CHAPTER 8

General discussion

It is critical to gain knowledge in the underlying mechanisms that control human cardiovascular developm ent, which helps us to understand the onset of congenital cardiovascular diseases, and to develop optimal culture methods for efficient *in vitro* cardiomyocyte differentiation from hPSCs, which are of interest for final translational applications including screening and efficacy assays for disease modelling, drug discovery and development, personalized medicine, and perhaps the regeneration of cardiovascular tissues for therapeutic purposes. In this thesis, we show how genetic manipulation of human pluripotent stem cells (hPSCs), resulting in the genomic integration of a fluorescent protein encoding sequence at the locus of a key cardiac transcription factor, allows us to visualize and isolate early pre-cardiac progenitors subpopulations, and to study the molecular mechanisms involved in their further differentiation to cells of the cardiac lineage, including smooth muscle cells, endothelial cells, and cardiomyocyte subtypes.

IN VITRO MODELLING OF HUMAN CARDIAC DEVELOPMENT AND DIFFERENTIATION USING HUMAN PLURIPOTENT STEM CELLS:

HOW DO FLUORESCENT GENE REPORTERS CONTRIBUTE?

Temporal gene expression analysis upon cardiac differentiation

Multiple fluorescent PSC reporter lines have been generated to visualize the derivation of cardiomyocytes and to isolate PSC-derived cardiac cells, including cardiac progenitor populations and subtypes of cardiomyocytes (reviewed in **Chapter 2**). Most *in vitro* fluorescent reporter PSC lines described in literature so far, contain a construct that follows the expression of a single gene during differentiation, such as cardiomyocyte reporters NKX2-5$^{eGFP/w}$, NCX-1-eGFP, or alpha-MHC-mCherry human embryonic stem cell (hESC) lines[1, 2] [3]. These lines allow visualization and isolation of the reporter-expressing cells, cardiac progenitors and cardiomyocytes. On the basis of a fluorescent marker, this allows screening molecules for their role in cardiomyocyte differentiation or the identification of subtype specific cell surface markers. This was used for example to identify SirpA and VCAM1 as cell surface markers for early cardiomyocytes[3, 4]. In **Chapter 3** we describe the generation of a dual fluorescent cardiac reporter MESP1^{mCherry}/wNKX2-5$^{eGFP/w}$ hESC line, that allows the derivation of early pre-cardiac mesoderm progenitors to be monitored and their further lineage-committed differentiation to the cardiac lineage, marked by NKX2-5-eGFP expression. Comprehensive gene expression analysis of MESP1 cardiac derivatives in **Chapter 4** showed how cardiac differentiation of these progenitors recapitulates the early steps of *in vivo* heart development, through the sequential expression of key cardiac transcription factors and the expression of functional and structural genes in definitive cardiomyocytes, providing evidence of how such a dual fluorescent cardiac reporter line is useful for studying early human cardiac differentiation. Several previous studies have shown global gene expression patterns upon cardiac differentiation from hPSCs[5, 6]. However, in our study, we could follow the formation of cardiac cells, deriving from an early pre-cardiac-committed lineage, and decreasing the risk for upregulation of genes that are not associated with the cardiac lineage, and thus increasing the predictive value of this model. Putative (co)-regulators of cardiac lineage commitment were identified based on their temporal enrichment levels compared to MESP1 negative derivatives, their DNA-binding properties, and their predicted interaction with other established key regulators. We identified homeobox domain containing HOXB2 as potential interactive transcriptional partner for MEIS1, MEIS2, and PBX3. This was strengthened by a similar expression level pattern throughout cardiac differentiation. MEIS1, MEIS2, and PBX3 all share regulating roles in either heart looping and chamber septation stages, and cardiac lineage commitment. Moreover, MEIS1, MEIS2, HOXB2, and PBX3 were all upregulated upon Mesp1 induction in mouse ESCs, indicating that they all act downstream of Mesp1 (REF). Other DNA binding transcription factors that showed significant stage-specific expression levels upon cardiac differentiation, included zinc finger protein containing transcription factors ZNF503, ZFHX3, ZFPM1, and ZFPM2, that may have co-regulatory roles for established cardiac transcription factors or repressive roles on transcription of key genes involved in other lineages, such as skeletal[7]. Such a repressive role of other lineages could perhaps be of importance for enhancing cardiac lineage commitment.

