Molecular therapies for cardiac arrhythmias
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THE PAST, PRESENT, AND FUTURE OF PACEMAKER THERAPIES

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ABSTRACT

Although the concept of electrical stimulation dates back to ancient Rome, its application to cardiac arrhythmias originated in the 18th century. Incorporation of electronic pacing into routine clinical practice goes back to the 1960s and is critically depended on an increased understanding of bradycardias and advancements in pulse generators and battery technology. Since its introduction into clinical practice, electronic pacing has saved many lives and is a true triumph of 20th century medicine. Despite the continuous improvements of electronic pacemakers, these devices are still not without important shortcomings, which stimulated the development of biological alternatives. In this concept, pacemaker function is generated by genes or cells with the potential of providing a cure for bradycardias. Over the past years, significant improvements have been made in biological pacemakers, but issues remain in relation to long-term outcomes and safety, both of which require further investigation. On the other hand, efforts to improve electronic pacemakers have also intensified. The coming decades therefore will witness either improvements in electronic pacing to a point that removes the need for alternatives and/or significant advances in biological pacing that merit its application to man.
INTRODUCTION

Despite significant successes of electronic pacemakers, some important shortcomings remain. These range from functional problems such as inadequate autonomic responsiveness to hardware problems such as limited battery life. Additional downsides of electronic pacing include: pacemaker pocket/lead infections, lead fractures, electromagnetic interference, pacing from non-physiological sites associated with remodeling, and challenges for application in pediatric patients. Facilitated by an increased understanding of endogenous pacemaker function and the availability of gene and cell therapy, biological pacemakers have been developed in the past decade as a potential alternative to electronic pacing.

This thesis described some important steps made in attempting to improve biological pacemaker function. Part I (Chapter 1-2) introduced the various methods of gene and cell therapy that may be employed in heart rhythm disorders. Part II focused on the identification and exploration of optimized gene targets and delivery methods in the setting of bradycardia treatments. Chapter 3 showed the usefulness of lentiviral gene transfer in biological pacemaker engineering. This work provided important proof-of-concept in an effort to induce long-term pacemaker function. One other avenue which we explored was the development of a novel in vitro system to test cardiac gene and cell therapy. To this end, we reported the use of an organ explant system in Chapter 4. Due to the low-to-absent intrinsic spontaneous activity, this system appeared particularly useful in testing various methods relevant to biological pacing. One of the approaches tested here was the usefulness of cardiac myocyte progenitor cells (CMPCs; described in Chapter 5). When loaded with hyperpolarization-activated cyclic nucleotide-gated channel (HCN)4, these cells coupled to myocytes in organ explants, thereby generating autonomically-controlled pacemaker activity.

Our results in these initial studies, together with the findings of others, suggest that biological pacing purely based on HCN overexpression are not sufficient to generate the required level of function (i.e., basal beating rates of 60-90 bpm, robust sensitivity to autonomic modulation, and low dependence on electronic back-up pacing). We therefore started to explore alternative methods. Based on its importance in sinoatrial node (SAN) development, we selected overexpression of the T-box transcription factor TBX3 as a potential alternative to HCN-based biological pacing. In Chapter 6, we described the outcomes of reprogramming atrial and ventricular cardiac myocytes towards pacemaker-like cells using this method. We found that TBX3 by itself did not induce ectopic pacemaker activity. Yet, TBX3-based reprogramming did induce an efficient switch from the working myocardial expression profile to that of pacemaker myocardium and may therefore be used to improve stem cell-based or HCN-based pacemaker function.

Part III describes several large animal studies in the framework of biological pacing. In Chapter 7, we investigated the sensitivity of HCN2-based biological pacemakers to emotional arousal. Here, we found that food presentation after an overnight fast induced a significant increase in beating rate and heart rate variability. This work represents...
the first demonstration of a biological pacemaker responding to emotional arousal stimuli. In Chapter 8, we explored a novel approach to gene-based biological pacing via overexpression of the Ca\textsuperscript{2+}-stimulated adenylyl cyclase AC1. Overexpression of AC1 increases intracellular cAMP which impacts on a variety of pacemaker mechanisms, including the pacemaker current $I_f$.\textsuperscript{16} When AC1 was overexpressed in the left bundle branch (LBB) of AV-block dogs, efficient biological pacing was generated with faster basal beating rates and greater sensitivity to autonomic modulation than HNC2.\textsuperscript{17} Finally, in Chapter 9, we investigated the outcomes of biological pacing based on HCN2/SkM1. We hypothesized that HCN2-induced function would be improved by hyperpolarizing the action potential (AP) threshold via coexpression of SkM1. This study indeed demonstrated robust biological pacing with baseline beating rates in the 80 bpm range, brisk autonomic responsiveness, and a complete elimination of electronic back-up pacing with the latter being persistently required in previously explored strategies. In this study, we further showed function of HCN2/SkM1 to be critically dependent on a reduction in AP threshold and injection into the LBB.\textsuperscript{18}

This discussion chapter readdresses the outcomes of the above-mentioned studies together with the ongoing advances in the field of biological pacing. Furthermore, it places these proceedings into context with the past, present and future of electronic pacemaker engineering.

