips placed into 24-well plate in a final volume of 700 μ L.
 - Refresh half of the volume of the medium (350 μ L) 24 h after dissociation with fresh LI-BPEL medium and keep cells in culture for at least 5 d until patch clamp experiment.
 - This delay is needed as cells require time to recover from dissociation. Although cells may appear recovered after 1-2 d, plasma membranes

result too fragile for patch clamp recordings. Keeping cell in culture for longer than 10 d will lead however to some cell death.

- For single-cell immunostaining: resuspend the cell pellet in the appropriate volume of LI-BPEL medium and plate $25\text{--}50 \times 10^3$ cells per cm^2 seeding density on Matrigel-coated glass coverslips. Refresh the cells 24 h after dissociation with fresh LI-BPEL medium. Cells need time to recover from dissociation so keep the cells in culture for at least 3–5 d, before proceeding with immunostaining protocols.

? TROUBLESHOOTING

Box 2 Characterization of ECs by FACS analysis TIMING 1.5 h for sample preparation plus 15 min for sample acquisition

1. Dissociate ECs as described in 'Cardiac endothelial cell differentiation, isolation, culture and cryo-preservation' section (Step 16 Avii-xi)
2. Take an aliquot of cell suspension (approximately 250,000-300,000 cells) and transfer it into a FACS tube.
3. Centrifuge the cells at 300g for 3 min at room temperature, aspirate and discard the supernatant.
4. Resuspend the pellet in 1 mL of FACS buffer and split 500 µL of cell suspension into two FACS filter-cap tubes, passing the cells through the filter-caps.
5. Centrifuge the cells at 300g for 3 min at room temperature, and discard the supernatant.

CRITICAL STEP This additional step of centrifugation in FACS buffer is required to remove the residual culture medium, before proceeding with the staining.

6. Resuspend the cells in one tube with 100 µL of FACS buffer containing 1 µL of directly labelled antibody (Anti-CD31-APC, dilution 1:100). Resuspend the cells in the other tube with 100 µL of FACS buffer containing 1 µL of the correspondent isotype control (IgG1-APC, dilution 1:100).
7. Incubate the cells for 1 h at 4 °C in the dark. After incubation, wash the cells with 500 µL of FACS buffer.
8. Centrifuge the cells at 300g for 3 min at room temperature, aspirate and discard the supernatant.
9. Resuspend the cells in 200 µL of FACS buffer and proceed with flow cytometry analysis, adjusting the gates according to the isotype control, and analyze the data in FlowJo software (Supplementary Figure 1).

Box 3 Characterization of ECs, EPI and CFs by immunofluorescence TIMING 2 d

1. Dissociate ECs, EPI and CFs as described in Step 16 Avii-xii and re-plate the cells on FBN- (for ECs and EPI: 1.0×10^4 cells) or VTN- (for CFs: 1.0×10^4 cells) coated black-walled 96-well plates with clear bottom in LI-BPEL medium supplemented with 50 ng/ml VEGF (for ECs), 10 µM SB431542 (for EPI) and FGM3 medium (for CFs).

CRITICAL STEP The use of opaque black-walled 96-well plates with clear bottom is crucial for immunofluorescence assays to reduce fluorescent signal crosstalk and background among different wells.

2. After 2-3 d in culture, aspirate the medium and wash twice with 50 µL of PBS^{+/+} to completely remove residual culture medium.

3. Fix the cells with 50 μ L of 4% (vol/vol) PFA solution for 15 min at room temperature.
4. Aspirate the fixative solution and wash three times (5 min each) with PBS^{+/+} to completely remove residual fixative solution.

PAUSEPOINT Fixed cells can be stored in 100 μ L of PBS^{+/+} at 4 °C up to 2 -3 weeks, sealing the plate with parafilm, before proceeding with immunostaining.

5. Permeabilize the cells with 50 μ L of permeabilization solution 0.1% (vol/vol) Triton X-100 for 10 min at room temperature.
6. Block to minimize unspecific binding of the primary antibody by adding 50 μ L of blocking solution for 1-2 h at room temperature.
7. Stain with 30 μ L of blocking solution containing primary antibodies: CD31 for ECs; WT1, TBX18, ZO-1 and CX43 for EPI cells; CX43, COL1A1, VIM, GATA4 for CFs (see recommended dilution in ‘Materials’ section).
8. Incubate the plate overnight at 4 °C on a rocking platform, with gentle shaking.
9. The following day, wash three times with 50 μ L of PBS^{+/+}, each time for 10 min on a rocking platform, with gentle shaking.
10. Stain with 30 μ L of blocking solution containing secondary antibodies (see dilution in “Materials” section). Incubate the plate for 1 h at room temperature in the dark on a rocking platform, with gentle shaking.
11. Wash three times with 50 μ L of PBS^{+/+} in the dark, each time for 10 min on a rocking platform, with gentle shaking.
12. Stain with 30 μ L of DAPI at 1:500 dilution in PBS^{+/+} for 10 min at room temperature in the dark on a rocking platform, with gentle shaking.
13. Wash once with 50 μ L of PBS^{+/+} to remove residual DAPI solution.
14. After completion of immunostaining, proceed immediately with image acquisition or store the plates at 4 °C in the dark, sealing the plate with parafilm.

PAUSEPOINT Stained cells can be stored in 100 μ L of PBS^{+/+} at 4 °C for up to 1-2 months, sealing the plate with parafilm, before proceeding with image acquisition.

Box 4 Characterization of cardiomyocytes (CMs) by flow cytometric analysis for the cardiomyocyte marker, cardiac troponin T (cTnT)

1. Dissociate CMs as described in Box 1 steps 6-9 or Step 16 Cix-xiii, take an aliquot of cell suspension (approximately 250,000-300,000 cells) and transfer it into a FACS tube.
2. Centrifuge the cells at 300g for 3 min at room temperature. Discard the supernatant.
3. Resuspend the cell pellet in 500 µL FACS buffer. Filter the cell suspension using a 5 mL tube with a 35 µm cell strainer cap.
4. Pellet the cells at 300g for 3 min at room temperature and discard the supernatant.
CRITICAL STEP Resuspending and pelleting the cells in FACS buffer is important to remove residual culture medium.
5. Resuspend the cells in 50 µL Fixation medium. Briefly vortex to ensure the cells are completely resuspended.
6. Fix the cells for 15 min at room temperature. Flick the tube after ~7.5 min to ensure the cells remain in suspension.
7. Dilute the Fixation medium with 1 mL FACS buffer. Pellet the cells at 300g for 3 min at room temperature. Discard the supernatant.
PAUSEPOINT Fixed cells can be stored in 1 mL of FACS buffer at 4 °C in a sealed tube for up to 7 d. When ready to continue, pellet the cells and proceed with next step.
8. Resuspend the cells in 1 mL of FACS buffer. Divide the cell suspension evenly into 2 tubes, filtering the cells through a 35 µm cell strainer cap.
9. Pellet the cells at 300g for 3 min at room temperature and discard the supernatant.
10. Resuspend the cells in one tube with 50 µL of Permeabilization medium containing 5 µL of directly labelled antibody (Anti-cTnT-VioBlue, dilution 1:50. Resuspend the cells in the other tube with 50 µL of Permeabilization medium containing 5 µL of the correspondent isotype control (REA Control (I)- VioBlue, dilution 1:50).
11. Briefly vortex to ensure the pellet is properly resuspended and incubate the cells for 20 min at room temperature in the dark.
12. After incubation, wash the cells with 1 mL of FACS buffer.
13. Pellet the cells at 300g for 3 min at room temperature, aspirate and discard the supernatant.
14. Resuspend the cells in 200-500 µL of FACS buffer and run the samples on a flow cytometer. Analyze the data using FlowJo software, adjusting the gates according to the isotype control (Supplementary Figure 2).

Table 4. Troubleshooting advice

Step	Problem	Possible reason	Solution
9: hiPSC thawing	Poor attachment and survival of hiPSCs	hiPSC medium was not supplemented with RevitaCell Imperfect coating	Add RevitaCell to hiPSC medium before plating the cells Passage hiPSCs to properly coated new plates
9: hiPSC expansion	Spontaneous differentiation of hiPSCs hiPSCs are proliferating very slowly	Plating density was not optimal (cells too sparse or too dense) hiPSCs should be passaged several times to completely recover from thawing Mycoplasma contamination Karyotype rearrangements	Adjust passage ratio/plating density Passage hiPSCs more Test for Mycoplasma Perform karyotyping analysis
10A(viii) and 10B(x): Passaging hiPSCs	Many dead cells after re-plating	EDTA/enzyme incubation time was too long	Monitor the cells under a microscope and stop dissociation earlier
15: Cardiac mesoderm induction	Massive cell death during the first 24 h of cardiac mesoderm induction	Poor quality of hiPSC culture Timing of mesoderm induction was not optimal Seeding density was not optimal Cytokines/small molecules were not added or added incorrectly in the medium Cytokines/small molecules were too old or not properly handled/stored	See above problems and solutions related to issues during hiPSC expansion Do not use hiPSCs passaged more than 50-60 times To improve differentiation efficiency, start cardiac mesoderm induction ~ 23-24 h after the seeding of hiPSCs Adjust and optimize the seeding density at day -1 Carefully add the correct molecules (double-check concentration/dilution) to the medium at day 0 of differentiation Adjust the cytokine concentration based on the specific activity of the lot in use Thaw new aliquots of cytokine/small molecule stocks at the correct temperature and store them no longer than indicated
16A(xiv): EC differentiation	Low efficiency of differentiation and	Seeding density of hiPSCs was not optimal	Adjust and optimize the seeding density at day -1
16B(vi): EPI cell differentiation		Cytokines/small molecules were not added or added incorrectly in the medium during cardiac mesoderm induction	Carefully add the correct molecules (double-check concentration/dilution) to the medium exactly after 72h from mesoderm induction
17A(viii) and 17B(viii): CF differentiation		Cytokines/small molecules were too old or not properly handled/stored	See above solution for mesoderm induction problems

Step	Problem	Possible reason	Solution
16C(xii): CM dissociation	Incomplete dissociation of CM clusters	Insufficient pipetting during dissociation	Examine a small sample of cell suspension under the microscope to check whether dissociation has taken place properly
	High cell death	Cell line is particularly sensitive to dissociation	Repeat gentle pipetting if necessary Use 100 units/mL DNase (see Reagents and Reagent setup) to reduce viscosity during dissociation
16C(xvi) and Box 1: CM differentiation	No monolayer confluency at day 6 of differentiation	Seeding density of hiPSCs was not optimal	Adjust and optimize the seeding density at day -1
		Cytokines/small molecules were not added or were incorrectly added in the medium during cardiac mesoderm induction	See above solution for problems in the other cell type differentiations
	No spontaneously contracting cells at day 10-12 of differentiation	Monolayer confluency at day 6 of differentiation was not optimal	See solutions above for monolayer confluency problem
		Cells were not properly fed and nutrients in the medium are exhausted	Refresh LI-BPEL medium every 2-3 d after day 6 of differentiation and increase the amount of medium per well if medium turns yellow. If cells are still not beating, discard the differentiation and start a new one.
18A(x): CM thawing and re-plating	Many dead cells after thawing and re-plating	Freezing medium was unsuitable	Use 90% (vol/vol) KSR with 10% DMSO (vol/vol) for freezing CMs
		Freezing process damaged CMs	Pipette CMs carefully and gently to preserve cell membrane Always use isopropanol freezing cryoboxes and freeze at -80 °C. Store the cryovials in liquid nitrogen tank after 24-48 h
		Thawing process damaged CMs	Thaw CMs as quickly as possible but add medium slowly to minimize osmotic shock Revitacell (dilution 1:100 to 1:200) can be added to the medium to improve recovery.

Step	Problem	Possible reason	Solution
26: Cardiac MT generation	Cardiac MTs are not compact and/or are not beating well	CM differentiation efficiency was too low	Only use differentiated cell populations with > 80% TnT positive cells
		Cell dissociation was too harsh	Monitor cell dissociation under the microscope Carefully and gently pipette the cells and do not centrifuge at more than 300g
		96-well plates were not centrifuged after plating	Plates need to be centrifuged for 10 min at 300g to promote cell aggregation
		MTs were not properly fed and nutrients in the medium are exhausted	Refresh medium every 3 d Add growth factors to LI-BPEL just before refreshing medium
		Extensive medium evaporation occurred	Check cell incubator settings to maintain proper humidity Do not seed MTs in the outermost wells of the plate. Add 50uL of PBS to the outermost wells of the plate to prevent medium evaporation
27A(xviii): Immunostaining of whole 3D cardiac MTs	Immunostaining of cardiac MTs is not homogenous	Under- or over fixation of cardiac MTs	Fix the cardiac MTs under the correct conditions: 1h at 4° C
	High fluorescent background in immunostained cardiac MTs	Inadequate permeabilization step	Avoid under- or over permeabilization (30 min-1 h at room temperature is the optimal condition)
		Insufficient washing	Wash cardiac MTs no less than three times (20-30 min each)
27C(iv): Video analysis of calcium transients	Cardiac MTs are not properly and homogenously loaded with Fluo-4 AM fluorescent calcium dye	Fluo-4 AM loading solution was not properly mixed	Vortex Fluo-4 AM solution before loading cardiac MTs to ensure homogenous distribution of the fluorescent dye
		Fluo-4 AM loading solution concentration and incubation conditions were incorrect	Carefully dilute the correct amount of Fluo-4 AM stock to LI-BPEL medium. Ensure that incubation time (30 min) and temperature (37 °C, 5% CO ₂) are correct.
		High fluorescence background of cardiac MTs after Fluo-4 AM	Do not exceed 30 min as incubation time. Do not exceed 5 µM as concentration of Fluo-4 AM loading solution
		Incubation time was too long or Fluo-4 AM loading solution concentration was too high	
		Fluo-4 AM loading solution was not properly washed out after incubation	Carefully wash out and remove any dye not specifically associated with cardiac MTs

Step	Problem	Possible reason	Solution
27D(xvii): Metabolic analysis using Seahorse	Injections do not show expected results	Incubation time too short	Longer exposure to drugs is necessary, rerun assay with more measurement points and wait for curve to flatten out
		Drug concentrations are not optimal	Depending on cell line, drug concentrations might need additional adjustment. Note that concentrations can be both too low and too high. A broad screen is recommended
		Injection ports are clogged	Make sure to use medium without large proteins, such as albumin. These can cause the small injection ports to clog resulting in the drug not being added to the medium
	MTs detach after assay	Aspiration of medium too disruptive	Use a pipette to carefully remove medium manually
	Normalisation has very low signal	Assay not sensitive enough	Total cell count per well is low, decrease lysis buffer used to increase concentration or use a different assay. Picogreen assay has necessary sensitivity
27F(xi): Dissociation of cardiac MTs at single-cell level	Many dead cells after re-plating	Enzymatic dissociation was too harsh	Carefully and gently pipette the cells to preserve cell membrane Reduce or increase the rounds of dissociation, and reduce the mechanical pipetting

TIMING

Steps 1-9, Thawing and expansion of hiPSCs: 10 min plus 3-4 d for cell expansion

Step 10A, Passaging hiPSCs in E8 medium/VTN-coated plate: 10 min

Step 10B, Passaging hiPSCs in hiPSCs in StemFlex medium/laminin-coated plate: 10 min

Steps 11-15, Cardiac mesoderm induction with growth factor: 5 d

Step 16A, Cardiac endothelial cell differentiation, isolation, culture and cryopreservation: 6 d

Step 16B, Epicardial cell differentiation, culture and cryopreservation: 9 d

Step 16C, Cardiomyocyte differentiation, lactate purification, and cryopreservation: 15-17 d

Step 17A, Cardiac fibroblast differentiation from freshly dissociated epicardial cells: 16 d

Step 17B, Cardiac fibroblast differentiation from cryopreserved epicardial cells: 10 min for thawing plus 3-4 d for expansion of EPI cells, and 16 d for CF differentiation

Step 18A, Thawing and re-plating of cardiomyocytes (CMs): 10 min plus 5 d for cell recovery

Step 18B, Thawing and expansion of endothelial cells (ECs): 10 min plus 3 d for cell expansion

Step 18C, Thawing and expansion of cardiac fibroblasts (CFs): 10 min plus 3 d for cell expansion

Steps 19-26, Generation and culture of 3D cardiac microtissues: 2h for cardiac microtissue generation plus 21 d for cardiac microtissue culture

Step 27A, Immunofluorescence staining: 3 d

Step 27B, Video analysis of contraction: 2-3 min per cardiac microtissue

Step 27C, Video analysis of calcium transients: 1 h for dye incubation and 2-3 min for analysis of single cardiac microtissue

Step 27D, Metabolic functionality of whole MTs using Seahorse XFe96: 6 h for plating plus 3 h for measurements

Step 27E, Sharp electrode recordings: 10-15 min for recording of single cardiac microtissue

Step 27F, Dissociation of cardiac microtissues into single-cells: 2-3 h

ANTICIPATED RESULTS

For successful cardiac MT generation, highly efficient differentiation of all three input cell types is essential. For this reason, high-quality undifferentiated hiPSC cultures are required to start the differentiation protocols. This can be achieved in various ways with different hiPSC culture media and coatings (E8/VTN and StemFlex/laminin) that provide optimal growth and expansion of undifferentiated hiPSCs, retaining typical hiPSC morphology (Fig. 3). Quality control of the differentiated cell types is necessary to determine the efficiency of differentiation and purity of the final cell populations, before proceeding with cryopreservation and further incorporation of the cells into MTs.

