Creation of a Biological Pacemaker by Cell Fusion

Hee Cheol Cho, Yuji Kashiwakura, Eduardo Marbán

As an alternative to electronic pacemakers, we explored the feasibility of converting ventricular myocytes into pacemakers by somatic cell fusion. The idea is to create chemically induced fusion between myocytes and syngeneic fibroblasts engineered to express HCN1 pacemaker channels (HCN1–fibroblasts). HCN1–fibroblasts were fused with freshly isolated guinea pig ventricular myocytes using polyethylene-glycol 1500. In vivo fused myocyte–HCN1–fibroblast cells exhibited spontaneously oscillating action potentials; the firing frequency increased with β-adrenergic stimulation. The heterokaryons created ectopic ventricular pacemaker activity in vivo at the site of cell injection. Coculture of nonfused HCN1–fibroblasts and myocytes without polyethylene-glycol 1500 revealed no evidence of dye transfer, demonstrating that the $I_f$-mediated pacemaker activity arises from heterokaryons rather than electrotonic coupling. This nonviral, non–stem cell approach enables autologous, adult somatic cell therapy to create biopacemakers.

Cardiac rhythm disorders are caused by malfunctions of impulse generation and/or conduction. Present therapies for deficient impulse generation, eg, electronic pacemakers, remain palliative. Here we continue to develop the alternative concept of biological therapy for cardiac arrhythmias; the objective is to achieve functional reengineering of cardiac tissue, so as to alter a specific electrical property of the tissue in a salutary manner. In this study, engineered cells were introduced to create a biological pacemaker in normally quiescent myocardium. A key ionic current present in sinoatrial nodal pacemaker cells, but largely absent in atrial and ventricular myocytes, is the pacemaker current, $I_f$.1 We used polyethylene glycol–induced fibroblast–myocyte fusion as a method to deliver $I_f$ to myocardium to create a biological pacemaker.

Materials and Methods

For in vitro fusion experiments, HCN1–fibroblasts and myocytes were incubated in prewarmed (37°C) 40% polyethylene glycol 1500 (PEG) (Roche Applied Science, Indianapolis, Ind) in PBS for 2 to 4 minutes. Cells were rehydrated with high potassium solution (same solution as used after myocyte isolation) for 5 to 10 minutes and then washed with normal Tyrode’s solution.

An expanded Materials and Methods section is in the online data supplement at http://circres.ahajournals.org.

Results

Guinea pig lung fibroblasts stably expressing HCN1 channels with a green fluorescence protein (GFP) reporter (HCN1–fibroblasts) and loaded with calcine acetoxyethyl ester were fused with freshly isolated guinea pig ventricular myocytes using PEG. Within 3 minutes, the HCN1–fibroblasts fused with ventricular myocytes, as verified by the sudden introduction of calcine fluorescence from the fibroblast cytoplasm into the myocyte cytoplasm (Figure 1A, left). To extend these in vitro observations, we focally injected HCN1–fibroblasts suspended in 50% PEG into the apex of guinea pig hearts. Langendorff isolation of ventricular myocytes from the site of injection revealed GFP-positive myocytes (Figure 1A, right). Such myocyte/HCN1–fibroblast heterokaryons formed by in vivo fusion displayed spontaneous action potentials with a slow phase 4 depolarization (Figure 1B, left), characteristic of pacemaker cells. Spontaneous activity was not observed in myocytes fused with control fibroblasts expressing GFP only; in such cells, action potentials could be elicited by only external electrical stimulation (Figure 1B, right). The maximum diastolic potentials of the heterokaryons formed with HCN1–fibroblasts were only modestly depolarized (−76 ± 9 mV, n = 9) relative to the resting membrane potentials of the heterokaryons formed with control fibroblasts (−80.5 ± 2 mV, n = 7). Subsequent voltage-clamp recordings with external Ba$^{2+}$ (1 mmol/L) to block $I_{Ca}$ revealed the heterologously expressed pacemaker current, $I_f$ (Figure 1C, left), which was not detectable either in ventricular myocytes alone or in myocytes fused with control fibroblasts (Figure 1C, right). Freshly isolated heterokaryons formed by in vivo fusion between myocytes and HCN1–fibroblasts expressed robust pacemaker current with a conductance of $-770\pm7\ pS/pF$ (n = 9, Figure 1D), an $I_f$ density $>2$-fold that reported in isolated rabbit sinoatrial nodal cells.2 The $I_f$ expressed from heterokaryons exhibited normal HCN1 activation kinetics (Figure 1E, left) with a potential of half-maximal activation of $-73.1\pm2.2\ mV$ (Figure 1E, right). The chemically induced in vivo fusion events did not alter the main excitatory ionic current, $J_{Na}$, of the heterokaryons (Figure 1F; $-22\pm3\ nA\ [n=9]$ at $-40\ mV$ for myocytes fused with HCN1–fibroblasts versus $-20.8\pm3\ nA\ [n=7]$ for myocytes fused with GFP-alone control fibroblasts). Cell fusion should be accompanied by an increase in total cell surface area, a parameter that can be indexed by measurements of electrical capacitance. Indeed, GFP-positive heterokaryons exhibited a larger membrane capacitance than the GFP-negative...
myocytes (124±14 pF, n=9 and 97±8 pF, n=15, respectively, \( P<0.05 \)), supporting the concept of in vivo fusion events. The increased cell capacitance, in effect, would dilute the density of the hyperpolarizing-current, \( I_{K1,b} \), by 20%. Thus, the robust \( I_f \) conductance, combined with the decreased \( I_{K1} \) conductance, drives the spontaneous pacemaking in the heterokaryons.

