

# A Large-Scale Investigation of Hypoxia-Preconditioned Allogeneic Mesenchymal Stem Cells for Myocardial Repair in Nonhuman Primates

## Paracrine Activity Without Remuscularization

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**Rationale:** The effectiveness of transplanted bone marrow mesenchymal stem cells (MSCs) for cardiac repair has been limited; thus, strategies for optimizing stem-cell-based myocardial therapy are needed.

**Objective:** The present study was designed to test our central hypothesis that hypoxia-preconditioned MSCs (HP-MSCs) are more effective than MSCs cultured under ambient oxygen levels for the treatment of myocardial injury in a large-scale (N=49), long-term (9 months), nonhuman primate (Cynomolgous monkeys) investigation.

**Methods and Results:** MSCs were engineered to express green fluorescent protein, cultured under ambient oxygen or 0.5% oxygen (HP-MSCs) for 24 hours and then tested in the infarcted hearts of Cynomolgus monkeys ( $1 \times 10^7$  cells per heart). Hypoxia preconditioning increased the expression of several prosurvival/proangiogenic factors in cultured MSCs, and measurements of infarct size and left-ventricular function at day 90 after myocardial infarction were significantly more improved in monkeys treated with HP-MSCs than in monkeys treated with the control vehicle; functional improvements in normal cultured bone marrow mesenchymal stem cells-treated monkeys were not significant. HP-MSCs transplantation was also associated with increases in cardiomyocyte proliferation, vascular density, myocardial glucose uptake, and engraftment of the transplanted cells and with declines in endogenous cell apoptosis, but did not increase the occurrence of arrhythmogenic complications.

**Conclusions:** Hypoxia preconditioning improved the effectiveness of MSCs transplantation for the treatment of myocardial infarction in nonhuman primates without increasing the occurrence of arrhythmogenic complications, which suggests that future clinical trials of HP-MSCs transplantation are warranted. (*Circ Res.* 2016;118:970-983. DOI: 10.1161/CIRCRESAHA.115.307516.)

**Key Words:** Cynomolgous monkeys ■ hypoxia preconditioning ■ mesenchymal stem cells ■ myocardial infarction

Bone marrow mesenchymal stem cells (MSCs) have improved cardiac performance when administered after acute myocardial infarction (MI) in both large-animal models and in patients.<sup>1,2</sup> However, the results from randomized controlled

clinical trials have been less impressive; the authors of one meta-analysis concluded that left ventricular ejection fractions (LVEFs) increased by just 2.92% in response to cell therapy.<sup>3</sup> Nevertheless, MSCs remain attractive for the treatment of

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### Nonstandard Abbreviations and Acronyms

<b>DMEM</b>	Dulbecco's Modified Eagle's Medium
<b>GFP</b>	green fluorescent protein
<b>HP</b>	hypoxia preconditioning
<b>HP-MSCs</b>	hypoxia-preconditioned bone marrow mesenchymal stem cells
<b>LVEFs</b>	left ventricular ejection fractions
<b>MI</b>	myocardial infarction
<b>MSCs</b>	bone marrow mesenchymal stem cells
<b>NHP</b>	nonhuman primate model
<b>N-MSCs</b>	normal cultured bone marrow mesenchymal stem cells
<b>TUNEL</b>	terminal deoxynucleotidyl transferase dUTP nick end labeling

myocardial disorders because they are easy to obtain, self-replicating, multipotent, and only mildly immunogenic after transplantation.<sup>4</sup> Thus, researchers continue to search for techniques that may improve the efficacy of MSCs therapy.

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Only a small percentage of transplanted cells are retained and survive at the site of administration in infarcted myocardial tissue, and this low engraftment rate is believed to be one of the primary barriers to the effectiveness of cell therapy.<sup>5</sup> However, we have shown that the engraftment rate of MSCs in rodent models of myocardial injury can be improved by incubating the cells under hypoxic conditions before administration,<sup>6,7</sup> and infarct sizes were significantly smaller in rats treated with hypoxia-preconditioned MSCs (HP-MSCs) than in rats treated with MSCs cultured under ambient (ie, normoxic) conditions (N-MSCs). HP-MSCs transplantation has also been investigated for the treatment of left ventricular (LV) remodeling after acute MI in pigs.<sup>8</sup> However, the effect of transplanted HP-MSCs on myocardial recovery has yet to be evaluated in a nonhuman primate (NHP) model.

Although the results from both preclinical and early-phase clinical trials have consistently indicated that MSCs transplantation is safe, only large-animal studies can evaluate a novel therapeutic with enough detail to ensure that patients receive the maximum possible benefit while minimizing the risk of adverse events.<sup>9</sup> For example, stem cell–derived cardiomyocytes have not been associated with arrhythmogenic complications in mice, rats, or guinea pigs, but when the dose was scaled for delivery to macaques, the treated animals experienced arrhythmias.<sup>10</sup> Here, we present the results of the first large-scale, randomized, partially double-blind (for assessments of LV function and infarct size), preclinical investigation of MSCs therapy for the treatment of cardiac injury in an NHP model. MI was surgically induced in Cynomolgus monkeys, and the animals were treated with HP-MSCs, N-MSCs, or control medium (Dulbecco's Modified Eagle's Medium [DMEM]). Our results suggest that treatment with HP-MSCs led to significant improvements in LV function and infarct size.

### Methods

A more detailed description of the experimental methods is available in the Online Data Supplement.

### Ethics Statement

Experiments involving live animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

### HP-MSCs/N-MSCs Culture Conditions and In Vitro Studies

Cynomolgus monkey MSCs were isolated as described previously<sup>11</sup> and then engineered to express green fluorescent protein (GFP) via lentivirus transfection. Hypoxia preconditioning (HP) was performed by placing the cells in a well-characterized, finely controlled ProOx-C chamber system (Biospherix, Redfield, NY) for 24 hours. The oxygen concentration in the chamber was maintained at 0.5%, with a residual gas mixture composed of 5% CO<sub>2</sub> and balanced N<sub>2</sub>. N-MSCs were incubated under 21% oxygen and 5% carbon dioxide for 24 hours in complete culture medium. The effect of HP on MSCs multipotency, tube formation, and apoptosis was investigated in vitro.

### NHP MI Model, Treatments, and Analyses

Myocardial infarction was surgically induced in 49 adult Cynomolgus monkeys (male, 5–6 years old, 5–7 kg body weight); the monkeys were obtained from Suzhou Xishan Zhongke Laboratory Animal Co, Ltd, which has been certificated by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The animals were anesthetized with intramuscular injections of ketamine (5 mg/kg) plus midazolam (0.2 mg/kg) and ventilated with room air on an animal ventilator (Matrx model 3000 USA). A left thoracotomy was performed, and the left anterior-descending coronary artery was permanently ligated distal to the first branch with a 4-0 silk suture. Thirty minutes after ligation, animals in the HP-MSCs group were injected with a solution of 1×10<sup>7</sup> allogeneic HP-MSCs in 1 mL DMEM, animals in the N-MSCs group were injected with 1×10<sup>7</sup> allogeneic N-MSCs in 1 mL DMEM, and animals in the DMEM group were treated with 1 mL DMEM; the injections were delivered with a 29-gauge syringe to 5 sites in the peri-infarct region. A fourth group of animals (the normal group) underwent all surgical procedures except the ligation step and recovered without any of the experimental treatments. Heart function and infarct size were evaluated via cardiac magnetic resonance imaging; cardiac tissue glucose uptake was evaluated via positron emission tomography; electric stability was evaluated via continuous telemetric electrocardiographic (ECG) monitoring and programmed electric stimulation; engraftment was evaluated via quantitative RT-PCR measurements of GFP expression; apoptosis was evaluated via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining; and vascular density, arteriole density, proliferation, and endogenous progenitor-cell activation were evaluated via immunofluorescence analyses of marker expression.

### Statistical Analysis

All data are presented as mean±standard error of the mean (SEM). Comparisons among groups were analyzed for significance with 1-way or 2-way analysis of variance. A value of *P*<0.05 was considered significant. Statistical analyses were performed with Sigmaplot software (version 12.0).