**A BRIEF HISTORY OF PACEMAKER THERAPIES**

The initial medical use of electro-therapy has been assigned to Scribonius Largus of Sicily (3 B.C. – 54 A.D.). In his *Compositiones Medicae* (46 A.D.), Scribonius suggested the application of live electric ray to relieve pain in conditions such as headache and gout.\textsuperscript{1} As with many medical therapies this one was purely based on empirical knowledge that indicated anesthetic and analgesic effects after contact with these rays. A more physical approach to electricity came from Thales of Miletus (6\textsuperscript{th} century BC) who described experiments in which rubbing amber with cat’s fur resulted in the generation of static electricity.\textsuperscript{19} Although Thales mistakenly interpreted his outcomes as being comparable to the magnetic properties of lodestone, it has been the Latin translation “electricus” (like amber) for Greek “\textepsilon\textlambda\textkappa\textomicron\texttau\textomicron\nu” (elektron) - introduced by the 16\textsuperscript{th} century English scientist William Gilbert - that gave electricity its now common name. After millennia of little to no progress, Gilbert revived the interest in electrical phenomena with his studies that carefully dissected the differences between static and magnetic electricity (*De Magnete* 1600). The subsequent initial appearance of the words “electric” and “electricity” in English writing is generally assigned to the work of Thomas Browne in his *Pseudodoxia Epidemica* (1646).\textsuperscript{20}

Significant progress that followed these early works occurred with the discovery of the Leiden jar (1745), as this apparatus provided the first capacitor that allowed for storage of static electricity in a relatively small glass container.\textsuperscript{21} In 1752, Benjamin Franklin used the Leiden jar to collect static electricity from thunderstorm clouds in his famous kite experiment, which helped him in proposing the concept of positive
and negative charge. The succeeding understanding of electricity operating within biological systems importantly came from the experiments of Luigi Galvani (1781). This work showed that a frog’s leg twitches when brought into metallic contact with its crural nerves. It remains uncertain if this serendipitous observation was made by a laboratory assistant or by Galvani’s wife while preparing frog legs for supper. However, regardless of its discoverer, the outcome spurred further experimentation by Galvani, leading him to conclude that animals could produce electricity for which he considered fluids of brain and nerve as the primary source. Alessandro Volta proposed the metal (used in Galvani’s experiments) and not the animal’s nervous system was the source of electric energy. This theory encouraged him to build a column of copper and zinc discs with each pair being separated by saline soaked cardboard; the so called “Voltaic pile”. This apparatus was capable of generating electric current and was later recognized as the first battery. In the end, both men were right and their efforts marked a great step toward electricity-based therapies. Even without the electricity debate being resolved, contemporaries of Galvani and Volta had started to use the Leiden jar (as a source of energy) connected to an electrometer (to set the energy level) and two metallic strings (functioning as electrodes) to shock humans in settings requiring resuscitation.

In 1849 Hoffa and Ludwig showed that the application of strong constant currents to canine hearts caused an abrupt cessation of the normal heart beat and induced arrhythmic contractions that were insufficient to maintain normal blood pressure. In attempting to terminate this lethal arrhythmia, Hoffa and Ludwig found that some hearts resumed regular contractions after the injection of cold calf serum into the coronary arteries. These experiments inspired McWilliam in 1887 to further study the concepts of ventricular fibrillation in which he started applying faradic (electric) currents to the hearts of various animals. After confirming the findings of Hoffa and Ludwig, he found that single shocks - applied directly to the exposed heart or delivered trans-thoracically - could induce single contractions without sending the auricles or ventricles into fibrillation. Two years later, McWilliam conceptualized transthoracic pacing as a therapy for cardiac resuscitation yet not without recognizing the following: “It is, of course, only in a very limited number of the cases of cardiac failure that the question of artificial excitation of the heart beat becomes one of practical importance” — clearly indicating that he did not expect a very broad application of this therapy. In any case, to test the concept of cardiac pacing, he proceeded with the application of single shocks to the exposed hearts of anesthetized cats in the setting of vagal stimulation-induced bradycardia.

It took another forty years before the first portable external pacemaker systems were reported by Lidwell and Hyman. Lidwell was an anesthesiologist from Sydney who studied the electrograms of dying patients in whom he noted SAN dysfunction and conduction system failure as potential causes of death. Together with the physicist Booth he started building a device intended for the delivery of electrical stimuli during cardiac resuscitation. Their apparatus consisted of a pulse generator, a
skin pad-electrode soaked in saline, and a needle-electrode intended for positioning into myocardium. Using this device they were in 1929 the first to successfully pace the heart of a stillborn infant. Ten minutes later the heart continued beating without electrical assistance and the newborn survived.\textsuperscript{31}

Hyman, a physician from New York who teamed up with his physicist brother, was the first to patent the “artificial pacemaker” in 1933. Interestingly, the Hymans did not build their device to treat conditions such as SAN dysfunction or complete heart block; they were spurred by the “stopped heart”, a condition that frequently occurred in electrical power workers who had died suddenly after accidental electrocution.\textsuperscript{32} Apparently both Lidwell and Hyman were more focused on the resuscitation setting and did not connect their pacemaker therapies to bradycardia and syncope that had already been recognized for centuries as potentially lethal conditions. Noteworthy in this respect are the early descriptions by the 18th century Italian anatomist and physician Giovanni Battista Morgagni\textsuperscript{33} and by the 19th century Irish physicians Robert Adams and William Stokes.\textsuperscript{34, 35} Unfortunately, due to a combination of technical difficulties and a general opinion against these types of interventions, both teams (Lidwell & Hyman) did not succeed in bringing the pacemaker to clinical practice.

