Multimodality Evaluation of the Viability of Stem Cells Delivered Into Different Zones of Myocardial Infarction

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Background—We tested the hypothesis that multimodality imaging of mouse embryonic stem cells (mESCs) provides accurate assessment of cellular location, viability, and restorative potential after transplantation into different zones of myocardial infarction.

Methods and Results—Mice underwent left anterior descending artery ligation followed by transplantation of dual-labeled mESCs with superparamagnetic iron oxide and luciferase via direct injection into 3 different zones of myocardial infarction: intra-infarction, peri-infarction, and normal (remote). One day after transplantation, magnetic resonance imaging enabled assessment of the precise anatomic locations of mESCs. Bioluminescence imaging allowed longitudinal analysis of cell viability through detection of luciferase activity. Subsequent evaluation of myocardial regeneration and functional restoration was performed by echocardiography and pressure–volume loop analysis. Using 16-segment analysis, we demonstrated precise localization of dual-labeled mESCs. A strong correlation between histology and magnetic resonance imaging was established ($r=0.962$, $P=0.002$). Bioluminescent imaging data demonstrated that cell viability in the remote group was significantly higher than in other groups. Echocardiography and pressure–volume loop analysis revealed improved functional restoration in animals treated with mESCs, although myocardial regeneration was not observed.

Conclusions—Multimodality evaluation of mESC engraftment in the heterogeneous tissue of myocardial infarction is possible. Magnetic resonance imaging demonstrated accurate anatomic localization of dual-labeled mESCs. Bioluminescent imaging enabled assessment of variable viability of mESCs transplanted into the infarcted myocardium. Echocardiography and pressure–volume loop analysis validated the restorative potential of mESCs. Although mESCs transplanted into the remote zone demonstrated the highest viability, precise delivery of mESCs into the peri-infarction region might be equally critical in restoring the injured myocardium. (Circ Cardiovasc Imaging. 2008;1:6-13.)

Key Words: heart failure • myocardial infarction • magnetic resonance imaging • stem cells, embryonic

Stem cell transplantation has emerged as a promising therapeutic alternative for the restoration of the injured myocardium.1 Although bone marrow–derived stem cells have shown possible restorative capability of the infarcted myocardium, attention has focused on embryonic stem cells (ESCs) as a potential source of cardiomyocytes.2–7 Mouse ESCs (mESCs) have been shown to differentiate into cardiomyocytes and to form stable intracardiac grafts.2 Transplantation of these grafts into murine myocardial infarction (MI) models has demonstrated functional restoration and subsequent limitation of heart failure progression.8–10

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With the use of superparamagnetic iron oxide (SPIO)–labeled cells, magnetic resonance imaging (MRI) has emerged as a primary modality to detect the ex vivo–labeled mESCs and to assess functional restoration of the injured myocardium.11–13 However, in vivo MR monitoring of stem cell transplantation is mostly limited to cell localization and functional restoration of the myocardial tissue. Fundamental biological information on cell viability cannot be obtained.14 To address this limitation, multimodality imaging has been implemented to assess stem cell engraftment in vivo.15 In
addition to MRI, echocardiography and pressure–volume (PV) loop analysis have been implemented to assess myocardial function and regeneration, and bioluminescent imaging (BLI) has been used to detect molecular signal of cell viability. These enhanced capabilities enable a comprehensive assessment of stem cell engraftment and recipient tissue, including cellular location, viability, myocardial regeneration, and functional restoration.

The complementary strengths of the multimodality approach have been investigated systematically in a murine MI model to address the following: (1) localization of SPIO-labeled transplanted mESCs delivered into 3 different zones of MI, assessed with MRI; (2) viability of mESCs delivered into 3 different zones of the injured myocardium, assessed with BLI; and (3) functional restoration and regeneration of the myocardium, assessed with echocardiography and PV loop analysis.

