CHAPTER 7

Summary, conclusions and future perspectives

Summary and conclusions

The general introduction of this thesis, ***Chapter 1,*** describes normal cardiac function and in more detail, the mechanisms of action potential initiation and propagation in the healthy heart. Furthermore, the effects of ischemic heart disease are discussed, the current treatment options and their limitations, followed by the potentials of regenerative medicine for cardiac disease, and the current status and problems that need to be overcome in order to bring these therapies a step closer to clinical practice. The aim of this thesis was to explore from a mechanistic point of view, cellular modification processes associated with heart disease, as well as harnessing cellular modification for treatment and prevention of detrimental electrophysiological consequences of heart disease.

Chapter 2 provides a comprehensive description for the generation of iPSCs from fibroblasts derived from four different species, namely mouse, rat, pig and human. In order to capitalize on the potential of pluripotent stem cell-based applications, such as patient-specific disease modelling or regenerative medicine, it is of vital importance to be able to create iPSCs with high quality and efficiency. We therefore selected four widely used model species and described an efficient method for generating iPSC lines of high quality.

Chapter 3 describes the result of a high throughput screen for cytokines and signalling molecules to induce cardiac lineage specification. To this end, a transgenic mouse embryonic stem cell (ESC) reporter line with the base promotor and cardiac enhancer region for Nkx2.5 coupled with eGFP, was used. A panel of 44 cytokines, growth factors and signalling molecules were tested by exposure of cells in a dose-response curve. Cardiac differentiation was assessed by flow cytometry quantification of $eGFP^+$ cells, representing $Nkx2.5^+$ cardiac progenitor cells (CPCs). This screen identified insulin-like growth factors (IGF1, IGF2 and insulin) as positive regulators of cardiac differentiation. Further analysis revealed that temporal stimulation with IGF resulted in selective proliferation in the $Brachyury^+$ early mesodermal cell population, thereby selectively expanding cardiac precursor cells. This effect was not seen during simultaneous inhibition of Akt or mTOR, known downstream signalling molecules in the IGF pathway. Elucidating the intricate steps of signalling pathway activation and inhibition can be mimicked during the dynamic process of cell lineage specification. Early timed IGF pathway activation was shown to direct cells toward a cardiac lineage, and could ultimately lead to more efficient cardiac differentiation. Robust cardiomyocyte differentiation is a prerequisite to meet the large demand for transplanted cells in regenerative therapies.

In ***Chapter 4*** the electrophysiological effects of forced cellular fusion of human ventricular scar cells (hVSCs) with neonatal rat ventricular myocytes (NRVMs) was investigated. Scar cells were isolated from post-myocardial infarction scars of human left ventricles and characterized by immunological assay. Cultured hVSCs were predominantly (myo-)fibroblasts and approximately 40% of cells showed characteristic α-SMA striation. Upon co-culturing hVSCs with NRVMs (20%/80%), cultures showed action potential duration (APD) prolongation, increased APD dispersion and a markedly greater number of early after-depolarizations at 1 Hz pacing compared to non-hVSC containing NRVM cultures. Transduction of hVSCs with fusogenic *vesicular stomatis virus-G* (VSV-G) allowed partially controllable cell membrane fusion with surrounding cells. This resulted in heterokaryonic cell clusters, which retained electrophysiological properties from hVSCs as well as NRVMs, but mostly resembled NRVMs. Importantly, in contrast to hVSCs, heterokaryons were electrically excitable and had large repolarization reserve. Patch clamp measurements confirmed presence of greater outward K_v current in heterokaryons compared to NRVMs and hVSCs. Fused co-cultures showed increased Cx43 expression, indicating better coupling, as well as a more negative membrane diastolic potential compared to unfused cultures. Fused hVSC/NRVM co-cultures showed an electrophysiologically favourable phenotype (cancellation of APD prolongation and dispersion, decreased incidence of EADs). This study provides proof of principle that forced cellular fusion is able to undo detrimental arrhythmogenic effects resulting from the presence of fibroblastic cells as occurs in diffuse fibrosis. Whether forced cellular fusion could ever become a therapeutic modality remains unlikely. However, fusion of electrophysiologically distinct cell types, and thereby removing their intercellular barriers, creates a less arrhythmogenic substrate and is therefore an interesting model to gain new insights into fibrosis-mediated arrhythmia.

In ***Chapter 5*** the results of complimentary *in silico* and *in vitro* models are presented in order to study the mechanisms underlying re-entry initiation and dynamics in remodelled cardiac tissue, characterized by fibrosis and conduction slowing. A common effect of cardiac injury is (local areas of) conduction slowing, usually due to tissue remodelling, such as fibrosis and decreased Cx43 expression. Therefore, NRVMs were cultured with 30% fibroblasts and cells were transduced with lentiviral vectors encoding short-hairpin RNAs directed against Cx43 in different dosages. Degree of Cx43 knock-down inversely correlated with culture conduction velocity. Upon rapid pacing, spatially discordant APD alternans was observed. Discrete areas of APD alternans were organized throughout the culture, and termed alternans phase islands (APIs), given their island-like patterns on culture maps. Wavebreaks occurred at borders between APIs of opposite polarity (APIs with long-short conformation APD alternans; L-S, lying adjacent to APIs with short-long alternans conformation; S-L). Extent of conduction slowing correlated with API formation, and ultimately re-entry complexity. This study provides

mechanistic insight into arrhythmia initiation and dynamics in a model of conduction slowing and fibrosis, representing remodelled ventricular tissue.

