Biological pacing by gene and cell therapy

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At present, cardiac rhythm disorders such as sick sinus syndrome (SSS) or AV nodal block (AVB) are usually treated by electronic pacemakers. These devices have significant shortcomings, including lack of autonomic modulation, and the need for repetitive procedures for battery replacement or lead repositioning. Biological pacemakers as replacement or complement to electronic pacemakers have been the subject of increasing research interest. This research has resulted in many encouraging preclinical studies. Various approaches in the field of gene and cell therapy have been developed by different groups and this combined effort makes it increasingly realistic that this therapy will eventually find its way to clinical applicability. (*Neth Heart J* 2007;15:318-22.)

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Electronic pacemakers are of great value in the therapy of cardiac conduction disease. Although these devices have become more and more sophisticated over the past years, shortcomings such as lack of autonomic modulation, limited battery life, unstable electrode position, and electronic or magnetic interference remain. If bio-engineered pacemakers are combined with an electronic pacemaker, improvements

could be obtained by adding autonomic modulation and extending the battery life of the combined entity. Eventually, biopacemakers might progress into a reliable monotherapy if full functional stability can be achieved. Given the excellent safety standards of the electronic pacemaker, quality standards for the bio-engineered version will be high. However, in the end, biopacemakers might provide a cure rather than palliation for the underlying cardiac disease.

The complex architecture and electrophysiology of the sinoatrial (SA) node is not easily imitated by gene or cell therapy. However, functional aspects have been incorporated in different strategies. The SA node is a heterogenous structure composed of specialised cardiomyocytes and a high level of connective tissue. Activity in this node is driven by a spontaneous change in the membrane potential, called the slow diastolic or phase 4 depolarisation. This membrane depolarisation results in the formation of action potentials (APs), thereby triggering the contraction of the heart (figure 1). A major current underlying this process is the 'funny current' or I_f .¹ A family of hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels is believed to underlie this inward current. There are four HCN isoforms, which are all expressed in the heart, but expression levels vary among regions.^{2,3} In the rabbit SA node, HCN4 is the dominant transcript representing more than 81% of total HCN mRNA.⁴ The activity of HCN channels is controlled by cyclic adenosine monophosphate (cAMP)-binding which allows alteration of activation kinetics by β -adrenergic and muscarinic stimulation. By this mechanism, channel activity might be increased or decreased. This plays an important role in the autonomic regulation of heart rate.⁵ However, I_f is not the only current contributing to the pacemaker cell diastolic depolarisation. Other inward and outward currents are involved as well. Essentially, any increase in inward and/or decrease in outward current may initiate or accelerate the process of phase 4 depolarisation. Based on this principle, different gene and cell therapy strategies have been developed.⁶

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Transforming normal cardiac myocytes into pacemaker cells

To transform normal working cardiac myocytes into cardiac myocytes with a pacemaker phenotype, two different strategies have been applied. In these strategies, normal stable resting

Figure 1. The contribution of I_f to sinoatrial (SA) node action potentials and autonomic modulation of heart rate. (A) Action potentials in the SA node at baseline, and after β -adrenergic stimulation. The process of slow diastolic depolarisation is mainly initiated by I_f , which is activated upon hyperpolarisation. HCN channels are the proteins underlying this current. When the threshold is reached, the action potential starts as a result of the opening of T-type and L-type calcium channels. Repolarisation occurs mainly due to the opening of K^+ currents. (B) Illustration of an SA node cell and its intracellular cyclic adenosine monophosphate (cAMP) signalling pathway. Alterations in cAMP result in regulation of HCN channel activity. β_1 -adrenergic receptor stimulation increases cAMP levels, as a result of G-protein coupled regulation of adenylyl cyclase (AC) activity, whereas the opposite is a result of M_2 -muscarinic receptor stimulation. Cyclic AMP binds to HCN channels near the amino terminus and it accelerates activation kinetics. (From Biel et al. with permission).³¹

membrane potential (RMP) is disturbed either by the down-regulation of an outward hyperpolarising current or the up-regulation of an inward depolarising current. Both will result in the spontaneous generation of APs.

Down-regulation of the outward hyperpolarising current I_{K1} that inhibits spontaneous depolarisation was achieved by the overexpression of a dominant negative construct of the inward rectifier potassium channels. These channels are abundantly expressed in the working myocardium of the atrium and ventricle, but not in the SA node, and they play an important role in repolarisation and RMP stabilisation. A

functional knock down of these channels results in RMP depolarisation, which liberates endogenous pacemaker activity.⁷ The first in vivo proof-of-concept of this approach was provided by Miake and colleagues in 2002.⁸ The dominant negative effect in this approach was a result of the replacement of three amino acids in the pore of Kir2.1. Multimerisation of dominant negative Kir2.1 subunits with wild-type subunits results in the formation of tetrameric inward rectifier potassium channels with reduced function. With adenoviral (Ad) vectors, the left ventricular cavity of guinea pigs was targeted. Three to four days after the transduction, Kir2.1AAA overexpressing myocytes demonstrated a reduction of about 80% in I_{K1} which results in spontaneous activity in these cells. Electrocardiograms of animals treated with Kir2.1AAA confirmed ectopic pacemaker activity originating from the left ventricle. One major concern regarding this strategy is that reduction in repolarising currents may result in excess prolongation of repolarisation, which constitutes a pro-arrhythmic effect, potentially causing torsade de pointes ventricular tachyarrhythmias that could degenerate to ventricular fibrillation.⁹