Additionally, in our study, shortly after temporal MESP1 expression, we also identified significant enriched temporal expression levels of DNA binding genes with an unknown role in heart development at present, including ZBTB16, RUNX1T1, and TSHZ2. The exact role of such putative regulators, which may include lineage commitment, but could also govern cell cycle regulation, proliferation, and/or survival, has to be determined in functional studies, such as *in vitro* inducible knock down studies in MESP1-lineage specific derivatives, or downstream target gene analysis using chip sequencing. Functional studies are a prerequisite for definite conclusions about gene function and role.

In the study of Paige et al.[5], they show how temporally regulated gene expression levels are accompanied by programmed temporal alterations in chromatin structure, which distinguishes key regulators of cardiovascular development from other genes. Currently, further studies are being performed to look into chromatin patterns and dynamics of genomic regions of transcription factors that we identified in this thesis, which would strengthen their role in cardiac differentiation and/or lineage commitment. This will be discussed briefly below.

Future Directions: integrative (epi)genetic analysis upon cardiac lineage commitment.

Despite increasing knowledge on key cardiac transcriptional regulators, signalling molecules and interacting pathways, the role of epigenetic alterations, such as chromatin dynamics and histone modification patterns, are largely unknown, but are proposed to play crucial roles in gene regulation during mammalian heart development and differentiation[5, 8].

Recent studies have indicated that large-scale reorganization of the genome, and epigenetic mark transitions, such as histone modification patterns, coincide with the commitment of pluripotent cells to differentiation[5, 8, 9]. A large integrative study on (epi)genetic and transcriptional regulation during early cardiac differentiation, dual fluorescent cardiac gene reporter MESP1$^{mCherry/w}$-NKX2-5$^{eGFP/w}$ hESCs would therefore be useful, and is currently being performed. It allows the isolation of defined cardiac (progenitor) populations in order to study gene- and cell type-specific chromatin dynamics, histone modifications patterns, RNA expression levels, and enhancer regions. The increasing development and technical improvements of molecular genomic technologies, such as ATAC-sequencing (studying chromatin availability), ChiP-sequencing, and Chromosome Conformation Capture Hi-C (studying chromatin conformations) will allow us to integrate large genomic and epigenetic information at stage-specific cardiac cell types, in order to contribute to the identification of stage-specific (distal) enhancer- and/or promoter elements and unidentified (co)-regulators of cardiac lineage commitment, and potentially also of cardiac subtype diversification. Current pitfalls that require improvement: high costs are made, and high cell numbers are required for such studies. Nonetheless, interest into large integrative (epi)genetic studies is growing.

Screening signaling molecules upon cardiac lineage commitment

From a large number of differentiation studies we know that the inhibition of Wnt-pathway signalling, using distinct Wnt-pathway inhibiting molecules such as Xav939, is crucial for efficient differentiation of pre-cardiac mesoderm towards the cardiac lineage[10-12]. MESP1 expressing mesoderm progenitors may differentiate towards distinct lineages, including skeletal, hematopoietic, and cardiac lineages[13]. In order to determine how the presence and/or absence of distinct signaling molecules may induce pre-cardiac MESP1 expressing mesoderm stage progenitors to further segregate towards the cardiac lineage, dual fluorescent cardiac gene reporter MESP1$^{mCherry/w}$-NKX2-5$^{eGFP/w}$ hESCs is of excellent utility. In **Chapter 3**, we studied a selection of signaling molecules, which included small molecule Wnt-pathway inhibitor Xav939[12], TGF-β/act/Nodal antagonist SB431542, and BMP inhibitor Dorsomorphin. Besides the strong role for Wnt-pathway inhibition in efficient cardiomyocyte differentiation from mesoderm stage, none of the other inhibitors led to an increase in NKX2-5-eGFP expression, where TGF-β/act/Nodal inhibition even seemed to decrease cardiomyocyte differentiation, in contrast with previous literature describing an inhibitory role for TGF-β/act/Nodal signaling in mesoderm to cardiomyocyte differentiation[14, 15]. Such diverse effects could be highly dependent on ligand or small molecule concentration and timing, and in the case of growth factors, specific activity, which is rarely measured. In addition, growth factor effects on cardiomyocyte sublineage commitment, may depend on the combinatorial presence of other cytokines so that it will be necessary to study the effects of a large number of growth factors or small molecules at middle- or high-throughput level (in 96-, 384- or 1536 well plates). For *in vitro* differentiation assays, the additional use of cardiomyocyte subtype reporters, resulting in a multi-color differentiation study may be ideal. This would allow us to follow hPSC differentiation to mesoderm, followed by cardiac specification, and definite subtype derivation. Examples of cardiomyocyte subtype reporters, established in the NKX2-5$^{eGFP/w}$ hESC line, are discussed below (**Chapter 6 and 7**).

