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Stem Cell Based Biological Pacemakers From Proof of Principle to Therapy: a Review

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Abstract

Electronic pacemakers are the standard therapy for bradycardia related symptoms but have shortcomings. Over the past 15 years experimental evidence has demonstrated that gene and cell-based therapies can create a biological pacemaker. Recently, physiologically acceptable rates have been reported with an adenovirus-based approach. But adenovirus-based protein expression does not last more than 4 weeks, which limits its clinical applicability. Cell-based platforms are potential candidates for longer expression. Currently there are two cell based approaches being tested: 1) Mesenchymal stem cells used as a suitcase for delivering pacemaker genes and 2) Pluripotent stem cells differentiated down a cardiac lineage with endogenous pacemaker activity. This review examines the current achievements in engineering a biological pacemaker, defines the patient population for whom this device would be useful and identifies the challenges still ahead before cell therapy can replace current electronic devices.

Keywords

cell therapy; gene therapy; arrhythmia therapy; pacemaker

Introduction

Stem cell-based therapy has gained increasing attention over the last 10 years, with most efforts focusing on cardiac repair after myocardial infarction (MI). A 2012 review of 50 studies including 2625 patients with ischemic heart disease has demonstrated a significant long-term improvement in cardiac parameters (left ventricular ejection fraction, infarct size, end diastolic diameter and end systolic diameter) and a possible decrease of the cardiac mortality in patients treated with stem cells derived from bone marrow (1). Alternatively, stem cell based therapy for arrhythmias or conduction disorders is still in its infancy and has

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Disclosure of interest

We have no disclosure of interest to declare.

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never been tested in humans. Several studies performed in vitro and in large animal models have provided proof of concept that both gene-based and cell-based therapies are effective platforms to re-create a biological pacemaker. But none of these strategies has fulfilled the high safety and quality requirements needed for clinical translation. In this review, we will focus on stem cell based biological pacemaker engineering from proof of principle to therapy. To better address the field 5 questions are considered: (a) why is a bio-engineered pacemaker needed, (b) what is a biological pacemaker, (c) what lessons have we learned from gene-based biological pacemaker engineering (d) which stem cells can be used and finally (e) what requirements must a biological pacemaker fulfill to compete successfully belly-to-belly with its electronic counterpart in human RCT?

Why is a biological pacemaker needed?

Electronic pacemakers are currently the standard therapy for symptomatic bradycardia-related symptoms or in the presence of heart failure associated with wide QRS and severe left ventricular dysfunction (2). In the 1950's, electronic pacemakers were cumbersome external devices associated with high complication rates. Device miniaturization, transvenous insertion, demand rather than fixed rate function and battery-life improvement were followed by wide spread use of the electronic pacemakers for symptomatic high degree atrio-ventricular block (AVB) in the late 1960's. Despite the formidable progresses achieved in electronic pacemaker engineering and implantation techniques, there are still shortcomings:

- a. Electronic pacemakers "per se" have no physiological autonomic responsiveness leading to a lack of rate adaptability during stress, emotion or exercise. Rate-adaptative pacemakers have improved the exercise tolerance of patients but are not a substitute for autonomic responsiveness.
- b. There is a need for regular pacemaker unit testing and replacement.
- c. Potentially lethal complications can occur during both the perioperative period (pneumothorax, hemothorax, hemopericardium) and after (pacemaker related infective endocarditis, lead fracture imposing a lead replacement). Right ventricular apical pacing can lead to pacing induced cardiomyopathy (3). Septal and infundibular approaches are promising but the long-term effects are still unknown.
- d. Electromagnetic interferences with pacemakers in medical and non-medical environment have been reported (4).
- e. There is no ideal pacemaker for children. Thus the same-size unit designed for adults is implanted. In addition a surgical procedure for epicardial lead placement can be required for low weight children. This procedure is painful and associated with a higher risk of lead failure (5).

The primary biological pacemaker: the sino-atrial node

The rhythm of the human heart resides in the cardiac myocytes. This means that all the channels and transporters necessary to initiate and sustain pacemaker activity are resident in the heart. The autonomic nervous system modulates heart rate but does not initiate it. During

the 80 years-lifetime of a human being the heart beats roughly 3.5 billion times. In normal conditions most of these beats are initiated in a highly specialized and heterogeneous structure called the sino-atrial node (SAN) (6). The electrical impulse is then transmitted to the atrias and from them to the ventricles through the atrio-ventricular node (AVN). Conduction through the AVN is very slow which allows atrial contraction to help in filling the ventricles, thus optimizing cardiac output.

