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

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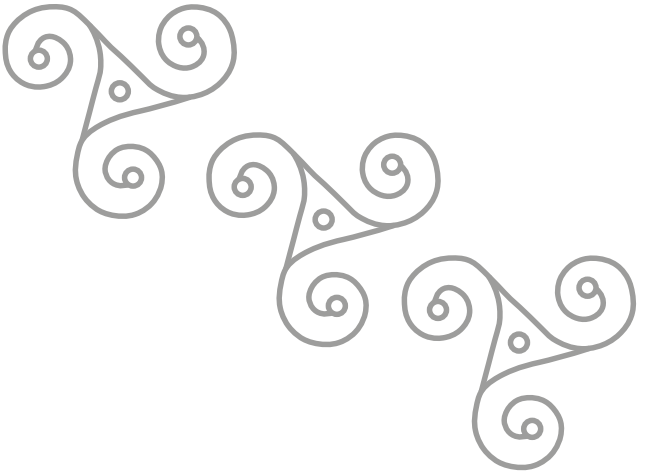

Chapter 8:
General Discussion

Cardiac diseases are, despite continuous improvement of surgical techniques and improved therapy, still a major cause of morbidity and mortality and according to the World Health Organization account for approximately 30% of all deaths worldwide. This indicates that innovative alternatives leading to improved therapeutic strategies are highly needed. Particularly during the last decade, efficient differentiation of human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) in a dish has enormously increased our in-depth knowledge of human heart development, genetic and acquired cardiac diseases and has also opened promising alternatives for traditional drug screening methods performed in experimental animals or transgenic cell lines (Braam et al., 2010; Davis et al., 2011; Doyle et al., 2015).

Continuous optimization of differentiation and purification protocols for the derivation of hPSC-CMs allows reliably production of human CMs. In chapter 2, we describe two of the most widely used methods for generation of hPSC-CMs: the spin-embryoid body (spin-EB) and monolayer-based protocols (Mummery et al., 2012). Spin-EBs more closely resemble embryonic development with regard to their spheroid, three-dimensional (3D) shape. Importantly, EBs can be manipulated during differentiation by interfering with signaling pathway, which are known for their essential role during human embryonic development. However, equal distribution and penetration of signaling compounds into these cell aggregates may be difficult, typically resulting in heterogeneous cell populations (including also non-CMs). Additional disadvantages of spin-EBs are limited scalability, as well as the difficulty of reproducibility. In contrast, monolayer protocols efficiently produce CMs with high purity and show improved reproducibility between experiments. However, while spin-EBs efficiently differentiate into CMs without the inhibition of WNT signaling by treatment with XAV939 at day 3 of differentiation (chapter 2, 3, 4 and 5), in monolayer protocols inhibition of WNT is a prerequisite for efficient cardiac differentiation (Giacomelli et al., 2017).

In order to separate CMs from non-CMs, several approaches have been developed, antibody- or fluorescent-based sorting (Tomlinson et al., 2013), metabolic selection (Tohyama et al., 2013) and magnetic beads. In chapter 2, we established magnetic bead-based selection of VCAM1-positive CMs. While purifications yielded highly pure CMs, efficiency was rather low because a high fraction of CMs remained in the negative population. Nonetheless, selected CMs could successfully be applied for transcriptional analysis, as

well as immunostaining. Recently, new approaches were reported, such as the combination of VCAM1-PE antibodies with pre-coupled anti-PE magnetic small-sized nano-beads, which allow selection of CMs with higher efficiency (Giacomelli et al., 2017). However, nano-beads adhere permanently to CMs. Nonetheless, functionality of selected CMs was validated by electrophysiological characterization of single CMs after nano-bead-based isolation and demonstrated comparable characteristics before and after isolation (Giacomelli et al., 2017). Whereas hPSC-derived ventricular CMs (hPSC-VMs) have successfully been implemented in studying human cardiac development, disease modeling and safety pharmacology, less attention has been paid to hPSC-derived atrial CMs (hPSC-AMs).

