# CHAPTER 1

## General introduction and outline of thesis

## Background

The heart is a vital organ, which is of great importance for the maintenance of tissue homeostasis. Millions of years of evolution have culminated in the heart in its current state, a well-orchestrated and efficient pump that supplies the body with nutrient- and oxygen-rich blood. While evolutionarily well-designed, the heart is also a delicate organ with very limited regenerative capacity. This implies that the slightest injury or adverse effect can have serious detrimental outcomes, such as acutely lethal arrhythmias or chronically lethal heart failure. Recent advances in the field of cardiology, such as the development of percutaneous coronary interventional procedures, ventricular assist devices and implantable cardioverter defibrillator devices, have led to major breakthroughs in preventing or limiting the extent of these adverse effects. This has resulted in a drastic lowering of heart disease related mortality and morbidity. Despite of these tremendous developments, cardiovascular disease still has the highest mortality rate worldwide.[1] It is therefore important to continue developing new and improved therapeutic modalities. A promising strategy for treating heart disease is by harnessing the potentials of molecular medicine, such as genetic or cellular therapies.

## Normal cardiac function

The adult human heart consist of about five to ten billion cells. These comprise the smallest building blocks, of which the most important cardiac cell types are the cardiac muscle cells (cardiomyocytes), smooth muscle cells, endothelial cells, (myo)fibroblasts, Purkinje fibers, sinoatrial pacemaker cells and atrioventricular nodal cells. Each individual cell type has its unique contribution to normal pump function. In order for the rhythmic contractions to occur, cells need to be electrically activated at the right moment. This allows synchronous mechanical contraction and relaxation to occur in a timely fashion, which is important for optimal and efficient blood movement through the organism. The electrical activation of the heart is a hierarchically structured and coordinated process, which starts in the cardiac pacemaker cells of the sinoatrial node in the right atrium. These nodal cells derive their pacemaker activity from their funny current channels ($I_f$), an inward current which opens upon hyperpolarization, as well as the sodium-calcium exchanger (NCX) current, which produces a net inward current. These currents contribute to the membrane clock (contribution of ion channels in plasma membrane) and calcium clock (contribution of calcium release from sarcoplasmic reticulum) respectively, which both allow for spontaneous depolarizations, thereby starting the action potential in the heart. From here, the electrical wave is transmitted to the surrounding cardiomyocytes in the atria and reaches the atrioventricular node. This specialized tissue slows down conduction,

allowing sufficient time for full contraction and blood expulsion from atria through the mitral and tricuspid valves into the left and right ventricles, respectively. The action potential then travels through the Purkinje fibers, which consist of specialized cells that rapidly transmits the electrical wave down the His bundles to activate the ventricles. This sequential multistep process culminates in ventricular contraction, propulsion of deoxygenated blood through the pulmonary valve into the pulmonary arteries on the right side of the heart, and oxygenated blood through the aortic valve into the aorta on the left side of the heart.

## Electrical Wave Propagation

In order for electrical wave propagation to occur, there is an extensive intercellular communication system through gap junctional coupling. These gap junctions form intercellular channels, which, amongst others, allow for electrotonic interaction through low-resistance trafficking of ionic currents from cell to cell. Gap junctions are formed when one hemichannel (i.e. connexon) of one cell is coupled to one hemichannel of an adjacent cell. Connexons consist of an assembly of six proteins of the connexin family. The contributing connexin (Cx) subtypes can vary per connexon, and are spatially organized in the heart, so that each specific cardiac tissue type has its own unique biophysical properties.[2-4] In the human heart, the distribution pattern is mainly (from highest to lowest) Cx43 and Cx45 in the ventricles and Cx40, Cx43 and Cx45 in the atria.[5, 6] Once an activation front reaches an excitable cell, an elaborate mechanism of multiple steps produces an action potential in the cell. This process of excitation consists of five distinct phases. During phase 0, transient depolarization by movement of ionic current through gap junction channels from an adjacent cell activates fast sodium ($Na^+$) channels, leading to influx of $Na^+$ ions ($I_{Na}$) and rapid depolarization. At phase 1, the depolarization is self-limiting as fast $Na^+$ channels become inactivated and there is transient net outward current of potassium ($K^+$) and chloride ($Cl^-$) ions carried by the $I_{to}$ channels. Phase 2 is also known as the plateau phase, as activation of L-type calcium ($Ca^{2+}$) channels causes inward movement of $Ca^{2+}$ ions, while simultaneously there is a counterbalancing effect of outward $K^+$ current through $K_v$ channels such as slow delayed rectifier $K^+$ channels ($I_{Ks}$), thereby offsetting the effect of the inward movement of $Ca^{2+}$ current. This creates the plateau in the action potential. The high intracellular $Ca^{2+}$ concentration makes $Ca^{2+}$ ions more readily available for $Ca^{2+}$-binding troponin molecules of the tropomyosin complex in the contraction apparatus of cardiomyocytes. Therefore, it is during this phase (phase 2) that the cell is able to contract. Meanwhile, the NCX channel, as well as $Na^{2+}/K^+$-ATPase begin to restore intra- and extracellular ion concentrations. Phase 3 is characterized by the rapid repolarization phase of the action potential, due to inactivation of the $Ca^{2+}$ channels, while $I_{Ks}$ channels remain open. This results in a net outward