Chapter 6 describes a new approach to terminate re-entry once it has been established. To this purpose, atrial cardiomyocytes isolated from neonatal rats were cultured and transduced with lentiviral vectors encoding CatCh, a light-sensitive ion channel. These channelrhodopsins were originally isolated from algae and have recently become widely used in the optogenetics field, where protein behaviour can be modulated with high spatiotemporal control by exposure to light stimulation. Brief light pulses (10 ms blue light, 470 nm wavelength) were able to generate enough depolarizing current to evoke action potentials. After stable re-entry induction by rapid electrical pacing was established, cells were exposed to a brief 500 ms blue light pulse, which resulted in rapid re-entry termination in 100% of cases in CatCh-transduced cultures compared to 0% termination in eGFP-transduced control cultures. Mechanistically, rotor core size was shown to increase, thereby destabilizing the phase singularity which caused it to drift and collide with culture boundaries or phase singularities of opposite chirality. These results demonstrate the feasibility of arrhythmia termination by optogenetic engineering of cardiomyocytes, which equips cells with highly controllable ion channels, which, upon an external cue are able to intrinsically generate a defibrillating current.

Future perspectives

Tremendous efforts have been made so far in order to bring regenerative therapies such as cellular or genetic interventions a step closer to clinical practice. These studies have shown that a lot more hurdles need to be overcome in order to achieve this important goal. First, differentiation protocols need to be further refined for efficient and reproducible cardiomyocyte generation from pluripotent stem cells. Many lessons can be learned from cardiac lineage specification and maturation in the developing embryo. Heart development is a complex multistage process, where cells are rapidly being exposed to molecular cues. This results in precisely timed activation of specific signalling pathways, which induce a specific set of transcription factors. Unravelling the spatiotemporal sequence of activation and inhibition of the signalling pathways and downstream transcription factors can give much insight into cardiac lineage specification, which can be mimicked *in vitro* by precise exposure of differentiating stem cells to signalling molecules in order to direct cardiac development. Given the complex nature of this process, a simpler, but laborious approach would be to conduct detailed screening for signalling molecules associated with cardiac development. Since signalling pathways can be active during very brief developmental steps, it will be important to identify at higher resolution cell lineage markers of intermediate developmental stages. This

can aid the molecular guidance of cells towards mature cardiomyocytes. These studies could potentially also lead to an improved set of factors that need to be overexpressed in order to achieve direct reprogramming into reprogrammed cardiomyocytes that are indistinguishable from native cells. This will also be important in order to attain cardiac cells that are mature, both electrophysiologically as well as in terms of contractility. In addition, improved cellular transplant homing and graft survival will be necessary to achieve sustained transplantation effects. Cellular guidance and alignment into the existing cardiac syncytium would allow for optimal graft function and simultaneously reduce arrhythmic activity associated with the graft. Further studies should give more insight into cellular integration into the host tissue, a process that is also disturbed in cardiac fibrosis. The arrhythmogenic substrate of cardiac fibrosis likely shares characteristics of transplanted cells, which are also poorly aligned and not integrated within the host tissue. Control over guided cellular alignment could be achieved by molecular gradients, such as in the developing embryo, or using a tissue patch of extracellular matrix containing scaffold, on which the cells are aligned by micro-patterning. Exact host tissue architecture can be visualized through advanced 3-dimensional imaging techniques. These images can subsequently be reconstructed in the lab by 3-dimensional printing of scaffolds to create an exact fit of transplantable cells. Micro-surgery could then be performed to create a perfectly aligned tissue patch.

Transplanted cells need to be well connected to surrounding host cells, a process that would likely benefit from cellular alignment. Suboptimal connection could lead to conduction slowing, thereby creating a vulnerable substrate for re-entry initiation through occurrence of action potential duration alternans phase islands (APIs). Future research should focus on the molecular mechanisms related to API formation. If, for example, APIs are related to alterations in an ionic current, this could become a promising drug target in order to prevent re-entry-based arrhythmia.

Lastly, cell grafts could be protected from the detrimental effects of arrhythmia by equipping them with an intrinsic defibrillator. The most elegant solution would be if cells could sense arrhythmic activity and activate an ion channel, thereby creating a defibrillating current. Although our studies provide proof of principle for cells to be able to generate an intrinsic defibrillating current, still many more steps are still to overcome in order to achieve cells with artificial intelligence that is able to self-defibrillate in case of arrhythmia. It is likely that an intricate molecular engineering process would lie at the basis of this solution, where an existing or transgenic ion channel would sense rapid membrane potential fluctuations and thereby activate itself or another ion channel, which could provide a current sufficiently strong to overcome the arrhythmic activity. Cellular and genetic interventions for heart disease have met many drawbacks which have so far prevented swift translation to clinical practice. Recent efforts have provided many clues that need to be resolved, but simultaneously give new hope for tailored therapies at the cellular and molecular level.