Bioenergetic and Functional Consequences of Cellular Therapy
Activation of Endogenous Cardiovascular Progenitor Cells

Qiang Xiong,* Lei Ye,* Pengyuan Zhang, Michael Lepley, Cory Swingen, Liying Zhang, Dan S. Kaufman, Jianyi Zhang

**Rationale:** The mechanism by which endogenous progenitor cells contribute to functional and beneficial effects in stem cell therapy remains unknown.

**Objective:** Utilizing a novel $^{31}$P magnetic resonance spectroscopy–2-dimensional chemical shift imaging method, this study examined the heterogeneity and bioenergetic consequences of postinfarction left ventricular (LV) remodeling and the mechanisms of endogenous progenitor cell contribution to the cellular therapy.

**Methods and Results:** Human embryonic stem cell–derived vascular cells (hESC-VCs) that stably express green fluorescent protein and firefly luciferase (GFP$^+/Luc^+$) were used for the transplantation. hESC-VCs may release various cytokines to promote angiogenesis, prosurvival, and antiapoptotic effects. Both in vitro and in vivo experiments demonstrated that hESC-VCs effectively inhibit myocyte apoptosis. In the mouse model, a fibrin patch–based cell delivery resulted in a significantly better cell engraftment rate that was accompanied by a better ejection fraction. In the swine model of ischemia-reperfusion, the patch-enhanced delivery of hESC-VCs resulted in alleviation of abnormalities including border zone myocardial perfusion, contractile dysfunction, and LV wall stress. These results were also accompanied by a pronounced recruitment of endogenous c-kit$^+$ cells to the injury site. These improvements were directly associated with a remarkable improvement in myocardial energetics, as measured by a novel in vivo $^{31}$P magnetic resonance spectroscopy–2-dimensional chemical shift imaging technology.

**Conclusions:** The findings of this study demonstrate that a severely abnormal heterogeneity of myocardial bioenergetics in hearts with postinfarction LV remodeling can be alleviated by the hESC-VCs therapy. These findings suggest an important therapeutic target of peri-scar border zone and a promising therapeutic potential for using hESC-VCs together with the fibrin patch–based delivery system. (*Circ Res.* 2012;111:455-468.)

**Key Words:** myocardial infarction ■ stem cells ■ metabolism ■ ischemia ■ swine

A transmural myocardial infarction, left ventricular (LV) remodeling with chamber dilation, and hypertrophy occur to compensate for the loss of contracting myocardium. Although stable LV remodeling may be achieved for a period of time, progressive myocardial dysfunction can develop and ultimately lead to overt congestive heart failure (CHF). The mechanisms that contribute to the transition from the compensated state to CHF remain unclear but may be related to progressive contractile dysfunction in the region of viable myocardium that surrounds the infarct (border zone, BZ).1,2 We have recently demonstrated that BZ myocardium has a severely reduced energetic capacity, operates at a very low energetic state and is therefore more vulnerable to oxidative and other stresses.2 We hypothesize that chronically elevated systolic wall stress in the BZ surrounding a myocardial infarct results in progressive abnormalities of oxidative phosphorylation and contractile dysfunction in this region and that in the absence of treatment, the energetic and contractile abnormalities of the BZ continue to expand radially to involve the entire left ventricle, thereby leading to global LV dysfunction and the development of CHF.1,2

Currently, the available therapeutic options for heart failure due to transmural LV infarct are limited. Several exciting...
recent studies have shown that tissue specific stem cells may have the ability to generate cells of tissues from unrelated organs.\textsuperscript{3–16} Whether this unexpected plasticity constitutes “transdifferentiation” or whether a small population of resident cardiac progenitor cells (CPC) persists in postnatal heart remains unknown. Although it is a consistent observation in the literature that cell transplantation improves LV contractile function,\textsuperscript{7,11–15,17,18} the cell engraftment rate a few weeks after transplantation is ligated with a 6.0 surgical silk suture. Fifteen minutes after ligation, the surviving animals were randomly assigned to the groups receiving the respective treatment as follows: sham control group (SHAM, n = 5 per group) and day 7 (total 15, n = 5 per group) and day 7 (total 15, n = 5 per group) for histological evaluations. Another 6 sham mice were used for FACS analysis. Ten mice died during the surgery of myocardial infarction or during the follow-up. The total number of mice used for this study is 86. More detailed procedures for the animal model are included in the Online Data Supplement.

In Vivo Swine Study
The swine model for myocardial infarction (MI) procedure were as reported previously.\textsuperscript{22} Briefly, 12-week-old, immunodeficient NOD/SCID/γc\textsuperscript{−/−} (NSG) mice (Jackson Laboratory, Bar Harbor, ME) were used in the current study. A left thoracotomy was performed to expose the heart and the left anterior descending artery for the animal model are included in the Online Data Supplement.

Analysis of Cytokines Released from hESC-VCs
ECs or SMCs (0.4 million) were harvested from the normal growth media, washed 3 times using PBS, and then cultured in T75 flasks with 8 mL basal medium. The cell cultures were then subjected to either normoxic or hypoxic conditions for 48 hours. Supernatants were then harvested and analyzed by ELISA array (Human C series Angiogenesis Array, RayBiotech, Norcross, GA). Basal media exposed to identical conditions was used as blank controls to remove any background signals.

In Vivo Mouse Study
Details of the mouse model of myocardial infarction (MI) procedure were as reported previously.\textsuperscript{22} Briefly, 12-week-old, immunodeficient NOD/SCID/γc\textsuperscript{−/−} (NSG) mice (Jackson Laboratory, Bar Harbor, ME) were used in the current study. A left thoracotomy was performed to expose the heart and the left anterior descending artery was ligated with a 6.0 surgical silk suture. Fifteen minutes after ligation, the surviving animals were randomly assigned to the following groups to receive respective interventions: saline (MI, n = 8); intramyocardial injection of hESC-VCs (MI+C, n = 8; 0.125 million each of hESC-ECs and SMCs); fibrin patch without cells (MI+P, n = 7), or hESC-VCs (0.125 million each of hESC-ECs and SMCs) seeded in fibrin patch (MI+P+C, n = 9). Eight mice were used to experience identical open-chest surgery but without MI (SHAM, n = 8). These 5 groups (40 mice) were followed up for 28 days. Additional 30 mice from groups of MI, MI+P, and MI+P+C were used and euthanized at day 2 (total 15, n = 5 per group) and day 7 (total 15, n = 5 per group) for histological evaluations. Another 6 sham mice were used for FACS analysis. Ten mice died during the surgery of myocardial infarction or during the follow-up. The total number of mice used for this study is 86. More detailed procedures for the animal model are included in the Online Data Supplement.

methods
An expanded Methods section is available in the Online Data Supplement.

All experiments were performed in accordance with the animal use guidelines of the University of Minnesota, and the experimental protocol was approved by the University of Minnesota Research Animal Resources Committee. The investigation conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH publication No. 85–23).

Generation of Vascular Cells From Human Embryonic Stem Cells
Vascular cells (VCs) were derived from the human embryonic stem cell (hESC) line H9 (Wicell, Madison, WI) and consisted of 2 cell types: endothelial cells (EC) and smooth muscle cells (SMC). Details of hESC culture and differentiation are described in our recent publications\textsuperscript{20,23} and are briefly described in Online Data Supplement. The methods of cell characterization as well as in vitro cytotoxicity are also included in the Online Data Supplement.

