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Enhanced Effect of Combining Human Cardiac Stem Cells and Bone Marrow Mesenchymal Stem Cells to Reduce Infarct Size and to Restore Cardiac Function After Myocardial Infarction

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Background—Because mesenchymal stem cells (MSCs) induce proliferation and differentiation of c-kit⁺ cardiac stem cells (CSCs) in vivo and in vitro, we hypothesized that combining human (h) MSCs with c-kit⁺ hCSCs produces greater infarct size reduction compared with either cell administered alone after myocardial infarction (MI).

Methods and Results—Yorkshire swine underwent balloon occlusion of the left anterior descending coronary artery followed by reperfusion and were immunosuppressed after MI with cyclosporine and methylprednisolone. Intramyocardial combination hCSCs/hMSCs (1 million cells/200 million cells, n=5), hCSCs alone (1 million cells, n=5), hMSCs alone (200 million cells, n=5), or placebo (phosphate-buffered saline; n=5) was injected into the infarct border zones at 14 days after MI. Phenotypic response to cell therapy was assessed by cardiac magnetic resonance imaging and micromanometer conductance catheterization hemodynamics. Although each cell therapy group had reduced MI size relative to placebo ($P<0.05$), the MI size reduction was 2-fold greater in combination versus either cell therapy alone ($P<0.05$). Accompanying enhanced MI size reduction were substantial improvement in left ventricular chamber compliance (end-diastolic pressure-volume relationship; $P<0.01$) and contractility (preload recruitable stroke work and dP/dtmax; $P<0.05$) in combination-treated swine. Ejection fraction was restored to baseline in cell-treated pigs, whereas placebo pigs had persistently depressed left ventricular function ($P<0.05$). Immunohistochemistry showed 7-fold enhanced engraftment of stem cells in the combination therapy group versus either cell type alone ($P<0.001$).

Conclusions—Combining hMSCs and hCSCs as a cell therapeutic enhances scar size reduction and restores diastolic and systolic function toward normal after MI. Taken together, these findings illustrate important biological interactions between c-kit⁺ CSCs and MSCs that enhance cell-based therapeutic responses. (*Circulation*. 2013;127:213-223.)

Key Words: heart failure ■ mesenchymal stem cells ■ myocardial infarction ■ stem cells

After myocardial infarction (MI), scar tissue replaces lost myocardium, leading to chamber remodeling with depressed left ventricular (LV) systolic and diastolic function, the substrate for heart failure and sudden cardiac death.¹ Historically, the heart has been considered a postmitotic organ lacking capacity for self-renewal after injury. Observations that adult cardiomyocytes reenter the cell cycle and form new myocytes after MI have challenged long-held dogma that the adult heart is a terminally differentiated organ.² The identification of stem cell niches in the adult heart and the ability to isolate c-kit⁺ cardiac stem cells (CSCs) from a small heart biopsy have generated enormous enthusiasm for the potential to develop safe and effective cell-based therapies to treat ischemic cardiomyopathy.^{3,4}

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Endogenous proliferation of cardiomyocytes after MI is a rare event and does not lead to full replacement of the large number of lost cells.² To overcome the limited endogenous regenerative capability of the adult heart, there is intense interest in developing cell-based therapies to promote tissue repair and to reverse remodel the injured myocardium. In this regard, several approaches have been used in which in vitro cultured stem cells are transplanted into post-MI hearts with the goal of repopulating scar tissue with new cardiomyocytes and ultimately restoring cardiac function. The bone marrow (BM) has been the most widely used source of stem cells to repair the

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heart, and BM-derived mesenchymal stem cells (MSCs) are particularly attractive candidates because of their multilineage potential, their immunomodulatory properties, and their ability to secrete a host of growth factors that may support endogenous repair mechanisms.⁵⁻⁹

Intramyocardial injection of MSCs has been shown to be safe in large-animal models, and initial data from human trials demonstrate reverse remodeling and improved regional contractility of scarred areas, effects predicted to produce clinical benefits.¹⁰⁻¹² Several mechanisms have been proposed for MSCs to reduce scar size and to improve cardiac function in large-animal models of MI, including engraftment and differentiation into functional cardiomyocytes,¹¹ paracrine signaling,⁷ and stimulation of endogenous CSC proliferation.¹⁰ Additionally, MSCs have been shown to regulate the hematopoietic stem cell and CSC niches.^{10,13} More recently, clinical trials reported early results of transplanted cell products prepared from the heart itself. Both the Cardiac Stem Cell Infusion in Patients With Ischemic Cardiomyopathy (SCIPIO) and Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction (CADUCEUS) trials used *ex vivo* amplification of cell preparations from biopsied heart tissue.^{14,15} When readministered to patients, these *ex vivo* expanded cell products improved ejection fraction (EF), reduced MI size, or both.

An optimal cell therapeutic has not yet been identified. On the basis of observations that MSCs can promote the formation of stem cell niches in diverse organ systems, we hypothesized that cell-cell interactions could be harnessed for therapeutic purposes by combining different cell types. To address this hypothesis, we tested the prediction that combining human (h) MSCs with hCSCs would amplify the phenotypic response of CSC therapy by producing greater infarct size reduction and enhanced ventricular performance after MI.

Methods

All animal protocols were reviewed and approved by the University of Miami Institutional Animal Use and Care Committee. The porcine model of MI and intramyocardial stem cell injection was performed as recently described.¹⁶ Briefly, Yorkshire swine (35–40 kg) underwent experimental MI followed by stem cell (n=15) or placebo (n=5) injection. Cardiac magnetic resonance imaging (CMR) and micromanometer conductance catheter pressure-volume (PV) loop analysis were conducted to assess structural and functional changes.^{17,18} For all procedures, anesthesia was induced with intramuscular ketamine (33 mg/kg) and maintained with inhaled isoflurane 2% to 4%.

Myocardial Infarction

A closed-chest, ischemia/reperfusion protocol to generate a model of anterior wall MI was used. Using angioplasty techniques, we performed balloon occlusion of the left anterior descending coronary artery immediately beyond the first diagonal branch for 90 minutes to induce MI, and full reperfusion was confirmed by angiography after balloon deflation. Aspirin 81 mg/d was given orally for the duration of study.

CMR Studies

CMR studies were conducted on a Siemens Trio 3-T Tim (Erlangen, Germany) scanner with Syngo magnetic resonance software using a 16-channel body surface coil with ECG gating and short breath-hold acquisitions. All animals underwent CMR at baseline, 2 weeks after

MI (preinjection), 4 weeks after MI, and 6 weeks after MI (before death).

Steady-state free-precession cine images in 2-chamber short-axis planes (slice thickness, 8 mm; field of view, 280–300 mm; matrix, 192×100; repetition time, 40 milliseconds; echo time, 1–2 milliseconds; number of averages, 2; bandwidth, 965 kHz; flip angle, 60°) were obtained. At end diastole and end systole, semiautomated epicardial and endocardial borders were drawn in contiguous short-axis cine images covering the apex to mitral valve plane with QMass Software (Medis, Leiden, Netherlands) to calculate LV mass, end-diastolic volume (EDV), end-systolic volume, stroke volume, and EF.

