Quantifying functional phenotypes in human pluripotent stem cell derived cardiomyocytes for disease modelling and drug discovery

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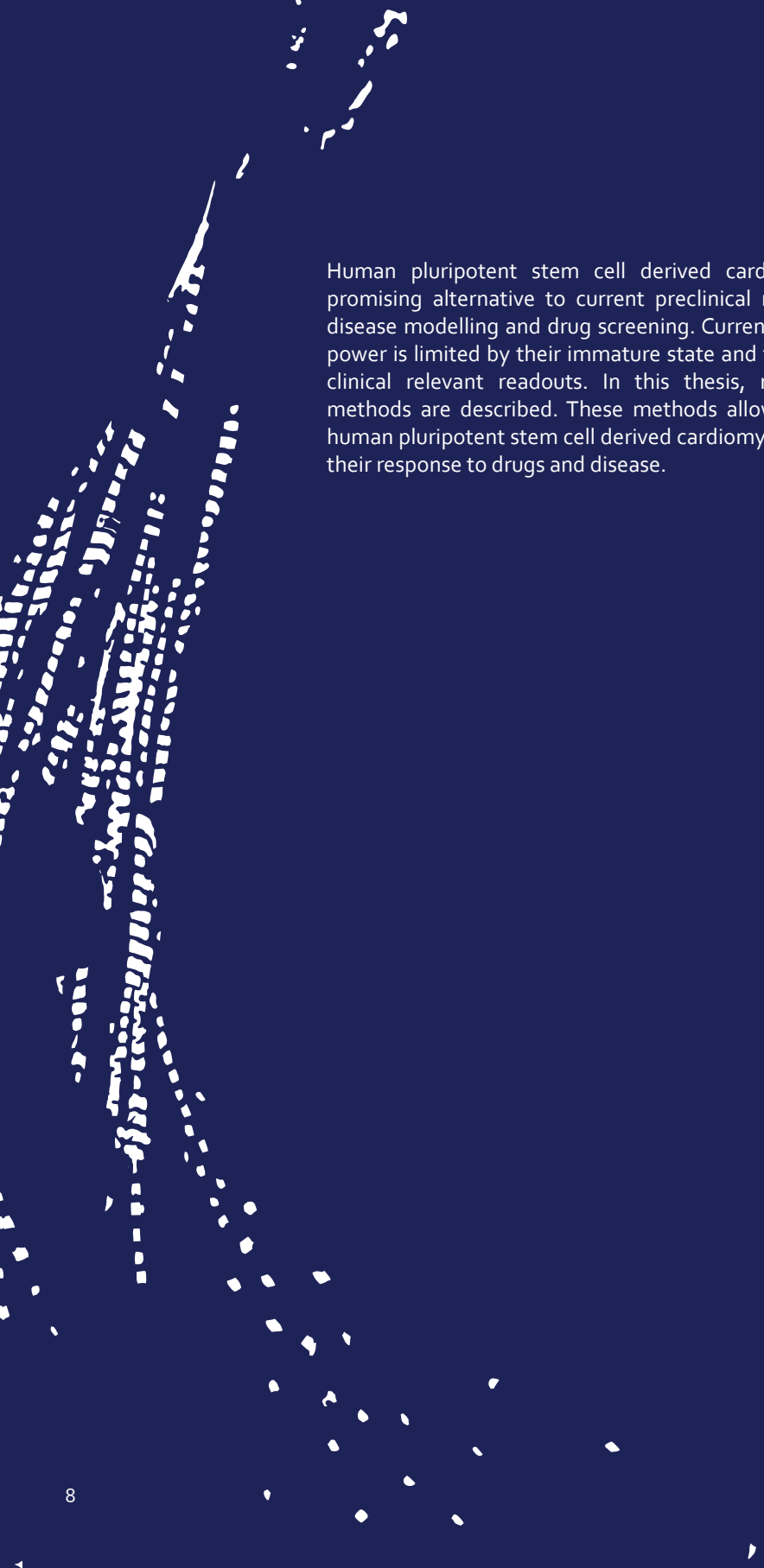

Abstract

Human pluripotent stem cell derived cardiomyocytes are a promising alternative to current preclinical models for cardiac disease modelling and drug screening. Currently, their predictive power is limited by their immature state and the lack of scalable clinical relevant readouts. In this thesis, new measurement methods are described. These methods allow quantification of human pluripotent stem cell derived cardiomyocyte maturity and their response to drugs and disease.