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
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<td>BZ</td>
<td>border zone</td>
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<td>CHF</td>
<td>congestive heart failure</td>
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<td>CPC</td>
<td>cardiac progenitor cell</td>
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<td>CSI</td>
<td>chemical shift imaging</td>
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<td>EC</td>
<td>endothelial cell</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>hESC</td>
<td>human embryonic stem cell</td>
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<td>I/R</td>
<td>ischemia reperfusion</td>
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<td>IZ</td>
<td>infarct zone</td>
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<td>Luc</td>
<td>firefly luciferase</td>
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<td>LV</td>
<td>left ventricle</td>
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<td>MRS</td>
<td>magnetic resonance spectroscopy</td>
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<td>ROI</td>
<td>region of interest</td>
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<td>RZ</td>
<td>remote zone</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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<tr>
<td>TR</td>
<td>repetition time</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick-end labeling</td>
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<td>VC</td>
<td>vascular cell</td>
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An expanded Methods section is available in the Online Data Supplement.
were used and euthanized at day 3 (n=9, 3 each group), and day 7 (n=12, 4 each group) after surgery for histological evaluations. Five animals died of lethal arrhythmia during surgery or early in the follow-up. The total number of animals used in this study is 52. More detailed methods for surgery and resuscitation, cardiac MRI, and histology are also included in Online Data Supplement.

Fibrin Patch–Based Cell Delivery
The methods of fibrin patch–based cell delivery were as previously described in detail.21,24 ES-derived vascular cells (endothelial and smooth muscle cells, 2 million each) were harvested freshly from cell culture immediately before transplantation, resuspended in 1 mL total volume of fibrinogen solution (25 mg/mL). This solution was then coinkjected with catalytic thrombin solution (75 NIH units/mL) supplemented with 4 mL CaCl2 and 2 mmol/L e-aminocaproic acid onto the epicardium of the infarcted area. A plastic holder was placed on top of the heart to serve as a mold for fibrin patch. Usually within 1 minute, the mixture would be solidified, resulting in a circular fibrin patch with approximately 3 cm in diameter and 2 to 3 mm in thickness.

In Vivo Myocardial Energetic Mapping
In vivo myocardial energetic mapping was achieved using 31P MR spectroscopy with 2-dimensional chemical shift imaging (31P-2DCSI). MR measurements were performed on a 65 cm-bore 9.4-T magnet interfaced with a Vnmrj console (Varian, CA).25 Radiofrequency transmission and magnetic resonance spectroscopy (MRS) signal detection were performed with a 28-mm-diameter, double-tuned (1H and 31P) surface coil. The proton signal from water was tuned (1H and 31P) surface coil. The proton signal from water was used to adjust the position of the animal in the magnet so that the coil was at the magnetic isocenter, and to shim the magnetic field.31P MR spectra were acquired with adiabatic half passage pulse to minimize flip angle variation due to B1 inhomogeneity from the nearby myocardium and to shim the magnetic field.31P MR spectra were acquired with adiabatic half passage pulse to minimize flip angle variation due to B1 inhomogeneity from the surface coil. 31P-2DCSI uses 10x8 phase encoding steps to cover a field of view of 5x4 cm2, resulting in a spatial resolution of 0.5x0.5 cm2. 31P MR acquisition was gated according to both the cardiac and respiratory cycle, with an average repetition time (TR) of 2.7 seconds. Each phase encoding step utilized 12 repetitive scans, resulting in a total data acquisition time of 43 minutes for 31P-2DCSI. The raw data were subject to Fourier series windowing reconstructions26 on a home-built Matlab program, and the peaks of PCr and ATPy from each voxel were integrated. The ratio of PCr to ATPy (PCr/ATP) was then corrected based on 2 global spectra of the surface coil. 31P-2DCSI sequence and the results of phantom studies are shown in Online Figure I.

Statistics and Data Analysis
Statistical analyses were performed using Sigmastat version 3.5 (San Jose, CA). All data are expressed as a mean±SD. Data were analyzed with 1-way analysis of variance for repeated measures. A value of P<0.05 was considered significant. When a significant result was found, individual comparisons were made using the method of Scheffé.

Results
Derivation of Endothelial and Smooth Muscle Cells From hESC
Vascular differentiation of hESCs was initiated when cocultured with M2–10B4 stromal cells, giving rise to a population of CD34+/CD31+ cells as previously described (Figure 1).20,21,27 From these CD34+/CD31+ cells, both endothelial cells (hESC-ECs) and smooth muscle cells (hESC-SMCs) were derived by culture under appropriate conditions.20 The 2 cell populations assumed typical phenotypes and expressed cell-specific markers corresponding to ECs and SMCs, respectively, and formed tube-like structures in vitro. The cells with endothelial and smooth muscle phenotype that have not yet formed tube-like structures were used for transplantation into the infarcted hearts.

Ant apoptotic Effects of hESC-VCs
To investigate the protective effects of hESC-VCs in vitro, cardiac myocytes (HL-1) were cultured in normal growth medium, serum-free medium, or hESC-VC-conditioned media for 24 hours. The conditioned media from hESC-VCs demonstrated protective effects on HL-1 cells in vitro as a result of reduced apoptosis (Online Figure II).

The antiapoptotic capabilities of hESC-VCs in vivo were tested on both mouse and swine models of myocardial infarction (Figure 2). At either day 2 (mouse) or day 3 (swine) after surgery, animals were euthanized and the infarct center of LV myocardium was subject to terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, where apoptotic cells would be visualized as red nuclei. Compared with both MI and MI+P groups, the MI+P+C group of both mouse and swine models showed a significant reduction in apoptosis, indicating that hESC-VCs were able to protect the nearby myocardium from infarction.

To confirm that the hESC-derived VC were capable of cytoprotection via paracrine effects, we investigated the cytokines released from hESC-VCs using an ELISA array; the results are summarized in Online Table I. A total of 19 cytokines were detected in the supernatants of the cell cultures, including those that promote angiogenesis, cell proliferation, inhibition of apoptosis, and the induction of cell migration.28,29

Enhanced Cell Delivery Using Fibrin Patch
Using the myocardial infarction mouse model and hESC-VCs that were genetically modified to stably express green fluorescent protein (GFP) and firefly luciferase (GFP/Luc+), we compared the efficacy of different cell delivery methods. The same dose of hESC-VCs was transplanted into infarcted mouse hearts using either a fibrin patch or intramyocardial injection. The temporal change of cell retention was examined using in vivo bioluminescent imaging, which detected luciferase expressing hESC-VCs. Interestingly, a significantly higher cell retention rate was observed in the fibrin patch–based delivery method (Figure 3B, P<0.05). The engraftment rate of fibrin patch–based hESC-VCs transplantation in swine hearts was evaluated based on immunostaining against GFP (Online Data Supplement). The fibrin patch–based delivery resulted in an engraftment rate of 2.6±0.6% at week 4 after transplantation, based on an initial cell number of 4 million.

At 4 weeks after surgery, cardiac function was assessed using echocardiography (Figure 3C). The hESC-VC-patch–transplanted group (MI+P+C) showed remarkably better contractile function (P<0.05 versus MI+C and MI+P), supporting the effectiveness of the fibrin patch cell delivery system versus direct injection.

Myocardial Protection From hESC-VC Patch Transplantation
Groups of mice with MI, MI+P, and MI+P+C, were studied and euthanized at 1 week after surgery to examine the
The mechanistic basis of myocardial repair from hESC-VC patch transplantation. Left anterior descending coronary artery ligation resulted in a severe transmural scar in the LV myocardium, which was characterized by the loss of myocytes. In response to hESC-VC-patch transplantation, nearby myocardium was protected, resulting in a significant increase in thickness of the LV wall (Online Figure II). The week 1 cardiomyocytes were negative for GFP or BrdU colocalization (Online Figure II), indicating a mechanism of myocardial protection which spared myocytes that would otherwise have undergone apoptosis. These findings, together with the anti-apoptosis and cytokine release results, demonstrate a cardiac protection effect from the hESC-VC patch transplantation.

