Chapter 7

Discussion and Future Perspectives
Discussion

Heart Failure

With a prevalence of 1-2% and a very poor prognosis with a five-year mortality rate of 75%, heart failure represents a major unmet clinical need.[1,2] New treatments that have become available over the past few decades have led to incremental improvements in the survival of heart failure patients, however the prevalence remains high and with an aging population an epidemic of heart failure seems inevitable.[3,4] An improved understanding of the pathophysiological underpinnings of heart failure is essential for the development of new treatments to fundamentally improve the outcome of heart failure patients. In this thesis, we have endeavored to increase our understanding of the biology of heart failure by using human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) and the novel BASiC method to investigate iron deficiency induced cardiac dysfunction and doxorubicin (DOX) induced cardiotoxicity.

Functional Phenotyping of Human Pluripotent Stem Cell-Derived Cardiomyocytes

Over the past few decades, human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) have emerged as an attractive model to study human cardiovascular disease. hPSC-CMs partly recapitulate the physiology of in vivo cardiomyocytes. They express most of the cardiac-specific ion channels and currents, have a functional contractile apparatus and respond to cardioactive drugs.[5] To unlock the full potential of hPSC-CMs, the development of methods for the detailed functional phenotyping of these cells is of major importance.

To date, the simultaneous assessment of calcium cycling, action potential characteristics, contractile kinetics, and force generation has been challenging. Current methodologies geared at assessing myocardial contractility of adult CMs typically rely on edge detection techniques that directly assess CM shortening [6] or the change in sarcomere striations over time.[7] These approaches require cells with clearly defined cellular borders and distinct striations, limiting their applicability to less mature cell types.[8] Likewise, current TFM approaches for the measurement of contractile force of single myocardial cells require the use of fluorescent microspheres, limiting their capacity for use in conjunction with other fluorescence-based physiological assays.[9,10] Other approaches, including optical flow analysis, yield a unit-less or dual peaked curve rather than standard fractional shortening and force generation curves.[11-13]

The first part of this thesis describes the development of a method for the integrated analysis of hPSC-CM function that addresses the limitations of existing methods. Chapter 2 lays out a method that allows for the quantitative analysis of contraction kinetics by analyzing the changes in cell morphology over time, this method was termed Baseline Adjusted Similarity Comparison (BASiC). A similarity matrix describing the similarity between frames in movies of a contracting rod-shaped hPSC-CM is generated. From this matrix a signal of contractility is derived, as expressed in fractional shortening. Importantly, the outcome of this novel method of generating fractional shortening curves was extensively validated versus manual measurements in hPSC-CMs. Additionally, the
fractional shortening curves were validated in adult mouse CMs that were isolated by Langendorff heart perfusion. The BASiC method presented in Chapter 2 showed very high degrees of agreement for fractional shortening curves with edge detection and sarcomere length measurements, the current golden standard methods of assaying adult CM contractility.

Fractional shortening is an interesting readout of the contractility of CMs and indeed it has often been used as such. However, fractional shortening is heavily influenced by the stiffness of the CM’s surroundings. When in suspension, CMs encounter very little resistance when contracting and will display higher rates of fractional shortening. Conversely, when CMs are attached to common lab materials such as polystyrene or glass they display hardly any fractional shortening. [6] Rather, they display a twitching motion with each attempt at a contraction. Chapter 2 describes the culture of hPSC-CMs on a flexible PDMS substrate that allows for fractional shortening of the cells up to 10-20% with each contraction. Other reports have also made use of flexible substrates that allow hPSC-CMs to shorten.[14] However, these methods use substrates of an undefined stiffness. Importantly, the stiffness of the PDMS substrate used in Chapter 2 has a defined stiffness of 5kPa. [15] The contraction curve generated by the BASiC method is subsequently combined with the stiffness of the PDMS substrate and a biomechanical model to concurrently calculate the force generated by hPSC-CMs with each contraction as they deform the flexible substrate. Force generation is the primary function of the heart, allowing for the pumping of blood into the body’s circulation against the aortic blood pressure. Therefore, the calculation of force generation forms a great advantage compared to other methods that assess contractile kinetics only. The biomechanical model to calculate force generation was again successfully validated against the golden standard of Traction Force Microscopy using fluorescent microbeads.[10]

Notably, the BASiC method allowed for the analysis of contractile kinetics and force generation without the use of fluorescent microbeads or probes. This permitted the use of fluorescent probes to simultaneously analyze the membrane potential and intracellular calcium levels of hPSC-CMs. This combination of approaches allowed for simultaneous analysis of the major readouts of CM function: contractile kinetics, force generation, electrophysiology, and calcium handling. In accordance with other reports, hPSC-CMs in our model showed appropriate dose-dependent responses to isoproterenol and verapamil. [12,16-19]

