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CHAPTER 9

General discussion
Cardiomyocyte maturation

An important yet unsolved problem in the hPSC-CM field while using these cells as models of disease is their immature phenotype. Enhancement of the functional phenotype of hPSC-CMs towards a more mature state is thus one of the main aspects of this thesis. In chapter 2 we provided a detailed overview of the features of immature versus mature cardiomyocytes in terms of morphology, electrophysiology, calcium handling and metabolism in combination with experimental strategies that promote hPSC-CM maturation such as prolonging culture time, electrical stimulation, mechanical strain, co-culture with other cell types, addition of small compounds, adjustment of substrate stiffness and ectopic expression of key genes associated with higher function in cardiomyocytes. The quantitative and qualitative comparison provides a useful benchmark for studies on hPSC-CM maturation. The outcome of this comparison indicated that the best strategies for enhancing maturation of hPSC-CMs combined several different cues at the same time, not just one. In one of our studies for instance, we combined multiple hormones and small molecules simultaneously. Nunes et al [1] made a similar conclusion combining mechanical strain with electrical stimulation and addition of non-cardiomyocyte cells in a 3D format. This gave an impressive improvement in AP phenotype.

Our efforts focused on improving bioenergetics/metabolism, calcium handling, electrophysiology and contraction force, but instead of pursing a combinational approach at the outset, we first examined the role of mitochondria biogenesis. During heart development as the heart tissue matures, there is a large increase in mitochondrial numbers in cardiomyocytes to cope with the energy demand of the growing heart tissue. However, this coincides with an increase in ROS levels which are detrimental to ion channel function [2]. Although cells can counteract this effect by increasing expression of antioxidant enzymes that suppress ROS [3], we found that these coping mechanisms were unable to reduce ROS levels in hPSC-CMs. In chapter 3, we showed improved AP and calcium transient phenotype when ROS levels were substantially lowered after knockdown of PGC-1α, the key regulator of mitochondrial biogenesis [4]. This illustrated the importance of effectively controlling ROS concentrations in hPSC-CMs. In fact, by adding antioxidants to hPSC-CMs and reducing oxygen concentrations (3%), we managed to attain enhanced calcium transients with increased amplitude and systolic [Ca^{2+}].

The importance of the glucocorticoid signaling pathway in hPSC-CM maturation was examined in chapter 4. As opposed to other compounds such as T3
hormone that have been shown to exert pro-cardiac maturation effects [5,6], how glucocorticoids act had not been investigated in hPSC-CMs prior to our study. We showed that glucocorticoids affect calcium transient properties, accelerating transient decay and that this is mediated through enhanced SERCA and NCX activities. The expression of the genes transcribing these ion channels/exchangers was not changed upon glucocorticoid treatment. The precise link between the glucocorticoid pathway and the enhanced function of these proteins therefore remains to be answered in further studies. In addition, the genetic link between glucocorticoids and features of the molecular mechanism behind contraction also needs further study in the context of the increased forces of contraction. Gene expression analysis of dexamethasone treated hPSC-CMs for genes relating to sarcomeric, other contraction components and the calcium transient could shed light on this issue.

Although dexamethasone by itself did not affect the electrophysiological phenotype of hPSC-CMs, it was interesting to see that the combination of T3, IGF-1 and dexamethasone (TID) did improve AP properties (as well as contraction force) as described in chapter 5. Based on our results that IGF-1 and dexamethasone induce a synergistic effect on the bioenergetics of hPSC-CMs (increased basal respiration rates, ATP production rates and glycolytic rates), it is possible that the beneficial effect on electrophysiology and contraction might be exerted through enhanced energy production. Addition of some non-cardiomyocyte cells along with TID did not enhance AP properties any further. However, in basal conditions, non-CMs were shown to improve AP characteristics as described in chapter 8. Defining the identity of these cells and benchmarking different CM to non-CM cell ratios should be pursued in future studies.

**Disease modeling**

Using hiPSC-CMs to model sodium channel related cardiac diseases is challenging since the functional parameters for comparison and assessment are the upstroke velocity of the AP and the sodium current density which are both very low compared with adult cardiomyocytes. Changes are thus difficult to detect. This is especially true when using the upstroke velocity as a readout. The voltage dependent availability of sodium channels is an important aspect of studies such as our own (chapter 6) focusing on the AP profile. If the maximum diastolic potential was different in control and patient hiPSC-CMs we could not have used the upstroke velocity as a parameter of comparison. This would be true even if the control lines were isogenically matched with the patient lines by means of
genetic correction. Another challenging aspect of our study was the use of the readthrough promoting compounds in hPSC-CMs. When used in overexpression systems such as HEK cells modeling disorders relating to homozygous nonsense mutations, a small percentage of restored protein can produce large effects on functional output [7]. In our study however, we focused on heterozygous mutations since patients carrying homozygous non sense mutations in the SCN5A are rare. Therefore, a small percentage of restored protein would carry only a minor impact on the function of the sodium channel that we cannot even detect, given the sensitivity limitations of our experimental setup. In any event however, this potential small percentage is of poor clinical value. Nonetheless, the potential of our system to model sodium channel related cardiac diseases is evidenced by the clear distinction of electrophysiological phenotype between control and patient hiPSC-CMs. Coupled with gene editing techniques this potential becomes even greater. As seen in chapter 7, after genetic correction of the non-sense mutation W156X, only a slight enhancement on the sodium current density and the up-stroke velocity of the AP was observed. This indicates that the W156X mutation might not have such a large impact on the functional phenotype of hiPSC-CMs as originally thought and suggests that exploring additional genetic causes, which might be linked with the pathology of the patient, may be of value.

