Guide to the heart: Differentiation of human pluripotent stem cells towards multiple cardiac subtypes

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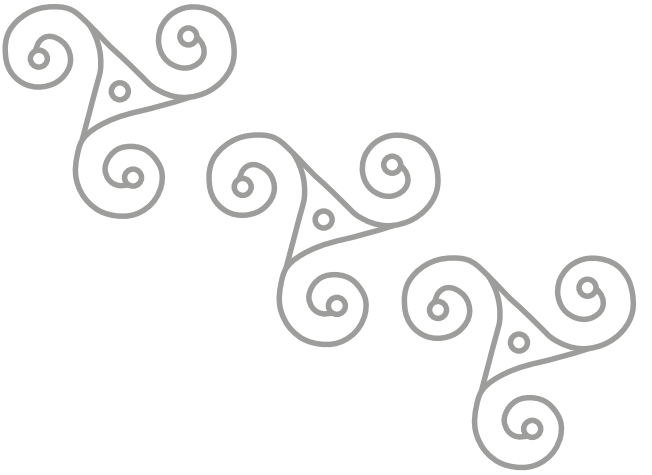

Chapter 9:
Summary
Samenvatting
Zusammenfassung

Summary

The heart is the first functional organ formed by complex re-arrangements during embryonic development. Human pluripotent stem cells (hPSCs), both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have the exceptional abilities to self-replicate indefinitely and differentiate into all cells of the three germ layers by recapitulating steps from *in vivo* embryonic development. Since the first isolation of hESCs from the inner cell mass of human blastocysts in 1998, these properties have been valuable for studying developmental biology, as well as disease modeling in vitro, drug screening and discovery and regenerative medicine. In 2007, hiPSCs were generated by reprogramming somatic cells into PSCs for disease modeling and personalized medicine purposes. The derivation of cardiac progenitor cells (CPCs) and cardiomyocytes (CMs) from hPSCs has significantly advanced our knowledge about cardiac specification and disease development in human. HPSC-derived CMs have rapidly been considered as a promising alternative in safety-pharmacology and pre-clinical drug discovery. A long-term goal of hPSCs and their derivatives are cell-based repair and tissue engineering. While hPSC-CMs pose the possibility for cardiac repair, technical challenges, such as (1) generation of high numbers of defined cardiac subtype-specific cells with high purity and (2) creating the biological niche that mimics native heart to boost in vitro maturation towards an adult phenotype need to be solved. Core of this thesis was generation of multiple hPSC-derived cardiac subtypes for understanding human cardiac development and their application in selective pharmacology and cardiac repair.

Since efficient and reproducible generation of pure populations of hPSC-CMs is crucial for regenerative medicine, disease modelling and drug screening, in **chapter 2** of this thesis, two widely used methods for the differentiation of hPSCs to contracting CMs within 10 days are described. Despite high efficiencies, cardiac differentiations typically generate heterogeneous populations comprised of both CMs and uncharacterized non-cardiac cell-types. Therefore, a protocol for magnetic bead-based purification of hPSC-CMs was developed.

In **chapter 3**, hPSC-derived CMs with atrial identity (hPSC-AMs) were differentiated by modulating retinoic acid signaling during differentiation. HPSC-AMs were transcriptionally and functionally characterized and

applied as pre-clinical pharmacological tool for testing atrial selectivity of anti-arrhythmic drugs targeting the atrial enriched ion channels $K_v1.5$ or Kir3.1/3.4. In response to multiple ion channel blocker, vernakalant and $K_v1.5$ blocker, XEN-D0101, hPSC-AMs but not hPSC-VMs showed action potential (AP) prolongation due to a reduction in early repolarization. In hPSC-AMs, XEN-R0703, a novel Kir3.1/3.4 blocker restored the AP shortening caused by carbachol. Furthermore, it was demonstrated that these ion channel genes are regulated by COUP-TF transcription factors.

