State of the heart : the promise of pluripotent stem cell-derived cardiomyocytes in disease modelling, differentiation and development

Berg, C.W. van den

CHAPTER 7

General Discussion

The research presented in this thesis describes the application of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) to studying cardiac diseases and development. Several protocols for efficient cardiomyocyte differentiation based entirely on defined reagents were developed, various mouse and human iPSC lines were derived including disease models and cardiac reporters, and pluripotent stem cell (PSC) derived cardiomyocytes were compared with the human foetal heart in terms of gene expression.

Mouse and human PSCs for modelling a complex genetic cardiac disease

The first part of this thesis focused on the use of pluripotent stem cells in modelling genetic cardiac diseases as an alternative to animal models. Almost all of our knowledge on genetic-based cardiac diseases comes from animal models and even though the mouse is quite similar to human (~85 % of the protein coding regions are identical [1]), differences also exist, particularly in the physiology of the heart and its electrophysiological properties. Most notably the heart rate in mice is ~500 beats per minute (bpm) compared to ~60 bpm in humans. Additionally, the action potential in humans is much longer, has a plateau phase due to a prolonged calcium influx and different potassium channels are responsible for action potential repolarisation (**chapter 2**). The possibility to differentiate human PSCs (hPSCs) to specialized cells, such as cardiomyocytes, offers the unique opportunity to investigate human diseases using bona fide human cells in vitro.

One group of diseases that has received a lot of attention are genetic-based cardiac diseases. There have now been numerous publications demonstrating that PSCs can model ion channelopathies [2]. In **chapter 3** of this thesis we demonstrated that it was possible to model a complex channelopathy where a mutation in the Na^+ ion channel gene (*SCN5A*^{1795insD}) can lead to either a loss- or gain-of-function. The function of the sodium ion channel in the heart is conserved between mice and human, with the opening of the channel leading to an influx of Na^+ and depolarisation of the cardiomyocyte's membrane potential, which corresponds to the rapid upstroke of the action potential. This means mice carrying the murine equivalent of the human *SCN5A*^{1795insD} mutation display characteristics similar to the human phenotype [3]. Our aim was to investigate if the iPSCs from the mouse model could indeed mirror the disease phenotype in vitro. We also compared the iPSC-derived cardiomyocytes to the cardiomyocytes derived from the genetically modified ESCs that were originally used to generate the mice. This offered the unique opportunity to determine if both types of PSCs could accurately model the disease phenotype. An important and unique aspect of our study was that we could validate the results from the cardiomyocytes generated in vitro by comparing them to the primary cardiomyocytes that were isolated from adult heterozygous mice. Such an evaluation cannot readily be performed in humans unless a biopsy is obtained, which is usually very rare [4]. Our study concluded that a complex overlap syndrome indeed could be modelled in vitro in these mouse cells, and that the differences observed between wild type and mutant cardiomyocytes were due to the mutation.

After finding this clear relationship we then derived human iPSCs (hiPSCs) from a patient with the *SCN5A*^{1795insD} mutation. As all the major aspects of the disease could be detected in mouse cardiomyocytes, this gave us confidence that we would be able to model the disease using hiPSCs. Also here we found significant differences between the control and the patient cells. A possible caveat to these findings was that the control hiPSCs were derived from an unrelated individual, and thus are not the most suitable control due to the broad electrophysiological variability observed between different cell lines [4,5]. Ideally one would use isogenically-matched cell lines where either the genetic mutation has been corrected in the patient-derived iPSCs, thus restoring the healthy phenotype, or the mutation has been introduced into a control PSC line. This obviates the influence of genetic background on the observed disease phenotype [6]. The burden of proof in these types of models has increased over the last several years and now studies investigating genetic diseases using PSCs are expected to correct the mutation or perform a rescue experiment. While classical gene targeting approaches in hPSCs have been very labour intensive due to the low targeting efficiency, the new clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 nuclease system has revolutionized this field of research. This new targeting strategy involves introducing targeted double strand breaks at the locus of interest, which improves the chances of homologous recombination occurring. While the frequency of off-target insertions or deletions needs to be further explored, this new technique for gene editing of pluripotent cell lines enables the generation of isogenic mutated and healthy cell lines with a higher efficiency [7].