The VC patch area was also extensively revascularized as evidenced by vessel staining in Figure 3D. Similar results were also observed in swine hearts (Figure 7F2). This reestablished circulatory system inside the fibrin patch could provide nutrition to support the long-term engraftment of transplanted hESC-VCs, further strengthening the efficacy of cardiac protection. To examine the origin of these reestablished vessels, human-specific CD31 antibody was used to evaluate the histology of these sections. The results indicate that majority of the well-resolved vessel structures are human-specific CD31 negative, only a few of the vessels are human-specific CD31 positive, such as shown in the Figure 3E. These data suggest that fibrin patch–enhanced delivery of hESC-VCs induced a significant increase of neovascularization, the majority of which are secondary to angiogenesis and only small portion of the new vessels structure are result of vasculogenesis.

Mobilization of Endogenous CPCs
hESC-VC-patch transplantation also induced recruitment of c-kit+ progenitor cells. Representative c-kit staining of the hESC-VC-patch area is shown in Figure 4A, where ~25 c-kit+ cells were recruited. Quantification of c-kit+ cells revealed that MI+P+C hearts had a significantly greater number of c-kit+ cells as compared with the MI group (Figure 4A2). Interestingly, the c-kit+ cell density in the hESC-VC-patch area was ~40 fold higher than that of the MI hearts, further supporting the hypothesis of c-kit+ cell recruitment by the hESC-VCs. The proliferation activity of these c-kit+ cells was assessed using a 7-day BrdU incorporation protocol as well as Ki67 staining (Figure 4B and 4C). Quantification of BrdU+/c-kit+ and Ki67+/c-kit+ cells suggested that the hESC-VC-patch transplantation was also associated with elevated proliferation activity of endogenous c-kit+ cells.

Assessment of Endogenous CPCs
To evaluate the contribution of hematopoietic progenitors to the cardiac c-kit+ cell pool, additional in vivo and in vitro
Figure 2. hESC-VCs reduce cardiomyocyte apoptosis in vivo. A through C, Representative TUNEL staining of the infarct center in mouse (A and B) and swine hearts (C), respectively. Panels a1–a4 (MI mouse) and b1–b4 (MI+P+C mouse) illustrate the individual channels and merged images of TUNEL staining with higher magnification. Panels C1–C3 illustrate individual channels and merged images of TUNEL staining from MI, MI+P and MI+P+C pig hearts, respectively. Animals were euthanized at day 2 (mouse) or day 3 (swine) after surgery. Colors shown are: cardiomyocytes (cTnl, green), apoptotic cells (TUNEL, red), nuclei (DAPI, blue), and merged TUNEL and DAPI-positive cells (apoptotic nuclei, pink). D, Quantification of TUNEL staining on both mouse and pig hearts in terms of percentage of TUNEL+ cell number relative to total cells number (TUNEL+%). TUNEL quantification was based on 5 fields (infarct center) per heart and 3 hearts per group. CMs indicates cardiomyocytes. The MI+P+C group showed a significant reduction in TUNEL+ % in myocardium of both mouse and pig hearts. *P<0.05 versus MI; #P<0.05 versus MI+P.
Figure 3. Fibrin-patch delivery improves the efficacy of cellular therapy for myocardial repair. A, Bioluminescent imaging time course of mice receiving 0.25 million hESC-VCs (Luc<sup>+</sup>, GFP<sup>-</sup>) either via fibrin patch or intramyocardial injection. B, Quantification of bioluminescent data from A. *P<0.05 versus injection method at the same time. C, Ejection fraction analysis at week 4 after surgery measured using echocardiography. *P<0.05 versus MI; #P<0.05 versus MI+P; ‡P<0.05 versus MI+C. D, The hESC-VC patch was extensively revascularized at week 1 after transplantation but negative for cardiac regeneration, evidenced by costaining of smooth muscle actin (SMA) and alpha-sarcomeric actin (αSA). Figure inset shows von Willebrand factor (vWF) and SMA double staining from the adjacent section, indicating the maturation of these vessels. E, Representative Immunostaining of human-specific CD31 (hCD31) from pig heart (MI+P+C) at 4 weeks after transplantation. In the same field, there are another 4 vessels (black arrows) from the host that were not positive in hCD31 staining (serve as negative controls). Figure insets e (e1: hCD31 channel; e2: DAPI channel) illustrate transplanted endothelial cells (arrowheads) that have not yet integrated into vascular structure. Figure insets f (f1: hCD31 channel; f2: DAPI channel) illustrate one vessel generated from transplanted hCD31<sup>+</sup> cells (red arrows).
experiments were performed to examine the fraction of c-kit\(^+\) cells that were also CD\(^+\). Flow cytometry analysis of myocyte-depleted single-cell preparations based on 6 sham mouse hearts yielded a percentage of 12.7 ± 1.8% for CD45\(^+\)/c-kit\(^+\) cells within c-kit\(^+\) cell pool (Figure 4D), which is consistent with previous reports.\(^{30}\) This result was further confirmed by histological analysis (costaining of CD45 and c-kit, data not shown) performed on 5 MI + P + C mouse hearts at 1 week after MI. Over 100 c-kit\(^+\) cells were evaluated for each heart. The fraction of CD45\(^+\)/c-kit\(^+\) cells within the c-kit\(^+\) population was estimated to be 10 ± 2%.

**In Vivo Swine Study**

hESC-VCs was then used, with a swine model of I/R (Figure 6A and 6B) and fibrin patch–based transplantation of hESC-VCs that was also associated with substantial cell engraftment, extensive neovascularization, and recruitment of endogenous c-kit\(^+\) cells (Online Data Supplement).

**Myocyte Turnover Level in Response to Cell Transplantation**

Recently, there is strong evidence supporting the concept that there is a certain level of myocyte turnover rate in normal heart and that this rate is increased in response to myocardial injury.\(^{31-34}\) To evaluate the cardiomyocyte cycling activity and its relationship with the endogenous cardiac progenitors, 8 swine (n = 4 for each group of MI and MI + P + C) were subjected to 7-day BrdU incorporation protocol (starting from the day of myocardial infarction) and euthanized at either week 1 (time point BrdU is discontinued) or week 4 (21 days after the BrdU had been discontinued). The percentage of BrdU-labeled myocytes was quantified and summarized in Figure 5. The cell-treated hearts (MI + P + C) showed significantly higher percentage of BrdU\(^+\) myocytes than nontreated hearts (MI), indicating an elevated myocardial regeneration level in response to cell transplantation.
hESC-VC Patch Improved LV Contractile Function and Wall Stress

At 4 weeks after surgery, the LV contractile performance and wall stress were examined. The LV chamber function, in terms of ejection fraction (Figure 6G), was significantly improved in the hESC-VC patch–transplanted swine (MI/P+C, P<0.05 versus MI and MI/P). In addition, significant improvement of the regional myocardial systolic thickening fraction in both peri-infarct border (BZ) and infarct zones (IZ), was also observed in hESC-VC-patch–transplanted hearts but not in the fibrin patch only group (Figure 6H). Similarly, the systolic LV wall stress was significantly reduced in hearts receiving the hESC-VC patch treatment (Figure 6I).

hESC-VC Patch Attenuated Myocardial Hypertrophy

At 4 weeks after surgery, animals were euthanized, and cardiac hypertrophy, in terms of left ventricle weight over body weight (LV/BW), was examined. The severe cardiac hypertrophy secondary to myocardial infarction was attenuated in hESC-VC patch–treated hearts (Figure 6C). The attenuation of cardiac hypertrophy was further supported by hematoxylin and eosin staining of BZ myocytes, which showed the smallest myocyte cross-sectional area in MI+P+C group (Figure 6D through 6F).