### Results

#### HP Does Not Significantly Alter Marker Expression or Differentiation Potential in MSCs

Bone marrow MSCs were isolated from Cynomolgus monkeys, incubated under hypoxic or normoxic conditions as described earlier, and tested for surface-marker expression and differentiation potential. Both HP-MSCs and N-MSCs expressed MSCs markers, such as CD29, CD90, CD105, and CD166, but did not express markers for hematopoietic cells, such as CD34 and CD45 (Online Figure IA), and HP-MSCs and N-MSCs were

similarly capable of differentiating into osteogenic, adipogenic, and chondrogenic lineages (Online Figure IB). Thus, we observed no obvious differences between HP-MSCs and N-MSCs in surface-marker expression or differentiation potential.

### HP Promotes the Paracrine Activity of Cultured MSCs

Previous studies have shown that bone marrow MSCs secrete multiple cytokines that promote angiogenesis and survival<sup>12</sup>; thus, we evaluated the effect of HP on pro-survival protein expression in MSCs via Western Blot. Erythropoietin, hypoxia-inducible factor 1 $\alpha$ , and angiopoietin-1 protein levels were significantly greater in HP-MSCs than in N-MSCs (Online Figure IC and ID). Furthermore, *in vitro* tube formation analyses indicated that tube length was significantly greater when human umbilical vein endothelial cells were cultured in conditioned medium collected from HP-MSCs than in medium from N-MSCs (Online Figure IE and IF), and when N-MSCs and HP-MSCs were cultured with serum-free medium under 0.1% oxygen to induce apoptosis, subsequent TUNEL staining assessments indicated that HP protected the MSCs from cell death (Online Figure IG and IH). Collectively, these data demonstrated that HP may enhance the paracrine activity of MSCs.

### HP-MSCs Transplantation Improves Cardiac Function in an NHP MI Model

We tested our hypothesis that transplanted HP-MSCs would improve recovery after ischemic myocardial injury in an NHP model (*Cynomolgus* monkey) of MI. A total of 49 monkeys underwent the study protocol and were randomly assigned to treatment with 10 million allogeneic HP-MSCs (HP-MSCs group;  $n=16$ , bodyweight:  $5.30\pm 0.17$  kg), 10 million allogeneic N-MSCs (N-MSCs group;  $n=16$ , bodyweight:  $5.61\pm 0.15$  kg), or DMEM (DMEM group;  $n=15$ , bodyweight:  $5.36\pm 0.35$  kg); animals in the normal group underwent all surgical procedures except for the permanent ligation step (normal group;  $n=2$ , bodyweight:  $5.30\pm 0.50$  kg). Two animals in the HP-MSCs group, 4 in the N-MSCs group, and 5 in the DMEM group died during or shortly after MI induction and treatment because of ventricular arrhythmia or acute heart failure. Ten animals (4 in the HP-MSCs group and 3 in both the DMEM and N-MSCs groups) were euthanized on day 3 after injury for analyses of cell engraftment and histological observations, and 14 animals (4 in the N-MSCs group and 5 in both the DMEM and HP-MSCs group) were euthanized on day 28 after injury for cardiac functional and histological analyses. Ten of the 12 (83.3%) animals in the HP-MSCs group, 9 of 13 (69.2%) in the N-MSCs group, and 7 of 12 (58.3%) in the DMEM group survived for at least 28 days after MI, and no animals died from day 28 to day 270 (Figure 1A and 1B; Online Figure II). Thus, the survival rate appeared to be higher among animals in the HP-MSCs group than those in N-MSCs or DMEM group, although the number of animals included in the study was not great enough to determine whether the differences between groups were significant.

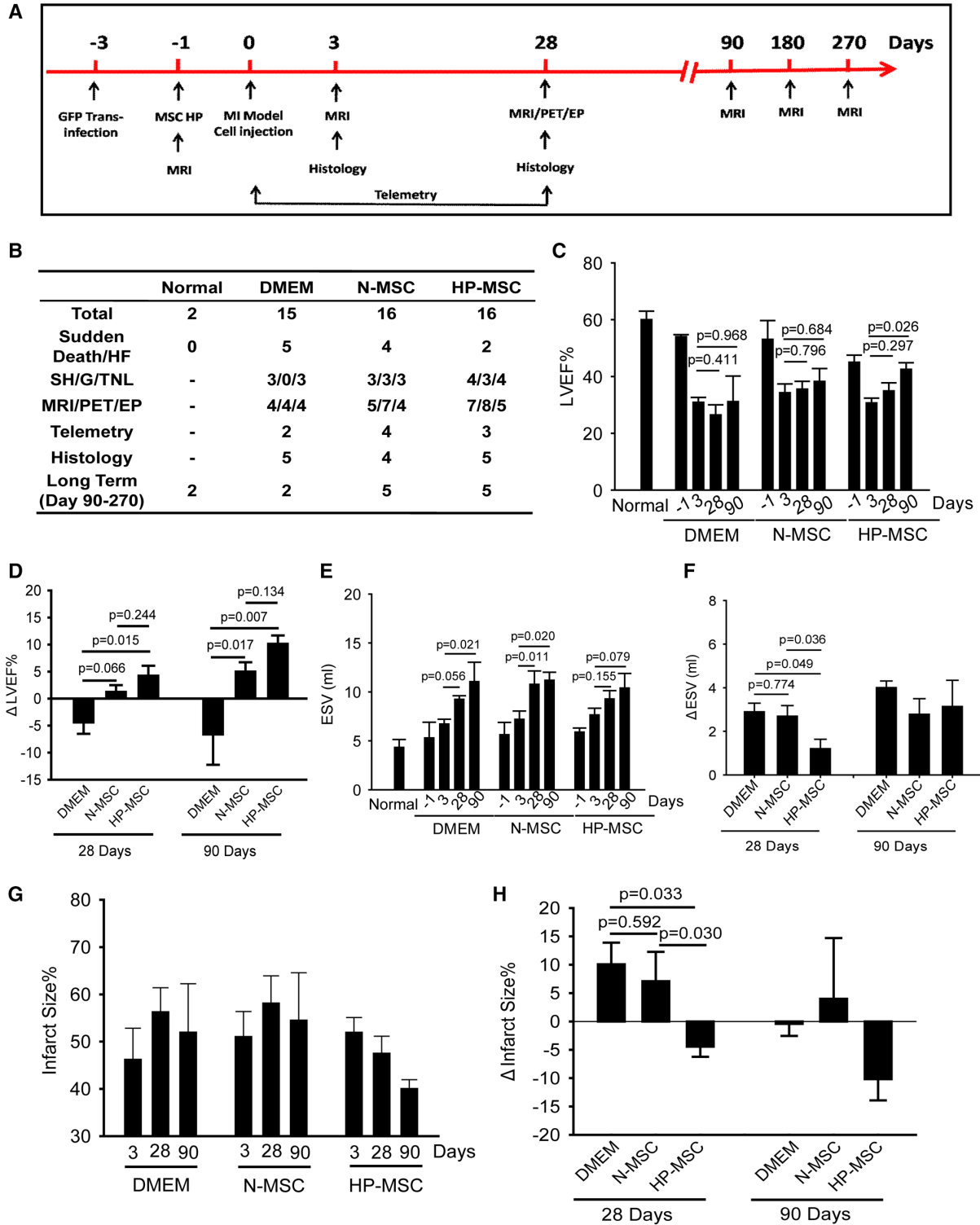
Cardiac function was measured via magnetic resonance imaging assessments of LVEF and end-systolic volume performed 1 day before injury and 3 days, 28 days, and 90 days afterward. LVEF was unchanged (or slightly smaller) on day 28 and day 90 than on day 3 in DMEM and N-MSCs animals,

but measurements in the HP-MSCs group increased significantly from day 3 to day 90 (Figure 1C); furthermore, the change in LVEF ( $\Delta$ LVEF) from day 3 to day 28 and from day 3 to day 90 was significantly greater in the HP-MSCs group, but not in N-MSCs group, than in animals treated with DMEM (Figure 1D). End-systolic volume measurements increased significantly from day 3 to day 90 in the DMEM and N-MSCs groups, but were unchanged in HP-MSCs animals, and change ( $\Delta$ ) in end-systolic volume was significantly smaller in HP-MSCs hearts than in DMEM or N-MSCs hearts from day 3 to day 28 ( $P<0.05$ ), which suggests that HP-MSCs transplantation limited cardiac dilatation (Figure 1E and 1F). HP-MSCs transplantation also appeared to be associated with smaller scar sizes at day 28 after injury (Figure 1G), and measurements of the change in infarct size ( $\Delta$ IS) indicated that scar size improved significantly more in HP-MSCs hearts than in either DMEM or N-MSCs hearts from day 3 to day 28.  $\Delta$ IS from day 3 to day 90 was also better in the HP-MSCs group than in either DMEM or N-MSCs group animals, but the differences between groups did not reach statistical significance (Figure 1H). Collectively, these results indicated that HP-MSCs are more effective than N-MSCs for promoting the recovery of cardiac performance and for limiting adverse remodeling after myocardial injury in NHPs.