The severity of cardiac ion channel diseases is thought to be dependent on the genetic background of the individual with additional genetic modifiers playing a role. Research in two mouse strains (FVB/N and 129P2) demonstrated that this *Scn5a* mutation was more severe in 129P2 mice due to their smaller conduction reserve [8]. We also investigated the possibility to model the overlap syndrome in miPSC-derived cardiomyocytes from the FVB/N mice with the intention to ultimately compare them to the cardiomyocytes derived from miPSCs from the 129P2 mice. If the differences in disease severity were also reflected in the miPSCs from the two strains, this would provide a model to more readily investigate the contribution of additional genetic variants to the disease phenotype. However, the iPSCs from the FVB/N mice were difficult to maintain in a pluripotent state as well as to differentiate to cardiomyocytes. This could be due to incomplete reprogramming of these iPSCs. Possibly serum free methods for culturing the mouse PSCs could assist in maintaining them in an undifferentiated state or differentiate them to cardiomyocytes [9,10]. It will be of great interest to know whether these genetic differences observed in the mice are captured in their derivative miPSC cardiomyocytes.

Differentiation of PSCs to cardiomyocytes without line-to-line optimization

Much of the work in this thesis focussed on the differentiation of hPSC to cardiomyocytes. There were two reasons for this: (1) we wished to have an efficient protocol for differentiation that was effective for hPSC grown in an undifferentiated state in fully defined medium, and: (2) we wished to have a defined protocol that was equally effective for both hiPSC and human ESC (hESCs) so that we could compare the cardiac differentiation of these directly without any differences in culture reagents impacting the analysis. At that time no cardiac differentiation protocols existed to differentiate multiple PSC lines following the same procedure.

The first cardiomyocytes derived from hESCs were generated in aggregates by co-culture with a mouse visceral endoderm-like cell line called END-2 that promoted cardiogenesis [11]. Despite many aggregates in the culture visibly beating, they only contained a few cardiomyocytes. Factors that were involved in inducing the differentiation by the END-2 cells, such as PGI2, have been identified [12], but other factors were likely to also be involved. One important finding was that the presence of Insulin or IGF-1 biased the differentiation towards neurectoderm [13], illustrating that it was not only important to add cytokines but also in some cases to omit them. The development of new enzymatic dissociation solutions and the adaptation of hPSCs for passaging using these reagents opened possibilities to differentiate them toward the cardiac lineages as aggregates (or embryoid bodies (EB)) using defined numbers of cells [14]. These methods provided the opportunity to generate cardiomyocytes under defined conditions where the cells were treated with cytokine combinations that mimic signalling pathways important in embryonic heart development [15]. There is nevertheless high variability in the efficiency of EB-differentiation protocols, and with any protocol to date, the cardiomyocytes are relatively immature compared to adult cardiomyocytes. In terms of electrophysiology, the cardiomyocytes derived from PSCs have different Ca^{2+} handling properties reflected by a more positive maximum diastolic potential, and show a slower upstroke velocity compared to mature cardiomyocytes. Furthermore, unlike PSC-derived cardiomyocytes, adult cardiomyocytes present well-developed sarcomeric structures, are multinucleated, larger and more elongated and do not beat spontaneously.

Some protocols start from hPSCs maintained in undefined conditions, such as mouse embryonic fibroblasts (MEFs) and/or serum. These are potential sources for the variability in cardiac efficiency observed with different hPSC lines. MEF- and serum-free conditions have reduced variability but generally result in the cells that do not form EBs [15]. Instead of generating cardiomyocytes in 3D as in the aggregate/EB methods, hPSCs grown in 2D have also been shown to be able to differentiate to the cardiac lineages [16]. The cytokines and small molecules [17,18] used for this are similar to those in the EB-protocol, but monolayer-based differentiation offers the opportunity to obtain large numbers of cells and is thought to be more efficient because the differentiation-inducing factors are equally available to all cells.

In this thesis a monolayer-based protocol for cardiomyocyte differentiation was developed for undifferentiated hPSCs cultured in defined xeno-free medium (**chapter 4**). We found that the protocol could be applied to hPSCs maintained in the undifferentiated state in any of four different methods, with beating cardiomyocytes generated within 7–10 days of differentiation. Two methods described the differentiation from MEF-based cultures; the other protocols were for cell lines maintained in defined commercially-available media without feeder cells. The protocols worked efficiently and reproducibly for several hPSC lines. Whilst several differentiation protocols have recently been described that are based on a chemically defined medium using small molecules [18,19], we have found variability between differentiations using these methods. The small molecules target specific signalling pathways and probably these protocols will improve further in the future, perhaps in combination with the method developed in chapter 4 in this thesis.

