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

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Abstract

Over the past 15 years, human pluripotent stem cell derived cardiomyocytes (hPSC-CMs) have been extensively explored as a new model to study human responses to cardiac drugs and disease. To become a fully-fledged alternative to current animal or non-cardiac *in vitro* models, hPSC-CM based micro physiological systems (MPSs) need to (i) exhibit a mature (postnatal or adult) cardiac phenotype – at least for the features of interest in each particular study – and (ii) output clinically or mechanistically interpretable data. These two critical requirements would allow these models to be designated as accurate *in vitro* models of native human myocardium.

In this thesis several tools were developed towards fulfilling these requirements. First of all, a new measurement method to quantify cardiac contractility from bench to bedside was introduced. Whilst challenging to develop, the method is straightforward in use, was validated across a wide range of contractile models and made widely available for use in standard laboratory settings. To gain mechanistic insight into the complete excitation-contraction (EC) coupling cascade, a non-invasive optical measurement method was also developed to quantify the action potential, calcium flux and contraction of hPSC-CMs simultaneously in response to drugs and disease. In addition to the measurement technique as such, new analysis methods based on statistics and known cardiac physiology were developed that can automatically identify the mechanism of action of cardioactive drugs. The application of the system was further demonstrated in a blinded, multi-centre evaluation of drug-induced contractility changes. Next, aiming to further improve maturation of hPSC derived models using non-chemical cues, a modular Organ-on-Chip (OoC) device was developed in which a wide range of features could be built to enable for example cyclic stretch and cardiomyocyte anisotropic alignment. Finally, looking critically at polydimethylsiloxane (PDMS), the current standard material used to make many MPSs, its absorption of cardioactive drugs and compounds was investigated and solutions to mitigate this problem were explored. In this final chapter the results presented in this thesis and future perspectives are discussed.

Chapter 9

General discussion and future perspectives

Mimicking human cardiac function with hPSC-CMs

Although hPSC-CMs are promising models for cardiotoxicology screening, drug development and disease research, their limited maturity remains a hurdle to full use. As summarized in Chapter 2, many of their functional and structural properties remain underdeveloped *in vitro*. It is not unsurprising, therefore, that there are many efforts ongoing worldwide to address this and improve this maturation state[1–4]. It is not believed that there is an intrinsic block to maturation of hPSC-CMs since in the right microenvironment *in vivo* after transplantation to the heart (of animals) maturation appears to take place[5,6]. Sensitive measurement tools however, might be the most immediate solution to mitigate poor maturity at least in part (e.g. low force of contraction might be detectable with more sensitive quantification methods) but more fundamental limitations are encountered when cardiac mechanisms present in adult cardiomyocytes are not fully developed in hPSC-CMs[7]. For example, this might be the reason for the lack of cAMP-related effect in the PDE3 inhibitors tested in Chapter 5 and 6. Apart from the cellular immaturity, the interaction of cardiomyocytes with different cell types in the *in vivo* microenvironment is important to take into consideration. For example, the absence of sympathetic nervous system is a very likely reason explaining why no effect was found in hPSC-CMs in response to atenolol (Chapter 5 and 6)[8]. In addition, the mechanical environment of cardiomyocytes is important. The OoC device 'Cytostretch' developed in Chapter 6 can be used to investigate whether an environment that resembles the mechanical properties of the heart more closely by including for example cyclic strain and topological cues for alignment will improve hPSC-CM maturation. While the dimensions of certain features currently used in the device might be suboptimal[9] or perhaps irrelevant[10], it was demonstrated that the fabrication of the modular device is feasible. In fact, this flexible design can be used easily and adapted to exploit important maturation cues reported[11]. In addition, electrical stimulation has recently been shown to mature hPSC-CMs significantly[3] and can be applied via the integrated electrodes. Furthermore, the PDMS on the device can be tuned to have viscoelastic properties that mimic the walls of the heart which is reported to influence cardiomyocyte function[12] and might improve hPSC-CM maturation although this has not yet been formally shown[13].