Chapter 1

General introduction

Models to study heart failure

Arguably, the heart is the most important organ of the human body. Cardiac failure remains a leading cause of death in the (Western) world[1] despite high investments in cardiac healthcare[2]. Although unhealthy lifestyle and diet are still major causes of heart failure (HF), the role of genetic defects resulting in metabolic diseases, high propensity for arrhythmias or high risk to develop congestive heart failure (CHF) should not be underestimated[3]. Consequently, secondary effects of CHF such as limited oxygen supply to tissue have also become more important to study and resolve. Apart from inherited susceptibility to HF, unexpected drug induced cardiotoxicity might trigger cardiac failure. For example, on September 30, 2004 the pharmaceutical company Merck voluntarily withdrew the drug rofecoxib from the market after it was found to induce myocardial infarction[4]. Rofecoxib was approved by the Food and Drug Administration in 1999 for treatment of arthritis, acute pain and menstrual cramps and used daily by 2 million patients. Such unexpected cardiovascular related toxicity is accountable for 45% of drug withdrawals from the market[5] and is still an important side effect of widely used chemotherapeutic drugs like doxorubicin[6] and trastuzumab[7]. Finding ways to prevent detrimental effects on the heart or alternative drugs without these effects is therefore still highly relevant in drug discovery and development.

To study cardiotoxicity and cardiac disease, *in vitro* models are used to mitigate the high risks, limited throughput and costs associated with *in* vivo testing. Animal models are used as well as non-cardiac human cells genetically modified to express critical cardiac features mediating toxicity. Examples of such features include the human Ether-à-go-go-Related Gene (hERG) ion channels to model risk of arrhythmia[8]. However, both type of models lack accuracy[9]. A critical reason underlying the lack of predictivity for both positive and negative drug- and disease effects in current models of the heart becomes clear by comparing their functional parameters to those of human cardiac tissue. Ventricular cardiomyocytes are functionally defined by the excitation-contraction coupling (EC-coupling) which key events are (i) the action potential (AP), which leads to (ii) an increase of free cytosolic calcium (Ca^{2+}) that in turn triggers (iii) the contraction through shortening of the cell's sarcomeres. This tightly orchestrated cascade is present in both animal and human cardiomyocytes, but the timing and shapes of these transient parameters exhibit high interspecies variation mainly due to different ion currents underlying the action potential[10]. It is thus no surprise that animal models often do not provide mechanistic insight in human cardiac function. Moreover, since most drugs and disease states affect ion channels or structural proteins of which animals and humans often express different isoforms, responses to them are highly species dependent. Non-cardiac cell based models transiently transfected with ion channels exhibit the correct currents, but are devoid of the complexity of the complete EC-coupling cascade which again leads to a lack of predictive power especially due to false-positives[11].

Stem cell derived cardiomyocytes

An emerging alternative class of *in vitro* models recapitulating both the complexity of EC-coupling and the accuracy of correct ion channel expression are based on human pluripotent stem cell derived cardiomyocytes (hPSC-CMs). Pluripotent stem cells

(PSCs) can divide indefinitely and are capable of differentiating to any cell type of the human body[12]. They are typically characterized by the expression of pluripotency associated genes Sox2, Oct-4 and Nanog[13], cell surface proteins recognized by TRA-1-60 and TRA-1-81 antibodies[14], and a high nuclear to cytoplasmic ratio. By inhibiting signalling pathways associated with pluripotency and activating pathways associated with lineage commitment during embryonic development it has become possible over the last decade to direct differentiation of PSCs to many specific tissue cell types with high efficiency[15–18]. The first human PSCs were isolated in 1998 from late blastocyst stage embryos generally donated for research after becoming surplus to requirements for treating infertility. Since this raises ethical issues because the embryo is destroyed during the procedure, many efforts were made to identify alternative sources for these embryonic. The greatest and most significant breakthrough in this context was the discovery of induced PSCs (iPSCs) in 2007 by Takahashi and Yamanaka[19]. iPSCs are derived from somatic cells from healthy or diseased individuals by reprogramming with the pluripotency associated transcription factors (e.g. Sox2, Oct-4, cMyc and Klf4) originally identified as characteristic of embryonic stem cells. Somatic cells from many different sources have been used including blood[20], skin[21] and urine[22]. This makes them widely available and moreover, they have a genotype of which the adult (disease or healthy) phenotype is known which theoretically enables personalized medicine.

