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GENE AND CELL THERAPIES
IN THE TREATMENT OF BRADY-
AND TACHYARRHYTHMIAS

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ABSTRACT

Morbidity and mortality of cardiac arrhythmias are major international health concerns. Drug and device therapies have made inroads, but because of their shortcomings alternative approaches are being sought. For example, gene and cell therapies have been explored for treatment of brady- and tachyarrhythmias, and proof-of-concept has been obtained for both biological pacing in the setting of heart block and gene therapy for treating ventricular tachycardias. This chapter discusses the state-of-the-art developments with regards to gene and cell therapies for cardiac arrhythmias. In doing so, it will provide a general introduction to this thesis.
INTRODUCTION

Cardiac arrhythmias are a major burden on society in developed countries. Ventricular tachycardia/ventricular fibrillation (VT/VF) are the predominant causes of cardiac arrest, accounting for approximately 340,000 deaths annually in the United States alone.\(^1\) Anti-arrhythmic drugs have largely failed to reduce mortality.\(^2\) Radiofrequency catheter ablation has provided a more targeted approach, but success rates are moderate and recurrence rates high. Therefore, only implantable cardioverter/defibrillators provide a reliable safeguard to otherwise lethal arrhythmias. However, these devices are associated with significant morbidity as they terminate rather than prevent arrhythmias, and they are a physical and psychological burden to many patients.\(^2\)

The slow heart rates that characterize bradyarrhythmias necessitate nearly 200,000 electronic pacemaker implantations annually in the United States.\(^3\) Although such pacemakers are mature therapies, important limitations remain, including an inadequate response to autonomic modulation, and impulse initiation from non-physiological sites that may induce significant cardiac remodeling.\(^4, 5\) Furthermore, they are inappropriate for many pediatric patients, and up to 5% of all pacemaker implantations result in serious complications that require surgical or endovascular revision.\(^6\) Hence, in the fields of both brady- and tachyarrhythmias, there is a pressing need to improve currently available therapies.

The inadequacy of conventional therapies to treat arrhythmias stems in part from problems related to targeting therapies to the appropriate sites within the heart. For example, electronic pacemakers are typically implanted in the right ventricular apex at sites that optimize stable lead position, but these sites induce a non-physiological contraction pattern of the cardiac muscle, which may result in adverse remodeling of the heart. Different pacing sites (including His, para-His and the right ventricular outflow tract) to generate a more physiological contraction wave have been explored for years. Yet, these approaches are still hampered by difficult placement procedures, unstable lead positioning and higher risks for complications. Pacing from the right ventricular apex has therefore remained the standard method of care.\(^7\) On the other hand, antiarrhythmic drugs typically target ion channels that are expressed throughout the heart. Such an approach lacks specific targeting to the pathophysiologic substrate and has been complicated by proarrhythmic side-effects. Gene and cell therapy may provide important improvements because virtually every site within the heart is readily accessible for construct injection. Furthermore, in contrast to approaches that use radiofrequency (RF) catheter ablation to locally destroy arrhythmogenic areas, gene and cell therapy have the potential to prevent arrhythmias without doing further damage, and they may even regenerate cardiac muscle.

To better understand the rationale behind the various molecular therapies proposed for cardiac arrhythmias, I begin this chapter by discussing the basic concepts of cardiac rhythm and the various tools available for gene and cell therapy. Next, I summarize achievements in the area of arrhythmia research. Finally, I will address the major goals in this thesis.
BASIC CONCEPTS IN THE GENERATION AND MAINTENANCE OF CARDIAC RHYTHM

In the right atrium, close to the entry site of the superior vena cava, a small group of cells present in the sinoatrial node (SAN) depolarizes spontaneously providing a nidus of impulse initiation to pace the heart (Figure 1A). Activation spreads from the SAN through the atria and activates the atrioventricular node (AVN), where impulse propagation is delayed. This delay allows the ventricle to fill during atrial contraction. From the AVN, activation proceeds rapidly through the His-Purkinje system to activate ventricular myocardium.

The spontaneous action potentials generated in the SAN drive the heart throughout life in healthy individuals with only rare moments of failure. In the diseased heart, however, bradycardia may occur due to SAN dysfunction or due to failure of impulse propagation from atria to ventricle. In these instances, residual pacemaker activity in the AVN and the distal conducting system may be revealed, as these tissues have intrinsically slower firing frequencies than the SAN. Although these secondary pacemakers maintain a cardiac rhythm, in most instances the bradycardia which develops requires implantation of an electronic pacemaker. To better understand the electrical phenomena behind cardiac impulse initiation and propagation, major processes in cellular electrophysiology will be discussed.

The greatest electrophysiological differences among cardiac myocyte types are those between SAN and ventricular cells (Figure 1B). In SAN cells, spontaneous action potentials (APs) are generated by slow diastolic depolarization. Important contributors to this process include the hyperpolarization activated cyclic nucleotide-gated (HCN) channels encoding the pacemaker current (“funny” current, $I_f$) and the T-type Ca\textsuperscript{2+}-channels. A more complete discussion on the various mechanisms contributing to SAN pacemaking is provided below. When the threshold for activation of voltage-gated L-type Ca\textsuperscript{2+} channel opening is reached, phase 0 depolarization commences which initiates the regenerative action potential in the SAN cell and is a stimulus for propagation of impulses to neighboring cells. Impulse propagation from a cell to its neighbors primarily occurs through low-resistance intercellular connections provided by gap-junction channels. Following depolarization of the membrane, voltage-gated K\textsuperscript{+} channels open and generate an outward current that repolarizes the cell to its maximal diastolic potential (MDP; Figure 1C).