The shortcomings of hiPSC-CMs as a model system.

The main caveat of hiPSC-CMs as with all stem cell derivatives [8] is their immature functional phenotype, resembling that of fetal cardiomyocytes. This often raises critique on the value of hiPSC-CMs to accurately model the complexity of the human heart cell. Every cell type of the adult human heart (pacemaker cells, atrioventricular nodal cells, Purkinje fiber, atrial myocytes, ventricular myocytes) has a distinctive electrophysiological profile resulting from the presence of a variety of ion channels on their membrane. Even within a given cardiac subtype, for example ventricular cells, the electrophysiological profile can differ from one ventricular region to another (for instance the right ventricular outflow track compared to other ventricular regions). The distinct identity of cardiac cells whether epicardial, mid-myocardial and endocardial from the same section of a ventricular wall increases the heterogeneity in AP phenotypes that can be recorded from adult cardiomyocytes. Until recently, most differentiation protocols generated hiPSC-CMs that consisted of a mix of cardiac subtypes with heterogeneous AP profiles. In most cases they could be designated as ventricular/atrial/nodal-like. Two major issues remain to be addressed before hiPSC-CMs can robustly recapitulate all cardiac arrhythmias: (i) Differentiation protocols must generate specific cardiac subtypes [9,10,11] that mimic the complexity of electrical het-
erogeneity of the human heart and (ii) the functional phenotype of generated cells must be enhanced so that key sub-organelle and ultrastructural features such as gap-junctions, intercalated disks and T-tubules are evident. Combinational approaches that impact the functional profile of hiPSC-CMs in many aspects such as metabolism, electrophysiological profile, calcium handling and sarcomeric ultrastructure are therefore likely of value.

In the introduction of this thesis, I described all of the studies to date that used hiPSC-CMs to model cardiac diseases. In general, these recapitulated salient features of the disease. In chapters 7 and 8 I presented our findings in this context of the phenotype in hiPSC-CM derived from a Brugada syndrome patient. However, it is important not to overstate the abilities of hiPSC-CMs to recapitulate disease since many diseases only arise in adulthood or there are other features not captured in vitro. The exact pathophysiology of Brugada syndrome for instance, is rather complex [12,13] and may involve structural abnormalities in combination with an aberrant function of the cardiac sodium channel (recorded in 10-30% of Brugada patients). The correct statement is therefore that hiPSC-CMs at present can recapitulate some aspects of the pathophysiology of cardiac diseases. Despite their limitations however they do have the potential to provide new clinical insight on each particular mutation and with the advent of new technologies such as “tissue engineering” and “organ-on-chip” they might begin to mimic the patients pathophysiology even closer.

**Future perspectives**

Culturing hiPSC-CMs in a three-dimensional (3D) format in such a way that micro cardiac tissues are formed is among the recent technologies in development. The goal of cardiac tissue engineering resides in converging towards true fidelity of the human heart through accurate reproduction of the cardiac niche in vitro. Attention is increasingly given to various biophysical, biochemical and bioelectrical features of cardiac tissue. The primary structural support of engineered tissues for instance, can be provided by either hydrogels [14], decellularized hearts [15] or bioprinting [16], all techniques showing great promise. The extracellular matrix (ECM) composition is another key aspect since cell-ECM interactions in the native heart tissue modulate the heart’s elasticity and contraction properties. Complex mixtures of ECM proteins (laminins, collagens) such as Matrigel [17,18] or ECM aggregates from decellularized hearts [19], provide the suitable microenvironment for cardiomyocyte 3D culture. Arranging the spatial distribution of hPSC-CMs in such formats (cardiomyocytes have cell to cell contacts with on average eleven other cardiomyocytes in the native heart [20]) so that molecular
diffusion or optimal vascularization for oxygen/nutrient delivery is achieved, remains challenging. Fine tuning of substrate stiffness and mechanical stress as well as the cyclic stress due to the hemodynamic work load [21,22] must also be taken into consideration.

The functional output of engineered cardiac micro-tissues, in terms of force generation and cell contraction can be evaluated without difficulty [23,24]. A problem however occurs during analysis of the electrophysiological output: to acquire the precise action potential parameters as well as ion current properties and calcium handling capabilities (calcium transients) requires disruption of the high order structure to the single cell level. We then immediately interfere with the “matured” phenotype of these cells. The development of hPSC lines genetically encoding fluorescent voltage and calcium sensors [25,26] might circumvent this problem and at the same time provide high-throughput readings for electrophysiological analysis. Several technical limitations (camera capture speeds, quantum mechanical properties of voltage dyes etc.) remain to be addressed however before realizing this prospect.

The pinnacle of hPSC-CM technology would be reached if hPSC-CMs could eventually be used for modeling adult-onset cardiac diseases such as ischemic heart disease. Nonetheless, exploring new approaches towards hPSC-CM maturation will not only benefit the hPSC-CM field. Knowledge of how the human heart develops and matures postnatally would provide insight into this equally important aspect, which relates to finding the causes of several congenital heart diseases. Although a quite distant prospect in personalized medicine, future myocardial repair procedures in the clinic would benefit considerably if hPSC-CMs shared the electrical and mechanical properties of the native myocardium.
References