In vitro differentiation of embryonic or induced hPSCs towards cardiomyocytes starts by inducing nascent mesoderm formation then cardiac mesoderm development which is regulated by three families of growth factors: bone morphogenetic proteins, wingless/INT proteins (WNTs) and fibroblast growth factors[23]. Next, WNT is inhibited to derive cardiac progenitors which develop towards ventricular-like cardiomyocytes[24] after removing the WNT inhibitor protein. Recently, different protocols have been established to derive different cardiac subtypes such as atrial-like cardiomyocytes using retinoic acid[25] and cardiac endothelial cells using vascular endothelial growth factor[26].

Characteristics of cardiomyocytes

Adult human cardiomyocytes are rod-shaped, often bi- or poly-nuclear cells highly optimized for cyclic contraction. The EC-coupling cascade of AP, increase of cytosolic Ca^{2+} and contraction is facilitated by a large number of cardiomyocyte specific processes, ion channels and structural proteins. The AP arises from an interplay of various passive and active ion transporters (i.e. channels, pumps and exchangers) that regulate the traffic of Na^{+}, Ca^{2+} and K^{+} across the membrane. The most important ion transporters for the AP of ventricular cardiomyocytes are encoded by the genes SCN5A, CACN1C, KCND2/3/4, KCNH2 (hERG), KCNQ1, KCNJ2/12/4 and a splice variant of SLC8A1 and produce the currents I_{Na}, $I_{Ca,L}$, I_{to}, I_{Kr}, I_{Ks}, I_{K1} and I_{NCX}, respectively[27]. $I_{Ca,L}$ and I_{NCX} increase the free cytosolic Ca^{2+} in the second phase of the AP mainly by triggering the fast release of stored Ca^{2+} in the sarcoplasmic reticulum (SR) via the ryanodine (RYR2) receptors. Finally, the contractile apparatus is activated which is made up of myofilaments, sarcomeres and their regulatory components[28]. Among the most important proteins involved in the assembly of the contractile apparatus are myosin light chain (MYL), myosin heavy chain (MYH), cardiac actin (ACTC1), tropomyosin (TPM), troponin (TNN), titin (TTN), cardiac α-actinin (ACTN2) and myosin binding protein-C3 (MYBPC3). Many

steps involved in EC-coupling have a high energy demand which is met by a large ratio of mitochondria to cell volume[29].

During embryonic development, the protein isoforms that are expressed in the human heart change in many cases. For example, ratios of MYL2/MYL7, MYH7/MYH6 and TNNI3/TTNI1 are higher than 1 in adult myocardium, while they are below 1 in prenatal myocardium[30]. Specific isoform transition of such structural proteins in adult myocardium is associated with sarcomeric dysfunction[31]. In general, mutations in genes encoding proteins involved in cardiomyocyte function are highly associated with cardiac diseases such as Brugada syndrome (mutation in SCN5A[32]), Long QT syndrome (mutation in KCNQ1[33], KCNH2[34], etc.; different types exist and are dependent on the specific mutation[35]), catecholaminergic polymorphic ventricular tachycardia 1 (CPVT1; mutation in RYR2)[36] and hypertrophic cardiomyopathy (mutation in MYBPC3)[37]. Cardiomyocytes originating from iPSC cells derived from patients with such mutations can be used to model the diseased phenotype *in vitro*[38–40].