Voltage-gated Na\textsuperscript{+}-channels (primarily encoded by the α-subunit SCN5A) are the predominant driver of phase 0 membrane depolarization in cells of the working myocardium. Following the AP notch (caused by initial repolarization carried by the transient outward current $I_O$), ventricular myocytes manifest a plateau phase during which inward (Ca\textsuperscript{2+}) and outward (K\textsuperscript{+}) currents are largely in balance. Next, membrane repolarization is driven by voltage-gated K\textsuperscript{+} channels (Figure 1D).

There are a number of additional differences between SAN and ventricular myocytes: 1) The inward rectifier current ($I_{K1}$) in ventricle generates a more hyperpolarized and stable resting membrane potential than does SAN, in which $I_{K1}$ is virtually absent.
Figure 1. Basic properties in cardiac electrophysiology. A, Schematic drawing of cardiac anatomy and its functional relation with the electrocardiogram (ECG). B, Differences in action potential morphology in cells isolated from sinus node, atrium and ventricle and their relationship to the ECG. C-D, Schematic drawing of the action potential shape and underlying currents of in sinus node (C) and ventricular myocyte (D). Modified from reference 155, with permission.
This difference primarily stems from the higher expression levels of the Kir2 family of genes encoding $I_{K_1}$ in ventricle compared to SAN. The strong $I_{K_1}$ in ventricle clamps the membrane at a negative potential and limits the membrane depolarization that would be induced by inward currents.\textsuperscript{11-13} 2) $I_f$ current density in ventricular cells is much smaller than in cells of the SAN. This difference is the result from both differences in expression levels of HCN channels and differences in activation kinetics of $I_f$. In SAN cells, HCN expression levels are much higher and $I_f$ activates at more positive potentials than in ventricular cells. The positively shifted activation kinetics stem in part from the elevated baseline cAMP levels in SAN versus ventricle, but other contextual factors contribute as well, e.g., the β-subunit minK-related protein.\textsuperscript{1,14} 3) $I_{Na}$ only minimally contributes to phase 0 depolarization of SAN cells, as the depolarized membrane potentials in SAN cells inactivate the voltage dependent $I_{Na}$. The low expression levels of SCN5A in central SAN cells further increase this difference with ventricular myocytes.\textsuperscript{10} 4) Intracellular Ca\textsuperscript{2+}-handling also differs. In SAN cells, spontaneous local Ca\textsuperscript{2+} release events importantly contribute to spontaneous activity. In ventricular myocytes, these local release events occur randomly and generally do not generate spontaneous activity.\textsuperscript{15} Furthermore, the intracellular Ca\textsuperscript{2+}-apparatus in ventricular myocytes is linked to a well-developed sarcoplasmic reticulum (SR), which supports contraction. In SAN cells, this apparatus contributes to pacemaking, but is underdeveloped with regards to the generation of contractile force.\textsuperscript{16}

Other cardiac myocytes have phenotypes that are more or less in between the extremes of a SAN and a ventricular cell. As compared to their ventricular counterparts, atrial myocytes harbor fewer $I_{K_1}$ channels, but still have a hyperpolarized resting membrane potential and lack spontaneous activity. Cells within the specialized conduction system harbor slow spontaneous activity that gradually decreases in the proximal to distal direction while the membrane remains relatively hyperpolarized.\textsuperscript{17} Finally, a key difference between SAN and Purkinje cells is that the latter harbor abundant Na\textsuperscript{+}-channels and connexins. These differences facilitate the rapid conduction that characterizes the ventricular conduction system.\textsuperscript{10,18}

**Endogenous pacemaker function**

Decades of research have fueled our knowledge of mechanisms contributing to slow diastolic depolarization and pacemaker function within the SAN. Ever since the discovery of HCN channels and the $I_f$ current which they generate,\textsuperscript{19,20} high importance has been assigned to their role in SAN function.\textsuperscript{21,22} Indeed, these channels are predominantly expressed in the pacemaker regions of the heart\textsuperscript{23} and their biophysical properties predict the generation of an inward current that flows during diastole as these channels open upon hyperpolarization.\textsuperscript{19,20} Furthermore, HCN channels can bind cAMP, thus facilitating direct autonomic modulation.\textsuperscript{24}

The notion that $I_f$ is the unique determinant of pacemaker function (as shown in Figure 1C) has been challenged by the demonstration of a coupled-clock system.\textsuperscript{25,26} Ca\textsuperscript{2+} is cycled in a process of SR release and reuptake (designated “Ca\textsuperscript{2+}-clock”), which
in turn facilitates membrane depolarization via a variety of ion channels and exchangers in which the Na+/Ca2+-exchanger plays a significant role. Although there is agreement that both If- and the Ca2+-clock contribute to SAN activity, ongoing disagreement exists on the relative contributions of these systems and whether one of these systems can be considered primary rather than secondary to the other. This ongoing discussion is particularly fueled by dissimilar outcomes in different species (e.g., Ca2+-clock based mechanisms appear more important in guinea pig than in canine SAN), in different modes of pacemaking (i.e., in the absence or presence of autonomic stimulation/inhibition), and in different pacemaker tissues (e.g., If-based pacemaking appears to be predominant in Purkinje fibers). In addition, If and Ca2+-clock based pacemaker mechanisms may in fact be highly interlinked. Evidence in favor of this hypothesis was recently provided by Yaniv and colleagues in experiments on isolated rabbit SAN cells. Here, they found 3 μM ivabradine to specifically block If without having direct effects on other membrane currents or Ca2+ cycling. However, indirect effects associated with the reduced AP firing rate in the presence of ivabradine did appear
to orchestrate reduced Ca\(^{2+}\) handling and prolongation of the period of spontaneous local Ca\(^{2+}\) releases.\(^2\)