Subsequent breakthroughs occurred in the 1950’s. On the clinical side, Zoll revitalized the concept of cardiac pacing using transthoracic stimulation of the heart during resuscitation.\textsuperscript{36} On the engineering side, the commercialization of silicon transistors in 1956 critically contributed to the design and fabrication of battery-powered pacemaker systems. The first successful implantation of such a system using epicardial leads was performed in 1958 by Elmqvist and Senning in Sweden.\textsuperscript{37} At the same time Furman and colleagues were the first to pace the heart via the transvenous route.\textsuperscript{38} In the 1960s the employment of permanent transvenous endocardial pacing and specific pacing modes were introduced. The latter included the ventricular inhibited and ventricular triggered protocols.\textsuperscript{39-41} In the 1970s pacemaker lead fixation was importantly improved, titanium was used to hermetically seal the pulse generator, and lithium-iodine-based power sources became available. These remain the first choice today. When durable and safe pacemakers had been generated, further developments in the 1980s focused on more physiological pacemaking via the employment of dual chamber and rate adaptive systems (i.e., pacemaker systems that accelerate heart rates indirectly in response to activity – via motion sensors, or in response to increases in breathing frequency).\textsuperscript{41}

Advancements in pacemaker therapies are ongoing. With regards to electronic pacemakers, the most recent efforts focus on the development of leadless systems and power sources that allow transcutaneous recharge.\textsuperscript{42} As a potentially disruptive technology, we and others started to employ the use of gene and cell therapies to develop biological pacemakers.\textsuperscript{3-5, 43} If successful, advantages of such biological pacemakers would include: 1) a more physiologic rate in response to sympathetic and parasympathetic stimuli, 2) pacing from more physiologic sites within the heart, and 3) potential for life-long cure rather than temporary palliation.
IDENTIFYING TARGETS TO INDUCE/ENHANCE BIOLOGICAL PACING

HCN-based biological pacing

Exactly replicating all features of SAN pacemaker function may not be needed in approaches aimed at biological pacing. The goal is efficient pacemaker outcomes in conjunction with an optimal safety profile. The initial approaches therefore employed single gene transfer interventions and studied the level of function that could be generated.44-46 One of the most favorable strategies was the overexpression of HCN channels as these channels are directly sensitive to autonomic stimulation via binding of cAMP. The most extensive studies were performed in canines with complete heart block, in which adenoviral HCN2 was injected into the LBB (Chapters 7-9 and earlier.
references). In this setting, basal firing rates were 50-60 bpm, showed a modest response to pharmacological and emotional autonomic stimuli and overall accounted for ~50% of the beats (the remaining beats were generated by ectopic foci elsewhere in the heart or were electronically induced). Studies with viral or stem cell delivery of HCN4 suggest comparable outcomes with this isoform. However, optimal pacemaker properties were targeted to have basal beating rates of 60-90 bpm, strong sensitivity to autonomic stimulation, and low-to-absent dependence on electronic back-up pacing. The recognition that the initial biologic pacemakers did not meet these requirements spurred the search for causes of dysfunction and alternatives aimed at improvement.

Current-to-load mismatch in relation to biological pacemaker function

One hypothesis in relation to biological pacemaker dysfunction is that current-to-load mismatch could contribute to the relatively slow beating rates found in vivo. This hypothesis was supported by the finding that in settings without load (e.g., in single cells and completely transduced monolayers), beating frequency was faster and less unstable. However, important differences in the kinetics of overexpressed HCN2 exist when comparing ion channel function in neonatal vs. adult myocytes. In neonatal myocytes, $i_f$ activates at more depolarized potentials and therefore generates a more robust current, hence one would expect faster beating rates in this cellular environment. To further explore the concept of current-to-load mismatch, we therefore needed to keep HCN kinetics constant. To this end, we introduced electrical load of hyperpolarized myocardium to a monolayer of HCN4 expressing myocytes. We found that monolayers composed of a central area of HCN4 overexpressing myocytes surrounded by unmodified myocytes exhibited slower and unstable beating rates (Figure 1) as compared to homogeneously transduced monolayers (Chapter 3; Figure 6B). Although this may not conclusively proof a contribution of current-to-load mismatch to in vivo dysfunction of HCN-based biological pacemakers, it helped us conceptualize methods for enhancement of function. We assumed that biological pacemakers would be improved by approaches that further facilitate diastolic depolarization or reduce hyperpolarizing electrical load. The gene targets that have helped understanding and testing this theory and their relation to other approaches are discussed below and are summarized in Figure 2.