To investigate in vivo fusion events histologically, HCN1–fibroblasts were transduced with adenovirus expressing cytoplasmic \( \beta \)-galactosidase (Ad-lacZ). 5-Bromo-4-chloro-3-indolyl \( \beta \)-D-galactoside staining of the heart sections at the site of cell injection revealed \( \beta \)-galactosidase activity in the longitudinally oriented ventricular myocytes at the border between myocytes and injected HCN1–fibroblasts, as well as in some HCN1–fibroblasts that had not undergone fusion with myocytes (Figure 2, top left). Because most cardiomyocytes are multinucleated, detection of extra nuclei from HCN1–fibroblasts as evidence for cell fusion in the heterokaryons was not feasible. Instead, immunohistochemistry against \( \beta \)-galactosidase and myosin heavy chain colocalized the 2 proteins in regions of the myocardium (Figure 2, bottom right), indicating fusion of cytoplasm from HCN1–fibroblasts (containing \( \beta \)-galactosidase) and cardiomyocytes.

To examine ectopic pacemaker activity generated by the in vivo fusion events, the heart rates of guinea pigs were slowed with methacholine injection. Electrocardiograms recorded less than 1 to 22 days after HCN1–fibroblast injection revealed ectopic ventricular beats that were identical in polarity and similar in morphology to those recorded during bipolar pace-mapping of the apex in the same animal (Figure 2B, top; \( n=5 \) of 13). Occasionally, junctional escape rhythms (Figure 2B, bottom, horizontal arrows) could be overtaken by ectopic ventricular pacemaker activity. Such ectopic beats were not observed in animals injected with control fibroblasts expressing GFP only (data not shown, \( n=9 \)).

\( \beta \)-Adrenergic stimulation is a potent physiological mechanism to accelerate physiologic cardiac pacing.\(^{3}\) We sought to determine whether heterokaryons formed between HCN1–
fibroblasts and myocytes could respond to the β-adrenergic agonist isoproterenol. As demonstrated in Figure 3A, 1 μmol/L isoproterenol increased the spontaneous beating rate of isolated heterokaryons by 25 ± 10% (n = 4). Thus, chronotropic responsiveness is an intrinsic feature of fusion-engineered biopacemakers. Furthermore, the spontaneous action potential (AP) oscillations could be blocked by an I_f-specific blocker ZD7288 (Figure II in the online data supplement).

To exclude the possibility of gap-junctional coupling between fibroblasts and myocytes as an alternative mechanism of pacemaker activity, HCN1–fibroblasts were loaded with calcine and mixed with nonloaded myocytes. The dye did not diffuse from loaded HCN1 pulmonary fibroblasts to neighboring myocytes, indicating the absence of cell–cell coupling (Figure 3B, top). On the other hand, calcine transferred efficiently from cardiac fibroblasts to myocytes, consistent with the known ability of such fibroblasts to couple via gap junctions (Figure 3B, bottom). Thus, the I_f-mediated pacemaker activity arises from heterokaryons rather than electrotonic coupling between myocytes and fibroblasts.