High quality EC populations (Box 2, 3) should display typical endothelial (cobblestone) morphology (Fig. 4a,b) and be positive for endothelial markers, which can be assessed by flow cytometry and immunofluorescence (as VE-cadherin, CD31⁺ cells > 90%) (Fig. 4c,d). The quality of the EPI population is also crucial since it represents a key intermediate stage required for successful CF differentiation. During differentiation, EPI cells undergo epithelial-to-mesenchymal transition to

form CFs and brightfield images at different time points (Fig. 5a) show the change of cell morphology from epithelial to elongated fibroblast-like shape. Epicardial differentiation should give rise to cells exhibiting characteristic cobblestone-like morphology (Fig. 5a) that are positive for epicardial cell markers E-cadherin, ZO-1, and CX43 (junctional localization), as well as nuclear WT1 and TBX18, as seen by immunofluorescence (Fig. 5b). Efficiency of cardiac fibroblast differentiation is assessed by immunofluorescence for markers such as Vimentin, CX43, COL1A1, and GATA4 (Fig. 5c) (Box 3).

In this protocol, we describe two alternative and equally efficient methods for CM generation, one based on cytokines starting from the same common mesoderm to obtain the other cell types¹¹ and the other using a modified protocol based on small molecules⁶⁶ (Step 16C and Box 1, respectively). Notably, the differentiation method does not influence MT characteristics, although high CM differentiation efficiency is crucial and should be at least 80%. As shown in Box 4, the presence of a homogenous beating monolayer of cells starting from day 8-10 of differentiation is evidence of optimal CM differentiation (Fig. 6a and Supplementary Video 1, 2). Differentiated CM also show positive staining for the sarcomeric marker Troponin T (cTnT⁺ cells > 80-90%) when analysed by flow cytometry (Fig. 6b).

Cellular components of MTs can be produced, characterized and stored frozen in large batches, for rapid use post-thaw in MTs. After MT generation, time-lapse video shows the rapid cell aggregation of the three cell types in compact round-shaped spheroids reaching a cell density of approximately 600-900 cells/mm³ within 24-48 h (Fig. 7 and Supplementary Video 3) and with the MTs starting to beat normally within 1-4 d after initial formation. We characterized the structural and functional parameters of MTs 21 d after their generation and the results obtained using this protocol provide evidence of extensive CM maturation, with significant enhancement of structure and contractile, metabolic, and electrophysiological function¹¹. Representative immunostaining of 3D whole MT at low magnification shows the overall morphology and homogeneous distribution of the three different cell types which stain for the cardiac marker Troponin I (TNNI), endothelial marker CD31 and fibroblast marker Collagen Type I (COL1A1) (Fig. 9a).

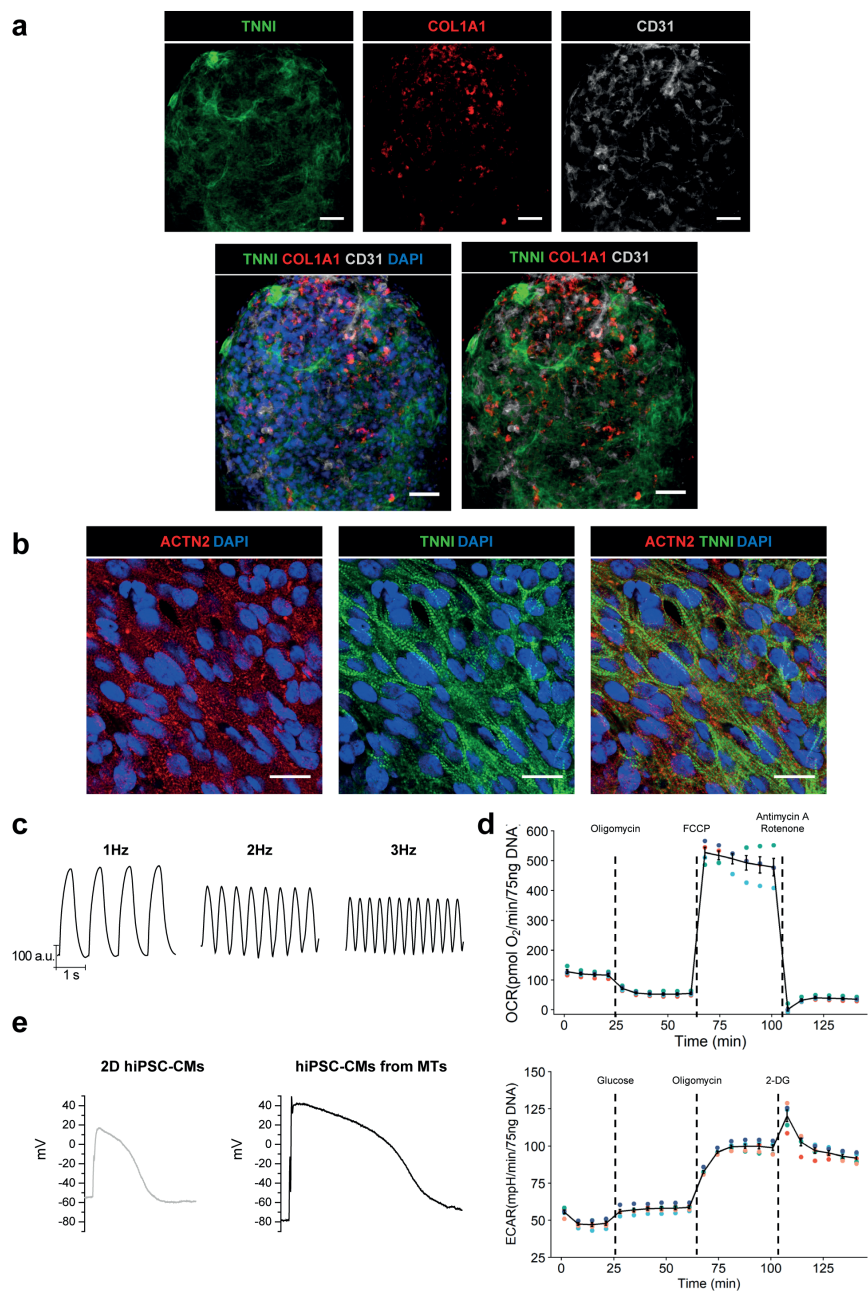


Fig. 9: Characterization of 3D MTs after 21 d in culture.
(a) Immunofluorescence analysis of cardiac- (TNNI, green), fibroblast- (COL1A1, red) and endothelial-(CD31, grey) markers in d21-MTs. Nuclei are stained in blue with DAPI. Scale bar, 50 μ m. (b) Representative

immunofluorescence images for cardiac sarcomeric proteins ACTN2 (red) and TNNI (green), showing the organization of sarcomere structures in 3D MTs. Nuclei are stained in blue with DAPI. Scale bar, 20 μm . (c) Representative contraction traces from MTs stimulated at 1 Hz, 2 Hz and 3 Hz, as indicated. (d) Representative traces of mitochondrial stress test (oxygen consumption rate, OCR, upper panel) and glycolytic stress test (extracellular acidification rate, ECAR, lower panel) from Seahorse measurements of MTs. $N = 16$ MTs/4 wells. Data are shown as mean $\pm$ SEM of 4 wells for the line and mean for each well of 4 MTs for the scatter. (e) Representative action potential (AP) traces recorded from hiPSC-CMs from standard 2D culture (left panel) and dissociated from MTs (right panel). The cells were not artificially hyperpolarized and APs were recorded using the perforated patch clamp technique in current clamp mode.

Notably, CD31 positive cells display a vascular-like network organization within the MT architecture (Supplementary Video 4). Immunofluorescence images of MTs for cardiac sarcomeric proteins TNNI and alpha-2 Actinin (ACTN2) at high magnification shows highly organized sarcomere structures (Fig. 9b). Expected values and variability of sarcomere length and alignment are shown in Table 2, with sarcomere length being the most reproducible parameter among different batches and lines.

To evaluate mechanical properties of MTs, contraction video analysis can be performed on spontaneously beating MTs or stimulated at increasing pacing frequencies (Fig. 9c and Supplementary Video 5) and different parameters can be quantified by MUSCLEMOTION software⁶¹. Virtually all MTs formed with wild type cells can be expected to follow pacing at 1-2 Hz, whereas only around 50% follow at 3 Hz¹¹. Higher pacing frequency was not tested. To characterize Ca^{2+} handling in MTs, Ca^{2+} transients can be assessed by video analysis of MTs loaded with Ca^{2+} -sensitive dye Fluo-4 AM and paced at 1.5 Hz (Supplementary Video 6). Of note, we developed a method to perform metabolic analysis using Seahorse XFe96 assay to measure the metabolic activity on whole 3D MTs¹¹. Usually, MTs are dissociated and re-plated as single-cells which is a less than ideal reflection of the metabolic environment. In this protocol, OXPHOS and glycolytic activity of the cells can be characterized under the same conditions used during regular tissue culture, allowing much more accurate probing of cellular metabolism (Fig. 9d). Electrical maturity of MTs can be determined in whole MTs in 3D using sharp electrode analysis or after single-cell dissociation and single-cell electrophysiology. The action potential (AP) parameters are typically characterized by more hyperpolarized resting membrane potential compared to 2D cultured hiPSC-CMs and by the presence of an AP notch in the majority (> 50%) of the cells/clusters (Fig. 9e). The expected AP parameters from single CMs dissociated from MTs and their expected batch-to-batch and line-to-line variability is shown in Table 2.

In summary, by following this protocol contracting 3D MTs should be obtained in which CMs are characterized by an enhanced maturation. Because differentiated cellular components of MTs can be frozen in large batches, rapid availability for large-scale production of MTs with high reproducibility across different lines and batches, as shown in Table 2, is expected.

DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available in Giacomelli et al. 2020¹¹ and from the corresponding authors upon request.

CODE AVAILABILITY STATEMENT

MUSCLEMOTION is freely accessible on the web at <https://gitlab.com/bjvanmeer/MUSCLEMOTION> or <https://github.com/l-sala/MUSCLEMOTION/>. The license is a GNU GENERAL PUBLIC LICENSE version 3 (GPL v3), which can be found here: <https://github.com/l-sala/MUSCLEMOTION/blob/master/LICENSE>. After downloading the macro, users can access the code through a text editor or directly through FIJI. When using MUSCLEMOTION, users are encouraged to read Sala, L. et al., 2018 PMID: 29282212 (<https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.117.312067>)⁶¹.

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AUTHOR CONTRIBUTIONS

G.C. and V.M. designed and performed the experiments, analysed the data, wrote the manuscript. E.G developed and optimized the cardiac microtissue protocol, acquired and analysed the data, wrote the manuscript. R.v.H. and L.Y acquired and analysed the data and wrote the manuscript. R.P.D.

contributed in drafting the manuscript and revising it for important intellectual content. M.B., V.O. and C.L.M. conceived, designed and supervised the protocol, acquired the funding, wrote and approved the final manuscript.

COMPETING INTERESTS

C.L.M. is co-founder of Pluriomics (now Ncardia) bv.