Our overall aim was to be able to compare pure populations of hiPSC- and hESC-derived cardiomyocytes directly to investigate their similarities and differences. This could determine whether hiPSCs are a suitable alternative for hESCs in applications where hESCs cannot be used, for example for ethical reasons. To exclude external factors as contributors to any differences observed, we required that both cell lines (from different genetic backgrounds) would differentiate using the same media and cytokine concentrations. Even though our protocol is based on the use of cytokines that could vary from batch to batch in contrast to small molecules, the protocols we developed starting from completely defined and xeno-free E8 medium proved to be highly reproducible in generating cardiomyocytes from different hPSC lines and did not require optimization for different hPSC lines. We used E8 medium supplemented with 1 % DMSO [20] to reduce the variability between separate differentiations and between cell lines. At that time we passaged the cells using EDTA that resulted in differences in seeding density due to the formation of clumps. Now, PSCs can be passaged as single cells in E8 medium supplemented with a selective rho-kinase inhibitor combined with molecules that have antioxidant and free radical scavengers properties, offering higher differentiation reproducibility due to more precise seeding of cell numbers per well.

The newly developed defined protocol in chapter 4 could now be used to specifically compare the cardiomyocytes derived from ESCs and iPSCs directly without any difference in culture reagents. We targeted GFP to the *NKX2-5* locus in a control hiPSC line using the same construct as previously used in hESCs [21]. Both lines were maintained and differentiated using the same defined media and protocols. In **chapter 5** we showed that the reporter cell lines were useful for isolating the cardiomyocytes at early and later stages of differentiation from both hiPSCs and hESCs. The resulting cardiomyocytes had very similar gene expression profiles during early phases of differentiation and a maximum of only 182 genes were significantly different at the latest time point analysed. This suggested that regardless of their genetic background, hiPSCs were essentially equivalent to hESCs in their differentiation to

cardiomyocytes as intermediate cell types and that the differentiation kinetics were also very similar. This study demonstrates that these hiPSCs are a suitable replacement for hESCs, at least for investigating the early stages of cardiac differentiation, and that this cell line offers the possibility to isolate specific populations of iPSC-derived cardiomyocytes.

Unfortunately, as with previous differentiation protocols, the monolayer protocol still resulted in immature cardiomyocytes as determined by electrophysiology but with the additional caveat that the cells were fragile and difficult to patch for electrophysiology. Culturing the cells in one of the maturation media currently under development could assist in the maturation of these cells, while pre-passaging the PSCs enzymatically may make them less fragile for patch clamp electrophysiology.

The resulting cardiomyocytes were largely ventricular-like cells as determined by electrophysiological measurements [15,22]. Differentiation protocols that lead to the formation of each specific cell type of the heart are of particular interest since most methods predominantly generate ventricular like cells. Interestingly methods for obtaining atrial [23-25] or pacemaker cells [26] have recently been described. In addition, cardiac progenitor cells have rarely been shown to proliferate in vitro and certainly not for long periods. Recently ectopic expression of *MYC* has been shown to allow the expansion of these cardiac progenitors, which can then further differentiate into cardiomyocytes [26]. This offers the possibility to expand the cells to the large numbers necessary for drug development and disease modelling and perhaps regenerative medicine in the future. It would be of interest to know whether these approaches would also apply to the defined conditions for cardiomyocyte differentiation described in this thesis and whether the cardiac phenotypes are comparable.

Modelling heart development in a dish

During the differentiation, developmental processes are mimicked and we find similar gene expression profiles as cardiomyocytes formed during development in vivo [27]. The cardiomyocytes we generate from PSCs resemble foetal cardiomyocytes, both electrophysiologically as well as based on gene expression (**chapter 6**). We aimed to investigate the differences and similarities in the gene expression profiles and maturation state between hPSC-derived cardiomyocytes in culture and in vivo. We made use of a rare set of separated atrial and ventricular foetal heart samples from both first and second trimester and designed a database containing the gene expression profiles of these samples. We included commercially available reference samples for normalization of future samples and present this database as a resource for other laboratories. Cardiomyocytes generated from PSCs using improved protocols for maturation can be compared to the heart samples in this database for benchmarking and characterization of their maturation state.