The persistent function of the SAN under a large variety of pathological and experimental conditions suggests significant redundancy within the contributing systems. Indeed, regardless of which system is considered as primary, it is the interaction among mechanisms that contributes to the final pacemaker outcome. These mechanisms involve regulatory elements executed by G proteins and cyclic nucleotides, exchangers (the Na\(^+\)/K\(^+\) exchanger is particularly important in secondary pacemakers) and several ion channels including the L- and T-type Ca\(^{2+}\)-channels, Na\(^+\)-channels and K\(^+\)-channels.\(^3\)

The involvement of Na\(^+\)-channels largely depends on a hyperpolarized membrane potential. These channels critically contribute to the excitation of the SAN periphery, the atrial and ventricular muscle and the His-Purkinje system.\(^3\) In the more depolarized cells of the central SAN and AVN, Ca\(^{2+}\)-channels are the primary charge carriers for membrane excitation (Figure 1C).

The involvement of K\(^+\)-channels is complex as reductions in outward current during diastole will facilitate phase 4 depolarization. However, increases in outward current during phases 2 and 3 of the action potential will accelerate repolarization; a resultant shorter action potential duration in the setting of a constant phase 4 depolarization slope has the potential to increase the rate of impulse initiation. The most important processes contributing to SAN pacemaking are summarized in Figure 2.

A final key element to cardiac pacing is the electrical coupling between the SAN and surrounding atrial cells. Appropriate cell-to-cell coupling is important, because too-weak coupling will result in failure to excite adjacent atrial cells, while too-strong coupling would transmit too much hyperpolarizing current from atrial cells, which would tend to silence pacemaker activity.\(^3\) Protecting the SAN from hyperpolarizing effects of surrounding atrial cells is provided by its partial encapsulation with connective tissue and blood vessels, limiting the number of exit pathways.\(^3\) Furthermore, absence of the fast-conducting connexins Cx40 and Cx43 within the SAN together with presence of the more slowly conducting connexins Cx45 and Cx30.2 (mice) or Cx31.9 (human) contribute to a lesser degree of cell-cell coupling within the node and at the interface with atrial myocardium.\(^3\)

**Mechanisms underlying tachyarrhythmias**

In general, three mechanisms are considered important in the pathophysiology of tachyarrhythmias: 1) reentry, 2) triggered activity, and 3) abnormal automaticity. Reentry is the leading cause of arrhythmias complicating ischemic heart disease.\(^4\) The occurrence of reentry is determined at the tissue level, where functional barriers (e.g., tissue that is coupled electrically with cardiomyocytes, but unexcitable such as fibroblasts, or myocardial tissue that remains unexcited at certain heart rates or activation patterns) or structural barriers (e.g., infarct scar) may cause the activation wavefront to persist in a circular pattern. Further requirements for reentry include the
Figure 3. Mechanisms of cardiac arrhythmias. A, Upper left panel shows normal impulse propagation under healthy conditions. Upper right panel shows moderate conduction slowing as indicated by the crowding of isochrones around the black bar that represents myocardial tissue damage. For reentry to occur, another prerequisite is the occurrence of unidirectional conduction block represented by the black jagged line in the lower left panel. The subsequent lower panels show the initiation and maintenance of a tachycardia circling around the area of myocardial damage. Modified from reference 157, with permission. B, Early afterdepolarizations in cardiac Purkinje fibers. In the left panel, the solid line represents a normal action potential (AP), the dashed line (arrow) represents AP prolongation that can predispose to single afterdepolarizations (EADs, arrow; middle panel) or a train of EADs (arrow; right panel). C, Left panel, stimulated APs followed by subthreshold delayed afterdepolarizations (DAD; arrow). Right panel, when the stimulation cycle length is shortened, DAD amplitude increases to reach threshold and induce triggered activity (arrow). In B and C, each trace shows a drawing based on original recordings. Modified from reference 38, with permission.
occurrence of unidirectional conduction block in combination with a path length and speed of conduction that allow for recovery of excitability before the circulating wave front returns (Figure 3A). Important determinants for the speed of impulse propagation include the availability and biophysical characteristics of the voltage-gated Na\(^+\) and Ca\(^{2+}\)-channels, availability and distribution of gap-junctions and the presence of fibrosis (excess of collagen). Recovery of excitability on the other hand is primarily determined by the duration of repolarization; hence, voltage-gated Na\(^+\), Ca\(^{2+}\), and K\(^+\) channels are also involved.

Triggered arrhythmias may develop either based on early afterdepolarizations (EADs) or delayed afterdepolarizations (DADs).\(^3^5\) During an EAD, depolarization reinitiates before AP repolarization has completed. The occurrence of EADs is therefore facilitated by factors that prolong repolarization (e.g., electrolyte imbalance, inherited ion channelopathies, and slow heart rates; Figure 3B). In addition, increased availability of Na\(^+\) and Ca\(^{2+}\)-channels may also contribute to the occurrence of EADs as these channels contribute inward current to membrane depolarization.\(^3^6\), \(^3^7\) DADs, on the other hand, occur after cellular repolarization has completed. Here, elevations in cytosolic Ca\(^{2+}\) activate the electrogenic Na\(^+\)/Ca\(^{2+}\)-exchanger (NCX), which carries net inward current that drives membrane depolarization. Calcium leakage from the SR Ca\(^{2+}\) stores, such as occurs during heart failure, may importantly contribute to increases in cytosolic Ca\(^{2+}\) and the occurrence of DADs.\(^3^8\) Although EADs and DADs are cellular phenomena, in the intact heart they need to acquire sufficient cellular mass to allow for impulse propagation to surrounding tissue. Similar to the spread of pacemaker activity, partial uncoupling (e.g., due to fibrosis or connexin heterogeneities) may importantly contribute to the propagation of triggered activity.