Furthermore, Chapter 2 describes a proof-of-principle experiment underscoring the importance of the integrated analysis of hPSC-CMs for cardiotoxicity screening. After exposure to dofetilide, a known cardiotoxic drug, hPSC-CMs displayed clear electromechanical dissociation. In some hPSC-CMs the action potential signal was unchanged while the contractile activity was severely disturbed. Quantification of this phenomenon clearly demonstrated an increase in sensitivity for the detection of cardiotoxicity by combining functional readouts. Over the past decades, numerous drugs development projects have been halted by concerns over cardiotoxicity. Additionally, numerous drugs had to be recalled from the market after approval. Because significant differences exist between humans and rodents, hPSC-CMs have been suggested as a promising alternative to rodent adult CMs. Several studies using hPSC-CMs for cardiotoxicity screening have been performed with different readouts of toxicity, such as increased action potential duration and the occurrence of early after depolarizations. [20,21] Chapter 2 demonstrates the rapid detection
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of drug-induced cardiotoxicity by assessing contractility and action potential simultaneously. The finding that excitation-contraction coupling may be perturbed under toxic conditions suggests that simultaneously assessing action potential characteristics and contractile behavior may improve the sensitivity of drug-induced cardiac toxicity screening. In fact, dofetilide was initially approved for the treatment of atrial fibrillation. However, reports of fatal Torsades de Pointes in patients prompted the producer Pfizer to voluntarily withdraw this approval.[22]

Chapter 3 sets out a protocol for the use of the method described in Chapter 2. A drawback of the methodology described in Chapter 2 is the limited amount of rod-like CMs available for study. When hPSC-CMs are seeded onto PDMS substrates at a low density, many cells assume irregular shapes. Moreover, many hPSC-CMs arrange in clusters of random size and shape. A widely used approach to control the shape of cells microcontact printing. With this technique, extracellular matrix proteins are deposited on substrates in a user-defined pattern.[9] However, hPSC-CMs were plated on stiffer substrates that did not allow for physiologic amounts of fractional shortening during contraction, making these approaches less suitable for the analysis of contractile function. At the same time, microcontact printing on soft substrates is technically very challenging due to the sticky nature of soft substrates such as the PDMS 527 used in Chapter 2. Therefore, Chapter 3 also describes a method for microcontact printing of proteins on a soft substrate. The described method is adapted from Yu et al.[23] Non-toxic polyvinyl alcohol (PVA) films are created and microcontact printing is initially performed on these films. The protein micropattern is subsequently transferred to the soft PDMS substrate through conformal contact. Next the film is dissolved using PBS washes, absolving the need to remove the PVA film from the sticky PDMS substrate. Microcontact printing of 100 x 20 μm rectangles using this method allows for the generation of a large number of rod-like CMs. Alternatively, by using a micropattern with larger rectangles of 240 x 60 μm, integrated anisotropic cardiac microconstructs can be generated.

Cardiovascular Disease Modelling: Iron Deficiency

Chapters 4 and 5 focus on the application of the methods developed in Chapters 2 and 3 for cardiovascular disease modelling. Chapter 4 examines the effect of iron deficiency on hPSC-CMs. Iron deficiency is a highly clinically relevant co-morbidity, present in 40% of patients with chronic heart failure, even in non-anaemic patients,[1,24-26] and is related to impaired exercise capacity, reduced quality of life and a worse prognosis.[27-30] In addition to its key role in oxygen uptake and transport as a part of haemoglobin, iron has an important role in cellular oxygen storage and metabolism, redox cycling and as an enzymatic cofactor. Therefore, maintaining a normal iron homeostasis is crucial for cells that have a high energy demand such as cardiomyocytes. Chapter 4 demonstrates that iron deficiency directly affects human cardiomyocyte function, impairing mitochondrial respiration, and reducing contractility and relaxation. A rescue of this phenotype by restoration of intracellular iron levels is also shown. The data presented in Chapter 4 is supported by other findings in the literature. An upregulation of the transferrin receptor (TfRC) was found in response to intracellular iron deficiency. This is corroborated by the finding of Melenovsky et al., demonstrating that cardiac explants of patients with end-stage heart failure with the lowest iron content had the highest expression of TfRC.[31] Additionally, Seahorse Mito Stress testing
showed that intracellular iron deficiency resulted in a reduced OXPHOS chain activity affecting basal respiration, ATP synthase-linked respiration and maximal respiration. This was driven by a reduced OXPHOS chain activity of complexes I, II and III, which are highly dependent on iron–sulfur clusters for their functioning. These findings are in accordance with the published literature. Indeed, reduced activity of complexes I–III in states of iron deficiency has also been found in both animal and human myocytes.[31,32] Depletion of the intracellular iron in hPSC-CMs also resulted in reduced contractile function. A similar finding was reported by Haddad et al., showing that iron-deficient mice exhibit a reduced contractile reserve during dobutamine challenge due to a declining phosphocreatine/ATP ratio.[33] Moreover, it has previously been shown that such a loss in contractile reserve (as demonstrated by the loss of a positive force–frequency relationship) might also occur in heart failure patients with iron deficiency during exercise.[34] Furthermore, iron deficiency induced a switch in the hPSC-CM model presented in Chapter 4 from oxidative phosphorylation towards anaerobic glycolysis. A similar shift has been found in the failing heart.[31] Finally, the hPSC-CM model shows that the effects of iron deficiency are mostly reversible. This is in line with the results of three clinical trials, showing that repletion of iron stores with intravenous ferric carboxymaltose reduces heart failure hospitalizations and mortality.[35]

