Studying cardiac diseases using human stem cell-derived cardiomyocytes
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Chapter 8

General discussion and future perspectives
In this thesis, we applied human \textit{in vitro} disease modeling to overcome the disadvantages of non-human preclinical models that are applied in translational research. With suitable human \textit{in vitro} disease models, human diseases can be studied in human cells in a highly controllable environment. Consequently, pathogenesis can be studied from a very early stage to be followed over time. To this end, we have designed \textit{in vitro} models to study the mechanisms underlying several cardiac pathological processes. In this thesis, we study acquired pathologies that arise due to non-genetic causes (part I) and due to a (putative) genetic predisposition (part II). Specifically, we have studied the effects of iron deficiency and mechanical stress on cardiomyocyte function and adaptation in part I. In part II, we have addressed the underlying mechanism of peripartum cardiomyopathy (PPCM) with a clinical, bioinformatic and mechanistical approach.

**PART I**

**Reviewing the current state of cardiac \textit{in vitro} disease modeling**

In chapter 2 of this thesis, we have reviewed the current means and methods applied to model \textit{in vitro} cardiomyopathies. \textit{In vitro} disease modeling is a rapidly evolving field of research in which state of the art techniques are introduced and improved constantly. Great efforts are made to apply current two-dimensional models in a three-dimensional environment through tissue engineering. However, as insights into genetic and epigenetics involvement in cellular mechanisms also steadily increase, we become more aware of the limitations of \textit{in vitro} models. The main issues arise when the cause of a disease is expected to be genetic, but a specific mutation has not been identified. In contrast, a disease could also be caused by an epigenetic aberration, which is nearly impossible to study in hiPSC. Once a pathological factor is identified, modern genetic editing techniques allow for recapitulation in \textit{in vitro} and \textit{in vivo} models to study the associated phenotype. Alternatively, the respective phenotype can be repaired by correcting the genetic variation in patient-derived hiPSC by employing CRISPR-Cas9-mediated gene editing. All of these aspects have to be considered when designing experiments and projects. Based on this review of current approaches and available techniques, we concluded that fundamental experiments regarding subcellular mechanisms (e.g. studies regarding miRNA/protein expression, binding targets and kinetics etc.) can be performed in a monolayer-based setup, while intercellular experiments that depend on cell-cell interactions can now be studied with elegant and feasible tissue engineering methods. Moreover, genetic variations can now easily be corrected in hiPSC or, vice versa, can be introduced in standardized cell lines.
The effects of iron deficiency on human cardiomyocytes

Iron status is an important predictor for disease outcome and iron deficiency was found to be a major risk factor for a worse prognosis\(^1\). Subsequently, administration of intravenous iron supplements has been shown to be beneficial for heart failure patients\(^2\). To determine which cellular processes are reversibly impaired in iron deficient cardiomyocytes, we have investigated the subcellular effects of iron deficiency on human cardiomyocytes in chapter 3. For this model, we have utilized deferoxamine (DFO) to chelate cellular iron. Intracellular iron levels were determined based on ferritin levels, which were gradually reduced over 4 days. After 4 days of incubation with DFO, ferritin levels were reduced by 84% and genes involved in iron internalization were greatly induced. Notably, these ferritin levels were lower than what can be expected in patients. We expected that cellular deficiency of such magnitude would critically impair other tissues (e.g. hematopoietic stem cells) before cardiac tissue is affected. However, this model provides novel insights into the coping mechanisms that are activated in mitochondria-rich cells (i.e. cardiomyocytes) that heavily rely upon iron-dependent processes. Previous studies have determined the effects of genetic ablation of mediators in iron homeostasis in the mouse heart (e.g. the transferrin receptor, hepcidin, and iron regulatory proteins)\(^3-5\). In addition, our findings highlight the importance to study cellular iron levels in addition to circulating iron markers such as transferrin saturation; i.e. intracellular iron levels provide functional information in addition to circulating biomarkers. Specifically, our observation pertaining to ER stress and Fe-S cluster-based mitochondrial dysfunction contribute to the characteristics of cellular iron deficiency. The latter supports the heme-sparing mechanism as was hypothesized for heart failure patients. Khechaduri et al. demonstrated that heme levels are maintained in the human failing heart, whereas non-heme iron levels were reduced\(^6\). Interestingly, clinical studies showed that iron supplementation is beneficial for exercise capacity and symptoms\(^2,7\). After inducing iron deficiency, we have restored intracellular iron levels by the supplementation of transferrin-bound iron, which restored most of the parameters within the experimental time frame. The observed plasticity of cardiomyocytes to recover from such severe iron deficiency substantiates the use of iron supplements during heart failure as it may improve exercise tolerance through improved mitochondrial function. Therefore, the development of an in vitro model identified previously unconfirmed aberrant mechanisms in iron deficient cardiomyocytes that may contribute to the progression of heart failure.