A final arrhythmia mechanism to consider is abnormal automaticity. In contrast to enhanced normal automaticity, which may arise from (close to) normal levels of membrane potential, abnormal automaticity is initiated from a reduced membrane potential.\(^3^9\) When the resting potential of atrial or ventricular myocardial cells is reduced to less than about –60 mV, spontaneous diastolic depolarization may occur. Channels that generate inward current or reductions in outward current may depolarize the membrane until threshold for action potential initiation. Underlying conditions such as heart failure and ischemic heart disease are associated with a loss of membrane potential and the occurrence of abnormal automaticity.\(^4^0\), \(^4^1\)

**AVAILABLE GENE AND CELL THERAPY TOOLS**

The field of regenerative therapy as applied to arrhythmias typically includes three of the following components: 1) a target for modifying electrical activity; 2) the vector or cell used to deliver the therapy, and 3) the genetic component selected as modifier of electrical activity.\(^4^2\)-\(^4^4\) Alternatively, pure stem cell-based approaches may be used to regenerate the cardiac muscle after myocardial infarction and in doing so prevent arrhythmias. The following paragraphs will outline the most important gene and stem cell therapy systems currently available.
Gene therapy

The general concept of gene therapy is that designated vehicles are used to deliver genetic information to specific target cells. In the heart, such targeting may be achieved by injecting vectors into specific areas or infusing vectors via the coronary vasculature. In these settings, viral vectors are typically used for direct myocardial gene transfer. Adenoviral vectors are useful candidates because they are easily produced, transduce myocardium efficiently, and have a large insert capacity allowing for the investigation of a broad range of genetic targets. A downside of this system is that gene expression only persists over a period of weeks due to the fact that the gene is not integrated into the host genome and the limited stability of gene expression due to the inflammatory response to adenoviral gene products.45, 46 This makes these vectors suitable for proof-of-principle studies, but their clinical value is limited. Long-term myocardial gene transfer is typically generated by adeno-associated viral vectors (AAV). Because all the viral genes have been removed from this vector, they only induce a minimal inflammatory response, and myocardial gene transfer has been shown to persist for up to 12 months.47 In addition, these vectors have been shown to be safe while being tested clinically in patients with failing hearts.48, 49 AAV therefore provides the first choice platform when developing novel gene therapies for the heart. However, because AAV-based gene transfer remains largely episomal, it is uncertain how expression will be maintained over the longer term (>years). In addition, a significant downside of AAV is the limited insert capacity.50 Consequently, alternatives such as lentiviral vectors are actively being explored. Lentiviral vectors are derived from the human immunodeficiency virus and, similar to this virus, they integrate into

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<th>Major area of application</th>
<th>Adenovirus</th>
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<th>Lentivirus</th>
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<tr>
<td>Viral Genome</td>
<td>dsDNA</td>
<td>ss or ds DNA</td>
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<tr>
<td>Cloning capacity</td>
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<td>Vector genome</td>
<td>episomal</td>
<td>~90% episomal &amp; ~10 integrated</td>
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Figure 4. Major gene therapy vehicles used in cardiovascular applications. A, Scaled cartoons of viruses. From left to right: particles of adenovirus, adeno-associated virus (AAV) and lentivirus. B, Summary of important properties of these viral vectors. ds, double strand; ss, single strand.
the host genome.\textsuperscript{51} This probably has the best potential for very long-term gene expression, but gene integration also comes with safety concerns regarding insertional mutagenesis. Because of these concerns and a more laborious production process, lentiviral vectors are now primarily used clinically for the \textit{ex vivo} modification of stem cells.\textsuperscript{52} Yet, the first clinical trial using lentiviral vectors for direct gene transfer into brain tissue has been initiated\textsuperscript{53, 54} and it appears likely that, over time, these studies will extend to other organs including the heart. Properties of the most important viral gene therapy vectors are further summarized in Figure 4.

\textbf{Cell therapy}

Myocardial regeneration and repair have been goals of scientists for many years (Figure 5).\textsuperscript{55-57} Research using stem cells to achieve this end has been performed extensively in the clinic and has shown some improvement in function, but often in terms of statistical significance rather than physiological effectiveness.\textsuperscript{58} Nonetheless, it has been established that the administration of autologous human stem cells, including cardiac progenitors, is safe.\textsuperscript{59-61} Additional data indicate safety for delivery of human mesenchymal stem cells administered allogeneically.\textsuperscript{62, 63}

Among the various types of stem cells, it is important to make a distinction between cells that are applied undifferentiated and those that are differentiated towards a cardiac myocyte phenotype before application. The undifferentiated cells may be obtained from various sources including bone marrow, adipose tissue and myocardium, with the latter source generating the so-called cardiac progenitors. In the classical approaches aimed at myocardial repair after infarction, the undifferentiated cells isolated from bone marrow have been shown to impact positively on cardiac function despite low engraftment and minimal potential for the formation of new cardiac myocytes. The predominant mechanism of action therefore appears to involve the secretion of paracrine factors that mobilize endogenous stem cells for repair (Figure 6A).\textsuperscript{64, 65}