Short-axis and 2-chamber long-axis delayed-enhancement images (slice thickness, 8 mm with no gap; field of view, 280–300 mm; matrix, 256×100; repetition time/echo time, 500 milliseconds; bandwidth, 250 kHz; and flip angle, 20°) were acquired 8 minutes after intravenous infusion of gadolinium 0.3 mmol/kg. Scar size was calculated from the short-axis delayed-enhancement images covering the apex to the mitral valve plane. Using QMass software, we drew epicardial and endocardial contours of the LV with a semiautomated tool. The signal intensity of normal and scarred myocardium was calculated by the software, and infarct size was quantified by full-width at half-maximum intensity.

Cell Isolation and Culturing

To obtain c-kit⁺ hCSCs, explanted cardiac tissue was harvested from the core of apical tissue removed during implantation of an LV assist device in a single human male donor. CSCs expressing c-kit were isolated from enzymatic dissociated myocardial sample through the use of magnetic microbeads coupled with specific (anti-human CD117) antibodies. After dissociation, cells were plated at high density, amplified, harvested, and cryopreserved. A BM aspirate was obtained from the iliac crest of a human male donor to obtain hMSCs. hMSCs were isolated from other BM cells by Ficoll density centrifugation and plastic adherence as previously described.¹⁹ hMSCs were amplified, harvested, and cryopreserved. Iron oxide (Molday ION; BioPal, Worcester, MA) was used to label cells for imaging with CMR as recently described.²⁰ Iron oxide experiments were performed in additional animals (n=2) to detect accurate delivery and retention and thus did not affect the main results of the cell comparisons.

Thoracoscopy-Guided Intramyocardial Injection

At 14 days after MI, animals underwent thoracoscopy-guided direct transepical stem cell (hCSC/hMSCs, n=5; hMSCs, n=5; hCSCs, n=5) or placebo (n=5) injection. A left minithoracotomy was created with a small 4- to 5-cm incision in the fifth anterior/lateral intercostal space, and the left plural cavity was entered under direct visualization. A 5-mm port was placed in the sixth or seventh intercostal space, and a 5-mm endoscope (Karl Storz, Tuttlingen, Germany) was inserted into the left chest cavity. The pericardium was opened, and the infarct area was identified by wall motion abnormalities and correlation with coronary anatomy. A curved 27-gauge needle was inserted tangentially into the myocardium, and 10 separate injections were administered to the infarct border zone. A 12F chest tube was inserted into the left pleural cavity via the port incision and tunneled through the chest wall. All incisions were closed, and the chest tube was placed to –20 cm of underwater suction to evacuate the pneumothorax. Fluoroscopy was done to confirm lung expansion, and the chest tube was removed before extubation. Animals were recovered and provided adequate postoperative analgesia with a transdermal fentanyl patch (75 µg/h) for 3 days and buprenorphine (0.03 mg/kg IM) immediately after the procedure.

On the morning of stem cell injection, cells were thawed, washed, and resuspended in phosphate-buffered saline (PBS). For hMSC alone injections, 200 million hMSCs were suspended in 6 mL PBS; for hCSC alone injection, 1 million hCSCs were suspended in 3 mL PBS. For the combination c-kit⁺ hCSC/hMSC injections, 1 million hCSCs and 200 million hMSCs were suspended in 6 mL PBS and mixed before injection. For placebo injections, 6 mL PBS was administered. All cell or placebo injections were divided into 10

equal-volume aliquots and injected transeptically with a 27-gauge needle. All animals were euthanized at 6 weeks after MI.

Micromanometer Conductance Catheterization

LV invasive PV loops were obtained immediately before intramyocardial stem cell or placebo injection and at 4 weeks after injection to assess hemodynamic changes. A PV loop transducer (Millar Instruments Inc, Houston, TX) was zeroed and balanced in warm saline and guided to the LV apex by fluoroscopy via a peripheral artery introducer with the chest closed. Steady-state PV loops were acquired during a short breath hold. A Fogarty balloon was guided through the venous introducer to the inferior vena cava/right atrium junction and inflated to occlude lower body venous return to the heart while a series of occlusion PV loops were recorded during a short breath hold. The PV loop volumes were calibrated to corresponding CMR-derived EDV and stroke volume using the point-and-difference technique, which calibrates the conductance volume signal to the independently obtained CMR volume assessment.¹⁶

Histology

Hearts were fixed for >24 hours in 10% buffered formalin and sliced transversely into 4-mm-thick slices with a commercial meat cutter. Slices 4 through 8 were embedded in paraffin (formalin fixed) and processed for confocal analysis. Engraftment of human stem cells in swine hearts was assessed with DNA fluorescence in situ hybridization using a fluorescence in situ hybridization probe specific for the human Alu repetitive DNA elements. Immunofluorescence costaining was performed with anti-porcine c-kit (mouse monoclonal, clone 2B8/BM; kindly provided by Dr Revilla, Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria; Madrid, Spain), anti-human c-kit (rabbit polyclonal, c-kit PharDx; DAKO, Carpinteria, CA), and tropomyosin (mouse monoclonal; Abcam, Cambridge, MA) antibodies. Representative samples were selected from the infarct zone, left and right border zones containing infarct and nonscarred tissue, and remote zone obtained from the posterior non-infarcted LV wall for engraftment quantification. The total number of positively stained cells was quantified per slide to calculate the number of cells per unit volume (cm³) from each sample. Morphometric analyses were performed with a custom research package (Image J; National Institutes of Health, Bethesda, MD). Microscopic evaluations and image acquisitions were performed with a Zeiss LSM-710 confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY).

Immune Suppression

All swine, cell treated and placebo treated, were placed on cyclosporine and methylprednisolone therapy to prevent rejection of human stem cell transplants. Twelve days after MI, animals were started on cyclosporine 400 mg PO twice daily. Serum trough levels were measured at least once a week and titrated to maintain a serum level of 125 to 225 ng/mL. Methylprednisolone 250 mg IM was administered the morning of intramyocardial injection (14 days after MI) and tapered to 125 mg PO daily over the first 2 weeks after transplantation, and all animals were continued on that dose for the duration of the study.

Statistics

The impact of cell therapy on phenotypic changes measured over time was evaluated with the use of ANOVA with repeated measures. Postinjection time was used as the repeated factor, and differences within groups were described at each time point. Multiple testing between groups in the repeated measures ANOVA models used a Bonferroni correction; actual *P* values are reported and unadjusted. Preinjection versus postinjection PV loop hemodynamic parameters were compared by use of the paired Student *t* test. Statistics were assessed with SAS software (version 9.2; SAS Institute Inc, Cary, NC), and GraphPad Prism (version 4.03; GraphPad Software Inc, La Jolla, CA) was used to plot graphs. All values are expressed as mean±SD unless otherwise stated. A value of *P*<0.05 was considered statistically significant.