The cellular composition of the adult myocardium is not limited to cardiomyocytes. In fact, only around 30% of all cells are myocytes, while the other 70% is a combination of cardiac endothelial cells, cardiac fibroblasts and vascular stromal cells[14]. Incorporating these different cardiac cellular subtypes is likely to increase the predictive power of hPSC-CMs since it can capture indirect effects of other cells on cardiomyocytes (e.g. endothelial cells that are reported to play a major role in trastuzumab-induced cardiotoxicity[15]) but also because the correct (cardiac) cellular niche has been reported to play a role in maturing the myocytes themselves[16,17]. In most work reported in this thesis, these carefully controlled and composed multicellular cardiac cultures were not used although it is most likely that even with the highly efficient differentiation protocols used, all cardiomyocyte batches not specifically purified contained an (undefined) non-myocyte population. This was the case for both in-house differentiated hPSC-CMs but also for the commercially available Pluricyte hPSC-CMs also used in this thesis. Although more expensive, the benefit of commercial cells in general should not

be underestimated. Large batches of frozen cells allow multiple experiments at defined moments after thaw using a single differentiated population, thus decreasing assay-to-assay variation. Moreover, the quality control (QC) incorporated in the production process of commercial suppliers ensures high reproducibility and less batch-to-batch variation. Freezing methods for hPSC-CMs are becoming more widely available and using this for large batches of in-house differentiated cell populations does provide researchers (particularly as in this thesis, with complex downstream experimental set-ups) with more flexibility. It is thus an approach to decreasing inter-experiment variation without extra expense. However, the QC in most laboratories still largely relies to fluorescence-activated cell analysis or sorting to define the cellular composition of a differentiated population of cells. This could and should where possible be extended to functional readouts. MUSCLEMOTION in combination with a simple imaging tool such as a smartphone or in-incubator microscope might be used to implement such QC for contractility of hPSC-CMs in addition to the protein expression profile (Chapter 3).

Apart from batch-to-batch variability, hPSC line-to-line variability is important to take into account when designing and executing assays for drugs and disease. Even when hPSC-CMs are cultured and measured in similar conditions, they still show variability in functional parameters such as action potential duration 90%[18]. This line-to-line variation emphasizes the importance of using isogenic controls wherever possible when studying human induced pluripotent stem cell (hiPSC) derived from patients as models for disease phenotype. Alternatively, a set of well-described cardioactive drugs could be tested in each experiment as reference data for that particular line. Especially when testing drugs (blinded identity for operator) reference compounds offer a solution by comparing results of each line to their line-specific response to the reference compounds.

As hPSC-CM based models continue to advance and more closely resemble adult myocardium, their accuracy and predictivity is also expected to increase. However, even the best human models will inherently be a simplification of the reality making it important to select and use the right model for each given task. Nevertheless, even the output of fully matured human models is not easily translated to clinic relevance; the models often require a completely different measurement approach than used in patients and drug and compound metabolism, bioavailability and breakdown may strongly depend on the intact human body. The aspects addressed in this thesis mainly concern standardizing measurement methods.

Measuring cardiac function in hPSC-CMs based models

Measurement methods determining physical features of hPSC-CMs are typically used to gain insight in the state of maturation or drug- and disease-induced effects. The phenotypic changes of hPSC-CMs associated with these conditions are essentially reflected in (a combination of) a wide range of parameters related to the structure, action potential, calcium handling and -storage, contraction or metabolism. The level at which these changes take place can differ and can range from changes in gene and protein expression and protein trafficking to purely mechanical and electrical alterations. Ultimately, the most salient feature of cardiomyocytes is their cyclic

contraction and to quantify this MUSCLEMOTION was developed in Chapter 3. However, MUSCLEMOTION is a relative measure of contractility and is not able to quantify the force of contraction which gives more insight in the contractile power and is measured in more complex setups[2,19]. Although absolute values of force of contraction are valuable, care has to be taken in comparing the force generated by hPSC-CM in different setups since they might be subjected to different load. MUSCLEMOTION, but also the Triple Transient Measurement (TTM) system developed in Chapter 5 are all optical systems. While this often circumvents the need for complex substrates, making its applicability versatile, and reduces the invasiveness of the measurement method on the cell, the use of fluorescent sensors can increase experimental variation due to bleaching, loading differences and internalization of the sensor in cellular sub-compartments such as the mitochondria. In part, this problem could be mitigated by using ratiometric fluorescence sensors in the TTM system, but this would require more fluorescent channels to be multiplexed and would decrease the effective sampling frequency. Furthermore, even though less invasive than for example patch-clamp electrophysiology, these sensors should not be considered "non-invasive" for the cells. First of all, light – especially high power light at small wavelengths – induces reactive oxygen species in the cell culture media[20]. Secondly, the sensors themselves affect cellular behavior. For example, calcium sensitive dyes might have a buffering effect since calcium ions bind to and sequester the dye molecules which reduces the contractility depending on the concentration used. However, the kinetics of contraction and relaxation have been reported not to change[21].