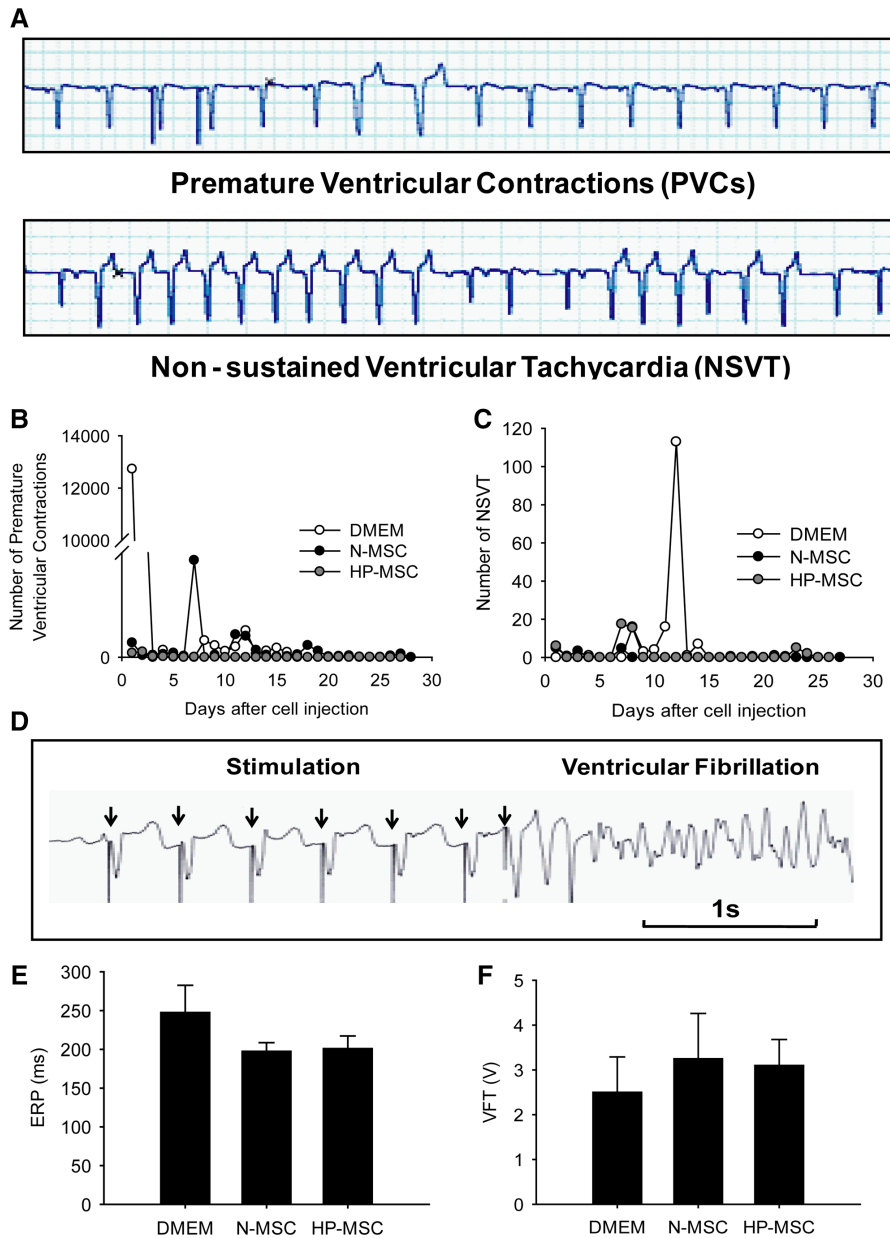
Measurements of LVEF at later time points (day 270) tended to be more improved in HP-MSCs animals (day 3 to day 270,  $\Delta$ LVEF= $2.81\pm 1.87$ ) than in N-MSCs animals (day 3 to day 270,  $\Delta$ LVEF= $-0.21\pm 3.35$ ), but the difference between groups was not significant, likely because the number of animals evaluated for each group (HP-MSCs:  $n=5$ , N-MSCs:  $n=3$ ; day 270) was small. Day 270 measurements are not reported for the DMEM group because only 2 animals were available. Assessments will continue to be performed over longer follow-up periods in all remaining animals.

### HP-MSCs Transplantation Is Not Associated With Arrhythmogenic Complications

Spontaneous arrhythmogenesis is one of the primary risks associated with stem cell therapy for the treatment of cardiac disorders; however, the results from recent large-animal studies have been inconsistent. Arrhythmogenic complications were observed after 1 billion stem cell-derived cardiomyocytes were delivered to the infarcted hearts of macaques,<sup>10</sup> but not when 10 million stem cell-derived cardiomyocytes were administered in a swine MI model.<sup>13</sup> Thus, we examined whether the HP protocol used here might influence the onset of cardiac arrhythmia by continuously monitoring the ECGs of 2 animals in the DMEM group, 4 animals in the N-MSCs group, and 3 animals in the HP-MSCs group from shortly after MI injury until day 28 for evidence of spontaneous arrhythmogenic complications, including premature ventricular contractions and nonsustained ventricular tachycardia. ECGs for the HP-MSCs animals were essentially normal throughout the entire follow-up period, whereas spontaneous arrhythmias were observed during the acute recovery phase (ie, between day 0 and day 14) for animals in both the DMEM and N-MSCs groups, but not at later time points (Figure 2A–2C). Furthermore, measurements of the effective refractory period and the ventricular fibrillation threshold on day 28 after MI



**Figure 1. Hypoxia-preconditioned bone marrow mesenchymal stem cells (HP-MSCs) transplantation improves cardiac function after myocardial infarction (MI).** **A**, Schematic representation of the study design and schedule of events. **B**, Number and disposition of animals in each experimental group. MRI assessments of left ventricular ejection fraction (LVEF) were performed at the indicated time points (**C**), and the change in LVEF ( $\Delta$ LVEF) was calculated by subtracting measurements taken on day 3 after MI from measurements taken on day 28 and day 90 (**D**). **E**, MRI assessments of left ventricular end-systolic volume (ESV) were performed at the indicated time points. **F**, The change in ESV ( $\Delta$ ESV) was calculated by subtracting measurements taken on day 3 after MI from measurements taken on day 28 and day 90. MRI assessments of infarct size were performed at the indicated time points (**G**), and the change in infarct size ( $\Delta$  Infarct Size) was calculated by subtracting measurements taken on day 3 after MI from measurements taken on day 28 and day 90 (**H**). EP indicates electrophysiology; G, green fluorescent protein (GFP) immunostaining; HF, heart failure; MRI, magnetic resonance imaging; N-MSCs, normal cultured bone marrow mesenchymal stem cells; PET, positron emission tomography; SH, short term (ie, day 3) histological assessments; sudden death, death caused by lethal ventricular tachycardia during the open-chest operation; TNL, TUNEL staining; and TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.



