Long-Term Improvement in Postinfarct Left Ventricular Global and Regional Contractile Function Is Mediated by Embryonic Stem Cell–Derived Cardiomyocytes

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Background—Pluripotent stem cells represent one promising source for cellular cardiomyoplasty. In this study, we used cardiac magnetic resonance to examine the ability of highly enriched cardiomyocytes (CMs) derived from murine embryonic stem cells (ESC) to form grafts and improve contractile function of infarcted rat hearts.

Methods and Results—Highly enriched ESC-CMs were obtained by inducing cardiac differentiation of ESCs stably expressing a cardiac-restricted puromycin resistance gene. At the time of transplantation, enriched ESC-CMs expressed cardiac-specific markers and markers of developing CMs, but only 6% of them were proliferating. A growth factor–containing vehicle solution or ESC-CMs (5 to 10 million) suspended in the same solution was injected into athymic rat hearts 1 week after myocardial infarction. Initial infarct size was measured by cardiac magnetic resonance 1 day after myocardial infarction. Compared with vehicle treatment, treatment with ESC-CMs improved global systolic function 1 and 2 months after injection and significantly increased contractile function in initially infarcted areas and border zones. Immunohistochemistry confirmed successful engraftment and the persistence of α-actinin–positive ESC-CMs that also expressed α-smooth muscle actin. Connexin-43–positive sites were observed between grafted ESC-CMs but only rarely between grafted and host CMs. No teratomas were observed in any of the animals.

Conclusions—Highly enriched and early-stage ESC-CMs were safe, formed stable grafts, and mediated a long-term recovery of global and regional myocardial contractile function after infarction. (Circ Cardiovasc Imaging. 2011; 4:33-41.)

Key Words: cardiac MRI embryonic stem cells left ventricular remodeling left ventricular wall motion myocardial infarction

Restoration of contractile function to the infarcted myocardium is the ultimate goal of cellular cardiomyoplasty. To achieve this goal, cell-based therapies have been proposed to replace some, or even a majority, of the myocytes lost to infarction. Several major unresolved issues remain, including the optimal cell type for effecting improvement of function and the most useful method for assessment of contractile function.

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Unlike most adult stem or progenitor cells, pluripotent stem cells derived from embryos (embryonic stem cells, ESCs) or experimentally from somatic cells (induced pluripotent stem cells) provide a nearly-unlimited source of cardiomyocytes (CMs) for cellular cardiomyoplasty. However, constraints to the use of human-derived ESCs for cell therapy include ethical barriers and potential immunogenicity of ESC progeny.1 These concerns can potentially be overcome by induced pluripotent stem cells, which are generated in vitro by transcription factor–mediated reprogramming, but problems still remain with respect to interline heterogeneity and incomplete epigenetic remodeling.2–4 Because of this variability, ESCs still represent one of the best model systems to study critical issues in cellular cardiomyoplasty.

The adult myocardium, however, is not suited for guiding the cardiac differentiation of ESCs in situ, and teratomas commonly form in immune-competent syngeneic hosts as well as in immune-deficient hosts.1,5 Alternatively, cardiac
Differentiation can be robustly induced in ESCs to derive bona fide CMs in vitro by formation of embryoid bodies, but purification of CMs from a mixed cell population is challenging owing to the lack of suitable CM surface markers. In the past, CMs ranging from a low to a moderately high enrichment obtained from ESCs of murine and human origin were shown to form grafts and improve global function. In a mixed cell population, survival of CMs could be promoted by noncardiac cells (for example, fibroblasts), but the risk of tumor formation from these noncardiac cells poses a safety concern. In addition, regional myocardial contractile function was not directly characterized in those studies, which might add further insight into the mechanisms of functional recovery.

To overcome these latter 2 limitations, kinematic analysis of myocardial wall motion can be used to estimate regional and intramural contractile function in a more detailed manner compared with that from left ventricular ejection fraction (LVEF). Importantly, these quantitative measurements have been achieved in both humans and mice. In our previous study, a significant improvement in LVEF and fractional shortening at infarct borders was observed after injection of undifferentiated ESCs; however, tagged magnetic resonance imaging (MRI) revealed a lack of contraction inside the graft, an observation consistent with infrequent cardiac differentiation in grafted cells. Hence, wall motion appears to be a more specific approach to evaluate regional improvement of myocardial contraction.

We recently described murine ESCs containing a cardiac-specific puromycin resistance gene that allows elimination of noncardiac cells by antibiotic treatment. By combining this system with cardiac magnetic resonance (CMR)--based wall motion measurements, we tested the hypotheses that highly enriched and early-stage ESC-CMs can be isolated in large numbers for in vivo studies and that such ESC-CMs injected into athymic rats 1 week after surgical induction of myocardial infarction (MI) would form grafts and improve global and regional contractile function by 2 months post-MI, as assessed by in vivo CMR.

