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Functional Integration of Electrically Active Cardiac Derivatives From Genetically Engineered Human Embryonic Stem Cells With Quiescent Recipient Ventricular Cardiomyocytes

Insights Into the Development of Cell-Based Pacemakers

Tian Xue, PhD*; Hee Cheol Cho, PhD*; Fadi G. Akar, PhD*; Suk-Ying Tsang, PhD; Steven P. Jones, PhD; Eduardo Marbán, MD, PhD; Gordon F. Tomaselli, MD; Ronald A. Li, PhD

Background—Human embryonic stem cells (hESCs) derived from blastocysts can propagate indefinitely in culture while maintaining pluripotency, including the ability to differentiate into cardiomyocytes (CMs); therefore, hESCs may provide an unlimited source of human CMs for cell-based therapies. Although CMs can be derived from hESCs *ex vivo*, it remains uncertain whether a functional syncytium can be formed between donor and recipient cells after engraftment.

Methods and Results—Using a combination of electrophysiological and imaging techniques, here we demonstrate that electrically active, donor CMs derived from hESCs that had been stably genetically engineered by a recombinant lentivirus can functionally integrate with otherwise-quiescent, recipient, ventricular CMs to induce rhythmic electrical and contractile activities *in vitro*. The integrated syncytium was responsive to the β -adrenergic agonist isoproterenol as well as to other pharmacological agents such as lidocaine and ZD7288. Similarly, a functional hESC-derived pacemaker could be implanted in the left ventricle *in vivo*. Detailed optical mapping of the epicardial surface of guinea pig hearts transplanted with hESC-derived CMs confirmed the successful spread of membrane depolarization from the site of injection to the surrounding myocardium.

Conclusions—We conclude that electrically active, hESC-derived CMs are capable of actively pacing quiescent, recipient, ventricular CMs *in vitro* and ventricular myocardium *in vivo*. Our results may lead to an alternative or a supplemental method for correcting defects in cardiac impulse generation, such as cell-based pacemakers. (*Circulation*. 2005;111:11-20.)

Key Words: stem cells ■ cardiac development ■ viruses ■ gene therapy ■ pacemakers

Human embryonic stem cells (hESCs) derived from the inner cell mass of human blastocysts can propagate indefinitely in culture while maintaining their normal karyotypes and pluripotency to differentiate into all cell types¹; therefore, hESCs may provide an unlimited supply of cells for transplantation and other cell-based therapies. Recent studies have demonstrated that spontaneously beating outgrowths of human embryoid bodies (hEBs) formed after *in vitro* differentiation of hESCs contain cardiomyocytes (CMs).²⁻⁶ These hESC-derived CMs express a number of cardiac-specific gene products and transcription factors. Functionally, hESC-derived CMs display chronotropic responses after adrenergic stimulation, as well as the 3 distinct types of rate-adaptive action potentials (APs) that are signatures of early-stage human nodal, atrial, and ventricular CMs.

Although it is presently known that human CMs can be derived from hESCs *ex vivo* and that electrical coupling does exist within hESC-derived beating outgrowths,⁶ their ability to functionally integrate with recipient cells after engraftment remains to be tested. Using a combination of electrophysiological, imaging, and lentivirus-mediated gene transfer techniques, we tested the hypothesis that donor hESC-derived CMs can form a functional syncytium with and therefore modify the excitability of recipient CMs *in vitro* and the intact ventricular myocardium *in vivo*.^{7,8}

Methods

Maintenance and Differentiation of hESCs

The hESC line H1 (Wicells, Madison, Wis) was maintained on an irradiated mouse embryonic fibroblast feeder layer and propagated as

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previously described.¹ In brief, the culture medium consisted of Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp) supplemented with 20% fetal bovine serum (HyClone), 2 mmol/L L-glutamine, 0.1 mmol/L β -mercaptoethanol, and 1% nonessential amino acids. Mouse embryonic fibroblast cells were obtained from 13.5-day embryos of CF-1 mice. For cardiac differentiation, the method of forming hEBs from enzymatically dispersed hESCs in suspension in Petri dishes in the absence of leukemia inhibitor factor and basic fibroblast growth factor was used.²⁻⁴ On day 6, suspended hEBs were plated onto gelatin-coated, 6-well plates. The medium, which contained 15% fetal bovine serum and 0.1 mmol/L nonessential amino acids in DMEM, was replenished daily during differentiation. Spontaneously beating outgrowths, which typically appeared \approx 7 days after plating of hEBs, were identified by visual inspection. Both continuously and episodically beating hEBs were observed. Although both groups were considered when assessing the percentage of spontaneously contracting hEBs present in the population, only the former was measured for beating frequency (in duplicate at $35 \pm 2^\circ\text{C}$) and transplantation experiments to minimize the variability in activity. The variability in beating frequency is presumably the result of electrical inhomogeneities.⁴

LentiV-Mediated, Stable Genetic Modification of hESCs

For stable genetic modification, we used the self-inactivating HIV1-based lentiviral vector (LentiV)⁹ for transgene delivery. LentiV was chosen as the vehicle because, unlike adenoviruses, it allows persistent transgene expression without eliciting host immune responses. With 6 of the 9 genes removed, including all of the crucial virulent factors of wild-type HIV1, LentiV is nonpathogenic. Furthermore, LentiV is effective even in mitotically inactive, terminally differentiated cells such as CMs,¹⁰ in contrast to other oncoretroviruses that also integrate transgene into the host genome. These unique properties make LentiV ideal for modifying hESCs and their cardiac derivatives. The plasmid pLentiV-CAG-green fluorescent protein (GFP) was created from pRRL-hPGK-GFP SIN-18 (generously provided by Dr Didier Trono, University of Geneva, Geneva, Switzerland) by replacing the human phosphoglycerate kinase-1 (hPGK) promoter with the composite CAG promoter. Unlike the cytomegalovirus (CMV) promoter, CAG does not lead to transgene silencing in hESCs. Recombinant LentiVs were generated by the 3-plasmid system¹¹ by cotransfecting HEK293T cells with pLentiV-CAG-GFP, pMD.G, and pCMV Δ R8.91. The latter plasmids encode the vesicular stomatitis virus G envelope protein and the HIV-1 *gag/pol*, *tat*, and *rev* genes, respectively, required for efficient virus production. For each 100-mm dish of HEK293T cells plated at 80% to 85% confluence, 5, 2.5, and 10 μg of pLentiV-CAG-GFP, pMD.G, and pCMV Δ R8.91 DNAs, respectively, were used for transfection. LentiV particles were harvested by collecting the culture medium 48 hours after transfection. The titer, measured in transducing units (TU) per milliliter, was determined by examining the percentage of green HEK293T cells obtained 48 hours after transduction with the corresponding serially diluted LentiV-containing supernatants by fluorescence-activated cell sorting (FACS) analysis (FACSscan or FACSort, Becton Dickinson). LentiVs generated with this protocol typically had titers in the range of 10^6 to 6×10^6 TU/mL. LentiVs were stored at -80°C before use.

