Cellular and Molecular Mechanisms of Arrhythmias in Cardiac Fibrosis and Beyond:
From Symptoms to Substrates towards Solutions

Chapter I

General Introduction

Background

Cardiac disease is a leading cause of morbidity and mortality throughout the world. Strongly represented among these diseases is structural heart disease that disrupts the normally highly organized 3-dimensional cardiac architecture as well as the electrophysiological functioning of the main cardiac functional unit, the cardiomyocyte (CMC). Adequate cardiac function relies on very tight regulation of structural organization and electrophysiology and concomitantly, a significant disruption of these parameters predisposes towards potentially lethal arrhythmias that are a major cause of sudden death. Due to the lethality and burden of arrhythmias, time, effort nor money have been spared to invent and improve therapeutic strategies to prevent and treat cardiac arrhythmias. Anti-arrhythmic drugs were among the first strategies to emerge and more recently, more sophisticated anti-arrhythmic strategies have surfaced, such as implantable defibrillators and radiofrequency catheter ablation. To an extent, these efforts have been successful in reducing the incidence of arrhythmia-related sudden cardiac death by focusing on symptomatic treatment, namely treatment for the arrhythmia itself. However, the incomplete understanding of pro-arrhythmic mechanisms and suboptimal anti-arrhythmic efficacy emphasize the necessity of investigating the underlying disease process, pro-arrhythmic mechanisms and substrate to be able to treat patients more adequately in the future. As cardiac arrhythmias are highly complex phenomena, its components need to be dissected and individually studied to increase our understanding of pro-arrhythmic mechanisms and accordingly, develop novel, more effective anti-arrhythmic strategies. To accomplish such a task, it is important to comprehend the basics of cardio-electrophysiology.

Basics of Cardiac Electrophysiology

For adequate cardiac function, rhythmic contraction and forceful extrusion of blood is achieved through careful structural organization of sequential contraction.[1] Contraction of CMCs is accomplished by a process termed excitation-contraction coupling, and the electrical cascades known as action potentials that are responsible for initiating, coordinating and regulating this process therefore lie at the core of cardiac function.[2]

The action potentials are in turn mediated by voltage-gated ion channels that allow selective passage of their respective an- or cat-ionic currents across the cellular membrane down their electrochemical gradient.[3] [4]The voltage difference across

the cellular membrane is the key regulating element of the conductance of these voltage-gated ion channels and is thereby the driving force of the cardiac action potential. By subtle changes in the trans-membrane voltage, ion channel activation and inactivation ports can rapidly change their confirmation, which alters conductance of the ion channel and subsequently changes ionic currents and the transmembrane voltage.[5] This in turn influences the conductance of other ion channels and their respective ionic currents, giving rise to an electrical cascade known as an action potential. To coordinate CMC contraction throughout the entire heart, action potentials need to be propagated between CMCs through gap-junctions.[6] These gap-junctions consist of hexameres of proteins called connexins that form transmembrane hemichannels (connexons) that connect to connexons of neighboring cells.[7-9]

Such gap-junctions may consist of different subtypes of connexins, of which the expression levels are tissue and site-specific to allow for specific modulation of conduction. In mammalian hearts, ventricles mainly express Cx43 and Cx45, whereas Cx40, Cx43 and Cx45 are mainly found in the atria and conduction system.[10] [11] Each of these subtypes has its own conductance for cat- or anions and low-molecular weight compounds below 1kDa, and responds differently to environmental changes which allows for complex biophysical behavior and modulation of cardiac action potential propagation.[12,13] CMCs are generally connected by gap-junctions in a head-to-tail fashion at intercalated discs that contain a high concentration of proteins involved in intercellular communications. Although CMCs express very high levels of connexins, other cardiac cell types such as fibroblasts also express connexins, albeit in lower quantities.[14,15] Gap-junctions are essential in intercellular communication and apart from action potential propagation, have been shown to be involved in regulation of other biological processes, such as cellular differentiation and embryonic development.[16,17] To overcome the high resistance posed by cellular membranes, gap-junctions form low-resistance intercellular channels that allow passage of ions and small molecules (<1 kDa). The resistance provided by the intercalated discs roughly approximates that of the CMC cytosol.[18] Due to rapid Na^+ influx during excitation, intracellular [Na+] rises and may therefore pass to neighboring, unexcited CMCs cells that are connected by gap-junctions. This passage of Na^+ ions can depolarize the coupled unexcited CMC beyond the excitation threshold and evoke excitation in this cell, thereby realizing action potential propagation.