The parameter that is theoretically the most limiting in the measurement is the voltage sensitive signal and more specifically the upstroke time (T_{rise}) which is related to the maximum upstroke (V_{max}) in classical electrophysiology. This potential limitation is due to the minimum sampling frequency for assessing the action potential which is calculated to be around 8.8 kHz (Chapter 2) in adult cardiomyocytes; this is more than 26 times higher than the 333 Hz used in the TTM system. However, hPSC-CMs have a lower V_{max} which makes the measurements of V_{max} at 333 Hz sufficient at their current maturation state. Extracellular field potential recordings as used in Cytostretch are easily made at sampling frequencies >10 kHz using the embedded electrodes. Compared to the TTM system and MUCLEMOTION, the advantage of integrating the culture environment and measurement tools is a reduction in readout apparatus complexity, ease-of-use and the potential for continuous monitoring. However, this comes at the expense of a higher price per data point, less flexibility in cellular configuration of the model and fixed predefined readouts. The choice for PDMS as material to develop these chips is also arguable. While the advantages of PDMS for Organ-on-Chip devices are evident (e.g. rapid prototyping, transparent, biocompatible, stretchable, tunable mechanic properties) the material absorbs many small molecules – and thus drugs – in high amounts (Chapter 7). This problem was mitigated to some extent by lipophobic coatings (also used for contact lenses) but is still lacking an off-the-shelf solution applicable in every lab. It was shown that the index for hydrophobicity (LogP) does not explain the level of absorption as is reported previously[22]. However, the inverse topological polar surface area seems to be a predictive measure.

Where possible, hPSC-CM features should be quantified in ways that are clinically

interpretable. MUSCLEMOTION was used both *in vitro* and *in vivo*, but head-to-head comparison of the results is still impossible due to the difference in cellular configuration and load, for example. The TTM system provides insight in the cellular physiology but is not easy comparable with the whole heart. In fact, most of our understanding of cardiac physiology at the cellular level derives from animal studies. It is often difficult to find studies performed on (adult or even fetal) human cardiomyocytes to examine interspecies differences.

Importantly, measurement tools developed in this thesis should be automated where possible. Apart from reducing the need for operator time and skills, this will further remove unintentional bias from the operator. For example, selecting measurement areas in the TTM system is now done by the operator while automation could have these randomly selected. Apart from the measurement method, automated analyses, such as the algorithm developed in Chapter 5 to identify mechanism of action in hPSC-CM drug responses, will eliminate bias in the interpretation of results. Automation can lead to unbiased, reproducible measurements with the potential to scale up. These are important requirements to make accurate human avatars for drug and disease research.

hPSC based models as human avatars

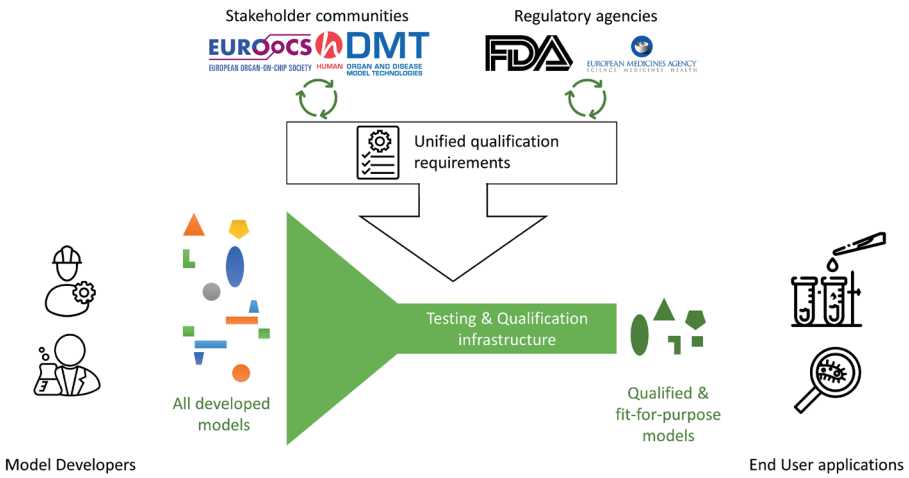
Together with advanced artificial intelligence computational modelling[23], hPSC based models and OoCs are likely to revolutionize the way pharma predicts, tests and screens potential drug candidates. As hPSC based MPSs (hPSC-MPSs) will increasingly capture the complete human complexity including unknown pathways or mechanisms, they could be used to check and validate predictions made by *in silico* models. Since this approach is fundamentally different from the current high-throughput *in vitro* method for identifying drug candidates applied in most cases, it is important to adopt the future hPSC-MPSs (or MPSs derived from other cell sources) to the new scenario instead of trying to replace the old. In practice, this means that the need for high-throughput MPSs or OoCs might be less important than often implied[24]. In turn, this allows for a higher price per data point and thus more complex models. Apart from pharma, academic research groups are likely to adopt hPSC-MPSs, especially if models emerge that have outputs that can be related to human physiology or clinical data directly. If MPS based assays can be made more widely available to non-expert laboratories and researchers they could be used as an 'plug-and-play' assay in the experimental palette. A portfolio of different devices is required since it is unlikely there will be one chip fit for all purposes. While emerging commercial OoC companies are instrumental in promoting this approach, the lack of flexibility in some commercial products is not beneficial for biological research where every organ may have unique requirements. Moreover, it is not likely that every hPSC based model needs an actual microfluidic chip, making MPS more favorable as a term over OoC. The emerging companies are however of great importance since they are the first to produce large batches of chips which is a step in reducing the lack of reproducibility found typically in in-house fabricated devices. Especially since relatively high variation is inherent to the field of biology, the technological part of the MPS should be made as reproducible as possible. The need for a wide portfolio of devices and models imposes difficulties on standardization and harmonization in the field. If anywhere these are most likely to be introduced in