positive current, which facilitates a negative change in the membrane potential, so that also other $K^+$ channels become active, like the rapid delayed rectifier $K^+$ channels ($I_{Kr}$) and inwardly rectifying $K^+$ channels ($I_{K1}$). This net outward positive current further repolarizes the cell. $I_{Kr}$ channels become inactivated around the membrane potential, while $I_{K1}$ is still conductive through phase 4, thereby establishing resting membrane potential. Meanwhile, ionic pumps like NCX and $Na^{2+}/K^+$-ATPase continue to restore ionic balances corresponding to resting state. Phase 4 is the resting membrane potential, which is maintained by activity of NCX and $Na^{2+}/K^+$-ATPase, as well as $I_{K1}$. Once a wavefront reaches the cell, electrotonic interaction by means of $K^+$ entering the cell through gap junction channels causes slight depolarization, thereby activating fast $Na^+$ channels, and the action potential starts again at phase 0.[7]

## Ischemic heart disease

Myocardium is an aerobic tissue, relying almost exclusively on aerobic oxidation for its energy consumption.[8] Ischemic heart disease is defined by an imbalance between myocardial oxygen demand and supply from the coronary arteries.[9] A myocardial infarction is a process where an atherosclerotic plaque in a coronary artery ruptures, exposing subendothelial collagen and thrombogenic necrotic material. Thrombocytes rapidly adhere and aggregate to form a platelet thrombus, thereby occluding the vessel lumen. This sudden loss of blood supply to the myocardium leads to rapid cardiomyocyte death, a process which is further aggravated by ischemia reperfusion injury when the vessel is opened.[10, 11] Cardiomyocytes are a permanent cell type, and possess very limited proliferation capacity, if any, at the adult stage.[12-16] In the event of injury, tissue integrity is maintained by an inflammatory response, where damaged or deceased cardiomyocytes are replaced by rapidly proliferating cardiac fibroblasts. This is followed by extensive tissue remodelling and myocardial scar formation, consisting of mainly fibrotic tissue.[17, 18] These changes have detrimental effects on both tissue contractility as well as excitability. As cardiomyocytes are the force-generating units of the heart, replacement of these cells by non-contractile fibroblasts leads to a reduction in contractile force, and thus a decreased ejection fraction. Furthermore, increased tissue stiffness causes diastolic dysfunction and these mechanisms can ultimately lead to heart failure.[19-21]

Cardiac fibrosis also has many detrimental effects from an electrophysiological point of view. The cardiac syncytium, which consists of well-aligned fibers of cardiomyocytes, surrounded by a meshwork of collagen-producing fibroblasts, is normally able to rapidly conduct action potentials over long distances. Cardiac fibroblasts are inexcitable cells and are poorly coupled to their excitable counterparts (*i.e.* cardiomyocytes), at least *in vitro*.[22-24] Fibroblasts are not

able to propagate action potentials over large distances.[25] Replacement of excitable and well-coupled cardiomyocytes by inexcitable and poorly-coupled fibroblasts therefore creates disruptions in the cardiac syncytium, which becomes interrupted by poorly aligned and poorly conducting cells. This causes asynchronous and inefficient electrical impulse propagation.[26] Ultimately, these effects cause conduction slowing and unidirectional block, which increases the likelihood of re-entrant activity.[24, 27-29]