The comparisons described in chapter 6 demonstrated that the foetal heart samples from first and second trimester have distinct gene expression profiles. Comparing the gene expression profiles at different points of gestation provided knowledge on genes that are important in human cardiac development and can be used to discriminate atria and ventricles. Unfortunately the number of samples was limited and we did not have the opportunity to include more samples in the analysis. For several developmental stages we only had one or at most two replicates that made statistical analysis for each time point separately impossible. More foetal heart samples could be added at a later time point, because commercially available reference samples are available for normalization. It would have been interesting also to have compared gene expression profiles of male hearts to female ones, as well as separate left and right ventricles and atria, but it was necessary to pool these in each trimester for statistical purposes.

We wished to compare the heart samples to hPSC-derived cardiomyocytes to investigate whether the hPSC-derived cardiomyocytes more closely resembled foetal heart samples from the first or the second trimester. We made use of hiPSC and hESC derived cardiomyocytes in multiple differentiation experiments collected at day 21 and compared these to the foetal heart samples. Even though the foetal heart samples were a mixed population of cells and the hPSC-derived were enriched for cardiac cells, we could demonstrate that these cardiomyocytes resembled first trimester more than the second trimester. Interestingly, when the cardiomyocytes were cultured in a medium that had been shown to mature cardiomyocytes [28], these cells more closely matched the second trimester samples, demonstrating the utility of this database for benchmarking the maturity state of hPSC-derived cardiomyocytes.

While we have only focused on heart samples from different stages and regions of the heart, a parallel study in our department has developed a tool (Keygenes) based on an algorithm for comparing RNA sequencing transcriptional profiles from samples to a whole set of organs or cell types [29]. Samples derived from hPSCs can be compared to this set to determine their cell identity as has been performed for atrial-like PSC-derived cardiomyocytes and iPSC-derived kidney progenitors recently [29,30]. Heart samples from atria and ventricles were included in this tool, though mainly from the later stages of development (week 16–22). Despite the fact that Keygenes is a very valuable tool for determining the atrial or ventricular identity from the PSC-derived cardiomyocytes and the stage of development (trimester 1 or trimester 2) in ventricular samples only, our study offers the possibility to determine the equivalent stage of the hPSC-derived cardiomyocytes in foetal heart development based on both atrial and ventricular samples of both stages.

Challenges in the field and future prospects

Although the use of hPSC-derived cardiomyocytes in research is increasing, several limitations or challenges still remain. For example, ESCs and iPSCs show similar characteristics in key pluripotency markers and morphology, but the efficiency and reproducibility of differentiation between pluripotent stem cell lines still varies and protocols need to be adapted and optimized for each cell line, mainly by altering the concentration and timing of cytokine and/or small molecule addition. The variability between cell lines could be how the cells have been maintained, their genetic background or their passage number and in the case of iPSC, the tissue of origin for the somatic cells and their epigenetic status. The epigenetic memory of the iPSCs can be caused by incomplete reprogramming from the original state of the cells. The cells then inherit some residual signatures (e.g. methylation pattern) of the somatic tissue that was reprogrammed [31]. Many transcriptome comparisons have been performed between undifferentiated iPSCs and ESCs. Some have concluded that the iPSCs should be referred to as another class of pluripotent stem cells [32], while others state that the ESCs and iPSCs show very few differences and it is not possible to distinguish iPSCs from ESCs using these variations at least provided enough cell lines are compared [33]. A variable that most consistently influences the reproducibility of the differentiation procedure in our hands was the quality of the starting population. It is important to ensure an homogeneous population of pluripotent stem cells during the maintenance phase before starting differentiation. An important advance has been the use of defined media that is already proving useful in maintaining the cells as a consistent pluripotent population, expressing high percentages of cells with characteristic pluripotency markers. Besides this, optimal intervals for passaging that prevent the cells from overgrowth are important and monitoring morphological or growth rate changes that could indicate abnormalities in the karyotype has also proven crucial. Although the protocols described in this thesis have been shown to be effective and robust in multiple cell lines, other cell lines may have distinctive characteristics and still require optimization of a differentiation protocol. A second challenge remaining is the immaturity of the hPSC-derived cardiomyocytes (and all other differentiated derivatives) in culture [34]. Generally the cardiomyocytes generated in vitro from hPSC resemble foetal- more than adult cardiomyocytes. They have immature electrophysiological characteristics, such as a higher resting membrane potential and slow upstroke velocities, and they express foetal cardiac genes [27,35]. No protocols yet describe the generation of mature cardiomyocytes that truly resemble adult heart cells [36], characterized by well-developed sarcomeric structures and distinct electrophysiological properties as mentioned above. Even though the efficiency of cardiac differentiation protocols has greatly increased in recent years [15], better methods to improve the maturity of the in vitro cardiomyocytes are required for recapitulating the adult phenotype. Some studies state that prolonged culture, patterned substrates or extrinsic stimuli (such as electrical or mechanical pacing) can improve the maturation of the cardiomyocytes. While long term culture of the