Through these gap-junctions, diffusion of ions occurs from the activated CMC, e.g. the source, to a coupled, resting CMC, e.g. the sink. As long as the electrical charge delivered by the source exceeds the charge required by the coupled CMC to become excited, propagation will be successful or otherwise stated the safety factor of conduction exceeds 1.[19] For cellular excitability, its capacitance that is mainly determined by cell size, its expression level of ion channels, their functional states as well as the resting membrane potential are crucial determinants. Source-sink mismatch that may cause the safety factor of conduction to fall below 1 at which conduction fails may develop by a myriad of permutations of the above mentioned factors that may exist in the source or sink cell. Without excitability of sink-cells, passive electrotonic propagation is slow and does not reach beyond 300 µm.[20] It is therefore no surprise that sodium channel expression in CMCs is highest near intercalated discs, to ensure that depolarization by Na^+ influx from an adjacent, coupled cell is sensed and responded upon as quickly as possible to maintain fast conduction.[21] Furthermore, while gap-junctional coupling can be reduced by 70% without affecting conduction velocity, even slightly reducing I_{na} slows conduction.[19,22] Beside the implications of a vast gap-junctional reserve, this also emphasizes the importance of excitability in maintaining fast conduction that in turn, is essential for proper sequential cardiac activation and contraction. Inversely, it is well established that slow conduction is a feature that predisposes towards heart rhythm disturbances known as arrhythmias.[6,18]

Arrhythmias

Cardiac electrophysiology is a complex phenomenon organized at the molecular, cellular, 2- and 3-dimensional tissue level. Therefore, alterations at any of these levels can disrupt cardiac electrophysiology and predispose towards arrhythmias. Arrhythmias comprise a wide range of conditions, each of which confers a disturbance of atrial or ventricular contraction rate. Although sinus bradycardia or tachycardia are a cause of morbidity, the most dangerous are the tachyarrhythmias that do not originate from the sinus node and exhibit exceptionally high activation frequencies that preclude diastolic filling of the atria or ventricles, which thereby may cause stroke, hemodynamic instability and sudden cardiac death. These tachyarrhythmias can be based on multiple patterns of activation, being focal, reentrant or fibrillatory.[23]

Focal tachyarrhythmias

Focal tachyarrhythmias are based on an ectopic focus or multiple foci that overrule the sinoatrial node and dominantly activate the myocardium. Clinically, the life-threatening "Torsade des Pointes" is a prime example of such an arrhythmia. A proposed mechanism of these arrhythmias is based on early afterdepolarizations (EADs). [24,25]

An EAD is reactivation of depolarizing force that may occur during phase 2 and phase 3 repolarization of the action potential and represents a potentially detrimental electrophysiological phenomenon.[24,25] Phase-2 EADs are considered to be based on reactivation of L-type calcium channels due to slow repolarization that allows the channels to de-inactivate and subsequently reactivate as the membrane voltage lingers within the so-called "window current".[26] If the criteria for a balance between de-inactivation and activation are met, this can lead to perpetual oscillations in membrane voltage and lead to a sustained focal tachyarrhythmia. Phase-3 EADs depend on Nav1.5-based mechanisms of reactivation and may play a role in EADs that are evoked by structural heterogeneity.[27] Since EADs typically occur before full repolarization, excitability is not fully recovered and therefore conduction is slowed. Moreover, apart from the decreased depolarizing force supplied by an EAD compared to normal action potentials, the downstream myocardium (sink) typically has not fully repolarized and therefore may be more difficult to excite. These changes in source and sink during EADs may therefore lead to a source-sink mismatch which may cause propagation to fail if these EADs are not generated in a synchronous fashion by sufficient amounts of CMCs. [28,29]

Thereby, heterogeneity of repolarization time throughout the cardiac tissue causes zones of conduction block, while other sites are able to propagate the EAD due to more favorable source-sink relationships because of shorter refractory periods that even in healthy hearts differ between different zones of myocardium.[30,31] This increased dispersion of repolarization is a feature that strongly predisposes towards arrhythmias.[32] As a result of increased dispersion of repolarization, unidirectional conduction block may develop, which forces activation in one direction around a refractory area that now functions as a non-conducting obstacle. If repolarization of the refractory area occurs while the activating wave front moves around this area, such critical timing would allow for the initiation of circular or reentrant activation, in which the activating front would chase its repolarizing tail.[18]

