From fetus towards adult : maturation and functional analysis of pluripotent stem cell-derived cardiomyocytes

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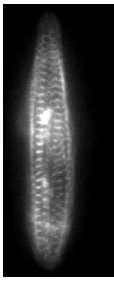
Chapter 8

General discussion and future perspectives



Summary

This thesis described how the symbiosis between biochemistry, biomaterials and stem cell technologies (**Chapter 2**) can be used to determine maturation and disease states of human pluripotent stem cell derived cardiomyocytes (hPSC-CM) (**Chapter 3**). The functional improvement inherent from this process enabled the revelation of a contractile defect caused by a MYBPC3 mutation in hiPSC-CM derived from patients with the disorder(**Chapter 4**). In order to further investigate how this can be attributed to this MYBPC3 mutation in human cardiomyocytes, several genetic tools were developed (**Chapter 5**) to mimic and analyse the underlying mechanisms (**Chapter 6**). The thesis ends with the development of a novel tool to expand cardiac progenitors and study their differentiation into the different cell types (**Chapter 7**).

Discussion

The use of human pluripotent stem cells (hPSC) to generate different organ-specific cell types creates enormous possibilities for developing disease modelling platforms, tissue engineering and drug toxicity studies. A major advantage of using hPSC is that functional human cell-types or tissue can in principle be generated with higher predictivity for disease modelling and drug responses when compared to non-human cell lines or animal models [1]. In the heart for example, human cardiomyocytes exhibit electrophysiological signals, which are very distinct from those, for example, in mice. Differences in the contribution of ion channels at different phases during the contraction-relaxation cycle are indicated by their differences in shape and duration of their respective action potentials (APs) and electrocardiogram (ECG) [2]. Mice have a tenfold faster beating rate and a much shorter AP, lacking a distinct plateau phase and have a shorter QT interval (100ms) when compared to that of the human heart (400ms). Variations in cardiac electrophysiological profiles between species translate into differences in susceptibility to drug-induced cardiotoxicity, caused by interference with ion channels and their respective ion currents, which has been identified as one of the major reason for drugs failing clinical trials[3]. Furthermore, these limitations not only affect drug toxicity assessment, but also the study of human cardiac diseases in which ion channel functions are impaired (i.e. channelopathies such as long-QT syndrome), which may be difficult to analyse in experimental animal models. Moreover, interspecies variations regarding other aspects of cardiac function complicate establishing reliable and predictable animal models of cardiac disease. For example, mice with only one copy of sarcomeric myosin binding protein c 3 (MYBPC3) do not develop cardiac hypertrophy whilst a non-sense mutation in one allele of MYBPC3, causing a decrease in the protein in humans, ultimately leads to hypertrophic cardiomyopathy and heart failure [4,5]. Additionally, despite specific human cardiac diseases being linked to coding/non-coding DNA variants through genome-wide association studies, the exact role of these gene variants have been difficult to assess due to absence of clear orthologs in mice or in other species [6].

Another advantage of hPSC technology is the ability to derive stem cells from any individual, enabling their generation from patients with specific genetic mutations and/or a disease phenotype (**Chapter 4**) [7,8]. The use of optimized and defined protocols for the production

of specialized functional cell-types, including those of the heart, enables in vitro modeling of patient diseases, making it feasible to study any associations between phenotype(s) observed in vitro and clinic symptoms. Additionally, the mechanism of disease can be further explored by the use of genome editing techniques such as Zinc finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) or the CRISPR/Cas system9. These techniques enable the correction of the disease causing mutation in order to create isogenic control cell lines or vice versa, introducing mutations in healthy control cell lines to directly analyse the functional and phenotypic consequences10.

