Xenografted Adult Human Mesenchymal Stem Cells Provide a Platform for Sustained Biological Pacemaker Function in Canine Heart

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Background—Biological pacemaking has been performed with viral vectors, human embryonic stem cells, and adult human mesenchymal stem cells (hMSCs) as delivery systems. Only with human embryonic stem cells are data available regarding stability for >2 to 3 weeks, and here, immunosuppression has been used to facilitate survival of xenografts. The purpose of the present study was to determine whether hMSCs provide stable impulse initiation over 6 weeks without the use of immunosuppression, the “dose” of hMSCs that ensures function over this period, and the catecholamine responsiveness of hMSC-packaged pacemakers.

Methods and Results—A full-length mHCN2 cDNA subcloned in a pIRES2-EGFP vector was electroporated into hMSCs. Transfection efficiency was estimated by GFP expression. \( I_{\text{HCN2}} \) was measured with patch clamp, and cells were administered into the left ventricular anterior wall of adult dogs in complete heart block and with backup electronic pacemakers. Studies encompassed 6 weeks. \( I_{\text{HCN2}} \) for all cells was 32.1 ± 1.3 pA/pF (mean ± SE) at −150 mV. Pacemaker function in intact dogs required 10 to 12 days to fully stabilize and persisted consistently through day 42 in dogs receiving ≥700 000 hMSCs (=40% of which carried current). Rhythms were catecholamine responsive. Tissues from animals killed at 42 days manifested neither apoptosis nor humoral or cellular rejection.

Conclusions—hMSCs provide a means for administering catecholamine-responsive biological pacemakers that function stably for 6 weeks and manifest no cellular or humoral rejection at that time. Cell doses >700 000 are sufficient for pacemaking when administered to left ventricular myocardium. (Circulation. 2007;116:706-713.)

Key Words: electrophysiology ■ gene therapy ■ heart block ■ pacemakers

In less than a decade, biological pacemakers have made the journey from drawing-board concept to experimental reality in a variety of animal and tissue models.1–6 They are now seen within the device field as a disruptive technology, with the potential for more physiological and durable function than their electronic counterparts. In the normal heart, an inward current (designated \( I_f \)) activates on hyperpolarization to initiate phase 4 depolarization and pacemaker activity. The molecular correlate of this pacemaker current is the HCN multigene family, of which there are 4 isoforms, designated HCN1 through HCN4.7,8 Our own approach to biological pacemaking has been to administer the murine HCN2 gene (mHCN2) to the heart, via either viral vectors4,9,10 or adult human mesenchymal stem cells (hMSCs)11 as a delivery system. In the latter setting, we electroporate hMSCs to load them with HCN genes, thereby avoiding the problems associated with viral vectors. When injected intramyocardially, the hMSCs form gap junctions with adjacent myocytes, and the coupled cells operate as a single functional unit to initiate spontaneous rhythms.11

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Despite the promise of biological pacemakers, review articles and editorials have raised concerns that the burgeoning field of cell and gene therapies may carry with it significant problems and toxicities.6,12–15 Hence, the purpose of the present study had 3 goals: to understand the “dose” of stem cells that might provide adequate pacemaker function, to
determine the extent to which these cells generate stable pacemaker function and catecholamine responsiveness, and to determine stem cell survival over a 6-week period. To this end, we electroprolated adult hMSCs with mHCN2 and implanted them into the hearts of dogs in complete heart block. Electronic pacemakers also were implanted to operate in a “tandem” mode as described by us and others. As shall be demonstrated, the biological and electronic components functioned in a complementary fashion over the 6 weeks; the minimal cell number for optimal effectiveness was 700 000 hMSCs, and there was no sign of cellular or humoral rejection at the end of this interval.

Methods

Experiments were performed using protocols approved by the Columbia University Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1996).

hMSC Maintenance and Transfection

hMSCs from Clonetics/BioWhittaker (Walkersville, Md) were cultured in MSC-growing medium (Poeitics MSCGM, BioWhittaker) in a humidified atmosphere of 5% CO₂. Each batch had been tested for purity by the company via flow cytometry and for the ability of the cells to differentiate into osteogenic, chondrogenic, and adipogenic lineages. We tested the 5 different batches obtained from them for the expression of cell surface markers. All demonstrated the same pattern of the expression of CD14, CD29, CD34, CD44, CD45, HLA-I, and HLA-II. We also tested the expression of the cell surface markers mentioned above in 5th- and 10th-passage hMSCs. The purity by the company via flow cytometry and for the ability of the product.

