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Reprogramming the conduction system: Onward toward a biological pacemaker

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Abstract

Diseases of the cardiac conduction system can be debilitating and deadly. Electronic pacemakers are incredibly effective in the treatment of sinus and AV node dysfunction, yet there remain important limitations and complications. These issues have driven interest in the development of a biological pacemaker. Here, we review experimental progress in animal models and discuss future directions, with a focus on reprogramming endogenous cells in the heart to treat defects of rhythm and conduction.

Reprogramming the conduction system: Onward toward a biological pacemaker

The incredible evolution of electronic cardiac pacemakers permits today's cardiologists and their patients the luxury of *almost* taking them for granted. Before the advent of cardiac pacing, atrioventricular block without an adequate ventricular escape rate was hopelessly debilitating and lethal among adults after myocardial infarction and in children after open heart surgery for septal defects. In the 1950's, Zoll described the first application of external ventricular pacing via subcutaneous leads among 14 patients suffering from Stokes-Adams attacks. Amazingly, five patients survived to come off pacing after the onset of an adequate

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idioventricular rate.[1, 2] At least three patients required narcotic sedation to withstand the pain of transcutaneous pacing, which Lillehei recognized made it impractical for use in children. When he and Bakken demonstrated temporary, epicardial ventricular pacing for transient postoperative heart block in 1957[3], they set into motion solutions from numerous investigators that ultimately led to the development of modern pacemakers. Their first pacemaker was external, bulky and powered from an electrical outlet. This was soon followed by a palm-sized pacemaker with a self-contained battery.[4] To solve the problem of ascending infection via the lead sites, Senning and Elmqvist developed and placed the first fully implantable system, although the battery quickly failed.[4] Chardack, Gage and Greatbatch followed with an implanted pacemaker whose battery could last five years.[5] Their 1960 New England Journal of Medicine paper prompted Bakken's brother-in-law to fly his own plane to Buffalo to buy their patents for their young company, Medtronic (<http://www.medtronic.eu/about-medtronic/our-story/growth-spurt/our-first-pacemakers/index.htm>). In the same period, Furman and Schweder proved the feasibility of transvenous, endocardial pacing, which ultimately obviated the need for thoracotomy to place epicardial leads.[6]

These earliest pacemakers provided single chamber pacing to the right ventricle. Over time, it came to be recognized that the consequent AV dyssynchrony could impair hemodynamics and eventually lead to ventricular dysfunction. The advent of dual chamber pacemakers providing synchronized AV delay improved cardiac function in these patients. More recently, the detrimental effects of ventricular dyssynchrony created either by chronic right ventricular pacing or in selected patients with left bundle branch block and heart failure have also come to be recognized. Biventricular electronic pacemaking can improve ventricular synchrony in these individuals, thus providing a potential treatment adjunct for congestive heart failure. However, the technology is expensive, and it is difficult to predict who will respond to the therapy.

Pacemakers have thus evolved over the years to substitute effectively for the sinus and AV nodes, to maintain atrioventricular and biventricular synchrony, and to provide some degree of heart rate responsiveness. Nevertheless, there remain important shortcomings, including the risks of lead failure, device and lead damage due to trauma, venous stenosis, infection, and the need to replace the generator every few years. In addition, pacemakers do not perfectly mimic the normal heart rate response to emotion and isometric or very high-intensity exercise. Furthermore, because electronic ventricular pacemakers do not directly activate the native His- Purkinje network, it will be difficult to achieve physiological biventricular synchrony. These shortcomings have motivated experiments in animal models that lay the groundwork for biological pacemakers. We review here early efforts, such as channel-based gene therapy and cell-based approaches, as well as more recent experiments that apply insights from cardiac developmental biology to reprogram cells in the postnatal heart.