Ischemic injury is associated with a massive inflammatory response, which activates cardiac fibroblast proliferation and differentiation. A fibroblastic cell subset transdifferentiates to a myofibroblast phenotype, due to certain pro-inflammatory signalling, such as the TGF beta pathway.[30, 31] These myofibroblasts mimick certain features of cardiomyocytes, such as having a contractile apparatus and expressing smooth muscle actin stress fibers. In addition, myofibroblasts have more abundant expression of connexins, which enable them to better couple to their surrounding cardiomyocytes.[32] However, this compensatory mechanism is to a great extent detrimental to the electrophysiological function of the heart, as myofibroblasts are inexcitable cells, and have a more positive resting membrane potential than cardiomyocytes.[33] Improved intercellular coupling causes partial depolarization of the surrounding cardiomyocytes, thereby leading to conduction slowing.[24, 32] Furthermore, coupling of cardiomyocytes with inexcitable cells prolongs the action potential duration because of a larger shared cell capacitance.[34, 35] A prolonged action potential duration is associated with increased incidence of early after-depolarizations, because of the prolonged time that the membrane potential is in the calcium window; the membrane potential window at which the $Ca^{2+}$ channels are active. This increases the chance of abnormally early influx of $Ca^{2+}$ ions into the cell, thereby creating a new ectoptic action potential from one or a few neighbouring cells. This irregular extra beat is out of phase with the normal wave fronts, and can therefore run into tissue that is still in its refractory phase. If other areas are repolarized faster, this could lead to unidirectional block and formation of a re-entrant circuit.[36-38] Increased coupling of (myo) fibroblasts to cardiomyocytes also decreases the source-sink mismatch normally present in cardiac tissue. This mismatch is protective, since its hierarchical nature causes synchronized action potential propagation and thereby prevents the occurrence of early or delayed after-depolarizations. In case of fibrosis, this source-sink mismatch is decreased, thereby contributing to increased early and delayed after-depolarizations and thus arrhythmogenicity.[28, 39]

In conclusion, ischemic heart disease causes cardiomyocyte damage and/or death. Due to limited regenerative capacity, cardiac remodelling occurs, which is characterized by replacement of dead or diseased cardiomyocytes by inexcitable, non-contractile fibroblasts. This leads to a reduction in contractile force, which is clinically translated to a decrease in

stroke volume. When the heart cannot meet the demands of maintaining adequate blood flow to the tissues in the body, there is a state of heart failure. Heart failure is usually a non-reversible, (slowly or rapidly) deteriorating process, with a high mortality rate.[40] Cardiomyocyte damage and fibrosis furthermore contribute to an arrhythmogenic substrate, which can lead to fatal arrhythmias like ventricular tachycardia (VT) or ventricular fibrillation (VF). One of the hallmarks of post-ischemic cardiac remodelling is changes in gap junctional coupling.[41-43] Damaged cardiomyocytes express lower levels of phosphorylated connnexin 43; the active protein which forms gap junctional channels. In addition, there is lateralization of connexins, meaning that connexins are predominantly expressed perpendicularly to cardiac myofiber direction, thereby decreasing their functional effectivity in electrical signal relaying.[44, 45]

## Current treatment options and their limitations

Recent scientific breakthroughs have revolutionized treatment of ischemic heart disease. Novel insight into the atherosclerotic process have contributed to improved preventive measures, such as the use of lipid lowering agents (e.g. statins), but also lifestyle adjustments such as smoking cessation, adhering a low-fat diet and regular exercise have all shown significant lowering effects on the incidence of ischemic heart disease.[46, 47] Furthermore, improved diagnostic tools have enabled clinicians to detect atherosclerotic lesions at an early stage. These lesions can be opened and stented by means of percutaneous coronary intervention (PCI) or bypassed by coronary artery bypass graft (CABG) procedures. During acute occlusion, these interventions are used to acutely re-establish blood-flow to the ischemic myocardium, thereby reducing infarct size, which has benefical effect on long-term prognosis, such as morbidity and mortality.[48-51]

Once an ischemic event has occurred, cardiac performance can be optimized by pharmacotherapy, thereby offsetting or delaying clinical manifestations of heart failure. Mainstay therapies are angiotensin converting enzyme inhibitors, angiotensin II receptor blockers, diuretics, beta blocking agents, all drugs that reduce pre- and afterload.[46, 52-55] Anti-arrhythmic agents are used to prevent or treat cardiac arrhythmias, but are frequently associated with paradoxical arrhythmogenic effects.[56] Recently catheter ablation techniques have been applied to treat scar-tissue associated arrhythmogenic foci.[57, 58] In addition, implantable cardioverter defibrillators (ICDs) are used to monitor patients' heart rhythm and apply a defibrillating shock when an arrhythmia is detected. Although a tremendous amount of progress has been made in the prevention and treatment of (the effects of) ischemic heart disease, morbidity and mortality still remains remarkably high. These numbers justify the search and exploration of better, more sophisticated treatment modalities.