In chapter 3, we have shown the robust differentiation of hPSC-CMs with transcriptional and functional characteristics of atrial CMs by modulating retinoic acid (RA) signaling. These hPSC-AMs have proven valuable for the pre-clinical screening of novel antiarrhythmic agents. 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. To allow identification and selection of hPSC-AMs, we then attempted to identify cell surface markers selectively expressed on atrial, but not ventricular CMs in chapter 4. Discrepancy between mRNA and protein expression of various selected surface markers precluded discovery of surface markers with a differential cardiomyocyte subtype expression pattern. Dynamic changes in transcriptional and protein expression may result in discordance between mRNA and protein (van den Berg et al., 2017). Another possible explanation is, that the fetal character of the cells hampered the identification of specific atrial and also ventricular surface markers, suggesting that more mature hPSC-derived cardiac subtypes are required to identify surface markers that allow separation of atrial and ventricular markers. In agreement, we have shown, in chapter 3, that hPSC-AMs resemble fetal atria of the second trimester. Even though several approaches to boost maturity in hPSC-VMs have been developed, cells typically gain some features of maturity, but fail to acquire an adult cardiac phenotype. In the future it will be very interesting to investigate whether pathways or approaches for the induction of maturation in hPSC-VMs, have similar beneficial effects on hPSC-AMs.

As an alternative approach to select hPSC-AMs, our aim was to generate an atrial fluorescent reporter line in chapter 5. In chapter 3, we demonstrated enriched expression of transcription factors chick ovalbumin upstream promoter transcription factor I (COUP-TFI or NR2F1) and COUP-TFII (NR2F2) in hPSC-AMs compared to hPSC-VMs. Moreover, we also demonstrated increased levels of COUP-TFII expression in human fetal and adult atrial cardiomyocytes when compared to ventricular cardiomyocytes. This prompted us to insert sequences encoding the red fluorophore mCherry into the genomic locus of COUP-TFII in the well-established human cardiac NKX2.5$^{eGFP/+}$ reporter (Elliott et al., 2011) using clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9-mediated genome editing in chapter 5. Fluorescent hPSC reporter cell lines have already proven valuable for studying temporal transcriptional changes during hPSC differentiation towards CMs *in vitro* (den Hartogh et al., 2016). Particularly for human embryonic development, little is known about transcriptional cues guiding atrial specification. Additionally, because of growing knowledge regarding the important role of epigenetic regulations during cardiac development (Wamstad et al., 2012) and postnatal maturation (Oyama et al., 2014) it would be worthwhile to explore epigenetic factors crucial for atrial specification. 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 mice, Coup-TFII has been shown to have a key role in atrial patterning (Pereira et al., 1999; Wu et al., 2013). Prominent selective enrichment of transcription factors COUP-TFI and COUP-TFII in hPSC-AMs prompted us to investigate the role of COUP-TFII in atrial CMs in chapter 3. Short hairpin RNA (shRNA)-mediated knockdown and chromatin immunoprecipitation (ChIP) in differentiated hPSC-AMs disclosed that both transcription factors COUP-TFI and COUP-TFII are able to regulate the atrial specific ion channel genes *KCNA5* (encoding K_v1.5) and *KCNJ3* (encoding Kir3.1). To determine whether COUP-TFII is required for specification of the atrial lineage during *in vitro* differentiation of hPSCs, we analyzed the atrial differentiation potential after CRISPR/Cas9-mediated knockout of COUP-TFII in chapter 5. Based on both molecular and functional profiling, complete knockout

of COUP-TFII did not affect the differentiation towards hPSC-AMs *in vitro*. As evidenced by studies in zebrafish, differences in COUP-TFII-dependent regulation of *KCNA5* and *KCNJ3* ion channel genes between COUP-TFII KO and knockdown may reflect a higher non-specific off-target effect of shRNAs in contrast to the high specificity of the CRISPR/Cas9 system (Schulte-Merker and Stainier, 2014). On the other hand, complete genetic knockout from the start of differentiation may be genetically compensated, while short-term effects upon shRNA-mediated knockdown in differentiated CMs may not (Rossi et al., 2015). The observation that COUP-TFII is dispensable for atrial specification of hPSCs *in vitro*, may not preclude its importance for formation of atrial chambers during *in vivo* development. However, differences in developmental expression of distribution of contractile proteins or ion channels (Kaese and Verheule, 2012; De Sousa Lopes et al., 2006) and cardiac physiology, such as heart beat rate, between rodents and humans are well known. Therefore, it is very well possible that loss of COUP-TFII might be compensated in human, but not murine atrial patterning.