patch-clamp studies of I_HCN2 expressed in hMSCs

The whole-cell patch-clamp technique was used to study membrane currents with an Axopatch-1D (Axon Instruments, Sunnyvale, Calif) amplifier. Patch electrode resistance was 4 to 5 MΩ before sealing. Cells were superfused with a gravitation perfusion system at 35 ± 0.5 °C. The pipette solution contained (in mmol/L) KCl 50, K-aspartate 80, MgCl₂ 1, Mg-ATP 3, EGTA 10, and HEPES 10 (pH adjusted to 7.2 with KOH). The external solution contained (in mmol/L) NaCl 137.7, KCl 5.4, NaOH 2.3, CaCl₂ 1.8, MgCl₂ 1, glucose 10, HEPES 5, and BaCl₂ 2 (pH adjusted to 7.4 with NaOH).

In Vivo Studies in Canine Ventricle

Stem cells were prepared as above. At the time of the in vivo experiment, cells were thawed, and a sample was counted in the presence of trypan blue to obtain the percentage of viable cells and the total number of viable cells. The percentage of viable cells ranged from 75% to 90%. The desired number of viable cells was then diluted in a final volume of 0.6 to 0.75 mL PBS solution. The cell count of trypan blue–negative cells measured just before injection ranged from 156 × 10⁶ to 1026 × 10⁶.

Under sterile conditions, mongrel dogs of either sex weighing 23 to 25 kg (Chestnut Ridge Kennels, Chippenssburg, Pa) were anesthetized with propofol 6 mg/kg IV, followed by inhalational isoflurane (1.5% to 2.5%). After anesthesia, a pacemaker lead (Flxteind lead, Guidant, St Paul, Minn) was introduced into the right ventricular apex via a jugular venous approach. An electronic pacemaker (Discovery II, Guidant) was placed in a subcutaneous pocket and set at VVI 60. After pacemaker implantation, the right or left femoral vein was catheterized, and complete heart block was induced via radiofrequency ablation of the atrioventricular node. After recovering for 2 days, the animals were anesthetized as above, and a thoracotomy was performed in the fifth left intercostal space under sterile conditions. hMSCs containing mHCN2/EGFP were injected subepicardially at 3 closely apposed sites in the left ventricular anterior wall 2 mm deep to the epicardium. Cells were administered in a total volume of 0.6 to 0.75 mL PBS solution via a 23-gauge needle. The region of injection was paced for 10 to 15 seconds via an epicardial electrode to facilitate ECG identification of the origin of any idiovenricular rhythm originating at or near that site during the follow-up period. Before closure, 5.0 proline sutures were placed near the injection region to mark it for histological studies.

The electronic pacemaker was then reprogrammed to VVI 35 bpm to operate in a “tandem” mode with its biological counterpart. ECG, 24-hour Holter monitoring, pacemaker log record check, and overdrive pacing at 80 bpm were then performed weekly for 6 weeks. To evaluate β-adrenergic responsiveness, on day 42, epinephrine (1 to 2 μg · kg⁻¹ · min⁻¹) was infused to an end point of a 50% increase in heart rate or ventricular premature beats or ventricular tachycardia, whichever occurred first. If none of the responses were observed, infusion was terminated after the maximal dose of 2 μg · kg⁻¹ · min⁻¹ had been administered for 10 minutes. Tissues were then removed for immunohistochemistry and histological study.

Histology

Left ventricular myocardium containing the injection sites was sampled with a portion of left ventricular myocardium from the cardiac apex (away from the injection site), fixed in formalin overnight, and embedded in paraffin. Serial sections were cut from each myocardial sample, and every 10th section was stained with hematoxylin and eosin to determine the distribution of hMSCs in relation to the needle tract and whether they were associated with inflammation.

Immunohistochemistry

Serial sections adjacent to those containing clusters of hMSCs identified by morphological means and sections of the corresponding left ventricular apices, which served as negative controls, were labeled with an immunoperoxidase staining kit (Vectastain, Burlingame, Calif) with antibodies to (1) green fluorescent protein (rabbit anti-GFP, 1:100, Dakopatts, Carpinteria, Calif) to confirm that these cells incorporated HCN2 and expressed current.
Results are presented as mean±SEM. Depending on the protocol, statistical significance was determined by Student t test for unpaired data or ANOVA for repeated measures. Linear regression analysis was also used. Values of P<0.05 were considered significant.

The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Statistical Analysis
Results are presented as mean±SEM. Depending on the protocol, statistical significance was determined by Student t test for unpaired data or ANOVA for repeated measures. Linear regression analysis was also used. Values of P<0.05 were considered significant.