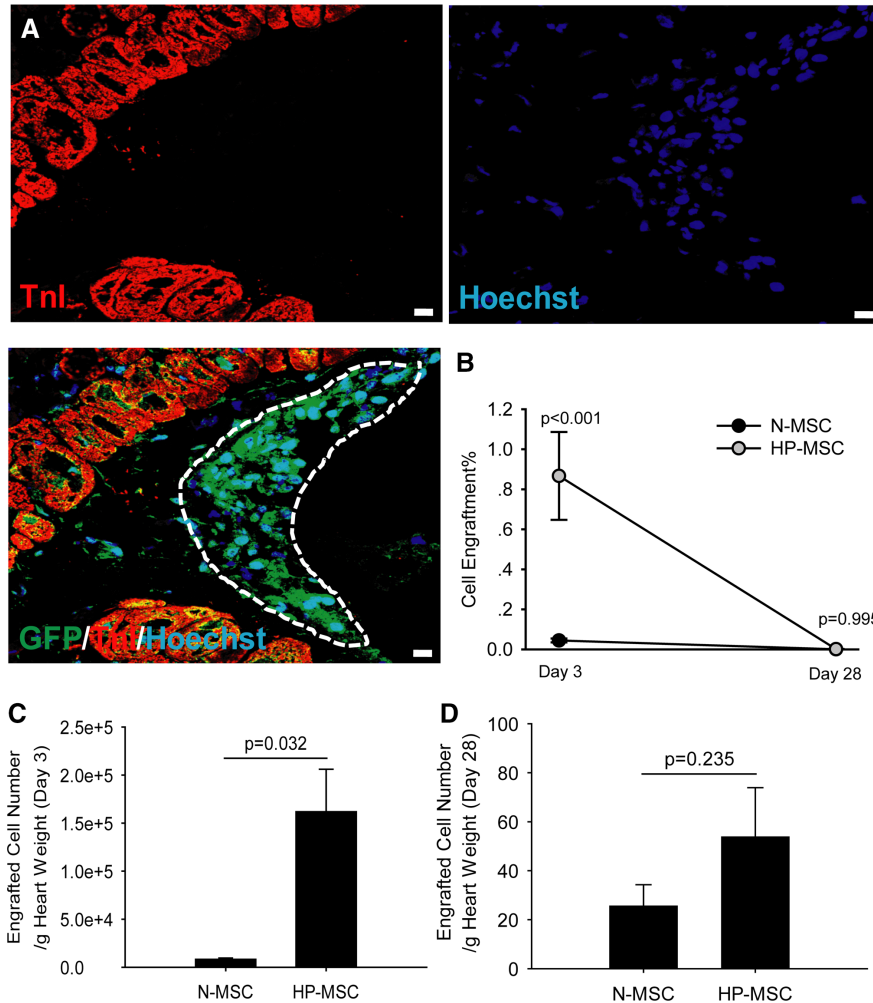
**Figure 2. Hypoxia-preconditioned bone marrow mesenchymal stem cells (HP-MSCs) transplantation is not associated with arrhythmogenic complications.** Electrocardiograms for animals in the HP-MSCs, N-MSCs, and Dulbecco's Modified Eagle's Medium (DMEM) treatment groups were monitored continuously (via telemetry) for evidence of premature ventricular contractions (PVCs) or nonsustained ventricular tachycardia (NSVT; **A**); representative tracings from a separate study (in macaques) are displayed. **B** and **C**, The average number of premature ventricular contractions (**B**) and NSVT (**C**) for animals in each treatment group was calculated daily from the moment of injury until day 28 afterward. **D–F**, Programmed electric stimulation experiments were performed on day 28 after myocardial infarction (MI) to determine the effective refractory period (ERP; **E**) and the ventricular fibrillation threshold (VFT; **F**) for animals in each treatment group.

in all 3 groups were similar (Figure 2D–2F). Taken together, these observations suggest that MSCs treated with the HP protocol used for the experiments described in this report do not impair the electromechanical stability of monkey hearts.

### HP-MSCs Are Grafted and Survive After Transplantation Into the Hearts of NHPs With MI

The efficacy of myocardial cell therapy is believed to be limited by the exceptionally small number of transplanted cells that become engrafted and continue to survive at the site of administration<sup>14</sup>; thus, we tested whether HP may improve

the engraftment/survival of transplanted MSCs. Because the MSCs were genetically engineered to express GFP, transplanted cells were identified in myocardial tissues from cell-treated animals via immunofluorescence staining for GFP expression, and the engraftment rate was calculated via quantitative PCR measurements of GFP DNA. Clusters of MSCs were observed in the border zone of ischemia on Day 3 after transplantation (Figure 3A), and the engraftment rate was  $\approx 20$ -fold greater for HP-MSCs ( $0.87 \pm 0.22\%$ ) than for N-MSCs ( $0.045 \pm 0.010\%$ ). Measurements in other organs indicated that the majority of



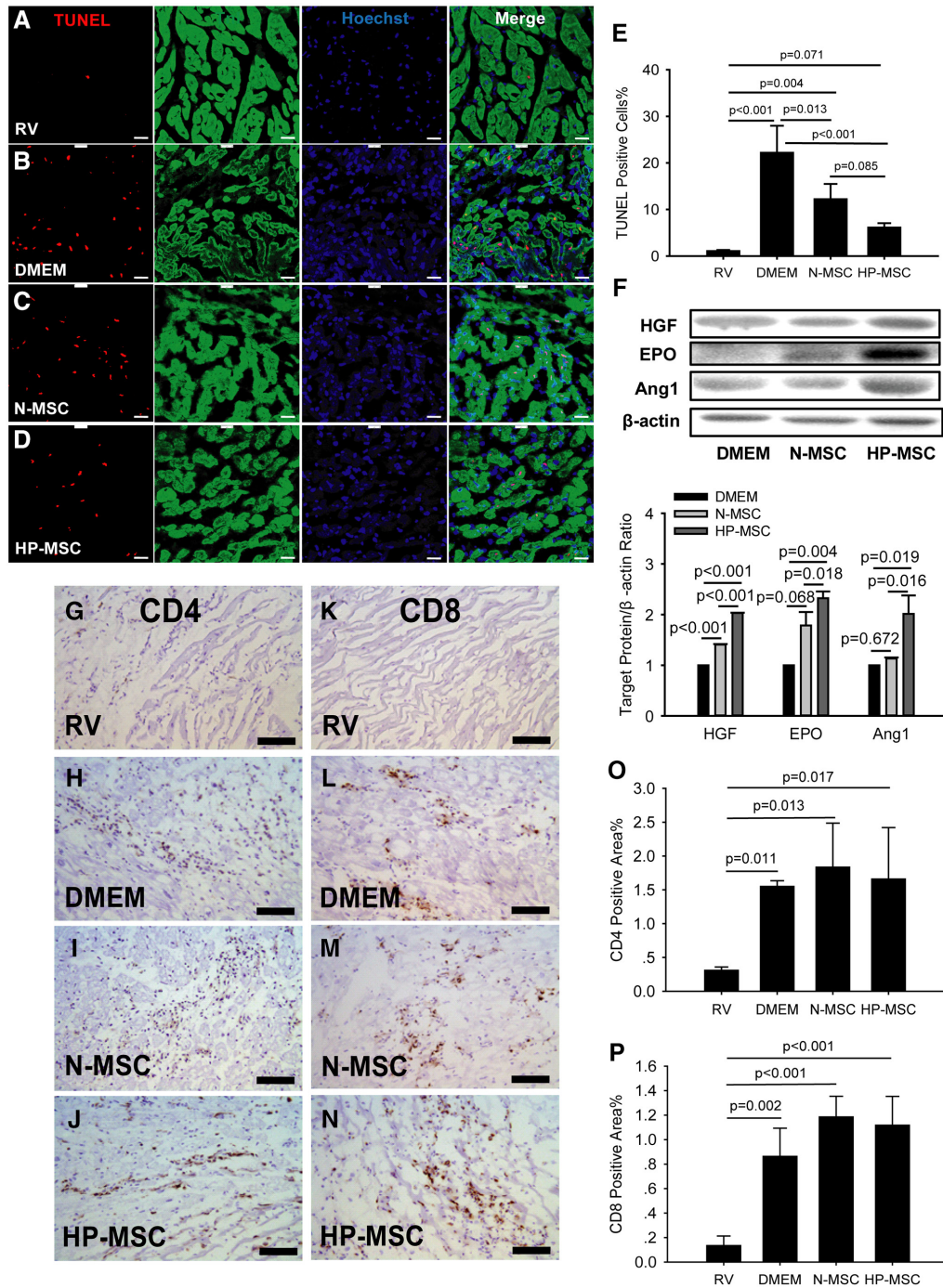
**Figure 3. Bone marrow mesenchymal stem cells (MSCs) are engrafted by the native myocardium after transplantation into the hearts of nonhuman primates with myocardial infarction (MI).** **A**, Engrafted cells were identified in sections stained for the presence of green fluorescent protein (GFP). The myocardium was visualized via fluorescent immunostaining for TnI, and nuclei were counterstained with Hoechst 33258, bar=50  $\mu$ m; a representative image from the heart of a hypoxia-preconditioned bone marrow mesenchymal stem cells (HP-MSCs)-treated animal euthanized 3 days after MI injury is displayed. **B–D**, The number of transplanted cells that were incorporated by the hearts of normal cultured bone marrow mesenchymal stem cells (N-MSCs)- and HP-MSCs-treated animals on day 3 and day 28 after injury was determined via quantitative PCR measurements of GFP mRNA levels in samples from the apex of the heart and presented as a percentage of the total number of cells administered (**B**) and the number of cells per gram of heart tissue on day 3 (**C**) and day 28 (**D**) after MI and treatment.

injected cells became sequestered in the lungs and liver (Online Figure III). By day 28, the engraftment/survival rate had declined by at least 2 orders of magnitude in both groups (HP-MSCs:  $0.0013 \pm 0.00061\%$ , N-MSCs:  $0.00045 \pm 0.00015\%$ ; Figure 3B–3D).