Results

Infarct Size Reduction and Restoration of EF After Stem Cell Injection

Global changes in LV function, chamber dimensions, and scar size are summarized in the Table. Delayed-enhancement CMR was used to measure changes in absolute scar mass and infarct size as a percentage of LV mass. Two weeks after MI, before injection, scar size was similar in all groups (*P*=0.811) and encompassed ≈18% of the LV mass (the Table). All stem cell-treated groups had significant reductions in infarct size 4 weeks after cell injection, whereas the placebo-injected group had unchanged infarct size from 2 to 6 weeks after MI (Figure 1). Although c-kit⁺ hCSC alone and hMSC alone therapy had comparable infarct size reductions (absolute scar mass) of 10.4±2.6% (*P*=0.010) and 9.9±1.7% (*P*=0.013), respectively, at 4 weeks after cell therapy, the combination of hCSCs and hMSCs produced a 2-fold-greater reduction in scar mass of 21.1±5.0% (*P*<0.0001). Furthermore, the infarct size measured as a percentage of LV mass decreased 36.8±3.3% in combination hCSC/hMSC-treated animals (*P*<0.0001), 25.6±3.2% in the hCSC alone group (*P*<0.0001), 23.6±4.4% in the hMSC alone group (*P*<0.0001), and 10.9±4.2% in the placebo group (*P*=0.036). Infarct size reduction by absolute mass was significantly greater in the combination hCSC/hMSC group compared with hCSCs alone (*P*=0.035), hMSCs alone (*P*=0.033), and placebo (*P*=0.0002). hCSCs (*P*=0.026) and hMSCs (*P*=0.030) had improved scar size reduction compared with placebo. The change in scar size between hCSCs alone and hMSCs alone was not different (*P*=0.964).

EF before MI was ≈40%, consistent with previous studies in Yorkshire swine,¹⁰ and similar between groups (*P*=0.412; the Table). Two weeks after MI, EF decreased ≈20% from baseline (Figure 2A). Interestingly, EF returned to baseline levels by 4 weeks after injection in the groups treated with combination hCSCs/hMSCs (*P*=0.001), hCSCs alone (*P*<0.001), and hMSCs (*P*<0.001) but remained persistently depressed in placebo-treated animals (*P*=0.885; Figure 2A). Correlation of the percentage change in EF relative to the percentage change in EDV (Figure 2B) and end-systolic volume (Figure 2C) showed attenuation of remodeling in all stem cell-treated pigs with the placebo-treated animals and was most pronounced in the combination hCSC/hMSC group. Furthermore, placebo-treated animals had significantly increased EDV (*P*=0.018) from 2 weeks after MI (preinjection) to 6 weeks after MI (Figure 2B).

Hemodynamic Changes After Stem Cell Injection

Next, we assessed the impact of cell therapy on LV diastolic and systolic performance, measured with conductance micromanometer catheterization immediately before stem cell injection and 4 weeks after injection. Preinjection hemodynamic parameters were similar in all groups.

The end-diastolic pressure-volume relationship, a measure of ventricular diastolic compliance, improved in the animals treated with combination hCSCs/hMSCs (*P*=0.005) and hMSCs alone (*P*=0.038), consistent with improved LV chamber compliance. Furthermore, the end-diastolic pressure-volume relationship in animals treated with combination hCSC/hMSC therapy

Table. Cardiac Magnetic Resonance Imaging Global Function and Scar Size

Parameter	Group	Baseline	At 2 wk (Preinjection)	At 4 wk	At 6 wk	Overall Within-Group P Value
LV mass, g	ckit ⁺ hCSCs/hMSCs	67.4±10.50	77.9±9.51	93.5±16.41	98.6±18.89	0.001
	ckit ⁺ hCSCs alone	64.7±8.26	82.9±8.96	92.2±11.93	100.4±18.96	0.011
	hMSCs alone	68.8±8.76	80.4±7.12	91.1±7.85	103.6±5.35	0.002
	Placebo	64.5±12.14	75.9±10.72	85.0±11.13	93.8±5.44	0.019
EDV, mL	ckit ⁺ hCSCs/hMSCs	66.5±8.49	93.1±11.99	97.7±12.23	95.0±14.90	0.481
	ckit ⁺ hCSCs alone	72.9±11.88	102±18.69	101.9±18.33	109.2±21.96	0.463
	hMSCs alone	78.9±10.35	93.2±15.47	94.3±17.69	105.1±23.99	0.163
	Placebo	75.4±7.81	87.8±15.55	90.1±18.54	106.1±21.17*	0.059
ESV, mL	ckit ⁺ hCSCs/hMSCs	38.7±7.35	61.2±7.98	56.8±11.95	53.0±7.91	0.141
	ckit ⁺ hCSCs alone	45.6±7.93	71.8±19.94	62.0±19.52	67.2±19.19	0.095
	hMSCs alone	49.6±8.74	67.5±13.3	59.2±17.01	67.4±22.46	0.309
	Placebo	43.2±3.91	58.8±12.8	59.1±21.17	74.38±20.52*	0.102
SV, mL	ckit ⁺ hCSCs/hMSCs	27.7±3.84	31.9±7.44	40.9±6.31	42.0±9.60	<0.001
	ckit ⁺ hCSCs alone	27.3±5.72	30.3±2.54	39.8±3.22	41.9±6.84	<0.001
	hMSCs alone	29.3±4.51	25.6±2.76	35.0±5.84	37.9±6.20	<0.001
	Placebo	31.6±7.93	28.18±7.34	31.0±8.59	31.7±0.72	0.369
EF, %	ckit ⁺ hCSCs/hMSCs	41.9±5.48	34.1±5.69	42.2±7.02	43.8±5.4	<0.001
	ckit ⁺ hCSCs alone	37.4±4.82	30.8±7.74	40.3±9.01	39.3±8.36	<0.001
	hMSCs alone	37.5±4.81	27.8±2.76	38.0±7.86	37.1±8.32	<0.001
	Placebo	41.5±6.86	32.38±7.02	35.4±12.17	30.0±4.61	0.945
Scar mass, g	ckit ⁺ hCSCs/hMSCs	0	14.1±2.40	12.3±2.66	11.2±2.58†	<0.001
	ckit ⁺ hCSCs alone	0	14.1±3.57	13.2±2.96	12.5±2.85	0.006
	hMSCs alone	0	13.8±2.74	12.0±1.77	12.4±2.51	0.015
	Placebo	0	15.1±6.95	16.1±8.02	17.2±6.83	0.430
Scar size, % LV mass	ckit ⁺ hCSCs/hMSCs	0	19.1±1.58	15.0±2.00	12.1±2.12†	<0.001
	ckit ⁺ hCSCs alone	0	17.5±3.11	14.5±2.07	13.0±1.92	<0.001
	hMSCs alone	0	17.8±3.11	14.3±1.55	13.5±2.61	<0.001
	Placebo	0	19.6±7.09	18.9±6.90	18.9±6.97	0.043

EDV, end-diastolic volume; EF, ejection fraction; ESV, end-systolic volume; hCSC, human cardiac stem cell; hMSC, human mesenchymal stem cell; LV, left ventricular; SV, stroke volume. Within- and between-group repeated measures ANOVA compares preinjection (2 weeks after MI) and postinjection time points. Values are mean±SD.

* $P<0.05$, within-group repeated measures ANOVA at 6 weeks.

† $P<0.05$, combination hCSCs/hMSCs versus hMSCs alone and hCSCs alone between-group repeated measures ANOVA at 6 weeks.