**Characterization of ESC-CMs**

**Enrichment**

The harvested cells were probed with antibody to cardiac troponin I (cTnI; Millipore, Billerica, Mass) and α-smooth muscle actin (SMA; Biomedia, Foster, Calif). The percentage of cTnI-positive cells was estimated either by microscopy from a total of 4313 cells in 3 experiments or by fluorescence-activated cell sorting in 3 experiments, each examining 10 000 cell events. Proliferation of ESC-CMs was evaluated by immunostaining for Ki-67 (Biomedia, Foster, Calif). The number of Ki-67 and α-actinin double-positive cells was counted by microscopy from a total of 3932 α-actinin-positive cells in 3 experiments to estimate the percentage of the Ki-67-positive population.

**Gene Expression Profile**

Genes related to pluripotency (OCT3/4 and Nanog) and those expressed at various stages of embryonic heart development (α- and β-myosin heavy chain), myosin light chain 2v, NKX2.5, NCX1, atrial natriuretic factor, and α-SMA were examined. RNA from ESCs, 16- to 17-day-old ESC-CMs, and isolated adult rat CMs was extracted. The primer sequences and reverse transcription--polymerase chain reaction procedure have been detailed in our previous report.

**Cellular Electrophysiology**

The whole-cell, patch-clamp configuration was used to record action potentials (APs) from ESC-CMs and adult mouse CMs with the use of hard borosilicate micropipettes with a resistance of 2 to 3 MΩ. APs were initiated by current pulses (0.2 to 0.3 ms, 400 to 500 pA) and recorded at 25°C with an Axopatch 200B amplifier (Axon Instruments, Foster City, Calif) under current clamp conditions. The pipette solution for recording APs contained (in mmol/L) potassium aspartate (80), KCl (50), MgCl2 (1), EGTA (10), HEPES (10), and Mg-ATP (3), with pH adjusted to 7.2. The bath solution contained (in mmol/L) NaCl (140), KCl (5.4), CaCl2 (1.8), MgCl2 (1), HEPES (10), and glucose (10), with pH adjusted to 7.4.

**Labeling With Superparamagnetic Iron Oxide Particles**

ESC-CMs were incubated with labeling medium containing 6.25 µg Fe/mL (Feridex, Berlex Laboratories) and 0.4 µg/mL poly-l-lysine overnight; labeling efficiency was determined from 3 experiments (total of 1490 cells).

**Methods**

**Production of Highly Enriched ESC-CMs by a High-Throughput System**

Murine R1 ESCs (clone syNP4) that stably express the puromycin resistance gene cassette under the cardiac-specific promoter of the sodium/calcium exchanger (NCX1) were seeded at a density of 10⁵ cells/mL into a spin flask (Integra Biosciences, Zizers, Switzerland) rotating at 60 rpm. Half of the ESC medium without leukemia inhibitory factor was replaced every other day. BMP2 (bone morphogenetic protein 2) (0.5 to 1 ng/mL; Sigma, St. Louis, Mo) was added to the medium on day 6 after seeding. Puromycin (2.5 µg/mL) was added at days 9 to 10 when contracting embryoid bodies were first observed. Embryoid bodies were then harvested and dissociated with collagenase on the following day. Monolayer culture continued for 7 days in the presence of puromycin, and all surviving cells were harvested at days 16 to 17. This high-throughput method routinely yielded 35 (±15) million ESC-CMs 16 to 17 days after the initial seeding of 25 million.

**In Vivo Studies**

**Surgery to Induce MI and Inject Cells**

All animal procedures were approved by the local institutional animal care and use committee. A reperfused MI was induced in female athymic nu/nu rats of 200 g (Frederick Cancer Center, Frederick, Md). When the infarct size estimated by CMR was in the specified range (10% to 30% of LV wall volume), the rat was assigned randomly to 1 of 2 groups: vehicle (n=16) or ESC-CM (n=17), which received 100 µL of vehicle or 5 to 10 million ESC-CMs suspended in 100 µL of vehicle, respectively. The vehicle solution contained a cocktail of growth factors dissolved in growth factor--reduced Matrigel (Collaborative Biomedical, Bedford, Mass). Intramyocardial injection was performed via a second lateral thoracotomy 7 days after MI. The lateral intercostal space was used as the entry site to avoid re-entry from the sternum. This strategy eliminated excessive bleeding from dissection of scar tissue that had formed after the first surgery and reduced surgery time and mortality. Vehicle or ESC-CM was injected into the midanterior LV wall in 2 locations.
CMR Imaging and Analysis
CMR was performed on a 4.7-T, horizontal-bore Varian INOVA system equipped with a 12-cm (internal diameter) gradient coil (25 gauss/cm). A volume transmitting coil (internal diameter of 70 mm) and a surface receiving coil (InsightMRI) were used. The animals were maintained under isoflurane (1% mixed with O₂) anesthesia via nose cone; ECG and respiration were monitored, and core temperature was maintained at 37±0.2°C (SA Instruments, Stony Brook, NY). All images were acquired under cardiac and respiratory double gating. CMR scanning required ≈1 hour, which included shimming and scout scans (15 minutes), cine (15 minutes), and a displacement encoding with stimulated echo (DENSE) protocol (2 minutes per set × 5 sets × 3 slices = 30 minutes). Initial infarct size was estimated 1 day after MI surgery by delayed hyperenhancement (DHE). Infarct size was calculated as a percentage of LV myocardial volume. Confirmation of intramyocardial delivery of ESC-CMs was achieved by T2*-weighted CMR 1 day after injection.