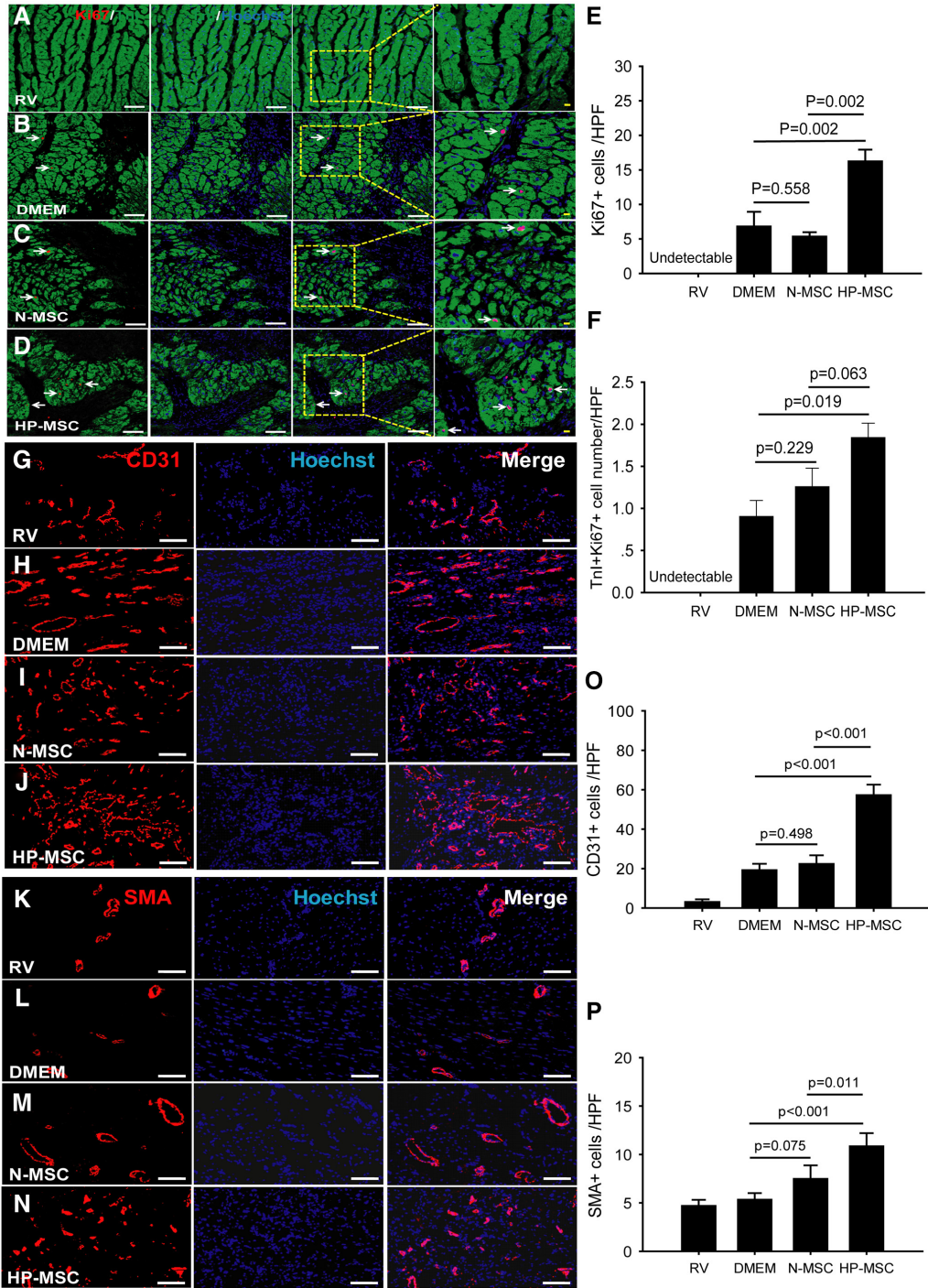
**Both HP-MSCs and N-MSCs Transplantation Reduce Cardiac Apoptosis After MI**

Because so few transplanted cells survived through day 28, we investigated whether the improvements associated with HP-MSCs transplantation occurred through the cells’ paracrine activity. The potential activation of cardioprotective mechanisms was evaluated by quantifying the number of apoptotic cells in the hearts of animals euthanized 3 days after MI and cell transplantation. Both HP-MSCs and N-MSCs administration were associated with significant declines in the number of apoptotic cells at the border zone of the infarct (Figure 4A–4E);

apoptotic cells also tended to be less common in hearts from the HP-MSCs group than in N-MSCs-treated hearts, but not significantly, and hearts from HP-MSCs animals expressed higher levels of the prosurvival proteins hepatocyte growth factor, erythropoietin, and angiopoietin-1 (Figure 4F). Furthermore, few CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes were found in the infarcted and border zone regions of hearts (Figure 4G–4N), and the number of T lymphocytes in the hearts of DMEM-, N-MSCs-, and HP-MSCs-treated animals did not differ significantly (Figure 4O and 4P), which suggests that the low rates of engraftment and survival were not caused by immune rejection of the transplanted cells. Thus, our observations indicate that HP-MSCs transplantation improved the survival of native cardiac cells, perhaps by activating endogenous cytoprotective mechanisms, and are consistent with previous reports that suggest MSCs are immune-privileged cells.<sup>15</sup>



**Figure 4. Bone marrow mesenchymal stem cells (MSCs) transplantation reduces cardiac apoptosis after myocardial infarction (MI).** **A–D**, Apoptotic cells were identified on day 3 after MI and treatment via TUNEL staining (red) in **(A)** sections of noninfarcted tissue from the right ventricle (RV) of animals in the hypoxia-preconditioned bone marrow mesenchymal stem cells (HP-MSCs) group and in sections from the border zone of the infarction in hearts from animals in the **(B)** Dulbecco’s Modified Eagle’s Medium (DMEM), **(C)** normal cultured bone marrow mesenchymal stem cells (N-MSCs), and **(D)** HP-MSCs groups. Cardiac tissue was visualized via fluorescent immunostaining for Tnl (green), and nuclei were counterstained with Hoechst 33258; bar=50 μm. **E**, Apoptosis was quantified as the percentage of cells that were positive for TUNEL staining. **F**, Expression of the pro-survival proteins, including hepatocyte growth factor (HGF), erythropoietin (EPO), and angiopoietin 1 (Ang1) on day 3 after injury were evaluated in tissues from the border zone of infarction via Western blot. β-Actin levels were also evaluated to serve as a control, and protein levels were quantified via densitometry analysis. **G–N**, Immune cells were identified on day 3 after MI by staining for CD4 (**G–J**) or CD8 (**K–N**) expression (brown) in sections of noninfarcted tissue from the right ventricle (RV) of animals in the HP-MSCs group (**G** and **K**) and in sections from the border zone of infarction in hearts from animals in the DMEM (**H** and **L**), N-MSCs (**I** and **M**), and HP-MSCs (**J** and **N**) groups (bar=100 μm). **O** and **P**, The immune response to cell transplantation was quantified as the percentage of the surface area that stained positively for CD4 (**O**) and CD8 (**P**).