The search for expandable early cardiac progenitor populations for translational applications

In order to generate large numbers of cardiomyocyte subtypes for many translational purposes, it would be advantageous to have cardiac lineage-committed progenitor populations, which can be expanded and further differentiated, under tight control, to large pure numbers of the required cardiac subtype. However, only a limited number of studies has shown the ability to maintain cardiac progenitors in their self-renewing developmental state, most with the limitation of a single expansion (reviewed in Birket et al.)[16].

There is a strong demand for the identification of specific cell surface markers that identify and can be used for isolation of hPSC-derived cardiac progenitor populations. In this thesis, we have identified the presence of previously described cardiac progenitors, based on ROR2, CD13 (ANPEP), PDGFRA and KDR, and SirpA and VCAM1 expression[4, 17]. However, the search for additional early cardiac-specific surface markers is still required, since ROR2, CD13, PDGFRA, and KDR showed a more broad mesoderm expression (**Chapter 3**). From our micro array analysis of MESP1-lineage derivatives in **Chapter 4**, we suggest potential novel surface marker proteins, based on their temporal-specific upregulated transcript levels. It will now be of importance to study their expression dynamics at protein level by antibody-binding experiments. Based on current knowledge of cardiac progenitor biology, a combinatorial expression of multiple surface markers may identify cardiac-lineage specific progenitors[4, 18, 19]. Thus, the exact genes and surface markers identifying critical cardiac progenitor populations remain to be defined. The next step will be to study their expandable capacity. In **Chapter 7,** we attempted to study the proliferative capacity of MESP1-mCherry expressing progenitors. A previous study described how they could expand an early hPSC-derived cardiovascular progenitor population (expressing MESP1). Their expansion protocol was based on the inhibition of BMP and activin/nodal signalling, and the activation of Wnt signalling[20, 21]. Even though both progenitors seem to be at a similar developmental stage, we could not reproduce MESP1 progenitor maintenance in our hands. Moreover, we attempted a variety of other cytokines, including those that were described to be involved in NKX2-5 progenitor maintenance[21].

The expansion capacity of cardiac progenitors may be based on their developmental stage and the extracellular niche that they may require. More studies into signals required for cardiac progenitor expansion will be necessary before they can actually be ready for translational purposes.

FLUORESCENT GENE REPORTERS FOR CARDIOMYOCYTE SUBTYPE DERIVATION

The identification of molecular regulators involved in cardiomyocyte subtype derivation.

In **chapter 5 and 6**, we describe the generation of two separate dual cardiomyocyte fluorescent hPSC reporter lines, either for the identification of ventricular or nodal-like cardiomyocytes, and to study molecular regulators important for their derivation. Both lines were generated in the previously described NKX2-5$^{eGFP/w}$ hESC line[3]. In chapter 6, we generated a reporter construct in which an atrioventricular-specific enhancer and a pan-cardiac enhancer, that were previously identified to synergistically activate TBX3 expression in the atrioventricular conduction system of the developing mouse embryo, were coupled to a promoter element and fluorescent protein mCherry. This reporter construct was randomly integrated into the genome of NKX2-5$^{eGFP/w}$ hESCs through lentiviral delivery.

The use of gene regulatory elements that have been identified through *in vivo* studies in other organisms, such as rodents, the zebrafish, and chick, is a common strategy for *in vitro* fluorescent reporter studies[22-24]. Likewise used the group of Laflamme, in order to identify PSC-derived nodal-like cardiomyocytes, a proximal promoter-enhancer region of the chicken GATA6 (cGATA6) gene that was earlier identified to be selectively activated in the atrioventricular (AV) node and the bundle of His of the adult mouse heart[24, 25]. hESC-cardiomyocyte cultures were transduced with a lentiviral vector in which cGATA6 drives expression of enhanced green fluorescent protein (EGFP), resulting in the identification of cardiomyocytes with a nodal-like phenotype, evidenced by their action potential. Furthermore, they showed how ErbB antagonist AG1478 could enhance the differentiation of hESC towards nodal-like cardiomyocytes. Therefore, in our study, we exposed dual reporter TBX3e-mCherry-NKX2-5$^{eGFP/w}$ hESCs to AG1478, shortly after mesoderm formation. However, we failed to identify TBX3e-mCherry expressing cells. Also preliminary screening with a variety of other cytokines and small molecules did not show any positive cells. Although we think that the combinatorial use of both TBX3 enhancers with cardiomyocyte marker NKX2-5 is of interest to study molecules involved in the derivation of nodal-like cardiomyocytes, we realized that our strategy could be biased by many biological and technical issues, including transgene silencing, mouse/human differences, or lack of activity of both enhancers in our system (technical issues are described below in more detail). It would be of interest to study a variety of other CCS-specific regulatory elements, or to report endogenous TBX3 expression through a knockin or fusion strategy.