Reentrant tachyarrhythmias

Reentrant excitation is a long known phenomenon that is highly relevant to cardiac electrophysiology, as reentry is an often observed propagation pattern of tachyarrhythmias.[33] During reentry, there is a constant spatial interaction between depolarization and repolarization that is crucial for the sustainability, stability and cycle length of the arrhythmia. Although reentry was first considered to mostly rely on anatomic pathways that facilitate circular conduction such as post-myocardial infarction scars or around the pulmonary veins,[34,35] reentry was also shown to be able to exist without anatomical obstructions, giving rise to the term "functional" reentry.[36] It was later shown that functional reentry takes the form of an Archimedean spiral that continuously depolarizes the cardiac tissue.[37] The continuous depolarization is maximal at the pivot point of reentry, or core that is therefore nonexcitable[38], and decreases towards the periphery.[39,40] Since functional reentry does not rely on anatomical fixation, such reentry may meander throughout the tissue which gives rise to polymorphic electrograms.[41] Inversely, anatomical reentry is mostly considered as a highly stable form of reentry that manifests itself with a monomorphic appearance on electrograms. Although it is tempting to view functional and anatomic reentry as separate phenomena, it is becoming increasingly clear that both phenomena exist at the edges of a spectrum of reentrant arrhythmias that may often exhibit features of both reentry types. This is illustrated by the observation that functional reentrant arrhythmias can pin to anatomic obstructions.[42]

Further adding to the complexity of reentrant arrhythmias is the possible presence of multiple rotors during arrhythmias. Moreover, focal and reentrant arrhythmias can occur simultaneously during the most complex arrhythmia type known as fibrillation. During fibrillation, complex interactions between multiple activating wave fronts cause a polymorphic aspect of electrograms. But despite the seemingly chaotic nature of fibrillation, its organization is still based on spatial and temporal availability of the electrophysiological function cardiac tissue.[43]As a result, slow conduction and refractory state of cardiac tissue are key elements that modulate the time windows for multiple activation fronts to interact and predispose towards reentrant arrhythmias.[44] Therefore, these parameters are often targeted in anti-arrhythmic strategies.

Current treatment of arrhythmias

The primary tool against arrhythmias has been symptomatic treatment by pharmacological modulation of ion channel function. Most of these anti-arrhythmic drugs exhibit an ion-channel blocking function and have been categorized according to the Singh Vaughan Williams classification.[45] These agents can block Na^+ channels, K^+ and Ca^{2+} channels, which slows conduction, prolongs refractory periods or shortens refractory periods, respectively. Interestingly, despite a predictably pro-arrhythmic effect of conduction slowing by Na^+ channel blockade, anti-arrhythmic effects of such pharmacological interventions can be observed, which may be explained by attenuation of phase-3 EADs by blockade of the late sodium current. [46,47] Moreover, sodium channel blockade may induce drift of reentrant tachyarrhythmias which may terminate these arrhythmias should the arrhythmia encounter unexcitable borders of cardiac tissue. [42,48] Potassium channel is known to prolong the refractory period of cardiac tissue and thereby decrease the propensity towards reentrant arrhythmias as reentry needs a refractory period that is short enough to favor reentrant excitation.[18,49] However, prolongation of action potential duration can be pro-arrhythmic by predisposing towards EADs and increased complexity of reentrant arrhythmias.[50,51] Blockade of L-type calcium channels was also reported to yield anti-arrhythmic effects in context of acute myocardial infarctions,[52] but its effects on vascular tone and inotropy make it an undesirable agent for a large portion of patients with pro-arrhythmic substrates, that often also suffer from mechanical cardiac dysfunction and are prone to become hemodynamically comprised.[53] As all of these pharmacological agents can have either anti- or pro-arrhythmic effects, it is unfortunately unsurprising that their therapeutic efficacy at preventing sudden cardiac death is disappointingly low.[54] Such results may be explained by our incomplete understanding of pro-arrhythmic mechanisms, and provided incentive to invent different anti-arrhythmic interventions, such as the Implantable Cardioverter-Defibrillator (ICD). This implanted device monitors heart rhythm and rate and delivers electrical shocks or anti-tachy pacing to terminate arrhythmias should it detect abnormal heart rates. While effective at preventing sudden cardiac death, it constitutes a purely symptomatic treatment that does not positively affect the underlying pro-arrhythmic mechanisms. [55,56] Moreover, shocks are experienced as very stressful by patients and inappropriate shocks remain an issue with potentially lethal effects.[57,58] In addition, recurrent ventricular arrhythmias remain a problem.[59] Therefore, a technique that aims to more specifically target the underlying pro-arrhythmic substrate instead of symptoms known as radio-frequency catheter ablation was developed. During such a procedure, the aim is to damage pro-