Increasing diastolic depolarization via cation channels

Original improvements of gene-based biological pacemakers have been made via targeted mutagenesis of selected HCN isoforms. One hypothesis was to shift activation kinetics positively in order to increase channel availability during diastole. In this respect, two mutant channels have been studied, the point mutation construct HCN2-E324A and the HCN1 deletion construct (235–7EVY; designated HCN1-ΔΔΔ). HCN2-E324A showed improved sensitivity to catecholamine stimulation, yet overall function in vivo remained comparable to wild-type HCN2. A confounding factor in the comparison between wild type HCN2 and HCN2-E324A was the lower expression efficiency of the latter. With HCN1-ΔΔΔ, physiological beating rates were obtained in porcine atria; however, a significant dependence on electronic back-up pacing (~15%) remained.
A downside of HCN1-based biological pacing is that this isoform is only minimally sensitive to stimulation by cAMP. This concern led to the design of the chimera construct HCN212 that incorporates strong sensitivity to cAMP based on the C terminus of HCN2 and fast activation kinetics based on the transmembrane region of HCN1. Unfortunately, this combination induced excessive increases in beating rates leading to bursts of ventricular tachycardia exceeding 200 bpm.\textsuperscript{51} \textit{In vitro} and \textit{in silico} studies later revealed potential mechanisms contributing to this burst pacing behavior of HCN212.\textsuperscript{52} They showed that the faster activation kinetics in HCN212 vs wild-type HCN2 were most likely responsible for the faster beating rates in the chimera. The irregularities in the HCN212-induced rhythms may in part be explained by the non-equilibrium properties of the channel. For example, during consecutive pacing at rapid frequencies, the activation kinetics slow to such an extent that the time-dependent diastolic HCN current decreases. This type of negative feedback was less pronounced in HCN212. In addition, as a result of slower deactivation, HCN2 exhibited a more pronounced instantaneous current at slower beating rates. Hence, the frequency dependence of both time-dependent and instantaneous current generates stronger negative feedback in HCN2 than HCN212. These differences in negative feedback may have provided the basis for uncontrolled increases in heart rate in HCN212. At rapid rates, other mechanisms such as overdrive-suppression may have been responsible for the abrupt pauses in pacemaker activity. These studies suggest that channels with properties intermediate to HCN212 and HCN2 may generate more favorable outcomes.\textsuperscript{52}

Because HCN-based biological pacing may be hampered by unpredictable results relating to heteromultimerization with endogenous channels,\textsuperscript{53, 54} the Marban group sought an alternative channel to generate inward current. To this end, they mutated the K\textsubscript{v}1.4 potassium channel in the pore to obtain a hyperpolarization-activated nonselective inward ion channel.\textsuperscript{55} Although this strategy appeared free of heteromultimerization and in some specific mutants generated physiological beating rates, 24h pacemaker stability was not reported. A downside of these channels is that they do not incorporate direct sensitivity to autonomic control. At this stage, work on this approach appears to have been discontinued.

**Increasing diastolic depolarization via β-adrenergic/cAMP pathways**

The roles of β-adrenergic receptor (βAR) stimulation in enhancing cardiac inotropy and chronotropy have long been understood.\textsuperscript{56} This understanding facilitated the conceptualization of using β-adrenergic receptor overexpression as a novel therapy for cardiac chronotropic incompetence.\textsuperscript{44} In the heart, β\textsubscript{1}AR and β\textsubscript{2}AR stimulation are both importantly involved in the augmentation of beating rates.\textsuperscript{56} However, transgenic mice experiments suggested inferior outcomes in respect to enhancement of cardiac chronotropy when comparing overexpression of β\textsubscript{1}AR to β\textsubscript{2}AR.\textsuperscript{57, 58} Edelberg and colleagues therefore overexpressed the β\textsubscript{2}AR gene in spontaneously active cardiac myocytes and murine right atria (including SAN) in which they found increased beating rates.\textsuperscript{44} They next proceeded with catheter delivery of the β\textsubscript{2}AR construct into the SAN of the pig and again confirmed enhanced chronotropy.\textsuperscript{59} These studies were followed...
by overexpression of adenylyl cyclase 6 (AC6), a downstream target in the β-adrenergic signaling cascade. Here, an AC6 carrying adenovirus was injected into the left ventricular free wall. Ectopic activity could be induced by rapid electronic pacing and infusion of isoproterenol. A significant downside of both these approaches is the persistent dependence on adrenoreceptor stimulation for the induction of function.

Finally, in an effort to improve HCN2-based biological pacing, the combination of β2AR and HCN2 overexpression has been studied. To this end, Charpentier and coworkers injected HCN2 and β2AR constructs (in a ratio of 3:1 and mixed with tetronic 304, a poloxamine block copolymer) into the left ventricular free wall of mice that were made bradycardic 5 days later via His bundle ablation. They also injected HCN2 alone, but this only generated a minimal (insignificant) increase in ventricular ectopy activity, as compared to sham treated animals. The HCN2/β2AR group demonstrated ectopic activity at a rate of ~175 bpm ten to fifteen days after construct implantation, and showed significant sensitivity to autonomic stimulation as induced by isoproterenol. Although this rate of biological pacing would be too rapid for human application, it was interesting that the tetronic-based transfection method persisted over more than a month. This is particularly relevant, given the ease of construct preparation via this method. However, efforts to translate this approach to other animals such as Syrian hamsters and canines have thus far been unsuccessful, raising questions regarding its applicability to humans.

From a functional viewpoint, β2AR overexpression appears an efficient approach to enhance HCN2-based biological pacing, although further optimization would likely be necessary to titrate the basal beating rates. A downside of this approach, comparable to what has been discussed above, is that the improvement of HCN2-based function critically depends on beta-adrenergic stimulation. This may be particularly problematic in settings where beta-adrenergic stimulation is low such as during sleep or other inactivity.

