Cardiomyocytes from human induced pluripotent stem cells: capturing disease severity of LQT2 syndrome and the impact of chromosome aberrations

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General Discussion

GENERAL DISCUSSION

Cardiomyocytes from human induced pluripotent stem cells (hiPSCs) beat spontaneously in culture and express the full complement of ion channels present in primary human cardiomyocytes, albeit at ratios that resemble that in the foetal heart rather than the adult. This means that they have the potential to model human channelopathies caused by mutations in ion channel genes (reviewed in Brandão *et al*, 2017)[1]. In this thesis we focus on challenging the hiPSC-cardiomyocyte model to depict in vitro differences in LQT2 severity when only single point mutations are introduced into the ion channel gene, *KCNH2*. The primary aim here was to see whether more subtle phenotypic differences caused by different mutations could be detected using established technology, and to determine in parallel whether genetic drift commonly observed during hiPSC culture would affect the physiology of derivative cardiomyocytes.

SETTING THE STAGE

We first revisited hiPSC cardiac disease models and considered their present value in capturing disease phenotypes based on current methods in widespread use. We critically evaluated whether treatments suggested by the published reports could be translated (eventually) to clinical practice, and we also highlighted their relevance in identifying novel disease mechanisms that could potentially lead to testing new therapeutic compounds and strategies to advance precision medicine initiatives. While appreciating the highly positive impacts that hiPSCs and hiPSC-CM differentiation has had on understanding heart development, we concluded that it was important to consider shortcomings of these models with respect to disease modelling and other clinical applications. Aside from widely discussed issues like the immaturity of hiPSC-CMs (and of other differentiated hiPSC and hESC derivatives in

general), another important point to consider when using hiPSC as disease models, is line-to-line variability. This could be the result of different reprogramming methods, genetic backgrounds, as well as somatic tissue sources used for reprogramming (blood, skin, urine etc.). Several reports have shown key disease phenotypes being faithfully recapitulated using patient hiPSC-CM models; nevertheless, many of those studies would have benefitted from higher n among the controls or using isogenic pairs with the “disease” and “control” hiPSC lines coming from the same genetic background; either through genetic repair of the mutation or introduction of the mutation(s) in a “healthy” control line.

Even when hiPSC-CMs are cultured and measured in similar conditions, variability can arise through the presence of genetic modifiers, variants of unknown significance (VUS), or gender, all of which can affect functional parameters. In this context, genome editing technologies have become a particularly powerful tool for creating isogenic lines and more reliable controls. hPSCs from a patient could be edited and corrected prior to CM differentiation to rescue a specific disease phenotype or, using a “wild type” line from a healthy control individual, a variant could be inserted to confirm it as a disease phenotype trigger. While the first approach seems well-suited for precision medicine, it remains a line derived following complex genetic manipulation in which multiple other (unintended) changes could have taken place. In addition, it is currently financially unfeasible (reprogram and modify each patient hPSC line) and technically challenging (time consuming techniques) to do this at scale on a large population of individuals. However, the second approach, whereby different variants are introduced into a single cell line, could still impact precision medicine with the development of centralized cell banks and better methods to assess genetic modifiers and VUS. In this thesis, we adopted this concept to create several LQT2-hiPSC-CM lines that were

used as a proof-of-concept of how hiPSC isogenic lines could be exploited for assessing disease severity. For this, we developed a detailed protocol (chapter 3) that established a robust method for introducing and screening heterozygous mutations in hiPSC lines. While the creation of knockout mutations in hiPSCs are relatively robust, knock-in experiments with precise and scarless monoallelic targeting remains a challenge. In our protocol we focused in evaluating sgRNAs on-target efficiency to determine the best delivery method to be used. For example, with very efficient sgRNAs it can be difficult to achieve heterozygous targeting, indicating that a lipofectamine based transfection would be desirable. Likewise, sgRNAs with low on-target activity, a more efficient transfection such as electroporation would be necessary if no other options of sgRNA are available.

FROM A POINT MUTATION TO A FLAT LINE

Although presented as a monogenic disorder, LQT2 is a very complex channelopathy due to its uncertain variability and differential drug response. First, several studies have demonstrated the location of the mutation within the affected ion channel is an important determinant of arrhythmic risk in patients. In chapter 4 we have also showed that the position of the mutation had an impact on arrhythmic events in hiPSC-CMs with pore-loop mutation conferring greater susceptibility those events to occur. Further complicating, there are genetic modifiers that can alter disease phenotype, little about which is known. We have demonstrated the impact of K897T polymorphism on channel kinetics for hiPSC-CMs harbouring a primary LQT2-causing mutation (chapter 5). A recent study also identified two potential genetic modifiers on different genes that affect disease severity in LQT2 patients[2]. A point mutation in *REM2* (G96A), a member of the *Ras* superfamily of GTPases which regulates voltage-gated calcium channels, increased $I_{Ca,L}$ that in combination with a