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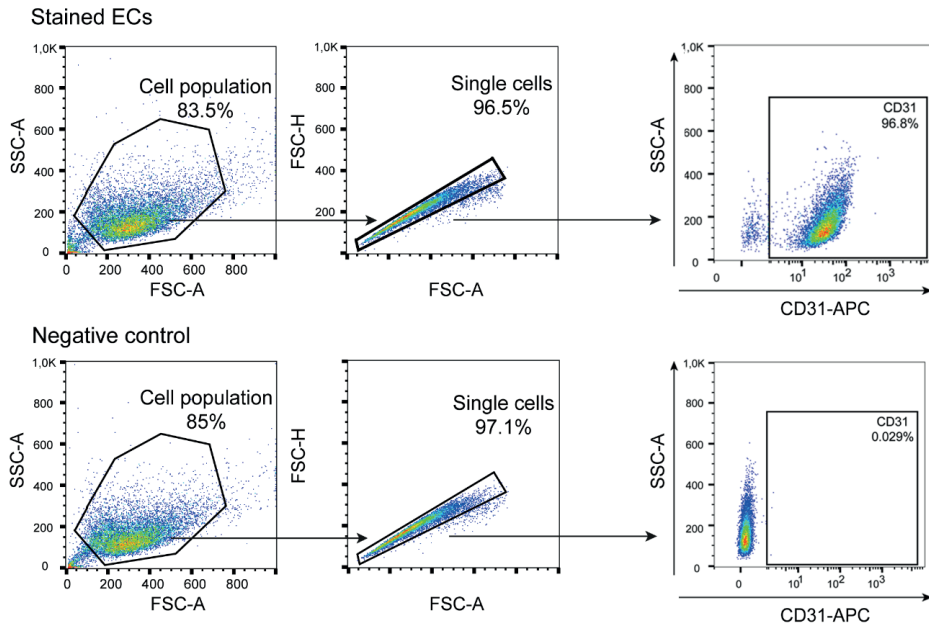
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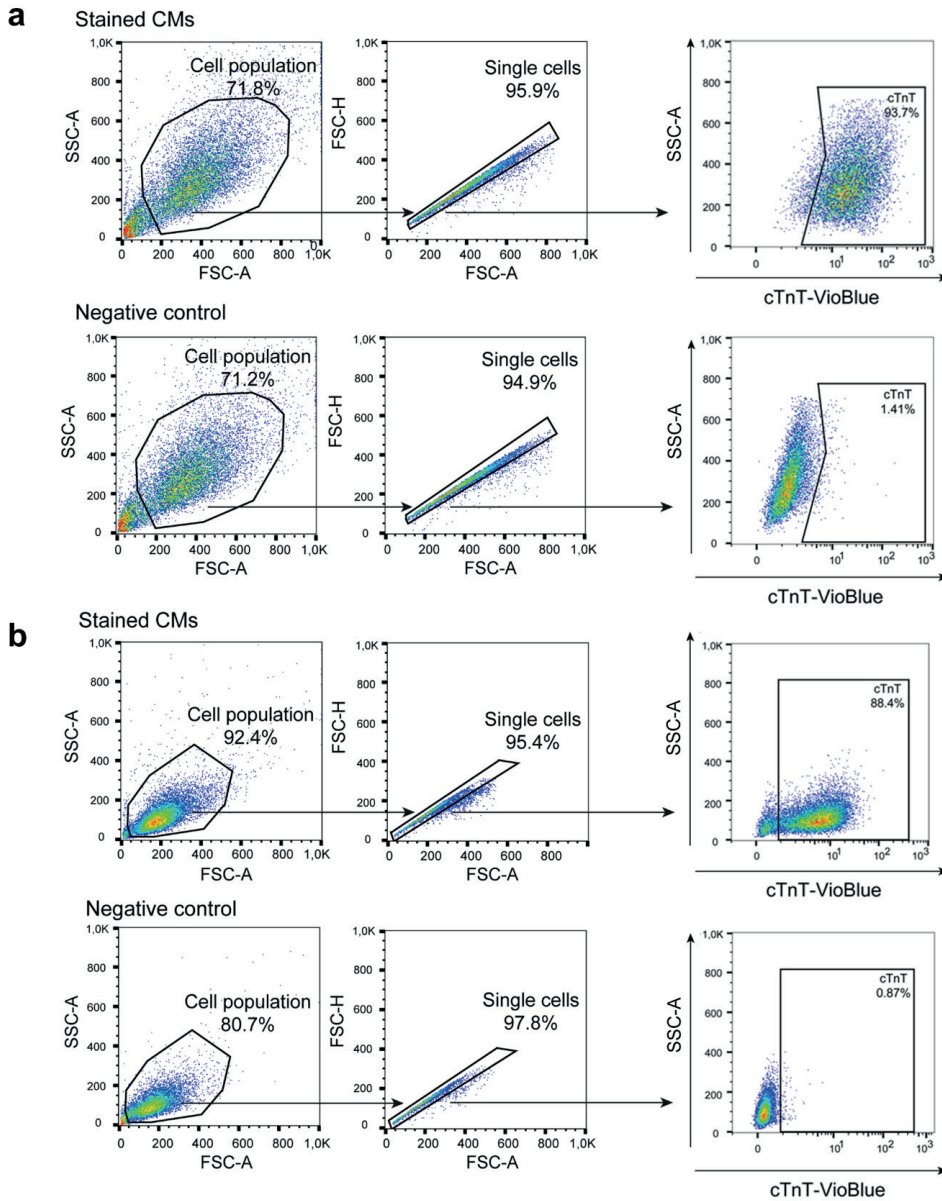
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SUPPLEMENTARY INFORMATION



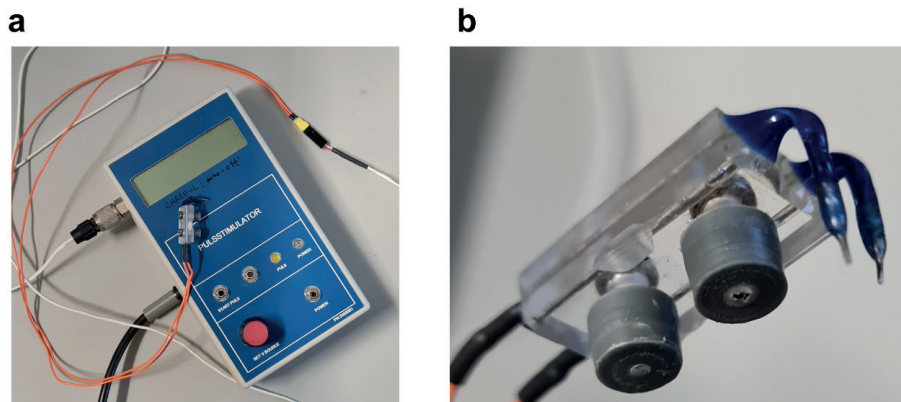
Supplementary Figure 1: Gating strategy used for FACS analysis of hiPSC-ECs.

(a) Forward and side scatter dot plots showing the starting cell population, (b) the gating to discriminate single cells and (c) the gating to isolate CD43-APC positive cells with the help of cells stained with isotype control (APC). The percentage of the relevant cell populations are provided. Cells were acquired with a MACSQuant VYB (Miltenyi Biotech) and analyzed with FlowJo Software.



Supplementary Figure 2: Gating strategy used for FACS analysis of hiPSC-CMs.

(a) Forward and side scatter dot plots showing the starting cell population, (b) the gating to discriminate single cells and (c) the gating to isolate cTnT-VioBlue positive cells with the help of unstained cells (mBEL) or cells stained with isotype control (VioBlue). The percentage of the relevant cell populations are provided. Cells were acquired with a MACSQuant VYB (Miltenyi Biotech) and analyzed with FlowJo Software.



Supplementary Figure 3: Equipment used for electrical pacing of cardiac MTs.

(a) Pulse stimulator connected to custom-made electrodes fitting the V-shaped 96-wells and (b) details of custom-made electrodes.

Supplementary Video 1: Beating monolayer hiPSC-CMs after 21 d of differentiation using LI-BPEL protocol with lactate purification

Supplementary Video 2: Beating monolayer hiPSC-CMs after 21 d of differentiation using mBPEL protocol

Supplementary Video 3: Time-lapse movie of the first 72 h of MT formation (time frame: 15 min)

Supplementary Video 4: Movie of representative MT showing CD31 positive endothelial cells organized in a vessel-like structure (in red) and cardiomyocytes stained with ACTN2 (in green) (15 f.p.s.)

Supplementary Video 5: Contracting MT paced at 1 Hz at after 21 d in culture

Supplementary Video 6: Contracting MT loaded with Fluo-4, paced at 1.5 Hz after 21 d in culture

Supplementary Table 1: Raw data for results summarized in Table 3. Table contains morphological, contraction and single-cell electrophysiological parameters measured in LUMC0099iCTRL04 and LUMC0020iCTRL06 hiPSC lines.



CHAPTER

On-chip analysis of glycolysis and mitochondrial respiration in human induced pluripotent stem cells

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ABSTRACT

Recent advances in microfluidic engineering allow the creation of microenvironments in which human cells can be cultured under (patho-)physiological conditions with greater reality than standard plastic tissue culture plates. Microfluidic devices, also called Organs-on-Chip (OoC), allow complex engineering of the cellular compartment, yielding designs in which microfluidic flow can be precisely controlled. However, it is important that cellular physiology is not only controlled but can also be monitored in these devices. Here, we integrated oxygen and pH sensors into microfluidics, allowing close monitoring of the extracellular flux from the cells, enabling constant assessment of features such as glycolysis and mitochondrial oxidative phosphorylation *in situ*. Using human-induced pluripotent stem cells (hiPSCs) as an exemplar of a highly metabolic and relatively challenging cell type to maintain, we showed that monitoring the extracellular environment allowed rapid optimization of the seeding protocol. Based on the measurements, we implemented earlier and more frequent media refreshment to counteract the rapid acidification and depletion of oxygen. The integrated sensors showed that hiPSCs in the devices exhibited mitochondrial and glycolytic capacity similar to that measured with the Seahorse extracellular flux system, the most widely used standard for these types of assays in conventional cell culture. Under both conditions, hiPSCs showed greater reliance on glycolysis than mitochondrial OXPHOS and the absolute values obtained were similar. These results thus pave the way for the assessment of cell metabolism *in situ* under conditions of fluidic flow with the same precision and relevance as current standard static cell cultures.

Keywords: Extracellular flux, human stem cells, Sensors, Metabolism, Organ-on-Chip, Oxygen, pH

Highlights

- Integrated sensors on a microfluidic chip allow accurate assessment of metabolic flux
- Monitoring pH and dissolved oxygen in organ-on-chips give insight into the metabolic cellular state
- Optimization of tissue-seeding in organ-on-chip can be rapidly optimized by monitoring pH and oxygen

Abbreviations

Human induced pluripotent stem cells (hiPSCs);
 Organs-on-Chip (OoC);
 Oxygen consumption rate (OCR);
 Extracellular acidification rate (ECAR);
 Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP);
 2-deoxy-D-glucose (2-DG);
 Oxidative phosphorylation (OXPHOS);
 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES);
Dulbecco's Modified Eagle Medium (DMEM);
 4-(5,5-difluoro-7-(4-hydroxyphenyl)-1,9-diphenyl-5H-5λ4,6λ4-dipyrrrolo[1,2-c:2',1'f][1,3,5,2]triazaborinin-3-yl)-N-dodecylbenzamide (OHC12);
 2-(*N*-morpholino)ethanesulfonic acid (MES);
 Tris(hydroxymethyl)aminomethane (TRIS);
 Platinum(II)meso-tetra(4-fluorophenyl) tetrabenzoporphyrin (Pt-TPTBPF)

INTRODUCTION

How cells use different metabolites is organ-dependent and can change significantly in different disease states. It is now widely accepted that metabolic shifts can rapidly and profoundly alter cellular behaviour and phenotype by directly regulating the epigenome¹⁻⁵. Parameters like oxygen consumption, changes in pH and glucose consumption can provide information on cellular ATP production, giving insight into the viability and metabolic state of cultured cells. Several commercial assay systems are available to address this in conventional static cell cultures^{6,7}. One of the most widely used systems is the Agilent XF Analyzer, known as the Seahorse™⁶. Since its launch in 2006, more than 5000 publications have reported data on cells acquired using Seahorse™ (Fig. A.1).

However, from the biological perspective, microfluidic systems are now being explored to create more physiologically relevant environments for cultured cells. These miniaturized microfluidic devices, often referred to as Organs-on-Chip (OoC) are designed to mimic conditions encountered by cells in whole organs, particularly those of the vasculature^{8,9}. They often rely on fluid and gas perfusion combined with 3D tissue cultures and thus are not compatible with assays designed for static 2D cell culture on standard tissue culture or customized plates. For many questions, however, tools that monitor cellular metabolism in real-time would be valuable for understanding complex cellular behaviour under conditions of fluid flow in OoC¹⁰. In addition, when stem cells or their derivatives are the cellular input for OoC, information can be obtained on how different states of differentiation,

maturation, disease or drug treatments impact metabolism^{8,9}. Furthermore, metabolic monitoring involves the continuous assessment of relevant culture parameters like oxygen, pH and nutrients. These parameters could be used for rapid optimization of cell culture protocols in OoC, since these devices are typically closed and maintaining cell culture is often affected by the limited availability of oxygen and nutrients. While this can be tuned by altering the flow rate, the current OoC do not have the relevant metabolic readouts to provide the necessary feedback.

Integration of sensing elements inside a microfluidic chip is the first step toward monitoring metabolism *in situ*, and optical chemical- or electrochemical sensors within a microfluidic channel can be used for this. Optical chemical sensors are based on changes in luminescence properties of immobilized dyes in the presence of the analyte; they enable contactless, *in situ*, real-time measurements of metabolically relevant parameters like oxygen consumption rate (OCR), extracellular acidification rate (ECAR) or detect specific metabolites such as glucose and lactate^{11–14}. The sensing elements (dye and support matrix) can be miniaturized to fit microfluidic channels allowing seamless integration into the devices without interfering with the tissue¹⁵. Optical oxygen and pH sensors have already shown versatility within microfluidic chips^{16–18}. They have been used to assess the OCR and ECAR of cultured cells inside microfluidic systems and the influence of drugs or nanoparticles on these parameters^{10,16}. However, these studies did not compare on-chip data with more widely used standard assays for assessing metabolic rates, such as the Agilent Seahorse XFe Analyzers™, widely used on static cell cultures.

Here we describe the integration of oxygen and pH sensors into an existing commercial chip and use it to monitor the concentration of dissolved oxygen and pH during cell culture and to measure the OCR and ECAR of human-induced pluripotent stem cells (hiPSCs) during stop-flow measurements. These (non-transformed) cells are highly proliferative, have high metabolic rates and are notable for their reliance on glycolysis^{19,20}. We compared metabolic rates obtained under dynamic conditions in the chip with results obtained in the Seahorse system™. On both platforms, we carried out the two most widely used assays for which the Seahorse system was designed: the mitochondrial stress test and the glycolysis stress test. We showed that the on-chip measurements could capture the metabolic activities of the hiPSCs within a microfluidic channel and that the outcome did not differ from experiments performed on the Seahorse system.

2. MATERIAL AND METHODS

2.1 Sensor integration

Liquid sensor formulations were first prepared for both sensors. The oxygen indicator dye platinum(II)meso-tetra(4-fluorophenyl) tetrabenzoporphyrin (Pt-TPTBPF) was synthesized as previously described¹². Poly-tert-butylstyrene particles (ptBS particles) were stained with 2 % (w/w) Pt-TPTBPF as described elsewhere¹⁴. 7% Hydromed D7 (AvanSource biomaterials), a polyurethane-based hydrogel, was prepared in a mixture of ethanol-water (9+1). The Pt-TPTBPF stained particles were mixed in the hydrogel resulting in a 2.5% (w/w) dispersion. The mixture was homogenized by vigorous stirring for 5 min.

Preparation of pH sensors was adapted from Müller et al.¹⁶. Hydromed D4, a polyurethane-based hydrogel, was purchased from AvanSource biomaterials. The aza-BODIPY pH indicator dye 4-(5,5-difluoro-7-(4-hydroxyphenyl)-1,9-diphenyl-5H-5λ4,6λ4-dipyrrolo[1,2-c:2',1'-f][1,3,5,2] triazaborinin-3-yl)-N-dodecylbenzamide (compound 5; OHC12)¹¹ and microcrystalline powder of silanized Egyptian Blue¹² were synthesized in our lab as previously described.

0.1 mg aza-BODIPY indicator was dissolved in 100 µl THF. This solution and 16.8 mg Egyptian Blue were suspended in 415 mg 8% Hydromed D4 solution in ethanol-water (9+1). The suspension was homogenized using a sonifier (Branson) with ten pulses at 25% of maximum amplitude for one-second with a pause of 10s between each pulse. The sensor formulation was used within an hour of preparation.

Open Vena8 CGS chips, containing 8 channels with dimensions of 800 µm (W) x 80 x µm (D) x 28 mm (L) were purchased from CelliX Ltd. Both sensor formulations were integrated into the microfluidic compartment of the chip via microdispensing. A microdispenser MDS3200+ from VERMES Microdispensing GmbH equipped with a tungsten tappet with a tip diameter of 0.7 mm and a nozzle diameter of 200 µm was used. The liquid sensor formulation was dispensed in the reservoir, and pressurized air at a pressure of 200 mBar (oxygen) or 400 mBar (pH) was applied. Printing parameters for both oxygen and pH sensors are given in Table A.1. Four sensors were integrated into each channel with a distance of 4 mm between the centre of the sensor spots. Either three oxygen sensors with one pH sensor in a middle position (Fig. 1) were integrated in one channel or the other way round with three pH sensors and one oxygen sensor. Chips with more oxygen sensors were intended for use in the mitochondrial stress tests, whereas chips with more pH sensors were used for the glycolysis stress test. All sensors were allowed to dry for more than 2 h before the chips were sealed with the coverslip provided by CelliX.