**Figure 5. Hypoxia-preconditioned bone marrow mesenchymal stem cells (HP-MSCs) transplantation promotes cardiomyocyte proliferation and angiogenesis after myocardial infarction (MI).** **A–D**, Proliferating cells were identified on day 28 after MI and treatment by staining for expression of the proliferation marker Ki67 (red) in **(A)** sections of noninfarcted tissue from the right ventricle (RV) of animals in the HP-MSCs group and in sections from the border zone of the infarct in hearts from animals in the Dulbecco’s Modified Eagle’s Medium (DMEM; **B**), normal cultured bone marrow mesenchymal stem cells (N-MSCs; **C**), and HP-MSCs groups (**D**). Cardiac tissue was visualized via fluorescent immunostaining for TnI (green), and nuclei were counterstained with Hoechst 33258 (bar=100  $\mu$ m); the boxed regions of merged images are also displayed at higher magnification (yellow bar=50  $\mu$ m). **E**, Proliferation was quantified as the number of Ki67+ cells per high-power field (HPF), and **(F)** cardiomyocyte proliferation was quantified as the number of cells that expressed both Ki67 and TnI per HPF. **G–J**, Endothelial cells were identified on day 28 after MI and treatment by staining for expression of the endothelial marker CD31 (red) in **(G)** sections of noninfarcted tissue from the right ventricle (RV) of animals in the HP-MSCs group and in sections from the border zone of the infarct in hearts from animals in the DMEM (**H**), N-MSCs (**I**), and HP-MSCs groups (**J**); nuclei were counterstained with Hoechst 33258 (bar=100  $\mu$ m). **K–N**, Smooth muscle cells were identified on day 28 after MI and treatment by staining for expression of smooth muscle actin (SMA; red) in **(K)** sections of noninfarcted tissue from the right ventricle (RV) of animals in the HP-MSCs group and in sections from the border zone of the infarct in hearts from animals in the DMEM (**L**), N-MSCs (**M**), and HP-MSCs groups (**N**); nuclei were counterstained with Hoechst 33258 (bar=100  $\mu$ m). Vascular density was quantified as the number of CD31+ cells per HPF (**O**), and arteriole density was quantified as the number of SMA+ cells per HPF (**P**).



### HP-MSCs Transplantation Enhances Cardiomyocyte Proliferation After MI

Proliferating cells were identified via immunofluorescent staining for the proliferation marker Ki67. Four weeks after injury, Ki67<sup>+</sup> cells were significantly more common in the border zone of HP-MSCs–treated hearts than in the border zones of hearts from DMEM- and N-MSCs–treated animals (Figure 5A–5E), whereas cells that expressed both Ki67 and Troponin I were significantly more common in the HP-MSCs group than in the DMEM group (Figure 5A–5D and 5F). Thus, HP-MSCs transplantation appeared to promote the proliferation of endogenous cardiac cells, including cardiomyocytes. To determine whether the effect of HP-MSCs transplantation on cardiomyocyte proliferation (and other paracrine mechanisms) could have been mediated by the activation of cardiac progenitor cells, which are stimulated by MSCs transplantation in infarcted swine hearts,<sup>16</sup> expression of the progenitor cell marker *c-kit* was evaluated in the hearts of animals euthanized 28 days after MI injury and treatment. Border-zone *c-kit*<sup>+</sup> cells tended to be most prevalent in HP-MSCs–treated hearts, but the differences between groups did not reach statistical significance (Online Figure IVA–IVD).

### HP-MSCs Transplantation Promotes the Angiogenic Response to MI

To determine whether increases in vessel growth could have contributed to the functional improvements observed in HP-MSCs–treated hearts,<sup>17</sup> cells that expressed the endothelial cell marker CD31 or smooth muscle actin were identified in the border zone of infarction 4 weeks after injury. CD31<sup>+</sup> and SMA<sup>+</sup> cells were significantly more common in HP-MSCs–treated hearts than in hearts treated with either N-MSCs or DMEM (Figure 5G–5J and 5O and Figure 5K–5 N and 5P). Collectively, these observations suggest that HP can promote the ability of MSCs to stimulate angiogenesis in native myocardial tissues.

### HP-MSCs Transplantation Modulates Myocardial Metabolism After MI

The progressive decline in myocardial performance that often occurs after myocardial injury has been linked to adverse changes in myocardial metabolism, and the results from a recent study suggest that the functional improvements associated with HP-MSCs transplantation in the hearts of rats are accompanied by metabolic improvements.<sup>18</sup> To determine whether HP-MSCs can also improve the metabolic profile of injured NHP hearts, we evaluated glucose uptake in the hearts of HP-MSCs, N-MSCs, and DMEM animals via [<sup>18</sup>F]-fluorodeoxyglucose positron emission tomography; measurements were performed in the infarct zone, the border zone of the infarct, and in uninjured regions (ie, the remote zone) of the hearts 4 weeks after MI injury (Figure 6A). Myocardial glucose uptake was significantly higher in the border zone of hearts from animals in the HP-MSCs group than in the border zones of hearts from animals in the DMEM and N-MSCs group, but measurements in the infarcted and remote zones of all 3 treatment groups were similar (Figure 6B). Furthermore, Western blot analyses indicated that the expression of proteins involved in fatty acid metabolism (carnitinepalmitoyl transferase 1B

and thioesterase superfamily member 2) or in the inhibition of glucose metabolism (pyruvate dehydrogenase kinase and isozyme 4) declined significantly in the remote zone, but not in the border zone, of HP-MSCs–treated hearts on day 28 after MI (Figure 6C–6F). Collectively, these observations demonstrate that HP-MSCs transplantation increases the viability of ischemic myocardium by modulating cardiac energy supply and metabolism.

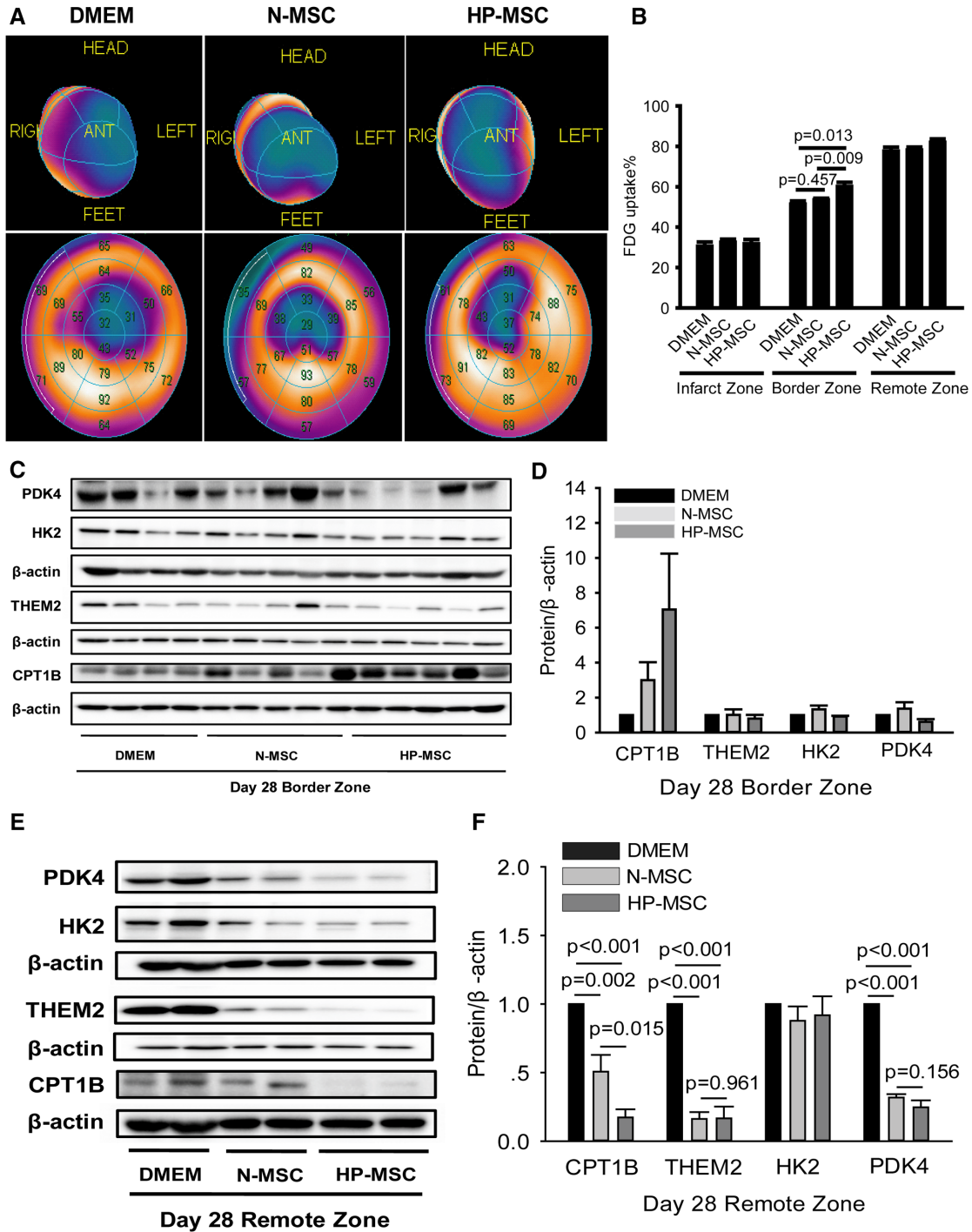
### HP-MSCs Transplantation Alters the Expression of Inflammatory and Chemo-Inducible Proteins After MI