primary *KCNH2* haploinsufficiency mutation led to a severe deficiency in repolarization. The second modifier was present in another potassium channel in which the variant KCNK17-S2G may protect patients by a compensatory current in the presence of a primary mutation in *KCNH2*[2]. Altogether, these features make it difficult, and often inconclusive, to interpret the functional consequences of potential disease-causing variants. Therefore, being able to create a range of LQT2-hiPSC lines in a single genetic background could not only shed some light into mechanisms for variable expressivity and incomplete penetrance, but also, they could be used as a platform to test alternative drugs, improve treatment strategies, and build opportunities to develop tailored therapies for patients.

We have conceptualized proof-of-concept studies that could be used to evaluate the extent to which hiPSC-CMs are able to reflect intragenotype differences in disease risk such as observed between LQT2 patients. In addition, we investigated the possibility of developing a scoring system to estimate the arrhythmogenic risk to E-4031 (a hERG channel blocker) addition for the different *KCHN2* variants. By genetically modifying a control hiPSC line we have created hiPSC lines harbouring heterozygous mutations within the pore-loop domain (KCNH2-A561T) or in the cytoplasmic tail (KCNH2-N996I) of hERG (chapter 4); lines harbouring the same heterozygous mutations but with the K897T polymorphism either on the same or opposite allele to the primary *KCNH2* mutations (chapter 5); and finally, lines lacking the expression of wild type hERG but with the pore and tail mutation present in the one allele (chapter 6) (Table 1). Molecular and functional comparisons of these edited lines confirmed not only that the hiPSC-CMs could recapitulate the key features of LQT2 (i.e., prolongation in cell repolarisation, susceptibility to arrhythmias) but also that differences due to the primary mutation as well as the linkage phase of a single nucleotide polymorphism (SNP) could be identified in the

cell lines. Dissimilarities in channel functionality were caused by these variants: while the primary mutations resulted in impaired glycosylation of the channel affecting trafficking of hERG to the membrane (chapter 4), functional analysis of the heterozygous compound mutations in KCNH2-K897T lines also revealed changes in the biophysical properties of the channel, with KCNH2-K897T cis exhibiting faster activation and deactivation kinetics than KCNH2-K897T trans hiPSC-CMs (chapter 5). Furthermore, the differences in I_{kr} densities detected between pore and tail variants indicated distinct disease mechanisms, with the tail mutation leading to haploinsufficiency and the pore mutation causing a dominant-negative phenotype (chapter 4). Being able to pinpoint these genotype-disease mechanism relationships using isogenic hiPSC-CM lines could further our understanding of the molecular pathology in LQT2, especially when the action of modifiers may exacerbate severity via a different disease mechanism. The subsequent lines harbouring a primary KCNH2 mutation in one allele and a disruption of the reading frame in the other allele (chapter 6) also corroborate that relationship and it goes further by using additional molecular studies to predict keys steps on possible regulation pathways and recovery mechanisms. Although more investigation is required, it already highlighted how Ca^{2+} channels and proteins linked to its release could have a real impact on LQTS by offering a new way to approach the disease and finding different targets for possible treatments.

Another important aspect revealed by our work was the ability to recapitulate the variable disease severity *in vitro*. Not only did all our edited lines exhibit electrophysiological changes in action potential (AP), but lines harbouring a pore mutation showed longer AP and field potential durations (FPD) compared to tail mutated lines. Moreover, a prolonged repolarization phenotype in pore mutant lines combined with E-4031 revealed greater susceptibility to proarrhythmic effects

compared to the tail mutation. These subtle differences between mutations are promising and highlight the potential of hiPSC-CMs to predict risk and to assist in stratification of patients regarding potential treatment. Scoring systems are useful for risk prediction besides aiding in establishing clinical diagnosis of LQTS. In chapter 4, we proposed a system based on previous work for evaluating the cardio safety of pharmacological compounds to estimate the arrhythmogenic risk to E-4031 for hiPSC-CMs with KCNH2 variants. As expected, pore mutations gave higher susceptibility to develop early afterdepolarisations (EADs) and "fibrillation" than tail mutation but, more interestingly, we showed that altered risk was evident even between lines with the same primary mutation differing only on chromosome orientation of the KCNH2-K897T polymorphism (chapter 5). Such a scoring system using hiPSC could facilitate better drug dosing, cardiotoxicity assessment and new drug studies. However, it remains necessary to challenge the models with different compounds to further validate their predictive power. Furthermore, our model would also benefit from analysis of additional lines with mutations at different positions within *KCNH2* to further strengthen evidence for the hypothesis that mutation location determines disease phenotype. And, more importantly, it could potentially identify in vitro the anomalies resulting from those diverse mutations.