Myocardial Perfusion and Vascular Density

Utilizing gadolinium-based delayed enhancement and first-pass perfusion MRI,35 regional myocardial perfusion was assessed noninvasively. The infarct was visualized using a delayed-enhancement MRI (Figure 7A), where regions of interest corresponding to IZ, BZ, and RZ were drawn and applied to first-pass perfusion images (Figure 7B) to measure the time-dependent change of regional signal intensities. Fermi model fitting of the regional signal intensity curves was used to quantify myocardial blood flows (Figure 7C), which are summarized in Figure 7D. The myocardial blood flow rate was significantly lower in the IZ and BZ of MI hearts. In response to the hESC-VC patch transplantation, the myocardial blood flow significantly improved in both IZ and BZ (P<0.05 versus MI and MI+P). The structural basis of the observed improvement of myocardial perfusion was examined by immunostaining for arteriolar and vascular density, respectively (Figure 7E and 7F and Online Data Supplement). Consistent with the perfusion data, I/R severely damaged the IZ vessel structure, which was partially ameliorated in hESC-VC patch–treated hearts (Figure 7E and 7F).

hESC-VC Patch Improved BZ Myocardial Energetics

In vivo mapping of myocardial energetics was achieved using a novel method of 31P MRS in combination with...
2-dimensional chemical shift imaging (2DCSI). Superior spatial localization of 2DCSI sequences was demonstrated in vitro using phosphate phantoms (Online Figure I). A typical in vivo myocardial energetic map of an infarcted swine heart is shown in Figure 8A, where the $^3$P spectra corresponding to different regions of the IZ, BZ, and RZ are color-coded and highlighted in Figure 8B. Prominent abnormalities of myocardial energetics were detected in both IZ (depletion of high-energy phosphates) and BZ (reduction of PCr/ATP). The depletion of high energy phosphates in IZ is consistent with the fact that the scar area consists of fibrotic tissue and no high-energy phosphates. The in vivo bioenergetic mapping was measured in the three groups of MI, MI$+/P+$C, and sham (n=4 each). Myocardial infarction resulted in severe reduction of PCr/ATP ratio in the BZ myocardium. The abnormality was significantly alleviated in response to hESC-VC patch transplantation (Figure 8E).

### Discussion

We have developed a novel in vivo 2DCSI MRS method, which demonstrates a therapeutic target of energetically vulnerable BZ in hearts with postinfarction LV remodeling (Figure 8). The new findings of the present study demonstrate that the fibrin patch–based delivery of hESC-VCs results in the improvements of myocardial bioenergetics and perfusion (Figures 7 and 8) at BZ of hearts with postinfarction LV remodeling. These functional improvements are accompanied by significant increase of BZ vascular density, particularly the resistant vessels (Figure 7E3), and recruitment of endogenous CPCs for cardiac repair (Figures 4 and 5).

### Myocyte Turnover Rate and Activity of Endogenous CPCs in Response to Cell Transplantation

Although the quantitative levels remain to be controversial, there is strong evidence to support the concept that heart is not a postmitotic organ. These studies demonstrate that a certain myocyte turnover rate exists in normal adult heart, and this cell proliferation is increased in response to myocardial injury. To examine the short term activation, mobilization, and differentiation of endogenous CPCs, additional mouse study was completed. The additional mouse study yielded a density of $0.8 \pm 0.3$ c-kit$^+$ cells/mm$^2$ for MI$+/P+$C mice at day 2 after surgery, which is not significantly different from that of sham mice ($0.6 \pm 0.2$ c-kit$^+$ cells/mm$^2$). CPCs increased significantly at day 7 after acute myocardial infarction and increased further more at day 14.

To determine whether the newly formed cells were derived from host CPCs, the pulse-chase BrdU labeling technique...
was used. hESC-VC transplantation was accompanied by significant recruitment of c-kit⁺ cells (Figure 4). We previously reported that resident Sca-1⁺/CD31⁺ CPCs exist in the adult mouse heart.²² Similarly, it has been reported that the numbers of c-kit⁺ CPCs are increased in hypertrophied and cardiomyopathic hearts.³⁸–⁴⁰ In the present study, cardiomyo-
cyte cycling activity was identified by an increased number of endogenous c-kit+ CPCs that were committed to myogenesis. Recent reports suggest that this reparative mechanism of CPCs exists throughout life in normal hearts. The data from the present study suggest that without interventions, this rate of regeneration is very modest and not sufficient to prevent severe structural remodeling and a decreased ejection fraction after infarct (Figures 2 and 6). In response to hESC-VC transplantation, the number of myocardial c-kit+ cells was significantly increased with a significant portion costaining positive for BrdU and Ki67 (Figure 4), suggesting that cardiomyocyte replacement occurs in hearts receiving this cell treatment.

The increased myocyte cycling activity in response to patch-enhanced cell transplantation was directly evidenced by BrdU incorporation of myocytes (Figure 5). The hESC-VC–transplanted hearts have a significantly higher percentage of BrdU+ myocytes than MI hearts, suggesting an elevated myocardial regeneration level in response to cell therapy.

Cytokine-Associated Myocardial Protection and Neovascularization

Findings from both in vitro and in vivo experiments demonstrate that hESC-VCs release cytokines that can prevent myocyte apoptosis (Figure 2, Online Figure II, and Online Table I). These secreted factors probably contribute to the beneficial antiapoptotic effects (eg, FGF, IGF), neovascularization promotion (eg, VEGF, FGF; Figure 7E and 7F), and mobilization of endogenous CPCs (eg, IL, IGF, EGF; Figure 4). Interestingly, the secretion of these factors was significantly increased under hypoxic conditions and was only observed in hESC-EC but not hESC-SMC (Online Table I), suggesting that EC may play an important role from the endocrine perspective. A remarkable protection of myocytes was observed by evaluating the apoptosis frequency in both in vitro and in vivo experiments. These findings are consistent with previous studies using other progenitor cell types. It is a rather consistent finding in the literature that (1) only a very small percentage of transplanted cells show long-term engraftment in recipient myocardium.
and (2) an even smaller fraction of the engrafted cells appear transdifferentiate into cardiomyocytes or vascular cells.\textsuperscript{14,42,43} These findings have led to the concept that early postimplantation paracrine interactions between the transplanted cells and native cardiomyocytes and vascular cells (and possibly cardiac and vascular progenitor cells) are the basis of much of the benefit observed after cell transplantation initiated quite early after an ischemia-reperfusion (I/R) event.\textsuperscript{44,45} Indeed, there are many reports that cell transplantation performed shortly after an I/R event is associated with decreased early apoptosis of injured cardiomyocytes. This sparing of native cardiomyocytes that would have otherwise died after the initial I/R insult presumably reduced infarct size and this accounted for the decreased subsequent LV remodeling and dysfunction observed in the treated animals.\textsuperscript{14,15}

Myocardial Perfusion

hESC-VCs transplantation was accompanied by significant improvements in myocardial perfusion in both the infarct and border zones in addition to a significant increase in vascular density (Figure 6), suggesting that vessels associated with hESC-VC transplantation and regeneration are functional. It is possible that because of increased BZ wall stress, flow reserve in this region is inadequate to support energy demands during periods of increased cardiac work. Hence, to support LV contraction at basal and high cardiac work states, an increased vascularity may be required in the BZ. Myocardial perfusion by MRI provides direct evidence that regional perfusion is significantly improved by cell transplantation (Figure 6) and is supported by the structural evidence of increased vascular density. The histological staining methods applied in Figure 3B of the present study only pick up the arterioles, which are the resistant vessels that control the autoregulation. These are the smallest muscular vessels (50–150 μm) that regulate the myocardial perfusion. Each arteriole supports capillaries where the exchanges of oxygen and carbon substrates occur. We have previously reported that hypertrophied and remodeled LV is associated with the subendocardial ischemia during the increased cardiac work states.\textsuperscript{46,47} Therefore, the increase of the density of the resistant vessels as illustrated in Figure 7E3 is the important structural basis of the improved BZ myocardial blood flow rate measured by cardiac MRI (Figure 7D). The improvement of myocardial flow probably contributes to the reduction of apoptosis and improvement in LV contractile performance that observed in the present study.