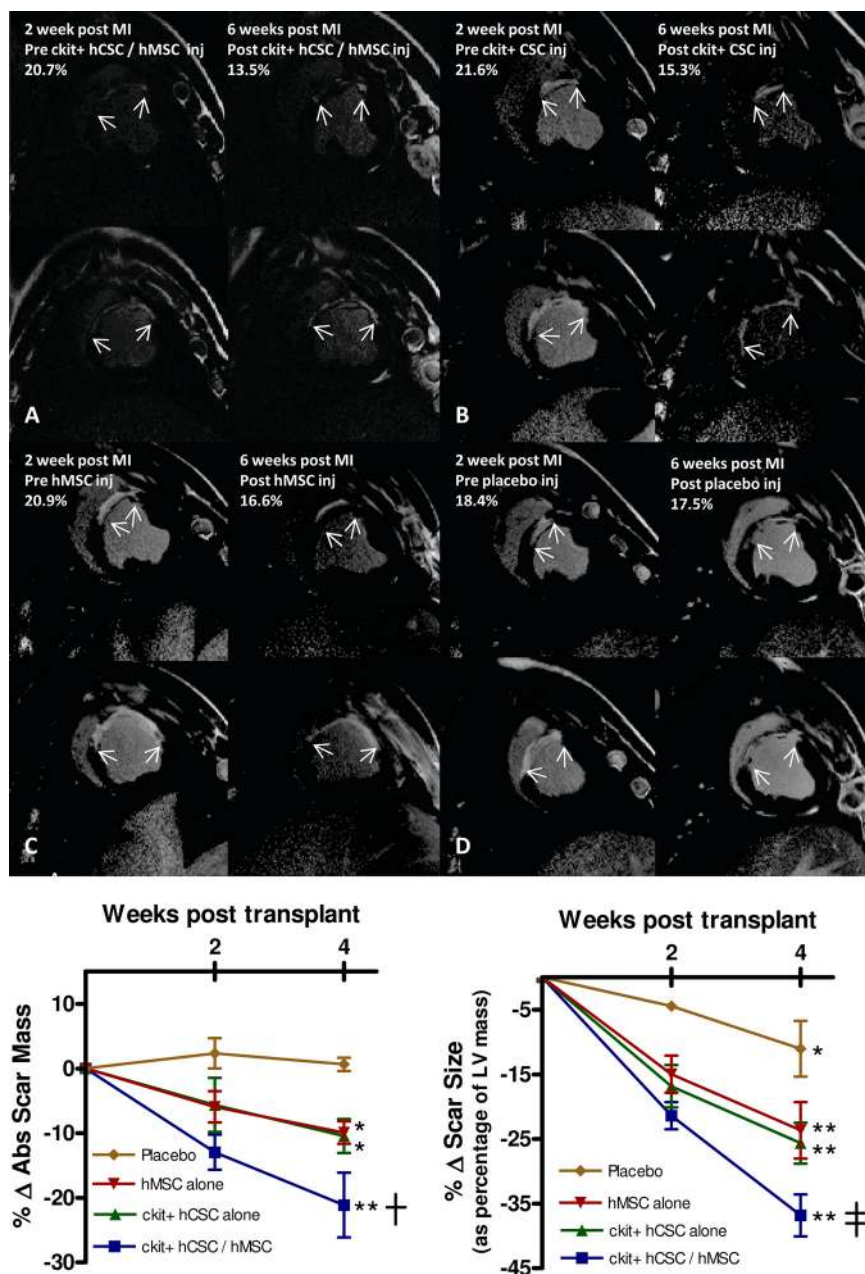
was significantly better compared with placebo-treated animals at 4 weeks after injection (Figure 3E). A flatter curve (smaller slope) showed that for any EDV the heart functioned at a lower end-diastolic pressure (Figure 3C and 3D). Isovolumetric relaxation time, measured by τ and an index of active ventricular relaxation, was also significantly lower at 4 weeks after injection in the combination c-kit⁺ hCSC/hMSC group and the c-kit⁺ hCSC group compared with the placebo group (Figure 3F). Additionally, the combination therapy group had significantly improved rate of pressure change during diastole (dP/dt_{min}) at 4 weeks after injection compared with all other cell therapy- and placebo-treated groups (Figure 3G). Taken together, these hemodynamic changes suggest that combining hMSCs with c-kit⁺ hCSCs significantly improves diastolic function to the greatest degree after MI, affecting both active and passive phases of diastolic relaxation.

There was no change in measures of preload or afterload in any of the groups except for larger EDV in the hCSC alone group (Figure 4B). However, EDV measured by CMR showed

no difference in the group treated with c-kit⁺ hCSC alone (the Table). The combination hCSC/hMSC-treated group demonstrated improved measures of contractility, including the maximal rate of pressure change during systole (dP/dt_{max}; $P=0.018$; Figure 4D) and preload recruitable stroke work ($P=0.001$; Figure 4E); interestingly, the hMSC alone group also showed improvements in preload recruitable stroke work ($P=0.024$; Figure 4E).

Integrated measures of cardiac performance—EF, cardiac output, stroke volume, stroke work, and heart rate—integrate all the measures of contractility, preload, afterload, and lusitropy. All stem cell-treated groups demonstrated improved EF and stroke volume (Figure 5A and 5B). Stroke work (Figure 5C) improved in the combination hCSC/hMSC group ($P=0.023$) and hMSC alone group ($P=0.023$). Cardiac output (Figure 5D) improved in the hCSC alone ($P=0.015$) and hMSC alone ($P=0.018$) groups and had a trend toward improvement in the combination therapy group ($P=0.064$). Heart rate was unchanged in all groups ($P=NS$; Figure 5E).

Figure 1. Infarct size reduction after human cardiac stem cell (hCSC) and bone marrow mesenchymal stem cell (hMSC) therapy. Delayed-enhancement cardiac magnetic resonance images showing pre-injection scar and 4-weeks-postinjection scar changes (white arrows indicate infarct extension) in pigs treated with (A) combination c-kit⁺ hCSCs/hMSCs (n=5), (B) c-kit⁺ hCSCs alone (n=5), (C) hMSCs alone (n=5), and (D) placebo (n=5). Reduction in (E) absolute infarct size and (F) infarct size as a percentage of left ventricular (LV) mass shows that all stem cell-treated pigs had reduced infarct size compared with pigs treated with placebo, and combination therapy had substantially greater scar size reduction compared with pigs treated with either hMSCs or hCSCs alone. MI indicates myocardial infarction. Graphs represent mean±SEM. **P*<0.05 and ***P*<0.0001 within-group repeated measures ANOVA; †*P*<0.05, combination hCSC/hMSC vs both hCSCs and hMSCs alone, between-group repeated measures ANOVA; ‡*P*=0.02, combination hCSCs/hMSCs vs hMSCs alone and *P*=0.06, combination hCSCs/hMSCs vs hCSCs alone, between-group repeated measures ANOVA.



Engraftment and Host Immune Response to Transplanted Human Cells

All groups had similar therapeutic cyclosporine trough levels throughout the study (178.3±19.4 versus 233.6±40.0 versus 225.2±52.6 versus 183.1±12.1, combination hCSCs/hMSCs versus hCSCs alone versus hMSCs alone versus placebo, respectively; *P*=0.55), and all were treated with methylprednisolone per protocol. Postmortem tissue samples were taken from serial sections of each heart, and a cardiac pathologist analyzed hematoxylin and eosin- and Masson trichrome-stained sections from the infarct zone, border zone, and remote zone (30 specimens per heart) from each animal. All animals showed structural changes of postreperfusion necrosis, including stages of fibrosis, vascular proliferation, and foci of calcification, which could occasionally be identified as calcified cardiomyocytes. There was minimal

evidence of rejection of human stem cells (administered in combination or alone) as demonstrated by the presence of rare, small lymphoreticular aggregates in all groups. Interestingly, 1 animal in the combination hCSC/hMSC group and 1 animal in the hMSC alone group had prominent foci of nodular lymphoreticular aggregates. Additionally, 1 animal in the placebo-treated group had diffuse mononuclear cell infiltrate.