The origin of the heartbeat has been one of the most exciting cardiac fields of research for more than a century. The anatomical description of the SAN by Keith and Flack (7) in 1907 and the application of the Hodgkin and Huxley model from the squid axon to the cardiomyocyte roughly 50 years later (8) have been critical steps towards understanding pacemaker activity and conduction of the cardiac action potential. In the early 1980's the "outward potassium-conductance-decay" theory was replaced by the view that an inward funny current I_f (also called the pacemaker current) was activated during pacemaker depolarization (phase 4) of the SAN action potential (9,10). Nevertheless this I_f -focused theory is widely debated (11). Experimental and computational evidence (12) have stressed the importance of other currents such as the late or transient calcium current ($ICaT$ or $ICaL$ respectively) (13), the persistent tetrodotoxin sensitive sodium current (14), the rapidly delayed potassium current (15), and the Na/K pump (16). The development of submembrane calcium imaging performed simultaneously with the patch clamp technique (17) led some authors to hypothesize that the pacemaking process results from a complex interplay between both the currents generated by the membrane channels ("voltage clock") and the calcium homeostasis ("calcium clock") (18).

Importantly, when the SAN or the AVN fail, the heart does not (always) stop. It is usually driven by a secondary biological pacemaker whose rate varies depending of its location: an impulse originating from the ventricles will be slower than one coming from the atria or the AVN. This is primarily determined by the distribution and the biophysical properties of the inward current I_f and the opposing outward background current I_{K1} . The activation curve of I_f is negatively shifted in the ventricles (19) while I_{K1} current is larger compared to the AVN leading to a more negative maximum diastolic potential, less net inward current and a reduced or absent pacemaker depolarization as one proceeds distally in the ventricular conducting pathway (20). One requirement for biological pacing (whether native or induced) is the existence of net inward current. The smaller the inward currents or the larger the outward currents are, the slower the spontaneous rate is.

The gene-based biological pacemaker: a built road

The gene-based biological pacemaker provided proof of concept as well as *in vivo* evidence that a biological pacemaker was feasible. The 3 initial approaches consisted of (a) overexpression of β -adrenergic receptors (21), (b) down-regulation of the outward, hyperpolarizing current I_{K1} (22) and (c) overexpression of inward depolarizing current I_f (23).

Edelberg et al. used a healthy pig model and atrial injection to overexpress the β_2 -adrenergic receptor. This increased sinus rate by 50% (21). This strategy enhanced the risk of

worsening supra-ventricular arrhythmias (particularly in the setting of sinus sick syndrome where atrial bradycardia and atrial tachycardia coexist) and was *a priori* not pursued since it required a functional native biological pacemaker as the starting point.

Miake et al. were the first to employ ion channels as a biopacemaker target. They reduced the outward current I_{K1} by expressing a dominant negative subunit. This converted a quiescent ventricular preparation with no net current flowing during phase 4 to one with net inward current creating a spontaneous depolarization to the activation threshold for an action potential (24). Effectively, the injection in the left ventricular cavity of guinea pigs induced ventricular arrhythmias in 50% of the cases while isolated transduced cells showed a 90% reduction of their I_{K1} current and spontaneous depolarization of the membrane potential during phase 4. The major concern of the study was the prolongation of the QT consistent with the phenotype of the type 7 inherited long QT syndrome (Anderson-Tawil syndrome), which increases the risk for lethal ventricular arrhythmias (25).

As stated above, the alternative to reducing outward current is to increase inward current. This approach focused on I_f overexpression. Since I_f rapidly deactivates on depolarization it had little or no impact on the action potential duration. The HCN gene which forms the alpha subunit of the I_f channel has a cAMP binding site and is thus responsive to the autonomic nervous system. Its autonomic responsiveness and its lack of effect on the action potential duration make this approach attractive. The first success delivering I_f to cardiac myocytes was obtained *in vitro* when Qu et al reported that neonatal rat ventricular myocytes infected with an adenoviral HCN2 had a spontaneous beating rate faster than control cardiomyocytes (26). This same group then demonstrated that canine left atrial injection of adenovirus containing HCN2 + GFP induced a faster atrial escape rhythm compared to controls (GFP alone) during a transient vagally induced asystole (27). Similar results were obtained when the construct was injected into the canine left bundle branch (28). The group injected with the adenovirus expressing HCN2 + GFP had a faster junctional rhythm (matching the site of injection) than control dogs during a transient vagally-induced AVB. Finally the I_f -strategy was tested in a canine model of induced AVB implanted with an electronic pacemaker set at 45 bpm. The dogs injected with the wild type or mutant HCN2 (mE324A) channel had significantly less electronic back-up beats than control dogs (GFP alone) (26% vs 36% vs 83%, respectively) and a greater increase in HR following epinephrine injection especially in the E324A group (29). Autonomic responsiveness was also revealed by the analysis of the heart rate variability after presenting food (30). Importantly, in the previous studies, the presence of the HCN protein/ I_f expression was systematically demonstrated through histological, immuno-histochemical or electrophysiological analysis of the explanted heart. The potential applicability of the I_f engineering strategy was supported by a report from another group that injected an HCN1 mutant into the left appendage. This injection was able to reduce the percentage of atrial pacing from 69% to 14% in a porcine model of sinus sick syndrome instrumented with a dual chamber pacemaker set at 60 bpm (31).