Of note, the cardiac microconstructs as described in Chapter 3 were not able to withstand the iron deficiency protocol, therefore iron deficiency was induced in monolayers. This could be due to the very severe iron deficiency that was induced, with for example decreases in ATP production of 74%. It appears as though monolayers had a higher threshold before apoptosis was induced, possibly due to the shuttling of iron from relatively iron-rich hPSC-CMs through gap junctions to the most severely affected cells.

Cardiovascular Disease Modelling: Doxorubicin Induced Cardiotoxicity

Chapter 5 examines the effect of several different treatments of DOX on hPSC-CMs. Cardiotoxicity has been observed for decades after the use of DOX for cancer treatment. These therapeutics induce cardiotoxicity due to direct, non-selective damage to the myocardium. As a result, the risk of cardiovascular morbidity and mortality among survivors remains increased until decades later.[36] Chapter 5 concludes that the cardiotoxic effects of DOX are mediated by mitochondrial dysfunction and impaired contractility. Differential responses after exposure of hPSC-CMs to a single low doses, two low doses, and a single high dose of DOX were observed. Interestingly, a large difference in cardiotoxicity was observed between a single low dose and two low doses of DOX. The fractional shortening of hPSC-CM derived cardiac microconstructs, a measure of their contractility, was significantly impaired after two low doses of DOX while not being impaired after a single low dose of DOX. This phenotype could be explained by severe mitochondrial dysfunction and loss of sarcomeric integrity, both of which were not observed after a single low dose of DOX. Conversely, after a single high dose of DOX a large amount of oxidative stress and cell death was observed. Previous reports have ascribed the cardiotoxicity of DOX to cell death and alternatively cardiomyocyte dysfunction.[37-44] Chapter 5 demonstrates that these differing results might be due to different doses of DOX being used, with low doses leading to cellular dysfunction and high doses leading to cell death. Indeed, previous studies involving hPSC-CMs have shown increasing amounts of
apoptosis with increasing doses of DOX.[38-41] Cancer treatment as received by patients is more closely mimicked by repeated low doses than a single doses of DOX of various concentrations, therefore Chapter 5 concludes that the cardiotoxic effects of DOX are predominantly mediated by impaired mitochondrial dysfunction and loss of sarcomeric integrity leading to impaired contractile function of CMs.

Further study is required to solidify the findings of Chapter 5. Additionally, further study is needed to elucidate the primary mechanism by which DOX causes cardiotoxicity. In this way, interventions in this mechanism could be explored to find options to prevent DOX induced cardiomyopathy.

**Maturation Status of Human Pluripotent Stem Cell Derived Cardiomyocytes**

One major limitation to any endeavor to model human heart disease using hPSC-CMs is the immature state in which these cells reside. For example, hPSC-CMs are often round and much smaller than adult CMs. Additionally, hPSC-CMs have shorter sarcomeres with protein isoforms that are found in fetal CM and as a result produce much less force than adult CMs. The electrophysiology, calcium handling, and metabolism similarly display fetal-like characteristics. To overcome this obstacle, various approaches have been applied to mature hPSC-CMs, such as long-term culture, biochemical stimulation, electrical stimulation, and mechanical stimulation.[45-48] Most papers describe significant increases in the maturation state of hPSC-CMs after the respective interventions, however it is often unclear how these incremental improvements compare to adult CMs. To put it simply, two questions arise: i) “where is the finish line?” and ii) “where are we?” Addressing these questions could help the field move forward in a more methodical way and shed light on the progress that has been made in solving this obstacle thus far. We try to answer these questions in Chapter 6. The natural maturation program is compared with the immature state of hPSC-CMs. Unlike other reviews on the topic which offer a general comparison, we have quantified specifically the maturation of fetal, neonatal, infant, and adult CMs *in vivo*. In doing so, we provide a framework for a semi-quantitative comparison with hPSC-CMs. Using this framework, we have developed the Maturation Score and used it to assess the success of various maturation strategies. We are in the process of developing a web-based tool that other researchers can use to assess the maturation state of their hPSC-CMs and compare the results to other published articles and adult CMs.