CMR imaging was used to detect retention of injected human stem cells labeled with iron oxide and confirmed appropriate delivery of cells (Figure 6A–6G). Engraftment of human stem cells in swine myocardium was assessed with confocal immunofluorescence. Clusters of hCSCs and hMSCs were localized in the infarct zone and border zone by immunohistochemical staining for Alu, an antibody that binds to a specific sequence of DNA found only in human

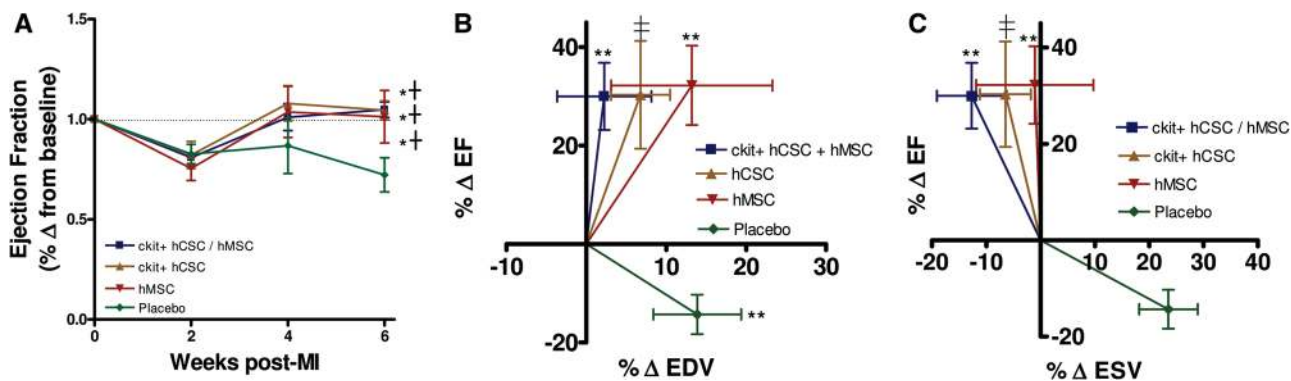


Figure 2. Restoration of left ventricular (LV) function and attenuation of remodeling with human cardiac stem cell (hCSC) and human mesenchymal stem cell (hMSC) therapy. **A**, Ejection fraction (EF) was restored to baseline level in animals treated with combination c-kit⁺ hCSC/hMSC (n=5), c-kit⁺ hCSCs alone (n=5), and hMSCs alone (n=5) but remained depressed in placebo-treated animals (n=5) during follow-up. Percent changes in LVEF plotted against the percent changes in **(B)** end-diastolic volume (EDV) and **(C)** end-systolic volume (ESV) at 4 weeks after injection demonstrate attenuation in remodeling with hCSCs and hMSCs compared to placebo, which was more pronounced in combination c-kit⁺ hCSC and hMSC therapy compared to either cell type administered alone. MI indicates myocardial infarction. Graphs represent mean±SEM **P*<0.01 within-group repeated measures ANOVA; ***P*<0.05, paired *t* test, before vs 4 weeks after injection; †*P*<0.05 vs placebo, between-group repeated measures ANOVA; ‡*P*=0.06, paired *t* test, before vs 4 weeks after injection.

cells, not swine cells (Figure 6H). Additionally, transplanted human stem cells engrafted into vessels (Figure 6I). Corresponding hematoxylin and eosin histology and gross section showed no evidence of host inflammatory response to engrafted human stem cells (Figure 6J and 6K). The engraftment of hCSCs and hMSCs in the combination group was 7-fold greater than in either cell group administered alone (Figure 6L).

Discussion

Here, we show that hMSCs and c-kit⁺ hCSCs reduce infarct size and improve LV function in post-MI swine. When both cells are injected together, there is a 2-fold-greater reduction in scar size compared with either cell administered alone. Scar size reduction in all stem cell-treated pigs was accompanied by substantial recovery in cardiac diastolic and systolic function, which was not as robust when either cell was administered alone. Taken together, intramyocardial injection of hMSCs with hCSCs as a combined cell therapy enhances scar size reduction and improves hemodynamic function in post-MI swine, suggesting that this combination represents an effective and robust cell therapeutic strategy. The improvement in both structural and functional parameters has important clinical implications for the use of this combination therapeutic for heart failure.

The quest for the ideal cell-based therapeutic strategy is intensifying. After encouraging preclinical studies, the field has recently transitioned to the conduct of early-phase clinical trials. Strategies have included the use of both mesenchymal cells from a variety of tissue sources (BM, adipose)⁹ and CSCs obtained from myocardial specimens.^{14,15} Recent mechanistic insights that MSCs stimulate CSC recruitment and lineage commitment suggest that combining cells may offer therapeutic advantages and could overcome factors inhibiting or offsetting the efficacy of current approaches.¹⁰

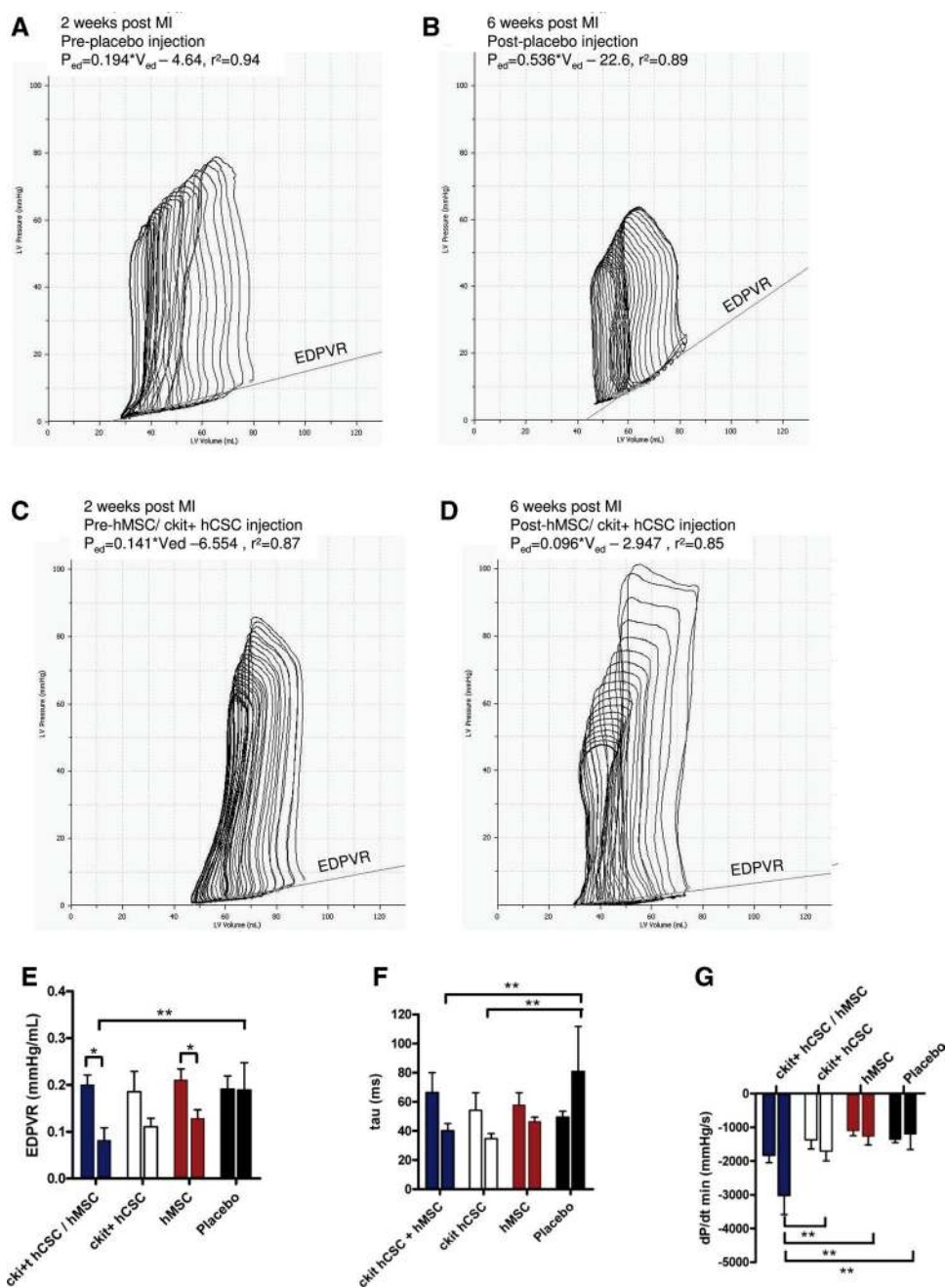
In terms of functional improvement in LV performance, we showed that all stem cell-treated animals had recovery of EF to near-baseline levels, whereas the placebo-treated animals had persistently depressed LV function. Accordingly,