In designing specific arrhythmia treatments, adult mesenchymal stem cells have also been loaded with ion channels to provide an alternative delivery vehicle for ion channel function. Many of the undifferentiated cells express cardiac connexins and couple with cardiac myocytes via the formation of gap junctions. In so doing they provide an alternative vehicle for functional delivery of the ion currents. In this respect, mesenchymal stem cells (MSCs) have been most extensively studied for the delivery of HCN2-based pacemaker function (Figure 6B).\textsuperscript{66-68}

Differentiated stem cells have also been considered both for direct myocardial repair and the induction of pacemaker function (Figure 6C-D). Important examples of this approach include the use of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPS). Both cell types are pluripotent, meaning that they can differentiate into virtually every cell of the human body (including cardiac myocytes and pacemaker cells), however their source of origin is remarkably different. As implied by their name, ESCs are obtained from embryonic tissue which brings significant drawbacks
The iPS cells, on the other hand, may be obtained from various adult tissue sources and therefore have the potential of autologous application. However, iPS cells come with other issues that relate to their method of production. Originally, Takahashi and Yamanaka used retroviral vectors to deliver the cocktail of reprogramming factors (KLF4, OCT4, SOX2 and C-MYC), pushing skin fibroblast back into the pluripotent state. Yet, especially in this setting of high cell turnover, integrating vectors may increase the risk for malignant transformation. Ongoing efforts therefore focus on the use of non-integrating vectors and improvement of other techniques involved in the generation of a large and pure population of well-differentiated cells.

**GENE AND CELL THERAPIES TARGETING BRADYARRHYTHMIAS**

Robust proof-of-concept has been obtained for treatment of complete heart block. Early studies focused on the transplantation of SAN tissues or the SAN on a pedicle to the ventricle. All met with failure. Yet the combination of intellectual interest in pacemaker function and recognition of the shortcomings of electronic pacing created an environment of persistent interest in developing “biological pacemakers” that might be used as replacements for or adjuncts to electronics. It was reasoned that biological solutions might be better adapted to the needs of children’s hearts, might be more physiologically autonomically responsive, might confer better opportunities for improving cardiac output, and might be longer-lived than electronics. If these objectives were met, then the biologic solution would be an attractive alternative to electronics.

The history of proof-of-concept with biological pacemakers is straightforward and from the beginning has incorporated elements of gene and cell therapy. The earliest gene therapy reports of interest come from Edelberg et al., who introduced the β₂-adrenergic receptor in a plasmid to the atrium of the mouse and later of the pig. The result was an increase in basal rate and in catecholamine responsiveness. Clearly, this was not a clinically viable approach, but it paved the way to use gene therapy to modulate pacemaker function. The first cell therapy approach involved the implantation of fetal canine atrial cells, including those identified as SAN cells, into the ventricles of two adult dogs that were then put into heart block. Rhythms were mapped to the injection site and had rates of about 70 bpm. Certainly, this suggested viability of a cellular approach to therapy, although the use of fetal cells for such a purpose creates a number of issues including long-term incorporation into myocardium and rejection of the fetal cells by the host.

**Virally-delivered Gene therapy**

The first report of virally-delivered gene therapy to provide biological pacing was provided by Miake et al., who used a dominant-negative approach to reduce the hyperpolarizing current, I_{K1}, in guinea pig ventricular myocardium. While this clearly provided proof-of-concept using viral vectors and modifying an ion current, it also led to prolongation of repolarization (a potentially pro-arrhythmic change) and was largely...
abandoned for that reason. Subsequent gene therapy approaches have focused on the HCN family of genes responsible for the pacemaker current, $I_{f}$. Proof-of-concept of pacemaker activity—again using adenovirus—was established first in the canine atrium and then via injection into the bundle branch system of dogs in transient or complete heart block. This approach has shown basal rates of 50-60 bpm and sympathetic and parasympathetic responsiveness. The optimal pacemaker rate would be 60-90 bpm in the resting heart, with sympathetic responsiveness increasing rate to the range of 130-160 bpm. In an effort to optimize biological pacemaker rates, various HCN mutants have been studied. However, mutations on HCN1 and HCN2 have shown outcomes more subtle than substantive. For example, the HCN2 mutant E324A was originally selected because of its shifted activation kinetics—rendering

Figure 5. Different sources of stem cells that are currently being developed for myocardial repair. The time scale at the bottom gives an approximation of when clinical testing was or will be initiated. Modified from reference 57, with permission.
more channels open within the physiological range of membrane potentials—which while maintaining intact sensitivity to cAMP. However, this intervention did not improve dependence on the electronic backup pacemaker, nor did it improve basal heart rates. A chimeric channel, HCN212, was designed to incorporate the pore-forming unit of HCN1 (which has more favorable activation kinetics than HCN2) and the cAMP binding site of HCN2 (which is more robust than that of HCN1). However, HCN212 had an excessive effect, inducing ventricular tachycardias with rates above 200 bpm. An alternative approach was explored by Marban et al. who mutated a Kv1.4 channel to produce a HCN-like current. This approach generated biological pacing at beating rates ranging from 60 to 150 beats per minute, depending on the specific mutation. A downside of this approach is that these channels are not directly regulated through autonomic signaling.

As an alternative to gene transfer of ion channels, overexpression of transcription factors may be employed to transdifferentiate working myocardial cells towards cells with a pacemaker phenotype. Initial proof-of-concept for this approach was provided by Hoogaars and colleagues. Using transgenic mice studies, they showed that overexpression of the T-box transcription factor TBX3 during early embryogenesis imposes a pacemaker phenotype upon atrial cells. More recently, these studies were followed by experiments that tested biological pacemaker function based on adenoviral overexpression of TBX18 in guinea pig left ventricle. In isolated AV-block hearts, this approach generated heart rates of around 160 bpm and harbored significant sensitivity to adrenoceptor stimulation. Yet, at this stage it remains difficult to assess the value of this approach as the obtained beating rates are too slow for guinea pigs, but too rapid for humans.