To gain new insight into which paracrine factors and molecular mechanisms may contribute to the beneficial effects associated with HP-MSCs transplantation in injured NHP hearts, quantitative proteomics analyses were performed with tissues from the peri-infarcted regions of hearts from HP-MSCs, N-MSCs, and DMEM animals euthanized 3 days after injury. The expression of numerous inflammatory and chemo-inducible proteins, such as chemokine ligand 24 (CCL24), chemokine ligand 26 (CCL26), interleukin 16 (IL-16), interleukin-1 receptor antagonist (IL-1RA), macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein 2 (MCP-2), and nucleobase transporter 3 (NT-3), declined substantially in response to HP-MSC transplantation (Figure 7A–7 L), and these observations were corroborated by ELISA measurements of IL-1 $\alpha$ , IL-16, and MCP-2 levels (Online Figure VA–VC). HP-MSCs–treated hearts also tended to have the fewest number of cells that expressed the macrophage marker CD68, but the differences between groups did not reach statistical significance (Figure 7M and 7N). Collectively, these observations suggested that the functional benefits associated with cell therapy seem to be accompanied by a decline in myocardial inflammation.

## Discussion

Although the safety and feasibility of MSCs transplantation for treatment of ischemic myocardial disease has been well documented in both preclinical and early-phase clinical trials, improvements in myocardial function have been unremarkable.<sup>1,2,15,17,19</sup> Nevertheless, MSCs are easy to obtain, can be readily maintained and expanded in culture, and modulate the immune system, which may have a beneficial effect on the inflammatory response to myocardial injury and cell administration. Thus, researchers continue to search for techniques that may improve the effectiveness of MSCs therapy. HP is among the most promising techniques being tested because it stimulates paracrine mechanisms that may improve the survival, migration, and angiogenic activity of transplanted MSCs,<sup>6,7,20</sup> while avoiding the safety concerns associated with genetic or pharmacological approaches.<sup>21–24</sup> The potential benefit of HP is also supported by the results from our recently completed, phase I, randomized controlled China Acute Myocardial Infarction (CHINA-AMI) trial<sup>25</sup>; however, studies in an NHP model are needed to adequately characterize the safety profile and mechanisms of action associated with HP-MSCs therapy before these cells can be administered to large numbers of patients.

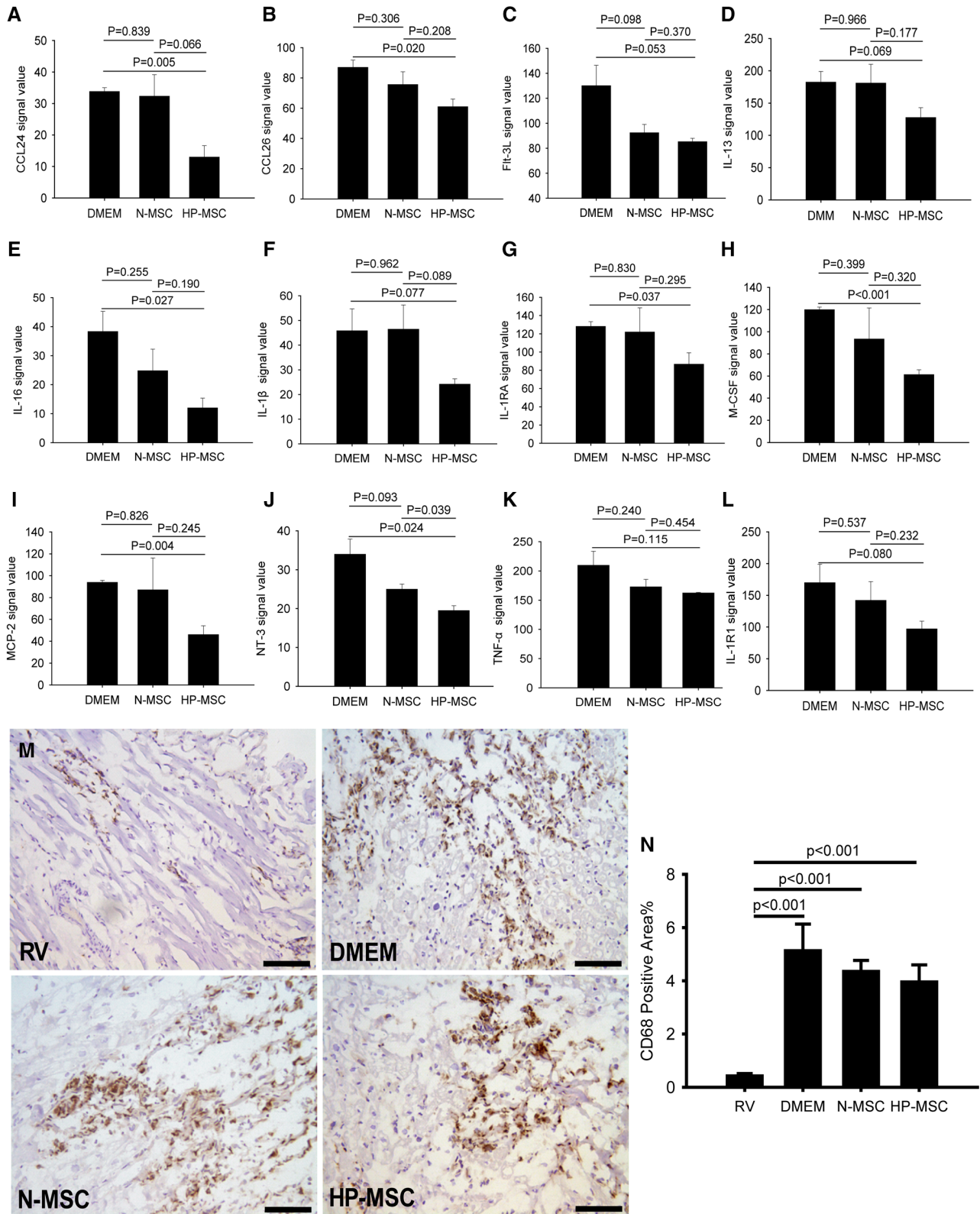
Here, we present the first large-scale (N=49) investigation of HP-MSCs administration for the treatment of MI in



**Figure 6. Hypoxia-preconditioned bone marrow mesenchymal stem cells (HP-MSCs) transplantation modulates myocardial metabolism after myocardial infarction (MI).** **A**, On day 28 after MI and treatment, animals in the Dulbecco's Modified Eagle's Medium (DMEM)-, N-MSCs, and HP-MSCs-treatment groups were injected with radiolabeled glucose ( $^{18}\text{F}$  fludeoxyglucose), and their hearts were imaged via positron emission tomography. Data are presented as a bulls-eye image; regions with high glucose uptake appear bright yellow, and images with low glucose uptake appear blue. **B**, Glucose uptake was quantified in the infarcted region, at the border-zone of the infarct, and in the noninfarcted (ie, remote) region of the heart. **C–F**, Expression of the metabolic proteins carnitinepalmitoyl transferase 1B (CPT1B), thioesterase superfamily member 2 (THEM2), hexokinase 2 (HK2), and pyruvate dehydrogenase kinase, isozyme 4 (PDK4) on day 28 after MI and treatment was evaluated in tissues from the border-zone of infarction (**C**) and the remote (noninfarcted) zone via Western blot (**E**).  $\beta$ -Actin levels were also evaluated to serve as a control. Protein levels in the border-zone of infarction (**D**) and the remote (noninfarcted) zone were quantified via densitometry analysis (**F**).

an NHP model. Our results suggest that HP substantially improved the therapeutic potency of MSCs: improvements in infarct size and LVEF from day 3 to day 28 or day 90

were significantly greater in HP-MSCs-treated monkeys than in monkeys treated with N-MSCs or DMEM, whereas differences between the N-MSCs and DMEM groups did