Channel-based Gene Therapy

The nature of the ionic currents that underlie distinct action potentials in pacing and contractile cardiac myocytes has long been well understood. Early work in biological

pacemakers attempted to induce ectopic automaticity via adenoviral-mediated transfer of genes expressing ion channels. In one of the earliest attempts, Miake, Marban and Nuss[7] injected a dominant negative mutant construct of Kir2.1 to suppress the I_{K1} current. Ventricular automaticity did increase when the mutant construct was injected into the left ventricular chamber of guinea pigs under aortic cross clamp. Rosen and colleagues alternatively targeted the pacemaker current I_f using an adenovirus that expressed a mutant HCN2 channel. They similarly demonstrated increased automaticity within the left atrium or left bundle branch when the construct was injected at either site in dogs[8, 9]. Both groups provided important proofs-of-principle for biological pacemaking, but this work also highlighted significant limitations to this type of approach. The first is the relatively transient nature of pacemaking activity, likely due to suppression of the transduced gene or to the clearance of virally-transduced cells by the immune system. In addition to fully recapitulate pacemaker function, it seemed likely that a broader complement of ion channels and associated genes such as receptors would be more physiologic.

Exogenous Cell-based Approaches

Transplantation of pacemaking cells into the heart could provide that complete and persistent function. In the first demonstration by Ruhparwar and colleagues in 2002[10], canine fetal atrial cardiomyocytes, including the sinus node, were injected into the left ventricle of adult dogs. Three weeks later, the AV node was ablated and an epicardial pacemaker was placed to ensure a minimal backup rate. Dogs that were injected with fetal atrial cardiomyocytes developed a ventricular escape rhythm of 70 bpm that originated from the injection site, whereas dogs injected with fibroblasts were pacemaker-dependent. Immunohistochemical studies suggested that the donor fetal cardiomyocytes were electrically coupled to recipient myocytes via gap junctions. This study showed that cell transplantation of a biological pacemaker is possible, but did not explore how long the transplant would survive beyond the electrophysiologic study performed immediately after AV node ablation.

Rosen and colleagues showed that transplanted human mesenchymal stem cells that express HCN2 could survive at least six weeks after injection into the left ventricle of dogs without immunosuppression.[11] The AV node was ablated and a backup electronic ventricular pacemaker was implanted. Cells were injected two days later; the number injected ranged from 200,000 to 1 million. A stable ventricular rhythm originating from the implantation site arose 10–12 days after injection. The rate increased with an epinephrine infusion, a sign of the autonomic responsiveness of the cells. Of note, cell engraftment was unreliable below 700,000 cells, a problem that could limit the effectiveness of cell-based approaches.

Transplanted cells as a source of a biological pacemaker could also pose serious complications. The risk of arrhythmias is clearly illustrated in a study by Murry and colleagues[12], where human embryonic stem cells (hESCs) were injected into the infarct border zone of non-human primates to study myocardial repair. During the two week period between infarction and hESC injection, no arrhythmia was seen. Following injection of hESCs, however, all the animals developed ventricular arrhythmias. Ventricular arrhythmias were not observed during similar studies by these investigators in smaller animal models,

such as guinea pig.[13] The larger primate heart may permit the development of reentrant loops, especially in the setting of poorly vascularized and hence ischemic, transplanted cells.

Direct Reprogramming to an Induced Conduction-like Phenotype

Although the electrophysiologic properties of various types of cardiac myocytes have been studied for decades, the relative inability to observe the embryonic development of the conduction system had previously hampered efforts to study its specification and electrical programming. Kupersmidt and Roden provided a key breakthrough in 1999 when they demonstrated that the sinus node, central and peripheral conduction systems in the *minK* (*Kcne1*) knockin mouse express the bacterial gene that replaced the *Kcne1* coding sequence. [14] Any cell that expresses the bacterial *lacZ* turns blue in the presence of a chromogenic substrate, permitting visualization of the entire conduction system without difficult and tedious histologic methods. Using the *minK-lacZ* mouse as a tool enabled the discovery of the roles of *Nkx2-5* and *Tbx5* in the development of the AV node, proximal bundle branches and Purkinje system.[15, 16] With these initial studies, the concept was born that postnatal conduction defects could arise directly from abnormal embryonic transcriptional programming. In the subsequent decade, roles for more than a dozen transcription factors and developmental signaling molecules have now been described to be important for the formation and postnatal function of the pacemaking and conduction systems.[17]

Recent efforts to develop a biological pacemaker have focused on reactivating developmental pathways to convert one postnatal cardiac cell type into another. Once a cell is “reprogrammed”, it should no longer require the continued expression of genes that induced the new cellular phenotype. Notch signaling was demonstrated to regulate central conduction system development in zebrafish and mouse[18, 19], and the concept of a developmental pathway enabling transdifferentiation of cardiomyocytes into another cell type was demonstrated in mouse and cell culture experiments in 2012, where activation of Notch signaling in ventricular cardiomyocytes reprogrammed them to adopt a Purkinje-like phenotype.[20]