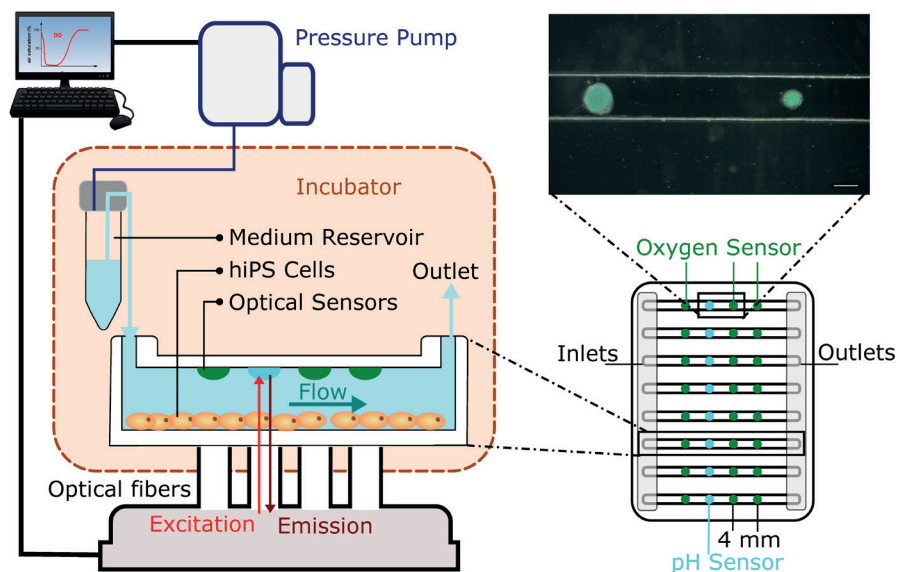


Figure 1) Schematic design of the experimental setup.

The microfluidic chip features 8 channels with 800 µm diameter. Optical oxygen and pH sensors are integrated into the top of the chip, with each channel containing a total of 4 sensors (Scalebar = 500 µm). The hiPSCs are cultured on the bottom, and optical read-out of the sensors is performed through the cultured monolayer. The assay is performed inside a tissue-culture incubator, while the pressure pump is kept outside the incubator.

2.2 Chip preparation

The chips with integrated sensors were sterilized with UV light for 20 min in a laminar flow hood. The channels were coated overnight at room temperature with fibronectin in PBS (50 µg/ml, bovine plasma, Sigma). This fulfilled two purposes: i) the sensors were hydrated, and ii) the channel was coated with fibronectin to promote cell adherence. Next, the chips were placed in a desiccator and a vacuum applied for 30 min to remove air bubbles that formed in the chip overnight. The PBS in the chip was then aspirated, and the chips were used immediately for experiments.

2.3 Sensor calibration

The sensor calibration was performed in the same setup as the final experiments. The chip was placed in a custom-made holder in the incubator (37°C, 0% CO₂) to align 2 m polished plastic optical fiber (1/2.2 mm) with the integrated sensor spots. 32 optical fibres guided the excitation light and collected the emitted light to the 48-channel phase fluorometer (PyroScience) as a readout¹⁷.

The oxygen sensor material was the same as that described earlier¹⁴. Two-point calibration at 37°C

was performed. Anoxic conditions were induced in one of the channels using 2% sodium sulphite solution. The resulting calibration value was used for all sensors throughout the experiments. Individual calibration of the sensors in air-saturated conditions was performed on the day of the experiment. The oxygen sensor was left to equilibrate in air-saturated PBS or medium at 37°C for the calibration value of the air-saturated conditions. An optical temperature microsensor (PyroScience) was used to compensate for temperature changes.

The pH sensors were calibrated in the same medium used for the assays (see section 2.5). The medium was supplemented with 10 mM of MES (2-(*N*-morpholino)ethanesulfonic acid) (Sigma Aldrich) or TRIS (tris(hydroxymethyl)aminomethane) (Sigma Aldrich) and adjusted to 6 different pH values between 5.4 and 9.0 using 1 mM NaOH or 1 mM HCl at 37°C. The buffers were passed through filters (0.2 µm Whatman) to ensure sterility. The buffers were flushed manually through the system and each was allowed to rest for 15 min to obtain stable calibration data. The chip was flushed with sterile water before injecting the next buffer. After calibration, the chip was washed with the assay medium. The data obtained were fitted to a Boltzmann equation for calibration (Eq. 1), where A is the top value, B is the bottom value, pKa is the apparent pK and the slope at the pKa value ¹⁶.

$$\cot(\delta\phi) = \frac{A - B}{1 + 10^{\frac{pH - pK_a}{\text{slope}}}}$$

Equation 1 Boltzmann equation.

2.4 hiPSCs maintenance and cell-seeding

hiPSCs (hPSCReg: LUMCi028-A) were maintained in 12-well tissue culture plates on human recombinant vitronectin-coated substrates and were refreshed daily with E8 medium (ThermoFisher). The hiPSCs were passaged biweekly using 0.5 mM EDTA in PBS without Ca⁺ and Mg⁺ (PBS⁻). For cell-seeding in either the microfluidic device or Seahorse plate, cells were washed with PBS⁻ and dissociated using TrypLE 1x (ThermoFisher) and washed in E8 medium containing 0.5x RevitaCell supplement (ThermoFisher). Cells were subsequently counted and seeded at the density specified for each assay.

2.5 Quantification of oxidative and glycolytic metabolic capacity measured by Seahorse XF-96 Analyzer

hiPSCs were seeded in the Seahorse 96-well plate pre-coated with 10 µg/ml fibronectin (Bovine, Sigma) at a density of 70k cells per well in the appropriate pre-made assay medium with the addition

of 0.5x RevitaCell. The cells were allowed to recover for a period of 3 hours before commencing the assay. The assay medium for the mitochondrial stress test consisted of DMEM without bicarbonate (Sigma) supplemented with 5 mM glucose (Sigma), 2 mM L-glutamine (ThermoFisher), 1 mM pyruvate (ThermoFisher), 5 mM HEPES (Sigma), and with 0.25% fatty-acid free BSA (Bovogen), followed by pH calibration to 7.4. For the glycolysis stress test assay, the same medium was used without glucose. The mitochondrial stress test was performed by serial injection of oligomycin (1.5 μ M, Sigma), Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 0.75 μ M, Sigma), and finally, antimycin A and rotenone together (2 μ M, Sigma). The glycolysis stress test was performed using injections of glucose (5mM), oligomycin (1.5 μ M), and finally, 2-deoxy-D-glucose (2-DG; 10 mM, Sigma). For both assays, the same conditions and measuring profiles were applied, with a measuring period of 4.5 min and a 4 min period of mixing or flow between each measurement. The data was normalized by staining live cell nuclei with HOECHST (ThermoFisher) and assessing the total cell number per well.

2.6 Quantification of oxidative and glycolytic capacity in a perfusable microchannel chip

The chip was placed in the incubator at 37°C and 0% CO₂ and allowed to equilibrate for 15 min. Cells were subsequently seeded by making a cell suspension of 25×10^6 cells/ml in the assay medium with 0.5x RevitaCell and injecting 3 μ l of the cell suspension into the channel from both sides. The cells were allowed to attach for 30 min before adding 12.5 μ l of assay medium in the reservoirs on one side of the channel, allowing the fresh medium to flow through the channel. Every 30 min, 20 μ L of assay medium was added until 3 hours after seeding when the assay would commence.

The assay was performed by connecting the chip to the tubing using blunt-end Luer lock syringe needles 20G (Techcon). The microfluidic setup consisted of a pressure pump (Fluigent, FLPG Plus) connected to the microfluidic flow control system (Fluigent, LineUP Flow EZ). The controlled flow was connected to a 15 mL Falcon (Greiner) tube with a P-CAP (Fluigent) to pressurize the tube. By pressurization, the medium passed via the liquid outlet into the tubing connected to a flow rate sensor (Fluigent, FLOW UNIT size L ± 1000 μ l/min). The pump was controlled from a laptop with the Fluigent A-i-O (v1.0.43) and Fluigent OxyGEN (v1.0.1.0) software. The pump pressure was regulated to maintain a set flow rate over the flow unit of 20 μ l/min. The flow was subsequently split into 8 channels, resulting in a flow rate of 2.5 μ l/min per channel. The pressure pump drove the medium from the reservoir and split via an 8-way manifold with additional 85 μ m resistors (Elveflow), allowing equal flow rates through each channel. The assay was started by perfusing all the channels at the predefined flow rate with the equilibrated assay medium for 7.5 min, ensuring

that pH and oxygen had reached baseline levels within each channel. Subsequently, four baseline measurements of 4.5 min were performed under static conditions, followed by reperfusion of 4 min. Subsequent drug exposures were done by disconnecting the chip and perfusing the tubing with the assay medium containing the final drug concentration. The chip was subsequently reconnected, and three measurements were performed according to the same scheme as the basal measurements for each condition.

3. RESULTS & DISCUSSION

3.1 Experimental setup and sensor characterization

The setup consisted of the Cellix Vena8 microfluidic chip with eight channels. The chip features ports at both ends of the channels. These ports can be used either as reservoirs or connected to a pressure-driven pump, allowing the application of a controlled flow within the system. Each channel featured four sensor spots for either oxygen or pH measurement (Fig. 1). The sensor matrix consists of a biocompatible polyurethane-based hydrogel (Hydromed D4 or D7) doped with either Pt-TPTBPF stained sensor particles for oxygen measurement or Egyptian blue and OHC12 dye for pH sensors. Previous studies have shown that neither sensors are detrimental to cells ¹⁶.

Furthermore, the sensors were integrated on the top of the microfluidic channel, whereas the cells were cultured on the bottom, ensuring no direct contact and interaction between the sensors and the cells but still allowing proper measurement of pH and oxygen through the medium. The integrated sensors had diameters of less than 600 μm (oxygen) and 700 μm (pH) (Fig. 1). The chip was mounted on a custom-made holder aligning the optical fibres for the sensor readout from the bottom. The indicator dyes used were excited with red light and emitted in the near infrared spectrum, allowing sufficient penetration through the cultured cell monolayer and signal generation (data not shown). Furthermore, the measurements were based on luminescence lifetime measurement, which is not affected by inhomogeneities in the light path. Therefore, reliable measurement through the cultured tissue is possible. The oxygen sensors used had been shown to remain stable during cell culture at 37°C in previous studies ^{10,21,22}. The pH sensors showed a reproducible response to pH with an apparent pKa (point of inflexion of calibration curve) of 7.30 ± 0.01 and a resolution of 0.004 at 37°C after the six-point calibration (Fig. A.2). The dynamic range of the sensor was from pH 6.3 to pH 8.3, which is a suitable measurement range for the tissue culture conditions from pH 6.3 to 7.5. The response time (t_{95}) was approximately 50 s for a change from pH 7.0 to 7.5 via manual flushing of the system (Fig. A.2), showing a rapid response time in the relevant range. The long-term stability of the pH-sensor was not assessed as it showed little drift over 7 days in previous studies ¹⁶.

3.2 Optimization of cell-seeding using pH-sensors to monitor tissue-culture acidification

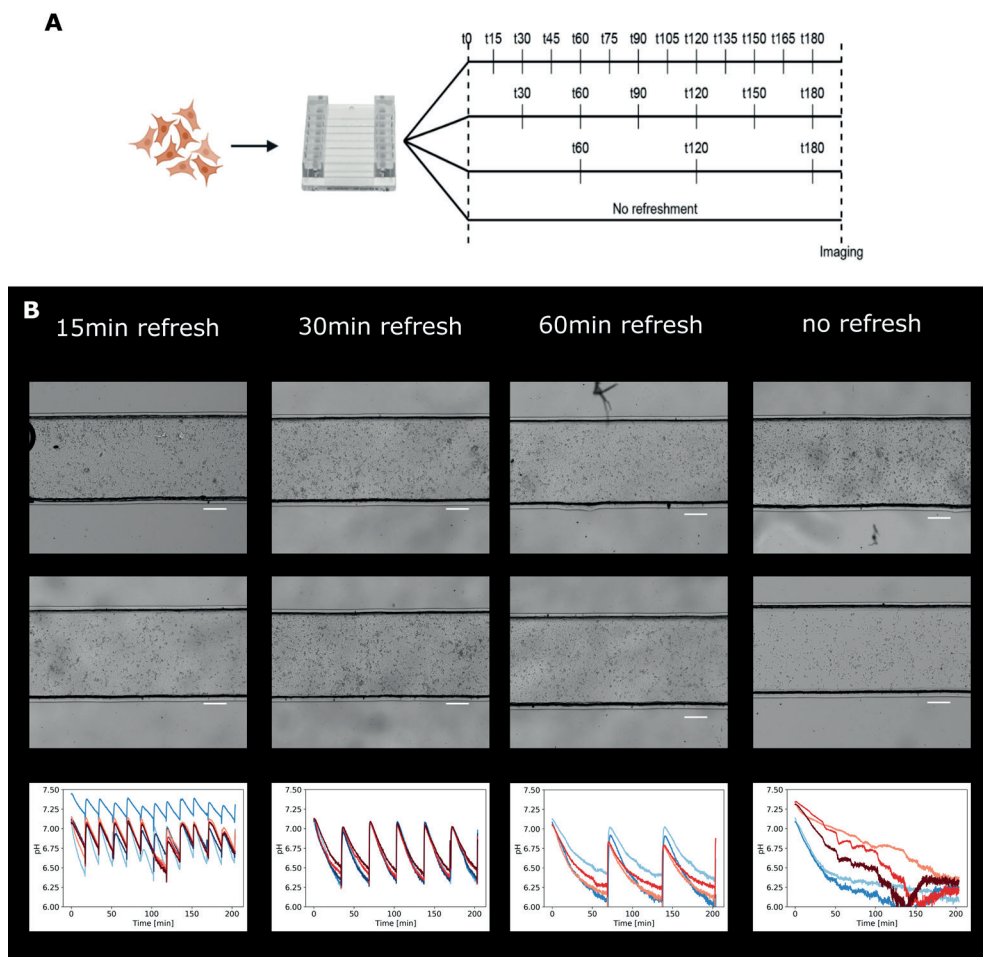


Figure 2) Optimisation of seeding conditions using the sensors.

A) During the attachment of the cells, the medium was refreshed by adding 12.5 μ l medium at 15-, 30-, and 60-min intervals. In an additional control channel, the medium was not refreshed. B) The effect on the cells was investigated using microscopy and the read-out of the pH sensors. Each panel shows duplicate images acquired at the end of the experiment and the read-out of the pH measurements. Scale-bar = 250 μ m.

After the cells were seeded, monitoring of dissolved oxygen concentration and pH of the medium was started directly using the integrated sensors. Cell attachment took up to three hours, during which the microfluidic pumps were not connected to the chip and there was no flow in the channel. pH

levels dropped drastically during this period, to less than pH 6.2, outside the measurement range. This acidification of the medium led to cell death and prevented proper formation of a cell monolayer. Therefore, gentle flow was introduced at different time intervals by adding 12.5 μ l of medium to one of the reservoirs. Time intervals of 15, 30, and 60 min were tested, during which the sensor readout was followed to find the best culture conditions (Fig. 2). After three hours, the cells were examined using bright-field microscopy (Fig. 2B). As before, pH levels dropped to less than pH 6.2 without medium refreshment and leading to variable cell confluence. Medium refreshment every 60 min also led to a drop in pH below 6. However, in samples refreshed every 15 or 30 min, the pH did not drop below 6.2. The pH difference was greater with refreshment intervals of 30 min rather than 15 min (Δ pH 0.75 ± 0.02 and 0.40 ± 0.07 , respectively) but refreshment every 15 min reduced the cell number in the centre of the channel, possibly because cells were disrupted during attachment and were then washed away. Only with refreshment intervals of 30 or 60 min were the cells able to form a confluent monolayer. However, the pH dropped below 6.2 in the 60 min interval preventing accurate measurements. Thus, the 30 min interval was chosen for further experiments.