cardiomyocytes has been occasionally reported to result in a more mature morphological structure, such as increased sarcomere length, multinucleation, increased cell size, more organized myofibrillar morphology, and these cells also showed expression of *MYH6*, *MYH7* and *SERCA2*, they still were not identical to adult cardiomyocytes [37]. Gene expression profiles from differentiated hESC-CMs compared to the adult heart have been reported to show 74 % similarity and after another 4 weeks of culture this increased to 78 % [35]. Most likely, part of the difference in the expression profiles could be explained by the presence of other cell types in the heart samples, but not all because the majority of the cells in the foetal heart are cardiomyocytes. It is only after birth that the other cell types such as endothelial cells or fibroblasts proliferate faster than cardiomyocytes [38]. Another important variable is the substrate for culturing PSC-derived cardiomyocytes. The use of the 'sandwich method', in which Matrigel is used to cover the differentiating pluripotent stem cells, increased the differentiation efficiency as well as the maturation of the cells. The electrophysiological properties of the cardiomyocytes matched cardiomyocytes that were cultured for 60–90 days as EBs, while they were only in culture for 30 days, suggesting that the sandwich methods stimulates more rapid maturation compared to long term EB-cultures [39], however again still not similar as adult cardiomyoyctes.

The heart is not only composed of cardiomyocytes but in the case of the adult, endothelial cells and cardiac fibroblasts. Perhaps co-culture of cardiomyocytes with these other cell types found in the heart or culture of cardiomyocytes in a 3-dimensional format using gels or polymer substrates could provide a more physiological environment or niche. Media containing extrinsic stimuli such as thyroid hormone as a main component [28], or thyroid hormone combined with IGF-1 and dexamethasone [40] have been developed that apparently promote maturation of cardiomyocytes. Culturing hPSC-derived cardiomyocytes in the first medium suggested that the cardiomyocytes develop further, and when compared to foetal heart samples, showed they more closely resembled second trimester cardiac cell types than first trimester [27]. Additionally their electrophysiological characteristics also improved [28].

If it were possible to generate more mature cardiomyocytes recapitulating the adult cardiomyocyte phenotype, it would be less challenging to assess the disease phenotype related to for example ion channelopathies and cardiomyopathies. Some molecular and cellular mechanisms of these diseases can only be assessed when more mature cardiomyocytes are generated. Models based on PSC-derived cardiomyocytes are mostly suitable when the disease has early onset, e.g. during development, but this is rare and it is much more challenging when the disease develops late in adulthood. A more mature, adult like cardiomyocyte would be vital to reproduce certain disease phenotypes in vitro and perhaps in the future these cells could be used to predict the onset and the development of the disease. They may even allow the investigation of ways to prevent the disease by delaying the onset of symptoms.

The prospect of using human iPSC-derived cardiomyocytes for testing drugs is one of their promises and it is possible they will become a preferred tool for drug development and discovery. The number of animal models currently used could then be limited in the future, also because the differences between human and animal physiology mean that many cardiac disorders lack an appropriate animal model. Not only are these mature cardiomyocytes attractive for investigating toxic effects of drugs, these cells could also be used for personalized medicine applications. Cardiomyocytes derived from iPSCs carry the genetic background of the patient for example and would be an excellent model for drug testing either to assess drug sensitivities or look for cures. These drugs could be screened on subsets of patients with any given mutation that may help in the identification of markers to monitor disease progression and in the development of therapies. Also the combination of drugs can be easily assessed that target different disease mechanisms. Drug testing combined with cells carrying the genetic background of the patients would allow us to develop personalized disease treatment by predicting the response of the patient to the administered drug in vitro. Therefore the availability of mature cardiomyocytes would certainly open up a world of new opportunities for researchers in the field.