Nevertheless, although the majority of hPSC-CMs in this differentiation protocol have an atrial identity, these cultures also contain low percentages of other cell types, including hPSC-VMs. Therefore, aim of **chapter 4** was to identify atrial- and ventricular specific surface markers for the separation of atrial and ventricular CMs. To identify human subtype-specific surface markers for the separation of atrial and ventricular CMs from mixed hPSC-derived cultures, transcriptional profiling of hPSC-CMs by whole genome microarray analysis was performed. Potential atrial and ventricular surface markers were validated by quantitative-polymerase chain reaction. Gene expression demonstrated enhanced transcriptional levels of selected candidate cell surface markers in atrial CMs, while selected ventricular cell surface markers were enriched in ventricular cells. Unfortunately, immunostaining and flow cytometric analysis of the same cell surface markers did not display significant differential expression patterns between cardiomyocyte subtypes. Strategies to induce a more adult phenotype may be important for the identification of subtype-specific surface markers.

As an alternative approach to select hPSC-AMs, an atrial fluorescent reporter line was generated by CRISPR/Cas9-mediated knockin of red fluorescent mCherry into the genomic locus of atrial-enriched COUP-TFII in the well-established NKX2.5$^{eGFP/+}$ hPSC line in **chapter 5**. The dual atrial NKX2.5$^{eGFP/+}$-COUP-TFII$^{mCherry/+}$ reporter line allowed identification and selection of GFP$^+$ (G$^+$)/mCherry$^+$ (M$^+$) CMs following cardiac differentiation. These cells exhibited transcriptional and functional properties of atrial CMs, whereas G$^+$/M$^-$ CMs displayed ventricular characteristics. Pure populations of human atrial and ventricular CMs might support the identification of surface markers specifically expressed on either atrial or ventricular CMs required for the selection of atrial CMs differentiated from patient-specific induced PSCs. Moreover, selection of hPSC-AMs may support the development of more predictive human *in vitro* disease models for atrial diseases, such as atrial

fibrillation (AF) and improve our knowledge about underlying causes in the future. In addition, via CRISPR/Cas9-mediated knockout, we demonstrated that COUP-TFII is not required for atrial specification in hPSCs. Based on both molecular and functional profiling, complete knockout of COUP-TFII did not affect the differentiation towards hPSC-AMs *in vitro*.

Replacement of large areas of muscle may be required to regenerate the heart of patients following myocardial infarction (MI). Thus, in **chapter 6**, it was determined whether doxycycline (DOX)-inducible (Tet-On-MYC) hPSC-derived cardiac progenitor cells (CPCs) could be directed to proliferate followed by differentiation in vivo in a drug-regulated manner in conjunction with fibroblast-growth-factor (FGF) activation, and thus achieve large-scale remuscularization and coincident revascularization of the heart. Transplanted CPCs expanded robustly in cardiac myocardium and a non-cardiac niche under the skin using the antibiotic-inducible transgene system together with FGF. Upon withdrawal of these self-renewal factors, CPCs differentiated with high efficiency at both sites into the major cardiac lineages including cardiomyocytes, endothelial cells and smooth muscle cells. We further described the potential of hPSC-derived CPCs to improve ventricular remodeling and fibrosis after transplantation to the heart after acute MI in mice.

In **chapter 7**, the progressive build-up of the cardiac extracellular matrix (ECM) during embryonic development, the ECM of the adult human heart, as well as natural and synthetic materials with regard to their beneficial effect on hPSC-CM maturity during generation of 3D cardiac tissues from hPSC-CMs was reviewed. HPSC-CMs generally have a fetal-like phenotype. During embryonic development, the cardiac ECM experiences a gradual build-up of matrix proteins that changes along the maturation of CMs. Mimicking these dynamic stages may contribute to hPSC-CMs maturation *in vitro*.

Chapter 8 is a final discussion to link and discuss each chapter of this thesis. Future perspectives on disease modeling, as well as personalized medicine approaches including cardiac repair and tissue engineering are outlined. This thesis ends with a historical view on clinical developments, such as the time for routine application of vaccination, as well as an outlook on opportunities for the hPSC field with regard to new therapies and the conclusion that hESCs and hiPSCs have already made quick progress on their route to the clinic, despite various hurdles that still need to be crossed.