3.3 Assessing oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) by measuring oxygen and pH

To measure OCR and ECAR accurately during the assays within the chip, the same measuring protocol was used as within the Seahorse. The Seahorse system measures by lowering a probe into each of the wells, creating a tightly sealed micro-well of 1.6 μ l. The smaller volume of the micro-well ensures more rapid depletion of dissolved oxygen and acidification of the medium, which in turn allows accurate assessment of the metabolic rates. The oxygen tension and pH measurements are made every 16 sec for a total of 4.5 min, after which the measurements stop. Subsequently, the airtight seal created by the probe is broken, mixing the medium and returning oxygen and pH levels to baseline. After this mixing period, the probe is again lowered into position and measurements are resumed. Each of these measuring periods is used to generate a single OCR and ECAR datapoint by fitting a first-order polynomial and taking the derivative.

To assess whether we could apply the same principle to measurements acquired from the sensors on our chip, optimal timing for the flow- and static cultures were first determined (Fig A.3). Perfusion intervals of 1-5 minutes were tested to determine which timespan was sufficient to reoxygenate the channel and reset the medium pH back to baseline. The period for static conditions was optimized from 1-10 min to allow an analogous drop in oxygen and pH as in the Seahorse. OCR and ECAR were derived by analyzing the data during periods of static culture, fitting a first-order polynomial and taking the derivative. Fig. 3A demonstrates the assessment of ECAR by comparing one background

channel without cells to three channels containing an equal number of cells. ECAR measurements remained stable during this period, while even unintentionally failed reperfusion allowed estimation of ECAR. However, failed reperfusion led to a decrease in ECAR over time; therefore, these data were excluded from further evaluation. Using this method to determine the ECAR, a stop-flow protocol was designed to mimic the Seahorse protocol, where 4.5 min measurements under static conditions were performed. These measurements were followed by 4 min of reperfusion to allow oxygen and pH to reset to baseline values. We verified that this yielded similar results to a Seahorse experiment with identical settings for measurement and mixing. Fig. 3B shows oxygen depletion from the medium is linear in our chip, like that observed in the Seahorse. Oxygen depletion during the measurement phase in the chip is approximately 10 mmHg, while the Seahorse showed depletion of approximately 5 mmHg in the same time period. Seahorse does not continue measurement of oxygen during mixing, while our chip setup allows continuous monitoring, even during perfusion. We found an increase in oxygen concentration in our reference channel and the Seahorse system blank wells during the static periods. There are several possible explanations – or a combination of them – for this phenomenon. One is that the medium is not entirely equilibrated with the ambient conditions in the incubator. This could involve slightly too low temperature due to the large temperature capacity of the medium and insufficient pre-heating. An increase in the temperature of the medium in the channel would lead to an elevated signal from the oxygen sensor while the actual oxygen concentration within the medium remains the same. Additionally, a slight difference in the oxygen concentration in the media and the ambient air in the incubator could lead to re-equilibration during the flow stop. Another possibility could be the oxygen storage capacity of the thermoplastic chip material. When thermoplastics are heated, they release some of their stored oxygen, which also increases the oxygen concentration in the channel. All of these effects would presumably also be present in the channels with cells but not evident as the cells consume more oxygen than the increase in the reference channels showed. Before further evaluation, the reference measurement was used as baseline correction for the cell measurements, thereby eliminating the measuring error induced by the observed effect.

ECAR measurements in the chip also resembled the Seahorse (Fig. 3C). The measurement showed a significant decrease in the pH of 0.15 during the measurement time, while Seahorse showed a smaller decrease in pH of 0.1. Here we found that the reference channels in the chip and the background wells in the Seahorse showed no significant differences in pH during the measurement or flow periods. Overall, we obtained outcomes from sensors in the chip which resembled the Seahorse assay closely. We note that the reference channel values are important to correct measurements in the channels containing cells.

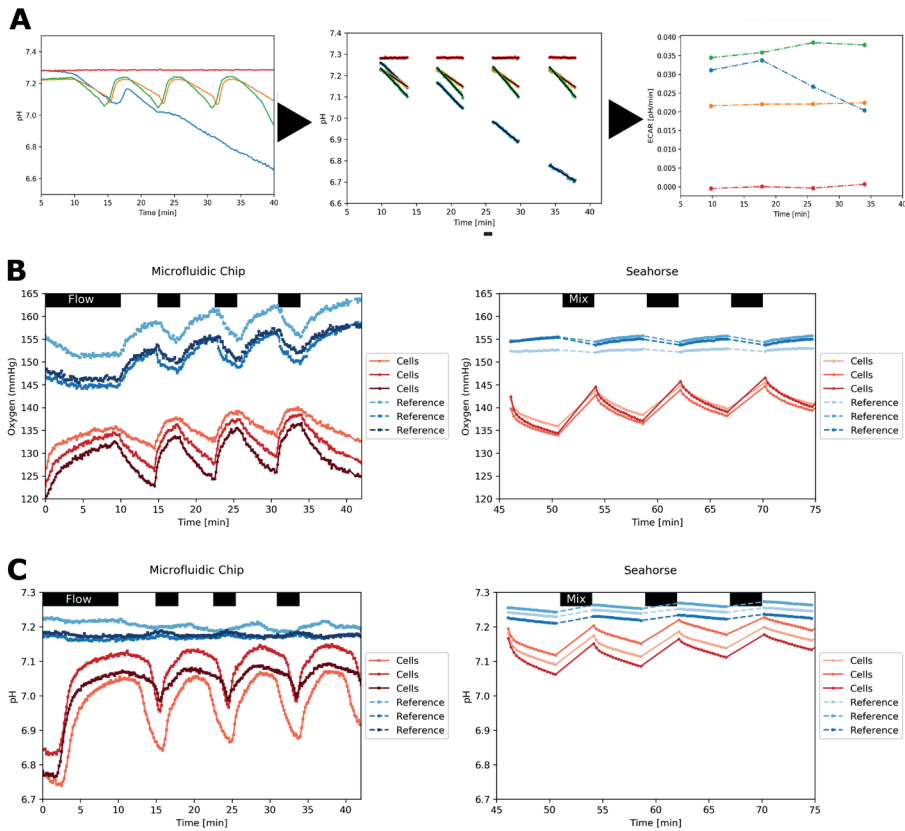


Figure 3) Derivation of ECAR and raw data comparison from chip and Seahorse.

A) On the left, complete data acquisition during the stop-flow protocol. The colour-to-data assignment shown in this panel also applies to the following panels. The middle panel shows the isolated periods without flow and the applied polynomial fits (black lines) from which the extracellular acidification rate (ECAR) can be calculated, plotted in the right-side panel. B & C) Comparison of Raw Data of measurements in the chip (left) and the seahorse system (right) during baseline measurement for mitochondrial assay (B) and glycolysis assay (C). Flow/Mixing intervals are marked in black. The measurement interval of 4 min shows changes in oxygen partial pressure and a decrease of pH in the chip and the Seahorse for measurements with cells. References are empty channels without cells.

3.4 Mitochondrial respiration and glycolytic acidification can be assessed by on-chip monitoring of pH and oxygen

The mitochondrial- and glycolysis stress test assays were performed on the chips and the Seahorse. These tests are the most commonly used in the Seahorse system and allow assessment of the metabolic capacity of cells to generate ATP via glycolysis or oxidative phosphorylation (OXPHOS) (Fig. 4).

The mitochondrial stress test was performed by serial injection of oligomycin (1.5 μM), blocking OXPHOS complex V and allowing quantification of OCR dedicated to ATP production, followed by FCCP (0.75 μM) injection, uncoupling the inner-mitochondrial membrane and assessing maximal respiration capacity. A final injection of antimycin A and rotenone (both 2 μM), blocking complex III and complex I, respectively, allows quantification of the proton leak and non-mitochondrial oxygen consumption (Fig. 4A). Performing this assay in the Seahorse on hiPSCs results in an expected profile, with both the injection of oligomycin and antimycin A with rotenone resulting in a strong decrease in OCR, while the injection of FCCP leads to an increase in OCR (Fig. 4B). Calculating the contribution of OCR in the hiPSCs under the various conditions revealed that the contribution of OCR towards ATP production is lower than the basal consumption, caused by a high proton leak (Fig. 4C). Proton leak is the result of protons leaking over the inner membrane of the mitochondria, where they react with oxygen to generate H_2O and release further energy in the form of heat. This process does not contribute to the charging of ADP into ATP and is, therefore, inefficient. Additionally, injection of FCCP did not increase the OCR above the basal levels, thus suggesting that the OXPHOS capacity of the hiPSCs is already used at full potential under basal conditions^{23–25}. These results match with previously published work and show that hiPSCs predominantly rely on glycolysis for their metabolic and energetic needs^{19,20}.

The glycolysis stress test (Fig. 4D) was performed by basal measurements in a medium containing no glucose, the first measurements set a baseline, assessing non-glycolytic ECAR. Glucose (5 mM) was subsequently added to the medium, restarting glycolysis in the cells and allowing a measure of the basal level of glucose utilization via glycolysis. Subsequently, oligomycin (1.5 μM) was added, blocking ATP production via OXPHOS, and forcing the cells to compensate by upregulating glycolysis; this results in the maximal glycolytic rate of the cells. Finally, 2-DG (10 mM) was added, which acts as a competitive inhibitor of the enzyme phosphoglucosomerase, blocking glycolysis in the second step. This drug was used to verify the absence of glycolysis in the first measurements of the assay. Performing this assay in the Seahorse on hiPSCs resulted in the expected profile, with the addition of glucose and oligomycin leading to a significant, approximately 7-fold, increase in ECAR (Fig. 4E,F). Quantification showed that both the injection of glucose and oligomycin contributed to increases in ECAR, revealing that hiPSCs have additional (spare) glycolytic capacity, which is not used during normal basal conditions^{19,20,26}.

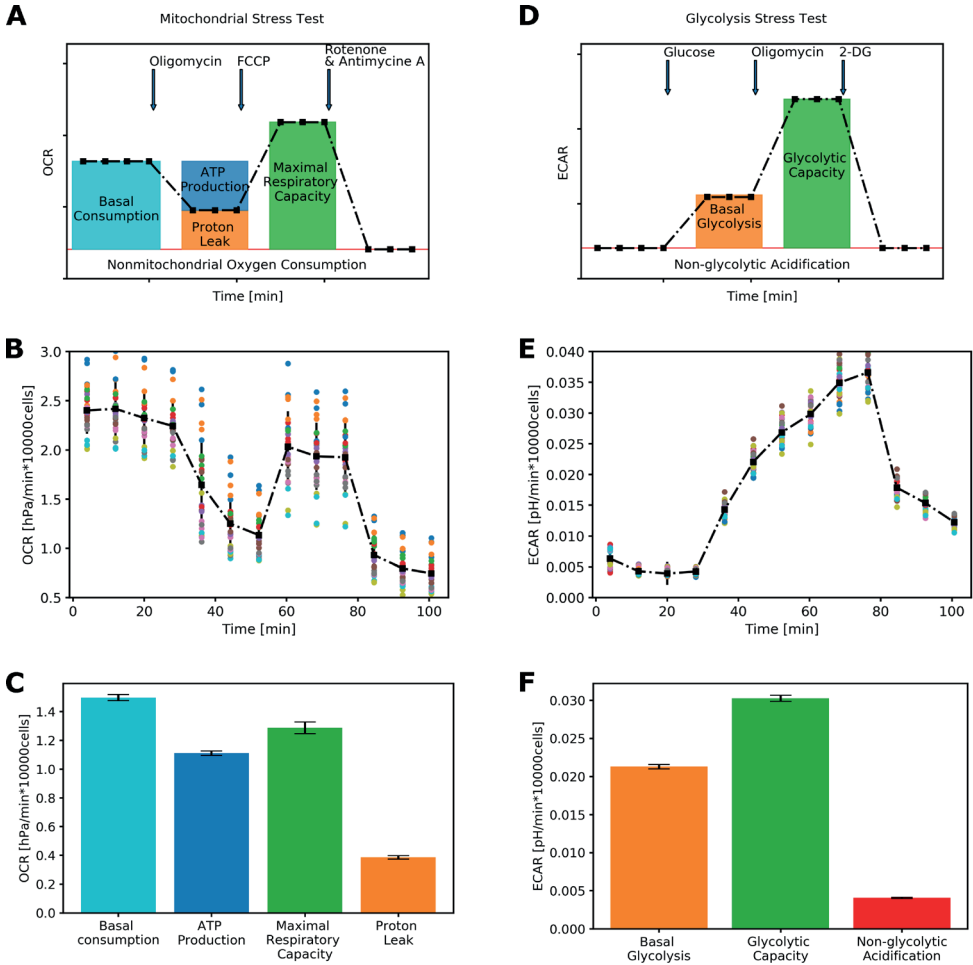


Figure 4) Representative Seahorse experiment and data.

A-C) The mitochondrial stress test, with a graphic representation (A), representative data obtained from hiPSCs (B), and the quantification of the data (C). D-F) Glycolytic stress test, first depicted as a graphical representation (D), a representative experiment with hiPSCs (E), and final quantification from the experiment (F). Data mean $\pm$ standard deviation, with 28-30 replicates per condition and a single experiment. Oxygen consumption rate (OCR) is defined as the depletion of oxygen (hPa) per minute, normalised by total cell count. Extracellular acidification rate is the decrease in pH per minute, normalised by total cell count. Arrows note the moment when a drug compound is injected into the plate.

We performed the assays in parallel on both Seahorse and the chip to assess whether the sensors on the chip could replicate the measurements obtained by Seahorse on the same batch of cells and medium and on the same day. Cells in both the Seahorse and chip assay were treated with the same final concentrations of the drugs, where the Seahorse made use of a 10x concentrated injection while the chips were perfused with the medium containing the compound at the final concentration.

Fig. 5 A and B show the OCR measured in the chip and the Seahorse during the mitochondrial stress test assay, respectively. It is evident that the OCR was similar between the two systems after correcting for total cell count. Additionally, the expected effects of adding the drugs were observed in both assays. Quantification of the assay showed no significant differences between the two systems for the OCR at basal respiration, ATP production and proton leak, while maximal respiration was increased on the chip (Fig. 5C).

The time course in Fig. 5D and E show the kinetics during the glycolysis stress test in both the chip and the Seahorse. Like the mitochondrial stress test, the measured flux on the chip resembled the traces observed in the Seahorse system, with quantification showing no significant difference between non-glycolytic acidification and glycolytic capacity and an increase in basal glycolysis on the chip (Fig. 5F). Of note is the relatively low glycolytic capacity observed in these experiments, which were similar to the basal glycolysis in both the chip and the Seahorse. These results suggested that the hiPSCs use their maximal ATP production rate under the culture conditions present, in contrast to our Seahorse results initially obtained which showed a large glycolytic capacity. However, the hiPSCs demonstrated this behaviour in both platforms, indicating it is not an inaccuracy of the measuring method. Differences in cellular phenotype were, however, observed between the methodologies. The hiPSCs plated in the Seahorse displayed a lower maximal respiratory capacity and basal glycolytic rate. With the similarities in absolute values of OCR and ECAR, we estimate that the differences are most likely to be explained by differences in tissue-culture plate, plastic versus glass and the additional administration of medium perfusion. A previous study has indicated transcriptional differences between hiPSCs derived in tissue-culture plates when compared to hiPSCs derived in a microfluidic device²⁷. To better understand these differences, more metabolic studies are needed, comparing different culture methods and including perfusion to decipher the contribution of methods used to a difference in metabolic phenotype.