hESCs were transduced by adding purified LentiVs to cells at a final concentration of 10 000 TU/mL with 8 $\mu\text{g}/\text{mL}$ polybrene to facilitate transduction. The multiplicity of infection was \approx 5 for each round of transduction. After 4 to 6 hours of incubation with LentiV-CAG-GFP, 2 mL of fresh medium per 60-mm dish was added. Transduction was allowed to proceed for at least 12 to 16 hours. Cells were washed twice with phosphate-buffered saline (PBS) to remove residual viral particles. To obtain a homogeneous population of LentiV-CAG-GFP, green portions of hESC colonies were microsurgically segregated from the nongreen cells, followed by culturing under nondifferentiating conditions for expansion. This process was repeated until a homogeneous population of green hESCs, as confirmed by FACS, was obtained.

Assessment of Functional Coupling After In Vitro Transplantation

Monolayers of neonatal rat ventricular myocytes (NRVMs) were prepared as previously described¹² and plated at a density of $\approx 1.5 \times 10^3$ cells/ mm^2 . Typically, some preparations of NRVMs became electrically quiescent without any spontaneous contractions after 10 to 14 days in culture. Only absolutely quiescent NRVMs were chosen for experiments. Ca^{2+} transients were recorded by incubating cells at 37°C under 5% CO_2 with rhod-2AM (Molecular Probes) by laser-scanning confocal microscopy. GFP was excited at a wavelength of 488 nm from a Kr-Ar laser and visualized with a 525-nm bandpass emission filter. The rhod-2 signal was generated by excitation at 568 nm coupled with a 600-nm bandpass emission filter. In vitro multielectrode array (MEA) recordings were performed at 37°C by simultaneously recording from 60 microelectrodes arranged in an 8×8 layout grid with an interelectrode distance of 200 μm . The raw signals were collected at 25 kHz, bandpass filtered, and amplified (Multi-channel Systems), followed by analysis with MC Data Tool V1.3.0 to generate a conduction map based on the time differences at which signals were detected at each of the microelectrodes. For high-resolution optical mapping of monolayers, cells were incubated in 10 $\mu\text{mol}/\text{L}$ RH237 (Molecular Probes) for 15 minutes at 37°C in DMEM without phenol red in a 5% CO_2 environment, followed by washing to remove the dye and then recording on a custom-built system.

Immunostaining

A spontaneously beating hEB cocultured with a monolayer of NRVMs was fixed in 4% paraformaldehyde for 5 minutes at 21°C , washed 4 times with PBS, and blocked with 10% bovine serum albumin with 0.075% saponin in PBS for 2 hours at 21°C . Fixed cells were incubated with primary antibody against connexin43 ($\times 800$ dilution; Chemicon) overnight at 4°C . Cells were visualized by incubation with anti-mouse Alexa Fluor 595 ($\times 500$ dilution; Molecular Probes) and observed by laser-scanning confocal microscopy.

In Vivo Transplantation and Optical AP Mapping

For in vivo transplantation, 5 microdissected, beating hEB outgrowths prepared as described earlier were injected subepicardially with a 21-gauge needle into the left ventricular (LV) anterior wall (≈ 1 to 2 mm deep) of adult breeder guinea pigs after thoracotomy. The area of injection (anterior epicardium, midway between the apex and base) was chosen because it is the most suitable for mapping owing to minimal heart curvature; thus, motion artifacts can be suppressed by gentle stabilization. A small suture in the immediate vicinity of the injected area was typically introduced to further assist our identification of the region of interest during the mapping experiments. Furthermore, a parallel optical port was designed to enable visualization of the exact mapped area, which was centered on the injected region. The optical port also allowed visualization of the suture as well as the GFP signal on excitation with light at 400 nm.

Injected animals were allowed to recover from the surgical procedure for 48 to 72 hours before further experiments were performed. No tumor, inflammation, or immune rejection was observed during the time course of our experiments. To determine whether electrically active, hESC-derived CMs could indeed capture the myocardium, we implemented a novel experimental model that enabled us to investigate in detail the functional electrophysiological interaction between transplanted human cells and recipient ventricular myocardium. This model was designed to specifically test the ability of hESC-derived CMs to modulate the intrinsic rhythm of the intact organ by direct impulse propagation from the injected exogenous cells to the surrounding myocardium. In preliminary experiments, we had found that the average RR interval of Langendorff-perfused guinea pig hearts under our experimental conditions (36°C) was 435 ms, corresponding to a heart rate of 138 bpm, which is significantly greater than the in vitro beating rate of hESC-derived CMs in culture (cf Figure 1C). Therefore, we needed to suppress the heart rate of these preparations to avoid overdrive suppression of electrical activity of hESC-derived CMs by the intrinsic guinea pig