Cyclic mechanical stretch to induce in vitro hypertrophy

Cardiac hypertrophy is part of adaptive compensatory mechanisms in response to various types of cardiac stress. Under physiological conditions, this process is beneficial and reversible. However, once damage has become too severe, this mechanism can become maladaptive, resulting in progressive heart failure. Therefore, obtaining more insight into
the molecular processes underlying hypertrophy would be beneficial for future treatment strategies that address the first stages of HF. In chapter 4, we applied cyclic mechanical stretch to hESC-derived cardiomyocytes in order to induce hypertrophy as an in vitro model for wall stress. Major hallmarks of hypertrophy were observed, as well as increased sarcomere length, cellular stiffness, and decreased contractility. Hypertrophy has been studied extensively in various cell types, primarily by addition of phenylephrine, angiotensin II, or similar drugs. A recent study by Rupert et al. indicated that phenylephrine induced a gain of surface area of cells at the expense of cell height (i.e. cellular volume remained the same). In the present study, we demonstrated that mechanical stretch also resulted in increased surface area, while cellular height remained similar to static control cells. Consequently, cell volume increased. After confirmation that mechanical stretch induces hypertrophy in cardiomyocytes, we sought to dissect the molecular mechanisms involved in hypertrophy by transcriptome sequencing after mechanical stretch. As a result, we identified the SP6-FSTL3 pathway as a major mediator of hypertrophy. Short hairpin RNA-mediated inhibition of either of these genes nullified the hypertrophic response to mechanical stretch. While these findings contribute to the expansion of what is known about the mechanisms of hypertrophy, it remains a topic of discussion whether inhibition of hypertrophy would be beneficial for the patient. Indeed, hypertrophy is a first mechanism of remodeling in a stressed heart, but at that stage, hypertrophy can be regarded as beneficial to cardiac function. Whereas, when stress persists or becomes severe enough, maladaptive remodeling occurs. The transitional stage from adaptive to maladaptive remodeling has been a subject of research for many years, because it is postulated that heart failure might be avoided when this transition can be prevented. However, despite the induction of hypertrophy in an in vitro system, it is not possible to compare our model to adaptive and maladaptive remodeling as observed in vivo. Our study indicates that SP6-FSTL3 pathway may drive hypertrophy, but we cannot conclude whether inhibition of this pathway may prevent maladaptive hypertrophy exclusively. Consequently, in vivo studies need to be performed to clarify to what extent the SP6-FSTL3 pathway is involved in maladaptive vs. adaptive hypertrophy. Following such studies, specific genes of proteins may be targeted to prevent maladaptive hypertrophy specifically. Similar to gene silencing by shRNA in vitro, future therapies may include administration of anti-sense oligonucleotides that prevent expression of specific genes by premature targeted RNA breakdown. Such approaches are currently being tested for neuromuscular diseases like Duchenne muscular dystrophy, but could also be applied for other diseases, such as gene-driven cardiomyopathies.
PART II