**Future Perspectives**

Science often progresses in leaps and bounds. Striking examples of this phenomenon are the development of the printing press by Johannes Gutenberg in 1439 and the invention of an alternating current motor by Nikola Tesla in 1888, giving rise to respectively the widespread distribution of information and electricity. This subsequently caused dramatic advances in many unrelated scientific fields. More recently, the discovery of induced pluripotent stem cells and CRISPR-Cas9 gene editing technology have similarly paved the way for an array of major biological discoveries.[49] As science and history in general rarely progress in a linear fashion for prolonged periods, it is very challenging to predict events occurring beyond the very near future with any certainty. Discoveries in fields
wholly unrelated to medicine can have major impact on the practice of medicine. One classic example of this phenomenon is the incidental discovery of X-rays by Wilhelm Roentgen in 1895. News of this discovery rapidly spread across the world. Nikola Tesla was among the first scientists to test this technology in the US that same year and shortly afterwards surgeons were using the technology to guide their work.[50] Similarly, future discoveries in fields wholly unrelated to the biology of cardiovascular disease of which at present we have no conception, could hold the key to solving the major issues facing the effective treatment of heart failure today. However, based on the trends in heart failure epidemiology and stem cell-based cardiovascular disease modelling that we have observed over the past few years and assuming a linear trajectory of these trends, certain predictions can still be reasonably made.

In the near future heart failure will pose an even larger problem to societies worldwide than it does now. With the aging of the population in the Western world the prevalence of heart failure will increase further. Moreover, improved outcomes of myocardial infarctions with the widespread use of PCI technology in the developing world will rapidly increase the prevalence of heart failure in these countries as well. Therefore, the world is currently heading towards a heart failure epidemic that will heavily burden healthcare systems worldwide. Large advances in the treatment and prevention of heart failure are needed to stave off this epidemic.

Significant advances in the pharmacological treatment of heart failure have been made over recent decades with the introduction of beta-blockers, ACE inhibitors, angiotensin II receptor blockers, and mineralocorticoid receptor antagonists.[51] However, recent clinical trials of new drugs for heart failure have often failed to demonstrate improvement on the current standard of care. As a result, the introduction of new drugs for heart failure has waned over the past 20 years. Unfortunately, this is not due to having reached a finish line where treatment of this disease cannot be further improved, as evidenced by the still poor prognosis of heart failure at present. More likely, the lack of new drugs being approved for heart failure is due to the fact that new drugs being tested are often based on the same paradigms of the currently approved drugs for the treatment of heart failure.

The current categorization of heart failure patients based on ejection fraction is likely a great oversimplification of a wide array of underlying pathophysiology. To stave off a future heart failure epidemic, it is crucial that we gain a better understanding of the pathophysiology of heart failure grounded in basic science to inform the development of new treatment options. For example, a better understanding of cardiotoxicity of various compounds, myofilament isoform switching, cardiac remodelling, and aberrant cardiomyocyte metabolism could inform the development of new drugs to counter these processes involved in heart failure. Moreover, the lack of efficient pharmacological treatment for the large group of patients with heart failure with a preserved ejection fraction underlines the importance of better understanding basic mechanisms that lead to heart failure.

An improved understanding of the pathophysiology of different forms of heart failure will lead to more tailored therapy which will be steered by much more information than we use today. For example, personal genomic data, a more holistic measure of cardiac function, and perhaps even the inclusion of big data based on the information gathered through our mobile devices could help
to inform personalized preventive measures, pharmacological treatment, exercise programs, and device therapy.

We envision that in the near future, hPSC-CMs will be used even more than today for cardiotoxicity assays, cardiovascular disease modelling and drug discovery assays within academic research. Moreover, with improvements in standardization of hPSC-CM cell culture and high-throughput assays of hPSC-CM function, we expect that biotech companies and the pharmaceutical industry will start to fully leverage this technology for drug development purposes.

hPSC-CM based cardiovascular disease modelling will likely be a very important avenue of research that will lead to a better understanding of heart failure. In this regard, CRISPR-Cas9 gene editing technology and hPSC-CMs will also play an important role. We believe that CRISPR-Cas9 will be used more broadly to understand the effect of specific mutations on various aspects of hPSC-CM function. Additionally, using CRISPR-Cas9 to repair a mutation in hiPSC-CMs can demonstrate more specifically the role of that mutation in causing the disease affecting the patients from which the hiPSC-CMs were derived. Ultimately, such proof of concept approaches for gene therapy in a dish could provide the basis for clinical trials using the same approach.