Global function was measured 1 day after MI and 1 and 2 months after cell injection; the left ventricle from base to apex was imaged in 11 to 13 contiguous short-axis images (each 1 mm thick). LV end-diastolic and end-systolic volumes and LVEF were derived from cine images.

Regional contractile function was measured 2 months after cell injection with a DENSE imaging sequence adapted from Kim et al. In our sequence, the read-out and phase-encoding directions were not swapped, and only 1 set of reference images was acquired. DENSE data sets were acquired from 3 short axis slices (each 1 mm thick) from the midventricle to apex with a 1.5-mm gap and the following parameters: field of view (FOV) = 128 mm × 128 mm, matrix = 128 × 128, slice thickness = 1.5 mm, k = 0.5 cycle/mm (0.156 cycle/pixel); 7 time points were captured evenly over 140 ms, which included >75% of the rat cardiac cycle (160 to 170 ms). The k-space raw data were analyzed offline with the MATLAB program (Mathworks, Natick, Mass) as detailed in the online-only Data Supplement to derive intramyocardial displacements and normal lagrangian strains (radial strain Er, circumferential strain Eθ, and shear strain Eτ).

Two segmentation schemes were used to present regional function: (1) the I/B-L/B-S/R scheme, in which the infarct (I), lateral border (B-L), and septal border (B-S) of the infarcted area and the remote region (R) were defined on DENSE images. These regions were then transferred to corresponding DENSE images to estimate the Er, Eθ, and Eτ, and (2) the S/A/L/P scheme, in which the myocardial wall on the DENSE image was divided into septal (S), anterior (A), lateral (L), and posterior (P) segments.

Immunohistochemistry to Characterize Grafts and Estimate Scar Size
All rats were euthanized 2 months after transplantation. The heart was embedded in paraffin or OCT. Each heart was cut in a short-axis orientation (from the base to apex) into 30 μm between adjacent segments. Five sections (each 10 μm thick) per level were obtained for the following analyses: (1) hematoxylin/eosin staining for general morphology and identification of teratomas; (2) Prussian blue (PB) staining to visualize superparamagnetic iron oxide particle (SPIO)-labeled cells; (3) immunofluorescence with primary antibodies against α-actinin, α-SMA, and connexin-43 (all from Sigma, St. Louis, Mo) to visualize grafts; (4) host macrophages identified by CD68 staining; and (5) scar tissue as visualized by Masson’s trichrome staining on 1 section per segment for all segments. The areas of scar tissue and myocardium were measured in ImageJ (http://rsbweb.nih.gov/ij/index.html).

Statistical Analysis
Data are presented as mean±SD in the text and figures. Student’s t test was used to compare 2 groups. A probability value of <0.05 was considered significant. To test the hypothesis of intervention effects on global function at various time points, a 3-stage linear model analysis, consisting of MANOVA followed by ANOVA and post hoc contrasts, was performed in SAS/STAT PROC GLM. In the first stage, a separate MANOVA was performed 1 day after MI and 1 and 2 months after cell injection. Each multivariate analysis included 3 within-subject cardiac outcomes, LVEF, LV end-diastolic volume, and LV end-systolic volume, and the 3 between-subject groups: ESC-CM, vehicle, and normal (noninfarcted). When the result of the MANOVA was significant by the Wilks’ criterion, it was followed by a 3-group, s-way ANOVA for each of the 3 cardiac outcomes. The third stage consisted of Tukey post hoc contrasts to identify significant intervention effects for each outcome at each time point.

Results
In Vitro Characterization of ESC-CMs
Immunostaining for cTnI suggested that cells harvested at the end of a 16- to 17-day differentiation protocol were highly enriched in ESC-CMs: the enrichment was 98±1.7% by microscopy and 92±3% by fluorescence-activated cell sorting (Figure 1, A and B). Approximately 98% of the cells were double-positive for cTnI and α-SMA, and 2% were α-SMA–positive only (arrowheads in Figure 1C). The gene expression profile suggested that ESC-CMs expressed mature cardiac markers, including NCX1, α- and β-myosin heavy chain, and myosin light chain 2v, as well as early markers such as α-SMA (Figure 1K). Pluripotency markers were not expressed in ESC-CMs. Ki-67 and α-actinin double-positive staining was observed in 5.7±2.3% of α-actinin–positive cells (Figure 1D, arrows). Ki-67–negative ESC-CMs showed typical striation patterns (Figure 1F), which were absent in positive ESC-CMs (Figure 1E). The SPIO labeling efficiency was 20.7±9.8% (Figure 1G–J). Ventricle-, atrium-, and pacemaker-like cells were identified as subtypes of ESC-CMs by single-cell electrophysiology studies (Figure 1, L–N), and the percentages were similar to those previously published. Ventricle-like ESC-CMs exhibited a pronounced plateau phase with a longer AP duration at 90% repolarization but a lower AP amplitude compared with adult ventricular myocytes (Figure 1, O), suggesting that they resemble embryonic/fetal rather than adult ventricular myocytes.