In **chapter 6**, we aimed to monitor and isolate hESC-derived ventricular cardiomyocytes by the generation of dual cardiomyocyte reporter MYL2$^{w/T2A\text{-}mCherry}$-NKX2-5$^{eGFP/w}$ hESCs through an endogenous fusion strategy. Although from electrophysiology data on cardiomyocyte cultures we know that PSC differentiation often results in a predominantly ventricular fate, we could identify MYL2-mCherry expressing cardiomyocytes, only at low levels. This agrees with previous studies using mouse and human MYL2 PSC reporter lines[22, 26] and from our microarray analysis of MESP1 derivatives (**Chapter 3**) we know that MYL2 starts to be significantly expressed around day 14 of differentiation. Moreover, as MYL2 levels in the adult heart are >2 fold upregulated compared with hPSC-CMs, and as MYL2 levels in hPSC-CMs increase upon maturation (Ribeiro et al.[27] and **Chapter 6**), MYL2 may also be an interesting marker for measuring the degree of ventricular cardiomyocyte maturation.

Although recent protocols, based on retinoic acid signalling, have been shown to drive hPSCs towards atrial fate[28, 29], fluorescent atrial reporter lines have not yet been developed. Genes of interest to target could include atrial-specific sarcolipin or COUPTF genes[29-31]. This would allow monitoring of a variety of other molecules possibly involved in atrial fate differentiation, and may contribute to optimize growth factor concentration and timing. The availability of high numbers of hPSC-derived atrial cardiomyocytes will be of interest for assay development for atrial-specific associated disease modelling and drug discovery and/or toxicity[29].

Another strategy to obtain atrial- or ventricular specific cardiomyocytes could be based on a panel of surface markers being expressed, without the requirement of genetic modifications of hPSC lines. The primary use of fluorescent reporter lines allows the isolation of cardiomyocyte specific cell types, which then may contribute to the identification of cell type specific surface markers that could be used for future subtype isolation.

The optimal strategy for the generation of a cardiac fluorescent reporter line should be carefully considered: biological/technical hurdles and new perspectives.

As mentioned before, prior to the generation of a fluorescent reporter line in hPSCs, it is important to consider the technical and biological hurdles that may be faced upon establishment of such a line. Comprehensive details are reviewed in **Chapter 2**. In this thesis, we show the generation of four distinct reporter lines, using different modification strategies. In **Chapter 3**, we generated a MESP1mCherry/w reporter line through the replacement of one allele with the coding sequence of fluorescent protein mCherry, allowing reporting the activity of the endogenous MESP1 promoter. mCherry is monomeric, has a reasonable brightness, and a fast maturing folding efficiency at 37 °C and a high photostability, optimal for long-term imaging studies[32]. Targeting was performed in the previously by Elliott et al. generated NKX2-5eGFP/w hESC line[3]. MESP1-mCherry expressing progenitors could be distinguished from NKX2-5-eGFP cardiac progenitors, due to their non-overlapping timing of expression, and the non-overlapping excitation and emission spectra of mCherry (excitation at 587 nm) and eGFP (excitation at 488 nm). In a similar matter, we made use of these distinguishing spectra when we generated the dual cardiac reporter hESC lines TBX3e-mCherry-NKX2-5$^{eGFP/w}$ in **Chapter 5** and MYL2$^{w/T2A\text{-}mCherry}$-NKX2-5$^{eGFP/w}$ in **Chapter 6.**

Although we could not detect molecular abnormalities upon cardiomyocyte differentiation from MESP1$^{mCherry/w}$NKX2-5$^{eGFP/w}$ hESCs, generating a knockin reporter line through gene replacement brings along the risk for haploinsufficiency defects. Therefore, we generated the MYL2w/T2A-^{mCherry}NKX2-5$^{eGFP/w}$ hESC line through a fusion knockin strategy, where we made use of the small viral T2A fusion peptide[33], which, upon activation of the MYL2 promoter, results in the generation of one transcript, containing both MYL2 and mCherry coding sequences, and is later translated into two separate proteins. This latter strategy seems to have a great potential in stem cell fate reporter technology, especially with the eye on the generation of multicolour reporter lines. However, in hPSCs, there are few reports on the use of these small fusion peptides, thus technical hurdles may still exist. Although in our study, we show the presence of MYL2-mCherry expressing cardiomyocytes, the translation efficiency of both proteins was undefined. Another strategy would be to fuse a fluorescent protein directly the targeted gene, although this may affect gene function.