References

1 National Human Genome Research Institute. Why Mouse Matters, *http://www.genome.gov/10001345* (2010).

2 Yang C., Al-Aama J., Stojkovic M., Keavney B. et al. Concise review: Cardiac disease modeling using induced pluripotent stem cells. *Stem Cells* 33: 2643-2651 (2015).

3 Remme C.A., Verkerk A.O., Nuyens D., van Ginneken A.C. et al. Overlap syndrome of cardiac sodium channel disease in mice carrying the equivalent mutation of human SCN5A-1795insD. *Circulation* 114: 2584-2594 (2006).

4 Knollmann B.C. Induced pluripotent stem cell-derived cardiomyocytes: boutique science or valuable arrhythmia model? *Circulation Research* 112: 969-976; discussion 976 (2013).

5 Sinnecker D., Goedel A., Laugwitz K.L., Moretti A. Induced pluripotent stem cell-derived cardiomyocytes: a versatile tool for arrhythmia research. *Circulation Research* 112: 961-968 (2013).

6 Bellin M., Casini S., Davis R.P., D'Aniello C. et al. Isogenic human pluripotent stem cell pairs reveal the role of a KCNH2 mutation in long-QT syndrome. *The EMBO Journal* 32: 3161-3175 (2013).

7 Merkle F.T., Neuhausser W.M., Santos D., Valen E. et al. Efficient CRISPR-Cas9-mediated generation of knockin human pluripotent stem cells lacking undesired mutations at the targeted locus. *Cell Reports* 11: 875-883 (2015).

8 Remme C.A., Scicluna B.P., Verkerk A.O., Amin A.S. et al. Genetically determined differences in sodium current characteristics modulate conduction disease severity in mice with cardiac sodium channelopathy. *Circulation Research* 104: 1283-1292 (2009).

9 Nichols J., Silva J., Roode M., Smith A. Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. *Development* 136: 3215-3222 (2009).

10 Hartman M.E., Librande J.R., Medvedev I.O., Ahmad R.N. et al. An optimized and simplified system of mouse embryonic stem cell cardiac differentiation for the assessment of differentiation modifiers. *PLoS One* 9: e93033 (2014).

11 Mummery C.L., Ward-van Oostwaard D., Doevendans P., Spijker R. et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation* 107: 2733-2740 (2003).

12 Xu X.Q., Graichen R., Soo S.Y., Balakrishnan T. et al. Chemically defined medium supporting cardiomyocyte differentiation of human embryonic stem cells. *Differentiation* 76: 958-970 (2008).

13 Freund C., Ward-van Oostwaard D., Monshouwer-Kloots J., van den Brink S. et al. Insulin redirects differentiation from cardiogenic mesoderm and endoderm to neuroectoderm in differentiating human embryonic stem cells. *Stem Cells* 26: 724-733 (2008).

14 Ng E.S., Davis R.P., Stanley E.G., Elefanty A.G. A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. *Nature Protocols* 3: 768-776 (2008).

15 Mummery C.L., Zhang J., Ng E.S., Elliott D.A. et al. Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: a methods overview. *Circulation Research* 111: 344-358 (2012).

16 Batalov I., Feinberg A.W. Differentiation of cardiomyocytes from human pluripotent stem cells using monolayer culture. *Biomark Insights* 10: 71-76 (2015).

17 Lian X., Zhang J., Azarin S.M., Zhu K. et al. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/beta-catenin signaling under fully defined conditions. *Nature Protocols* 8: 162-175 (2013).

18 Burridge P.W., Matsa E., Shukla P., Lin Z.C. et al. Chemically defined generation of human cardiomyocytes. *Nature Methods* 11: 855-860 (2014).

19 Lian X., Hsiao C., Wilson G., Zhu K. et al. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proceedings of the National Academy of Sciences of the United States of America* 109: E1848-1857 (2012).

20 Chetty S., Pagliuca F.W., Honore C., Kweudjeu A. et al. A simple tool to improve pluripotent stem cell differentiation. *Nature Methods* 10: 553-556 (2013).