Cell therapy
Three approaches have been used for cell therapy. One has involved the implantation of human embryonic stem cell-derived cells as biological pacemakers into the ventricles of pigs with complete heart block and immunosuppressed to prevent rejection. The stem cells had been coaxed into a lineage with pacemaker properties and drove the ventricles effectively during the period of study. The obvious concern about proceeding in a clinical direction with embryonic stem cell-derived pacemakers is the need for immunosuppression: even with their attendant issues, electronic pacemakers are a superior option to immunosuppression for the lifetimes of patients needing pacemakers. Another approach focused on the use of adult human mesenchymal stem cells (hMSCs) as a platform—loaded with HCN genes—to deliver pacemaker current. This has been done in dogs with complete heart block and without immunosuppression, based on literature suggesting these cells are “immunoprivileged”. In fact, effective pacemaker current has been shown for 6-week periods, during which time rejection or apoptosis have not been issues. These stem cells have been shown to couple to canine myocytes via gap junction proteins and to deliver their current robustly. The major issue that has arisen is the propensity of stem cells to migrate elsewhere, once implanted in the heart. For this
reason, efforts are underway to encapsulate the cells in biomaterials that might retain them in position while still permitting gap junction formation to occur. An alternative to cell encapsulation has been tested: this is fusion of HCN-overexpressing cells to adjacent cardiac myocytes using polyethylene glycol.\(^92\) The third cell therapy approach involves the use of iPS cells as biological pacemakers. Several groups have reported the derivation of cardiogenic and functioning pacemaker cell lineages from these iPS cells.\(^93,\ 94\) An exciting feature as this area continues to advance is that the cells are obtained autologously, alleviating fears of rejection. However, many questions remain, and in vivo testing has not yet been reported.

**Other approaches to complete heart block**

Whereas biological pacemakers placed in the ventricle would offer treatment to a subset of patients in complete heart block, another subset has normal sinus node function and requires pacemaker therapy because of atrioventricular (AV) block alone. These patients now benefit from AV sequential pacing. The obvious alternative here would be to build an AV bypass tract to permit the normal sinus nodal impulse to propagate to the ventricle. Attempts here have been reported, although all are still rudimentary. They range from overexpressing connexins in cells in culture that are then used to bridge islands of myocytes, to preliminary reports of implanting conducting cells between atrium and ventricle.\(^95-97\) While this approach has not achieved the success of biological pacing, its requisites are far more rigorous, including the optimizing of the AV delay, programming in autonomic responsiveness, and initiating activation at a site in the ventricle that recruits as normal a ventricular activation pathway as possible. It is likely that the final approach will utilize both a cell matrix and a biomaterial or hydrogel to carry it. But much more time will be needed to solve this issue.

**GENE AND CELL THERAPIES TARGETING TACHYARRHYTHMIAS**

Two types of tachyarrhythmias have been studied with an eye toward their treatment with gene or cell therapy: atrial fibrillation (AF) and VT/VF. These will be considered individually. However, common to both has been the extent of dissatisfaction with existing therapies. This reflects the inadequacies of antiarrhythmic drugs (which as individual therapies remain useful in atrial fibrillation and less so in VT/VF), the complexities and fragilities of cardioverter-defibrillators, and—for the atria—the mixed successes with ablation techniques to maintain the heart in sinus rhythm.\(^98-103\) The difficulty in bringing these gene and cell therapy approaches to clinical reality lies in part in the preparation of effective constructs. As shown below, some effective constructs have already been identified, but delivery to an opportune site is still complex. Hence, advances in specialized methods that localize regions suitable for treatment and allow for accurate and efficient therapy delivery, are crucial. This is especially important because it will determine the effectiveness of the therapy; and in some cases, the benefit over globally applied pharmacological interventions.
Atrial fibrillation

Both rhythm control and rate control have been goals here. For rate control, the major cellular approach has been the implantation of fibroblasts (~320 injections) in the AVN region of dogs. This has resulted in scarring of the nodal region and prolonged atrium-to-His delays. In yet another approach, overexpression of Gαi2 in the AVN of porcine was used to prolong the effective refractory period (ERP) and reduce the ventricular rate during AF. In this case, adenoviral vectors given through the AVN artery locally suppressed adenylyl cyclase activity and thereby L-type Ca2+ current. Similarly, L-type Ca2+ currents could also be suppressed by overexpressing Gem, a GTP-binding protein within the Ras superfamily, which reduces trafficking of the α subunit of the channel to the plasma membrane. Importantly, these strategies are all intermediate alternatives to generally well-tolerated drug treatments and clinically available RF ablation procedures. It therefore remains to be seen whether these novel strategies become a method of choice.

For rhythm control, the initial approach has been to prolong the effective refractory period (ERP) throughout the atria. Proof-of-concept was provided by Levy et al. who administered the mutant ion channel Q9E-hMiRP1 to the atria of pigs via plasmid gene transfer. This mutant ion channel is associated with drug-induced long QT syndrome and therefore allowed for the delivery of a “caged” therapy. Clarithromycin had to be used to trigger the local prolongation of repolarization. Another approach has been described by Donahue et al. who improved on the efficiency of global atrial gene transfer. They combined the use of adenoviral vectors with a painting method involving 20% pluronic (to form a gel that adheres to the myocardium) and 0.5% trypsin (to facilitate viral penetration through the first layers of connective tissue). Using this method, they reported transmural gene delivery and demonstrated regional prolongation of monophasic action potential (MAP) and ERP with gene transfer of the long QT-associated ion channel, HERG-G628S. In a subsequent study, they were able to maintain sinus rhythm in HERG-G628S overexpressing animals for periods during which control animals developed burst pacing induced AF. A different approach to rhythm control in AF is being tested in experiments that speed conduction. Here, Cx43 gene transfer has been applied via adenoviral vector injection in combination with electroporation, which appeared effective in the protection against burst pacing-induced AF. A subsequent study used the above described atrial painting method and indicated potential superiority for Cx43 vs Cx40 in preventing burst pacing induced AF.