A less labour-intensive method to develop fluorescent reporter lines is the lentiviral delivery of a reporting construct. In **Chapter 5** lentiviral transfection of NKX2-5$^{eGFP/w}$ hESCs, resulted in the random integration of the TBX3e-Hsp68-mCherry reporting construct into the hESC genome. However, as mentioned before, lentiviral integration into active genome sequences is random, and can be highly susceptible to transgene silencing due to surrounding regulatory elements or upon cardiac lineage differentiation (shown in **Chapter 7**). Positive controls to select correctly targeted clones is a prerequisite, but also labour-intensive.

Therefore, stem cell reporter technology requires technical improvements, including more efficient and less labour-intensive locus-specific targeting strategies (perhaps through the use of the new advances in CRISPR/CAS technology), and through the identification of loci in the human genome that are not silenced upon cardiac-specific lineage differentiation[34-36]. However, it may not be a guarantee that such a “docking locus” may be active in every cardiac sub lineage that will be studied.

HUMAN PLURIPOTENT STEM CELL-DERIVED CARDIAC PROGENITORS AND CARDIOMYOCYTES: TRANSLATIONAL APPLICATIONS

The establishment of hPSC technology and advanced cardiomyocyte differentiation protocols has opened a new platform for cardiac development, tissue engineering, disease modelling, and drug efficacy and toxicity testing. There are still many hurdles that need to be taken before stem cell based therapies find its way in the clinic for the treatment of heart failure. In recent years the recognition for using hPSC-derived cardiomyocytes for disease modelling, drug discovery, and drug toxicity screenings has increased significantly. hPSC-technology aims to develop highly reliable animal-free pre-clinical drug discovery and safety pharmacology models that closely mimic the physiological human environment[37]. In particular, the availability of hiPSC models now makes it possible to generate human cardiomyocytes *in vitro* from both healthy individuals and from patients

with cardiac abnormalities[38]. A wide range of scientific studies has shown how *in vitro* hiPSC-cardiomyocyte models closely mimic their *in vivo* disease phenotype[39-41]. This allows the pharmaceutical industry to design and develop compounds on disease-specific human *in vitro* models[38]. Minimal qualifications that are necessary for hiPSC-CMs to be of interest for pharmaceutical industry is the high quality of cells, high purity, and high yields; all contributing to the high predictability and cost-effectiveness of the screening models to be developed. Advanced protocols to yield high numbers of cardiomyocytes are present and constantly subject to improvements[10, 11]. Furthermore, current issues that are being studied, which may be crucial for reliable prediction of drug efficacy and/or toxicity, are the immature nature of these cardiomyocytes[42], the lack of cardiomyocyte subtype purity[43], and the physiological conditions of cardiomyocyte models that may not reflect the *in vivo* adult heart.

The combinatorial use of fluorescent reporters and high-content analysis in high-through-put screening assays

For cost-effective drug discovery, safety and toxicity screening assays, high-throughput screenings and high content analysis are required. Detailed opportunities for fluorescent reporters in such assays are described in **Chapter 2**. In **Chapter 7** we show how a live fluorescent proliferation reporter in PSCs may be used to detect cardiac progenitor proliferation, available for high-content detection in a cardiomyocyte monolayer differentiation assay. Additionally, such an assay could be useful for studying cardiomyocyte proliferation, which could for example be of interest for therapies of the damaged heart. To note, the development of assay-specific automated imaging analysis software is a prerequisite for high-content screenings. The combinatorial use of such technologies may together be a powerful opportunity to develop biochemical and cell-based assays for compound efficacy and/or toxicity screenings.

Future physiological relevant cardiac assays

The development of predictive physiological relevant *in vitro* cardiac assays for drug discovery and safety pharmacology is a whole new era to be explored in the short-term, especially since legislation to decrease adverse drug reactions (pharmacovigilance) are being strengthened (Pharmacovigilance Legislation EU, effective since 2012), and new legislation is being developed to reduce the use of animal research in drug efficacy and risk management studies (Directive 2010/63/EU, effective since 2013).