regulatory settings such as safety and toxicology testing.

The field would be best focused on developing MPS of critical organs in combination with essential human-specific processes such as the immune and inflammatory system (triggers to many diseases) in environments that provide the correct chemical and/or mechanical cues to organize the cells as in native tissue. This process of organization is a trade-off between directed bioengineering and developmental self-assembly which will require close collaboration and communication between engineers and biologists to find the right balance for each MPS. To develop disease specific MPS of these critical organs, data banks with well-documented hiPSC lines are essential and emerging at acceptable cost and these can be used to identify and predict disease severity based on genetic background. In addition, using CRISPR/CAS9 it is becoming increasingly possible to introduce a large number of different mutations efficiently in a single hiPSC cell line to investigate the mutation-specific effects of, for example, long QT syndrome where multiple mutations are described for same disease[25].

Future perspectives

Use of hPSC derived cells as models of disease, for drug discovery and the identification of toxic responses in organs and tissues is coming of age but is widely expected to become "adult" in the near future due to further integration of biology and engineering which will enable complex, biologically mature and patient-specific MPS. As costs to produce hiPSC lines from patients and their subsequent differentiation beginning to decrease and cell banks with associated patient information come online worldwide, it seems increasingly likely that stem cell-based models might become personalized or semi-personalized using hPSC of genetically similar subpopulations. What we have now are readily accessible options for scaled production of well-defined differentiated stem cells that allow high throughput bioassay screens on hundreds of well-plates with each



well containing an identical cell population. What we (academics and pharma) would increasingly like to have though, is to have these well-plates filled with differentiated stem cells from hundreds of different individuals or patients. This would make the technology personalized. More research is clearly required to develop reproducible and fit-for-purpose MPS with readouts that are clinically relevant and give insight in human physiology but at the same time are scaled down to reduce the cost of each data point. This would make it feasible for pharma to carry screens on large compound libraries across whole populations rather than just a few individuals. This could best be done using advanced robotic or liquid handling systems based and several of the quantitative bioassay assays developed in this thesis could be implemented in these pipelines.

Coming-of-age of the field will also require infrastructure suitable to bridge the gap from academia to end user applications. Such infrastructure should be technological (e.g. standardized platform technology, reproducible fabrication, automation software) but also biological (e.g. large batched of hPSC derivates, large biobanks for population screenings, testing facilities) and even mathematical (e.g. *in vitro* to *in vivo* relationship modelling). Such multidisciplinary infrastructure needs to be built and supported by a large network of developers, end users and other stakeholders in close collaboration with each other. A possible model for this is shown in Figure 1. This shows a infrastructure design to take the technology forward such that it can be implemented by regulatory bodies as alternatives to current technologies which often miss human (patho)physiological relevance.

Figure 1: Organ-on-Chip infrastructure is needed to bridge the gap from model developers to end user applications.

This infrastructure should be able to select, test and qualify models making them directly suitable for End Users. Importantly, the qualification requirements should be defined in close collaboration with both stakeholder communities and regulatory agencies to ensure wide support but also take steps towards incorporation of such models in the drug approval process. Image credits (Noun Project): engineer by Esaú Aguilera, Scientist by parkjisun, Job Description by SBTS, testing by priyanka, bacteria observation by Egorova Valentina.

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