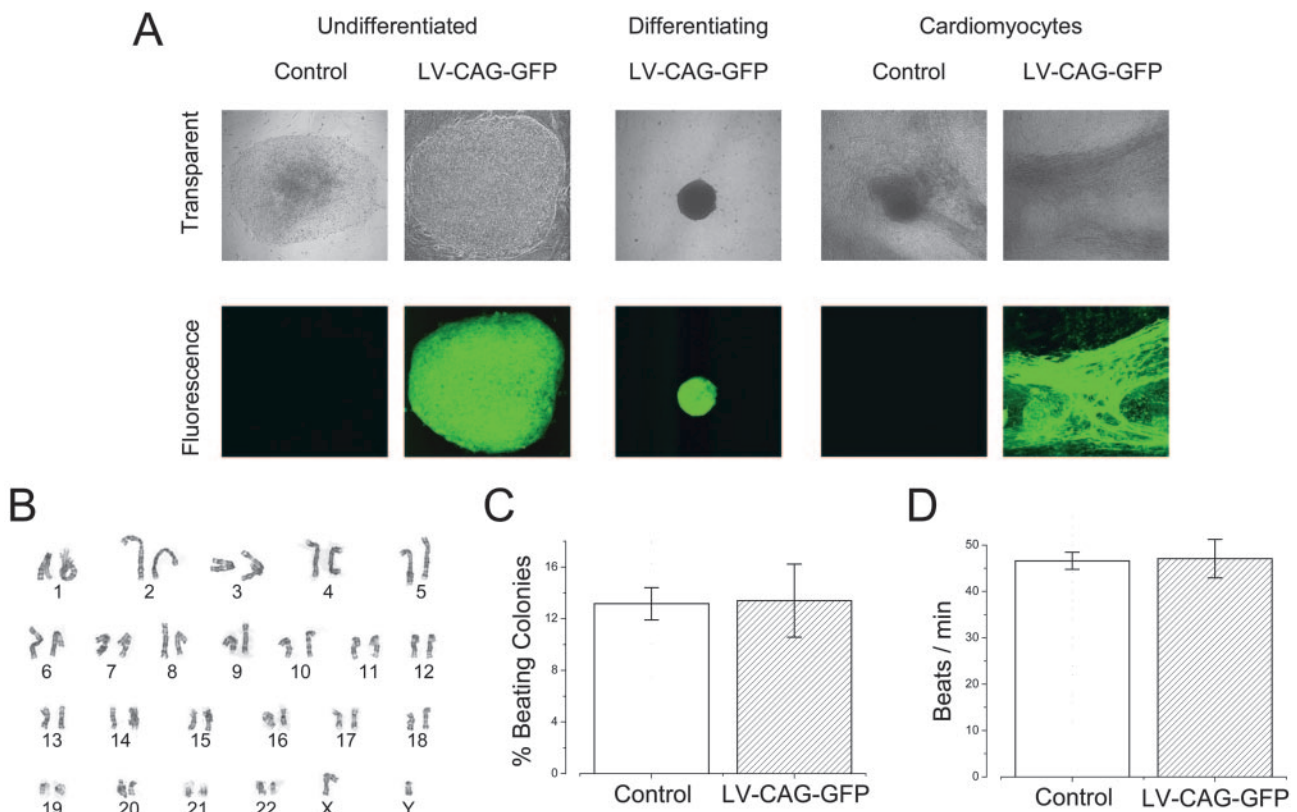


Figure 1. Synchronized beating of hESC-derived CMs and NRVMs. A, Representative confocal images (top, phase-contrast; bottom, green fluorescence) of control and lentivirus (LV)-CAG-GFP-transduced, undifferentiated hESC colonies, differentiating hESCs, and spontaneously beating hEB outgrowths, as indicated. B, LV-CAG-GFP-transduced hESC line displayed a normal karyotype. C, Tabulated distribution of individual hEB (open symbols) and averaged (shaded) data summarizing percentage of hEBs containing contracting outgrowths on day 10 after attachment. Each data point represents individual differentiation reaction. D, Spontaneous beating rate measured in bpm was identical for control and LV-CAG-GFP-transduced groups of contracting hEBs. All other abbreviations are as defined in text.

heart rhythm. In addition to having a relatively fast heart rate, isolated, perfused, and stained guinea pig hearts consistently exhibit complete heart block, as evidenced by clear dissociation of the P waves and QRS complexes on the volume-conducted ECG (data not shown). Because the junctional escape rhythm was too fast relative to the rate of transplanted hES-derived CMs, an alternative approach, based on performing atrioventricular nodal cryoablation in the isolated guinea pig heart, was developed. In brief, after surgical dissection of the right atrium, a custom-designed cryoprobe was inserted into the right ventricular (RV) cavity and placed in contact with the high septum, 1 mm below the base of the heart. LN₂ was then rapidly and continuously passed through the probe with use of a commercially available cryogun (Brymill Inc) for 2 minutes, resulting in ablation of the RV-facing septum and the endocardial surface of the basal RV free wall but not the LV, as assessed by histological examination of the preparation and the lack of change in electrophysiological measurements, including epicardial AP duration and morphology, LV epicardial and endocardial diastolic pacing thresholds, and effective refractory periods (Table). Cryoablated

hearts of preinjected (n=6) and noninjected (n=6) animals were stained with the voltage-sensitive dye di-4-ANEPPS and placed in a custom-designed, temperature-controlled imaging chamber for optical AP measurements, as previously described.¹³

Statistics

All data reported are mean±SEM. Statistical significance was determined for all individual data points and fitting parameters by 1-way ANOVA and Tukey's honestly significant different post hoc test at the 5% level.

Results

Generation of a Stably Transduced, GFP-Expressing hESC Line

To achieve stable genetic modification of hESCs, we first constructed LentiV-CAG-GFP, which directs GFP expression under the control of CAG, an internal composite constitutive

Summary of Electrophysiological Parameters Recorded From Control Guinea Pig Hearts Before and After Right Atrium Removal and Cryoablation

	Mean AP Duration, ms	Conduction Velocity (Longitudinal), cm/ms	Diastolic Pacing Threshold	
			Epicardial	Endocardial
Before cryoablation	179±8	56.2±5.3	0.3±0.1	0.5±0.2
After cryoablation	181±9	55.4±2.4	0.4±0.2	0.4±0.2
<i>P</i> , before vs after	0.7	0.8	0.2	0.7

promoter containing the CMV enhancer and the β -actin promoter. Transduction of hESCs with LentiV-CAG-GFP enabled generation of an hESC line that stably expressed GFP (Figure 1A) for facilitating the identification of hESC-derived CMs after engraftment. Undifferentiated LentiV-CAG-GFP-transduced hESCs were positively stained for molecular markers of pluripotency, such as Oct4, SSEA4, TRA-60, and TRA-80 (data not shown); remained green for >2 years; maintained a normal karyotype without detectable insertion, deletion, or rearrangements (Figure 1B); and propagated normally with a split cycle not different from that of non-transduced hESCs (ie, \approx 8 days).