Because the I_f -strategy was promising but unable to replace its electronic counterpart, efforts have focused on optimization by (a) creating HCN mutants (29,32) and/or (b) co-expressing another determinant of the pacemaker initiation (33–36). The most striking results have been

obtained by expressing both HCN2 and SkM1 in adenoviruses and then injecting them into the left bundle branch. With this strategy, dogs implanted with a VVI pacemaker set at 35 bpm had no dependence on electronic back-up, a resting rate around 80 bpm and a brisk response to autonomic influences (36). Nevertheless, the adenovirus-based protein expression is not expected to last more than 4 weeks and risks of neoplasia have been raised with the use of longer term expressing lentivirus.

Recently, another gene-based strategy has been explored. Kapoor and al reported that Tbx18-loaded adenovirus could reprogram rodent ventricular myocytes into spontaneously active cells indistinguishable from sino-atrial node cells (37). They investigated the potential *in vivo* applicability of this strategy in 2 ways. First, they injected Tbx2 into the apex of guinea pigs. 2–4 days later, upon inhibition of sinus node function with metacholine, they observed ectopic ventricular rhythm originating from the apex in 5/7 Tbx2-injected guinea pigs and 0/5 in the control group. Second, persistence of *in vivo* function 3–4 weeks after injection was investigated in a model of isolated perfused atrio-ventricular blocked heart. They found that 100% (8/8) of the injected perfused heart showed beats matching the site of implantation of the construct (37). Because a significant proportion of induced-SAN cells were still present 6–8 weeks after gene transfer, the authors hypothesized that Tbx18 could induce a genuine reprogramming. It is unknown if SAN-like cells observed 6–8 weeks after injection would result in *in vivo* pacemaker function. Finally, large-animal studies are needed before this strategy is considered a suitable strategy for biological pacemaker engineering.

At present in the best case scenario, adenoviral-based biological pacemakers (which become functional 24 to 48 hours after injection) could reduce the time needed for temporary external pacing when an infected device must be extracted. However, reprogramming with adenovirus would allow shorter term viruses to result in potentially longer term outcomes. If cells that are delivered are retained, it is the hope that cell-based approaches might permit longer term, safe expression that might overcome these limitations.

Which stem cells for what strategy?

The term “stem cells” refers to a subset of cells sharing the ability to renew and differentiate. However different stem cell types possess differences in lineage potential. For instance, embryonic stem cells (ESCs) can self-renew indefinitely *in vitro* and differentiate into derivatives of the 3 different germ layers while human mesenchymal stem cells (hMSCs) have only limited differentiation and regeneration capacities (38,39). Stem cells can be categorized according to their site of origin, their potency or the stage in development they come from. To date two strategies have prevailed to construct a stem cell based biological pacemaker: or (a) the injection of a cluster of inexcitable cells expressing an inward current that will depolarize the contiguous cells to their action potential threshold or (b) the injection of a cluster of excitable cells with the ability of generating spontaneous action potentials. In the first case, at least 2 cells (donor + host) are required to create a pacing unit while in the latter the delivered cells are the biological pacemakers. Both strategies assume that integration of delivered cells will occur through the formation of gap junctions between both donor cells themselves and between donor and target cells. These gap junctions are the basis

of intercellular current flow which allows the delivered cells to partake in the electrical activity of the newly created heterogeneous syncytium.