The developmental biology of the sinus node would obviously be most relevant to a biological pacemaker. Good evidence highlights the functions of the transcription factors *Shox2*, *Tbx3*, and *Tbx18*, and canonical Wnt signaling in the embryonic specification and maturation of the sinus node.[21–23] Kapoor and colleagues applied this knowledge to reprogram ventricular cardiomyocytes to an induced sinoatrial node (iSAN) phenotype by direct injection of a *Tbx18* adenovirus into the ventricular apex of adult guinea pigs.[24] The ectopic pacemaker was slower than the native sinus node, but its rhythmic activity was clearly observed once sinus rhythm was pharmacologically suppressed. The cellular morphology and electrophysiology of *Tbx18*-transduced ventricular cardiomyocytes resembled nodal cells with a flat, tapering spindle shape and a significantly shorter action potential duration and prominent diastolic depolarization. In addition, the iSAN demonstrated a physiologic response to isoproterenol and acetylcholine, as a normal sinus node would.

To demonstrate that the observed phenotypic changes remain stable in the absence of continued gene expression, the authors identified spontaneously beating myocytes in a

dissociated preparation. Of 5 beating cells, 4 expressed very low levels of TBX18. The cells carried epigenetic marks consistent with activation of the *Hcn4* promoter, a channel specifically expressed in the sinus node. One caveat of this result is that spontaneously beating isolated myocytes in control animals were not described. Therefore, it is formally possible that the effects could be due to adenoviral transduction alone. In addition, the extent to which the global cells fate has been switched by TBX18 versus induction of a specific gene cassette remains to be elucidated.

Most recently, the group has extended their work to a pig model, perhaps the most promising work for translation to date.[25] TBX18- or control-GFP adenovirus was injected into the posteroseptal region of the right ventricle via a percutaneous catheter rather than an open chest procedure. After ablation of the AV node, animals transduced with TBX18 had a higher heart rate, more diurnal heart rate variation, and decreased reliance on the backup electronic pacemaker. They also had an increased physiologic autonomic response to isoproterenol. While certainly promising, biological pacemaking peaked around day 7–8 and waned significantly by 2 weeks. Whether an immune response caused the transient success was not examined. Another caveat is the proximity of the injection site near the AV node, which may have led to reprogramming of conduction-like cells rather than of ventricular cardiomyocytes. Finally, ectopic activity as a result of inflammatory irritation, rather than from ventricular to iSAN reprogramming cannot be definitively ruled out. Although important questions remain, this was the first study to demonstrate successful biological pacemaking delivered via a minimally invasive, catheter-based approach in a clinically relevant large animal model.

In addition to work demonstrating reprogramming of cardiomyocytes in animal models, there has also been *in vitro* success with direct reprogramming of fibroblasts into an induced conduction-like phenotype. This was demonstrated by Smith et al., who cultured cells on distinct extracellular substrates during the reprogramming process to promote distinct cardiac subtypes.[26] Interestingly, while laminin and RGD coated hydrogels seemed to promote reprogramming to a working myocyte phenotype, they inhibited reprogramming to a conduction cell phenotype when compared with Matrigel coated polystyrene. Another study by Nam and colleagues demonstrated that a wide variety of cardiomyocyte subtypes could be induced directly from fibroblasts[27], including atrial and ventricular working cardiomyocytes, as well as pacemaker-like cells. Taken together, these studies suggest that when seeking to reprogram the conduction system for biological pacemaking, it may be possible to not only target healthy working myocardium, but also the fibroblasts that are likely to be found in scarred regions most in need of reprogramming, such as can be seen in the fibrotic replacement of the SAN in disease.

With any reprogramming strategy, it will be important to recapitulate the complex cellular and electrical physiology of the native SAN. While a full discussion is beyond the scope of this review, a few concepts are worth highlighting. To function as a pacemaker, the iSAN should express the ion channels necessary to recapitulate the complex depolarizing and repolarizing currents found within the native sinus node. The reprogramming strategy should also induce sufficient numbers of iSAN cells so as to maintain the appropriate degree of source-sink balance with the surrounding myocardium. An important contributor required

for maintaining source-sink balance within the native SAN structure is the surrounding conduction barriers.[28] The importance of cell coupling and substrate was also demonstrated by Rosen and colleagues in 2011 when they noted significantly different behavior of autologous SAN cells implanted into the canine RV when compared with the native sinus node.[29] Thus, the ideal reprogramming method should convert cells to an iSAN phenotype but also ensure that surrounding structures support the establishment and maintenance of the new pacemaker site, including appropriate vascularization, innervation, and surrounding fibrous tissue.