The further development of hiPSC-CM technology could eventually spark an era of truly personalized medicine in the pharmacological treatment of heart failure where the treatment of patients can be tailored to the response of hiPSC-CMs to various drugs. This approach however, does not appear possible nor valid in the very near future.

In which format hPSC-CMs will be utilized within academia and industry remains to be seen. More complex 3D-culture systems promise more physiological readouts, however they are usually less high-throughput by design. As such, more complex culture systems will have to be justified by demonstrating superior readouts within a specific experiment, especially for industrial purposes. It is likely that depending on the purpose of a study, hPSC-CMs will continue to be used in formats ranging from a monolayer to complex organ on a chip approaches with vascularized 3D cardiac tissue constructs containing other cardiac cell types. For cardiotoxicity screening the utility of hPSC-CMs has already been demonstrated by many recent studies, including Chapter 2 of this thesis. Therefore, we believe that a superiority over isolated animal CMs will be demonstrated in the near future and induce a move from the pharmaceutical industry towards cardiotoxicity models based on hPSC-CMs as their preferred method.

For hPSC-CMs to reach their full potential, methods for functional phenotyping of these cells will have to be further improved. One important factor of such methods that will need to be improved is the throughput. Within the BASiC method described in this thesis, we envision the possibility of further development into a fully automated high-throughput assay. All the steps necessary to create hPSC-CM based cardiac microconstructs as demonstrated in Chapter 5, are amenable to automation using robotics. As the cardiac microconstructs are all exactly sized to a micropattern and aligned in a roster, the imaging of these microconstructs could also be performed in an automated fashion. Subsequently, the analysis of obtained videos of beating cardiac microconstructs could be automated by employing machine learning to determine the edges of cardiac microconstructs as input for the Visible software used in the BASiC method. This would then lead to a fully automated, unbiased, high-throughput assay of hPSC-CM based cardiac microconstruct function. We believe
that in the future similar methods will be more widely employed to improve the throughput of other assays of hPSC-CM function.

Next to improvements in technologies to assay hPSC-CMs, improving the maturation status of hPSC-CMs will be crucial to fulfill the promise that hPSC-CMs hold. As demonstrated in Chapter 6, many advances in hPSC-CM maturation have been achieved using various strategies. Interestingly, most maturation markers can reach the fully adult level, displaying again the enormous potential of stem cell derived cardiomyocytes to mimic the adult heart. We envision that in the near future a combination of maturation strategies will lead to a level of hPSC-CM maturation that is sufficient for the vast majority of hPSC-CM applications in disease modelling, cardiotoxicity screening and drug discovery. In the more distant future, hPSC-CMs will likely achieve a maturation level which makes them indiscernible from adult CMs.

A very exciting possible future development is the clinical application of hPSC-CMs for regenerative medicine. hPSC-CMs could be administered to injured myocardium to integrate with the host tissue, thereby replacing CMs lost during a myocardial infarction. This treatment could then prevent or cure heart failure. Intriguing reports of hPSC-CMs integrating with infarcted myocardium in animal models offer hope that hPSC-CMs will one day be used to treat patients. However, as it stands now hPSC-CMs have shown electrophysiological coupling with the host tissue but no meaningful improvement in functional cardiac parameters.[52] Moreover, it has been speculated that minor improvements in cardiac function after administration of hPSC-CMs is more due to paracrine factors than the integration of functional hPSC-CMs in the injured heart.[53] In this regard, hPSC-CMs will also have to compete with the rapidly advancing field of bionics. Left ventricular assist devices have quickly developed over recent years. Their output has been improved while the form factor has been reduced. It remains to be seen which of these two approaches will be most widely adopted in the future. hPSC-CM treatment does have the potential to be less invasive, be more physiological, and be used as a secondary preventive measure, as compared to mechanical assist devices.

To summarize, heart failure will continue to be a major burden for patients and healthcare systems worldwide unless new treatment options are developed. An improved understanding of heart failure will be crucial to develop effective new treatments and hPSC-CMs have emerged as an effective tool to study the pathophysiology of heart failure. As such, hPSC-CMs might hold the key to steer the future towards more effective treatment and perhaps even prevention of heart failure.
References