To analyse the diverse aspects of the hiPSC-derived cardiomyocyte (hiPSC-CM) phenotype several technologies are widely used. Electrical activity, responsible for cardiomyocyte excitation, is often measured by single cell patch clamp, multi-electrode array (MEA) and most recently by optical mapping with the use of voltage sensitive dyes [11]. The electrical excitation induces the fast release of calcium from the sarcoplasmic reticulum, promoting force generation by the contractile machinery. Calcium transients can be measured using calcium sensitive dyes[12]. In the heart the contractile force production of each cardiomyocyte exerts the work necessary to pump the blood out of the cardiac ventricles into the circulation system. Methods available to measure contraction force in vitro vary from the use of force transducers for cardiac tissue strips measurements [13], micro-pillars [14] to beads embedded on acrylamide gels for single cardiomyocytes measurements (**Chapter 3**). Although, each measurement provides valuable information on each particular cardiac parameter, it would be of greater interest if multiple parameters could be determined simultaneously in a multiplexed system, comparable to the complex cardiomyocyte function in vivo. Such integration of simultaneous measurements in a single cell or tissue will enable the assessment of the interdependence and inter-regulation of the different cardiac parameters in each single beat. For example, assessing how does one slower/arrhythmic action potential influence the calcium transients and contraction force or does a defective contraction affect the next action potential and/or calcium transients? This information allows a deeper look onto the mechanisms through which channelopathies affect heart function or cardiomyopathies cause arrhythmias. Additionally, such analysis can be used as a sensitive and highly informative assay for drug cardiotoxicity screening. **Chapter 3** describes a platform that allows the assessment of the relation between different electrophysiological parameters and contractile forces, whereas in **Chapter 6** this platform is optimized for measuring calcium kinetics, sarcomere dynamics and contraction force in the same cardiomyocyte. Recently different platforms have been developed to simultaneously measure electrophysiology together with calcium transients [15,16] or calcium transients with contraction force [17]. Nevertheless, further development is needed to integrate more simultaneous measurements in a single cell or tissue. With the use of a polyacrylamide-based platform it is theoretically possible to measure five parameters in a single cardiomyocyte employing optical reporters as voltage sensitive dyes for electrophysiology, calcium indicator dye for calcium handling, the α-actinin-mCherry for the sarcomere dynamics and the fluorescent beads for contraction force, followed by single cell Q-PCR for gene expression analysis. This platform would create added value for understanding the interconnected mechanisms during development, disease or for drug-induced toxicity studies.

Despite the great potential of human stem cell-derived cardiomyocytes there are several hurdles that need to be overcome. One major issue is the immature or fetal-like phenotype of hPSC-CMs (**Chapter 3**). Micro-environmental aspects present in vivo, such as blood flow, cell-cell interactions, dynamic fluctuations of blood components, stretch, electrical fields, geometry of cells, softness of tissue, etc. are absent or suboptimal in cell-based in vitro assays; these are likely to affect differentiation and function of cells. It is therefore important to mimic the in vivo situation as closely as possible. Temporal and spatially-dependent activation of specific signalling pathways responsible for cell differentiation during development have been mimicked in vitro to successfully differentiate stem cells into different cardiomyocyte subtypes (**Chapter 7**). Nevertheless, these pathways are just few threads in the network of development signalling. Such incomplete input impairs the differentiation process, restraining cells from reaching an adult phenotype. In order to mimic more aspects of in vivo development, in **Chapter 2** we studied the use of biocompatible polymers with different stiffness (including conditions that mimic the stiffness of heart tissue) in combination with micro-contact printing of ECM proteins, enabling controlled geometry and cell shape. Although morphological and functional improvements towards a more adult phenotype were observed, the induction of cardiogenic promoting pathways in combination with soft substrates and controlled cell shape, were not sufficient to promote a fully mature state. However, we found that addition of T3 hormone in optimized cardiomyocyte maturation medium resulted in significant molecular, morphological and functional improvement of hPSC-CMs, comparable to human fetal primary cardiomyocytes from second trimester of gestation (**Chapter 3**). Moreover, in **Chapter 4** a fully defined medium containing T3, Insulin-like Growth Factor 1 (IGF1) and a synthetic glucocorticoid Dexamethasone (Dex) among other components was described to induce an increase in hPSC-CM contractile force to a level twice that in 2nd trimester human fetal cardiomyocytes (**Chapter 3** and **4**). Although these improvements of electrophysiological and contractile properties are still far from adult cardiomyocytes[18,19], the importance of this advance in maturation of cardiomyocytes is indicated by the fact that this optimized culture condition allowed us to detect contractile defects in patient-derived hiPSC-CMs carrying a mutation in MYBPC3, which were consistent with studies from patients[13].

To further characterize the phenotype of MYBPC3 deficiency in hPSC-CMs, a reporter line expressing an α-actinin-mCherry fusion protein, incorporated at the Z-disc of sarcomeres was produced in order to analyze changes in sarcomeric organization and sarcomere dynamics (**Chapter 5**). Congruent with previous observations in mouse models[20], the deficiency of MYBPC3 causes a reduction in the diastolic sarcomere length and a decrease in shortening and lengthening amplitudes. Moreover, this phenotype was accompanied by a decrease in ATP usage per contraction cycle. However, the changes in calcium handling or increase in cardiomyocyte area, characteristic of hypertrophic cardiomyopathy, were not observed in these cells (**Chapter 6**). This may be related to the relative immaturity of hPSC-CMs in a two dimensional in vitro setting, which contrasts with hypertrophic cardiomyopathy that normally emerges at a later stage in life in a three dimensional heart. Although important advances have been made, culture conditions need to be further improved in order to mimic physiologically revenant conditions and to ensure the ability to analyze

disease related phenotypes in vitro. Besides adjustment of culture conditions, generation of defined cardiac subpopulations need to accomplished. In **Chapter 7** we show for the first time an effective way to arrest cardiac differentiation and expand cardiac progenitors, which could be to further differentiated into endothelial cells, smooth muscle cells, working myocardium (atrium and ventricular-like NKX2.5 positive cardiomyocytes) and pacemaker-like cardiomyocytes depending on the downstream signaling events in defined differentiation protocols. A better understanding of these processes that control cardiac progenitors cells and direct differentiation to specific cell-types may have a great impact on further research of cardiac development and tissue engineering, as it allows the production of different "pieces of the puzzle" for reconstitution of the heart.