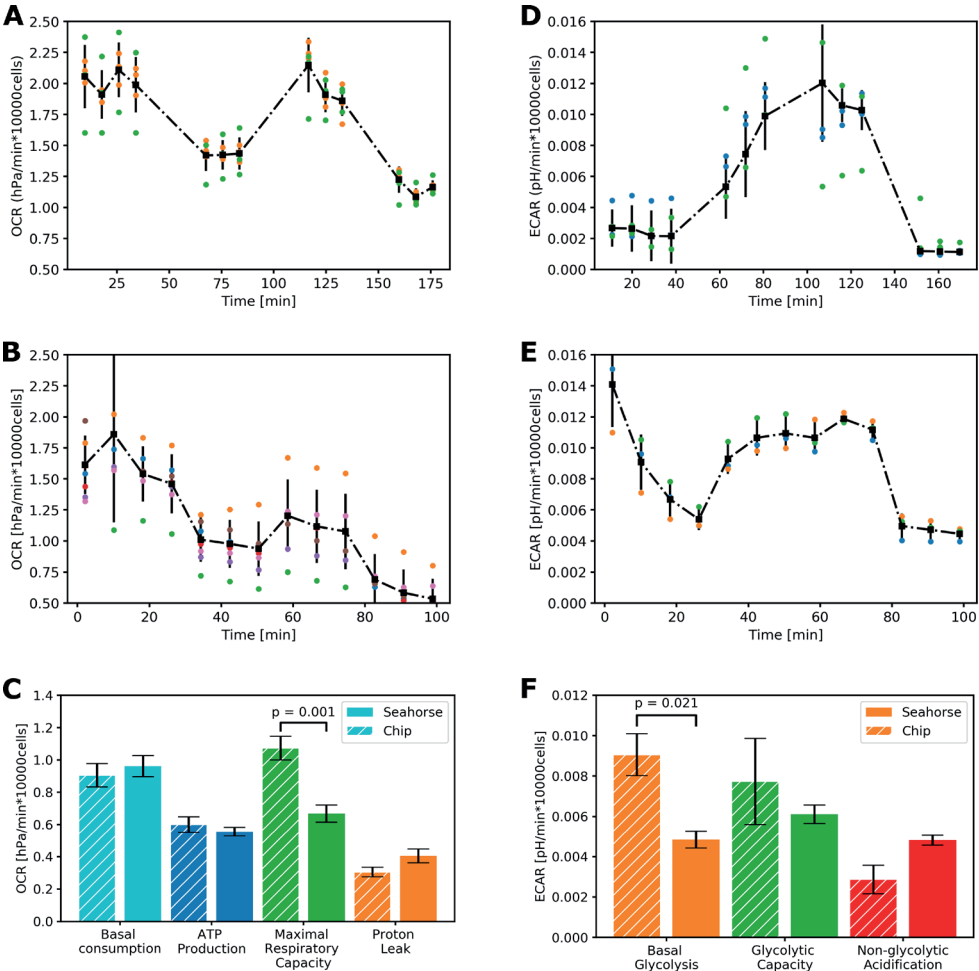


Figure 5) Comparison between measurements on-chip and in the Seahorse system.

A-C) The mitochondrial stress test assay with the chip (A, n = 6) is compared to the Seahorse (B, n = 7) and quantification of different respiratory states (C). D-F) The glycolytic stress test assay on the chip (D n = 4), compared to the Seahorse (E) and the quantification of the glycolytic capacity (F, n = 3), with the quantification of the glycolytic activity. All data represent mean $\pm$ standard deviation.

3.5 Comparison with existing systems

Recent studies have used either optical or electrochemical sensors to assess cellular metabolism inside microfluidic systems (Table 1). Most of these systems allow active perfusion of the cultured tissues and continuous refreshment of the culture medium. Some systems incorporated advanced 3D cell culture techniques, illustrating their ability to capture greater biological relevance than conventional 2D culture on plastic plates. Whilst the study here used 2D cell culture, the sensors have already been combined with 3D cell culture constructs¹⁰. Together, this shows flexibility for incorporating and accurately assessing more complex cultures under fluidic flow than in standard assays such as Seahorse.

Some sensor types used in commercial and custom-made systems interfere with the cells, making it challenging to assess cellular metabolism accurately. Intracellular sensors can directly interfere with the cells by sequestering intra-cellular oxygen, while electrochemical oxygen sensors actively consume oxygen while in operation, directly influencing the cell culture conditions and contributing to the depletion of oxygen. Within the small medium volumes often used in OoC-tissue culture, this oxygen consumption can directly impact the measurements and is, therefore, less reliable in assessing cellular metabolic flux (Table 1).

One of the significant advantages of the Seahorse system is the ability to carry out assays in 96-well plates in parallel. This scalability has made the system favoured for high-throughput data acquisition, important for rapid screening, statistical significance and, more importantly, biological relevance. Most published microfluidic systems do not allow easy scaling, which is increasingly required for OoC systems. The system here showed 8 microfluidic channels and 32 sensors in parallel. The readout device supports 48 sensors which, in principle, enables scale-up to 12 assays in parallel.

Table 1. Overview of previous publications using sensors for assessing metabolic flux.

System	Sensors	Perfusion	Tissue type	Platform	Throughput	Sensing principle	Sensor-tissue interaction	Intervention/medium exchange	Source
This work	Oxygen/pH	yes	2D	microfluidic chip	8-channels	Optical	no	yes/yes	This paper
Seahorse XF analyzer	Oxygen/pH	no	2D	self-contained	96-well	Optical	no	yes/no (treatment injection)	Ferrick et al., 2008 ⁶
MitoXpress	Oxygen/pH	no	2D	plate-reader/microscope	any well-plate	Optical	yes (for intra-cellular measurements)	no/no	Hynes et al., 2009 ⁷
HepaChip-MP	Oxygen	yes	Sinusoid	microfluidic chip	24 well	Optical	no	yes/yes	Busche et al., 2022 ¹⁷
Dornhof 2022	Oxygen/Glucose/Lactate	yes	Spheroids/3D	2-gel compartments	2-compartments	Electrochemical	yes (Sensors consume oxygen)	Yes/yes	Dornhof et al., 2022 ²⁸
Tanumihardja 2021	Oxygen/pH	No	2D	Chip	single-well	Electrochemical	yes (Sensors consume oxygen)	yes/no (treatment injection)	Tanumihardja et al., 2021 ²⁹
Liang 2019	pH	yes	2D	Microfluidic chip	single	Electrochemical	yes (Sensors consume oxygen)	yes/yes	Liang et al., 2019 ³⁰
Obeidar 2019	Oxygen/pH/Glucose/Lactate	no	2D/3D very small	Single-well chip	single	Electrochemical	yes (Sensors consume oxygen)	yes/no (treatment injection)	Obeidar et al., 2019 ³¹
Weltin, 2017	Oxygen/Lactate	no	3D	Electrical probe	96-well plate	Electrochemical	yes (Sensors consume oxygen)	yes/no (treatment injection)	Weltin et al., 2017 ³²
Bavli 2016	Oxygen/Glucose/Lactate	yes	Bioreactor	Reactor-on-chip	9 samples	Optical/ Electrochemical	No	Yes/yes	Bavli et al., 2016 ³⁴
Rennert, 2015	Oxygen	yes	2D	Microfluidic chip	9-channel	Optical	no	yes/yes	Rennert et al., 2015 ¹⁸

4. CONCLUSION AND OUTLOOK

In this study, we described a microfluidic chip with integrated optical oxygen and pH sensors that allowed monitoring of culture conditions and assessment of metabolic rates of cells with outcomes similar to that of standard assays in the Seahorse. We used hiPSCs as an exemplar to compare these systems directly since they are not transformed yet and have a relatively high metabolic rate. One of the challenges of OoC is the small culture volumes used in the microfluidic channels; the volume of culture medium available to the cells is much lower than in classical cell culture. A direct consequence is a more rapid depletion of oxygen and nutrients, the build-up of waste products and the changes in osmolarity due to evaporation and medium acidification, all of which can be detrimental to cells within OoC. Constant monitoring of pH and oxygen allowed us to monitor these medium changes during the initial setup of the microfluidic devices, providing essential information for optimizing protocols and ensuring consistent experimental conditions between repeated experiments. While initially challenged by inconsistent seeding outcomes within the chip, pH monitoring allowed us to identify an optimal protocol to create a consistent confluent monolayer of cells in the microfluidic channel.

In this work, we showed that optical sensors can accurately assess the metabolic flux of cells within a microfluidic device. Similar sensors have been used previously, which showed applications in revealing aspects of the biological responses of cells within OoC environments. However, none of the previous studies compared the outcome of metabolic assays in the cells head-to-head with more standard assays on the same cells. We assessed the metabolic rates of the cultured cells during stop-flow measurements and found that chip results were similar to the static measurements performed in the Seahorse. The mitochondrial- and glycolysis stress tests were also performed on both the chips and the Seahorse. We found very similar OCR and ECAR values in both systems, showing that the sensors can be reliably used in chips to assess ATP production rates from OXPHOS and glycolysis.

In sum, this study shows that our approach to combine optical sensors and stop-flow measurements can be used in microfluidic devices to obtain *in situ* data of similar relevance as the Seahorse system, with the added value that the cells can be cultured under physiological conditions with flow before performing the assay. This approach can be transferred to other microfluidic systems and OoC, as shown previously. This study sets the stage for follow-up studies using a multiplicity of differentiated hiPSCs as mono-cultures or multicell type combinations, and continuous quantification of metabolic activity in complex microfluidic cell culture devices.

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DECLARATION OF COMPETING INTEREST

The authors declare the following competing financial interest(s): T.M. is a founder, holds equity in PyroScience GmbH in Germany, and is the CEO of the Austrian branch, PyroScience AT GmbH. PyroScience is a developer, producer, and vendor of sensor technology. C.M. is co-founder of NCardia, a drug discovery company using hiPSCs.

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DATA AVAILABILITY

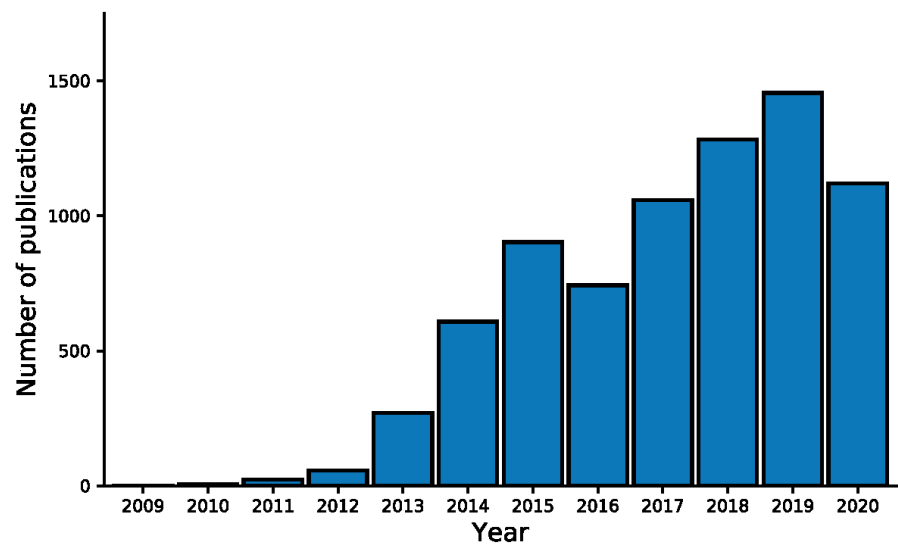
The raw data from this study are available from the authors upon request.

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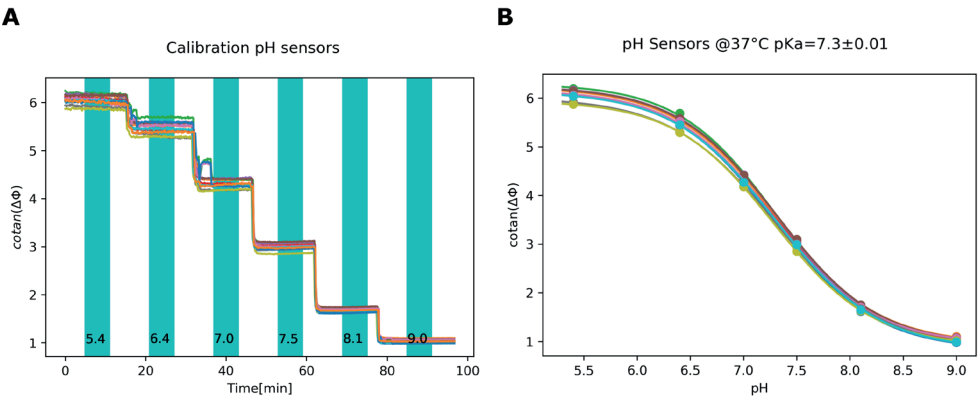
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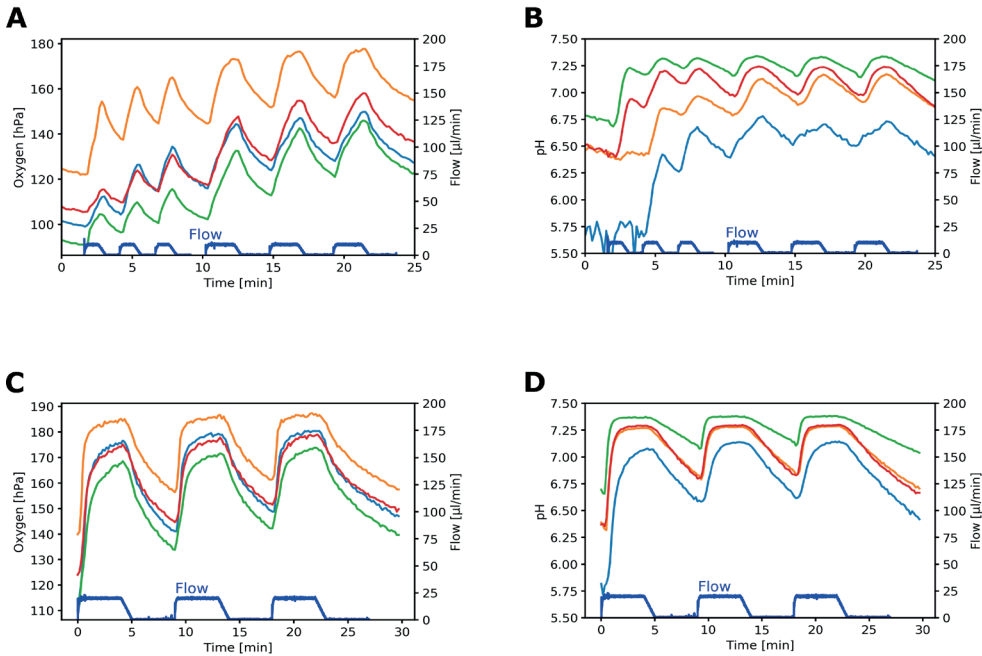
SUPPLEMENTARY DATA



Appendix Figure 1) Number of publications per year since the introduction of the Seahorse system³³.