Cardiotoxicity and safety pharmacology remain major concerns during drug development, with increased pro-arrhythmic potential being one of the main concerns. The pharmaceutical industry is challenged by the growing costs of research and development and cannot afford drug attrition in late phases of development or withdrawals of approved drugs[44]. hPSC-CM based assays are predicted as a new paradigm for assessment of cardiac risk in humans. Assays may include action potential analysis, contraction rates and features, specific ion-

current analysis, cell viability measurements, force of contraction measurements, sarcomeric organization, metabolic activity, cell size (e.g. hypertrophy), or activation of specific signalling pathways. Moreover, current protocols are mostly developed in a 2-dimensional matter. It may be of interest to develop complex 3-dimensional models, mimicking *in vivo* environment, consisting of a combination of multiple cell-types, such as endothelial cells, smooth muscle cells, fibroblasts, and cardiomyocyte subtypes. Also physical contraction and the presence of a haemodynamic blood flow may be of interest to develop in future assays. In addition, disease modelling and drug efficacy and toxicity may also be studied on organ-system level, in the presence of a blood flow system, with the interplay of important organs, including liver, heart, and kidneys.

The FDA CiPA Initiative

One of the factors that currently drives the potential use of hPSC-CMs in drug development, is the FDA (U.S. Food and Drug Administration) initiative for a Comprehensive In Vitro Pro-arrhythmia Assay (CIPA). Its proposal is to increase the understanding of the electrophysiological mechanism behind pro-arrhythmic risk of drugs by the use of hPSC-CMs and *in silico* prediction models[45-48]. hPSC-CM based assays should be a new paradigm for assessment of clinical potential of Torsades de Pointes (TdP), a specific type of abnormal heart rhythm that can potentially lead to sudden cardiac death. Recent withdrawals of prescription drugs from clinical use because of unexpected side effects on the heart have highlighted the need for more reliable cardiac safety pharmacology assays. Block of the human Ether-a-go go Related Gene (hERG) ion channel in particular is associated with life-threatening arrhythmias[45], but assaying concomitant block of multiple ion channels appears to improve predictability of TdP arrhythmia, indicating the involvement of more ion currents[46]. For standardization, hPSC-CM assays should show a higher specificity and give less “false positives” than the current hERG assay (hERG channel overexpression in HEK293 cells), leading to more sophisticated ECG modeling of drug effects in the future.

CONCLUDING REMARKS AND PERSPECTIVES

In this thesis, we aimed to decipher the molecular mechanisms important for early cardiac lineage commitment and further differentiation through the generation of human PSC fluorescent reporter lines. Here, the activity of cardiac progenitor and/or cardiomyocyte subtype specific genes was visualized and followed through fluorescent reporter proteins.
Knowledge of signals involved in cardiomyocyte differentiation from PSCs is growing rapidly, and efficient protocols are being developed. However, an in-depth understanding of key cardiac regulators, at epigenetic and genetic level, involved in cardiac (sub) lineage commitment is still sparse. The advanced development of new technologies and efficient differentiation protocols will allow us to discover how complex networks interrogate to form the developing human heart, composed of sublineages of cardiac cells. Cardiac developmental biology is the basis for understanding the onset of congenital heart disease, and the development of predictive complex physiologically relevant cell-based assays for disease modelling, drug discovery and pharmacokinetics. In the end, the development of animal-replacing and highly predictive hPSC-derived cell-based assays is under high attention and promise, and currently underway.