While hPSC-CMs have successfully been implemented in disease modeling and safety pharmacology, their clinical application for cardiac repair is still very challenging. Recently, it was shown that human embryonic stem cell (hESC)-derived CMs and allogeneic induced pluripotent stem cell (hiPSC)-derived CMs transplanted into non-human primate hearts post myocardial infarction (MI) had the capacity to remuscularize injured myocardium and to electrically couple to the host myocardium (Chong et al., 2014; Liu et al., 2018; Shiba et al., 2016). However, transplantation also caused transient non-fatal arrhythmias within an initial adaptation window, but not upon further *in vivo* maturation of the transplanted hPSC-CMs (Chong et al., 2014), which suggests that *in vitro* maturation of CMs before transplantation may prevent arrhythmogenic potential. Optimization and standardization of stem cell culture and differentiation protocols have improved considerably over the previous years and resolved some critical technical challenges, including the generation and purification of large numbers of hPSC-CMs. Nevertheless, the optimal route of cell delivery for high cell retention and integration in combination with the immature phenotype of hPSC-CMs generated *in vitro* still block the way to the clinic. In chapter 6, we performed a proof-of-principle experiment to show that doxycycline-inducible (Tet-On-MYC) hPSC-derived cardiac progenitors (CPCs) are able to expand and differentiate into multiple cardiac cells *in vivo* after intramyocardial injection in mice that were subjected to MI. In response to doxycycline

and fibroblast-growth-factor (FGF) CPCs efficiently proliferated to form large grafts. Upon withdrawal of doxycycline and FGF, CPCs successfully differentiated into major cardiac lineages including CMs, endothelial cells (ECs) and smooth muscle cells (SMCs) after treatment with the WNT-inhibitor XAV939. Importantly, while CPC grafts failed to improve cardiac function as quantified by the ejection fraction of the left ventricle, CPCs were able to vascularize the graft area and significantly reduced fibrosis in the infarcted area following acute MI. Moreover, as evidenced by evaluation of the wall thickness in the left ventricle by cardiac MRI in a 12-segment model of the left ventricle, CPCs attenuated left ventricular remodeling after MI. It is well known that species differences between mice and human prevent the coupling of human CMs to the murine host tissue, which is a pre-requisite for synchronized contraction. Thus, the lack of coupling between large human grafts and murine cardiac tissue might induce a conduction block and hinder cardiac function. Moreover, CPCs have been injected into hearts within a matrigel plug, a gelatinous mixture of laminin and collagen, which is known to hinder self-organization of cells, as well as coupling to host myocardium. In contrast, engineered cardiac patches from CMs or CPCs within carefully designed scaffold materials have proven superior because they support self-organization of transplanted cells and contractile function of CMs, vascularization, as well as matrix remodeling within the graft (Chen et al., 2010; Dvir et al., 2012; Tiburcy et al., 2017). In order to create the optimal environment for hPSC-derived CPCs and CMs it is important to mimic native extracellular properties. In chapter 7 we reviewed the native extracellular environment of CMs, as well as natural and synthetic materials with regard to their beneficial effect on hPSC-CM maturity during generation of 3D cardiac tissues *in vitro*.

Future perspectives

Disease modeling

Atrial fibrillation (AF) is the most common clinical condition with abnormal atrial rhythm affecting more than 33 million patients globally (Chugh et al., 2014). AF is linked to several serious comorbidities, including heart failure and stroke (Zoni-Berisso et al., 2014). Utilizing either long-term pharmacology, cardioversion, or surgical intervention by ablation, current

treatment options solely rely on control of the heartbeat, prevention of blood clotting and rhythm control (Yancy, 2017). Additionally, the majority of antiarrhythmic medications, such as beta blockers, are non-selective for the atria and risk fatal ventricular pro-arrhythmic events (Dobrev and Nattel, 2010). To improve our understanding of underlying causes of AF, several rodent, as well as larger animal models have been developed and used to establish mechanisms leading to AF (Nattel et al., 2005; Nishida et al., 2010). In many cases, AF is initiated by rapid ectopic atrial foci that trigger automaticity outside of the primary pacemaker of the heart. Structural remodeling of atrial tissue then forms the substrate for reentry of the electrical signal and persistent AF. Nonetheless, each model has its advantages and disadvantages and various differences between animals and humans, such as distribution of contractile proteins or ion channels, often complicate the translation to the clinic. Moreover, many years of research without effective treatment indicate that there is an urgent need to develop more predictable models. However, the development of atrial selective anti-arrhythmic drugs is only part of the solution in treating AF; additionally, it is important to develop human AF *in vitro* disease models. First progress in modeling AF was achieved with the development of a hESC-derived atrial *in vitro* model (Laksman et al., 2017). To study the role of genetic versus non-genetic AF or elucidating the role of external factors, such as 1) ectopic activity around the pulmonary vein or 2) cardiac remodeling with accumulation of collagen and fibroblasts as underlying causes of AF, more advanced 3D defined multicellular models are required.