Underlying mechanisms of peripartum cardiomyopathy

The pathophysiological processes that result in the dilated cardiomyopathy PPCM were reviewed chapter 5. In the recently proposed PPCM etiology, aberrant STAT3 function results in secretion of cathepsin D from the cardiomyocytes, which cleaves the hormone prolactin, the product of which induces neighboring endothelial cells to transfer miR-146a loaded exosomes back to the cardiomyocytes13-17. Ultimately, cardiomyocyte metabolism is impaired, which leads to cardiomyopathy (i.e. PPCM). Notably, PPCM is diagnosed based on exclusion, resulting in a heterogeneous disease group. Various theories regarding PPCM causes have been proposed (e.g. autoimmune disorders18, viral myocarditis19, sFLT1-mediated angiogenic imbalance16, familial dilated cardiomyopathy20,21, and TTN mutations22), but these may only contribute to the adverse progression of PPCM. While the proposed mechanisms are studied in cardiac-specific STAT3-knockout mice, this was not confirmed in patient cohorts23. As such, it may be expected that STAT3-related defects may not be the cause of PPCM in humans. This is highlighted by the observation that a global STAT3 knockout is lethal in the early embryonic phase24. Interestingly, activation of STAT3 has protective effects after acute myocardial infarction and pressure overload25,26. Moreover, STAT3 levels were found to be reduced in patients with a dilated cardiomyopathy27. These previous studies provided crucial insight into pathophysiological mechanisms involved in cardiomyopathy, especially PPCM. However, the previously mentioned findings imply that STAT3 is not a cause for PPCM. Moreover, ablation of a pivotal transcription factor in cardiomyocytes, like STAT3, is likely to result in susceptibility to develop heart failure at times as demanding for the cardiovascular system as childbirth. Defects in STAT3 levels or function may be a consequence of a cause that remains unknown, but these defects will certainly promote disease progression and should be studied in more detail.

Cathepsin D release is not specific to peripartum cardiomyopathy

Based on previous data regarding the central role of cathepsin D release in the pathophysiology of PPCM, we have investigated the specificity of cathepsin D release in heart failure patients in chapter 6. Interestingly, previously published clinical trials studying the protective effects of prolactin inhibition by bromocriptine administration were found to be (partially) successful28,29. In the study by Sliwa et al., small groups of PPCM patients were treated with bromocriptine for 8 weeks versus patients that received only standard treatment28. The study by Hilfiker et al. studied the effects of short term or long term bromocriptine administration29. The former study indicated a potential role for bromocriptine in PPCM, but 4 of 10 patients who were not treated with bromocriptine died during the study; the latter study lacked a control group (without bromocriptine administration). While promising results were obtained, decisive conclusions remain to be drawn. How-
ever, the role of cathepsin D in PPCM pathophysiology is believed to be pivotal for disease development. In light of this, we have studied circulating cathepsin D levels in a general heart failure patient population. We found that cathepsin D is released in a general population of heart failure patient. Cathepsin D serum levels were found to be associated with diabetes mellitus, CKD and higher levels of interleukin 6 and NT-proBNP. Moreover, high levels of circulating cathepsin D were found to be associated with adverse outcome in heart failure patients. While this observation does not disprove the currently accepted model for PPCM, it does indicate that cathepsin D release is not exclusive to PPCM etiology, while other steps in its complex pathophysiology may still be unique to PPCM. We have demonstrated that cathepsin D release occurred in response to mechanical stretch, which has been used to model wall stress as seen in heart failure patients, but also in pregnant women. In PPCM pathophysiology, the direct effect of cathepsin D release from affected cardiomyocytes is the cleavage of prolactin present in the blood to a 16 kDa fragment. More specifically, cathepsin D can produce four types of 16 kDa prolactin. All four of these products are members of an antiangiogenic class of molecules known as vaso-inhibins, which can be produced by various enzymes, an attribute not exclusive to cathepsin D. For example: matrix metalloproteases are abundantly expressed enzymes that also produce a form of 16 kDa prolactin. Moreover, plasma levels of cathepsin D correlated with fatty liver disease severity and was demonstrated to be a robust biomarker for hepatic inflammation. The role of cathepsin D in PPCM etiology is complex and we have demonstrated that circulating cathepsin D levels are associated to multiple comorbidities and to adverse outcome. We were unable to determine serum levels of cathepsin D in PPCM patients compared to other heart failure patients or healthy pregnant women. Therefore, we cannot conclude whether PPCM is the result of cathepsin D levels above a certain threshold in the presence of prolactin.