arrhythmic tissue and prevent recurrence of arrhythmias.[60] This technique has very strong anti-arrhythmic potential, especially in arrhythmias with a clear anatomical substrate such as Wolff-Parkinson-White syndrome or post myocardial infarction.[61,62] However, high success rates are often achieved by multiple procedures.[63] Moreover, due to the invasiveness of the technique it is prone to serious complications such as cardiac perforation.[64] Although radiofrequency catheter ablation selectively targets pro-arrhythmic tissue and thereby positively affects the underlying pro-arrhythmic substrate, it does not selectively target underlying pro-arrhythmic mechanisms. By furthering our understanding of pro-arrhythmic mechanisms of underlying substrates, we may be able to increase therapeutic efficacy even further without resorting to damaging cardiac tissue to stop and prevent arrhythmias.

Pro-arrhythmic substrates as anti-arrhythmic targets

As adequate cardiac electrophysiology relies on structural integrity and optimal ion channel functionality of CMCs, it is easily conceivable that a change in any of these parameters have pro-arrhythmic consequences. The most clearly identifiable pro-arrhythmic substrates of cardiac arrhythmias lie in the domain of the genetically inherited channelopathies, which are based on mutations in ion channel genes that affect the functionality of the associated translated protein.[65] As there are a myriad of ion channel genes, the patient population affected by these channelopathies is highly heterogeneous and may require different treatments depending on the particular mutation.[66] However, most arrhythmia-prone patients do not suffer from such a clear etiology of pro-arrhythmia, as a substantially larger group of patients suffer from arrhythmias due to genetic or acquired structural cardiac disease.[67] Cardiac hypertrophy, the adaptive response of CMCs to increase their size and number of sarcomeres to compensate altered pressure and strain profiles, is not widely associated with arrhythmias if hypertrophy is adequate and physiologic.[68] However, this adaptive response may become maladaptive or pathological hypertrophy, which is marked by re-initiated activity of the fetal gene program and predisposition for arrhythmias.[69-72] Pathological hypertrophy is the result of a response that fails to properly compensate the change in cell size with increased ion channel expression and other necessary electrophysiological alterations and can result in altered CMC excitability and action potential characteristics. [73-75] In addition, changes in connexin expression levels and distributions from head-to-tail connections at intercalated discs to a more side-to-side distribution can significantly affect conduction.[76,77] Moreover, hypertrophic CMCs have been shown

to be vulnerable to formation of EADs.[78] As a result, electrical remodeling that occurs in structural cardiac disease predisposes to cardiac arrhythmias, although its exact pro-arrhythmic mechanisms are still unclear. [79] Therefore, targeting mechanisms to prevent or reverse hypertrophy may prove to also be an anti-arrhythmic strategy.[80]

A factor that further complicates our understanding of the pro-arrhythmic mechanisms of pathological hypertrophy is the often coinciding fibrosis, which consists of an increase in the amount of fibroblasts and excessive extracellular matrix deposition. Fibrosis is a healing process that occurs in response to injury and is strongly associated with arrhythmias.[81-84] Since cellular proliferation and differentiation are opposite phenomena in biology, the highly differentiated phenotype of CMCs precludes any adequate amount of proliferation of these cells, even in response to injury.[85,86] When injurious stimuli occur, fibroblasts switch their phenotype to the phenotype of myofibroblast (MFB), a proliferative and secretory cell type. These injurious stimuli can be of chemical or mechanical nature. In normal hearts, mechanical stimuli do not reach the fibroblasts that are interspersed in the stable matrix network. However, disruption of the structural integrity of the heart, strong immune response or altered myocardial strain exposes the fibroblasts to mechanical or chemical stress that can activate them to become MFBs.[82,87] Then, MFBs secrete increased amounts of ECM to compensate the mechanical stress or loss of myocardium. As a result, loss of CMCs or CMC functionality is compensated by fibrosis to maintain structural integrity to such a degree that it even prevents cardiac rupture after myocardial infarction.[88] While beneficial at first, the increased thickness of the collagen fibers effectively separate cell-cell contacts between cardiomyocytes and may therefore disrupt slow or even block conduction by increasing intercellular resistance. Indeed, the increased matrix deposition in fibrosis has been shown to be cause pro-arrhythmic conduction slowing through zigzag courses that force conduction along a longer path of activation, thereby increasing myocardial activation times and increasing the propensity towards arrhythmias.[89] Moreover, interspersion of fibroblasts between CMCs may also contribute to anatomical mechanisms of conduction slowing.[90] In addition to the anatomically based pro-arrhythmic mechanisms of fibrosis, recent evidence suggests that the phenotypical switch to MFBs may cause them to modulate CMC electrophysiology more directly.[91] More specifically, this is suggested to be mediated through heterocellular gap-junctional coupling that may slow conduction, induce ectopic activity and predispose towards reentrant arrhythmias. [20,92-94] Paracrine-[95] and mechanical coupling mediated[96,97] pro-