Characterization of ESC-CM Grafts and Estimation of Final Infarct Size
Characterization of Grafts
SPIO-labeled ESC-CMs were injected into athymic hearts 1 week after MI, and no teratoma or teratoma-like structures were observed 2 months after injection in any of the hearts. Grafts were identified on hematoxylin/eosin–stained sections by morphology and confirmed by positive PB staining (Figure 2, A–C). Grafted ESC-CMs were elongated and striated (Figure 2B). They were double-positive for α-actinin and SPIOs (Figure 2, D and E) and for α-SMA and SPIOs (Figure 2, F and G) on adjacent sections. In host myocardium, α-SMA expression was limited to smooth muscle cells lining the vasculature (Figure 2F). Punctate connexin-43–positive sites were detected between host CMs and between grafted CMs (Figure 2, H and I) but infrequently (though unequivocally) between the grafted (green) and host CMs (Figure 2J). Grafts were identified in ≈70% of ESC-CM–treated rats (11 of 17). The graft extended from 5 to 10 transverse levels; the graft area on sections ranged from 0.009 to 0.86 mm², with an average of 0.14 mm². The maximal graft volume was...
Figure 1. A–O, Characterization of ESC-CMs in vitro. A and B, Enrichment estimated by immunostaining for cTnI by fluorescence microscopy (A) or fluorescence-activated cell sorting (B). Arrowheads mark cTnI-negative cells (A). Purple trace represents cTnI-positive cells; green trace represents control cells, which were stained only with fluorescein isothiocyanate–conjugated secondary antibody (B). C, Double staining for cTnI (green) and α-SMA (red). D–F, Proliferation status as assessed by double α-actinin (green) and Ki-67 (red) immunostaining. Boxes (with magnified views in E and F) show a Ki-67–positive and –negative cell, respectively. G–J, G and H represent the overlay of a differential interference contrast image of PB staining (I) and fluorescent α-actinin (J). SPIOs are shown surrounded by α-actinin (J). Differential interference contrast conveys a greyish appearance in G–H. Nuclei in A–J were counterstained with 4',6'-diamidino-2-phenylindole (DAPI). K, Gene expression profile of ESC-CMs, ESCs, and adult rat ventricular CMs detected by reverse transcription–polymerase chain reaction. β-Actin was used as the reference. *Water instead of rat CMs was used as a control in the polymerase chain reaction of SMA. L–O, Electrophysiologic properties. The AP of ESC-CMs of ventricle- (L), atrium- (M), and pacemaker-like (N) cells. The AP in ventricle- and atrium-like cells was evoked by current pulses. Electrophysiologic features of ventricle-like ESC-CMs and adult ventricular myocytes are shown (O). No statistics were performed for panel O owing to the small sample size in ventricular myocytes (n=3) and ESC-CMs (n=2). APD_{90} indicates AP duration at 90% repolarization.
estimated to be 0.24% of LV volume, with an average volume of 0.10 ± 0.086%.

**Juxtaposition of SPIO-Containing Cell Populations**

Triple staining for SPIO, α-actinin (or α-SMA), and CD68 (macrophage) revealed that 38 ± 18% of PB-positive cells were ESC-CMs and 62 ± 18% were macrophages, which were distributed around the graft (Figure 2, K–M). No host CMs were PB-positive. These data suggest that SPIOs were not uniquely associated with ESC-CMs; however, α-SMA was positive only in grafted ESC-CMs or in vascular smooth muscle in the host heart. Arrows mark α-actinin- or SMA-positive cells that contain SPIOs. D and F are adjacent sections. H and I, Immunofluorescence of connexin-43 (red) and α-actinin (green). Connexin-43–positive sites, appearing as punctate red dots or thin lines, were frequently identified between host CMs (H) and between the grafted ES-CMs (I). J, Overlay of differential interference contrast image of PB staining with connexin-43 (red) and α-SMA (green). Connexin-43–positive sites (thin arrows) were found between a grafted CM (green) and host CM (its border revealed by differential interference contrast). K–M, Evaluation of SPIO distribution. K, Differential interference contrast image of PB staining for SPIO particles (blue spots). L, Immunofluorescence of α-actinin (green) and CD68 (red) for macrophages. M, Overlay of K and L revealed SPIO located in ESC-CMs (arrows) or in macrophages (arrowheads). The graft is outlined with dashed lines. Nuclei in D–M were counterstained with DAPI. N–O, Estimation of scar tissue by Masson’s trichrome staining 2 months after cell injection. Sections at the level of the papillary muscle and at 3 adjacent levels toward the apex are displayed for an ESC-CM– (N) and vehicle- (O) treated heart.