Figure 2. In vivo fusion of myocardium with HCN1–fibroblasts and ectopic ventricular pacemaker beats generated by the fused heterokaryons. A, Immunohistochemistry with a primary antibody against β-galactosidase (green, top left) and myosin heavy chain (red, top right). The merged image (bottom right) indicates expression of β-galactosidases in the neighboring myocytes (highlighted with a white dotted circle). The transmitted image of injected HCN1–fibroblasts is shown as a cluster of dark cells with bright phase around them. B, ECGs from guinea pig hearts injected with HCN1–fibroblast cells. Top, The ectopic ventricular beats (diagonal arrows) are unleashed on slowing of the heart rate, which share the same polarity and morphology as the electrode-paced ECGs recorded at the site of HCN1–fibroblast injection. Bottom, In another animal, junctional escape rhythms (horizontal arrows) were overtaken by ectopic ventricular beats (diagonal arrows, 16 days after cell injection).

Figure 3. Guinea pig lung fibroblasts used for HCN1–fibroblasts do not form a gap-junctional communication with syngeneic myocytes. A, Heterokaryon formed by in vivo fusion of HCN1–fibroblast and myocyte displays spontaneous AP oscillations in normal Tyrode’s (top). Presence of 1 mmol/L isoproterenol in the external solution increased the frequency of spontaneous AP oscillation in the same heterokaryon (bottom). B (top), Preloaded HCN1–fibroblasts were coincubated for 1 hour with unloaded guinea pig myocytes. B (bottom), Guinea pig cardiac fibroblasts as a positive control.
PEC-induced membrane fusion events have served as a model system to study eukaryotic cell–cell fusion events and to deliver outward K⁺ currents into myocytes. Here, we used syngeneic fibroblasts expressing HCN1 channels as donor cells to induce spontaneous activity in normally quiescent ventricular myocytes after chemically induced cell fusion. The cell fusion biological pacemakers were functional for at least 3 weeks and as early as <1 day postinjection, as revealed by electrocardiography. Previous studies suggest that heterokaryons can maintain the nuclei from each fusion partner separately and stably for at least several months.8,9

Our approach differs conceptually from previous cell-based pacemakers, which rely on cell–cell coupling for transmission of the impulse from the introduced cells to surrounding myocardium.10 Such gap-junctional coupling may or may not be stable over time; many of the major forms of human heart disease, associated with increased arrhythmic risk, coincide with gap junction remodeling, and decreased cell–cell coupling.11 Furthermore, stem cells have been shown to proliferate and migrate once injected into myocardium. This may cause unpredictable patterns of pacemaker activity from regions of the heart other than the desired site. In contrast, the present approach creates a biological pacemaker specifically localized to heterokaryons formed by PEG-induced fusion. Furthermore, fibroblasts that did not undergo fusion with myocytes would not generate pacing from sites other than the site of injection because of the lack of cell–cell coupling.

We have demonstrated that the present approach is feasible, but we have yet to demonstrate consistent pacing in vivo, or long-term effectiveness in a large-animal model. Efforts to increase the efficiency of fusion events such as the use of different fusogens may increase the stability of pacing in vivo. Nevertheless, a number of limitations of previous approaches do not plague the present strategy: first, autologous cells (eg, skin fibroblasts) can be harvested and used; second, viral vectors and their complications can be avoided, as stable transduction can be achieved by routine plasmid transfection technology; third, stem cells are not required. For these reasons, the present methodology may merit exploitation in the future development of biological alternatives to device therapy.

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Disclosures

E.M. owns stock and provides consulting services to Excigen Inc. H.C.C. is an employee of Excigen Inc.