**Methods**

**Cell Culture and Cell Labeling**

**Cell Culture**

The stable mESCs transfected with Click Beetle Red luciferase reporter gene (mESC-luc², courtesy of Dr Joseph C. Wu, Stanford University, Stanford, Calif) were used. The mESCs were maintained in an undifferentiated state on a feeder layer of irradiated mouse fibroblasts with maintenance medium containing Dulbecco modified Eagle medium (Invitrogen, Carlsbad, Calif) supplemented with 15% fetal bovine serum (Hyclone, Logan, Utah), 0.1 mmol/L 2-mercaptoethanol (Sigma, St Louis, Mo), 0.1 mM nonessential amino acids (Invitrogen), 1% penicillin/streptomycin (Invitrogen), 2 mmol/L L-glutamine (Invitrogen), and 1000 μM mouse leukemia inhibitory factor (ESGRO, Chemicon, Temecula, Calif) at 37°C, as described previously.¹⁶

**Cell Labeling**

To label mESCs, we used a commercially available ferumoxides suspension (Feridex IV, Berlex Laboratories, Wayne, NJ) that contains particles ~80 to 150 nm in size and has a total iron content of 11.2 mg Fe/mL. Clinical-grade protamine sulfate (American Pharmaceuticals Partners, Schaumburg, Ill) was prepared as a stock solution of 1 mg/mL in distilled water. Ferumoxides (100 μg Fe/mL) was put into a tube containing serum-free RPMI 1640 medium (Invitrogen) containing 25 mmol/L HEPES and L-glutamine. Protamine sulfate was added to the ferumoxides solution at a concentration of 12 μg/mL and mixed for 5 to 10 minutes. An equal volume of labeling solution was added to the existing medium in the mESC culture and incubated for 12 to 24 hours. The final concentrations of ferumoxides and protamine sulfate in the cell culture medium were 50 μg Fe/mL and 6 μg/mL, respectively.¹⁶,¹⁷

**Animals**

Healthy female mice with severe combined immune deficiency (SCID) and male Balb/C mice (8 to 10 weeks of age; weight, 20 to 25 g) were housed at no more than 5 per cage in our American Association for Accreditation of Laboratory Animal Care–approved facility with 12/12-hour light/dark cycles and free access to standard rodent chow and water. Stanford University’s Administrative Panels on Laboratory Animal Care approved this protocol.

**Left Coronary Ligation**

Mice were anesthetized with inhaled isoflurane (2% to 3%). Mice were intubated and ventilated with a mouse respirator (model 687, Harvard Apparatus, Inc, Holliston, Mass), and anesthesia was maintained with inhaled isoflurane (0.75% to 1.5%). A left thoracotomy was performed in the fifth intercostal space, the left lung was retracted, and the pericardium was opened. The left anterior descend-
substrate luciferin is fast ($t_{1/2} = 3$ hours). The relationship between luciferase enzyme concentration and the peak height of emitted light in vitro is linear up to 7 to 8 orders of magnitude.\textsuperscript{19} The BLI system (In Vivo Imaging System, Caliper Life Sciences, Hopkinton, Mass) consists of a supersensitive, cooled (90°C) charge-coupled device camera mounted onto a light-tight imaging chamber. The charge-coupled device chip is 2.7 cm$^2$ and consists of 2048$\times$2048 pixels at 13.5 $\mu$m each for a spatial resolution of 3 to 5 mm.\textsuperscript{20} The system does not allow 3-dimensional imaging; hence, spatial resolution is limited to a compressed, 2-dimensional image for analysis. Images are acquired at 1- to 10-minute intervals until peak signal is observed. Both the In Vivo Imaging System and its imaging analysis software are commercially available (Caliper Life Sciences). High reproducibility (within $\pm$8% SD from the mean values) and detection sensitivity of this system for monitoring luciferase reporter gene expression have been demonstrated in vivo. Luciferase generates most light activity 15 to 20 minutes after intraperitoneal injection of substrate luciferin at a dose of 30 mg/kg body weight (Caliper Life Sciences).\textsuperscript{15}

**Echocardiography**

Echocardiography was performed with the General Electric Vivid7 Dimension imaging system equipped with a 13-MHz linear probe (GE, Milwaukee, Wis). Mice were assessed preoperatively and 4, 7, and 14 days after surgery. Animals were induced with isoflurane, received continuous inhaled anesthetic (1.5% to 2%) within the duration of the imaging session to maintain heart rate between 300 and 500 bpm, and were imaged in the supine position. Echocardiography was performed by an independent operator blinded to the study conditions. M-mode short-axis views of the LV were obtained and archived. Analysis of the M-mode images was performed with built-in GE analysis software. LV end-diastolic diameter, end-systolic diameter, wall thickness, and tissue Doppler imaging were obtained and used to calculate fractional shortening by the following formula: fractional shortening = (end-diastolic diameter - end-systolic diameter) / end-diastolic diameter.