**Initial Infarct Size and Global and Regional Contractile Function Estimated by In Vivo CMR**

**Initial Infarct Size**

Initial infarct size was measured on DHE images (Figure 3, A and B). Transmural infarct was observed in nearly every case and was consistent with a prolonged ligation time (45 minutes). The infarcted territory was usually localized to the anterolateral portion of the myocardial wall and extended from midventricle to the apex. Animals with an infarct size in the range of 10% to 30% of the LV wall volume were assigned randomly to 1 of 2 experimental groups, whereas others were excluded. As a result, a relatively uniform distribution of infarct size in both groups was achieved: 20.4 ± 3.9% in the ESC-CM group (n=17) and 19.4 ± 4.9% in the vehicle group (n=16), with no significant difference (P=0.5) between them. The initial infarcted, border, and remote regions identified on DHE images were transferred to DENSE images (Figure 3, C and D). The SPIO-related MRI signal 1 day after injection was sufficient for assessing the distribution of injected cells (Figure 3E). At 2 months after injection, the hypointense signal was reduced but still traceable (Figure 3F and inset of Figure 3H).

**Global Function**

LVEF in both groups was significantly depressed 1 day after MI compared with normal values (P<0.0001, Figure 3G). Over time, LVEF in both groups increased, suggesting that growth factors in the vehicle solution had a beneficial effect on post-MI remodeling. However, at both 1 and 2 months...
Figure 3. A–O, Initial infarct size and global and regional contractile function estimated by in vivo MRI. A and B, Infarction visualized by DHE MRI 1 day after MI from a heart in the ESC-CM (A) and vehicle (B) groups. For I/B-S/B-L/R segmentation, I was marked between the 2 arrows; B-S, between the 2 green lines; and B-L, between the 2 blue lines, were defined as 30° sectors neighboring the infarct segment, and R was the remaining myocardium. The segmentation was transferred to DENSE images at the corresponding level (C and D); the infarcted region is pseudocolored in white, B-S in green, B-L in blue, and R in red. The yellow circle in C identifies the SPIO-containing cells. E and F, Visualization of SPIO-containing cells 1 day (E) and 2 months (F, between arrows) after cell injection. G, Global function of normal (noninfarcted) heart, hearts, infarcted hearts 1 day after MI and 1 and 2 months after cell injection. #P<0.05 for ESC-CM or vehicle vs normal at day 1; *P<0.05 for ESC-CM vs vehicle. H–O, Displacement vectors overlaid on DENSE images (end-diastolic phase) of an ESC-CM (H) and a vehicle-treated (I) heart; residual SPIO signal in a reference image is identified by a green arrow (inset of H) with corresponding Ecc maps of the same hearts (J and K). Histograms of Ecc maps (L and M; the y axis is the number of triangular elements). Regional Ecc values plotted for ESC-CM and vehicle groups were based on I/B-S/B-L/R (N) or S/A/L/P schemes (O). AV indicates average for all segments. *P<0.05 for ESC-CM vs vehicle group.
after injection, the ESC-CM group achieved a significantly greater LVEF ($P=0.0003$ and 0.0007, respectively) and lower LV end-systolic volume ($P=0.0408$ and 0.0411, respectively) than did the vehicle group. Taken together, these data demonstrate that ESC-CMs mediated a significant improvement in global systolic function over that induced by soluble growth factors alone.

**Regional Contractile Function**

Greater intramyocardial displacements were observed in ESC-CM–versus vehicle-treated hearts (Figure 3, H and I). $E_{ac}$ maps and corresponding histograms revealed more vigorous contraction (that is, more negative values) in ESC-CM–versus vehicle-treated hearts (Figure 3, J–M). In the I/B-S/B-L/R scheme (Figure 3N), a significantly greater $E_{ac}$ in the initial infarcted and B-L regions was observed in the ESC-CM versus vehicle group ($P=0.0328$ and 0.0244, respectively). In the S/A/L/P scheme (Figure 3O), $E_{ac}$ in the anterior and lateral regions was greater in the ESC-CM than in the vehicle group ($P=0.0481$ and 0.0037, respectively). Results from both schemes are concordant, providing direct evidence that ESC-CMs mediated a substantial and regional improvement in myocardial contraction. The $E_{ac}$ value for the S/A/L/P scheme was $0.25\pm 0.09/0.29\pm 0.09/0.30\pm 0.09/0.31\pm 0.12$ for the ESC-CM and $0.27\pm 0.08/0.29\pm 0.07/0.30\pm 0.08/0.33\pm 0.09$ for the vehicle group, with no statistically significant difference.

**Discussion**

Highly enriched ESC-CMs were obtained in large numbers. The degree of differentiation of ESC-CMs was determined by the following analyses: (1) double-positive status for cTnI and α-SMA (Figure 1C); (2) expression of NKX2.5, NCX1, α- and β-myosin heavy chain, myosin light chain 2v, and α-SMA by reverse transcription–polymerase chain reaction (Figure 1K); and (3) double-positive status for α-actinin and α-SMA 2 months after engraftment (Figure 2, D–G). These data combined with the electrophysiology feature suggest a phenotype of early-stage cardiomyocytes. On tissue sections (Figure 2, F–G), SMA staining was clearly observed in SPIO-labeled ESC-CMs, suggesting the usefulness of the SMA marker for identifying grafted cells. Normally, α-SMA is expressed in smooth muscle cells or myofibroblasts, but in the heart, it either marks the earliest appearance of embryonic CMs during heart development or is a marker of dedifferentiation and hibernating myocardium. Because the ESC-CMs were positive for α-actinin, they were neither myofibroblasts nor smooth muscle cells. These data therefore suggest that the SMA marker may serve as a surrogate for surviving cells in the heart that do not readily mature or couple. Alternatively, the use of SMA as a marker of grafted cells could underestimate the coupling between grafted and host CMs, as a “mature” ESC-CM should have lost its SMA expression. This observation is important for 2 reasons: first, SMA can be used histologically as a marker to evaluate the potential of ESC-CM engraftment in the heart; and second, it can be used to identify conditions that may promote ESC-CM maturation in vivo (that is, loss of SMA expression).