REFERENCES

1. Ovchinnikov DA, Hidalgo A, Yang S-K, et al. Isolation of Contractile Cardiomyocytes from Human Pluripotent Stem-Cell-Derived Cardiomyogenic Cultures Using a Human NCX1-EGFPReporter. STEM CELLS AND DEVELOPMENT 2015;24(1):11–20.
2. Kita-Matsuo H, Barcova M, Prigozhina N, et al. Lentiviral Vectors and Protocols for Creation of Stable hESC Lines for Fluorescent Tracking and Drug Resistance Selection of Cardiomyocytes. PLOS ONE 2009;4(4):e5046.
3. 3 Elliott DA, Braam SR, Koutsis K, et al. NKX2-5eGFP/w hESCs for isolation of human cardiac progenitors and cardiomyocytes. NAT METH 2011;8(12):1037–1040.
4. Dubois NC, Craft AM, Sharma P, et al. SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. NATURE BIOTECHNOLOGY 2011;29(11):1011–1018.
5. Paige SL, Thomas S, Stoick-Cooper CL, et al. A Temporal Chromatin Signature in Human Embryonic Stem Cells Identifies Regulators of Cardiac Development. CELL 2012;151(1):221–232.
6. Beqqali A, Kloots J, Ward-van Oostwaard D, et al. Genome-Wide Transcriptional Profiling of Human Embryonic Stem Cells Differentiating to Cardiomyocytes. STEM CELLS 2006;24(8):1956–1967.
7. Berry FB, Miura Y, Mihara K, et al. Positive and negative regulation of myogenic differentiation of C2C12 cells by isoforms of the multiple homeodomain zinc finger transcription factor ATBF1. JOURNAL OF BIOLOGICAL … 2001;(276):25057–25065.
8. Wamstad JA, Alexander JM, Truty RM, et al. Dynamic and Coordinated Epigenetic Regulation of Developmental Transitions in the Cardiac Lineage. CELL 2012;151(1):206–220.
9. Denholtz M, Bonora G, Chronis C, et al. Long-range chromatin contacts in embryonic stem cells reveal a role for pluripotency factors and polycomb proteins in genome organization. CELL STEM CELL 2013;13(5):602–616.
10. Burridge PW, Matsa E, Shukla P, et al. Chemically defined generation of human cardiomyocytes. NAT METH 2014:–.
11. Lian X, Zhang J, Azarin SM, et al. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. NATURE PROTOCOLS 2013;8(1):162–175.
12. Willems E, Spiering S, Davidovics H, et al. Small-Molecule Inhibitors of the Wnt Pathway Potently Promote Cardiomyocytes From Human Embryonic Stem Cell–Derived MesodermNovelty and Significance. CIRCULATION … 2011.
13. Chan SS-K, Shi X, Toyama A, et al. Mesp1 Patterns Mesoderm into Cardiac, Hematopoietic, or Skeletal Myogenic Progenitors in a Context-Dependent Manner. CELL STEM CELL 2013;12(5):587–601.
14. Cai W, Albini S, Wei K, et al. Coordinate Nodal and BMP inhibition directs Baf60c-dependent cardiomyocyte commitment. GENETICS 2013;27(21):2332–2344.
15. Behfar A, Zingman LV, Hodgson DM, et al. Stem cell