Loss-of-function mutations in Kir2.1 are clinically manifested in Andersen-Tawil syndrome (also known as long-QT syndrome type 7). This syndrome consists of mild QT-interval prolongation, prominent U waves on the ECG, frequent ventricular ectopy and polymorphic ventricular tachycardia, in conjunction with extracardiac features such as periodic paralysis.^{10,11} Additionally, theoretical considerations predict that a repolarisation of RMP following from reduction in I_{K1} may result in membrane potential oscillations that may culminate in cardiac arrhythmias.

Depolarisation of RMP can also be achieved by introduction of the slow depolarising current I_f . This current is believed to be one of the primary pacemaker currents in the SA node, where it is mainly generated by HCN4, and, to a smaller extent, by HCN2 and HCN1. First effects of HCN overexpression in cardiac myocytes were reported by Qu et al. in 2001.¹² These investigators showed an increased beating frequency in neonatal ventricular myocytes after transduction with Ad-mHCN2. They also demonstrated that autonomic responsiveness was retained in HCN2 overexpressing cells. After this in vitro proof-of-principle, the same vectors were used for in vivo experiments. In these experiments, sinus arrest was induced by vagal stimulation. Ad-mHCN2 injections in the canine left atrium (LA) resulted in spontaneous LA rhythms during sinus arrest.¹³

To demonstrate the potential of combined bio-electronic-pacemaker therapy, different experimental studies were conducted. In a canine model of AVB, an electronic pacemaker (EPM; set at VVI with a lower rate of 45 beats/min) was implanted together with an Ad-HCN2 based biopacemaker injected into the left bundle branch. The biopacemaker reduced the amount of electronically paced beats to 26% compared with 83% electronically paced beats in the control group (figure 2A/B). To improve autonomic responsiveness, an engineered mutant of HCN2 (mE324A) was also tested in a separate group. Although an enhanced response to epinephrine administration was seen (figure 2C), these dogs required a somewhat larger amount of electronically paced

Figure 2. Tandem biological-electronic cardiac pacemaker. (A) Representative tracings demonstrating the interaction between the HCN2-based biological pacemaker and the electronic back-up pacemaker. (B) Percentage of electronically paced beats comparing saline injected hearts with hearts injected with Ad-mHCN2. Throughout the 14-day period, mHCN2 biopacemaker animals required significantly less electronically paced beats ($p < 0.05$ for comparison at each time point). Electronically paced beats of the mE324A mutant HCN2 biopacemakers, which were constructed to improve autonomic responsiveness, did not significantly differ from the wild-type HCN2 based biopacemakers and were therefore not shown. (C) Representative experiments of 1 µg/kg per minute epinephrine infusion given on day 14. Although the mE324A constitutive mutant did not require fewer electronically paced beats, higher basal rates and a more pronounced response upon epinephrine were seen. (Modified from Bucchi et al. with permission).¹⁴

beats (36%). Reduced protein expression of the constitutive mutant, which was demonstrated by Western blotting, could provide an explanation for the larger amount of electronically paced beats.¹⁴ Others demonstrated further improvements using a mutant of HCN1 in a porcine model for sick sinus syndrome. Using adenoviral vectors, they injected this engineered HCN1 construct into the left atrial appendage of animals that also received an EPM set at VVI 60 beats/min (figure 3A/B). Here, device supported atrial pacing was reduced from 69 to 14%.¹⁵

Clearly, an uncontrolled increase in heart rate, by whichever gene therapy strategy, may cause deleterious effects on cardiovascular function. This has been made particularly obvious by recent clinical trials of the prototype I_f blocker ivabradine. In these trials, ivabradine exhibited clinical benefits when used with the aim of preventing angina pectoris through heart rate reduction. Conversely, it may be envisaged that the availability of ivabradine may be exploited to fine tune the pulse rate when used in conjunction with HCN-based gene therapy for the creation of a biopacemaker.^{16,17}

Stem cell based biopacemakers

In the search for biological pacemakers, both embryonic stem cells (ESCs) and human mesenchymal stem cells (hMSCs) have been studied by several investigators. They hypothesise that the complexity of the SA node cannot adequately be reproduced by simple gene transfer methods. Building on this hypothesis, ESCs could provide excellent candidates, because these cells are pluripotent, i.e., they can differentiate into any cell type, including nodal cells. Human MSCs, on the other hand, might be closer to clinical application, because they have been used in several trials and there is the additional advantage of these cells possibly being immunoprivileged. Human MSCs have not elicited major immune responses so far.¹⁸ However, hMSCs are multipotent, i.e., they are, in contrast to ESCs, only able to differentiate into mesenchymally derived lineages. Therefore, these cells need genetic modification to incorporate pacemaker properties. In addition to pacemaker properties, both stem cell types obviously require electrical integration into the host myocardium to ensure propagation of pacemaker activity. This integration does not involve further engineering, because both ESCs and hMSCs form functional gap junctions with adjacent myocytes due to abundant connexin expression.