CONFOUNDERS IN HPSC DISEASE MODELLING

The derivation of hESCs and the reprogramming of somatic cells into hiPSC, combined with prolonged culture, introduces changes in the genome that culminates in genetic drift and confers selective growth advantage on the aberrant cells by upregulating growth-promoting genes. In addition, inherited epigenetic alterations have been shown to affect gene expression dosage, with loss of imprinting and DNA methylation changes being the main source of epigenetic

aberrations specifically in hiPSCs. While only a minor impact is expected for certain early onset diseases, epigenetic aberrations may be crucial in considering late onset conditions since environmental contributions affect long-term disease progression or may be responsible for exacerbation of the disease phenotype. Moreover, with the increase in genome-wide association studies (GWAS) and the identification of an increasing number of VUS, epigenetic aberrations should be carefully considered when investigating phenotypes resulting from genetic variants since they may lead to incorrect interpretation of what an observed phenotype in the model means for the disease.

Another shortcoming of hPSCs results from the very properties that make them valuable and unique: self-renewal and pluripotency. Both features contribute to their tumorigenicity and genomic instability. In chapter 7 we, as others[3], showed that following extended growth in culture, hiPSCs underwent selection through differential growth rates for an altered genotype. This was specifically a duplication in the q arm of chromosome 1, creating a temporary mosaic hiPSC population. Although the impact of copy number variations (CNVs) has been widely studied in hPSC, the situation can be more complex if this is an ongoing process leading to genetic drift and the cells under study are differentiated hiPSCs. Very few studies have addressed the impact of CNVs on the phenotype of differentiated cells where new gene networks are activated and the action of “hitchhiker” genes can become pronounced[3]. For example, the dup-1q31.3 we and others identified in hiPSCs, and the resulting higher proliferation rate, would impact cell cycle studies. However, the same dup-1q31.3 in hiPSC-CMs would result in a new expression profile that may increase and/or decrease expression of one of more genes during or at the end of hiPSC differentiation into CM. This would be misleading in understanding disease phenotypes in these models as we showed for hiPSC-CM contraction analysis. To our

knowledge, this is the first time that dup-1q31.3 has been shown to impact hiPSC-CM functionality. Other studies in which hPSC karyotypic abnormalities on chromosome 17q and 20q have been investigated, have also shown altered transcriptomic profiles leading to growth advantage; moreover, when these hPSCs are differentiated to the neuroectodermal lineage, they show either faster commitment- or impaired neuroectoderm differentiation, respectively. The prevalence and consequences of genetic aberrations in hPSCs are currently the subject of extensive discussions and are important not only for disease modelling but also for future clinical translation. For this reason, the ISSCR is currently establishing guidelines for stem cell researchers and notes culture-acquired genetic and epigenetic stability as being a significant risk in all research areas that warrants careful scrutiny (ISSCR Standardisation Guideline, 2022). The guidelines advise monitoring not only hPSCs that have undergone extensive expansion in culture for genomic anomalies, but also their differentiated products where this is possible.

Increasing understanding of the processes that control development in the embryo has made it possible to establish even more precise differentiation protocols and to increase the spectrum of human cell types that can be produced. As we also discussed in our literature review in chapter 2 of this thesis, most of the hPSC-CMs generated to date display ventricular-like phenotypes in electrophysiological or gene expression analyses. However, through a process of constant optimization and the use of defined culture supplements and signalling molecules for inducing differentiation, as well as cell-type specific purification protocols, it is now possible to generate most if not all other heart cell types. All of this contributes to producing the specific cardiomyocyte subtypes necessary for studying many more channelopathy subtypes. Nevertheless, more steps are needed for studying non-autonomous diseases, where the cell causing the heart phenotype might not be the

cell bearing the (disease) mutation. Purified cultures of hPSC-CMs lack the complexity of cardiac tissue. The adult myocardium contains only ~30% cardiomyocytes (although ±80% by volume), while the other 70% is a combination of cardiac fibroblasts, cardiac endothelial cells, and vascular stromal cells. As we and others have discussed[4–6], a cardiac cellular microenvironment reflecting as much as possible that *in vivo*, with heterotypic cell types present is expected to increase the predictive power of hPSC-CMs since (non)myocyte cell-cell communication positively impacts cardiomyocyte maturation.