Contractile Functional and LV Wall Stress Improvements in Relation to hESC-VC-Induced Structural Changes

We examined whether hESC-VC transplantation improves the LV overall structural and functional characteristics. The transplantation resulted in significant improvements of LV chamber and regional contractile function and reduction of myocardial wall stress, which in turn was accompanied by a significant reduction in myocardial hypertrophy (Figure 6). Recently, Suzuki et al\textsuperscript{31} reported that cell transplantation into stable, chronically hibernating swine myocardium resulted in a functional improvement in the hibernating region, accompanied by myocardial regeneration resulting from activation of resident c-kit\textsuperscript{+} cells in the myocardium. In the present study, the structural improvements of increased vascular density and reduction in hypertrophy were accompanied by remarkable functional improvements of myocardial perfusion (Figure 7), regional systolic thickening fraction, LV chamber function (Figure 6), and bioenergetics (Figure 8).

Myocardial Energy Metabolism

Previously, it has been impossible to examine heterogeneity of myocardial bioenergetics between different regions of the in vivo heart. The novel, spatially localized, \textsuperscript{31}P-2DCSI MRS experiments in the present study demonstrate that bioenergetic changes in hearts with postinfarction LV remodeling are remarkably heterogenous (Figure 8). The BZ of hearts with postinfarction LV remodeling is exposed to overstretching that may result in repetitive ischemia.\textsuperscript{47–49} The spatially localized \textsuperscript{31}P-2DCSI experiments demonstrate that the improvements in myocardial bioenergetics are most pronounced in BZ of hearts with postinfarction LV remodeling (Figure 8). Bioenergetic improvements are accompanied by structural evidence for improvement in vascular density (Figure 7E and 7F), reduction of hypertrophy (Figure 6), and a reduction of apoptosis in hearts with postinfarction LV remodeling (Figure 2). The structural improvements are accompanied by beneficial outcomes in perfusion (Figure 7), contractile function and LV wall stresses (Figure 6). The data from the present study suggests a beneficial feedback cycle that is triggered by the sparing of BZ myocytes from apoptosis and myocardial regeneration from endogenous CPCs in response to hESC-VC transplantation. When we plotted BZ wall stress against BZ PCR/ATP for each heart, a linear relationship was evident (Figure 8D), supporting the concept that cellular therapy induced a beneficial feedback cycle. The remarkably higher wall stress and worsened energetics in the BZ, which was alleviated by the cellular therapy (Figures 6 and 8), is in agreement with the concept that hESC-VC was effective therapeutic target for protecting the BZ myocardium of hearts with postinfarction LV remodeling.

In summary, the findings of the present study demonstrate that the fibrin patch–based enhanced delivery of hESC-VCs resulted in a significant improvement of LV chamber function, regional systolic thickening fraction, and a reduction of wall stress in hearts with postinfarction LV remodeling. These contractile performance improvements were accompanied by significant reductions in myocardial apoptosis, hypertrophy, and vascular rarefaction. These structural benefits were accompanied by functional improvements in both myocardial perfusion and bioenergetics. In addition, this fibrin patch–based enhanced delivery of hESC-VC resulted in a pronounced mobilization of endogenous cardiac progenitor cells into the myocardial injury site, suggesting a novel mechanism of hESC-VC–induced endogenous CPC mobilization that contributes significantly to the aforementioned beneficial effects.

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References


The fibrin patch–based delivery of human embryonic stem cell–derived vascular cells (hESC-VCs) results in improvements of perfusion and an increase in the density of resistance vessels. This was accompanied by an improvement in myocardial perfusion and an increase in the density of resistance vessels. These beneficial effects were associated with activation and mobilization of endogenous cardiac progenitor cells. Additionally, we developed a novel 31P MRS-2DCSI method and report for the first time 2D mapping of high-energy phosphates in the in vivo heart. These findings demonstrate a bioenergetic heterogeneity of hearts during postinfarction LV remodeling, with the most severe abnormalities in the BZ myocardium. These findings demonstrate that combined use of the fibrin patch with hESC-derived vascular cells provides an effective means to therapeutically target the BZ myocardium of hearts with severe postinfarction LV dysfunction.
Bioenergetic and Functional Consequences of Cellular Therapy: Activation of Endogenous Cardiovascular Progenitor Cells
Qiang Xiong, Lei Ye, Pengyuan Zhang, Michael Lepley, Cory Swingen, Liying Zhang, Dan S. Kaufman and Jianyi Zhang

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Bioenergetic and functional consequences of cellular therapy: activation of endogenous cardiovascular progenitor cells

Xiong – endogenous cardiovascular progenitor cells contributions in cellular therapy

SUPPLEMENTAL METHODS

hESC-VC generation
Human embryonic stem cells (hESCs) were co-cultured with M2-10B4 stromal cells to support differentiation and gave rise to a population of CD34^+CD31^+ cells as previously described. The vascular cells (VCs) were generated from hESC-derived CD34^+CD31^+ cells and consisted of two distinct cell types. These populations assumed typical phenotypes and expressed cell-specific markers corresponding to endothelial cells (EC) and smooth muscle cells (SMC), and worked synergically to form tube-like structures in vitro.

Characterization of hESC-VCs
Detailed methods for flow cytometry- and immunostaining-based characterization of hESC-VCs have been published previously. Briefly, for flow cytometry analysis, hESC-ECs were harvested and stained with fluorescently labeled (PE) antibodies
against CD31, CD34 and CD146 or isotypes (BD Pharmingen, San Diego, CA, USA).

For immunostaining of hESC-ECs, CD31 and VE-cadherin were detected using mouse anti-human antibodies (eBioscience, San Diego, CA, USA and BD Pharmingen), von Willebrand factor (vWF) was detected using mouse anti-human vWF (DAKO Carpinteria, CA, USA). Fluorescence was visualized using Cy3-conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch, PA, USA). The hESC-SMCs were examined for expression of α-smooth muscle actin (SMA), SM22 and calponin. SMA was detected using mouse anti-α-SMC actin Cy3-conjugated antibody (Sigma). SM22 and calponin were detected with primary goat anti-human SM22 (Abcam, Cambridge, MA, USA) and mouse anti-human calponin (Sigma), respectively, followed by visualization using species-matched secondary antibodies labeled with Cy3. ProlongGold + Dapi (Invitrogen) was utilized for slide preparation and nuclear visualization via fluorescent microscopy.

**Anti-apoptotic capability of hESC-VCs in vitro**

The cardiac myocyte line HL-1 (Claycomb Laboratory, University of Louisiana) was maintained in fibronectin/gelatin-coated flasks with growth media (Claycomb medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM norepinephrine, and 2 mM L-glutamine). To examine the anti-apoptotic capability of hESC-VCs, HL-1 cells were plated onto 12-well plates (5x10^4 per well) and cultured with growth media, serum free media (SF), or hESC-VC-conditioned media (SF+EC, SF+SMC and SF+both) for 24 hr. hESC-VC-conditioned media was collected from either EC (0.5 million) or SMC (0.5 million) cultures following 48 hours in serum.
free media. Quantification of HL-1 cell death was measured using the CyQUANT cell proliferation assay kit (Invitrogen) as per manufacturer instructions. The death percent of HL-1 cell was normalized to the cell number of normal growth medium condition. Specifically, the cell death rate was calculated using the following equation: HL-1 death % = 100 x (cell number of normal medium – cell number of other medium)/(cell number of normal medium). In matched groups, the change in cell mitochondrial electrochemical potential was visualized using a mitochondria staining kit (Sigma-Aldrich, St. Louis, MO).3, 4