**Figure 7. Hypoxia-preconditioned bone marrow mesenchymal stem cells (HP-MSCs) transplantation modulates the protein expression profile in nonhuman primate hearts with myocardial infarction (MI).** A–L, Protein array analyses were performed with tissues from the border zone of infarction in animals euthanized 3 days after MI and treatment. Results are displayed for 16 inflammatory factors and chemo-inducible proteins whose expression levels were lower in samples from HP-MSCs-treated hearts than in Dulbecco’s Modified Eagle’s Medium (DMEM)–treated heart samples. M, Inflammation was evaluated on day 3 after MI by staining for expression of the macrophage marker CD68 (brown) in sections of noninfarcted tissue from the right ventricle (RV) of animals in the HP-MSCs group and in sections from the border zone of infarction in hearts from animals in the DMEM, N-MSCs, and HP-MSCs groups (bar=100  $\mu$ m). N, Macrophage infiltration was quantified as the percentage of the surface area that stained positively for CD68.

not reach statistical significance. Furthermore, the observed improvements were not accompanied by evidence of MSCs differentiation, and <1% of the administered cells were engrafted by the native myocardium, which is consistent with the results from several other studies,<sup>26–28</sup> and suggests that the benefit of HP-MSCs transplantation occurred through the cells' paracrine activity, rather than through remuscularization of the infarcted region.<sup>10,29</sup> Our observations also illustrate the exceptionally low immunogenicity of MSCs because neither N-MSCs nor HP-MSCs administration was associated with increases in the number of T-lymphocytes or macrophages at the site of administration, despite the cells' allogeneic origin and the absence of concomitant immunosuppressive therapy.

### HP and MSCs Engraftment

MSCs reside in the bone marrow where oxygen levels are low, which suggests that MSCs may have an intrinsic tolerance for the oxygen-deprived microenvironment of ischemic myocardium. Furthermore, previous reports indicate that HP-MSCs are less likely than N-MSCs to be rejected by the immune systems of rats,<sup>30,31</sup> and our results indicate that HP increases MSCs migration and reduces MSCs apoptosis when the cells are cultured under 0.5% oxygen.<sup>20</sup> Thus, the ≈20-fold increase in engraftment associated with HP observed on day 3 could have evolved through declines in the host animal's immune response, increases in cell survival or, perhaps, because the number of cells that were lost to the peripheral circulation declined. However, HP-MSCs engraftment declined to just 0.0013% by day 28, which suggests that the beneficial effects associated with HP endure far longer than the initial (day 3) improvement in engraftment.

### Mechanistic Observations

Because the engraftment rates for N-MSCs and HP-MSCs were low, and we found no evidence to suggest that the transplanted cells differentiated into functional myocytes or endothelial cells, the benefit of HP-MSCs transplantation likely evolved through the secretion of paracrine factors that stimulated endogenous cytoprotective or regenerative mechanisms. The results presented here, as well as in our previous small-animal studies,<sup>6,7</sup> confirm that HP increases the production of cytokines, such as hypoxia-inducible factor-1, angiopoietin-1, and erythropoietin in MSCs, and HP-MSCs transplantation increased proliferation and angiogenesis, while limiting fibrosis (ie, infarct size) and the expression of a panel of inflammatory proteins in the native myocardial tissue. Furthermore, although HP has been shown to reduce glucose uptake in MSCs,<sup>18</sup> myocardial glucose uptake increased significantly at the border-zone of the infarct in response to HP-MSCs transplantation, which may protect against progressive LV dysfunction and dilatation,<sup>32,33</sup> while fatty acid metabolism<sup>34,35</sup> significantly declined in noninfarcted tissues. Notably, the effect of HP on MSCs-induced paracrine activity would have been most prominent during the first few days after administration, when the engraftment rate was ≈20-fold higher in HP-MSCs animals than in the N-MSCs group. Thus, the improvements in angiogenesis, proliferation, and glucose/fatty acid utilization observed in HP-MSCs animals on day 28,

after the engraftment rates in the 2 cell treatment groups had declined by 2 orders of magnitude, are consistent with current concepts that emphasize the importance of cytokine activity during the early phase of recovery from myocardial injury.

### Arrhythmogenic Complications

Arrhythmogenesis has been recognized as a prominent safety concern of myocardial cell therapy because the complication was first reported in clinical investigations with skeletal myoblasts.<sup>36,37</sup> Arrhythmia has rarely been reported in small-animal studies, but when human embryonic stem cells were differentiated into cardiomyocytes and administered to the hearts of macaques with ischemia-reperfusion injury, all 4 of the cell-treated animals experienced periods of premature ventricular contractions and tachycardia within 2 weeks after treatment administration.<sup>10,38</sup> The authors suggested that this apparent discrepancy between small- and large-animal studies may have been caused, at least in part, by the number of cells (1 billion) administered to the macaques. This exceptionally large dose produced grafts of transplanted cells that were at least 10-fold larger than the grafts observed in other species and, consequently, may have altered electronic signal transduction (re-entry concern). Thus, our present study was conducted with a much smaller cell dose (10 million cells/animal), which did not produce grafts of significant size. Furthermore, experiments in a rat MI model have shown that the conditioned medium from HP-MSCs, but not N-MSCs, can restore conduction velocity and prevent death caused by arrhythmia,<sup>39</sup> perhaps by reducing the formation of fibrotic tissue,<sup>40</sup> and the effect of HP-MSCs transplantation on fibrotic tissue formation was also evident in our current investigation because infarct sizes were significantly more improved in HP-MSCs-treated animals than in animals from the N-MSCs group at day 28. Collectively, the results presented here suggest that MSCs can be administered to NHPs with no apparent increase in the risk for arrhythmia, and that this observation may be attributable both to the number of cells administered and to the paracrine activity of the HP-MSCs.

### Study Limitations

The long-term studies associated with this investigation are ongoing. Thus, the outcome assessments related to structural and molecular changes are not reported for the time course of day 270 after MI and treatment. Furthermore, although the number of animals included was large compared with other studies in NHPs, previous rodent studies have been performed with much larger study groups, which may explain why animals in the N-MSCs group did not display the level of improvement that is typically associated with intramyocardial MSCs transplantation. Our study also omitted the standard clinical regimen of medical treatments for MI and, consequently, the therapeutic effect of MSCs transplantation in patients may differ from the observations reported here.

### Conclusions

In conclusion, this report presents the first large-scale pre-clinical investigation of HP-MSCs therapy for the treatment of myocardial injury in NHPs. Our results suggest that treatment with HP-MSCs led to significant improvements in

cardiac function and infarct size without increasing the risk for arrhythmogenic complications, and that these benefits were likely mediated by increases in the paracrine activity of the hypoxia-preconditioned cells. Collectively, these observations suggest that HP-MSCs transplantation can be feasibly and safely investigated in clinical trials of myocardial cell therapy.

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### Disclosures

None.

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## Novelty and Significance

### What Is Known?

- Results of several clinical trials suggest that the transplantation of bone marrow mesenchymal stem cells (MSCs) for cardiac repair is safe, although their efficacy remains uncertain.
- Studies in rodents and swine indicate that hypoxia preconditioning (HP) can increase the therapeutic effectiveness of transplanted bone marrow MSCs for the treatment of myocardial infarction.

### What New Information Does This Article Contribute?

- Hypoxia preconditioning promotes the engraftment of injected MSCs in infarcted hearts of monkeys.
- Cardiac function was significantly improved 90 days after myocardial infarction injury and treatment with HP-MSCs.
- HP-MSCs transplantation was associated with increases in vascular density and myocardial glucose uptake and with declines in endogenous cell apoptosis.
- HP-MSCs transplantation enhanced the proliferation of endogenous cardiomyocytes.
- HP-MSCs transplantation was not associated with arrhythmogenic complications.

MSCs are a promising agent for the treatment of myocardial disorders because they are easy to obtain, self-replicating, multipotent, and only mildly immunogenic after transplantation, but their effectiveness in randomized controlled clinical trials has been disappointing. Therefore, researchers continue to develop strategies to improve the therapeutic potency of transplanted MSCs. Here, we present results of our large-scale (N=49), long-term (9 months) investigation of HP-MSCs transplantation in a Cynomolgus monkeys myocardial infarction model. Hypoxia preconditioning promoted the engraftment of transplanted MSCs, and improvements in heart function after injury and treatment were significantly greater in HP-MSCs-treated monkeys than in monkeys treated with N-MSCs or the control vehicle. HP-MSCs transplantation was also associated with increases in cardiomyocyte proliferation, vascular density, and myocardial glucose uptake and with declines in endogenous-cell apoptosis, but not with an increase in the occurrence of arrhythmogenic complications. Collectively, these findings support future clinical investigations of HP-MSCs transplantation.