Personalized medicine: Regenerative medicine for cardiac repair or tissue engineering

Cardiac repair

Differences in the regenerative potential of the heart exist between species. In contrast to mammalian adult myocardium which fails to regenerate after injury and instead is replaced by fibrotic scar tissue, non-mammalian vertebrates, such as the zebrafish, are able to totally regenerate their myocardium after up to 20% of apical resection (Jopling et al., 2010; Poss et al., 2002). While the contribution of resident stem or progenitor cells is only very limited, regeneration occurs primarily by injury-induced proliferation

of pre-existing, differentiated CMs which underwent disassembly of the sarcomeric machinery to allow re-entry into the cell cycle (Jopling et al., 2010). Similar to the regenerative mechanisms in zebrafish, the neonatal mice heart has the ability to regenerate after surgical resection of less than 20% of the ventricular apex or after MI within a transient postnatal window of less than 7 days (Haubner et al., 2012; Porrello et al., 2011, 2013). A recent single clinical case report suggests that the human heart also has the capacity to regenerate after MI within a regenerative window of 1 day after birth (Haubner et al., 2016). Complete cardiac regeneration resulted in normal long-term heart function that was indistinguishable from healthy hearts (Haubner et al., 2016). This suggests that it may be possible to boost the intrinsic capacity for myocardial regeneration in human adults. Several approaches have been attempted and are under current investigation, including neuregulin signaling (Bersell et al., 2009), hypoxia (Nakada et al., 2016), microRNAs (Porrello et al., 2013) and the control of the Hippo-YAP pathway (Uygur and Lee, 2016). Combination of hPSC-derived CPC or CM transplantation with re-activation of the intrinsic proliferative capacity of host CMs may eventually lead to successful cardiac repair strategies applied routinely in clinical settings.

Tissue engineering

Protocols to generate major cardiac subtypes of the heart, including atrial, ventricular and nodal CMs (Birket et al., 2015; Protze et al., 2016), as well as epicardial, endothelial and smooth muscle cells (Iyer et al., 2015; Orlova et al., 2014; Witty et al., 2014) have been evolved in recent years. However, more information on distinct cardiac subtypes of left and right atria and ventricles, or endocardial endothelial cells is required to develop robust protocols for the generation of distinguished subtype-specific cell types. Moreover, an ideal microenvironment closely recapitulating cardiac extracellular matrix will be required to support complex multicellular networks, such as the heart. Utilizing defined cell types and extracellular scaffold materials together with advanced 3D bioprinting techniques that allow generation of tissue-like structures by deposition of cells and scaffold materials layer-by-layer according to detailed designs (Duan, 2016) may support generation of tissue engineered cardiac tissues in the near future. Bioprinting 3D artificial hearts with defined cellular subgroups enveloped

by an optimal microenvironment mimicking native heart may support the self-organization of hPSC-CMs, as well as endothelial cells and epicardial-derived fibroblasts and smooth muscle cells into complex tissues. Cardiac tissues will be worth for advanced disease modeling and pharmacology and ultimately provide a step towards an alternative for the shortage in available donor hearts.

Historical view

The field of hPSCs, especially hiPSCs, and their application for tissue repair is young. Most new therapies took at least 20 years to develop from experimental research to routine application, including vaccination, in-vitro-fertilization (IVF) and organ transplantation. From the initial concept of vaccination until the first vaccine 300 years elapsed, but led to complete eradication of the cowpox and other diseases (Plotkin, 2014). Similarly, initial IVF experiments started in the 1950s, however controversies and ethical issues only allowed the first IVF baby to be born in 1978. Today, more than 5 million IVF babies have been born to support infertile couples with the desire to have children (Brian, 2013).