The ESC-CMs mediated teratoma-free myocardial repair by forming grafts, reducing scar size, and improving global and regional contractile function significantly with respect to vehicle-treated controls. The gap junction protein, connexin-43, was observed frequently between grafted ESC-CMs but rarely between the grafted and host CMs (Figure 2J), suggesting that cellular coupling between grafts and host myocardium is far from optimal. Additional experiments will be required to address this question in the future with other ESC-CM populations isolated at various developmental stages.

Our study did, however, clearly demonstrate the power of noninvasive, quantitative imaging to assess functional recovery after cellular cardiomyoplasty. First, we addressed a critical shortcoming of many studies concerning large variations in infarct size after surgically induced MI in rodents. Such variations may arise owing to variability in rodent coronary artery anatomy and invisibility of the left anterior descending artery in a substantial portion of rats or mice, despite confirmation of myocardial ischemia upon ligation by ECG (for example, ST-segment elevation) and/or blanching of the affected region. Our approach of excluding subjects whose infarct size on day 1 was not within the specified range led to a relatively uniform distribution of infarct size in experimental groups, minimized the bias introduced by infarct size–related variations in post-MI remodeling, and allowed a therapeutic study to be completed with a reasonable group size ($n=17$).

Second, more vigorous contraction in regions corresponding to the initial infarcted and lateral border zones in ESC-CM–treated animals suggests that relatively small grafts (<1% of LV volume) could mediate a substantial improvement in contractile function. Both the presence of grafts and a paracrine effect of ESC-CMs might have contributed to the improvement. Despite its small size, the graft might be able to transmit contractile force to the surrounding host CMs through proper coupling, or it might reduce stiffness in the infarcted region. The grafted ESC-CMs might also mediate a paracrine effect by secretion of cytokines and growth factors, which might reduce apoptosis or autophagy of host CMs during post-MI remodeling. The significantly smaller scar size in the ESC-CM group (5.9% vs 12.1% in the vehicle group) is consistent with this mechanism.

Third, by lowering the SPIO labeling efficiency ($\approx 20\%$ cells were labeled), mild perturbation of the local magnetic field homogeneity was obtained 2 months after injection to avoid interference with the DENSE protocol, whereas a sufficiently hypointense MRI signal was obtained 1 day after injection to confirm intramyocardial delivery of the cells. The close proximity of 2 SPIO-containing populations (grafted cells and macrophages) could be useful, in that it provides clues as to localizing grafts. However, whether an SPIO-containing cell is an ESC-CM or a macrophage can only be established by histology. Hence, the SPIO-associated MRI signal itself may not represent a graft. In applications where biopsy is not an option, other methods (either a unique marker of grafted cells or an indirect method, such as contractile function recovery in that region) will be required to confirm the existence of the graft.
In summary, highly enriched and early-stage ESC-CMs engraft in the infarcted heart and significantly improve regional wall motion. CMR provides a powerful quantitative tool for assessment of cellular cardiomyoplasty and facilitates direct comparisons of functional improvements among scientiﬁc laboratories worldwide. These results, together with our ﬁndings on SMA, should foster improved strategies for cell-based therapies in the heart.

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Unlike most adult stem or progenitor cells, pluripotent stem cells derived from embryos (embryonic stem cells, ESCs) or experimentally from somatic cells (induced pluripotent stem cells) provide a nearly-unlimited source of cardiomyocytes (CMs) for cellular cardiomyoplasty. In this study, highly enriched CMs derived from murine ESCs were generated in large quantities for in vivo evaluation in a rat myocardial infarction model. Characterization of these cells suggests that they expressed all cardiac markers and resembled early-stage CMs. By cardiac magnetic resonance, we examined the ability of ESC-CMs to improve global and regional contractile function during postinfarction remodeling. Engraftment of ESC-CMs improved global systolic function of infarcted hearts 1 and 2 months after injection and increased regional contractile function. No teratoma was observed in any of the treated animals. Immunohistochemistry confirmed formation of grafts and revealed expression of α-smooth muscle actin in grafted ESC-CMs. Connexin-43-positive sites were observed between grafted ESC-CMs, but only infrequently between grafted and host CMs, suggesting suboptimal cellular coupling between grafts and host myocardium. In summary, cardiac magnetic resonance provides a powerful quantitative tool for assessment of cellular cardiomyoplasty and facilitates direct comparisons of left ventricular functional improvement among scientific laboratories worldwide. The approaches used in this study can be applied to induced pluripotent stem cells, which overcome major constraints associated with the use of human ESCs.
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SUPPLEMENTAL MATERIAL

Analysis of DENSE images to derive regional contractile function of the heart

The cosine and - cosine modulated displacement-encoded data sets were acquired to suppress the T1 relaxation echo and the complex conjugate echo. Additional circular Gaussian filters were applied in k-space to suppress the interference signal from artifact echoes. The resultant images are then phase-corrected by the background reference data. To reduce noise in strain maps, spatial filtering in the form of median filter is applied to displacement vector maps and to triangulated strain maps as well as described below.