EF may not be the optimal metric to assess outcomes after cell-based therapy. Previously, we demonstrated in humans with ischemic cardiomyopathy that intramyocardial injection of BM-derived stem cells dramatically reduced LV chamber sizes in parallel, thus obscuring improvements in global EF.¹² Accompanying this reverse remodeling seen in humans was improved regional contractility of the treated scar. Therefore, assessing metrics for scar size and chamber dimensions and specific measures of cardiac function such as contractility and lusitropy may provide better insight into the clinical effects of stem cells on scarred myocardium.

To assess more detailed analysis of LV function, we conducted micromanometer conductance catheter PV loop measurements to assess the impact of stem cell therapy on hemodynamic performance. Although all stem cell-treated groups had improved measures of integrated cardiac performance (EF, stroke work, and cardiac output), specific measures of contractility such as preload recruitable stroke work and dP/dt_{max} were preferentially improved in the group receiving combination hMSC/hCSC therapy. In addition to systolic impairment, diastolic dysfunction is an additional hallmark of the postinfarction LV.^{21–23} Important measures of passive and active diastolic performance such as LV chamber compliance (the slope of the end-diastolic pressure-volume relationship), isovolumetric relaxation time (τ), and minimal rate of pressure change (dP/dt_{min}) can be accurately measured with PV loops. In the combination hMSC/hCSC group, we demonstrated improved LV compliance after therapy and improved rate of pressure change during diastole compared with all other groups (Figure 3). The improved lusitropy and contractility with combination therapy suggest a complementary effect of hCSCs and hMSCs on hemodynamic performance. Moreover, the salutary effects of this cell combination on both phases of the cardiac cycle, coupled with enhanced reduction in the burden of myocardial scar, augurs well for the possibility that this combination could yield meaningful clinical benefits in patients.

Differentiation of MSCs and CSCs into adult cardiomyocytes and new blood vessels has been proposed as a potential

Figure 3. Impact of human cardiac stem cell (hCSC) and human mesenchymal stem cell (hMSC) therapy on diastolic function. Example of a placebo-treated animal before injection (**A**) vs after injection (**B**). End-diastolic pressure-volume relationship (EDPVR) shows a significant rise in slope, indicating that for any given end-diastolic volume (EDV), the heart operates at an increased end-diastolic pressure (EDP). Example of a combination hCSC- and hMSC-treated animal before injection (**C**) vs 4 weeks after injection (**D**) depicting lower EDPs for any given EDV, indicating improved left ventricular (LV) chamber compliance. Also shown in the pressure-volume loop curves is lower EDP after hCSC/MSC therapy and improvements in other hemodynamic parameters such as increased stroke work (area in curve) and increased developed pressure (ESP-EDP) 4 weeks after human stem cell therapy. Graphs showing (**E**) the slope of the regression curve for EDPVR; (**F**) τ , the isovolumetric relaxation time; and (**G**) dP/dt_{min} , the rate of pressure change during diastole. All graphs show preinjection (2 weeks after myocardial infarction [MI]) vs 4-week postinjection values. Graphs represent mean \pm SEM * $P < 0.05$, paired t test; ** $P < 0.05$, between-group repeated measures ANOVA.



mechanism of action to repopulate scar tissue after MI.^{11,24} Previous studies in swine demonstrated that intramyocardial injection of MSCs may also stimulate endogenous CSCs to regenerate myocardium via cell-cell interactions and stimulation of cardiomyocyte cell cycling.¹⁰ Furthermore, paracrine signaling likely plays an important role in stem cell therapy because it has been shown that MSCs secrete an array of growth factors, cytokines, and matrix metalloproteinases that may stimulate endogenous repair mechanisms and reverse remodeling.⁷ Indeed, the mechanism of action of stem cell therapy remains controversial. Numerous cell actions likely work in concert to improve cardiac structure and function after MI.

Emerging data from clinical trials of stem cell therapy for heart failure support the potential of regenerative medicine

as a new treatment modality.^{2,14,15,25} Concurrent with ongoing human studies, experimental animal models of cell therapy are crucial for detailed cardiac phenotyping and delineation of the biological effects of cells on important clinical outcomes. Human stem cell studies in animals have been limited mostly to genetically altered rodent models that allow tolerance to xenotransplantation.^{24,26} Swine are a well-accepted large-animal model for preclinical studies for regulatory approval,¹⁶ but their competent immune system makes xenotransplantation of human cells challenging. To address this, we developed an immunosuppressed swine model to provide a tolerant environment for human stem cell transplantation. An extensive postmortem review of each heart demonstrated minimal host inflammatory response to the transplanted human cells (Figure 6). Because all animals (placebo and stem cell treated)