Improving Delivery Vectors

Efficient gene delivery via viral vectors is a prerequisite for reprogramming.[30] An ideal viral vector should be available at high titers, have limited pre-formed immunity, and evade the host immune response to prevent dose-related toxicities. Most studies to date have used traditional adenoviral vectors, which can be made in high titers and which do not integrate into the genome, but pre-formed immunity to adenovirus, which causes the common cold, is common. To prevent viral clearance, techniques such as the incorporation of peptides that mimic native surface proteins into the viral capsid with the goal of minimizing phagocytosis of viral particles may be useful, similar to strategies described in cancer research.[31] Activation of specific components of the immune response may enhance the efficiency of cardiomyocyte reprogramming, as was demonstrated in studies where the introduction of four reprogramming transcription factors, Oct4, Sox2, Klf4, c-Myc, into a fibroblast induced epigenetic changes that confer pluripotency.[32] Activation of the Toll-like receptor 3 pathway, an innate immune response, by retroviruses stimulated more efficient epigenetic reprogramming.[33] Therefore, to achieve permanent cellular reprogramming, it may be important to selectively activate one immunologic response while repressing those that clear the viral vectors and the transduced cells. Systemic immunosuppression with corticosteroids would not provide selectivity and would increase unwanted side effects such as infection. Other strategies to avoid clearance of iSAN cells by the native immune system might employ “gutless” viruses, in which virally-encoded genes are excised and replaced by non-coding DNA to limit the immune response. This approach has had early success in oncologic applications.[34] Finally, adenoviral constructs that specifically target cardiomyocytes would minimize unwanted systemic spread and response, as shown by targeting CD40 for immunotherapy of prostate cancer.[35]

An important conceptual advance in biological pacemaking would be to demonstrate definitive reprogramming of targeted cardiomyocytes. Kapoor et al. recognized the importance of this concept when they analyzed the iSAN cells for epigenetic modifications associated with the normal sinus node. One experimental proof would be to engineer a viral vector in which the expression of the selected transcription factor could be turned on only in the presence of a drug, such as tetracycline.[36] After a prespecified reprogramming period, the antibiotic could be removed, thus turning off expression of the transcription factor(s). If the phenotypic and epigenetic changes persist after cessation of transgene expression, permanent reprogramming would be convincingly demonstrated.

Increasing reprogramming efficiency through combinatorial gene transduction

The regulatory networks that govern SA and AV node development and function are beginning to be defined. In the setting of SAN dysfunction the AV node often becomes the dominant pacemaker. Therefore, a better understanding of pathways regulating embryonic AV canal development and AV nodal maturation is relevant to our understanding of the pacemaking phenotype. Once defined, it should be possible to leverage these pathways to allow interconversion of diverse cell types in the adult heart for regenerative medicine approaches. Several transcription factors have been demonstrated to promote lineage commitment and maturation of nodal tissues, and each of these identified transcription factors may be required to regulate different aspects of the nodal gene program. Defining characteristics of SA and AV nodal tissue are increased automaticity, slow conduction velocity, and decremental conduction. Decremental conduction in the AV node prevents very rapid atrial impulses from conducting to the ventricles during atrial fibrillation. We have recently discovered that ectopic Wnt activation during development results in an AV junction and nodal-like phenotype within the ventricles.[37] Ectopic regions of ventricular myocardium demonstrate downregulation of working myocardial genes such as *Scn5a* (encodes Na_v1.5) and *Gja1* (encodes connexin 43), and upregulation of AV junctional proteins including Tbx3 and periostin. Ultimately, the most robust conversion of myocytes to functional nodal-like cells may require a combination of transcription factors and small molecules. This combinatorial strategy is analogous to that used in direct conversion of fibroblasts into induced-cardiomyocytes, where three transcription factors are sufficient for reprogramming, but additional factors have been shown to increase reprogramming efficiency.[38–40]