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Results
Transfection of hMSCs With mHCN2 and Functional Expression of Pacemaker Current
mHCN2-transfected hMSCs expressed a large time-dependent inward current activating on hyperpolarizations up to −160 mV and deactivating during the following step to 15 mV (Figure 1A). Figure 1B shows the I_{HCN2} activation curve constructed from tail currents recorded from mHCN2-transfected hMSCs (see the inset for voltage protocol). For experiments examining the dose-dependent effect of mHCN2-transfected hMSCs, several batches of mHCN2-transfected hMSCs using the protocol described previously were generated, and the expression of I_{HCN2} in these cells was compared. I_{HCN2} was measured at a test potential of −150 mV (2-second duration) from a holding potential of −40 mV (Figure 2A) and normalized to its cell capacitance. The current density in the 6 batches of mHCN2-transfected hMSCs that provided the material for all 8 intact animal experiments is summarized in Figure 2B and shows no significant difference among the groups of cells (P>0.05).

Intact Animal Studies
Pacemaker function became evident within 2 to 3 days of implantation, and its stabilization was a function of both time after implantation and cell number implanted. This is illustrated in Figure 3, which provides representative records from 1 dog that received 157 000 cells and 1 dog that received 766 000 cells. Note that for the former the rate on day 12 was only 38 bpm and that on day 42 the electronic pacemaker was firing at 35 bpm. In contrast, for the dog receiving 766 000 cells.

Figure 1. Activation of I_{HCN2} in hMSCs transfected with the mHCN2 gene. A, Activation of I_{HCN2} was demonstrated in hMSCs transfected with the mHCN2 gene. B, Fit by the Boltzmann equation to the normalized tail currents of I_{HCN2} gives a midpoint (V_m) of −90.0±0.8 mV and a slope (K) of 16.3±0.7 mV (n=10). I_{HCN2} was fully activated at −140 mV with an activation threshold of −50 mV. Inset of A, The voltage protocol was to hold at −40 mV and hyperpolarize for 2 seconds to voltages between −40 and −160 mV in 10-mV increments, followed by a 2-second voltage step to 15 mV to record the tail currents.

Figure 2. The expression of I_{HCN2} in different batches of mHCN2-transfected hMSCs. A, Representative trace of I_{HCN2} elicited at a test potential of −150 mV (2-second duration) from a holding potential of −40 mV. B, I_{HCN2} expression in different batches of mHCN2-transfected hMSCs. Numbers in parentheses are numbers of cells studied. There is no significant difference among the groups of cells (P>0.05).
cells, biological pacemaker rates were in the 50- to 60-bpm range on both days 12 and 42. The graphs in Figure 4 summarize data from all experiments. On days 3 (Figure 4A) and 7 (Figure 4B), there was no clear relationship between percent of electronic beats and number of cells implanted, indicating that stable pacemaker function was not achieved within the first week (and through 10 to 12 days [data not shown]) after cell implantation. Idioventricular rates for all dogs before implantation of the hMSCs was 39±2 bpm; at 14 days, the rate was 52±3 bpm for the dogs receiving >700,000 cells and 43±3 bpm for those receiving <600,000 cells (P>0.05).

After day 14, the rates for those animals receiving <700,000 cells remained unstable (Figure 4C, lower left). Specifically, 4 of the 5 animals showed an increase in electronic beats occurring through day 42, whereas 1 animal (represented by upright triangles) showed fewer electronic beats with time. In contrast, the 3 animals receiving >700,000 cells (Figure 4C, upper left) manifested very stable function and little time-dependent variation in electronic beats. This stability persisted through day 42. Summary data for the 2 groups of animals across weeks 2 to 6 (Figure 4C, right) show a significantly lower percentage of electronic beats occurring in animals receiving >700,000 cells compared with those receiving <600,000 cells.

Whereas Figure 4 presents the percent of beats that were electronic versus idioventricular over the 6-week study period, we also pace-mapped the origins of the nonelectronic beats that occurred. Figure 5A suggests a direct relationship between the percent of beats that were pace-mapped to the site of cell implantation and cell number. This relationship is

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**Figure 3.** Representative ECGs during weeks 2 and 6 from 1 animal receiving 157,000 hMSCs and 1 receiving 766,000 hMSCs. See text for discussion.