**In vivo mouse model of myocardial infarction**

The mouse model of the myocardial infarction (MI) has been previously reported in details.5 Briefly, twelve-week old, immuno-deficient NOD/SCID/γc−/− (NSG) mice (Jackson Laboratory, Bar Harbor, ME), weighing 20g were anesthetized with an intraperitoneal injection (ip) of sodium pentobarbital (35 mg/kg), intubated and mechanically ventilated using a small animal respirator (Harvard Apparatus). A left thoracotomy was then performed to expose the heart and the left anterior descending (LAD) artery was permanently ligated with a 6.0 surgical silk suture. Fifteen minutes post ligation, the surviving animals were randomly assigned to the following groups to receive respective interventions: saline (MI, n=8); intramyocardial injection of hESC-VCs (MI+C, n=8; 0.125 million each of hESC-ECs and SMCs); fibrin patch without cells (MI+P, n=7), or hESC-VCs (hESC-ECs and SMCs, each 0.125 million ) seeded in fibrin patch (MI+P+C, n=9). Eight mice were employed to experience identical open-chest surgery but without MI (SHAM, n=8). These 5 groups (40 mice) were followed up for 28
days. Additional 30 mice from groups of MI, MI+P and MI+P+C were employed and sacrificed at Day 2 (total 15, n=5 per group) and Day 7 (total 15, n=5 per group) for histological evaluations. Ten mice died during the surgery of myocardial infarction or the follow up. Another 6 SHAM mice were employed for FACS analysis. The total number of mice used for this study is 86. Longitudinal cell engraftment rates were determined using in vivo bioluminescent imaging that detects the light signals generated from transplanted luciferase-expressing hESC-VCs. At week 4 post surgery, cardiac function was examined using echocardiography and the animals were then sacrificed for histology. Mice sacrificed at day 2 post surgery were subject to TUNEL staining to examine cell apoptosis. In order to examine the cell cycling activity in response to hESC-VC transplantation, Bromodeoxyuridine (BrdU, 50 μg per 1 gram of body weight per day) was administrated to mice for 7 days starting from the day of surgery.

**Echocardiography**

Echocardiography was performed to assess the cardiac function using a Vevo 770 system (Visualsonics) at week 4 post surgery. Mice were lightly anesthetized with 1% inhaled isoflurane and then two-dimensional short axis views of the LV were obtained at the level of papillary muscles. The LV internal dimensions at end-diastole and end-systole were measured by the method as previously described in details, which were used to calculate ejection fraction (EF) and fractional shortening (FS).

**Bioluminescent imaging**
In vivo bioluminescent imaging was performed in order to assess the engraftment of transplanted hESC-VCs. Mice were given an ip injection of 3.75 mg sodium luciferin substrate (Gold Biotechnology, St Louis, MO) and lightly anesthetized using 1% inhaled isoflurane. Fifteen minutes after introduction of the substrate, bioluminescent images were obtained using the IVIS Imaging System (Xenogen, Hopkinton, MA) with a 5 min exposure time. Quantification of luciferase activity, using Living Image software (Xenogen), was achieved by measuring the luminescent signal in photons per second within a standard digital area. Control animals without cell injection were imaged at the same time to determine the background signal.

**Immunohistochemistry**

Immunostaining of LV tissue was performed according to previously published protocols. Briefly, for paraffin staining, heart tissues were fixed in 10% formalin, embedded in paraffin and then sectioned into slices in 5 μm thickness. Prior to immunostaining, paraffin sections were deparaffinized, rehydrated and then exposed to boiling citrate buffer for antigen retrieval. For cryo-staining, heart tissues were embedded in Tissue-TEK OCT compound (Sakura Finetek USA, Torrance, CA) and then subject to cryostat sectioning into 10 μm thick slices. Prior to immunostaining, cryosections were fixed in -20°C acetone/ethanol solution (50/50) and then subjected to non-specific blocking with ultra V (Thermo Scientific, Minneapolis, MN). Antigens were detected by incubation with primary antibodies followed by visualization using fluorophore-conjugated specie-matched secondary antibodies. A list of antibodies used for immunohistochemistry is summarized in Supplemental Table II.
Fluorescence-activated cell sorting (FACS) analysis

Fluorescence-activated cell sorting (FACS) was employed to analyze the contribution of c-kit⁺ cell population from hematopoietic lineage (CD45⁺/c-kit⁺). Myocyte-depleted (35 μm filter) single cell preparations were obtained from NOD/SCID/γc⁻/⁻ (NSG) mice (n=6) via sequential enzyme digestion. Aliquots containing 10⁶ cells were incubated with APC-conjugated anti-CD45 antibody (eBioscience) and PE-conjugated anti-c-kit antibody (Biolegend), or isotype controls for 20 min at 4 °C. Samples were analyzed using a FACS Aria instrument (BD Biosciences) and the percentage of CD45⁺/c-kit⁺ cells within c-kit⁺ cell population was calculated.

In vivo swine heart model of ischemia reperfusion

Female Yorkshire farm swine (~15 kg, Manthei hog farm, Elk River, MN) were employed for this study. The swine model of myocardial ischemia/reperfusion (I/R) has been described previously. Briefly, animals were anesthetized with inhaled 2% isoflurane, intubated and ventilated with a respirator and supplemental oxygen. Throughout the surgery, the animal body temperature, ECG, blood pressure, and arterial oxygen saturation were monitored. A left thoracotomy was performed and the root of the 1st and 2nd diagonal coronary arteries from the left anterior descending coronary artery (LAD) was occluded for 60 min and followed by reperfusion. The ischemia/reperfusion usually resulted in 10% LV mass damage. If ventricular fibrillation occurred, electrical defibrillation was performed immediately. Other drugs administrated during open-chest surgery included Lidocaine (2 mg/kg iv bolus before ligation followed by 0.05
mg/(kg·min) iv for 70 min) and Amoidorane (5 mg/kg iv bolus before ligation followed by 0.04 mg/(kg·min) iv for 70 min). The chest was then closed in layers and animals were allowed to recover. Standard post-operative care, including analgesia, was administrated until animals ate normally and became active. All animals received the clinical protocol amount of immuno-suppression for xenotransplantation with Cyclosporine (15 mg/kg/day, supplemented with food). The animals were randomly assigned to the groups receiving the respective treatment as follows: sham control group (SHAM, n= 6), animals were exposed to identical open-chest surgery but without I/R or any treatment); I/R + saline (MI, n=7); I/R+ fibrin patch without cells (MI+P, n=6); I/R + hESC-VCs (hESC-ECs and hESC-SMCs, 2 million each) seeded in fibrin patch (MI+P+C, n= 7). All these above mentioned 4 groups of animals were followed up for 28 days. Additional 21 animals of MI, MI+P and MI+P+C groups were employed and sacrificed at day 3 (n=9, 3 each group), and day 7 (n=12, 4 each group) post surgery for histological evaluations. Five animals died of lethal arrhythmia during I/R or within the first 3 days after the surgery. The total number of pigs used for the present study is 52.

**Evaluation of engraftment rate**

hESC-VC engraftment rate of swine study was evaluated based on immuno-staining against GFP (n=8, 4 each at time points of day 7 and day 28 post surgery). The left ventricle was sectioned into 6 short axis rings (thickness of ~1 cm each) from base to apex as previously described.\(^{11}\) Each ring was further sectioned into 12 blocks according to coronary anatomy.\(^{11}\) Every other block from every other ring was then subject to paraffin embedding followed by sequential sectioning of 5 um in thickness.
Slides from every 10 sections were evaluated for GFP$^+$ cell number counting. Mean GFP$^+$ cell density (number of GFP$^+$ cells per cm$^2$) were calculated for each sample block based on 5 slides. The total GFP$^+$ cell counts for each sample block were estimated as: 
\[ \text{Mean GFP cell density}^{\frac{3}{2}} \times \text{volume of sample block} \]
The total number of engrafted cells was calculated as the sum of GFP$^+$ cell counts for all samples and then multiplied by 2 (every other blocks evaluated) and 2 again (every other short axis ring evaluated). The GFP$^+$ cell engraftment rate at Week 4 post surgery is estimated to be 2.6±0.6 % based on an original cell number of 4 million.