## Regenerative therapy for ischemic heart disease

Since the heart is a poorly regenerating organ, a lot of effort has been invested into strategies and techniques that could eventually lead to effective cell replacement or tissue adaptation therapy. The ability to culture pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs), and subsequently differentiate them into specialized cell types raised anticipation for generating tissue in a dish. This transplantable graft could replace a patient's myocardium once it becomes damaged or diseased. This promise grew further with the discovery of a technique to induce a terminally-differentiated cell type (e.g. fibroblast) into an induced pluripotent stem cell (iPSC), which allowed the production of patient-specific pluripotent stem cells.[59, 60] This breakthrough discovery made possible that patient-specific cells could be used to eventually replace their own diseased heart, without the need for donor matching or immunosuppressive therapies. In addition, these developments laid the foundation for establishing cell fate switching by means of transdifferentiation. Here, terminally-differentiated cell types directly convert into a different terminally differentiated cell type (e.g. fibroblast to cardiomyocyte).[61, 62] This reprogramming of one cell type to another has also been shown *in vivo* by direct transdifferentiation in the heart.[63, 64]

Despite these advances, much of the early optimism has now been replaced by scepticism with the realization that many important hurdles need to be overcome before these techniques could one day lead to a significant therapeutic intervention.

Limited directed differentiation has precluded generation of vast amounts of the cell type of interest (e.g. cardiomyocytes). There is a considerable amount of inter-PSC-line variability in terms of cardiac differentiation potential. What is more, differentiated PSC-derived cardiomyocytes show generally an immature phenotype compared to endogenous cardiomyocytes for important characterstics, such as gene expression profiles, action potential morphology and force generation.[65, 66] These problems are even worse for cardiac transdifferentiation, where the current reprogramming protocols give rise to inefficiently and incompletely reprogrammed cells, (*i.e.* cardiomyocyte-like cells).[67, 68]

Early transplantation studies have shown transient beneficial effects at an early stage, but these effects were lost after a few weeks.[69, 70] Furthermore, cellular homing to the heart after systemic administration has been extremely limited.[71] Both systemic and local administration resulted in poor long-term cell survival, thereby supporting the hypothesis that the beneficial effects were mainly due to transient paracrine effects.[72, 73]

Recently, a study showed robust ESC-derived cardiomyocyte engraftment in a primate model of myocardial infarction.[74] However, ventricular tachycardia was an important and frequent adverse effect after successful transplantation, likely due to poor cellular alignment and integration in the host tissue.[75] Electrophysiological integration and prevention of arrhythmogenicity are therefore important problems to overcome.

In order for regenerative therapies to one day become clinical practice, it is important to address several issues. First, elucidating and controlling the factors that determine differentiation potential and cellular maturation would be an important step to generate sufficient amounts of cardiomyocytes necessary for tissue graft transplantation. Second, differentiation protocols should be modified to improve cardiomyocyte maturation, yielding cells that are capable of generating sufficient contractile force and also electrophysiologically resemble endogenous cardiomyocytes. Many lessons can be learned from the developing embryonic heart. Orchestrated expression of key transcription factors by spatial and temporal activation of signalling pathways underlies heart development.[76, 77] This process can be mimicked *in vitro* by timed addition and removal of growth factors or signalling molecules.[78-81] Unravelling this process in detail can greatly make the process of cardiac differentiation more efficient.

Third, efforts need to be made to increase cellular integration into host tissue in order to prevent arrhythmogenicity of engrafted tissue. Cells could be further modified by genetic engineering to equip them with properties for improved cellular function. This could be done by increasing their contractile force, but also tailoring their electrophysiological behaviour. One such function is the ability of cells to self-terminate arrhythmias once they occur. Therefore, further investigation is required in order to produce transplantable quantities of cardiomyocytes. Additionally, these cells could be genetically modified to enhance several functions, such as their functional integration into the host tissue, thereby decreasing transplant arrhytmogenicity or fitting these cells with the capacity to intrinsically terminate arrhythmias when they occur.