**Figure 4.** Percent of beats electronically initiated for all animals injected expressed as a function of total viable cells injected. The viable cells are those that were trypan blue negative. A and B. Results on days 3 and 7, respectively. C. Dogs on days 12 to 42. Left, Records for individual animals. Note that for those receiving <600,000 cells, there is a far more variable response from week to week than for those receiving >700,000 cells. Right, The percent of beats that were electronic was significantly greater in the animals receiving <600,000 cells.
tested in Figure 5B, which demonstrates that the animals receiving >700,000 cells had ~70% of nonelectronic beats originating from the implantation site, whereas those receiving <600,000 cells had only ~10% originating from that site ($P<0.05$). Assessing a minimum rate was difficult on Holter monitor; when the biological rate fell to 35 bpm, the electronic pacemaker fired at that rate. Therefore, animals were brought to the laboratory on a weekly basis, and their ventricular rates were recorded as they lay quietly for 45 to 60 minutes under direct investigator observation with the electronic pacemaker turned off. Figure 5C demonstrates that in this circumstance, asystole did not occur, but pace-mapped rhythms having rates as low as 30 bpm were seen. The fastest rates achieved by pace-mapped rhythms per 24-hour period were assessed on Holter monitor (Figure 5D). Rates in the 60-to-70-bpm range were regularly achieved; rates as fast as 90 bpm also were seen. Using linear regression analysis, Figure 5E demonstrates that the maximum rates of the pace-mapped beats increased with the numbers of cells administered. Data in Figure 5E are from day 42, but comparable results occurred in weeks 2 to 5 (data not shown). Treadmill exercise protocols have not yet been performed in this animal model, and these may reveal a higher maximum rate.

**Catecholamine Responsiveness**

We infused epinephrine $1 \mu g \cdot kg^{-1} \cdot min^{-1}$ into 8 dogs for 10 minutes. In 4 animals in which the rhythm pace-mapped to the injection site at the time of infusion, there was a rate increase from $37 \pm 3$ to $55 \pm 3$ bpm after $1 \mu g \cdot kg^{-1} \cdot min^{-1}$ epinephrine ($P<0.05$). In contrast, the other 4 animals did not manifest a pace-mapped rhythm at the time of infusion. In those animals, $1 \mu g \cdot kg^{-1} \cdot min^{-1}$ epinephrine increased the rate by $34 \pm 5\%$, in contrast to the rate increase of $54 \pm 4\%$ in the former group ($P<0.05$).

**Histological Studies**

The needle track of the injection site was found in all hearts and studied histologically at 6 weeks after injection. hMSCs formed small clusters between the preexisting myocardial fascicles (Figure 6A) and were further identified by the brown reaction product within their cytoplasm when labeled either for human CD44 (Figure 6B) or with the anti-GFP
antibody (Figure 6C) using the immunoperoxidase technique. There was no binding of canine IgG to the surface of hMSCs (Figure 6D) and no significant infiltration by CD3+ T lymphocytes (Figure 6E). The results in Figure 6D and 6E indicate that neither humoral nor cellular rejection of the hMSCs, respectively, was occurring. None of the hMSCs displayed the nuclear characteristics of apoptosis or labeled for cleaved/activated caspase 3 (Figure 6F). Finally, although only a single noninjected reference site was used (the apical portion of the left ventricle several centimeters from the injection site), no hMSCs were noted there.

**Discussion**

Our intent in these studies was to expand on our understanding of the viability of hMSC-based biological pacemakers: viability in terms of electrophysiological function and in terms of the persistence of the hMSCs themselves. We had considered that cells might be present yet lose their pacemaker function or that, through apoptosis and/or rejection, the cells themselves might be lost. Preliminary data (M.J.S.) suggest that, in some dogs 2 weeks after injection, apoptosis may be present as a result of mild cellular rejection. In contrast, we have found no evidence of rejection at 6 weeks after hMSC injection in the 8 dogs studied here. Because no biopsies were done at earlier time points, it is impossible to state whether cell loss occurred before 6 weeks in these animals.

At 6 weeks, the injected hMSCs were readily identifiable by both morphological and immunohistochemical means (anti-GFP labeling) and appeared to remain within and around the injection site with no evidence of migration to the left ventricular apex. The anti-GFP labeling seen here is consistent with the GFP immunofluorescence of hMSCs implanted in canine myocardium that we have reported previously.11 Whether migration occurred to other parts of the heart or the rest of the body is not yet known. At this time, there was no evidence for apoptosis or any significant infiltration of CD3+ T lymphocytes. Neither was there binding of immunoglobulins to the surface of hMSCs, all consistent with immunoprivilege. One explanation of this phenomenon has been that hMSCs may lack the HLA surface markers characterizing most other cell types.17 However, more detailed literature suggests it is likely that, through cellular secretion of prostaglandins or other soluble factors such as interleukin-1b, transforming growth factor β-1, or hepatocyte growth factor, the surface antigens present on the hMSCs may simply be masked.17–20 This observation, although clearly of benefit with regard to studies such as ours and in its implications with regard to allogeneic transplantation of hMSCs into human subjects, does carry with it a major note of caution; ie, hMSCs have the potential to differentiate into more mature cell types. If this occurs, it is reasonable to question whether the cells will maintain their immunoprivileged status. Given this possibility, it is important to ensure...
that hMSCs remain in an undifferentiated state through their lifetime of use as biological pacemakers and/or that evidence be obtained to determine whether maturation and differentiation cause immunoprotection to be lost and rejection to occur.