Indeed, several 3D cell culture technologies, such as Engineered Heart Tissues (EHTs), cells sheets and cardiac microtissues (cMT), have been developed to tackle this issue. These have given promising results on cell maturation which is highly relevant for disease modelling. For example, a recent study showed that cardiac microtissues can promote post-natal maturation in hiPSC-CMs by changes in genes expression profile leading to the *SCN5A* isoform switch, as seen in adult cells[7]. In chapter 7 of this thesis, we also showed that only when hiPSC-derived cardiac fibroblasts, endothelial cells and CMs were combined in a 3D cMT model were we able to detect altered contractility in the karyotypically abnormal hiPSC-CMs. Moreover, by changing the composition of the cell types in these cMTs, we could conclude that CMs are the sole culprit for the disease phenotype. Their interaction with other cell types seemed to be necessary to reveal that phenotype, possibly by enhancing the maturation of CM. However, our failure to observe contraction differences in 2D models using the same cells and mutant phenotypes could also have been the result of the type of substrates on which the cells were plated.

FUTURE PERSPECTIVES

Despite the challenges mentioned above, ongoing efforts among the scientific community have made it possible to tackle and solve some of the hPSC limitations listed here. Studies focusing on epigenetic and genetic aberrations during stem cell culture have led to discussion advocating the creation of safety guidelines when manipulating hPSCs. The continuous search for CM maturation and the fusion of new methodologies in cell biology and genetic engineering have greatly stimulated the field of disease modelling. Whilst we now can reach postnatal stages using a variety of technologies and methodologies, it is not yet possible to obtain fully mature adult cardiomyocytes and aged cardiomyocytes are likely even further away. From novel mechanistic insights into the disease pathology, passing through "cell pharmacology" to tissue engineering, the ensemble of work from many research groups are closing the gap between stem cell biology and precision medicine. However, the effort is far from complete; better and robust precision medicine strategy must be created for the study of cardiac arrhythmias. As mentioned before, focusing on individual patient-specific approaches are technically and financially unsustainable using present technology, and for this reason, the complexity of human population studies cannot yet be achieved using in vitro hiPSC models. Creating a diverse patient cell bank is one way to guarantee high quality controls and consistency; nonetheless, platforms taking advantage of new techniques in gene editing are a promising way to create diverse libraries of mutated hPSC lines, with the potential of introduce any VUS candidate for pathogenic analyses.

Table 1: Summary of hiPSC lines created for each chapter.

	Primary mutation	K897T	
$KCHN2^{WT/A561T}$	Pore		Chapter 4
$KCHN2^{WT/N996I}$	Tail		Chapter 4
$KCHN2^{WT/A561T-K897T}$	Pore	Cis	Chapter 5
$KCHN2^{WT-K897T/A561T}$	Pore	Trans	Chapter 5
$KCHN2^{WT/N996I-K897T}$	Tail	Cis	Chapter 5
$KCHN2^{WT-K897T/N996I}$	Tail	Trans	Chapter 5
$KCHN2^{A561T/-}$	Pore		Chapter 6
$KCHN2^{N996I/-}$	Tail		Chapter 6

REFERENCES

1. Brandão, K. O., Tabel, V. A., Atsma, D. E., Mummery, C. L. & Davis, R. P. Human pluripotent stem cell models of cardiac disease: from mechanisms to therapies. *Dis. Model. Mech.* **10**, 1039–1059 (2017).
2. Chai, S. *et al.* Physiological genomics identifies genetic modifiers of long QT syndrome type 2 severity. *J. Clin. Invest.* **128**, 1043–1056 (2018).
3. Halliwell, J., Barbaric, I. & Andrews, P. W. Acquired genetic changes in human pluripotent stem cells: origins and consequences. *Nat. Rev. Mol. Cell Biol.* **21**, 715–728 (2020).
4. Giacomelli, E. *et al.* Human-iPSC-Derived Cardiac Stromal Cells Enhance Maturation in 3D Cardiac Microtissues and Reveal Non-cardiomyocyte Contributions to Heart Disease. *Cell Stem Cell* **26**, 862-879.e11 (2020).
5. Lemoine, M. D. *et al.* Human iPSC-derived cardiomyocytes cultured in 3D engineered heart tissue show physiological upstroke velocity and sodium current density. *Sci. Rep.* **7**, 5464 (2017).
6. Ronaldson-Bouchard, K. *et al.* Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature* **556**, 239–243 (2018).
7. Campostrini, G. *et al.* Maturation of hiPSC-derived cardiomyocytes promotes adult alternative splicing of SCN5A and reveals changes in sodium current associated with cardiac arrhythmia. *Cardiovasc. Res.* (2022) doi:10.1093/cvr/cvac059.