LentiV Transduction Did Not Alter the Cardiogenic Potential of Pluripotent hESCs

Although a modified hESC line that stably expresses GFP has the ability to differentiate into hematopoietic cells,¹⁴ it is not known whether hESCs that have been subjected to similar sustained genetic manipulation also retain their ability to differentiate into the cardiac lineage. Given that transgene silencing has been observed in mammalian ESCs as well as several primordial cell types derived from them,^{15,16} we verified that genetic modification persists during and after cardiac differentiation (see Discussion for applications). To test whether the pluripotency of hESCs to differentiate into CMs was altered by LentiV-mediated genetic modification, we induced and compared in vitro cardiac differentiation of control and LentiV-CAG-GFP-transduced hESCs. Spontaneously beating outgrowths of hEBs, which contain CMs,²⁻⁶ could be readily obtained from control and LentiV-CAG-GFP-transduced hESCs. Figure 1A also shows that GFP expression remained robust during and after cardiac differentiation. Both groups behaved similarly with regard to the time course of the appearance of visibly beating hEB outgrowths (a plateau of \approx 13% in the entire differentiated hEB population was reached on day 10 under our experimental conditions) and the associated beating frequency (\approx 50 bpm; Figure 1C and 1D; $P>0.05$). These results demonstrate that persistent genetic modification of hESCs can be achieved without altering their cardiogenic potential.

Electrically Active Donor hESC Cardiac Derivatives Form a Functional Syncytium With Quiescent, Recipient Ventricular Cells In Vitro

To investigate whether donor GFP-expressing, hESC-derived CMs can functionally integrate with recipient CMs, we adopted an in vitro transplantation model. For each experiment, a single CM-containing beating outgrowth (\approx 500 μ m in diameter) was microsurgically dissected from an hEB differentiated from LentiV-CAG-GFP-transduced hESCs and transplanted onto a quiescent monolayer of NRVMs (see Methods). After coculturing for 2 to 3 days, synchronous rhythmic contractions of GFP-expressing hEB and NRVMs were observed at a rate of 49 ± 4 bpm ($n=14$; Figure 2A; also see Data Supplement Movie I). Note that this rate is similar to that of a spontaneously contracting hEB alone (cf Figure 1D) but much lower than that typically observed with beating NRVMs (\approx 200 to 300 bpm before they became quiescent). Furthermore, coculturing hESC-derived CMs and NRVMs

without direct contact did not lead to spontaneous contractions (data not shown), excluding the so-called field effect transmission¹⁷ and the possibility that paracrine factors of hESC-derived CMs influenced the excitability and/or contractions of NRVMs. Spontaneous Ca^{2+} transients, not seen in otherwise-quiescent NRVMs, could be recorded from cocultured NRVMs >1 cm away from the human cells (Figure 2B). Collectively, these observations suggest that the transplanted cells were electrically driving the recipient cells. Consistent with this notion, immunostaining the coculture with a primary antibody against the gap junction protein connexin43 showed expression of connexin43 throughout hEB and NRVMs and along their contact surface, suggesting a functional connexin-mediated coupling between the 2 cell types (Figure 2C; note that the 3-dimensional preparation shown was focused on the contact surface between the hEB and NRVMs but that connexin43 was also expressed throughout the 2 cell types; see Data Supplement Movie II). Indeed, MEA recording of extracellular field potentials revealed a coordinated pattern of electrical conduction: rhythmic signals generated from an early-activated region corresponding to the transplantation site (green) spread to the rest of the monolayer (Figure 3A). As anticipated,^{18,19} application of lidocaine (100 μ mol/L), a Na^+ channel blocker, significantly slowed electrical conduction of another preparation by \approx 3-fold (Figure 3B; note the crowding of the isochrones in the right panel).

High-resolution optical mapping further displayed a consistent time delay between APs recorded from the hEB and NRVMs remote from the transplanted site (Figure 4A through 4C; note that the AP morphologies were also different). The patterns of activation were comparable to those obtained from MEA recordings. Heptanol is known to block gap-junction proteins and interfere with electrical coupling.²⁰ Application of 0.4 mmol/L heptanol uncoupled the hESC-derived CMs from the NRVMs without suppressing the pacemaker activity of the human cells per se (Figure 4B, right). Such an uncoupling effect was readily reversible after washout of heptanol (data not shown).

β -Adrenergic stimulation is a potent physiological mechanism to accelerate cardiac pacing.²¹ We next sought to determine whether the functional syncytium formed between hEB and NRVMs could display a change in beating rates in response to the β -adrenergic agonist isoproterenol. Indeed, the beating frequencies of the coculture increased significantly, from 48 ± 5 to 63 ± 8 bpm, after washing in of 1 μ mol/L isoproterenol ($P<0.05$). These results were consistent with a previous finding that β -adrenergic receptors are already expressed in hESC-derived CMs³ and further indicate that stimulation of these receptors produces a positive chronotropic response that can be transmitted to the recipient NRVMs. Recently, the cardiac current I_h , encoded by the hyperpolarization-activated, cyclic nucleotide-modulated (HCN) channel gene family, which is known to play an important role in pacing,²² has been identified in hESC-derived CMs.²³ Unlike isoproterenol, addition of the bradycardic agent ZD7288, an HCN-specific blocker,²⁴ significantly reduced the beating activity of our coculture (Figure 4D; $P<0.05$). Addition of neither isoproterenol nor ZD7288