MSC and the 2 cells pacing unit theory

In 2004 Potapova et al. demonstrated that hMSCs can be transfected with one of the pacemaker genes (mHCN2) by nucleoporation and used as a suitcase to deliver an I_f -like current to the resident canine ventricular myocytes which lacked this current in the physiologic voltage range (38). HMSCs are relatively immunoprivileged, electrically quiescent and express two cardiac gap junctional proteins, connexins (Cx) 40 and 43 (40). By expressing mHCN2 in the hMSC, it was expected that an inward I_f -like current would be expressed. This current would flow into the ventricular cells electrically coupled to the transfected hMSCs. When the cardiac myocytes hyperpolarized between contractions, I_f would be activated, spreading inward current into the electrically coupled myocytes and depolarizing these cells to the threshold for action potential firing. This was confirmed in two ways. First HCN2-loaded hMSCs co-cultured with rat neonatal myocytes induced an increase in the spontaneous beating rate from 93 ± 16 bpm in control to 161 ± 4 bpm ($p < .05$) while morphological gap junction formation was demonstrated with immunostaining analysis (41). Second, Valiunas et al. quantitatively examined the critical role that the gap junctions play in a 2-cell pacing unit comprised of an HCN2 delivery cell and a target adult canine ventricular myocyte. Briefly, non-excitabile cells (3 types) expressing Cx43 (either constitutively or after transfection) were transfected with HCN2 and co-cultured with canine adult ventricular myocytes (42). Experiments employing the dual patch clamp technique coupled with pharmacologic inhibition demonstrated that if one blocked I_f in the delivery cell pacing stops and is restored on washout. Similarly if the intercellular electrical coupling created by the gap junctions was blocked pacemaker activity was also eliminated until the gap junction uncoupler was removed. These experiments also demonstrated that a threshold of intercellular connectivity ("gap junction conductance") of about 3nS was critical for the 2 cells pacemaker unit to function, while increasing this electrical connectivity had little or no effect on the pacemaker rate. The ability of hMSCs to create a biological pacemaker was demonstrated in a set of large animal model experiments. 4 to 10 days after sub-epicardial left ventricular injection of up to one million hMSCs expressing either HCN2 + EGFP or EGFP alone, a vagally mediated complete AVB was induced. The rhythm of the escape beats in the HCN2 + EGFP group was faster than the control group (61 ± 5 vs 41 ± 5 bpm, $p < 0.05$) (38). Finally, in 2007, Plotnikov et al. employed a model of canine induced AVB (backed-up with a single ventricular lead pacemaker set at 35 bpm) and created a biological pacemaker by delivering up to 1 million hMSC-HCN2 cells to the left canine ventricle and having them couple to resident ventricular myocytes (43). The biological pacemaker was functional during the 6-week follow-up period with a physiological efficiency correlated with the number of cells injected. Nevertheless the biological pacemaker provided at most only 70% of the recorded beats stressing the need for optimization. Two independent groups have recently reported the feasibility of the stem cell approach in a set of experiments that slightly differed from the above studies (44,45). Concerns have been raised and discussed about the risk of infection, neoplasia, and further differentiation over time (46). All that is

presently known is that the histological analysis of injected areas in the Plotnikov study at six weeks demonstrated no signs of inflammation or rejection (43).

Recreating a SAN function with pluripotent cells

The second stem cell strategy consists of delivering a cluster of excitable cells that will depolarize the contiguous resident cardiac myocytes to their action potential threshold potential. Structural and electrical complexity of the SAN makes it unlikely to be reproduced. Thus, research efforts have focused on the possibility of creating an homology to the SAN by inducing pluripotent stem cells to differentiate into spontaneously beating cardiomyocytes. Various differentiation protocols are available (47) providing heterogeneous cells populations comprised of atrial, nodal, and ventricular phenotypes whose electrophysiological properties change through maturation (48). Kehat et al. were the first to demonstrate that excitable hESC-derived cardiomyocytes obtained through embryoid body (Eb) differentiation could integrate *in vitro*, form gap junctions, and provide a sustainable autonomously responsive *in vivo* biological pacemaker in 50% of their AV node blocked pigs (49). Xue et al. also demonstrated the functional integration of the cardiomyocytes derived from hESC by co-culturing them with electrically quiescent cardiomyocytes but failed to demonstrate the potential applicability of this strategy in an isolated guinea pig Langendorff system (50). Due to the heterogeneous nature of the Ebs (41), some groups have focused their effort on specifically isolating sino-atrial nodal type cells (52). But attempts to autologously graft the SAN or inject SAN myocytes failed to provide sustained biological pacemaker activity, highlighting the critical role of the substrate (53,54).