**Myocyte turnover rate and endogenous CPCs**

In order to examine the myocyte regeneration in response to hESC-VC transplantation, 8 swine (n=4 for each group of MI and MI+P+C) were subject to 7-day BrdU incorporation protocol (140 mg/day) starting from the day of surgery. Animals were sacrificed at either week 1 (n=2 per each group) or week 4 (n=2 per each group). The coronary artery ligation site was marked with a suture to guild the tissue sampling of the explanted heart. LV tissue specimens (~ 1 cm$^3$ each) from border zone were subject to 5 μm transmural sectioning followed by immuno-staining of BrdU and myocytes as previously described$^{13}$. High power microscopic images (400X) were randomly taken and evaluated on every 20$^{th}$ slide throughout the section area. The sections with BrdU$^+$ myocytes were further double checked using consecutive sections to rule out the possibility of overlapping cells. Twenty images per section, and 5 sections each sample were evaluated in a double-blinded fashion.
Cardiac MRI

MRIs were performed to assess cardiac function outcome on a 1.5 Tesla clinical scanner (Siemens Sonata, Siemens Medical Systems, Islen NJ) using a phased-array 4-channel surface coil and ECG gating. Animals were anesthetized with 2% inhaled isoflurane and positioned in a supine position within the scanner. The MRI protocol consisted of: 1) localizing scouts to identify the long- and short-axis of the heart, 2) short- and long-axis cine for the measurement of global cardiac function, 3) first-pass perfusion MRI to measure the myocardial blood flow (MBF), and 4) delayed contrast-enhancement (DE) MRI for the assessment of scar size. Steady-state free precession “True-FISP” cine imaging used the following MR parameters: TR = 3.1 ms, TE = 1.6 ms, flip angle = 79°, matrix size = 256 x 120, field of view = 340 mm x 265 mm, slice thickness = 6 mm (4 mm gap between slices) and 25 phases were acquired across the cardiac cycle. Perfusion images were acquired with a cardiac-gated, T₁-weighted turboFLASH sequence with a saturation-recovery magnetization preparation (TR = 2.4 ms, TE = 1.2 ms, TI = 10 ms, flip angle = 18°, matrix size = 80 x 128, field of view = 300 mm, slice thickness = 6 mm). Contrast agent dosage of 0.05 mmol/kg Gd-DTPA (Magnevist, Berlex, Wayne, NJ) were bolus-injected, iv at a rate of 7 ml/s. DE MR images were acquired using an ECG-gated turbo-FLASH sequences along the short axis of LV from base to apex. Sequence parameters were: TR = 16 ms, TE = 4 ms, TI = 300 ms, flip angle = 30°, matrix size = 256 x 148, field of view = 320 mm x 185 mm, slice thickness = 6 mm (0 mm gap between slices) and two signal averages. The appropriate inversion time (TI) was chosen to adequately nullify the signal intensity from normal myocardium. Global function and regional wall thickness data were computed from the short-axis cine
images using QMASS (Medis Medical Imaging Systems, Leiden, The Netherlands) for the manual segmentation of the endocardial and epicardial surfaces at both the end-diastole and end-systole, from base to apex. Infarct size was calculated from the DE MR images using QMASS to manually segment regions of non-viable tissue. Infarct size was calculated as the ratio of the total scar area to the total LV area and summarized in Supplemental Figure VI. Perfusion imaging analysis was performed using QMASS software. Images were manually segmented into Infarct Zone (IZ), Border Zone (BZ) and Remote Zone (RZ) according to DE MRI. Signal intensity changes from individual zones, during the transit of contrast agent, were generated and plotted against that of the LV cavity which served as an input function. The regional myocardial blood flow (MBF) was then calculated by fitting the signal intensity changes into a Fermi model using an automatic program CIMRA (CSON Medical, Minneapolis MN). The anatomic information from cardiac MRI, together with the hemodynamic data, were employed to calculate the wall stress based on Laplace model:

\[
\text{Wall stress} = \frac{\text{Pressure} \times \text{radius}}{2 \times \text{thickness}}
\]

Open-chest surgery preparation for in vivo $^{31}$P magnetic resonance spectroscopy (MRS)

At one month post surgery, animals were subject to in vivo $^{31}$P MRS to examine their myocardial bioenergetics. Detailed open-chest surgery preparation for $^{31}$P MRS has been described previously. Briefly, animals were anesthetized with 2% isoflurane and ventilated with supplemental oxygen on a respirator. Polyvinyl chloride catheters (3 mm OD) were inserted into the ascending aorta (through the left external carotid artery) and
left ventricle (through the apical dimple) for hemodynamic monitoring. Once the heart was exposed via a sternotomy and suspended in a pericardial cradle, the LV catheter was introduced. Ventilation rate, volume, and inspired oxygen content were adjusted to maintain physiological values for arterial PO₂, PCO₂, and pH. Aortic and LV pressures were continuously monitored throughout the study and summarized in Supplemental Table III.

**In vivo $^{31}$P MRS 2D-chemical shift image mapping of the heterogeneity of myocardial bioenergetics**

In vivo myocardial energetic mapping was achieved using $^{31}$P MR spectroscopy with two-dimensional chemical shift imaging ($^{31}$P-2DCSI). MR measurements were performed in a 65 cm-bore 9.4 Tesla magnet interfaced with a Vnmrj console (Varian, CA). Radiofrequency transmission and MRS signal detection were performed with a 28 mm-diameter double tuned ($^1$H and $^{31}$P) surface coil, sutured directly to the epicardium of LV peri-infarct area, as indicated by 1-month MRI. The proton signal from water, detected with the surface coil, was used to adjust the position of the animal in the magnet so that the coil was at the magnetic isocenter, allow the magnetic field to be homogenized and acquire the anatomic information necessary to orientate the planning of $^{31}$P-2DCSI. $^{31}$P MR spectra were acquired with an adiabatic half passage pulse to minimize flip angle variation due to $B_1$ inhomogeneity from the surface coil. $^{31}$P-2DCSI employs 10x8 phase encoding steps to cover a field of view of 5x4 cm$^2$, resulting in a spatial resolution of 0.5x0.5 cm$^2$. $^{31}$P MR acquisition was gated according to both the cardiac and respiratory cycle, with an average repetition time (TR) of 2.7 sec. Each
phase encoding step utilized 12 repetitive scans, resulting in a total data acquisition time of 43 min for $^{31}$P-2DCSI. The raw data were subject to Fourier series windowing reconstructions on a home-built Matlab program, and the peaks of PCr and ATPγ from each voxel were integrated. The ratios of PCr to ATPγ (PCr/ATP) were then corrected based on two global spectra of TR=2.7 sec and TR=12 sec, to compensate for the partial saturation effects. A schematic view of $^{31}$P-2DCSI sequence and the results of phantom studies are shown in Supplemental Figure I.
Supplemental Figure I. $^{31}$P MR spectroscopy with two-dimensional chemical shift imaging ($^{31}$P-2DCSI). Panel A shows a schematic view of the 2DCSI sequence. An adiabatic half passage (AHP) pulse was employed for excitation, and 2DCSI data acquisition was gated for both cardiac and respiratory cycles. Panel B illustrates the experimental setup for a phantom study to validate the 2DCSI. Three tubes (1 cm diameter) containing 100 mM different phosphate solutions were placed underneath the coil. A global spectrum of the three tubes is shown in panel D, indicating three different chemical shifts corresponding to each phosphate phantom. A 2DCSI scanning of the phantom resulted in a matrix of spectra, where each phosphate tube was spatially resolved (Panel C). pe1 and pe2 indicate the first and second direction of phase encoding, respectively.
Supplemental Figure II. Cardiac protection from hESC-VC-patch transplantation.