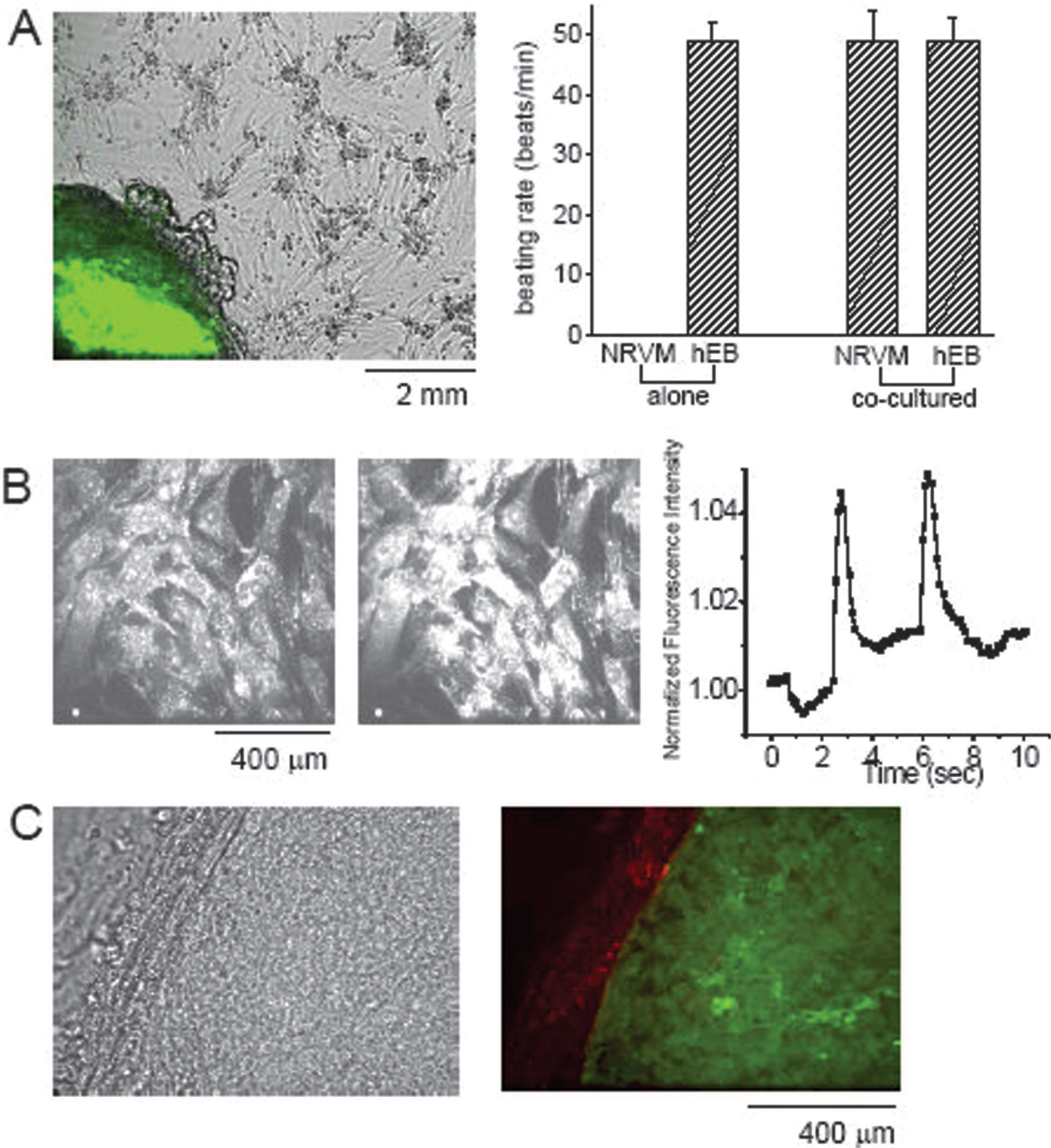


Figure 2. A, Spontaneously beating hEB outgrowth, which stably expresses GFP, was microdissected and transplanted onto quiescent monolayer of NRVMs (left). Beating rate of spontaneously contracting hEB exhibited similar beating rates before (alone; 47 ± 5 bpm) and after (co-cultured; 49 ± 4 bpm) transplantation onto monolayer of NRVMs. B, Ca^{2+} -transient recording from NRVMs located 1 cm away from transplanted, beating hEB, with rhod-2AM as indicator before (left) and during (middle) spontaneous contraction. Normalized fluorescence intensity was measured over 10 seconds in coculture (right). C, Immunofluorescence image with red fluorescence indicating presence of connexin43 at border between NRVMs and GFP-expressing, hESC-derived CMs. Although this image of 3-dimensional hEB was focused on contact surface between hEB and NRVMs, connexin43 was indeed expressed throughout both cell types (data not shown). Abbreviations are as defined in text.

affected quiescent NRVMs without engrafted hESC-derived CMs (data not shown).

At the end of each experiment, synchronous beating could be terminated by crushing or surgically excising the transplanted human cells ($n=17$; analogous to ablation), bolstering the notion that hESC-derived CMs were indeed the origin of

pacings. To further confirm that synchronous beating was not an epiphenomenon due to stretch-activated automaticity of NRVMs from the overlying, contracting, hESC-derived CMs, we studied the effect of 2,3-butanedione monoxime to uncouple the excitation-contraction coupling.²⁵ As anticipated from electrical integration of donor with recipient cells, the