Developing a Human In Vitro Model System for Mechanistic Studies

Testing candidate transcription factors in small and large animal models has many advantages. These include the ability to test many candidates in a high-throughput manner using a small animal model, and then hone techniques for delivery and validation in a more physiologically similar large animal model. What these approaches cannot offer, however, is validation that the transcription factor(s) will be effective in the human context. To this end, it may be useful to use organotypic human heart slices as a model system. Tissue slices from adult ventricular myocardium have previously been cultured for 28 days with high cellular viability and maintenance of expression of the major ion channels, as well as maintenance of the typical cardiomyocyte action potential characteristics.[41] Organotypic slice cultures also have important advantages over isolated human cardiomyocytes including the presence of a native tissue environment, allowing normal contacts with extracellular matrix and intercellular communication, which impacts the differentiation and maintenance of native morphology of cardiomyocytes. A multicellular model of the human heart that enables assessment of both gene expression and functional changes will be invaluable for precise refinement of reprogramming of adult atrial cardiomyocytes into induced-pacemaker cells.

Developing a Physiologically Relevant Animal Model

The results of cell or tissue culture or small animal experiments can lay the theoretical groundwork for a reprogramming method, but testing in a large animal model is still

necessary. The animal should have anatomical landmarks similar in size and spatial relationship to humans, and its sinus and AV nodal tissue should exhibit electrophysiologic properties that mimic human. When modeling therapies for biological pacemaking at the level of the AV node, an important consideration is the physiology and distribution of the His-Purkinje system, which has significant interspecies variability. For catheter-based delivery approaches, an animal model must also be selected that allows for conventional femoral or jugular access and traverses a distance similar to that of humans. When considering all these factors, porcine or canine models are likely best suited for investigations of sinus node dysfunction and therapy.

With regard to modeling the disease process itself, several studies have investigated transient sinus node suppression using pharmacologic or vagal stimulation methods, but very few studies have utilized durable models of sinus node dysfunction. One can envision that a model of sinus node ablation, such as that described by Hse et al.[42], might be a reasonable model to allow for more durable assessment of the persistence and efficacy of an iSAN within the atria. Given that catheter-based injection techniques for targeting the atria are more difficult owing to the heterogeneous geometry and thickness of the atria, many studies have targeted atrial tissues using open-chest techniques, either via direct viral targeting to the left atrium or left atrial appendage.[43] The left atrium, in particular, has been an attractive targeting site due to the ease of access with an open approach via left thoracotomy. In addition, when assessing the origin of an ectopic biological pacemaking site in a model of sinus node dysfunction, it is much easier to distinguish the native sinus node from a remote site within the left atrium versus a site within the right atrium. We would argue, however, that given the resolution of currently available mapping systems this distinction would be possible and injection and/or open techniques targeting the right atrium are worth investigating.

Summary

Electronic pacemakers are effective therapies, and continued advances in device technology will raise the bar for biological pacemaker therapy. Recent advances in cellular reprogramming may provide new opportunities for iSAN that may outperform electronic devices in clinically important ways, including the ability to couple with a patient's autonomic nervous system. Treating AV nodal and infra-Hisian block may also ultimately be feasible using biological approaches. For AV block, one could envision reprogramming cells of the slow AV pathway, which have a separate input to the His bundle, to increase automaticity.[44, 45] For infra-Hisian block, reprogramming ventricular myocytes to a fast-conduction phenotype or strategies to transplant engineered electrical conduction tracts could be considered.[46] Testing of newer approaches will require careful design of experimental models that closely recapitulate a well-described clinical pathophysiology and use a targeted, minimally-invasive, and permanent biologic therapy (see Figure 1). Early testing of biological pacemakers in translational large animal models and Phase I clinical trials will almost certainly employ a tandem approach, with electronic pacemakers providing a safety net. Eventually, however, we envision the possibility of a biological pacemaker that not only confers stability that outlasts that of an electronic pacemaker, but can also be individually tailored to the pathophysiology of each patient and can respond to their own

innate autonomic system. This will all need to be accomplished without any increased risk of rejection, neoplasia, or arrhythmias. Though many important hurdles to biologic therapies exist, given the exciting advances and increasing pace of innovation, we are optimistic that translation of this technology is on the horizon.

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Figure 1.
Proposed algorithm for optimizing cardiomyocyte reprogramming to an induced pacemaker-like phenotype