The epicardial and endocardial contours were manually defined for each cardiac frame. The phase of the displacement-encoded echo image was unwrapped using a quality-guided phase unwrapping algorithm, which was applied to pixels within the myocardial wall. Afterwards, the displacement vectors, representing the tissue movement from end diastole to later time points in the cardiac cycle, were obtained by dividing the phase of each pixel by \( -k_e \). A median filter was applied to the displacement vector map, which output the median value in the 7 by 7 neighborhood around the corresponding pixel.

Based on the pixel-wise displacement vector map, 2D Lagrangian strain analysis was carried out in the following steps:

1) Triangulate the vertices by Delaunay triangulation. This method ensures that the circumscribed circle of any triangle does not have pixels inside. The triangles with their centroids outside the contoured myocardium will not be computed for strains.
2) Considering a triangular element with nodes \( i, j, k \) illustrated in Figure S.1,

![Figure S.1. A triangular element for strain analysis.](image)

the displacement over each linear triangular element is expressed as weighted sum of nodal displacements:
\[ u = S_i U_{lx} + S_j U_{lx} + S_k U_{kk} \]
\[ v = S_i U_{ly} + S_j U_{ly} + S_k U_{ky} \]
\[ i.e. \begin{bmatrix} u \\ v \end{bmatrix} = \begin{bmatrix} S_i & 0 & S_j & 0 & S_k & 0 \\ 0 & S_i & 0 & S_j & 0 & S_k \end{bmatrix} \begin{bmatrix} U_{lx} \\ U_{ly} \\ U_{jx} \\ U_{jy} \\ U_{kk} \\ U_{ky} \end{bmatrix}, \]  
\[ [1] \]

where the shape functions \( S_i, S_j \) and \( S_k \) are
\[ S_i = \frac{1}{2A} (\alpha_i + \beta_i X + \delta_i Y) \]
\[ S_j = \frac{1}{2A} (\alpha_j + \beta_j X + \delta_j Y) \]
\[ S_k = \frac{1}{2A} (\alpha_k + \beta_k X + \delta_k Y) \]
\[ [2.1] \]

and
\[ \alpha_i = X_j Y_k - X_k Y_j \]
\[ \beta_i = Y_j - Y_k \]
\[ \delta_i = X_k - X_j \]
\[ \alpha_j = X_k Y_i - X_i Y_k \]
\[ \beta_j = Y_k - Y_i \]
\[ \delta_j = X_i - X_k \]
\[ \alpha_k = X_i Y_j - X_j Y_i \]
\[ \beta_k = Y_i - Y_j \]
\[ \delta_k = X_j - X_i \]
\[ [2.2] \]

Taking the derivatives of Equation [1] leads to the displacement gradient tensor:
\[ \begin{bmatrix} \frac{\partial u}{\partial x} \\ \frac{\partial u}{\partial y} \\ \frac{\partial v}{\partial x} \\ \frac{\partial v}{\partial y} \end{bmatrix} = \frac{1}{2A} \begin{bmatrix} \beta_i & 0 & \beta_j & 0 & \beta_k & 0 \\ 0 & \delta_i & 0 & \delta_j & 0 & \delta_k \\ 0 & \delta_i & 0 & \delta_j & 0 & \delta_k \end{bmatrix} \begin{bmatrix} U_{lx} \\ U_{ly} \\ U_{jx} \\ U_{jy} \\ U_{kk} \\ U_{ky} \end{bmatrix}, \]
\[ [3] \]

The finite Lagrangian strain in terms of displacement is expressed as
\[ E = \frac{1}{2} [\nabla u + \nabla u^T + \nabla u^T \nabla u], \]  

more specifically, \( E \) can be expressed in the tensor form as

\[
\begin{bmatrix}
E_{xx} & E_{xy} \\
E_{yx} & E_{yy}
\end{bmatrix} = \begin{bmatrix}
\frac{\partial u}{\partial x} + \frac{1}{2} \left( \frac{\partial u}{\partial x} + \frac{\partial v}{\partial x} \right) & \frac{1}{2} \left( \frac{\partial u}{\partial y} + \frac{\partial v}{\partial y} \right) \\
\frac{1}{2} \left( \frac{\partial u}{\partial y} + \frac{\partial v}{\partial y} \right) & \frac{\partial v}{\partial y} + \frac{1}{2} \left( \frac{\partial u}{\partial y} + \frac{\partial v}{\partial y} \right)
\end{bmatrix}.
\]

Therefore substituting displacement map \( U \) generated from MRI data into [3], and subsequently into [5] will compute the strain tensor \( E \) for each triangle element.