**Increasing pacemaker function via adenylyl cyclase-engaged pathways**

While looking into alternatives that would be partially receptor-stimulation independent, we considered Ca2+-stimulated adenylyl cyclases such as AC1 and AC8. We hypothesized that because of the intrinsically high cyclase activity and additional stimulation by Ca2+, gene expression would be able to generate a cAMP signal capable of inducing constant pacemaker function. Initial support for this hypothesis came from *in vitro* experiments investigating the role of Ca2+-dependent β-adrenergic signaling in modulation of HCN2. This study showed that AC1 increased baseline cAMP levels, positively shifted the activation V1/2 of overexpressed HCN2, and increased spontaneous beating in neonatal myocytes overexpressing HCN2. In a subsequent *in vivo* study (Chapter 8), an AC1 carrying adenovirus was injected into the LBB of AV-block dogs and showed robust pacemaker activity at baseline beating rates of ~60 bpm, low dependence on electronic back-up pacing (<2%), and >95% of the beats originating from the injected area. Furthermore, sensitivity to sympathetic modulation (as expressed by long-term beating rate variability) remained comparable
Figure 2. Summary of the various approaches employed to improve HCN-based biological pacing. Upper panels show schematic changes in action potential (AP) morphology based on the various strategies involved and compared to standard HCN2 overexpression (grey APs). A, In HCN2-E324A and HCN1-ΔΔΔ (blue AP) the activation-voltage relationship is shifted positively as shown in the left inset. In HCN212 activation kinetics are accelerated, as shown by the inset on the right, which resulted in ventricular tachycardia (red AP). B, AC1 overexpression (blue AP) increases intracellular cAMP which increases the $I_{\text{f}}$ current and likely also stimulates Ca$^{2+}$-based pacemaker mechanisms. HCN2/AC1 generated ventricular tachycardia (red AP). C, The Kir2.1-ΔΔΔ strategy, a dominant negative construct that blocks the inward rectifier current, reduces the maximal diastolic potential (red AP). D, SkM1 hyperpolarized the AP threshold (green AP) and thereby improved the rate of HCN2-based pacemaker function. The lower panel summarizes the study details, outcomes and anticipated next steps of the different strategies represent in the upper panels.

to HCN2, while sensitivity to parasympathetics (as expressed by short-term beating rate variability) was higher in AC1 than HCN2. When HCN2 and AC1 were co-expressed in this model, synergistic function was obtained with regard to basal beating rates, maximal beating rates, and sensitivity to sympathetic modulation (as expressed by long-term beating rate variability), yet to a degree that exceeded physiologically desirable ranges. This indicated that a potential benefit may be obtained from this combination, albeit not without further titrating individual gene expression levels.
Mechanistically, the AC1-based approach appears to stimulate both $I_f$-dependent and $I_f$-independent pacemaker function. Support for $I_f$-dependent pacemaker mechanisms came from the in vitro experiments discussed above. The interaction of AC1 with $I_f$-independent pacemaker mechanisms was demonstrated by experiments employing the use of the cAMP-insensitive mutant HCN2-R/E. In these experiments, carried out by Kryukova et al., it was shown that coexpression of AC1 with HCN2-R/E still resulted in a significant increase in beating rates without affecting kinetics of $I_f$.\textsuperscript{16} In vivo, the interaction between AC1 and $I_f$-independent pacemaker mechanisms was supported by the notion that the $I_f$-blocker ivabradine significantly slowed tachycardia in HCN2/AC1 to average beating rates of ~60 bpm, but did not completely terminate pacemaker activity (Boink et al.).\textsuperscript{17} This was in contrast to the finding that ivabradine completely silenced idioventricular activity in animals with tachycardia induced by the chimera construct HCN212 (Plotnikov et al.).\textsuperscript{51}

With regards to $I_f$-independent function of AC1, it appears likely that Ca$^{2+}$-based pacemaker mechanisms are at least in part responsible. Elevations in cAMP will stimulate protein kinase A–mediated phosphorylation which subsequently may enhance function of L-type calcium channels, phospholamban, ryanodine receptors, and K$^+$ channels. Yet, to what degree these various proteins are functionally stimulated by AC1 and to what degree phosphorylation-independent mechanisms are at play, awaits further investigation.

**Facilitating diastolic depolarization by hyperpolarizing AP threshold**

In our search for means to improve HCN-based pacemaker function, we realized that beating rates and stability should also improve when the AP threshold is hyperpolarized. In myocytes of the working myocardium and the ventricular conduction system, the AP threshold is importantly determined by the availability of Na$^+$-channels. In vitro studies indicated that overexpression of the cardiac Na$^+$-channel (SCN5A) has only a minor effect on cardiac myocyte Na$^+$-channel availability.\textsuperscript{64} This is because there is a large endogenous pool of Na$^+$-channels and channel availability is primarily limited by the kinetics of inactivation. Thus, overexpressing SCN5A is expected to have only a minor effect on the AP threshold. In contrast, the skeletal muscle Na$^+$-channel (SkM1) has inactivation kinetics 10-15 mV more positive than SCN5A.\textsuperscript{64} This difference in inactivation indeed appeared highly effective in restoring Na$^+$-channel availability in the depolarized epicardial border zone of the infarcted heart, which resulted in normalization of impulse propagation and protection against reentry arrhythmias.\textsuperscript{65} However the effect of SkM1 on AP threshold potential was not investigated in these studies.

The impact of increasing Na$^+$-channel availability on HCN2-based biological pacing was tested after injecting HCN2/SkM1 adenovirus into the LBB of AV-blocked dogs (Chapter 9). From day 4, when adenoviral gene expression plateaus, until day 7, at the time the study was terminated, biological pacing was highly stable and exhibited pacemaker properties well within the ranges previously defined as optimal.\textsuperscript{5} Beating rates were around 80 bpm during rest and increased during activity to maximal beating
rates of around 130 bpm; this completely eliminated the dependence on electronic back-up pacing.