Appendix Figure 2) Representative data of the response and calibration of the pH sensors.
A) Response of the pH sensor when exposed to buffered solutions at pH 5.4, 6.4, 7.0, 7.5, 8.1 and 9.0 at 37°C. B) Boltzmann fitted calibration curve on the obtained datapoints.



Appendix Figure 3) Optimisation of the microfluidic pump interval on the chip.

A-D) Measurements of oxygen depletion and resaturation within the microfluidic channels (A, C) and pH measurements showing the acidification and recovery (B, D) during different flow intervals. The blue line shows 3x flow intervals of 1 min on/off, followed by 2 min on/off with flow rates of 10 $\mu\text{l}/\text{min}$ (A, B), and stop-flow interval of 5 min on/off (C, D). The left side y-axis denotes the measured analyte, while the right side y-axis denotes the flow rate.

Appendix table 1: Microdispenser settings		
	Oxygen sensor	pH sensor
Tappet lift [%]	35	75
Rising time [ms]	0.2	0.3
Opening time [ms]	0.1	0.1
Falling time [ms]	0.15	0.1
Delay [ms]	0.1	0.1
Number of pulses	3	4
Number of repetitions	5	10



CHAPTER

General discussion

6

6. GENERAL DISCUSSION

Central to this thesis was the use of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) to model rare mitochondrial cardiomyopathy. In order to model this disease required the design of new methodologies to improve upon the current limitations of these cells as model systems, notably the variability in responding to differentiation cues and the immature state of the resulting cardiomyocytes (Sala *et al.*, 2017). The diseases we were interested in manifest soon after birth, but whether they initiate *in utero* is unknown, with only a single reported case of pre-natal pathology (Götz *et al.*, 2011). At the outset of the studies here, it was unclear, therefore, whether a phenotype would be evident in our standard 2D cultures when cardiomyocytes are at the equivalent of 16-18 weeks of gestation (Campostrini *et al.*, 2021b; Ulmer & Eschenhagen, 2020), or whether our 3D cardiac microtissue (MT) model would be required or could be refined to capture more salient features of the condition. Our focus was on the rare mitochondrial disease Combined Oxidative Phosphorylation Deficiency 8 (COXPD8) caused by mutation in the gene alanyl-tRNA synthetase 2 *AARS2*, where the mutation c.1774C>T seems to hold a founder effect. We refined our emerging 3D cardiac MT protocol (Giacomelli *et al.*, 2020) to improve robustness and reproducibility and reduce cost by basing differentiation on small molecules rather than growth factors (Campostrini *et al.*, 2021a) without altering the functionality of hiPSC-CMs. We also demonstrated that integrating pH and O₂ sensors in a micro-physiological chip was possible for the assessment of metabolic parameters under microfluidic flow. The overall goal of this thesis was to provide additional tools that would have utility in studying mitochondrial and other cardiac diseases.

Mitochondrial diseases are complex to study, in large part due to the heterogeneity of the pathology of the disease themselves, with thousands of known nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) mutations leading to a broad range of phenotypes affecting almost all tissue in the human body. Approximately 5-15 people per 100,000 are affected by some form of mitochondrial disease, but each pathology is exceedingly rare (Gorman *et al.*, 2016). This low prevalence makes studying the afflictions challenging, as large and diverse patient cohorts are difficult to recruit for trials and prospective studies. Animal studies do not provide suitable alternatives for investigating these pathologies, as many of the mutations found in human diseases cannot be recapitulated in mice. These difficulties are exemplified in cases of mitochondrial cardiomyopathies, where the pathology can differ greatly even with mutations affecting the same process (e.g. mutations in tRNA-synthetases and the tRNAs themselves).

Exploring mitochondrial cardiomyopathies in hiPSC-CMs models

In recent years, research using hiPSCs from patients has begun to contribute to studying rare familial diseases, as reviewed in **Chapter 1**. While many studies into individual cases of mitochondrial cardiomyopathies show promising results, further translation of these results into clinical applications for the patients has proven difficult. The lack of translation is in part due to hiPSC research often being underpowered. Many studies only shed partial light on mitochondrial impairments due to the low number of patient samples or, in a broader context, due to a lack of reproducibility in follow-up studies.

In **Chapters 2 and 3**, we investigated the application of hiPSC-CMs to model COXPD8 caused by mutations in *AARS2*. To ensure that we did not fall into the same pitfall as others by using only a single patient hiPSC line, we approached the problem by comparing two patients from the same family, their isogenic controls, and a control from a related individual. Whilst still relatively underpowered, the results of our study in derivatives of both patient lines showed hallmarks similar to other mitochondrial disease models, such as a reduction in oxidative capacity caused by altered mitochondrial function and a shift in metabolomic pathways revolving around NAD^+/NADH . Furthermore, we found reduced field potential duration in electrophysiological measurements and diminished calcium handling dynamics, leading to arrhythmia and altered contractile properties.

While significant work has been done to characterise mitochondrial behaviour in these types of pathologies, a lack of systemic nomenclature has made comparing results and interpretation difficult. Indeed, mitochondria are extensively malleable organelles, and a difference in any mitochondrial behaviour, often dubbed mitochondrial function or dysfunction, is not necessarily a pathological process; rather, it can be an adaptation to altered circumstances. In an attempt to work towards greater specificity without relying on all-encompassing terms such as function and dysfunction, Monzel, Enriquez, and Picard have written an extensive review that debates exactly this issue, ending with a proposed systematic classification system to describe all mitochondrial processes from higher- to lower level states (Monzel *et al.*, 2023). Their proposed system is based on distinguishing five levels to encompass the description of mitochondrial biology in a hierarchical manner. (1) cell-dependent mitochondrial properties, meaning characteristics that are only relevant in the full context of a cell, such as mitochondrial volume or density; (2) static molecular features, referring to molecular composition, protein dynamics, ultrastructure and morphology; (3) single-enzyme activities, such as the activity of enzymes of the ETC, membrane potential, protein or metabolite import and protein synthesis; (4) organellar functions, such as activities of the whole OXPHOS system, β -oxidation, Ca^{2+} regulation and ROS regulation, and (5) other behaviours seen on the scale of the whole organelle, such as fusion and fission, mobility and communication. In this thesis, we investigated 4 out of 5 of these categories, omitting only category #5. We found differences in all 4 of the levels measured, indicating that,

indeed, the *AARS2* mutations severely alter the mitochondria in this disease. Nevertheless, it remained difficult to identify the exact mechanism in which the mutation in *AARS2* effectuates these changes and how they cause further upstream functional defects at the level of the whole cardiomyocyte. The most immediate explanation would be that changes observed in the cell-dependent phenotypes and mitochondrial features, namely mitochondrial copy number, mitochondrial morphology and protein dynamics, lead to shifts in the mitochondrial membrane potential. These changes influence the observed shift in OXPHOS, NAD⁺/NADH concentrations and Ca²⁺ dynamics (Lan *et al.*, 2013; Moore *et al.*, 2023; Mosqueira *et al.*, 2019). These latter features have often been described in relationship to mitochondrial cardiomyopathies and hypertrophic cardiomyopathies (Kargaran *et al.*, 2021; Liu *et al.*, 2021; Yoon *et al.*, 2022).

However, while the majority of the observed phenotypes were evident in both patient lines, there were several assays in which patient 2 did not recapitulate phenotypes observed in patient 1, despite carrying the same mutation. The most striking of these were found in the calcium and contractile properties. Patient 1 showed altered calcium handling properties and an increased propensity towards arrhythmia in the calcium assays. However, patient 2 hiPSC-CMs did not differ from their isogenic control. Only once the cardiomyocytes were stimulated by electrochemical pacing were we able to distinguish the arrhythmic phenotype from the isogenic control for both patients. Other researchers have also observed that mitochondrial pathologies can present with a variety of different phenotypes, even when the mutations affect the enzyme or its substrate in a similar way. Many other mutations in the same gene can lead to a variety of phenotypes (Vafai & Mootha, 2012). However, this is unlikely to fully explain why we were not able to capture all observed phenotypes in both patient lines. Well-explored limitations of our 2D hiPSC-CMs models here, as mentioned above, cannot be ignored: these include the lack of most hallmarks of adult cardiomyocytes, absence of mature myofibers, ion-channels and mitochondrial genes, limited intracellular myofibre organisation as well as multicellular level organisation. In addition, immature cardiomyocytes rely mostly on glucose-derived ATP instead of β -oxidation (Campostrini *et al.*, 2021b; Guo & Pu, 2020; Lopaschuk & Jaswal, 2010; Ulmer & Eschenhagen, 2020). Therefore, the cardiomyocytes in this chapter had a phenotype resembling a foetal stage of development rather than post-natal, which partly could explain some of the observed discrepancies.

Increasing complexity of cardiac models

As the study of the mitochondrial disease progressed, it became evident there would be benefit in studying hiPSC-CMs in a more mature state. To that end, a method to promote the maturation of the hiPSC-CMs was developed, as presented in **Chapter 4**. In this chapter, we exploit the capacity of

cells to self-organise and promote their development when placed in a scaffold-free 3D organisation. We describe the protocols to create MTs consisting of 5000 cells, combining hiPSC-CMs, cardiac endothelial cells (cECs), and cardiac fibroblasts (cFBs). The protocol describes two different methods of inducing a “common” cardiac mesoderm, which can be used to derive all three cell types present in the MTs. The chapter further evaluates the structural and functional parameters of MTs 21 days post-generation, highlighting CM maturation. High-resolution immunostaining shows organised sarcomere structures. Mechanical properties are assessed through contraction video analysis, Ca²⁺ handling through Fluo-4 AM, and metabolic analysis using Seahorse XFe96. Electrical maturity is determined using sharp electrode analysis or single-cell electrophysiology. These MTs promote the development of the cardiomyocytes, cECs, and cFBs, leading to increased cell-to-cell cross-talk, and facilitated in part by increased cAMP signalling, which leads to increased sarcomeric organisation, mitochondrial OXPHOS, and the development of more adult-like electrophysiology and calcium handling (Giacomelli *et al.*, 2020).

Methods such as those described here, which promote the maturation of cardiomyocytes, can be utilised in modelling mitochondrial cardiomyopathies. However, it is important that each method be evaluated in the context of its strengths and weaknesses. The MTs described here offer clear advantages concerning their capability in modelling cell-specific phenotypes. The predetermined composition and separate differentiation of the three major cell types allow the creation of MTs in which a single subset of the cells carry the mutation. It is then possible to elucidate the effects of a mutation in each distinct cell population, increasing the confidence in determining which cell population contributes to the development of a pathology, as exemplified by the appearance of an arrhythmic phenotype when MTs were made using cFBs derived from patients with arrhythmogenic cardiomyopathy due to a mutation in the gene *PKP2* (Giacomelli *et al.*, 2020). An additional benefit is the relative ease of obtaining mature cardiomyocytes without the need to use high-tech Engineered Heart Tissues (EHTs), BioWires, bioprinting or Organ-on-Chip (OoC) constructs (Paloschi *et al.*, 2021; Stein *et al.*, 2021). This increase in maturation state was used in a study to elucidate the effect of a mutation in exon 6B of the gene *SCN5a*, an exon only expressed in mature cardiomyocytes. Here, the authors were able to elucidate the important function of MBNL1 as a crucial step in inducing the isoform switch, an effect only observed in the more mature cardiomyocytes obtained from MTs (Campostrini *et al.*, 2021a).

However, when studying diseases which primarily present with contractile defects, alternative methods to directly assess the contractile strength can be more relevant, such as the EHT platforms mentioned above, for which now many designs are available to allow miniaturisation (Mills *et al.*, 2017; Ribeiro *et al.*, 2022; Windt *et al.*, 2023), pacing (Ronaldson-Bouchard *et al.*, 2018) and vascularisation (Maoz *et al.*, 2017; Vollert *et al.*, 2014). Some also allow the application of afterload forces (Leonard *et al.*,

2018). Finally, there are now also models that allow the study of congenital and developmental heart defects. These heart organoids (or cardioids) derived by direct differentiation of hiPSC to heart cells mimic the development of the heart and result in a self-organised heart organoid resembling the ventricles of the heart (Branco *et al.*, 2022; Drakhlis *et al.*, 2021; Hofbauer *et al.*, 2021; Schmidt *et al.*, 2010; Volmert *et al.*, 2022). While these models are well-suited to the characterisation of early developmental defects also observed in infantile mitochondrial cardiomyopathies, we did not employ them within this thesis. Many of these models were still being developed when our research into mitochondrial cardiomyopathy started.

Further research into the phenotypes of our patient lines using these models could provide a new avenue for future investigation, allowing in-depth investigation of mechanisms underlying the functional phenotype from a developmental perspective, specifically the impact of the mitochondrial mutations on immature but nevertheless developmentally complete cardiac organoids. This might provide a platform for studying the relationship between (metabolic) cardiac maturation and the enhanced pathological phenotype observed in post-natal patients. Alternatively, 2D models in an OoC platform could perhaps provide more information on mitochondrial behaviour and metabolic output that might contribute to explaining the relationship of the mutations and the mitochondrial behaviour.

Finally, in **Chapter 5**, we investigated the use of optical oxygen and pH sensors for the continuous monitoring and evaluation of metabolic rates of cells in a microfluidic chip. One of the challenges in microfluidic OoC systems is the limited culture volume within the microchannels that leads to rapid depletion of oxygen and nutrients, accumulation of waste products, and changes in osmolarity due to evaporation and medium acidification. Continuous monitoring of pH and oxygen levels proved vital for optimising protocols and maintaining consistent experimental conditions in this study and others (Busche *et al.*, 2022). Our study demonstrated the effectiveness of optical sensors in accurately assessing cellular metabolic flux within a microfluidic device, matching results obtained from the gold-standard Seahorse assay.

While this study was a relatively simple demonstration, it does showcase the potential of continuous in-line monitoring during tissue culture. Clearly, continuous monitoring could prove crucial in uncovering more distinct phenotypes for metabolic and mitochondrial diseases, as the metabolic fluxes within the cell change over time due to the depletion of compounds from the medium. These differences in fluxes may be masked when relying on end-point assays, where the cells may exhibit similar phenotypes after several days in the same medium or when exposed to fresh batches of medium. This variation in fluxes is a well-known characteristic of yeast cultures, where for decades, the field has relied on bioreactors and continuous monitoring of pH and oxygen to provide cellular

mechanistic insights. Multiple studies have been reported which are already trying to incorporate these methods, relying either on read-outs of oxygen and pH (refs table from chapter 5), or more functional read-outs such as contractile strength of the cardiomyocytes on a platform such as the Cuore from Optix 11 (Iuliano *et al.*, 2023). Designing studies utilising these in-line measurements greatly enhances the fidelity of the disease model, enhancing the capacity to elucidate more complex behavioural differences that cannot be observed or deciphered with end-point assays.

Designing rare disease clinical trials in a dish

There is an important distinction between personalised (and the now more common term “precision”) medicine and a clinical trial-in-a-dish approach: each has its own important application in uncovering new avenues of treating patients (Ingber, 2022; Lam & Wu, 2021; de Korte *et al.*, 2020). The first focuses on discovering patient-specific phenotypes and sensitivities for treatments, increasing the specificity by which we can treat patients and improve treatment outcomes (Schork, 2015). This field of research focuses on the inherent variation between humans, which may be due to sex, race or socio-economic status, and endeavours to find which treatment would prove the most efficient for the individual patient: precision versus empirical medicine. These studies are dependent on disease models where a very specific phenotype can be observed and preferably linked to the success of treatment outcomes. Proof of principle for this approach has already been shown in colon cancer (van de Wetering *et al.*, 2015), cystic fibrosis (Beekman, 2016; Dekkers *et al.*, 2013) and steatohepatitis (Kimura *et al.*, 2022). However, for most diseases, such models do not yet (or never will) exist; thus, performing these studies has, thus far, been impossible. This is where (pre-)clinical trials in a dish can serve a purpose, both in identifying (novel) compounds and treatment avenues but most importantly, in solidifying the relationship between observed cellular phenotypes and the patient pathology.