hPSC-CM maturation

hPSC-CMs do not exhibit the same characteristics as adult cardiomyocytes. Instead, their state is more comparable to late embryonic or, at most, neonatal stages of development[30,41–43]. This lack of maturation is very likely to be limiting to the predictive power of hPSC-CMs as presently available since expected drug and disease responses might not be revealed if the cells do not recapitulate the adult state. For example, it has been shown that maturation is key in revealing the expected decrease in force of contraction in hiPSC-CMs derived from a patient with a mutation in MYPC3 leading to hypertrophic cardiomyopathy[44]. Since it limits the applicability of such models, improving hPSC-CM maturity is one of the major priorities in the field. Typically, this is pursued through biochemical stimulation with growth factors or hormones (e.g. triiodothyronine hormone, insulin-like growth factor 1 and dexamethasone[44]) or through the development of more complex heart models by introducing other cardiac cell types and organizing them in 3D geometries[43,45,46].

hPSC-CM immaturity is evident from looking at genomic, structural and functional level. The expression of certain cardiac specific genes remains rather low while genes associated with embryonic stages are upregulated (e.g. MYH7/MYH6 and TNNI3/TTNI1 ratios are typically below 1 [reference]). On a structural level, many signs of immaturity are present such as a low amount of bi-nucleated hPSC-CMs, a round shape and disorganized sarcomeres. Perhaps most importantly, many functional parameters of hPSC-CMs do not have similar levels. For example, the upstroke velocity of the AP in adult cardiomyocytes ranges from 150-350 V/s versus 10-50 V/s in hPSC-CMs, the resting membrane potential is reported to be around -85 mV versus -60 mV and the force of contraction is ranges from 40-80 mN/mm^2 versus 0.08-4 mN/mm^2, respectively [reference]. This issue of maturity and how to address it are considered more extensively in this thesis.

Functional phenotyping of cardiomyocytes

To gain insight in hPSC-CM performance, maturation and response to drugs and disease, accurate measurement methods are indispensable. Electrical activity is typically assessed using patch clamp, but this laborious method is limited by its low throughput and requires highly specialized operators. For higher throughput applications, multi electrode arrays can be used to measure the field potential of cardiomyocytes[47,48]. More recently, voltage sensitive fluorescent sensors originally developed for measuring neurons have been optimized and used for optical AP assessment[49]. Calcium flux is typically quantified using calcium sensitive fluorescent sensors[50]. For contraction, a wide range of measurement techniques exist[41], all varying in complexity and highly dependent on the configuration of the cellular model (e.g. single cells or monolayer of cells) which limits reproducibility across models and laboratories. The development of tools that can be applied in a wide range of cellular configurations is most important. Ideally, these measurement techniques would allow for measuring parameters not only *in vitro* but also *in vivo.* This would enable translational studies, which currently is a major challenge. For example, one important clinically used readout of contractility is ejection fraction output, which is impossible to measure *in vitro* since most hPSC-CM models are not configured to have cavities that pump out liquid, but are merely a planar monolayer cells exhibiting contractility.

Many of the current measurement methods are not suitable to assess hPSC-CM based models for pharmaceutical development applications, since they are limited by their throughput, operator dependency and number of readouts (i.e. only electrical activity, calcium or contraction). High-throughput assays that accurately quantify the complete EC-coupling are required to use hPSC-CMs for early cardiotoxicity screening in drug developmental pathways.

Increasing model complexity for the heart

Most differentiation protocols for deriving hPSC-CMs lead to heterogeneous cell populations with a high percentage of ventricular-like cells[24]. In recent years, biologists started to focus on more defined hPSC-CM cultures by increasing differentiation efficiency towards one cell type[25], purifying the cell type of interest[51] or culturing defined mixtures of purified cell types by putting them together in specific ratios[26], mimicking native myocardium. In the human heart, atrial and ventricular cardiomyocytes are the main cells responsible for the heart's contraction, but they rely on cell-cell interactions with, among others, nodal cardiomyocytes, cardiac endothelial cells[52] and cardiac fibroblasts[53] for their performance, including growth, electrical activity and contraction. It has therefore been hypothesized that cardiomyocyte maturation is dependent on biochemical or biophysical cues from these surrounding cell types. Besides co-culture of different cell types, researchers are increasingly adding biophysical complexity by developing 3D tissues[26,45,46,54] and including physical cues such as stretch[55,56] or flow using microfluidic channels[57]. Recapitulating the physiological environment of cardiomyocytes might not only induce maturation, but is likely to enable studying indirect cardiomyocyte failure due to affected endothelial cells or fibroblasts.