21 Elliott D.A., Braam S.R., Koutsis K., Ng E.S. et al. NKX2-5(eGFP/w) hESCs for isolation of human cardiac progenitors and cardiomyocytes. *Nature Methods* 8: 1037-1040 (2011).

22 Blazeski A., Zhu R., Hunter D.W., Weinberg S.H. et al. Electrophysiological and contractile function of cardiomyocytes derived from human embryonic stem cells. *Progress in Biophysics and Molecular Biology* 110: 178-195 (2012).

23 Zhang Q., Jiang J., Han P., Yuan Q. et al. Direct differentiation of atrial and ventricular myocytes from human embryonic stem cells by alternating retinoid signals. *Cell Research* 21: 579-587 (2011).

24 Josowitz R., Lu J., Falce C., D'Souza S.L. et al. Identification and purification of human induced pluripotent stem cell-derived atrial-like cardiomyocytes based on sarcolipin expression. *PLoS One* 9: e101316 (2014).

25 Devalla H.D., Schwach V., Ford J.W., Milnes J.T. et al. Atrial-like cardiomyocytes from human pluripotent stem cells are a robust preclinical model for assessing atrial-selective pharmacology. *EMBO Molecular Medicine* 7: 394-410 (2015).

26 Birket M.J., Ribeiro M.C., Verkerk A.O., Ward D. et al. Expansion and patterning of cardiovascular progenitors derived from human pluripotent stem cells. *Nature Biotechnology* 33: 970-979 (2015).

27 van den Berg C.W., Okawa S., Chuva de Sousa Lopes S.M., van Iperen L. et al. Transcriptome of human foetal heart compared with cardiomyocytes from pluripotent stem cells. *Development* 142: 3231-3238 (2015).

28 Ribeiro M.C., Tertoolen L.G., Guadix J.A., Bellin M. et al. Functional maturation of human pluripotent stem cell derived cardiomyocytes in vitro–Correlation between contraction force and electrophysiology. *Biomaterials* 51: 138-150 (2015).

29 Roost M.S., van Iperen L., Ariyurek Y., Buermans H.P. et al. KeyGenes, a Tool to Probe Tissue Differentiation Using a Human Fetal Transcriptional Atlas. *Stem Cell Reports* (2015).

30 Takasato M., Er P.X., Chiu H.S., Maier B. et al. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. *Nature* 526: 564-568 (2015).

31 Bilic J., Izpisua Belmonte J.C. Concise review: Induced pluripotent stem cells versus embryonic stem cells: close enough or yet too far apart? *Stem Cells* 30: 33-41 (2012).

32 Chin M.H., Mason M.J., Xie W., Volinia S. et al. Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. *Cell Stem Cell* 5: 111-123 (2009).

33 Guenther M.G., Frampton G.M., Soldner F., Hockemeyer D. et al. Chromatin structure and gene expression programs of human embryonic and induced pluripotent stem cells. *Cell Stem Cell* 7: 249-257 (2010).

34 Yang X., Pabon L., Murry C.E. Engineering adolescence: maturation of human pluripotent stem cell-derived cardiomyocytes. *Circulation Research* 114: 511-523 (2014).

35 Synnergren J., Ameen C., Jansson A., Sartipy P. Global transcriptional profiling reveals similarities and differences between human stem cell-derived cardiomyocyte clusters and heart tissue. *Physiological Genomics* 44: 245-258 (2012).

36 Veerman C.C., Kosmidis G., Mummery C.L., Casini S. et al. Immaturity of human stem-cell-derived cardiomyocytes in culture: fatal flaw or soluble problem? *Stem Cells and Development* 24: 1035-1052 (2015).

37 Lundy S.D., Zhu W.Z., Regnier M., Laflamme M.A. Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Stem Cells and Development* 22: 1991-2002 (2013).

38 Bergmann O., Zdunek S., Felker A., Salehpour M. et al. Dynamics of cell generation and turnover in the human heart. *Cell* 161: 1566-1575 (2015).

39 Zhang J., Klos M., Wilson G.F., Herman A.M. et al. Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method. *Circulation Research* 111: 1125-1136 (2012).

40 Birket M.J., Ribeiro M.C., Kosmidis G., Ward D. et al. Contractile Defect Caused by Mutation in MYBPC3 Revealed under Conditions Optimized for Human PSC-Cardiomyocyte Function. *Cell Reports* 13: 733-745 (2015).