**PV Loop Analysis**

PV loops were generated in anesthetized, instrumented mice 7 days after surgery. Briefly, mice were anesthetized as mentioned above. A 1.4F (0.46-mm) high-fidelity micromanometer catheter (Millar Inst.
Instruments, Houston, Tex) was inserted into the right carotid artery and advanced retrogradely into the LV chamber. Two pairs of miniature omnidirectional piezoelectric crystals were implanted in the endocardium of the LV to obtain short- and long-axis cavity dimensions throughout the cardiac cycle. Simultaneous LV pressure and dimensions were recorded at baseline and during the decrease in preload generated by gently pulling a suture to transiently constrict the inferior vena cava. The ventilator was disconnected during data acquisition to eliminate the effects of positive ventilation. All data were recorded digitally and stored on a computer for offline analysis.

**Statistical Analysis**

All data are expressed as mean±SD. The correlation between the anatomic location of SPIO-labeled mESCs and MRIs was analyzed by Spearman’s rank correlation coefficient. Comparison of BLI activities, fractional shortening, tissue Doppler imaging, and wall thickness from the same animals over time was made with the repeated-measures ANOVA technique. In making comparisons of PV loop analysis and echocardiography data between the mESC-treated and the control groups, we analyzed the data using Steel methods as a multiple comparison of nonparametric test. The distribution of the location of signal void by SPIO-labeled mESCs was compared among the 3 groups with χ² and Fisher exact tests. A value of P<0.05 was considered statistically significant.

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

**Results**

**In Vivo MRI of SPIO-Labeled mESCs**

One day after transplantation, the MRIs demonstrated dephasing signal at the SPIO-labeled mESC transplantation sites in the anterolateral wall of the LV walls of all mice (Figures 1A, 2A, and 2C). MRI could not detect areas of signal loss with unlabeled mESCs.

**Histological Correlation**

Nine mice were available for anatomic correlation between MRI and histology (labeled mESCs, n=6; unlabeled mESCs, n=3). Prussian blue staining of the SPIO-labeled mESC grafts demonstrated dense intracellular iron labeling in undifferentiated mESCs, with iron exclusion from the nucleus, as demonstrated in Figure 1C and 1E. Macrophages were present in the infarction and mESC transplantation sites, but most of the iron was localized to mESCs, as shown in Figure 1F. No iron stain was visible in the unlabeled mESC group. Using a 16-segment analysis, we demonstrated a strong correlation between the anatomic location of the negative signal void on MRIs and the SPIO-labeled mESCs by histology, as shown in Figure 2A through 2D (r=0.962, P=0.002).

**Accurate Localization and Quantification of SPIO-Labeled mESCs in the 3 Zones of MI With MRI**

In the in vitro setting, a significant linear correlation between the quantity of SPIO-labeled mESCs and the signal area by MRI was observed (r=0.987, P<0.0001; Figure 3B). Representative delayed-enhanced MRIs of 3 different delivery zones are shown in Figure 3C and 3E (intra-infarction, n=3; peri-infarction, n=3). The high-signal-intensity area indicat-
ing the injured myocardium distinguished the injured from the normal myocardium. Using 16-segment analysis, we detected the majority of the injured myocardium (anterolateral MI) in segments 1 through 6. The signal voids indicated that SPIO-labeled mESCs were observed in the following segments of the injured myocardium: peri-infarction, 1 through 3, 15, and 16 (Figure 3C and 3D); and intra-infarction, 4 through 7 (Figure 3E and 3F). Significant differences in the SPIO-labeled mESC distribution of 2 groups were found ($P<0.05$).

**In Vivo Optical BLI**

Representative in vitro BLI of mESCs-luc$^+$ is shown in Figure 4. In vitro analysis showed a significant linear correlation between the number of viable mESCs-luc$^+$ and BLI signal, as shown in Figure 4A and 4B ($r=0.982$, $P=0.0002$). Representative serial in vivo BLIs are shown in Figure 4C. Twenty-nine mice were examined for BLI (intra-infarction, n=9; peri-infarction, n=10; remote/normal, n=10). BLI activities of all groups significantly increased over time ($P<0.05$), and viability of mESCs in the remote/normal group was significantly higher than that of other groups, as shown in Figure 4D ($P<0.05$). There was no difference in survival rate between the mESCs in the intra-infarction and peri-infarction groups.