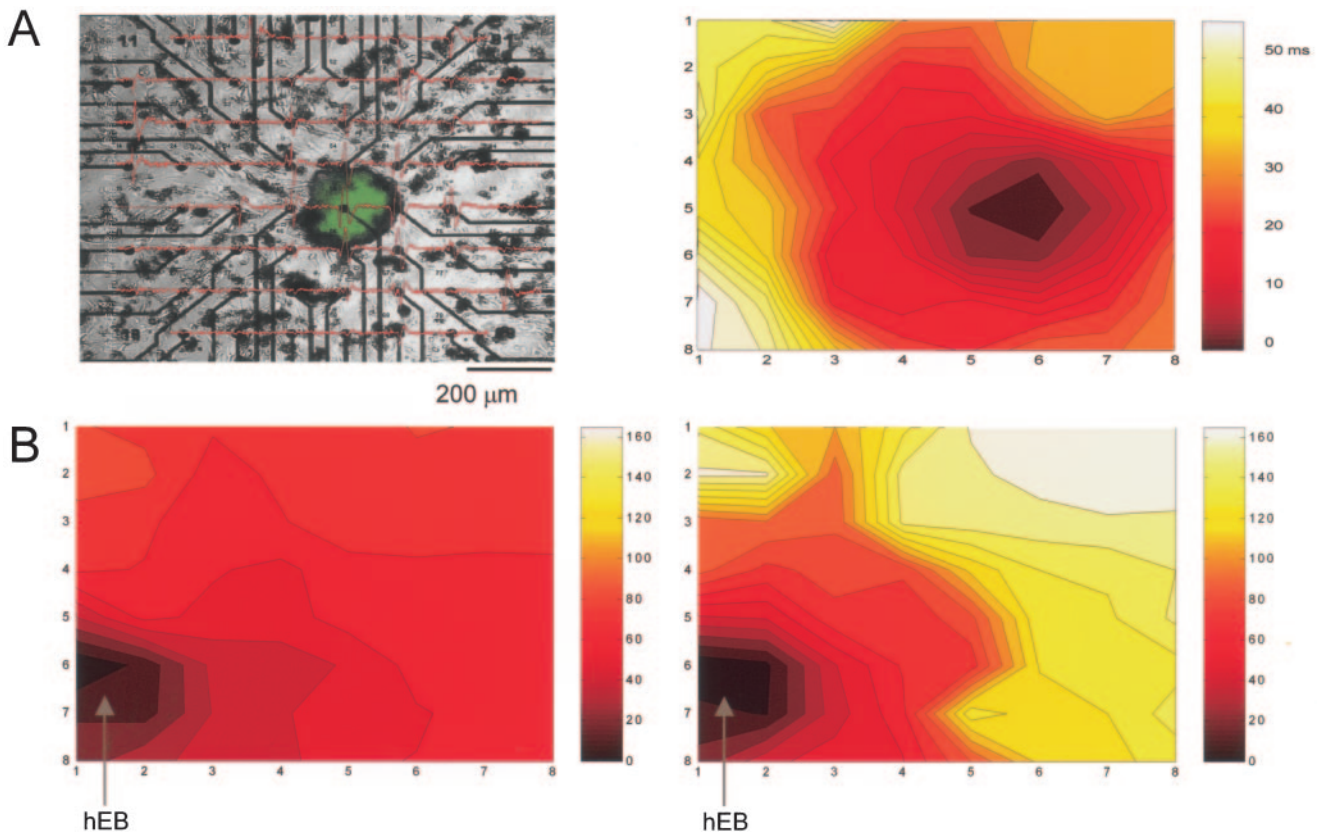


Figure 3. Spontaneous and rhythmic electrical signals were generated in and propagated from implanted hESC-derived graft. A, MEA recording of extracellular field potentials (left). Field potential as function of time contour map (right) located origin of potential wavefront as site of implantation of beating hEB outgrowth (black area). B, MEA recording from another coculture preparation before (left) and after (right) 200 $\mu\text{mol/L}$ lidocaine. Time contour map demonstrates delay of conduction from hEB (black area) after lidocaine application. Pacing origin also corresponded to site of implanted hEB. Abbreviations are as defined in text.

coculture ceased to contract after application of 1 mmol/L 2,3-butanedione monoxime, although optically mapped electrical conduction was unaffected (data not shown).

In Vivo Transplantation

Although the data presented thus far indicate that electrically active, donor hESC-derived CMs can engraft to and form an electrical syncytium with quiescent, recipient, ventricular CMs after in vitro transplantation, it is still necessary to demonstrate the functional efficacy of our approach in vivo. Therefore, optical AP mapping was performed in hearts of control (sham or noninjected) versus hESC-derived, CM-injected guinea pigs subjected to cryoablation (see Methods) that was designed to suppress the intrinsic heart rate. The functional consequences of the cryoablation protocol are shown in Figure 5, which demonstrates complete suppression of intrinsic heart rhythm by the ablation procedure in noninjected hearts (Figure 5C and 5F). Importantly, tissue damage incurred by this procedure was limited to the RV endocardium and RV septum without affecting the LV. In fact, successful LV pacing could be achieved with no change ($P>0.05$) in the diastolic pacing threshold with unipolar extracellular wires placed on the LV epicardium and LV endocardium (Figure 5D and 5E), suggesting that the procedure did not produce tissue damage in either region. To further discount the potential for tissue damage induced by

cryoablation, LV epicardial AP durations, morphologies, and longitudinal epicardial conduction velocity were also measured and compared. These parameters, as summarized in the Table, were identical before and after the procedure, further indicating the absence of changes in intrinsic electrophysiological properties in the myocardial region of interest where hESC-derived CMs had been transplanted.

Whereas ex vivo optical mapping of control, adult, cryoablated guinea pig hearts exhibited complete electrical silence throughout the entire LV ($n=6$; Figure 6A), relatively slow spontaneous APs could be readily recorded from the LV of animals that had been pretransplanted with spontaneously beating, hESC-derived CMs in vivo ($n=4$; Figure 6A). Shown in Figure 6B are isopotential contour maps recorded every 0.6 ms that depict the sequential spread of membrane depolarization across the anterior epicardial surface of a representative guinea pig heart injected with hESC-derived CMs before and after the cryoablation procedure. Before the procedure, the heart rate was relatively high and the spread of membrane depolarization was very rapid across the epicardial surface (Figure 6B, left; also see Data Supplement Movie III) and consistent with epicardial wavefront breakthrough from deeper intramyocardial layers, suggesting a normal ventricular activation sequence via the His-Purkinje conduction system and override of the transplanted hESC-derived CMs. Interestingly, after the cryoablation procedure, a slow heart