All of the chapters in this thesis and the points discussed above revolve around creating better models for mitochondrial cardiomyopathies: they form some of the bricks in the road towards clinical trials-in-a-dish. The points on the statistical power of our studies and the confidence in our models contribute to increasing the robustness of our observations and further elucidation of phenotypes and pathology. Whether this is achieved through maturation, improved measuring techniques, or the use of a properly defined study design, nonetheless, they do not address one of the most pertinent issues in the field of modelling rare diseases, which is reproducibility. There are two factors contributing to the difficulty in the reproduction of both results and conclusions. The first is the standardisation of methods, an ongoing and active debate in the field, which can seriously confound the reproducibility of results within- and between users. At the same time, the second is the absence of additional patient-derived materials, such as primary tissue, which complicates assessing whether the

model truly reflects a relevant phenotype due to a lack of potential biological replication of previous studies. Of more recent concern is the increasing focus on sharing patient-derived cell lines between academic researchers among each other or with industry, where most drug discovery takes place, and the extent to which informed consent rules will limit research in the future. This concern pertains particularly to the patient's right to withdraw consent, currently up to the time that reprogramming to generate hiPSC-lines starts, but proposed in some jurisdictions to be "at any time". This jurisdiction will further complicate the sharing of hiPSC-lines, as any academic researcher or industrial partner must keep exact track of who is in possession of any of their derived material, but more importantly, can have the basis for their assays and drug discovery pipelines undermined at any moment due to such a withdrawal.

These complications lead to a striking paucity of available patient material, even in the cases where such (surplus) material could or is available; use is often restricted to a single study, with no opportunities (or sometimes legal inability) to share material so that independent validation of results is possible. This lack of patient material makes it difficult to conclude which of the observed phenotypes truly contributes to the pathology of a disease and which of the phenotypes are cell line, sex, ethnicity or patient-specific. Furthermore, without reproducing previously obtained results in additional labs, preferably with different patient-derived lines, meta-analysis cannot be performed to elucidate which of the observed phenotypes are a robust characteristic of the pathology or mutation. Certainly, in the stem cell field, whether using hPSCs or adult organoids, large cohort studies have been performed on more common diseases. However, for the majority of rare diseases, the high number of hiPSC-lines necessary for conducting such a study is still lacking (Brandão *et al.*, 2017; Brunner *et al.*, 2023; Sandoe & Eggan, 2013). Overcoming this limitation and facilitating more labs to access multiple diseased hiPSC lines with the same mutation or pathology would be greatly beneficial. Patient advocacy groups play a crucial part in advancing the acceptability of this kind of progress, as has already been shown by advocacy groups for diseases such as ALS, cystic fibrosis, Alzheimer's Disease and Duchenne Muscular Dystrophy. However, while the overall frequency of mitochondrial diseases is quite large, their broad differences in presentation result in a lack of a single strong advocacy group, and most individual mitochondrial diseases do not have such one as such. More attention must be paid to these rare diseases to ensure that researchers have access to sufficient patient material. This was a major challenge to the work presented in this thesis. An attempt to address this is already being made within the EU Horizon framework, with funding now available to set up such studies. A more serious effort must be made in recruiting patient populations and connecting clinicians, academic researchers, and industry (Annemans *et al.*, 2017; Hedley *et al.*, 2023; Rare diseases, 2023). Fostering these connections and allowing researchers and industry access to patient material would greatly benefit the field and would foster a network where larger research cohorts can be set up for clinical trial-in-a-dish studies and a faster translation and adaptation into industry.

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APPENDICES

Nederlandse samenvatting

Curriculum vitae

List of publications

Dankwoord

A

SAMENVATTING

Het hart is het eerste orgaan dat gevormd wordt in het menselijke embryo: de eerste hartslag te horen is 12 weken na bevruchting. Dit benadrukt de essentiële rol die ons hart speelt in de ontwikkeling van ons lichaam, en in het behouden van de lichamelijke homeostase. Het gevolg hiervan is dat wanneer het hart een verminderde capaciteit heeft, of compleet faalt, dit directe en zware gevolgen heeft voor de rest van het menselijke lichaam. Hartfalen is daarmee een levensbedreigende ziekte die jaarlijks nog steeds tot de meeste doden leidt wereldwijd. Naast hartinfarcten zijn cardiomyopathiën een belangrijke oorzaak van hartfalen. Bij cardiomyopathiën is het functioneren van de hartspier verminderd met een verminderde pompfunctie en circulatie tot gevolg. Veel van deze cardiomyopathiën zijn erfelijk en worden veroorzaakt door mutaties in ons DNA. Een zeldzame vorm van deze ziektes treft ook kinderen, waarbij vaak de mitochondriën, de energiefabrieken van onze cellen, aangetast worden door deze mutaties.

Deze vorm van hartfalen, genaamd mitochondrial infantile cardiomyopathy, is erg moeilijk om te bestuderen, de patiëntengroep is klein en erg kwetsbaar, en de ziekte presenteert zich vaak pas wanneer deze al in een vergevorderd stadium is. Het is moeilijk om te achterhalen hoe de progressie van de ziekte is geweest en hier de juiste interventies voor te vinden. Ook is gebleken dat diermodellen de ziekte vaak niet goed kunnen nabootsen doordat de genen tussen muizen en mensen niet genoeg overeenkomen. Om deze redenen is er groeiende interesse in het gebruik van pluripotente stamcellen om menselijke ziektes na te bootsen. Deze cellen zijn in staat om te groeien en ontwikkelen naar elke cel in het menselijk lichaam, en kunnen worden gemaakt van ieder individu door het nemen van een klein huidbiopt, of wat bloed of urine. Met deze zogenoemde induced pluripotent stem cells (hiPSCs) kunnen hartcellen gemaakt worden van patiëntenpopulaties met zeldzame ziektes om zodoende het proces van de vorming van hartcellen naar het zieke hart te volgen. **Hoofdstuk 1** geeft een overzicht van de huidige stand van zaken in de literatuur en in **hoofdstuk 2 & 3** van deze thesis worden er hiPSCs gemaakt van twee kinderen met een zeldzame vorm van mitochondriële infantiele cardiomyopathie die heeft geleid tot hun dood binnen twee weken na geboorte, en hun moeder die dient als een gezonde controlepersoon. In deze hoofdstukken ligt de focus op het functioneren van deze hartcellen en de mitochondriële en metabole toestand van deze cellen. Er wordt gebruik gemaakt van een 2D *in vitro* systeem, waarbij de hartcellen die gemaakt zijn van de patiënt-stamcellen (hiPSC-CMs) gezamenlijk groeien in een vlakke plaat en hierop bestudeerd kunnen worden. Door middel van CRISPR/cas9 worden de mutaties die de ziekte veroorzaken in deze patiënten gecorrigeerd om vervolgens de door de mutatie aangedane hiPSC-CMs te kunnen vergelijken met de gecorrigeerde hiPSC-CMs. In **hoofdstuk 3** ligt de focus op het zoeken naar hoe de mutaties verandering tot stand brengen in de mitochondriën en in het functioneren van de hartspiercel zelf, van de generatie van het actiepotentiaal (het elektrische signaal van het hart) tot de samentrekking van de hartspiercellen.

De bestudeerde mutaties leiden in ons model tot een verminderde functie van de mitochondriën, waardoor de hartspiercellen minder energie tot hun beschikking hebben en er een tekort is van andere belangrijke metabole signaal moleculen. Dit heeft tot gevolg dat deze hiPSC-CMs slechter in staat zijn om samen te trekken als spiercel en ook spontane hartritmestoornis vertonen. Echter waren wij niet in staat om de ver gevorderde staat van deze ziekte aan te tonen, waarbij hypertrofie (een vergroting van het hart) een kenmerkend fenotype is. De reden dat pathologiën als hypertrofie nog slecht aantoonbaar zijn in deze 2D *in vitro* heeft ermee te maken dat hiPSC-CMs niet hetzelfde ontwikkelingsproces doorgemaakt hebben als de hartspiercellen in het hart; ze missen de juiste omgevingsfactoren om zich te ontwikkelen tot volwassen hartcellen.

In **Hoofdstuk 4** wordt een methode omschreven om de verdere maturatie van de hiPSC-CM's te bevorderen. In dit hoofdstuk benutten we het vermogen van cellen om zichzelf te organiseren en hun ontwikkeling te bevorderen wanneer ze in een 3D-organisatie worden geplaatst omringd door andere cellen die een ondersteunende functie in het hart spelen. We beschrijven de protocollen om zogenaamde microtissues (MTs) te maken van hiPSCs, bestaande uit de drie meest voorkomende cellen van het hart: de hartspiercellen zelf, cardiale endotheelcellen (cECs) en hartfibroblasten (cFBs). Het protocol beschrijft twee verschillende methoden voor het induceren van een "gemeenschappelijk" hartmesoderm, een precursor cel populatie waaruit deze drie celtypen af te leiden zijn. In dit hoofdstuk beschrijven we ook verdere methodes om de structurele en functionele parameters van deze MTs te karakteriseren en te kunnen gebruiken als mogelijk ziekte model.

Naast het plaatsen van hiPSC-CMs in een 3D-omgeving, in een niche zoals we die ook in het hart tegenkomen, is er ook een andere recente ontwikkeling geweest voor het bevorderen van de maturatie van hiPSC-modellen, de zogenaamde Organ-on-Chips (OoCs). Hierbij worden cellen in een omgeving geplaatst die beter lijkt op de fysiologische toestand van een specifiek orgaan, waarbij er niet alleen gekeken wordt naar de andere celtypen, maar ook naar de functionele vorm en naar de mogelijkheden van perfusie. Veel van deze modellen maken gebruik van microfluidische-kanalen en -perfusie en nieuwe meet- en detectie-methoden om continue te kunnen meten hoe het mini-orgaan zich gedraagt. In **Hoofdstuk 5** gebruiken wij een dergelijk model, waarbij de integratie van pH- en zuurstof-sensoren in een microfluidisch kanaal gebruikt wordt om live de metabole staat van hiPSCs te volgen. Door het volgen van deze metabole staat kunnen de weefselweekomstandigheden geoptimaliseerd worden. Dit is essentieel om ervoor te zorgen dat de cellen in dit soort OoC-modellen zich in de juiste toestand kunnen bevinden en zich daarbij het meest gedragen zoals ze dat ook zouden doen in het menselijke lichaam. Dit geeft meer vertrouwen in de wetenschappelijke resultaten die uit deze modellen behaald kunnen worden aangezien het waarschijnlijker is dat deze de daadwerkelijke toestand van de menselijke fysiologie juist nabootsen.

Tot slot zijn in **hoofdstuk 6** alle resultaten en perspectieven van deze thesis samengevat. De behaalde wetenschappelijke resultaten in deze thesis dragen enerzijds bij aan de kennis hoe mitochondriële infantiele cardiomyopathiën ontstaan en het doorontwikkelen van meer betrouwbare hartstamcelmodellen. Dit alles is deel van de weg die uiteindelijk moet leiden tot stamcelmodellen die de menselijke fysiologie zodanig kunnen nabootsen dat de ziekteprocessen nagebootst kunnen worden. Wanneer dit het geval is, kunnen deze modellen ingezet worden voor zogenaamde “clinical-trials-in-a-dish” en “personalised medicine”, waarbij deze stamcelmodellen, en niet dierproeven, de basis zullen vormen voor het testen van effectiviteit en specificiteit van nieuwe medicijnen.

CURRICULUM VITAE

Ruben Willem Joseph van Helden was born on march 6th 1990 in 's Gravenhagen in The Netherlands. He commenced his Bachelor's degree in Biomedical Sciences at Leiden University in 2010. He completed his bachelor's with a four-month internship in the Leiden University Medical Center (LUMC) at the Department of Parasitology under the supervision of prof.dr. Maria Yazdanbakhsh with the aim of studying the relationship between *Schistosoma*, the development of allergies, and serum immunoglobulins in a cohort of children from Ghana.

After his bachelor's, Ruben pursued a Research Master's in Biomedical Sciences at the LUMC where he started to develop his interest in stem cell research. His first foray into this was a five-month research internship at LUMC in the Department of Nephrology in the lab of prof.dr. Eelco de Koning, where he investigated the pancreatic duct niche for a potential primary stem cell candidate. Subsequently, he continued to work as a research assistant at the Department of Nephrology for prof.dr. Ton Rabelink, analysing electron microscopy images of the glomerulus. During this period, he was also a member of the Biomedical Sciences yearly student representation committee and a student member of the Education Committee for the study program. Finally, he completed his master's with an eight-month internship under prof.dr. Kevin Eggan at the Harvard Department of Stem Cell and Regenerative Biology. Here, he investigated the role of *SOD1* mutation in the development of amyotrophic lateral sclerosis in human induced pluripotent stem cell (hiPSC)-derived motor neurons and the development of the action-potential initiation segment in these motor neurons. For this work, he was awarded scholarships from the Dutch ALS fund, the Leiden University International Study Fund, the Minerva Scholarship Foundation, and the Jo Keur Fund. His research proposal was nominated for the Janne Fruin-help prize and his master's thesis was nominated for the LIFS prize.

Ruben's interest in stem cell research flourished, and he started his PhD in 2016 under the supervision of prof.dr. Christine Mummery, dr. Milena Bellin and dr. Valeria Orlova in the Department of Anatomy and Embryology at the LUMC. During this period he studied the effects of mutations leading to mitochondrial infantile cardiomyopathy in hiPSC-derived cardiomyocytes, specialised in metabolic assays, and developed an interest in developing a more complex *in vitro* model and the organ-on-chip field. During this period, he co-started and was a member of the early researcher committee for both the local and international reNEW consortium, funded by Novo Nordisk. He was additionally awarded a travel grant and presentation award at the Dutch-German cardiovascular meeting. In 2022 he continued his work as a post-doc and lab manager under prof.dr. Christine Mummery for the reNEW consortium.

Since 2023 Ruben has moved to Vienna to work as a senior scientist at the start-up company HeartBeat.bio working toward the development of a high-throughput 3D screening platform for heart diseases using self-organizing human cardiac organoids.

LIST OF PUBLICATIONS

1. Arslan U, Brescia M, Meraviglia V, Nahon DM, **Helden RWJ van**, Stein JM, Hil FE van den, Meer BJ van, Cuenca MV, Mummery CL, *et al* (2023) Vascularized hiPSC-derived 3D cardiac microtissue on chip. *Stem Cell Rep* 18: 1394–1404
2. Brandao KO, **van Helden RWJ**, van den Brink L, Blanch-Asensio A, Mol MPH, Kuipers T, Mei H, Mummery CL & Davis RP Investigating the molecular consequences of KCNH2 compound mutations in hiPSC-derived cardiomyocytes. Proefschrift (2023) Karina O. Brandão chapter 6
3. Campostrini G, Meraviglia V, Giacomelli E, **van Helden RWJ**, Yiangou L, Davis RP, Bellin M, Orlova VV & Mummery CL (2021) Generation, functional analysis and applications of isogenic three-dimensional self-aggregating cardiac microtissues from human pluripotent stem cells. *Nat Protoc* 16: 2213–2256
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