When looking into the mechanisms underlying the robust outcomes of HCN2/SkM1, we showed that injection into the LBB was indeed critically important for the obtained level of function. This was evidenced by the outcome of HCN2/SkM1-injected animals that received the construct subepicardially. In this case, basal beating rates, maximal beating rates and the dependence on electronic back-up pacing were all inferior to animals that received HCN2/SkM1 into the LBB. This outcome appears to result from specific LBB features such as presence of endogenous pacemaker function and a lower $I_{K1}$ current density. 66, 67 Secondly, we continued with the isolation of myocardial bundles and showed via microelectrode recordings that the AP threshold potential had been hyperpolarized as a result of SkM1 overexpression. In the presence of an unchanged maximum diastolic potential, the net result was that membrane potential was now closer to threshold. Thus, it appears that HCN2/SkM1 function is attributable to the bundle branch environment, slow diastolic depolarization induced by HCN2, and a hyperpolarized AP threshold facilitated by SkM1. 18

Reducing hyperpolarizing electrical load
The idea of blocking the inward rectifier current $I_{K1}$ to liberate endogenous pacemaker activity was one of the first approaches to biological pacing. 45 In this setting, $I_{K1}$ may be reduced by dominant negative proteins (e.g., Kir2.1-$\Delta$$\Delta$$\Delta$) 45 or by small interference RNA (siRNA). 68, 69 Yet, over the last years, this concept has largely been abandoned for its lack of direct modulation by autonomic stimuli. Recently, suppression of $I_{K1}$ function was revisited in the setting of co-expression of Kir2.1-$\Delta$$\Delta$$\Delta$ with HCN2. 70 Constructs were injected into the His bundle and induced efficient biological pacing at beating rates of 90-95 bpm. An unresolved concern with this approach remains the previously documented prolongation of AP duration induced by Kir2.1-$\Delta$$\Delta$$\Delta$. 71

REPROGRAMMING CARDIAC MYOCYTES TOWARD PACEMAKER-LIKE CELLS
Experiments by Hoogaars and colleagues intriguingly showed a critical role for the T-box transcription factor TBX3 in embryonic development of the SAN. 13 These studies inspired us and others to investigate the possibilities of using T-box transcription factors as a tool to transdifferentiate working cardiac myocytes towards pacemaker-like cells. To achieve this, we used tamoxifen-inducible transgenic mice and showed that expression of TBX3 in adult mice induced an efficient switch from the working myocardial expression profile to that of pacemaker myocardium (Chapter 6). 14 Important changes in gene expression included the suppression of genes encoding gap junction subunits (Cx40, Cx43), SCN5A, and inwardly rectifying K$^+$-channels (Kir genes). These changes resulted in reductions of $I_{Na}$ and $I_{K1}$ and a concurrent slowing of conduction. This occurred, however, without the induction of ectopic pacemaker
activity. As an addition to the tamoxifen-inducible transgenic mouse system, we also explored the phenotypic consequences of lentiviral TBX3 expression in neonatal cardiac myocytes (Chapter 6). Here, we found TBX3 to induce a variety of phenotypes including depolarized and spontaneously active cardiac myocytes. Based on the results of both these studies we concluded that the reductions in intracellular coupling and $I_{K1}$ are important reprogramming properties of TBX3. Thus, TBX3 expression is expected to reduce current-to-load mismatch, facilitate diastolic depolarization, and therefore may be applied to enhance biological pacing based on cell therapy or overexpression of HCN2.

After testing a variety of different T-box transcription factors, Cho and colleagues identified TBX18 as yet another promising candidate for transdifferentiation toward pacemaker-like cells. During embryogenesis, TBX18 plays a crucial role in differentiation of mesenchymal progenitors to pacemaker cells in the formation of the SAN head. When TBX18 was overexpressed in adult guinea pig myocardium, a subset of the transduced myocytes adapted morphological and electrophysiological characteristics of SAN cells including: reduced Cx43, reduced $I_{K1}$, increased HCN4, increased cAMP, and increased spontaneous Ca$^{2+}$ cycling. In isolated AV-block hearts, these cells generated baseline heart rates of ~160 bpm with significant sensitivity to autonomic modulation. The value of this approach is, however, difficult to assess as these rhythms are too rapid for human application and too slow for guinea pigs. It thus remains to be seen what level of function can be generated by TBX18 in more clinically relevant models such as pigs or dogs in complete heart block.

Although the outcomes with TBX3- and TBX18-based reprogramming are encouraging, a general concern is the incomplete success rate, meaning that a variety of different phenotypes may be generated. This was clearly illustrated by the TBX3 study in which at least 4 different phenotypes could be identified after reprogramming in vitro and in vivo. Furthermore, in the TBX18 approach, this type of heterogeneity was illustrated by the finding that only 13% of the TBX18 modified cells expressed HCN4. Also, when different cocktails of transcription factors were applied to healing myocardial infarcts, the efficiency of reprogramming fibroblasts towards ventricular cardiac myocytes ranged from 2-35%, further underlining the stochastic basis of this type of interventions. Additional studies are required to optimize the reprogramming efficiency and to establish to what extent a robust pacemaker phenotype that persists over time can be generated.