References

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Supplemental Data

Materials and Methods

Ventricular Myocyte Isolation

Guinea-pig left ventricular myocytes were isolated by Langendorff perfusion, as previously described\(^1\). After digestion, cells were stored at room temperature in a high potassium solution (mM: K-glutamate 120, KCl 25, MgCl\(_2\) 1, glucose 10, HEPES 10, and EGTA 1; pH 7.4) for 30 minutes. Myocytes were then placed on laminin-coated (20 µL/mL culture medium; Becton Dickinson Labware) cover slips in 6-well plates in medium 199 (Invitrogen, Carlsbad, CA) supplemented with 2% FBS (Invitrogen, Carlsbad, CA) and maintained at 37°C in a 5% CO\(_2\) humidified incubator for 1 hour.

Recombinant lentivirus production creation of stable fibroblast cell line expressing HCN1

Recombinant lentiviruses were generated by the 3-plasmid system\(^2\) by co-transfecting HEK293T cells with pLentiV-CAG-HCN1-IRES-GFP, pMD.G, and pCMVΔR8.91. The lentiviral construct expresses the pacemaker channel, HCN1, under the composite promoter CAG, and then expresses green fluorescent protein (GFP) after internal ribosomal entry site (IRES). Guinea-pig lung fibroblasts (ATCC, Manassas, VA) were grown to 80% confluency in 75 cm\(^2\) flasks in F12K media supplemented with 10% FBS (Invitrogen, Carlsbad, CA). The fibroblasts were stably transduced with pLentiV-CAG-HCN1-IRES-GFP at a final concentration of 10,000 TU/mL with 8 µg/mL polybrene to facilitate
transduction. The HCN1-GFP transduced fibroblasts were selected using fluorescence activated cell sorting (FACS). Flow cytometry was performed using a Facstar (Becton Dickinson, Bedford, MA) and analyzed using CellQuest (Becton Dickinson, Bedford, MA). Non-transduced guinea-pig lung fibroblasts were used as non-fluorescent controls. Green fluorescent protein (GFP)-positive cells were measured as those whose fluorescence intensity exceeded the fluorescence of 99.9% of the control cells (488/530 nm excitation/emission).

**Dye loading and *in vitro* cell fusion**

The fibroblasts stably expressing HCN1 (HCN1-fibroblasts) were loaded with Calcein-AM (2 µL/mL growth medium; 1 mmol/L stock solution in dimethyl sulfoxide; Molecular Probes, Eugene, OR) to introduce the cytosolic fluorescent marker. After staining, cells were trypsinized, centrifuged, and resuspended in 6 mL medium 199 supplemented with leukoagglutinin 40 µg/mL (Sigma-Aldrich, St. Louis, MO). The myocyte growth medium was exchanged with this HCN1-fibroblast suspension at 0.5 mL/well. One hour after co-plating, myocytes and HCN1-fibroblasts were fused with pre-warmed (37°C) 40% polyethylene glycol 1500 (PEG) (Roche Applied Science, Indianapolis, IN) in H₂O. After 2 to 4 minutes of exposure to PEG, cells were re-hydrated with high potassium solution (same solution that was used after myocyte isolation) for 5 to 10 minutes and then washed with normal Tyrode’s solution (see below).
**Electrophysiology**

Whole-cell electrophysiology recordings\cite{Hamill1981} was performed as described below. Experiments were carried out using standard microelectrode whole-cell patch-clamp techniques\cite{Hamill1981} with an Axopatch 200B amplifier (Axon instruments) with a sampling rate of 20 kHz and low-pass Bessel-filtered at 5 kHz. All experiments were performed at a room temperature. Cells were washed with a normal Tyrode’s solution containing (mmol/L) NaCl 138, KCl 5, CaCl\textsubscript{2} 2, glucose 10, MgCl\textsubscript{2} 0.5, and HEPES 10; pH 7.4. The micropipette electrode solution was composed of (mmol/L): K-glutamate 130, KCl 9, NaCl 8, MgCl\textsubscript{2} 0.5, HEPES 10, EGTA 2, and Mg-ATP 5; pH 7.2. Microelectrodes had tip resistances of 2 to 4 M\textOmega\ when filled with the internal recording solution. Voltage-clamp experiments were performed with an inter-episode interval of 2.5 seconds. Action potentials were either initiated by short depolarizing current pulses (2 to 3 ms, 500 to 800 pA) on myocytes fused with control (GFP alone) myocytes or recorded with I=0 mode on myocytes fused with HCN1-fibroblasts. Data were corrected for the measured liquid junction potential (-18 mV)\cite{Hamill1981}. A xenon arc lamp was used to view Calcein-AM fluorescence or GFP at 488/530 nm (excitation/emission).