differentiation requires a paracrine pathway in the heart. THE FASEB JOURNAL 2002;16(12):1558–1566.
16. Birket MJ, Mummery CL. Pluripotent stem cell derived cardiovascular progenitors--a developmental perspective. DEVELOPMENTAL BIOLOGY 2015;400(2):169–179.
17. Ardehali R, Ali SR, Inlay MA, et al. Prospective isolation of human embryonic stem cell-derived cardiovascular progenitors that integrate into human fetal heart tissue. PROCEEDINGS OF THE … 2013;110(9):3405–3410.
18. Yang L, Soonpaa MH, Adler ED, et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. NATURE 2008;453(7194):524–528.
19. Kattman SJ, Witty AD, Gagliardi M, et al. Stage-Specific Optimization of Activin/Nodal and BMP Signaling Promotes Cardiac Differentiation of Mouse and Human Pluripotent Stem Cell Lines. CELL STEM CELL 2011;8(2):228–240.
20. Cao N, Liang H, Huang J, et al. Highly efficient induction and long-term maintenance of multipotent cardiovascular progenitors from human pluripotent stem cells under defined conditions. NATURE PUBLISHING GROUP 2013.
21. Birket MJ, Ribeiro MC, Verkerk AO, et al. Expansion and patterning of cardiovascular progenitors derived from human pluripotent stem cells. NATURE BIOTECHNOLOGY 2015;33(9):970–979.
22. MULLER M. Selection of ventricular-like cardiomyocytes from ES cells in vitro. THE FASEB JOURNAL 2000;14(15):2540–2548.
23. Bondue A, Tannler S, Chiapparo G, et al. Defining the earliest step of cardiovascular progenitor specification during embryonic stem cell differentiation. THE JOURNAL OF CELL BIOLOGY 2011;192(5):751–765.
24. Zhu W-Z, Xie Y, Moyes KW, et al. Neuregulin/ErbB Signaling Regulates Cardiac Subtype Specification in Differentiating Human Embryonic Stem CellsNovelty and Significance. CIRCULATION … 2010;(107):776–786.
25. Davis DL, Edwards AV, Juraszek AL, et al. A GATA-6 gene heart-region-specific enhancer provides a novel means to mark and probe a discrete component of the mouse cardiac conduction system. MECHANISMS OF DEVELOPMENT 2001;108(1-2):105–119.
26. Huber I, Itzhaki I, Caspi O, et al. Identification and selection of cardiomyocytes during human embryonic stem cell differentiation. THE FASEB JOURNAL 2007;21(10):2551–2563.
27. Ribeiro MC, Tertoolen LG, Guadix JA, et al. Functional maturation of human pluripotent stem cell derived cardiomyocytes in vitro--correlation between contraction force and electrophysiology. BIOMATERIALS 2015;51:138–150.
28. Zhang Q, Jiang J, Han P, et al. Direct differentiation of atrial and ventricular myocytes from human embryonic stem cells by alternating retinoid signals. NATURE PUBLISHING GROUP 2010;21(4):579–587.
29. Devalla HD, Schwach V, Ford JW, et al. Atrial-like cardiomyocytes from human pluripotent stem cells are a robust preclinical model for assessing atrial-selective pharmacology. EMBO MOLECULAR MEDICINE 2015;7(4):394–410.
30. Josowitz R, Lu J, Falce C, et al. Identification and purification of human induced pluripotent stem cell-derived atrial-like cardiomyocytes based on sarcolipin expression. 2014.
31. Minamisawa S, Uemura N, Ishikawa Y, et al. Sarcolipin, a Novel Calcium Regulator, Is Preferentially Expressedin the Atrium and Is Downregulated in the Hypertrophic Heart. CIRCULATION JOURNAL : OFFICIAL JOURNAL OF THE JAPANESE CIRCULATION SOCIETY 2003;67:211.
32. Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. NAT METH 2005;2(12):905–909.
33. Trichas G, Begbie J, Srinivas S. Use of the viral 2A peptide for bicistronic expression in transgenic mice. BMC BIOLOGY 2008;6(1):40.
34. Costa M, Dottori M, Ng E, et al. The hESC line Envy expresses high levels of GFP in all differentiated progeny. NAT METH 2005;2(4):259–260.
35. Irion S, Luche H, Gadue P, et al. Identification and targeting of the ROSA26 locus in human embryonic stem cells. NATURE BIOTECHNOLOGY 2007;25(12):1477–1482.
36. Hockemeyer D, Soldner F, Beard C, et al. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. NATURE BIOTECHNOLOGY 2009;27(9):851–857.
37. Doherty KR, Wappel RL, Talbert DR, et al. Multi-parameter in vitro toxicity testing of crizotinib, sunitinib, erlotinib, and nilotinib in human cardiomyocytes. TOXICOLOGY AND APPLIED PHARMACOLOGY 2013;272(1):245–255.
38. Davis RP, van den Berg CW, Casini S, et al. Pluripotent stem cell models of cardiac disease and their implication for drug discovery and development. TRENDS MOL MED 2011;17(9):475–484.
39. Bellin M, Casini S, Davis RP, et al. Isogenic human pluripotent stem cell pairs reveal the role of a KCNH2 mutation in long-QT syndrome. EMBO J. 2013;32(24):3161–3175.
40. Moretti A, Bellin M, Welling A, et al. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. N ENGL J MED 2010;363(15):1397–1409.
41. Matsa E, Burridge PW, Wu JC. Human Stem Cells for Modeling Heart Disease and for Drug Discovery. SCIENCE TRANSLATIONAL MEDICINE 2014;6(239):239ps6–239ps6.
42. Yang X, Pabon L, Murry CE. Engineering adolescence: maturation of human pluripotent stem cell-derived cardiomyocytes. CIRCULATION RESEARCH 2014;114(3):511–523.
43. Blazeski A, Zhu R, Hunter DW, et al. Cardiomyocytes derived from human induced pluripotent stem cells as models for normal and diseased cardiac electrophysiology and contractility. PROGRESS IN BIOPHYSICS AND MOLECULAR BIOLOGY 2012;110(2-3):166–177.
44. Polak S, Pugsley MK, Stockbridge N, et al. Early Drug Discovery Prediction of Proarrhythmia Potential and Its Covariates. AAPS J 2015;17(4):1025–1032.
45. Braam SR, Tertoolen L, van de Stolpe A, et al. Prediction of drug-induced cardiotoxicity using human embryonic stem cell-derived cardiomyocytes. STEM CELL RESEARCH 2010;4(2):107–116.
46. Kramer J, Obejero-Paz CA, Myatt G, et al. MICE models: superior to the HERG model in predicting Torsade de Pointes. SCI REP 2013;3:2100.
47. Beattie KA, Luscombe C, Williams G, et al. Evaluation of an in silico cardiac safety assay: using ion channel screening data to predict QT interval changes in the rabbit ventricular wedge. J PHARMACOL TOXICOL METHODS 2013;68(1):88–96.
48. Mirams GR, Cui Y, Sher A, et al. Simulation of multiple ion channel block provides improved early prediction of compounds' clinical torsadogenic risk. CARDIOVASCULAR RESEARCH 2011;91(1):53–61.