Mitotically Inactivated Embryonic Stem Cells Can Be Used as an In Vivo Feeder Layer to Nurse Damaged Myocardium after Acute Myocardial Infarction : A Preclinical Study

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Mitotically Inactivated Embryonic Stem Cells Can Be Used as an In Vivo Feeder Layer to Nurse Damaged Myocardium after Acute Myocardial Infarction

A Preclinical Study

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Rationale: **Various types of viable stem cells have been reported to result in modest improvement in cardiac function after acute myocardial infarction. The mechanisms for improvement from different stem cell populations remain unknown.**

Objective: **To determine whether irradiated (nonviable) embryonic stem cells (iESCs) improve postischemic cardiac function without adverse consequences.**

Methods and Results: **After coronary artery ligation-induced cardiac infarction, either conditioned media or male murine or male human iESCs were injected into the penumbra of ischemic myocardial tissue of female mice or female rhesus macaque monkeys, respectively. Murine and human iESCs, despite irradiation doses that prevented proliferation and induced cell death, significantly improved cardiac function and decreased infarct size compared with untreated or media-treated controls. Fluorescent in situ hybridization of the Y chromosome revealed disappearance of iESCs within the myocardium, whereas 5-bromo-2′-deoxyuridine assays revealed de novo in vivo cardiomyocyte DNA synthesis. Microarray gene expression profiling demonstrated an early increase in metabolism, DNA proliferation, and chromatin remodeling pathways, and a decrease in fibrosis and inflammatory gene expression compared with media-treated controls.**

Conclusions: **As a result of irradiation before injection, ex vivo and in vivo iESC existence is transient, yet iESCs provide a significant improvement in cardiac function after acute myocardial infarction. The mechanism(s) of action of iESCs seems to be related to cell-cell exchange, paracrine factors, and a scaffolding effect between iESCs and neighboring host cardiomyocytes. (*Circ Res*. 2012;111:1286-1296.)**

Key Words: acute myocardial infarction ■ embryonic stem cells ■ irradiated stem cells

Intramyocardial or intracoronary delivery of numerous types of purified or unpurified adult bone marrow–derived or blood-derived hematopoietic stem cells (HSCs) and mesenchymal stem cells seems to improve cardiac function after infarction in animals,[1] as well as in humans trials,[2] but the mechanism involved remains unclear.[2] To date, 4 possibilities have been advanced: (1) lineage-specific differentiation of stem cells, especially HSCs, into endothelial and smooth muscle cells that contribute to vessel formation in the ischemic penumbra[3,4]; (2) transdifferentiation of adult mesenchymal and HSCs into cardiomyocytes[5–7]; (3) generation of cell fusion hybrids between HSCs and recipient somatic cells,[8–12] leading to replication of the heterokaryon; and (4) paracrine-mediated effects from the injected cells.[13] Although these mechanisms have been the source of debate,[14,15] under in vivo physiological conditions, transdifferentiation and cell fusion are probably rare and insufficient in vivo events to account for the reported improvement in cardiac homeostasis after adult stem cell injection.[2]

Editorial, see p 1250

Peri-infarct myocardium injected with embryonic stem cells (ESCs) also has been reported to result in improved

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Non-standard Abbreviations and Acronyms	
AMI	acute myocardial infarction
BrdU	5-bromo-2′-deoxyuridine
ESCs	embryonic stem cells
Gy	gray
HSCs	hematopoietic stem cells
iESCs	irradiated embryonic stem cells
MRI	magnetic resonance imaging

myocardial function.[16] However, injection of ESCs into either normal or ischemic myocardium may result in intracardiac teratomas, especially with higher doses of injected ESCs.[16–18] For this reason, the application of ESCs to repair myocardium has focused on ex vivo–directed differentiation of ESCs into cardiomyocytes before transplantation into the heart.

In animal models, transplantation of ESC-derived cardiomyocytes improves cardiac contractility and attenuates heart failure.[19] However, ex vivo–directed differentiation of ESCs into cardiomyocytes is a form of in vivo somatic cell replacement therapy, not in vivo stem cell therapy per se, and is complicated by inefficient ex vivo differentiation, expensive ex vivo quality control of sterility and purity, diminished posttransplant in vivo survival, unpredictable integration with host tissue, and requirements for immune suppression to prevent allogeneic ESC-derived cardiomyocyte rejection, and it is difficult to exclude with absolute certainty in vivo teratoma formation from a viable residual undifferentiated pluripotent stem cell.[20]

Herein, we use the novel approach of transplanting ESCs that are lethally inactivated with irradiation (irradiated embryonic stem cells [iESCs]) to prevent proliferation and avoid teratoma risk by ensuring a limited ex vivo and in vivo existence. We demonstrate that iESCs exist transiently both ex vivo and in vivo, but iESCs remain capable of improving cardiac function, suggesting a potent cardioregenerative iESC-mediated signaling to recipient cardiomyocytes and resident cardiac progenitor cells that are independent of persistent ESC presence and differentiation.