## Aims and outline of thesis

In order to meet the therapeutic promise of cellular modification and interventions for ischemic heart disease, it is important to overcome a number of challenges that preclude harnessing their full therapeutic potential, see ***Chapter 1***. Therefore, the aim of this thesis was to explore from an electrophysiological point of view, pathological cellular modification processes in cardiac disease, and to establish novel cell-modifying genetic interventions to prevent or treat

arrhythmia. In addition, cardiac differentiation was studied in an embryonic stem cell model, to gain new insight into cardiac lineage commitment. Improved *in vitro* cardiac differentiation can give rise to robust and mature cardiomyocytes, which could ultimately serve for cellular transplantation therapies.

In order to capitalize on induced pluripotent stem cell technology for regenerative applications, efficient reprogramming technique is a prerequisite to allow easy access to patient- or model animal-specific pluripotent stem cells. In ***Chapter 2***, the generation of iPSC by transcription factor reprogramming is described for fibroblasts of mouse, rat, pig and human origin, respectively.

iPSCs, like ESCs could be subsequently differentiated into any somatic cell type of interest, including cardiac lineage cells. A thorough understanding of the developmental processes that direct pluripotent stem cells towards the cardiac lineage is essential for efficient cardiac differentiation. Identifying which signalling pathways are active or repressed at specific time windows during cardiac development can be used for directed lineage induction and therefore greater cardiomyocyte yield. ***Chapter 3*** describes the results of a systematic screen of cytokines and signalling molecules for their ability to enhance $Nkx2.5^{+}$ cardiac progenitor cells in an ESC differentiation model. Insulin-like growth factors (IGFs) were identified to promote cardiac lineage induction by selective proliferation in early mesodermal lineage cells, which are precursors to the cardiac lineage.

Although these cells could be used for grafting viable myocardial tissue after removal of a patch of scar tissue, this approach becomes problematic in diffuse fibrosis, where large areas of the myocardium are generally affected. Fibroblasts exert detrimental effects in part due to their suboptimal integration into the myocardium. In ***Chapter 4*** these effects were counteracted by forced cellular fusion of human ventricular scar cells (hVSCs) with neonatal rat ventricular myocytes (NRVMs). Resulting heterokaryons derived from fusion of NRVMs and hVSCs showed enhanced repolarization force by increased outward $K_v$ current. This corrected fibrosis-mediated action potential duration (APD) prolongation and occurrence of EADs. In addition, fused cultures showed increased gap junctional coupling between cells, as well as a more negative membrane diastolic potential compared to unfused cultures. These results could provide a framework for understanding fibrosis-mediated arrhythmogenesis and lead to new strategies for its prevention.

Although prevention of cardiac arrhythmia is a holy grail, it is still far removed and cardiac arrhythmias are still everyday reality. Insights into re-entry initiation and dynamics in vulnerable tissue can improve therapeutic strategies, including genetic and cell therapeutic interventions. In ***Chapter 5*** complimentary *in silico* and *in vitro* models of conduction slowing were used to study both quantitatively as well as mechanistically, the process of re-entry induction and stability. Increased conduction slowing due to increased knock-down of Cx43, resulted in occurrence of spatially discordant APD alternans phase islands. Wavebreaks and phase singularities formed at boundaries of adjacent alternans phase islands of opposite phase. Increased re-entry complexity resulted from increased alternans phase islands. These results give new insight into the dynamics of re-entry formation and maintenance in remodelled ventricular tissue.

Once stable re-entry occurs, it can be terminated by pharmacotherapy or application of a biphasic electrical shock. Anti-arrhythmic drugs are often paradoxically associated with pro-arrhythmia, whereas electroshock therapy can cause serious patient discomfort and tissue damage. A more elegant approach is if the myocardium could self-terminate arrhythmic activity, without the need for an external current. A perquisite hereto would be for the cardiomyocytes to generate a current strong enough to meet the defibrillation threshold, and current activation should be highly controllable both spatially as well as temporally. Therefore, in ***Chapter 6*** the re-entry terminating ability of optogenetically engineered atrial tissue was investigated. To this purpose, atrial cardiomyocytes were equiped with a channelrhodopsin, an ion channel normally found in algae, thereby making them light-sensitive and allowing for high spatiotemporal control of depolarization with an intrinsic current.

Finally, ***Chapter 7*** describes the summary and conclusions of this thesis, as well as future perspectives of cellular and genetic interventions for treatment of heart disease.

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