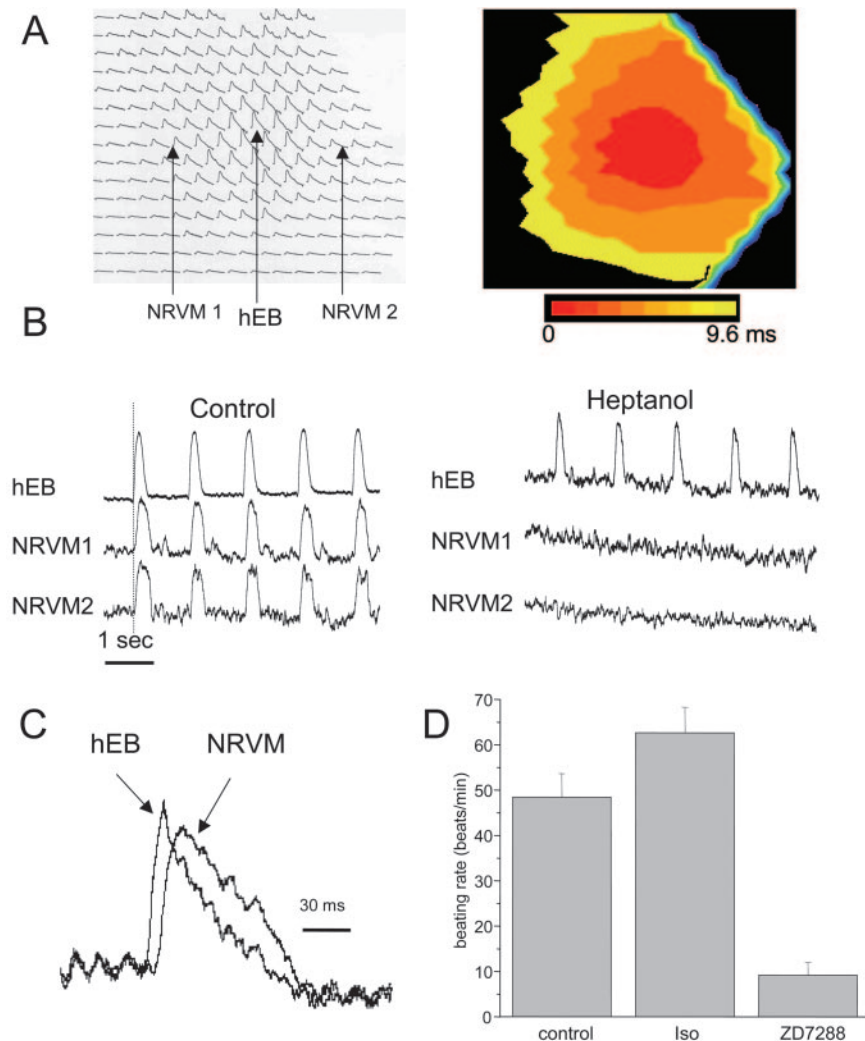


Figure 4. A, Optical APs were mapped with voltage-sensitive dye by photodiode array focused on region containing spontaneously beating hEB transplanted on quiescent NRVM monolayer. Right, conduction contour map generated from left image, demonstrating centrifugal propagation of AP wavefront from site of transplanted hEB (red) to periphery. B, Gap-junction uncoupler heptanol reversibly eliminated AP propagation to neighboring NRVM sites but did not affect APs in pacing origin of hEB. NRVM1 and NRVM2 represent 2 distinct sites at 3.2 and 3.6 mm, respectively, away from pacing origin. C, Superimposed optical AP profiles demonstrate delay of activation and slower rate of depolarization of NRVMs. D, β -Adrenergic stimulation with 1 $\mu\text{mol/L}$ isoproterenol (Iso) significantly accelerated spontaneous beating rate of hEB ($P=0.01$), whereas ZD7288 attenuated beating activity. $n=9$ for each group. All other abbreviations are as defined in text.

rate persisted exclusively in hearts preinjected with hESC-derived CMs but not in controls (cf Figure 6A). Importantly, the spread of membrane depolarization in these hearts was consistent with epicardial surface wavefront propagation and always proceeded from the site of transplantation, as identified by the GFP epifluorescence of hESC-derived CMs (Figure 6B, right; also see Data Supplement Movie IV). This

finding is consistent with that recently reported by Rubart and colleagues,²⁶ who demonstrated that fetal CMs transplanted into the LV of syngeneic adult mice fully integrated into the host tissue matrix and formed gap-junction proteins; our finding further demonstrates that exogenous transplanted cells can even electrically drive the surrounding myocardium. By contrast, injection of saline without hESC-CMs did not

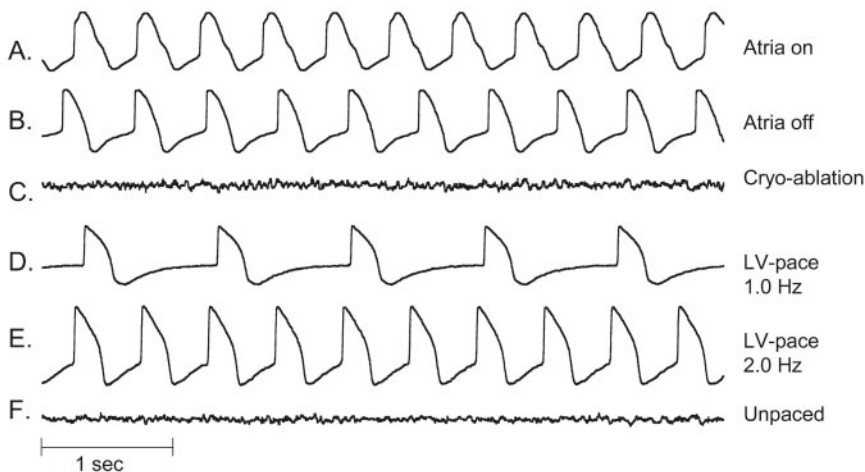


Figure 5. Effect of removing atria and atrioventricular nodal cryoablation (A–C) on representative control adult guinea pig heart with (D and E) and without external pacing (C and F). See text for details. Abbreviation is as defined in text.

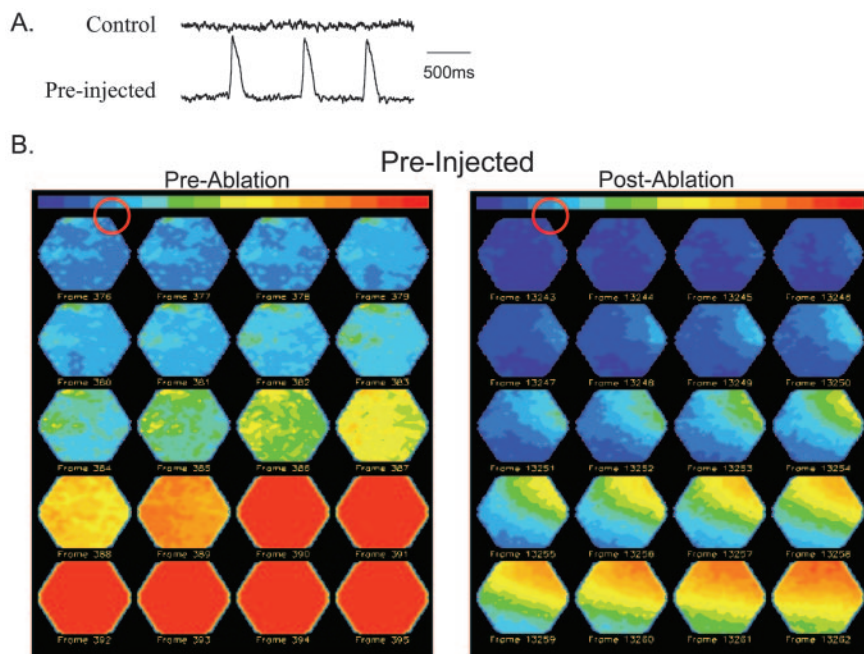


Figure 6. A, Effect of atrioventricular nodal cryoablation on control (top) and hESC-CM-injected (bottom) adult guinea pig heart without external pacing. B, Iso-potential contour maps of guinea pig heart preinjected with hESC-CMs before (left) and after (right) cryoablation. Note rapid epicardial wavefront breakthrough of preablation vs planar spread of depolarization of postablation. Right atrium had been removed in these recordings. Circles represent transplantation site, as identified by GFP epifluorescence of hESC-derived CMs (see Methods). In all measurements, area of earliest membrane depolarization was verified to correspond to site of injection. Recordings demonstrating progression of membrane depolarization from center but not corner of mapped region were also available. Abbreviations are as defined in text.

induce automaticity. As with control noninjected hearts, sham-injected hearts also exhibited complete electrical silence after cryoablation. Similar to the results of injection of mESC-CMs into mouse hearts,²⁷ hESC-CM injection into the guinea pig heart did not lead to tumor or arrhythmias (presumably because hESC-CM pacemaking activity was suppressed by the 2 to 3 times faster guinea pig heart rate) over the time course of our experiments.