Ventricular tachycardia/fibrillation

The antiarrhythmic approaches explored in AF, such as increasing ERP or speeding conduction, can also be applied to reentry-based VT/VF. The challenges here are different, and lie primarily in the identification of appropriate sites for the application of therapy. In most cases, this will require extensive mapping because of large inter-patient variations in anatomy and due to cardiac remodeling. Although these mapping techniques are already routinely applied in clinical VT ablation procedures, further
Figure 6. Different approaches to myocardial cell therapy. A, Undifferentiated stem cells isolated from various sources may be used to stimulate myocardial regeneration via the excretion of paracrine factors that stimulate endogenous pathways of repair. B, Undifferentiated stem cells may be loaded with ion channels to couple to cardiac myocytes through gap-junctions (GJ) and as such can locally modify the electrical substrate. C, Stem cell-derived cardiac myocytes may be used for myocardial repair after direct injection or infusion into damaged myocardium. D, Embryonic or induced pluripotent stem cells may be coaxed into a lineage of pacemaker cells and as such can be injected locally to couple to adjacent myocytes and compensate for bradycardia.
development will be required to combine them with the gene transfer methods discussed here.

**Myocardial infarction**

Proof-of-concept for the ERP-prolongation approach was provided by overexpressing the dominant-negative HERG mutant G628S in pigs. This strategy was tested in chronic (3-week old) infarcts, in which programmed electrical stimulation resulted in 100% monomorphic VT inducibility. Site identification issues were tackled by endovascular gene delivery at the site of initial balloon occlusion used to create the infarct. One week after gene transfer, HERG-G628S overexpressing animals showed increased ERP and VT was no longer inducible. In contrast, all control animals still demonstrated inducible VT.\(^{114}\)

Another approach to infarct-associated VT/VF is provided by interventions that improve conduction. The rationale is that even in the setting of tissue damage, and with no attempt to regenerate or replace the tissue, impulses might be made to conduct normally, and, in the process, prevent initiation of reentrant VT/VF. The most successful approach to date uses the skeletal muscle Na\(^+\) channel (SkM1; Nav1.4). This channel functions effectively at the depolarized membrane potentials (in the −65 mV range) seen in the border zones of myocardial infarctions.\(^{115, 116}\) At these potentials in the border zones of myocardial infarctions, the cardiac sodium channel, Nav1.5, is largely inactivated, and this contributes to conduction slowing/block.\(^{117}\) In the 5–7 days canine myocardial infarct, administration of an adenoviral vector carrying the Nav1.4 gene, SkM1, into selected border zone sites resulted in reduced electrogram fragmentation, an increased action potential upstroke velocity (\(V_{\text{max}}\)) of the depolarized myocardium (reflecting an increased inward Na current) and a significant reduction in VT/VF inducibility.\(^{118}\) In a related study, mapping techniques demonstrated that SkM1 overexpression improved longitudinal conduction in the border zone.\(^{119}\) Hence, SkM1 increases \(V_{\text{max}}\) of depolarized myocardium, speeds conduction, and reduces incidence of inducible sustained VT/VF in canine infarcts.

Another approach to reduce ventricular arrhythmias may derive from speeding conduction via endogenous or additionally introduced connexins. This approach was studied first using pharmacological modulation of gap junctions (via rotigaptide and analogs) and later using gene therapy. The rotigaptide approach indeed showed reduced spontaneous arrhythmias after ischemia/reperfusion in open-chest dogs\(^{120}\), and reduced arrhythmogenic dispersion of repolarization.\(^{121, 122}\) However, because rotigaptide prevents dephosphorylation of Cx43, it can only enhance function of connexins that are already present\(^{123, 124}\), while in some cases there might be the need for increased connexin presence.\(^{125-130}\) This perception fostered the development of techniques to increase connexin presence via post-transcriptional or overexpression pathways. In mice with myocardial infarction, an antisense inhibition of miR-1 restored Cx43 and Kir2.1 protein levels and thereby improved conduction and decreased arrhythmogenesis.\(^{131}\) We recently studied adenoviral delivery of Cx32 into the left
ventricle of mice and demonstrated a reduced incidence of ischemia/reperfusion arrhythmias. This strategy was comparably effective to SkM1, which was also tested in this model. The Cx32 isoform was selected because of its low sensitivity to acidosis, i.e., Cx32 channels largely remain open at low pH, whereas Cx43 channels close. Using Cx32 overexpression, maintenance of fast impulse propagation was demonstrated under conditions of metabolic stress and acidosis. 132

A concern with connexin enhancement strategies is that in settings of myocardial ischemia or infarction, they may lead to increased spread of injury mediators, resulting in a larger area of tissue damage. In line with these concerns, it has been demonstrated that gene knockdown of Cx43 133 or pharmacological inhibition of gap junction channels 134-135 show reduced infract size. In mice treated with adenoviral Cx32 overexpression, we indeed found an increase in infarct size 24 h after coronary artery ligation. 137