**In Vivo Echocardiography**

A total of 23 mice could be examined for echocardiography (intra-infarction, n=9; peri-infarction, n=10; control, n=4). The results of functional analysis by echocardiography are shown in Table 1. Functional evaluation by echocardiography at 14 days after the operation demonstrated a trend toward improved LV fractional shortening in animals treated with mESCs; similarly, tissue Doppler imaging at 14 days demonstrated a trend toward higher regional activity. The mean wall thickness of the anterior wall of both the control and intra-infarction groups significantly decreased over time ($P<0.05$), and that of the control group was significantly thinner at 14 days after the operation than in other groups treated with mESCs ($P<0.001$).

**PV Loop Analysis**

A total of 19 mice were examined for PV loop analysis at 7 days after operation (intra-infarction, n=4; peri-infarction,

### Table 1. Results of Echocardiographic Parameters

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Intra-Infarct Group</th>
<th>Peri-Infarct Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
<td>Day 14</td>
<td>Day 7</td>
</tr>
<tr>
<td>End-diastolic diameter, mm</td>
<td>3.7±0.2</td>
<td>3.6±0.7</td>
<td>3.4±0.3</td>
</tr>
<tr>
<td>End-systolic diameter, mm</td>
<td>3.0±0.4</td>
<td>2.8±0.8</td>
<td>2.3±0.5</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>20.7±10.4</td>
<td>22.8±9.5</td>
<td>33.3±12.6</td>
</tr>
<tr>
<td>Anterior wall thickness, mm</td>
<td>0.55±0.13</td>
<td>0.14±0.07†</td>
<td>0.73±0.15</td>
</tr>
<tr>
<td>Tissue Doppler imaging, cm/s</td>
<td>0.13±0.06</td>
<td>0.15±0.07</td>
<td>0.34±0.27</td>
</tr>
</tbody>
</table>

*P<0.05 vs control and intra-infarct groups.
†P<0.05 vs day 7 within the same animals.
The results of functional analysis by PV loop analysis are shown in Table 2 and Figure 5. Functional evaluation by PV loop analysis at 7 days after the operation demonstrated significant improved LV ejection fraction and LV dp/dt max in animals treated with mESCs (P<0.05), although no difference was observed in end-systolic elastance and E max (coefficient b of the curvilinear end-systolic PV relationship).

Histology
There was no evidence of mESC differentiation or regeneration by hematoxylin and eosin stain. However, significant evidence of teratoma formation was seen in most animals treated with mESCs, as demonstrated in Figure 1G (remote/normal, n=5 [71.4%]; intra-infarction, n=3 [75%]; peri-infarction, n=3 [75%]), in contrast to the mice without teratoma formation, as seen in Figure 1H. There was no difference in LV function parameters between treated animals with and without teratoma formation.

Discussion
The present study demonstrated multimodality in vivo imaging of stem cell engraftment. First, precise anatomic locations of SPIO-labeled mESCs transplanted into different zones of MI by MRI were validated. Second, differential survival of mESCs delivered into the 3 zones of MI and subsequent restorative potential based on the location of transplantation were determined. Third, the absence of myocardial regeneration and the presence of teratoma were observed.

Several studies have demonstrated successful use of stem cells to restore injured myocardium.13,21–25 Development of a sensitive, noninvasive imaging technology to track the engraftment of the transplanted stem cells will be critical in monitoring the therapeutic efficacy of cell therapy. MRI with SPIO-based cell labeling techniques is emerging as the main diagnostic modality to track the transplanted stem cells, and several groups have reported that the robust detection of ex vivo–labeled stem cells delivered into the myocardial tissue was possible with clinically available SPIO agents such as Feridex IV.11,13,26,27 Effective in vivo imaging techniques to monitor stem cell therapy should provide such information as accurate localization, quantification, and viability of the delivered cells. Our study addresses some of the key challenges to evaluating cell engraftment parameters after cell delivery with a multimodality approach.