Future directions

Integration of Biology and Engineering

Ultimately the best model of a human heart is the organ itself. Although current technology is not close to such an achievement, small steps have been made towards this direction. In 1997 the engineered heart tissue (EHT) concept was first introduced using chicken cardiomyocytes, but it was only until 2011 that hPSC-derived cardiomyocytes were used in three dimensional (3D) systems[21,22]. The 3D culture format has been shown to induce improvements in electrophysiology, cell size and orientation, structural organization and drug response of hPSC-CM[23,24]. Nevertheless, contraction force (0.37±0.29mN/mm2)[17] and electrophysiology parameters (RMP -49±1.2mV; Upstroke 10.4±1.2V/s)[22] of this 3D system are still far from the reality of the adult heart (Contraction force 44±11.7 mN/mm2; RMP -85mV; Upstroke 270V/s)[18,19], indicating that the 3D structure alone is not enough to direct hPSC-CM towards the adult stage. Other studies have demonstrated the importance of the contribution of non-cardiomyocyte populations of cardiac fibroblasts, endothelial and smooth muscle cells on the phenotype of hPSC-CMs in engineered tissues[25–27]. The main cardiac output measured in EHTs is calcium handling and contractile function in order assess drug responses or disease modeling[22,23,28]. However, in patients, disease symptoms or adverse drug effects in the heart are assessed through different parameters such as electrocardiography (ECG), ventricular wall thickness assessment, the ventricular volume intake, diastolic ventricular function left ventricular ejection fraction and blood pressure[29,30]. In order to progress towards more clinically relevant readouts it may have added value if the fluidic pump function of the heart can be mimicked in vitro.

3D Bioprinting

3D printing is a rapid evolving technology with an increasing number of applications. Recently, it was used for generation of biological material using viable cells and supporting substrates. This so-called 3D bioprinting technology potentially enables reconstruction of complex tissues by emulating the 3D architectural composition of the tissue of interest using the right cell types and supporting matrix components[31]. Currently there are different types of 3D bioprinters (e.g. Inkjet, Microextrusion and Laser assisted) that have been shown to generate not only multiple tissue types like skin[32], liver[33] and cartilage[34] but also diverse biological structures such as branched tumor models[35], vascular trees[36]

and aortic valves[37]. Despite the promising applicability of this technique in tissue engineering, there are major hurdles related to the materials and scaffolds that are currently used for 3D bioprinting. Generating complex functional tissues demands materials or scaffolds that are biocompatible, printable and have structural and mechanical properties that mimic the heart environment, which is very challenging[31].

Decellularized Heart

Another approach to build a 3D heart model is to start from a base structure which will serve as a fundament and an architectural blueprint for the heart. This can be achieved by decellularization of heart tissue[38]. This process involves the perfusion of cadaveric hearts with different types of detergents and/or enzymes that degrade and remove the intercellular content of the cells constituting the tissue, leaving the ECM structure intact for subsequent recellularization. The advantages of this approach are that the intact skeleton of the heart is being used to guide the assembly of the new cellular population, avoiding the hurdle of designing and building the correct heart structures and the use of complex synthetic scaffolds, together with non-inducing immunologic response of the native ECM structure on the new host in case of future transplantation application[38,39]. Nevertheless, the challenges of this approach are the poor delivery and low retention of repopulating cells together insufficient understanding how to produce and organize the different types and sub-types of cells in order to form a well-integrated functional multicellular heart tissue[40]. Some attempts have successfully been made on a smaller scale by repopulating decellularized mouse hearts with hiPSC-CM, which contracted and responded to adrenergic stimuli[40]. Perhaps in a short term basis the recellularization of single cavities of rodent hearts with hiPSC-CM can become a novel effective tool for drug development and toxicity studies.

Although at the long term any of these approaches may have the potential to successfully mimic the function of a pumping heart in vitro, most likely combinations of the above mentioned technologies could lead to formation of 3D functional heart model resembling the human heart in vivo, which will provide us more detailed information about drug responses and disease mechanisms. In the near future the hPSC-derived cardiac models will rise to a better and more predictive platform for drug discovery and development.

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