Finally, a cellular approach to treat myocardial infarction and related arrhythmias may be provided by attempts to regenerate cardiac muscle. This may be done with one of the various sources of stem cells that are currently available. However, there are concerns for potential proarrhythmia. 138 These concerns are particularly strong for skeletal myoblasts, because they do not couple electrically to adjacent myocardium and form isolated electrical barriers that may increase arrhythmogenicity. Although this problem may be met by overexpressing Cx43, the success rate of such an approach in reducing arrhythmias appears to vary among different models. 139, 140

**Congestive heart failure**

Gene therapy has also been applied to the treatment of arrhythmias associated with congestive heart failure (CHF). CHF can be complicated by prolonged repolarization associated with K⁺ channel down-regulation and attendant arrhythmogenic consequences. 141 This prompted the development of several gene and cell therapy strategies that enhance expression or availability of K⁺ channels/currents. 142-148 A general concern with these strategies is that because they accelerate repolarization, they also reduce Ca²⁺ cycling and contractility. 143 Therefore, in a subsequent study, Nuss et al. 149 overexpressed Kir2.1 and SERCA1 genes via intramyocardial injection in the guinea pig. In this way, the acceleration of repolarization induced by Kir2.1 and the attendant loss of Ca²⁺ cycling and contractility was averted. That is, repolarization was accelerated, which would reduce the risk of long QT-associated arrhythmias while maintaining contractility.

SERCA2a overexpression has further been used to improve contractile function in the experimental treatment of CHF. Overexpression of the cardiac isoform SERCA2a has shown reductions in hypertrophic remodeling and improvements in contractile function and survival. 150-152 In subsequent phase I/II clinical trials using AAV-1 mediated gene delivery in patients with end-stage CHF, safety and efficacy of the approach have been demonstrated, which supported the initiation of next stage clinical trials. 48, 49 Interestingly, SERCA2a overexpression also appeared to be antiarrhythmic against ischemia/reperfusion arrhythmias in rats and pigs. 153, 154 A potential mechanism that may underlie the antiarrhythmic effects of SERCA2a is provided by a significant
Table 1. Promising experimental approaches to treat cardiac arrhythmias and their expected follow-up.

<table>
<thead>
<tr>
<th>Arrhythmia</th>
<th>Therapy strategies</th>
<th>Next steps</th>
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<tbody>
<tr>
<td>Bradycardias</td>
<td>Focal HCNx gene/current transfer to atria or ventricle—to increase automaticity or create a de novo pacemaker</td>
<td>Obtain functional improvements using additional gene transfer or further engineered mutant genes *</td>
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<td></td>
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<td>Incorporate long-term functionality using dedicated viral vectors or encapsulated cells, and conduct associated testing *</td>
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<tr>
<td>AV-block</td>
<td>Focal modification of conduction in the AV-nodal region or implantation of cell constructs to create a de novo pathway for AV-conduction</td>
<td>Engineer more robust gene and cell based methods to restore conduction in the AV-nodal region</td>
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<td>Develop stem cell matrixes that allow for the transplantation of a targeted stem cell strand without eliciting an immune response</td>
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<td>Optimize function with regard to AV delay, autonomic responsiveness and activation initiation site in the ventricle</td>
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<tr>
<td>Reentry based AF</td>
<td>Global gene transfer to both atria of HERG-G628S, Cx40 or Cx43—to globally prolong, ERP or to speed conduction</td>
<td>Develop (minimal invasive) delivery methods to apply LV or AAV globally to both atria</td>
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<tr>
<td></td>
<td></td>
<td>Prepare long-term expression vectors and conduct associated testing</td>
</tr>
<tr>
<td>MI related VT/VF</td>
<td>Gene transfer of HERG-G628S or SkM1 to specific sites in and around the infarct—to locally, prolong ERP or speed conduction</td>
<td>Develop minimally invasive techniques, that allow for detection of sites suitable for gene transfer and subsequent application of viral vectors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prepare intermediate to long-term delivery systems (susceptibility to some post MI arrhythmias is transient) and conduct associated testing *</td>
</tr>
<tr>
<td>Congestive heart failure related arrhythmias</td>
<td>Global gene transfer of SERCA possibly in combination with K⁺ channel enhancement strategies</td>
<td>Further investigate effects of AAV based SERCA gene therapy on pump function and arrhythmias (ongoing in clinical studies)</td>
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<td></td>
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<td>Identify specific subsets of patients that can safely benefit from SERCA gene therapy</td>
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<tr>
<td></td>
<td></td>
<td>Establish potential benefit of adding K⁺ channel enhancement strategies to SERCA-based therapies</td>
</tr>
</tbody>
</table>

* Indicates next steps addressed in this thesis.

Resistance to action potential duration (APD) alternans, noted in single cells and intact guinea pig hearts. Reduced APD alternans likely stems from the increased diastolic Ca²⁺ uptake into the SR. Unfortunately SERCA2a overexpression may also imply a potential risks for proarrhythmia in the acute phase of permanent coronary artery occlusion. It will therefore be critical to establish which subsets of patients might safely benefit from SERCA2a gene therapy.
INTRODUCING THE NEXT STEPS

The next steps on the road towards clinical application for the most advanced gene and cell therapies in experimental electrophysiology and pacing are summarized in Table 1. In general, every successful gene and cell therapy has to achieve the following milestones before clinical application can be considered: 1) proof-of-concept, 2) optimization of genetic or cellular composition, 3) fabrication of vectors or cells that allow for long-term function, and 4) implementation of 2 and 3 into thorough toxicology and efficacy testing while using clinically applicable delivery methods. This thesis primarily focuses on the second and third milestones for the development of biological pacemakers and therapies targeting myocardial infarction related arrhythmias. The next chapter will more specifically introduce the research questions addressed.

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