Panel A, hESC-VC-conditioned medium (SF+EC, SF+SMC and SF+both, collected after 48 hr culture using serum free (SF) medium) effectively inhibited SF culture-induced death of HL-1
cells. *: p<0.05 vs. SF media. Panel B, Representative staining of the mitochondrial inner-membrane potential, suggesting that the cytoprotection effects of hESC-VCs are associated with reduced apoptosis. (B1) Normal growth media, (B2) serum free media, initial potential dissipation (green), an early indicator of apoptosis, was evident, (B3-B4) hESC-VC-conditioned medium. Panel C1, hESC-VC-patch transplantation (arrows) protected the nearby myocardium from apoptosis and scarring (arrowheads) at week 1. Panels C2 and C3, the protected cardiomyocytes were negative for GFP or BrdU co-staining, excluding cellular contribution from cardiac transdifferentiation or regeneration.
Supplemental Figure III. Myocyte turnover in mouse model from hESC-VC-patch transplantation as evidenced by BrdU incorporation. Mice were subjected to a 7-day BrdU administration protocol, starting from the day of surgery, and the hearts were harvested at week 4 post surgery for immunostaining. Both SHAM hearts (A) and remote region of infarcted hearts (B, MI-IZ) demonstrate low BrdU incorporation rates as compared to the BZ myocardium (Panels C-F). However, significantly more BrdU positive myocytes were observed in the BZ myocardium of hESC-VC-patch transplanted hearts (D-F, arrows) than in MI hearts (C), indicating the hESC-VC-patch transplantation is associated with increased cardiac regeneration.
Supplemental Figure IV. Tracking of hESC-VCs in swine heart (week 4). The transplanted hESC-VCs maintained their vascular lineage, expressed either vWF (endothelial cells) or SMA (smooth muscle cells), and contributed to neovascularization (arrows in Panels A and B). Figure inset of panel D showed a dividing GFP⁺/vWF⁺ human vascular cell (arrow), suggesting the proliferative capabilities.
Supplemental Figure V. hESC-VC patch improved the infarct zone vascular density of the recipient hearts. Heart tissues from locations of infarct zone (IZ) and remote zone (RZ) of MI, MI+P and MI+P+C groups were subject to immuno-staining of smooth muscle actin (SMA, red) and von Willebrand Factor (vWF, green) to visualize the vascular structure. Similar healthy vascular structures were observed in the RZ myocardium among all three groups. The IZ vascular structure was severely impaired due to ischemia/reperfusion damage. Compared to MI and MI+P groups, the hESC-VC-patch transplantation significantly increased the IZ vascular density.
Supplemental Figure VI. Infarct size (% infarct area over LV area) of swine and mouse models of myocardial infarction. Infarct size of swine study is measured using delayed contrast-enhancement (DE) MRI at week 1 (1WK) and week 4 (4WK) post surgery (A), where infarct is identified as hyper-intensity area (arrow). Panel B summarized the quantification based on 6 animals per each group. All three groups showed similar infarct size at week 1. However, the infarct size of MI+P+C group reduced significantly at week 4 ($p<0.05$), which is also significantly smaller than MI and MI+P groups at the same time. Panel C summarized infarct size of mouse study (n=5 per each group). The MI+P+C showed a significantly smaller infarct as compared to MI and MI+P groups. *: $p<0.05$ vs MI, #: $p<0.05$ vs MI+P.
Supplemental Table I. Cytokines released from human embryonic stem cell-derived vascular cells

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</tr>
<tr>
<td>PIGF</td>
<td>4205±125</td>
<td>4509±330</td>
<td>3851±51</td>
<td>4225±6</td>
</tr>
<tr>
<td>RANTES</td>
<td>5640±48</td>
<td>5505±75</td>
<td>4849±7</td>
<td>5067±54</td>
</tr>
<tr>
<td>TGF-β</td>
<td>4484±14</td>
<td>4301±45</td>
<td>4042±61</td>
<td>4412±15</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>35504±970</td>
<td>36433±1922</td>
<td>18633±617</td>
<td>31757±993</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>58675±1379</td>
<td>53703±2528</td>
<td>9944±361</td>
<td>47503±639</td>
</tr>
<tr>
<td>Thrombopoietin</td>
<td>4520±17</td>
<td>4411±23</td>
<td>4003±1</td>
<td>4044±6</td>
</tr>
<tr>
<td>VEGF</td>
<td>4089±14</td>
<td>4388±11</td>
<td>3864±38</td>
<td>4580±87</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>4615±8</td>
<td>4482±4</td>
<td>4107±8</td>
<td>4218±38</td>
</tr>
</tbody>
</table>

EC: endothelial cells; SMC: smooth muscle cells; norm: normoxia condition; hypo: hypoxia condition. Results were represented as Mean ± standard deviation (three measurements).
### Supplemental Table II: antibodies for immunohistochemistry

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Embedding</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse c-kit</td>
<td>Paraffin</td>
<td>Goat anti c-kit</td>
<td>Donkey anti goat</td>
</tr>
<tr>
<td>Pig c-kit</td>
<td>Paraffin</td>
<td>Rabbit anti CD117</td>
<td>Donkey anti rabbit</td>
</tr>
<tr>
<td>GFP</td>
<td>Paraffin</td>
<td>Goat anti GFP</td>
<td>Donkey anti goat</td>
</tr>
<tr>
<td>α-SA</td>
<td>Paraffin</td>
<td>Mouse IgM anti α-SA</td>
<td>Donkey anti mouse</td>
</tr>
<tr>
<td>BrdU</td>
<td>Paraffin</td>
<td>Rat anti BrdU</td>
<td>Rabbit anti rat</td>
</tr>
<tr>
<td>CD45</td>
<td>Paraffin</td>
<td>Rabbit anti CD45</td>
<td>Donkey anti rabbit</td>
</tr>
<tr>
<td>Ki67</td>
<td>Paraffin</td>
<td>Rabbit anti Ki67</td>
<td>Donkey anti rabbit</td>
</tr>
<tr>
<td>SMA</td>
<td>Paraffin/Cryo</td>
<td>Cy3-mouse anti SMA</td>
<td>NA</td>
</tr>
<tr>
<td>vWF</td>
<td>Paraffin/Cryo</td>
<td>Rabbit anti vWF</td>
<td>Donkey anti rabbit</td>
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<tr>
<td>Pig CD31</td>
<td>Cryo</td>
<td>Mouse anti CD31</td>
<td>Donkey anti mouse</td>
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<tr>
<td>hCD31</td>
<td>Cryo</td>
<td>Mouse anti human CD31</td>
<td>Donkey anti mouse</td>
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<tr>
<td>TUNEL</td>
<td>Cryo</td>
<td>Roch In Situ Cell Death Detection Kit</td>
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</table>

Supplemental Table III: physiological data of swine study

<table>
<thead>
<tr>
<th></th>
<th>HR (bpm)</th>
<th>LVSP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>Mean AOP (mmHg)</th>
<th>LVEDV (mL)</th>
<th>LVESV (mL)</th>
<th>ESTHK (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>103±7</td>
<td>101±6</td>
<td>7±3</td>
<td>72±4</td>
<td>53±7*#</td>
<td>24±4*#</td>
<td>10.4±1.0*#</td>
</tr>
<tr>
<td>MI</td>
<td>104±14</td>
<td>91±10</td>
<td>7±3</td>
<td>71±3</td>
<td>68±11</td>
<td>39±7</td>
<td>3.5±0.5</td>
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<td>MI+P</td>
<td>99±17</td>
<td>93±6</td>
<td>8±3</td>
<td>71±5</td>
<td>60±9</td>
<td>31±3</td>
<td>4.1±0.7*</td>
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<tr>
<td>MI+P+C</td>
<td>103±12</td>
<td>96±9</td>
<td>8±2</td>
<td>73±2</td>
<td>50±4*#</td>
<td>23±3*#</td>
<td>5.0±0.6*#</td>
</tr>
</tbody>
</table>

HR: heart rate. LVSP: left ventricular systolic pressure. LVEDP: LV end-diastolic pressure. Mean AOP: mean aortic artery pressure. LVEDV: LV end-diastolic volume. LVESV: LV end-diastolic volume. ESTHK: End-systolic LV wall thickness of the infarct zone. Data are mean± SD. *: p<0.05 vs. MI group. #: p<0.05 vs. MI+P group. †: p<0.05 vs. MI+P+C group.
REFERENCES