The use of ESCs to create biological pacemakers was first reported by Kehat and colleagues. They derived spontaneously beating human embryoid bodies (hEB) from cultured ESCs and implanted these into the ventricles of swine with complete AV block. Stable pacing was observed in about half of the animals. The remaining animals had, unfortunately, only frequent ectopic beats originating from the implantation site.¹⁹ Others also failed to demonstrate reliable pacing at a physiological rate using this strategy.²⁰ Besides feasibility problems, much has been written about the sociopolitical fear surrounding the use of these cells, and more technical concerns regarding the requirement for additional immunosuppressive treatment.^{21,22}

Figure 3. Left anterior view of 3D electroanatomical maps of experimental animals with sick sinus syndrome during RA pacing (A) and after Ad-HCN1- $\Delta\Delta\Delta$ injection (B). The intra-atrial septum was tagged with black dots and delineated with white dotted lines. During sinus rhythm, the earliest endocardial activation started in the junction between superior vena cava (SVC) and right atrium (RA) (not shown). During RA pacing, earliest activation started in the high anterolateral wall (red asterisk in A) and propagated to the LA appendage (LAA) and inferior vena cava (IVC). Spontaneous LA rhythms were observed after successful HCN1- $\Delta\Delta\Delta$ gene transfer. Earliest activation was mapped on the Ad-HCN1- $\Delta\Delta\Delta$ injection site marked by a white arrow and propagated to the RA appendage (RAA), SVC and IVC. (Modified from Tse et al. with permission).¹⁵

The use of hMSCs in the creation of biological pacemakers was first reported by Potapova and colleagues. These cells were loaded with HCN2 channels to incorporate pacemaker properties and were subsequently injected subepicardially into the left ventricular wall of dogs. During vagally induced sinus arrest, faster escape rhythms developed in the hMSCs-HCN2 injected dogs with a rate of approximately 50 beats/min.²³ Although hMSCs are possibly immunoprivileged, doubts remain regarding differentiation, migration and survival over time. It is possible that these cells differentiate into cardiac cells, but differentiation into other cells can not be ruled out.²⁴

Clinical perspective

If a biological pacemaker is to compete with current therapy, various requirements have to be fulfilled. To provide an improvement over electronic pacemakers, incorporation of

autonomic modulation is crucial. If one of the HCN channels is selected, direct autonomic modulation of channels activity will occur via adrenergic or muscarinic receptor pathways. In addition, the site of implantation is important. In electronic pacemakers, implantation sites are restricted to areas where stable lead positions can be obtained. With the gene and cell-therapy approaches, it is anticipated that there is much more freedom to choose a suitable position. This is an advantage if there is cardiac comorbidity, and arrhythmogenic substrates are present. Although in these patients, the best avenue with minimal arrhythmic potential could be selected via catheter-based intra-cardiac mapping, careful experimental investigations of different implantation sites should be performed first.

Two other issues are important: duration of effect and bio-safety. The functional duration of pacemaking should be comparable with current (and future) lifespans of the batteries

Figure 4. Human mesenchymal stem cell (hMSC) based biopacemaker. (A) Left panel shows an SA node cell connected via gap junctions to an adjacent myocyte. Action potentials (inset) are importantly initiated by slow diastolic depolarisation resulting from current flowing through HCN channels. Right panel shows HCN channels over-expressed in hMSCs. These channels are activated by membrane hyperpolarisation originating from the adjacent myocyte. Channel activation will result in excitation of the adjacent myocyte via gap junctions. This depolarising current results in action potential formation in the adjacent myocyte, but not in the hMSC, since hMSCs lack the set of ion channels which is required to create an action potential. (Modified from Rosen et al. with permission).⁶ (B) hMSC-based biopacemaker 7 days after implantation in the LV anterior wall epicardium of a dog. Left to right; ECG leads I, II, III, AVR, AVL and AVF. Left panel shows last two beats in sinus rhythm and sinus arrest resulting from vagal stimulation. Middle panel shows a regular idioventricular escape rhythm of approximately 50 beats/min. Right panel shows a postvagial sinus tachycardia. (Modified from Potapova et al. with permission).²³

that are used in electronic pacemakers. In a gene therapy approach, this requires stable and long-term expression of the transgene, which might be gained by the use of long-term expression vectors such as lentiviral vectors or adeno-associated virus (AAV) vectors. Both vectors have demonstrated sustained gene expression in a variety of organs including the heart.²⁵⁻²⁸ However, the feasibility of long-term biopacing with these vectors needs further investigation. When stem cells are used, more insights into the survival, migration and dedifferentiation after implantation are equally important. Recent advances in the fluorescent labelling of stem cells with nanocrystals might provide a solution by allowing fate mapping of these cells in vivo.²⁹ Nevertheless, long-term efficacy studies for both gene and cell therapy are required to demonstrate stable biopacing over extended periods of time. These studies are currently performed by various laboratories, including ours.³⁰

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