In 2006, Takahashi and Yamanaka first reported the possibility to reprogram adult mice fibroblasts into undifferentiated cells by introducing into their genome four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) (55). This discovery was welcomed with great enthusiasm as it carried the promise of autologous regenerative therapies without ethical debates and need for immunosuppression. Beside the therapeutic potential of these pluripotent cells, they also create another avenue of investigation for mechanistic understanding of a patient's specific disease further reinforcing the concept of "personalized medicine" (56,57). Since then, the technique has been expanded to human cells (58,59) and it has been demonstrated hESCs and iPSCs share many similarities including morphology, feeder dependence, surface marker expression, telomerase activity, and *in vivo* teratoma formation (60). Yet, ESCs and iPSCs are not identical differing in their *in vitro* differentiation potential or their epigenetic status (61).

To date there is no report of successful use of iPSCs to create a biological pacemaker *in vivo* but some authors have published promising data on their functional behavior. Mandel et al. demonstrated that iPSC and hESC derived cardiomyocytes were both spontaneously active, showed intrinsic heart rate variability, and were responsive to isoproterenol and carbamylcholine (62). Even if these SAN like rhythmic properties are promising, it remains to be seen whether they are a suitable cell type for *in vivo* use.

Requirements and challenges for a clinical translation

The potential for long-lasting expression of the transfected genes in the hMSCs and the spontaneous activity in the pluripotent stem-cells differentiated down a cardiac lineage present the possibility of a “cure” while the electronic pacemaker is only palliative. The gene-based therapy has been a wonderful proof of principle and demonstrated that genetic manipulation based on understanding the biophysical underpinnings of pacemaker activity is an effective building block strategy (36). In order to compete with electronic devices, biological pacemakers will have to fulfill higher safety and quality standards than have thus far been achieved. We propose at least the following minimum requirements before biological pacemakers can be considered a candidate for clinical trials:

- a. The construct must have stable functional expression for at least 1 year and must not migrate from the site of implantation.
- b. It must confer no risk of infection/neoplasia.
- c. It must have a lower rate of complications than electronic devices. This implies a safe technique of implantation. So far, due to the size of the construct, cell-based biological pacemakers have been delivered via a thoracotomy followed by an epicardial injection but modifications in catheter technology should allow transvenous access.
- d. Brisk and robust autonomic responsiveness. This is of critical importance for normal activities and for the response to stress.
- e. Absence of significant overdrive suppression. Although the sinus node is minimally overdrive suppressible, ventricular pacemakers are not. The biological pacemaker must not pause for more than 1 second independent of its implantation site.
- f. It must be implanted in a pre-specified ventricular site that is selected based on the etiology of the bradycardia. Ideally the site should be individualized to optimize cardiac output for each patient.
- g. It must not induce any arrhythmias. This requirement imposes a careful analysis of the gene and/or cell type and its expected effects on the action potential duration.
- h. Bio-engineered cells should be safely and easily accessible to catheter ablation. This is particularly meaningful if (I) a patient needs an “upgrade” of his pacing unit from a biological pacemaker to a multisite pacemaker with defibrillator in order to limit the competition of rhythm or (II) a biological pacemaker- related arrhythmia is suspected.

None of these criteria has been met unambiguously in the long term as testing of these candidate biological pacemakers has been limited to only a few months. Long term large animal studies are needed, coupled with extensive electrophysiological and histological analysis. It is also worth identifying the indications for biological pacing in an effort to delineate the future clinical inclusion criteria. It is of note that all studies performed with the AVB model have tested a single-site bioengineered pacemaker (either injected into the atria

or the ventricle) excluding (so far) any atrio-ventricular synchrony. Yet, we identify the potential candidates (as well as those to be excluded) as follows:

- a. The best candidates are the patients for whom atrio-ventricular synchronization is impossible to obtain. Typically this patient has high degree AV block associated with permanent atrial fibrillation. It would only be suitable when pulmonary vein isolation is not indicated.
- b. Additional potential candidates are patients for whom AV synchronization is not necessary. One such example is an elderly patient whose exercise was already restricted prior to the occurrence of AV block.
- c. *A priori* this strategy does not apply to patients for whom a synchronized AV contraction is hemodynamically needed and even less to patients who might benefit from multisite pacing. This group includes patients with cardiomyopathy for whom the atrial systole provides a significant improvement of ventricular filling and thus a better ventricular stroke volume.

Conclusion

Stem cell based biological pacemakers have demonstrated function *in vivo* but have not passed any of the longer term criteria required for translation to a meaningful therapy. Researchers have designed and tested a wide variety of gene constructs and a few stem cell approaches as well. The current results are imperfect and long-term trials are absent. However, advances in stem cell biology plus optimization of delivery systems should make long-term trials possible. Although not in our immediate future it is reasonable to suggest that biological pacemakers generated from stem cells may one day be the standard of care to treat symptomatic high degree atrio-ventricular block.

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