**ADVANCES IN CELL-BASED BIOLOGICAL PACEMAKERS**

In addition to the approaches discussed above, biological pacemakers may be fabricated via cell therapy. Conceptually, the cell-based approaches are divided into 1) strategies that generate cells with pacemaker-like properties or 2) strategies that use cells as a delivery vehicle for gene constructs. In the latter method, donor cells typically lack the machinery for cellular excitability, but couple or fuse to adjacent myocytes to form a functional pacemaker unit.
The usefulness of pacemaker-like cells

The initial approach exploring cells with pacemaker-like properties employed fetal atrial and SAN cells. Although these cells appeared capable of inducing idioventricular rhythms in AV-blocked dogs, they were never realistically considered for clinical applications. Nevertheless, these fetal cells generated important proof-of-concept for the exploration of other cell sources. One such source was provided by embryonic stem cells (ESC). These cells are typically cultured on an embryonic fibroblast feeder layer and can differentiate into a lineage of spontaneously active cells. Here, the ESC layer is dispersed into small clumps and cultured in suspension where the cell clumps further aggregate to form embryoid bodies. When spontaneously active embryoid bodies were transplanted into pigs with chronic AV block, they induced significant pacemaker function that persisted over a period of weeks. A downside of this approach was the continued need for immunosuppression, blocking further translation towards clinical application.

Methods to bypass the need for immunosuppression may be provided by autologous or undifferentiated (discussed below) cells. Initial approaches here included the use of progenitor cells that originally reside in the heart and that may be differentiated ex vivo towards spontaneously active cardiac myocytes. In addition, a major breakthrough has been provided by the development of induced pluripotent stem cells (iPS). In this approach, a cocktail of transcription factors (e.g., Oct3/4, Sox2, c-Myc, and Klf4) is used to dedifferentiate readily accessible cells (e.g., skin fibroblasts or hair keratinocytes) into a pluripotent state. Once the cells have become pluripotent, they can be induced toward cardiac myocytes using the embryoid body method similar to the differentiation of embryonic stem cells. Proof-of-concept for the use of iPS-derived cells in biological pacing is at this stage limited to in vitro studies, yet stable physiologically-controlled pacemaker activity could be demonstrated over a period of 15-days. It is therefore expected that at least during several weeks the in vivo level of function will be comparable to the ESC-based approach. The next test will come from long-term studies investigating safety and stability of function in a large animal model.

Cells as gene delivery vehicle

As a non-viral alternative to ion-channel delivery, the use of human mesenchymal stem cells (hMSCs) has been explored. The extensive safety data available for this approach would facilitate the clinical translational application in comparison to viral techniques. In this concept, the (undifferentiated) hMSCs are loaded with a pacemaker channel (e.g., HCN2), couple to cardiac myocytes via gap junctions, and deliver the \( I_f \) current to the host cell via electrical coupling. After in vitro proof-of-concept was obtained, cells were injected into the left ventricular free wall in AV-blocked dogs. Here, dose-dependent ectopic pacemaker activity was demonstrated exhibiting basal beating rates of 50-60 bpm and a moderate response to catecholamine infusion. A setback to this approach was that pacemaker function started to decrease after ~8 weeks. Loss of function may relate to migration of cells outside the injected area. This appears particularly likely as no signs of rejection or apoptosis were seen at the 6 week time...
point. Efforts to improve maintenance in the target area have therefore focused on biomaterials that anchor or encapsulate the cells, yet currently these have not been tested in vivo.  

Alternatively, stem cell maintenance may be improved by the use of cells that are native to the heart. To this end, we started working with cardiac myocyte progenitor cells (CMPCs).  

Bio-electronic pacing by pulses of light

The concept of cellular biological pacing was recently approached from a different perspective when Entcheva and colleagues modified a HEK cell line to express light sensitive ion channels.  

COMPARING FUNCTIONALITY OF THE MOST PROMISING STRATEGIES

Given the level of validation in large animal models and the outcomes in these studies, the following gene therapy-based approaches are at this stage considered most favorable: AC1, HCN1-ΔΔΔ, HCN2/AC1, HCN2/Kir2.1-ΔΔΔ, and HCN2/SkM1.  

As discussed above, each of these approaches has its individual strengths and weaknesses. Yet, it remains difficult to perform a direct comparison, because testing sometimes occurred in different animal models. Despite this limitation, it appears that the AC1- and HCN2/SkM1-based strategies offer the most robust outcomes when looking into the combination of basal function and sensitivity to autonomic modulation. In vivo studies suggest inferior sensitivity to autonomic stimuli for the HCN2/Kir2.1-ΔΔΔ- and HCN1-ΔΔΔ-based approaches.
Comparing the AC1 and HCN2/SkM1 gene transfer approaches to some of the more relevant stem cell or reprogramming strategies is at this stage difficult. This is because these latter alternatives have primarily been tested in vitro or in rodents, which makes the extrapolation to large animals difficult. However, the reprogramming approaches or methods that use iPS to obtain pacemaker-like cells probably have the strongest potential for the generation of cells that closely resemble properties of SAN myocytes. It remains to be seen if this will generate the most reliable biological pacemaker. Experiments with the transplantation of SAN cells into the right ventricle of AV-blocked dogs demonstrated suboptimal function with basal beating rates of 45-55 bpm and significant dependence on electronic back-up pacing. This suggests that SAN-like cells may not be the best impulse generator for application in ventricular tissue. On the other hand, approaches based on reprogramming of iPS-derived cells may generate biological pacemakers that operate at more depolarized potentials, which may be better suitable to pace the low $I_{K1}$ environment of the atrium.