Discussion

Although various lines of evidence suggest that adult stem cells possess a remarkable degree of plasticity by having the capacity to transdifferentiate into other lineages *in vivo*, this concept has been challenged by experiments showing that some results might have been misinterpreted by their fusion with existing cell types rather than by direct conversion.^{28,29} Two recent studies further indicate that hematopoietic stem cells adopt only hematopoietic fates and do not transdifferentiate into cardiac cells for myocardial regeneration.^{30,31} In contrast, hESCs have been proven to possess the potential to differentiate into multiple cell types, including CMs.¹⁻⁶ Here we have further demonstrated that hESCs retain their ability to differentiate into CMs even after stable genetic modification by a recombinant LentiV. Furthermore, differentiation of hESCs into the cardiac lineage does not result in transgene silencing. Given that mouse and human ESCs differ in a number of important aspects (eg, conventional methods of genetic manipulation, such as electroporation and transfection, that are highly effective for mESCs have been proven to be highly inefficient or even ineffective for hESCs; see Klug et al³² for review), this straightforward finding is critical for genetic methods such as use of a cardiac-specific promoter for driving the expression of a fluorescence reporter or an antibiotic-resistance gene are to be combined with LentiV-mediated gene transfer for deriving and selecting hESC-derived (chamber-specific) CMs.³²⁻³⁴

Normal heartbeats originate in the sinoatrial node, specialized cardiac tissue consisting of only a few thousand electrically active pacemaker cells that generate spontaneous rhythmic APs that subsequently propagate to induce coordinated muscle contractions of the atria and ventricles. Not surprisingly, malfunction or loss of pacemaker cells due to disease or aging necessitates the implantation of electronic pacemakers. Though effective, the use of such devices is also associated with significant risks (eg, infection or death), expense, and other disadvantages such as the need for periodic battery replacements. Using adenovirus-mediated, somatic gene transfer techniques, Miake and colleagues³⁵ demonstrated that “latent” pacemaker activity of normally silent ventricular myocytes can be unleashed to produce spontaneous firing activity by genetic inhibition of the inward rectifying K^+ current I_{K1} encoded by the *Kir2* gene family. However, the induced automaticity was ≈ 3 -fold slower than normal, and genetic suppression of I_{K1} does not provide a direct means to modulate the induced rhythm. Perhaps it is better to target a genuine pacemaker gene, such as the HCN channel. Although transient overexpression of HCN2 in the left atrium similarly induced an ectopic pacemaking activity, vagal suppression was required to observe the effect.³⁶ Furthermore, transgene expression in the heart in those experiments was sporadic. Clearly, the distribution of transgene(s) in the heart needs to be carefully targeted to achieve therapeutic goals because uncontrolled creation of ectopic pacemaker sites in the heart would lead to chaotic heartbeats. Localized transduction of the atrium, even if feasible, could be technically challenging. Furthermore, the creation of pacemakers by gene therapy relies on the conversion of preexisting heart tissue rather than the implantation of a new “surrogate node.” Recently, undifferentiated human mesenchymal stem cells have been tested as an alternative vehicle for delivering the *HCN2* gene into the heart.³⁷ However, these modified, undifferentiated, human mesenchymal stem cells

are incapable of pacing quiescent cells because the former are neither electrically active nor genuine cardiac cells. Because hESCs have been proven to possess the potential to differentiate into CMs, damaged sinoatrial nodal cells, at least conceptually, can be better replaced or supplemented with electrically active, hESC-derived CMs. Indeed, we have demonstrated in the present study the ability of electrically active, hESC-derived CMs to functionally integrate with and actively stimulate recipient, ventricular CMs in vitro and the ventricular myocardium in vivo. Maximal therapeutic flexibility could even be achieved by ex vivo genetic manipulation of hESC-derived CMs⁸ to exhibit a desired firing frequency. For instance, this can be accomplished by targeting the HCN gene.³⁸ Indeed, our laboratory has recently demonstrated that gene transfer of an engineered HCN1 construct, which better mimics the native heteromultimeric I_f channels than expression of a single isoform, to quiescent adult guinea pig ventricular cardiomyocytes can induce pacing with a normal firing rate,³⁹ unlike the 3-fold slower-than-usual rate unleashed by Kir2.1 suppression.³⁵ Furthermore, such an ex vivo approach is potentially advantageous over the in situ gene transfer approach by being able to first isolate clonal, genetically modified cell lines whose transgene location has been characterized to minimize the risk of inappropriate gene insertion (and thus, the associated oncogenesis). Our data also suggest that, unlike electronic devices, hESC-derived pacemakers retain their responsiveness to β -adrenergic stimulation as well as other ion channel modulators such as lidocaine and ZD7288.

Although the present study has established that donor hESC-derived CMs can functionally integrate with recipient ventricular CMs after in vitro and in vivo transplantation, additional experiments are still needed to explore in further detail the poorly defined, cellular electrophysiological profiles of hESC cardiac derivatives.²³ For instance, time-dependent electrical remodeling of hESC-derived CMs, such as changes in ion channel expression and AP duration after engraftment, could lead to arrhythmias. It is also necessary to determine the optimal graft size for in vivo transplantation, to test the long-term survival and functional efficacy of the grafted cells, to determine whether they can be sufficiently vascularized, etc. Furthermore, we have taken a simplistic proof-of-concept approach by using spontaneously beating outgrowths of hEBs. Although it remains a major technical challenge in the field of hESC research,^{32,34} derivation and purification of a homogenous population of nodal pacemaker cells will ultimately be necessary. This can be facilitated by combining what we have learned from classic mESC studies (eg, the aforementioned genetic method for selection) and other techniques that are more suitable for hESCs (eg, LentiV gene transfer). Nonetheless, the present study provides a platform for further investigating the possibility of modifying cardiac excitability with genetically engineered, hESC-derived cardiac derivatives. Our approach can also be applied to other multipotent stem cells (eg, cardiac resident stem cells).

We conclude that electrically active, hESC-derived CMs are capable of actively pacing recipient, ventricular CMs in vitro and in vivo. Our results may lead to a safer and less expensive

alternative or a supplemental method to implantable electronic devices for correcting defects in cardiac impulse generation.

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