Organs-on-Chips

To be able to mimic the native environment of cells even more closely, Organ-on-Chip (OoC) devices have been developed using soft materials with tuneable properties such as stiffness and viscoelasticity. Huh et al. cultured a alveolar-capillary interface in a Lung-on-Chip device fabricated from polydimethylsiloxane (PDMS) using primary alveolar epithelial cells and microvascular endothelial cells[58]. Even though being notorious for small molecule absorption which is a challenge for use in drug development trajectories, PDMS has been widely used by others as well, for example by Parker et al. to develop thin film membranes coated with isolated neonatal rat cardiomyocytes which were the first Heart-on-Chip devices[59]. More recently, multiple organs have been coupled together to form more complex models, including a model consisting of multiple organs to replicate the 28-day menstrual cycle of females[60].

By combining microfabrication technologies originally used in integrated chip fabrication with hiPSC biotechnology, OoCs might both increase cell maturity and enable high throughput readouts. Microfabrication enables creation of chips in the shape of vessels, flexible membranes, or other structures that recapitulate the native environment of the cellular niche while hiPSCs can be used to derive cell types of interest from healthy or diseased individuals. For hPSC-CMs, it is known that substrate stiffness highly determines the force of contraction[61], but cardiomyocyte shape induced by micropatterning also determines sarcomeric organization and functional output[62,63]. Additionally, by including sensors such as electrodes or force transducers in the substrate these devices can integrate readout systems that allow upscaling and high-throughput cellular measurements, making these models suitable for the early toxicity testing and drug screening.

Aim and scope of this thesis

The future of hPSC-CM applications in pharma for drug discovery will depend on their predictive power and their ability to recapitulate human disease states. In order to investigate this predictivity, several chapters in this thesis describe different aspects of the application of hPSC-CM in the context of drug and disease modelling. To understand the limitations of current measurement, methods for physiological responses of hPSC-CMs are described in Chapter 2. In addition, current multiplexed measurements of AP, Ca^{2+} flux and contraction and the application of biophysical stimuli are explored. In Chapter 3 a new software (MUSLCEMOTION) tool for measuring contraction is described that overcomes limitations of current techniques such as the cell configuration dependency. Instead, MUSCLEMOTION is used to quantify contraction in a wide range of cellular configurations *in vitro, in situ* in complex devices and even *in vivo*, enabling clinical translational studies. In addition, contractile response to drug and diseases in the different models is quantified. In Chapter 4 a detailed protocol for using MUSCLEMOTION is presented to enable every biologist and research group not specialized in measuring contraction to quantify contractility using standard equipment. MUSCLEMOTION is used in Chapter 5 in combination with fluorescent voltage and calcium sensitive sensors to develop a novel scalable non-invasive "Triple Transient Measurement System" for simultaneous high-speed assessment of cardiac

action potential, calcium handling and contraction. To demonstrate its potential for cardiotoxicity screening, disease modelling and drug discovery, hPSC-CM response to drugs and diseases is measured and quantified with automated analysis software. Next, the Triple Transient Measurement System is one of three platforms used in Chapter 6 for a blinded, multi-centre study to evaluate drug-induced contractility changes in hPSC-CMs. In Chapter 7, to integrate methods for maturation and hPSC-CM phenotyping, microfabrication methods are described to develop a modular OoC device (Cytostretch) based on PDMS. Cytostretch recapitulates the native environment of the heart by cell alignment on a soft substrate which can be periodically stretched to model the changing heart volume during a heartbeat. Furthermore, Cytostretch includes electrodes to measure the electrical activity of hPSC-CMs plated on the device. Chapter 8 elaborates on the notorious problem of the absorption of drugs by PDMS, which requires a solution before it can be used in drug response assays. The absorption of four cardioactive drugs is quantified and reduced by applying cell membrane-like lipid layers to the surface of PDMS. Finally, in Chapter 9, results and conclusions presented in the previous chapters are discussed, accompanied by a general outlook and future implications on the field.

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