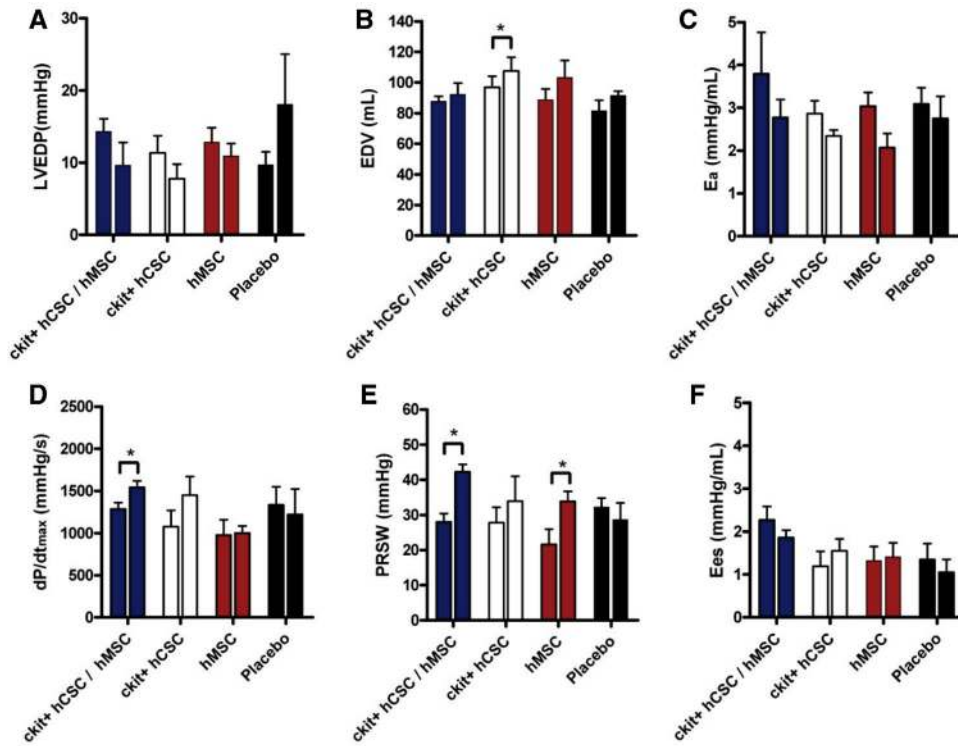


Figure 4. Preload, afterload, and contractility changes after human cardiac stem cells (hCSC) and human mesenchymal stem cell (hMSCs). **A**, Left ventricular end-diastolic pressure (LVEDP) and **(B)** end-diastolic volume (EDV) and afterload measured by **(C)** arterial elastance (Ea). Combination hCSC/hMSC therapy (n=5) improved contractility as measured by the **(D)** maximal rate of pressure change during systole (dp/dtmax) and **(E)** preload recruitable stroke work (PRSW), a preload-independent measure of stroke work. There was no change in **(F)** systolic elastance (Ees), the slope of the end-systolic pressure-volume relationship (ESPVR), in any of the groups. All graphs show preinjection (2 weeks after myocardial infarction) vs 4-week postinjection values. Graphs represent mean±SEM. *P<0.05, paired t test.

were administered the same immunosuppression therapy, any effects of the medications would be expected to manifest in the stem cell and placebo groups equally. Importantly, corticosteroid and cyclosporine therapy has been shown to have no effect on infarct size after MI.^{27,28}

Our study has important translational implications for cell-based therapy in post-MI patients. hMSCs and c-kit⁺ hCSCs

are adult stem cells easily isolated from a minimally invasive BM biopsy¹⁹ and cardiac endomyocardial biopsy,⁴ respectively. Recently, it has been shown that functional hCSCs can even be isolated from advanced heart failure patients.⁴ We have shown that combining c-kit⁺ hCSCs with hMSCs provides substantial enhancement to scar size reduction and improved hemodynamic function after MI. These improvements seen

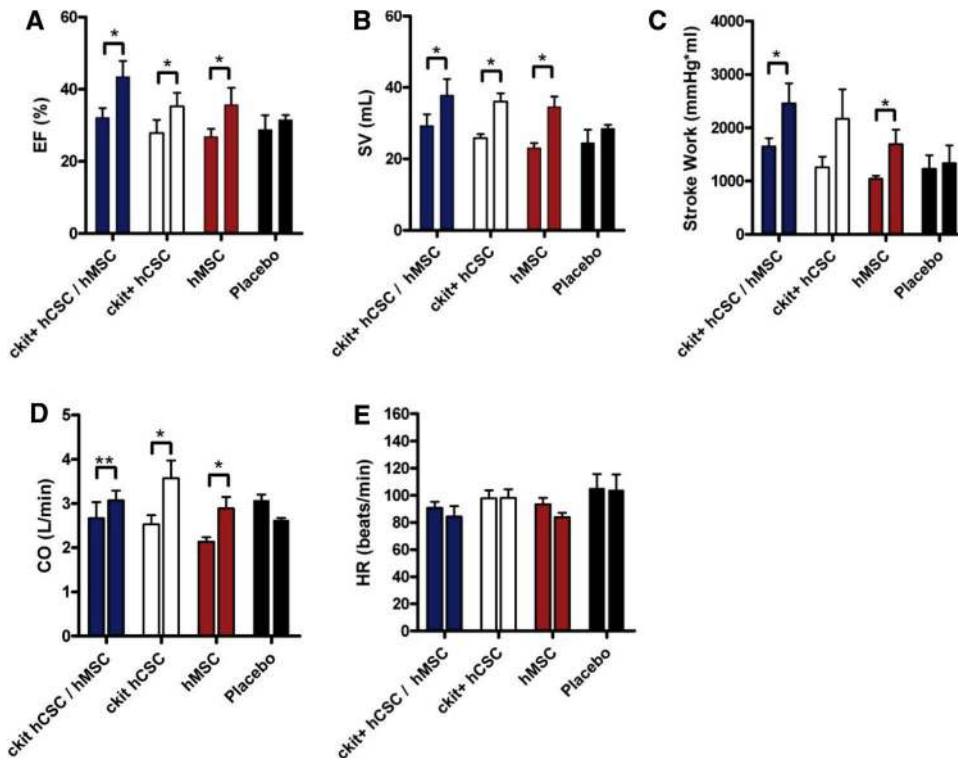


Figure 5. Integrated measures of cardiac performance improve after human cardiac stem cell (hCSC) and human mesenchymal stem cell (hMSC) therapy. All stem cell-treated groups demonstrated improved **(A)** ejection fraction (EF) and **(B)** stroke volume (SV), whereas the combination therapy (n=5) and hMSC alone therapy (n=5) improved **(C)** stroke work (SW), the area within the pressure-volume loop curve. The hMSC alone group (n=5) and hCSC alone group (n=5) had improved **(D)** cardiac output (CO), whereas there was a trend toward improved CO in the combination therapy group (n=5; P=0.06). The placebo-treated group (n=5) showed no change in any of the measures of integrated cardiac performance during follow-up, and all groups had **(E)** unchanged heart rate (HR). All graphs show preinjection (2 weeks after myocardial infarction) vs 4-week postinjection values. Graphs represent mean±SEM. *P<0.05 and **P=0.06, paired t test.