In vitro experiments indicated that cathepsin D is primarily released following mechanical stretch and in response to TNF-α. The former is a clear indication that volume and pressure overload results in cathepsin D release, and the latter suggests that events with an inflammatory component result in increased cathepsin D secretion. Remarkably, intracellular cathepsin D levels were decreased during hypoxia, but not in other conditions. This supports the protective role of cathepsin D as was seen during myocardial ischemia. Interestingly, cathepsin D knockdown during stretch result in a striking increase of TnT levels in the cell medium following mechanical stretch, which is an indication of increased cardiomyocyte death. In addition to previous studies, we demonstrated that cathepsin D is essential for cardiomyocyte coping mechanisms, while cathepsin D deficiency impairs cardiomyocyte survival after stress.
Elucidating PPCM pathophysiology using RNA sequencing in a familial case-control study

PPCM has been the subject of various studies using different approaches. In chapter 7, we studied PPCM pathophysiological molecular mechanisms in a patient with a typical diagnosis of PPCM, which was compared to a sister who went through multiple pregnancies without complications. We generated iPSC-derived cardiomyocytes from these individuals and applied mechanical stretch to these cardiomyocytes in order to reproduce wall stress, as observed during pregnancy. We performed transcriptome sequencing on these samples and found that sterol and lipid metabolism was impaired in PPCM cardiomyocytes. Interestingly, our observations so far are in line with the previous findings regarding the involvement of PGC-1α-mediated sterol and lipid metabolism-related pathways in the pathogenesis of PPCM. However, it remains unknown how apparently constitutively impaired sterol and lipid metabolic pathways result in PPCM at the characteristic time seen in patients. It has been reported that glycolysis is inhibited in pregnant women, whereas fatty acid oxidation rates had almost doubled in various animal models. It is known that pregnancy induces insulin resistance to a degree similar to diabetes mellitus, which indicates that the body has to activate coping mechanisms in order to endure reduced glycolysis during pregnancy. Insulin resistance is most severe during the last trimester. The time around delivery involves highly complex regulatory (hormone-based) mechanisms that drive the reversion from a pregnant state to a non-pregnant state of homeostasis. Since glycolysis is not a sustainable energy source for the heart, the heart needs to revert to fatty acid metabolism. We hypothesized that during this time, PPCM patients cannot revert effectively due to disrupted sterol and lipid metabolism. Consequently, metabolic pathway regulation may become dysfunctional and will ultimately lead to impaired cardiomyocyte function and heart failure (i.e. PPCM). Ultimately, the mechanisms found and described in this thesis may imply that PPCM pathogenesis is not a process that is exclusive to women, since impaired metabolism of sterol and lipids might also occur in men, albeit unrelated to pregnancy. In that scenario, our understanding and models regarding PPCM need to be adjusted. Importantly, these results were obtained from RNA sequencing of cardiomyocytes from a single PPCM patient. To validate these findings, cardiomyocytes were generated from a second patient and a familial control and genes involved in major sterol and lipid metabolic pathways were also found to be differentially expressed. Moreover, several of the top 10 genes (based on significance and fold change) were also validated by means of qRT-PCR. To further validate our findings, we determined expression levels of identified genes in mice with a cardiac-specific knockout for STAT3. Again, most of the genes were differentially expressed in STAT3 conditional knockout mice, confirming our findings in an independent model. Interestingly, the majority of affected pathways are related to sterol and lipid metabolism and SREBF1 was among the differentially expressed genes in PPCM. SREBF1 is a key regulator of lipid metabolism and was also affected in
mice with cardiac STAT3 ablation. This is a clear indication that fatty acid metabolism may be widely affected via defective regulation of involved pathways. A next step in this study could be to investigate the metabolic differences between cardiomyocytes derived from a PPCM patient and a healthy control and to determine whether these cells also show contractile dysfunction. Currently, further investigation is needed to determine the cause of PPCM, but we have shown that iPSC-derived cardiomyocytes obtained from PPCM patients show a gene expression profile that is common between two patients and an established mouse model.