arrhythmic mechanisms of MFBs further complicate the pro-arrhythmic substrate of fibrosis. More importantly, this complexity hampers our understanding and the development of more effective treatment strategies against fibrosis-associated arrhythmias. Therefore, investigation and targeting of substrate-specific pro-arrhythmic mechanisms may provide a powerful future anti-arrhythmic strategy against prevalent fibrosis-associated arrhythmias.

Aim and Outline of Thesis

To be able to more effectively treat arrhythmias, it is essential to comprehend the complexity of cardiac electrophysiology and its relation to cardiac structure as discussed in **Chapter I**.

Therefore, the aim of this thesis was to develop and utilize *in vitro* models of pro-arrhythmic substrates to investigate their pro-arrhythmic mechanisms to provide a rationale for future substrate-oriented anti-arrhythmic and preventive strategies.

In cardiac fibrosis, a dramatic increase in MFBs by proliferation occurs. As MFBs are key players in highly pro-arrhythmic fibrosis, limiting their numbers may counteract pro-arrhythmic effects of fibrosis. Therefore, it is tested in **Chapter II** whether anti-proliferative treatment of MFBs may confer anti-arrhythmic effects in an *in vitro* model of fibrosis with proliferating fibroblasts in cardiac cultures.

After attainment of the MFBs phenotype, these cells express relatively high amounts of Cx43.[91] In addition, MFBs have been shown to be pro-arrhythmic in cardiac cultures and have been demonstrated to electrically couple to CMCs. **Chapter III** is therefore an investigation of the anti-arrhythmic effects of Cx43 down regulation in MFBs.

Cardiac pathophysiology is a complex agglomerate of several processes that try to compensate structural or functional changes of the myocardium. As such, cardiac fibrosis and hypertrophy often coincide. As a result, no distinction can be made between fibrosis- and hypertrophy specific mechanisms. Therefore, **Chapter IV** set out to investigate differences between pro-arrhythmic mechanisms of fibrosis and of hypertrophy, and how this may influence anti-arrhythmic strategies.

Adequate pharmacological treatment of arrhythmias remains difficult due to its high complexity and potential lack of specificity of the drugs. Although action potential duration is a common parameter to increase to terminate reentrant arrhythmias, its efficacy may provide contradictory results. Therefore, **Chapter V** aimed to investigate the effect of the minimal action potential duration in an *in vitro* and *ex vivo* model of fibrillation and how it can be utilized to destabilize and terminate reentrant arrhythmias using a wide arrangement of pharmacological agents.

Cardiac fibrosis is a process that is initiated after loss of CMCs, due to their poor proliferative capability. Therefore, regenerative medicine has focused on replenishing these lost CMCs or enhancing survival and cardiac function with an external cell source. Although mesenchymal stem cells (MSCs) show limited capability to differentiate towards CMCs, their therapeutic use in cardiac fibrosis is gaining increasing attention and more focus is placed on increasing the amount of transplanted MSCs to maximize their therapeutic potential. However, due to the complexity involved in transplanting and integrating stem cells into the heart, potentially pro-arrhythmic consequences may arise by increasing the number of MSCs transplanted. Therefore, **Chapter VI** set out to investigate the pro-arrhythmic effects and mechanisms of MSC engraftment pattern and amount.

In **Chapter VII**, a novel method to limit fibroblast arrhythmogeneity was investigated. As fibroblasts may be pro-arrhythmic by several mechanisms, anti-arrhythmic reprogramming was attempted by forcing heterocellular fusion between human ventricular scar fibroblasts and neonatal rat cardiomyocytes.

To selectively target cell-type specific pro-arrhythmic mechanisms is difficult without also unintentionally modifying other cardiac cell types. Therefore, adeno-associated-viral vectors were developed that selectively force transgene expression in either the cardiomyocyte or the fibroblast in **Chapter VIII**.

In conclusion, **Chapter IX** summarizes the findings of this thesis. Moreover, results are discussed and placed in future perspectives.

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