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

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CHAPTER 6

General discussion

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 O_2 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 Ca^{2+} 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 near 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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