**FUTURE PERSPECTIVES**

**Cardiac in vitro disease modeling**

In this thesis, we have presented studies that highlight the applicability and feasibility of *in vitro* disease modeling related to cardiomyocyte dysfunction. Importantly, it is imperative to take the artificial nature of these models into account when designing an *in vitro* disease model. The degree of flexibility of study design *in vitro* experiments is considered to be an advantage as almost all conditions can be adjusted. A pivotal aspect of the applied (and discussed) methods in this thesis, is the use of two-dimensional cardiomyocyte cultures. As discussed in the introduction, culturing cardiomyocytes in three-dimensional format was demonstrated to improve various aspects of cardiomyocyte function, including alignment, sarcomeric organization, and contractility. Moreover, three-dimensional culturing is more representative to *in vivo* settings as engineered tissues consist of multiple cell types in a more physiological environment. The main motive to culture cardiac cells in engineered tissues is to improve cardiomyocyte maturity. However, the lack of a clear definition of cardiomyocyte maturity and unambiguous results has rendered it a popular topic of research. This issue is perpetuated by the unavailability of mature and healthy human cardiac tissue suitable for further investigation. It is expected that this issue will remain a major hurdle in the field of cardiac maturation, but resolving this issue can propel preclinical research to new frontiers by improving *in vitro* disease modeling. An interesting aim could be to develop new techniques to generate organoids, similar to what is currently being done in the field of neurology, nephrology, hepatology and gastroenterology[^37-43]. In contrast, it has proven to be difficult to obtain representative cardiac organoids. Recently, the group of prof. Kenneth Chien has demonstrated a method to generate cardiac tissues that include the cardinal cell types observed in the mature heart as these cells spontaneously migrate into the formed tissue[^44]. While very promising, this method relies on the generation of ventricular progenitor cells, similar to widely used cardiomyocyte differentiation protocols, as well as engraftment into the kidney of a mouse. Ultimately, the current state of *in vitro* cardiomyocyte differentiation is highly...
dependent on directed differentiation protocols, artificial culturing conditions, and often harsh purification protocols. Advanced techniques that could depend on self-assembly and less stringent protocols might result in more representative *in vitro* models that can be used to study cardiomyopathies.

**Unraveling the human pathophysiology of peripartum cardiomyopathy**

PPCM is a rare disease with possibly severe consequences. Many studies focusing on various aspects of the disease have been published, but the underlying mechanism causing the disease remains unknown. As opposed to previously proposed models, we have opted for an unbiased approach by performing expression analyses in two patients and an established mouse model. The results are intriguing and provide more insight into how PPCM may have developed and how specific pathways contribute to the susceptibility to developing the disease. It is a heterogeneous disease and our findings remain to be confirmed in other patients and possibly in larger cohorts of patients or patient databases. A next step towards elucidation of the underlying pathophysiology of PPCM may be to genetically screen multiple patients for the aberrations found in our primary patient and screening for commonalities between patients. Crucially, after hiPSC have been generated from these additional patients, the putative mutations could be corrected and specific mutations can directly be linked to PPCM etiology. Additionally, mouse models can be generated based on the results of *in vitro* studies. Alternatively, PPCM may be a disease of multiple cell types. It may therefore be plausible that complex multi-organ mechanisms cannot be studied *in vitro* and mouse models need to be generated. However, no single mutation has been identified. Therefore, it is not possible to generate a representative mouse model, resulting in a status quo. Interestingly, clinical observations indicated that the involvement of the coagulation cascade may play a pivotal role in PPCM development as a large percentage of PPCM patients showed ischemic events (e.g. stroke). This pathway might be closely linked to the previously suggested role of vasoinhibins and other anti-angiogenic mechanisms, and may be a detrimental event that has been overlooked in PPCM etiology. Specifically, it was recently published that the key coagulation factor PAI-1 facilitates 16 kDa prolactin uptake in endothelial cells. In that respect, pregnancy may induce aberrant plasma levels of PAI-1, which in turn facilitates vasoinhibin-mediated anti-angiogenic effects. Indeed, fluctuating plasma levels of cathepsin D or 16 kDa prolactin may not result in the development of PPCM, while acutely increased levels of PAI-1 might be the triggering event. A complex mechanism combining these factors is also feasible.

Overall, while the molecular mechanisms leading to heart failure are steadily elucidated, it actual cause that starts these mechanisms remains unknown. The *STAT3* and *PGC1α* genes are assumed to be involved, but without conclusive evidence for an actual causal role. These genes are major regulators of cardiac metabolism. Taken together, these
observations support our findings that aberrant cardiac metabolism may cause PPCM. Cardiac metabolism is a key field of research within cardiology and such methods may be detrimental to obtaining representative results. While this is beyond the scope of this thesis, there is a great need to elucidate these metabolic effects in the future. Metabolic pathways should be studied in PPCM patients immediately after diagnosis and should ideally be linked to defective gene expression levels and function parameters. However, as diagnosis is based on exclusion, means for PPCM diagnosis have to be improved greatly before patient selection will be reliable. Once the gene or pathway driving PPCM development has been discovered, a representative mouse model can be generated, putative drugs can be screened and selected. With an increased understanding of PPCM pathophysiology, a potential therapy can be designed.
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