This study validates in vivo MRI-guided anatomic localization of transplanted mESCs within the heterogeneous infarcted myocardium. Conventional reporter gene techniques (eg, green fluorescent protein) or a thymidine

Table 2. Results of the Steady-State Hemodynamic Parameters by PV-Loop Analysis

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Intra-Infarct Group</th>
<th>Peri-Infarct Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>357.5±11.2</td>
<td>353.2±6.3</td>
<td>351.9±12.0</td>
</tr>
<tr>
<td>LV end-systolic pressure, mm Hg</td>
<td>49.8±10.7</td>
<td>51.7±6.7</td>
<td>48.9±6.0</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mm Hg</td>
<td>12.5±2.5</td>
<td>12.2±3.7</td>
<td>11.2±3.2</td>
</tr>
<tr>
<td>End-systolic volume, μL</td>
<td>49.1±4.7</td>
<td>47.5±5.0</td>
<td>44.6±6.0*</td>
</tr>
<tr>
<td>End-diastolic volume, μL</td>
<td>54.4±5.0</td>
<td>54.2±7.8</td>
<td>52.3±7.5</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>9.8±2.5</td>
<td>12.0±4.6*</td>
<td>13.7±3.1*</td>
</tr>
</tbody>
</table>

*P<0.05 vs control.

Figure 5. The invasive parameters of cardiac contractility by PV loop analysis. The LV dP/dt, end-systolic elastance (Ees; the slope of the end-systolic pressure-volume relationship), and Emax (coefficient b of the curvilinear end-systolic PV relationship) are shown. The LV dP/dt in the peri-infarction group was significantly higher than in the other groups (P<0.05); however, there was no difference in either Ees or Emax among the 3 groups.
an analogue (eg, bromodeoxyuridine) to validate stem cell engraftment has depended mostly on postmortem immunohistological analysis. Through the use of BLI and positron emission tomography imaging techniques, survival of stem cells injected into the heterogeneous milieu of the infarcted myocardium has been demonstrated.\textsuperscript{15,28,29} Our results with the molecular reporter gene technique demonstrated that BLI activities significantly increased over time in all 3 groups and that mESCs transplanted into different zones of MI significantly altered cell survival. This observation suggests that the transplanted mESCs proliferated. However, this also could be consistent with teratoma formation found in 70% of the mice by histological analysis.\textsuperscript{29,30}

Despite a decreased rate of mESC survival, precise delivery into the peri-infarction region demonstrated improved LV functions of the injured anterolateral myocardium. The data from the present study suggest the importance of both cell survival and location of cell delivery for optimal myocardial restoration. Although there was no histological evidence of regeneration in the injured myocardium except for teratoma formation, significant functional restoration was observed. These findings might indicate the strategic importance of local paracrine effects, cellular support by teratoma formation, precise myocardial delivery, and cell viability for effective cell therapy.

Finally, this study demonstrated the feasibility of multimodality imaging of mESC survival and myocardial restoration. First, the delayed-enhancement MR technique accurately detected in vivo the anatomic location of transplanted SPIO-labeled mESCs within the injured myocardium. Combining this capability to accurately localize mESC transplantation, in vivo MRI also may track cell engraftment through quantitative assessment of functional recovery longitudinally.\textsuperscript{31} Furthermore, BLI demonstrated variable survival of the transplanted cells and echocardiography/PV loop analysis—measured resultant myocardial restoration.

Conclusions

Multimodality evaluation of mESC engraftment in the heterogeneous tissue of MI is possible. MRI accurately detected in vivo the anatomic location of transplanted stem cells in the injured myocardium after delivery. BLI enabled assessment of variable viability of mESCs transplanted into the heterogeneous tissue of the infarcted myocardium, whereas echocardiography and PV loop analysis validated the restorative potential of mESCs. Although the viability of transplanted stem cells is a biological prerequisite, precise delivery of mESCs into the peri-infarction region might be equally critical in restoring the injured myocardium.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Stem cell transplantation has emerged as a promising therapeutic alternative to restore the injured myocardium. Using iron oxide–based contrast agents, magnetic resonance imaging has emerged as a primary modality to detect ex vivo–labeled stem cells while assessing the functional effects of stem cell therapy on the injured myocardium. However, evaluation of the fundamental biology of transplanted cells is not possible with magnetic resonance imaging. This study investigated the feasibility of a multimodality approach combining magnetic resonance imaging and bioluminescence imaging to assess stem cell engraftment in the recipient tissue, including cellular location, viability, myocardial regeneration, and functional restoration. On the basis of these engraftment parameters, this study demonstrated that both the location and viability of transplanted stem cells are critical in restoring the injured myocardium.
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