Although baseline function and sensitivity to autonomic modulation are relevant benchmarks for the comparison of different biological pacemaker strategies, they need to be accompanied by reliable long-term function and an undisputable safety profile. Thus far, a detailed analyses of safety and long-term pacemaker stability are lacking. The HCN2/SkM1 approach will likely be followed up via delivery by lentiviral vectors or autologous stem cells such as MSCs or CMPCs. In addition, the HCN1-ΔΔΔ, HCN2/Kir2.1-ΔΔΔ, AC1 and HCN2/AC1 based biological pacemakers may be better delivered via adeno-associated virus (AAV) vectors. More definitive conclusions with regard to the most favorable biological pacemaker performance will therefore come from long-term animal trials that compare function between the different approaches. Such studies should also investigate safety and toxicity in the setting of both normal cardiac homeostasis and pathologic conditions such as ischemia, myocardial infarction, and heart failure. These studies should aid in the selection of the most favorable approach.

FROM EXPERIMENTAL STUDY TO CLINICAL TRIAL?

The field of biological pacing has shown intriguing advances over the past decade: a variety of concepts has been tested in different models. The function that is generated by some of the gene-based approaches has the potential of being superior to electronic pacing. In contrast, strategies employing reprogramming or cell therapy have not yet demonstrated this level of function and reproducibility. The reprogramming and stem cell-based approaches are therefore expected to have a longer road ahead before clinical testing can be considered. Yet, regardless of the approach, whether based on genes, transcription factors or cells, many important steps lie ahead, including optimization of well-controlled delivery methods and the demonstration of persistent and safe long-term function. Although selected systems such as AAV and lentiviral vectors have shown to be capable of introducing long-term gene expression in the heart, it remains unclear how working myocardium or bundle-branch myocytes
respond to persistent expression of pacemaker-related genes or transcription factors. In addition, serious concerns remain with regard to safety. When employing gene therapy, it would be particularly important to minimize off-target gene expression and demonstrate that the risk of insertional mutagenesis is indeed absent or very low. With regard to the use of cells, particular attention should be paid to the evaluation of potential neoplastic degeneration.

In any approach, thorough evaluation of potential proarrhythmia will be essential. Here, all strategies may have their individual downsides in relation to the introduction of structural heterogeneity or electrical heterogeneity. Kir2.1-ΔΔΔ has been demonstrated to prolong AP duration, a condition that is linked to lethal torsade de pointes arrhythmias. The elevation of cAMP induced by AC1 may impact on Ca\textsuperscript{2+} handling and could be a cause of problems in relation to Ca\textsuperscript{2+} overload and triggered activity. With the SkM1 construct, Ca\textsuperscript{2+} overload appeared not to be an issue and repolarization remained unchanged. Yet, given the bigeminal rhythms associated with bradycardias in AV-blocked animals that received SkM1/GFP, there may be a facilitation of early afterdepolarizations or reentry that becomes relevant at slow heart rates. The observation that arrhythmias did not occur in the HCN2/SkM1-based approach speaks for this strategy, yet it does not obviate the need for future efforts to analyze potential arrhythmia risks.

If safety and function can be assured, it becomes relevant to ask in which patients biological pacemakers should initially be applied. It is not expected that the biological approaches to bridge AV-nodal dysfunction will catch up any time soon with the success of biological pacing. Therefore, the initial application area will likely be that of demand ventricular pacing. Some investigators have suggested that biological pacemakers may be applied using adenoviral vectors in a setting of electronic pacemaker lead infection to bridge the time between lead extraction and re-implantation. A concern of this type of intervention is the potential facilitation of a dissemination of the ongoing infection as well as the function of adenoviral constructs to induce inflammation in their own right. Alternatively, we feel that biological pacemakers may best be utilized in a tandem approach with electronic pacemakers. This may provide the advantages of autonomically controlled biological pacing from a physiological site together with the proven back-up safety of an electronic pacemaker. An additional advantage would be the reduced power consumption of co-applied electronics, thus providing improved durability of the combined therapy. Early stage biological pacing may be applied to patients who are now primary candidates for lone ventricular pacing such as those suffering from permanent atrial fibrillation in combination with AV-block. Here, patients who have developed adverse remodeling from right ventricular pacing may particularly benefit from biological pacing generated in a proximal site of the ventricular conduction system.

What is the likelihood that some of the approaches to biological pacing will really reach the level of clinical testing? The answer to this question remains speculative, yet recent developments in the field of gene therapy suggest this to be a realistic
expectation. Firstly, advances in gene therapy for cardiac failure have been proven safe and effective in phase I/II trials. Secondly, even in complex multifactorial pathologies such as Parkinson’s disease, gene therapy has successfully completed phase II testing. Remarkably, this success was obtained in a biological approach of deep brain stimulation. Thirdly, last year’s EMA approval of Glybera (an AAV-based gene therapy for lipoprotein lipase deficiency) sparked enthusiasm in relation to the final step from successful clinical trial to market introduction of a gene therapy product. Finally, the demonstration of safety and perhaps some efficacy of cardiac progenitor cells in early stage clinical trials are another reason for optimism.

CONCLUSIONS

Developing biological pacemakers has been a scientific journey that has taught us a great deal about a variety of genes and cells, which may find its application in or outside the field of cardiac pacing. Just like McWilliam and the Hymans were not aware of their contribution to development of therapies for Adams-Stokes disease, the exact areas in which our work may be applied also remain unknown at this stage. Yet, despite potential heuristic returns, significant improvements in pacemaker function have been made, and given the current pace of progress, it is expected that the coming years will see successful biological pacing in long-term animal studies. If these studies demonstrate the pacemaker function that has already been obtained in short-term animal trials and safely provide pacemaker properties superior to those of the continuously improving standards of electronic pacing, then we are likely to see the initiation of clinical biological pacing.

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