3) Lagrangian strain tensor \( E \) is further diagonalized to yield the eigenvalues - maximal stretch \((E_1)\) and maximal shortening \((E_2)\). The radial \((E_{rr})\) and circumferential \((E_{cc})\) components of the strain tensor in the local cardiac coordinate are obtained by projecting \( E_1 \) and \( E_2 \) into the radial and circumferential direction. A second median filter, illustrated in Figure S.2, is applied on the triangulated strain map, where the strain value of each triangle is calculated as the median of this triangle and its 3 neighbors.

![Image of a 3-neighbor median filter applied on a triangulated strain map](image-url)

**Figure S.2.** A 3-neighbor median filter is applied on the triangulated strain map. The strain value of triangle 1 is the median of triangles 1-4.

**References**


SUPPLEMENTAL MATERIAL

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The epicardial and endocardial contours were manually defined for each cardiac frame. The phase of the displacement-encoded echo image was unwrapped using a quality-guided phase unwrapping algorithm 3, which was applied to pixels within the myocardial wall. Afterwards, the displacement vectors, representing the tissue movement from end diastole to later time points in the cardiac cycle, were obtained by dividing the phase of each pixel by \(- k_e\). A median filter was applied to the displacement vector map, which output the median value in the 7 by 7 neighborhood around the corresponding pixel.

Based on the pixel-wise displacement vector map, 2D Lagrangian strain analysis was carried out in the following steps:

1) Triangulate the vertices by Delaunay triangulation 4. This method ensures that the circumscribed circle of any triangle does not have pixels inside. The triangles with their centroids outside the contoured myocardium will not be computed for strains.

2) Considering a triangular element with nodes \(i, j, k\) illustrated in Figure S.1,

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the displacement over each linear triangular element is expressed as weighted sum of nodal displacements:
\[ u = S_l U_{lx} + S_j U_{lx} + S_k U_{kx} \]
\[ v = S_l U_{ly} + S_j U_{ly} + S_k U_{ky} \]

\[ \begin{bmatrix} U_{lx} \\ U_{ly} \\ U_{jx} \\ U_{jy} \\ U_{kx} \\ U_{ky} \end{bmatrix}, \]

where the shape functions \( S_l, S_j \) and \( S_k \) are

\[ S_l = \frac{1}{2A} (\alpha_l + \beta_l X + \delta_l Y) \]
\[ S_j = \frac{1}{2A} (\alpha_j + \beta_j X + \delta_j Y) \]
\[ S_k = \frac{1}{2A} (\alpha_k + \beta_k X + \delta_k Y) \]  \[ [2.1] \]

and

\[ \alpha_l = X_j Y_k - X_k Y_j \]
\[ \beta_l = Y_j - Y_k \]
\[ \delta_l = X_k - X_j \]
\[ \alpha_j = X_k Y_l - X_l Y_k \]
\[ \beta_j = Y_k - Y_l \]
\[ \delta_j = X_l - X_k \]
\[ \alpha_k = X_l Y_j - X_j Y_l \]
\[ \beta_k = Y_l - Y_j \]
\[ \delta_k = X_j - X_l. \]  \[ [2.2] \]

Taking the derivatives of Equation [1] leads to the displacement gradient tensor:

\[ \begin{vmatrix} \frac{\partial u}{\partial x} \\ \frac{\partial u}{\partial y} \\ \frac{\partial v}{\partial x} \\ \frac{\partial v}{\partial y} \end{vmatrix} = \begin{bmatrix} \beta_l & 0 & \beta_j & 0 & \beta_k & 0 \\ 0 & \beta_l & 0 & \beta_j & 0 & \beta_k \\ 1 & \delta_l & 0 & \delta_j & 0 & \delta_k \end{bmatrix} \begin{bmatrix} U_{lx} \\ U_{ly} \\ U_{jx} \\ U_{jy} \\ U_{kx} \\ U_{ky} \end{bmatrix}. \]  \[ [3] \]

The finite Lagrangian strain in terms of displacement is expressed as
\[ E = \frac{1}{2} [\nabla u + \nabla u^T + \nabla u^T \nabla u], \]  

more specifically, \( E \) can be expressed in the tensor form as

\[
\begin{bmatrix}
E_{xx} & E_{xy} \\
E_{yx} & E_{yy}
\end{bmatrix}
= \begin{bmatrix}
\frac{\partial u}{\partial x} + \frac{1}{2} \left( \frac{\partial u}{\partial x} + \frac{\partial u}{\partial x} + \frac{\partial u}{\partial x} \right) & \frac{1}{2} \left( \frac{\partial u}{\partial y} + \frac{\partial u}{\partial y} + \frac{\partial u}{\partial y} \right) \\
\frac{1}{2} \left( \frac{\partial v}{\partial x} + \frac{\partial v}{\partial x} + \frac{\partial v}{\partial x} \right) & \frac{1}{2} \left( \frac{\partial v}{\partial y} + \frac{\partial v}{\partial y} + \frac{\partial v}{\partial y} \right)
\end{bmatrix}.
\]

Therefore substituting displacement map \( U \) generated from MRI data into [3], and subsequently into [5] will compute the strain tensor \( E \) for each triangle element.

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\[ \text{Figure S.2. A 3-neighbor median filter is applied on the triangulated strain map. The strain value of triangle 1 is the median of triangles 1-4.} \]

**References**