That no humoral or cellular rejection was evident at 6 weeks indicates that in this setting a critical mass of cells is present to induce pacemaker function as long as the initial “dose” of cells is >700,000. However, more detailed information at 2 weeks and studies extending beyond 6 weeks are required to fully understand the events revolving around cell fate and their long-term implications for pacemaker function.

Stating that a minimum of 700,000 hMSCs needs to be injected is in some ways deceptive. It is possible that in some circumstances lower numbers of cells may provide an equally good response (eg, see the result with 257,000 hMSCs in Figure 3C). In addition, as stated in Methods, roughly 40% of cells incorporated HCN2. Hence, it is likely that, of 700,000 hMSCs injected into the left ventricular anterior wall subepicardium to generate the biological pacemaker rhythms observed, only ≈300,000 were uniformly loaded with HCN2. It is worth pointing out that our previous study demonstrated that hMSCs loaded with GFP but not the HCN2 gene generated no spontaneous rhythms at the site of injection. Moreover, depending on the proposed site of injection, the numbers of cells needed might be far smaller. For example, cells of the ventricular specialized conducting system have far less inward rectifier current than ventricular myocardium and possess native pacemaker current in the physiological range of potentials. For both of these reasons, we might speculate that injecting ≈1/10th the number of cells into the conducting system would be adequate to drive the ventricles as a biological pacemaker.

The observation of dogs in the resting state with electronic pacemakers turned off provided encouraging information in the sense that no asystole was seen. Nonetheless, rates of 30 bpm did occur and clearly are not desirable. This observation also emphasizes the need to use a tandem electronic-biological approach for testing biological pacemakers if and when they come to clinical trial. This intervention uses a biological pacemaker to provide an autonomically responsive driver for the heart, whereas the electronic unit provides a safety net and monitors the function of the biological unit. Delivering genetically engineered hMSCs in a tandem setting with an electronic pacemaker extends our previous research that used viral delivery as the biological portion of the tandem pacemaker. We envision this type of approach as necessary for any first generation of biological pacemakers.

Finally, the maximal biological rates achieved of up to 90 bpm are reflective of the responsiveness of HCN2 to autonomic intervention. We previously demonstrated autonomic responsiveness using a viral vector to implant a biological pacemaker. In considering the overall rate range seen with HCN2, we believe that an ideal biological pacemaker would have minimal rates in the 50s and be capable of achieving rates of 100 to 150 bpm under catecholamine demand. We and others are currently working with various mutant and chimeric channel proteins to attempt to optimize the rate range.

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Disclosures
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References
17. Zimmett JM, Hare JM. Emerging role for bone marrow derived mesenchymal stem cells in myocardial regenerative therapy. Basic Res Cardiol. 2005;100:471–481.


**CLINICAL PERSPECTIVE**

Biological pacemaking has been performed with viral vectors, human embryonic stem cells, and adult human mesenchymal stem cells (hMSCs) as delivery systems. Only with human embryonic stem cells are data available regarding stability for >2 to 3 weeks, and here, immunosuppression has been used to facilitate survival of xenografts. The purpose of the present study was to determine whether hMSCs provide stable impulse initiation over 6 weeks without the use of immunosuppression, the “dose” of hMSCs that ensures function over this period, and the catecholamine responsiveness of hMSC-packaged pacemakers. The study was performed by electroporating hMSCs with a full-length mHCN2 cDNA subcloned in a pIRES2-EGFP vector. Cells were administered into the left ventricular anterior wall of adult dogs in complete heart block and with backup electronic pacemakers. Pacemaker function in intact dogs required 10 to 12 days to fully stabilize and persisted consistently through day 42 in dogs receiving ≥700,000 hMSCs (~40% of which carried current). Rhythms were catecholamine responsive. Tissues from animals killed at 42 days manifested neither apoptosis nor humoral or cellular rejection. These results suggest that hMSCs provide a means for administering catecholamine-responsive biological pacemakers that function stably for 6 weeks and manifest no cellular or humoral rejection at that time. Cell doses >700,000 are sufficient for pacemaking when administered to left ventricular myocardium. Longer-term trials are required to assess the stability and potential untoward effects of such pacemakers before they can be considered for clinical trial.
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