The idea of a circulatory system arose in 1242 when Ibn al-Nafis was the first physician to describe the blood flow from the right chamber of the heart to the lungs for oxygenation and back to the left heart (West, 2008). Approximately 350 years later, in 1628, William Harvey proved existence of a heartbeat that continuously pumps blood throughout the body via a second arterial and venial connection, the capillaries (West, 2008).

The approach to repair diseased tissues by transplantation dates back to early 1597 when Gasparo Tagliacozzi reported about a nose replacement in 'The Surgery of Defects by Implantation' (Barker and Markmann, 2013). However, only in 1954, the first kidney transplant and in 1968 the first heart transplant were performed (Barker and Markmann, 2013). Limited knowledge about immune-system induced organ rejection resulted in poor survival of patients and only 20 years later, heart transplantation became a widely accepted clinical approach for the treatment of heart failure. Today, approximately 4000 heart transplants are performed each year.

Two decades following their first generation, hESCs and their derivatives are being tested in clinical phase I or II trials for severe disease conditions, such as age-related macular degeneration, diabetes, spinal cord injury and neurodegenerative diseases, Parkinson disease, as well as amyotrophic lateral sclerosis (ALS) (Trounson and DeWitt, 2016). Only a single decade after reprogramming somatic cells towards hiPSCs, hiPSC-derived retinal pigmented epithelium (RPE) have successfully been transplanted and interrogated vision loss in a single clinical case and were recently approved to enter larger clinical trials (Trounson and DeWitt, 2016). Despite various hurdles that still need to be crossed, hESCs and hiPSCs have already made quick progress on their route to the clinic.

Table 8.1: Clinical trials with hESC- and hiPSC- derivatives

Clinical trial GOV identifier	Cell type	Disease	Treatment	Patients	Status	Outcome
NCT01469832 or NCT01344993	hESC-RPM	Stargardt's Macular Dystrophy or Advanced Dry Age Related Macular Degeneration	Uniocular subretinal injection of hESC-RPE	12 or 13	Completed	Medium-term to long-term safety and graft survival. Vision-related quality-of-life measures increased for general and peripheral vision
NCT02903576	hESC-RPM	Outer Retinal Degenerations	Injection of hESC-RPE in suspension or seeded on a polymeric substrate	18	Recruiting	Estimated June 2019
NCT02320812	hiPSC-retinal progenitor cells	Retinitis Pigmentosa	Administration of a single dose of retinal progenitor cells	28	Completed	Not yet publicly available
NCT03482050	hESC-astrocytes	Amyotrophic Lateral Sclerosis	Spinal injection of a single or two consecutive doses of hESC-astrocytes	21	Recruiting	Estimated in August 2020
NCT03162926	hESC-insulin producing pancreatic progenitor cells	Type 1 Diabetes	hESC-insulin producing cells encapsulated into immune-protective device implanted subcutaneously	3	Completed	Cells can be maintained safely for up to 4 months
NCT03163511	hESC-insulin producing pancreatic progenitor cells	Type 1 Diabetes	hESC-insulin producing cells encapsulated into immune-protective device implanted subcutaneously	55	Recruiting	Estimated in December 2020
NCT02452723	Human partheno-genetic neural stem cells	Parkinson	Intracerebral injection to the striatum and substantia nigra	12	Recruiting	Estimated in December 2019
NCT02302157	hESC-derived oligo-dendrocyte precursor cells	Spinal cord injury	Single injection between 21 and 42 days post injury, inclusively, to subjects with subacute cervical spinal cord injuries	35	Active	Estimated in December 2018
NCT02057900	hESC-derived cardiac progenitors	Severe heart failure	Epicardial delivery of progenitor cells embedded within fibrin gel	10	Completed	Short- and medium-term safety. All patients were symptomatically improved with an increased systolic motion of the cell-treated segments

Conclusion

In conclusion, this thesis describes the *in vitro* generation of hPSC-derived subtype-specific atrial and ventricular CMs and their implication for drug screening and discovery, as well as understanding human cardiac development. In addition, it illustrates the *in vivo* expansion and differentiation of cryopreserved hPSC-derived expandable, multipotent cardiovascular progenitor cells towards multiple cardiac cells and their application for cardiac repair.

These achievements will ultimately support the development of more selective, targeted drug therapies, advanced human *in vitro* disease models and personalized medicine to defeat cardiac disease in the future.

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