**Adenovirus transduction of HCN1-fibroblasts and cell injection into guinea-pig heart**

The *E. coli* β-galactosidase encoded by *lacZ* gene was subcloned into an adenoviral shuttle vector pAd-Lox to generate pAd-Lox-LacZ by Cre-Lox recombination in Cre-4/HEK293 cells as described\cite{Cho2019}. HCN1-fibroblasts were transduced with Ad-*lacZ* for 6 hours prior to injection into a guinea-pig heart. Typically, 1 x 10\textsuperscript{5} HCN1-fibroblast cells were trypsinized...
(0.05%), resuspended in 100 µL of 50% PEG 1500, and injected intra-myocardially at the apex of a guinea-pig heart with a 30G1/2 needle.

**Electrocardiograms (ECGs)**

Animals were injected peritoneally with methacholine (0.1-0.5 mg per kg of body weight in saline, Sigma-Aldrich, St. Louis, MO) in order to slow down the heart rate prior to recording ECGs under general anesthesia (2% isoflurane, 98% O₂). ECGs were recorded using a 2-lead digital ECG system at 2 kHz (Lead 1 and Lead 3, BIOPAC Systems, Goleta, CA) and Lead 2 was off-line calculated by Einthoven’s triangle using Acqknowledge 3.7.3 software (BIOPAC Systems, Goleta, CA).

**X-gal staining and immunohistochemistry:**

Guinea-pig hearts were excised and frozen-sectioned in OCT (VWR Scientific) 5 µm slices. Alternating sections were used for either immunohistochemistry or staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The sections were fixed in 2% formaldehyde-0.2% glutaraldehyde for 15 min at room temperature, and stained for 6 h at 37 °C in PBS containing 1.0 mg/ml X-gal, 15 mM potassium ferricyanide, 15 mM potassium ferrocyanide and 1 mM MgCl₂. After staining, the slices were washed with PBS twice. For immunohistochemistry, 200-fold diluted rabbit polyclonal against β-galactosidase (FITC-conjugated, Abcam, Cambridge, MA) and 400-fold diluted mouse cardiac myosin heavy chain (MHC, Abcam, Cambridge, MA) were used for primary antibodies and AlexaFluor588 anti-mouse (diluted 200-fold, Invitrogen, Carlsbad, CA) was used for
secondary antibody against cardiac MHC. The sections were blocked with 10% goat serum + 0.01% TritonX-100 in PBS before the primary and secondary antibody incubation. All antibodies were diluted in 2% goat serum + 0.01% TritonX-100 and incubated on the sections for 45 min at room temperature.
References


Supplemental Data

Online Figure 1

Lung fibroblasts

Cardiac fibroblasts
Supplemental Data

Online Figure 2

10 μM ZD7288

Voltage (mV) vs. Time (s)
Supplemental Data

Online Figure 3
**Figure Legends**

**Online Figure 1**
Guinea-pig lung fibroblasts used to create HCN1-fibroblasts do not form gap-junctional connections with syngeneic fibroblasts after prolonged incubation (16 hours). Left: Guinea-pig lung fibroblasts were loaded with Calcein-AM and co-incubated with non-loaded lung fibroblasts. No evidence of dye transfer from dye-loaded fibroblasts to non-loaded fibroblasts is seen. Right: Dye transfer experiments using guinea pig cardiac fibroblasts as a positive control. Dye transfer is evident from calcein pre-loaded cardiac fibroblasts to neighboring non-loaded cardiac fibroblasts. Nuclei (blue) were labeled with Hoechst 43332 in fibroblast-fibroblast co-incubation.

**Online Figure 2**
Spontaneously oscillating action potentials from an in vivo heterokaryon can be blocked by an I\(_f\)-specific blocker ZD7288. A black arrow indicates the wash-in of 10 µM ZD7288. Representative of 3 experiments.

**Online Figure 3**
A quiescent heterokaryon formed by in vitro fusion of myocyte and HCN1-fibroblast can generate an action potential and simultaneous contraction (not shown) upon a 2 ms-stimulus of depolarizing current (0.5 – 1.0 nA) under current-clamp.