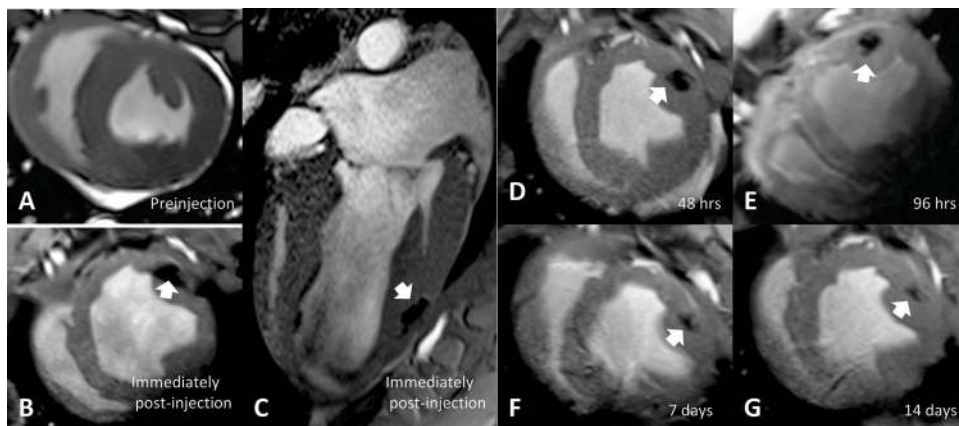
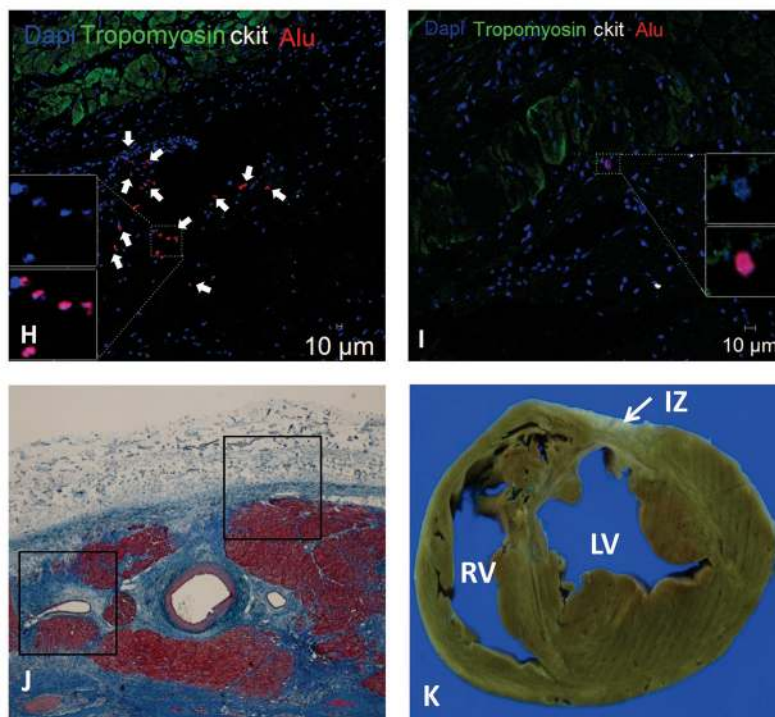
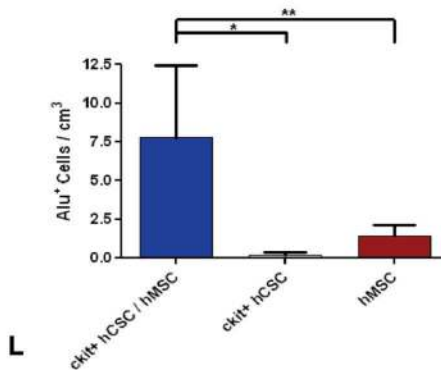


Figure 6. Retention and engraftment of human cardiac stem cells (hCSCs) and human mesenchymal stem cells (hMSCs) in post-myocardial infarction (MI) swine. **A** through **G**, Cardiac magnetic resonance images showing retention of iron oxide-labeled human stem cells by increased signal intensity (white arrow) in post-MI swine heart (representative of n=2). Immunohistochemical-stained images showing clusters of Alu-positive human stem cells (white arrows) engrafted in the (**H**) infarct territory and (**I**) vasculature at 4 weeks after transplantation. Engrafted human stem cells did not contain for c-kit. **J**, Masson trichrome-stained image depicting scar (blue) and islands of cardiomyocytes (red) with corresponding area of engraftment (black boxes) showing no cellular inflammation, which is also demonstrated in (**K**) gross section of heart (representative of n=5). **L**, Retention of Alu⁺ human stem cells was 7-fold higher when hCSCs and hMSCs were injected together compared with either cell type administered alone (n=3 analyzed per treatment group). Graphs represent mean±SEM. IZ indicates infarct zone; LV, left ventricle; and RV, right ventricle. *P<0.001 and **P<0.05, between-group 1-way ANOVA.



Retention of human xenografts at 4 weeks



in a large-animal model of MI would be predicted to provide similar effects in humans with ischemic cardiomyopathy, and immunosuppression may not be necessary if autologous cells are used. As the stem cell field advances, it may become apparent that using a single stem cell to treat the failing heart

is not the optimal cell-based strategy; rather, mixtures of complementary cells that provide synergistic effects may enhance therapeutic outcomes. Injecting a mixture of stem cells to damaged myocardium represents a new paradigm in the application of regenerative medicine to advanced heart failure.

Limitations

The major limitation of this study was the lack of dose escalation of each stem cell therapy. Previous work by our group has shown that higher-dose MSC therapy (200 million cells) provides significantly greater improvements in cardiac function and scar size than low-dose MSC therapy (20 million cells) in post-MI swine²⁹; thus, our standard dose for MSC therapy is 200 million cells. On the other hand, data from the POSEIDON trial suggest that lower doses or concentrations of cells may offer advantages over higher; these findings highlight the importance of optimizing doses and/or concentrations of cells in clinical trials.²⁵ The optimal dose for CSC therapy and when mixed with MSCs remains undefined and requires further investigation. Additionally, iron oxide labeling of cells for retention studies with CMR imaging may be confounded by efflux of iron oxide out of transplanted cells.³⁰ The iron oxide debris can be engulfed by macrophages, thus limiting the reliability of long-term imaging with iron oxide labeling for cell retention.

Conclusions

Intramyocardial injection of hMSCs with c-kit⁺ hCSCs enhances scar size reduction after MI, restoring diastolic and systolic function toward normal. Taken together, these findings illustrate the important interactions of hMSCs and c-kit⁺ hCSCs that result in substantial enhancement to cell-based therapy after MI. These findings have important clinical implications for future investigations of cell therapy for heart disease.

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Disclosures

Drs Hare and Heldman report owning equity in and are board members of Vestion, Inc. Vestion did not participate in funding this work. Dr Hare reports consulting for Kardina, and Drs Hare and Heldman report receiving research support from Biocardia, Inc. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

Stem cell-based therapy represents a potentially transformative new therapy for left ventricular dysfunction and other cardiovascular diseases. Bone marrow mesenchymal stem cells (MSCs) and cardiac stem cells (CSCs) are two of the leading candidates for cellular cardiomyoplasty. On the basis of earlier observations that MSCs interact with and promote survival and lineage commitment of CSCs, we tested whether combining MSCs with CSCs would augment a therapeutic response relative to either cell alone. Using xenotransplantation of human MSCs and c-kit+ CSCs delivered intramyocardially to swine after myocardial infarction, we show that combining MSCs and CSCs greatly enhances the reduction in infarct size and the improvement in left ventricular diastolic and systolic function achieved with either cell alone. These data support the idea that cell combination therapy is a practical and effective strategy to improve responses to cell therapy and support the conduct of clinical trials testing coinjection of MSCs and CSCs in humans with cardiac injury resulting from myocardial infarction and possibly other sources of left ventricular dysfunction.