Methods

Animals

Female ICR mice (25–28 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). NOG mice (NOD SCID gamma; NSG-NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) were purchased from Jackson Labs (Bar Harbor, ME). Rhesus macaque monkeys (3.5–4.5 kg) were purchased through Innovative Surgeries and Investigative Solutions (San Carlos, CA). Experiments were approved by the Institutional Animal Care and Utilization Committee.

Cells

Cardiomyocytes and splenocytes were obtained as primary cultures from ICR mice. Mouse cardiomyocytes were isolated using the cardiomyocyte isolation kit (Cellutron, Baltimore, MD) with Langendorff perfusion system. Mouse embryonic fibroblasts were obtained from Invitrogen, inactivated with γ irradiation (40 Gy), and cryopreserved. Murine ESCs were derived from 129/SvJX129/SV-CP male F1 hybrid 3.5-day mouse (H-2b) blastocysts. A supernumerary human ESC line was obtained from Cecolfes (Bogotá, Colombia). ESCs were irradiated using Gammacell 40 irradiator.

Viability, Proliferation, and Staining Assays

ESC proliferation and viability were monitored with 5-bromo-2′-deoxyuridine (BrdU) cell proliferation assay (Calbiochem) and CellTiter-GloTM luminescent cell viability assay (Promega, Madison, WI) kit, respectively. Primary antibodies for immunostaining were as follows: rabbit polyclonal anti-CD3 (diluted 1:100; Abcam) and rabbit polyclonal anti-CD31 (diluted 1:50; Abcam). Species-specific donkey secondary antibodies were labeled with fluorescein isothiocyanate or Cy5 (Jackson ImmunoResearch).

Cardiomyocyte Coculture With iESCs

iESCs were washed and resuspended in the condition medium that containing 50% AS medium (Cellutron, Baltimore, MD) and 50% ESC medium with leukemia inhibitory factor. Cardiomyocyte media was replaced with condition media 2 hours after seeding. iESCs were seeded at a density of 0.3×10^5 cells into cell culture inserts (pore size, 0.4 μm; BD) and placed immediately into the respective wells with cardiomyocytes. The condition medium was changed daily during the coculture assay. Non cocultured cardiomyocytes were included as controls. After 48 hours of coculture, the living cell cardiomyocytes were counted using a hemacytometer and trypan blue.

Acute Myocardial Infarction and iESC Transplantation

Mice underwent intraperitoneal anesthesia with a mixture of ketamine (100 mg/kg), xylazine (10 mg/kg), and atropine (0.04 mg/kg), and were ventilated via a rodent ventilator (Harvard model 687 mouse ventilator; Harvard Apparatus, Holliston, MA). Left thoracotomy was performed at the fourth intercostal space. The chest wall was retracted by the use of 5-0 silk or monofilament suture. Ligation proceeded with a 7-0 silk suture passed with a tapered needle underneath the left anterior descending branch of the left coronary artery <2 mm from the tip of normally positioned left auricle. A 1-mm section of PE-10 tubing was placed on the top of the vessel, and a knot was tied on the top of the tubing to occlude the coronary artery. The knot was cut after occlusion for 60 minutes to restore reperfusion. Three intramyocardial injections of 10 μL media±≈1×10^6 ESCs were injected into the infarction, border, and normal zones via a microsyringe. After chest wall closure, the mouse was removed from the respirator, endotracheal tube was withdrawn, warmth was maintained by a heating pad, and 100% oxygen via nasal cone was provided under intensive care until full recovery.

Monkeys were anesthetized with Telazol (0.2 mL intramuscular) and 0.8% isoflurane. A left thoracotomy was performed at the fourth intercostal space in a sterile operating theater. Ligation of the left anterior descending coronary artery at the distal edge of the left auricular appendix proceeded with a 3-0 silk suture knot over plastic tubing for 50 minutes, followed by knot release and injection of 10^6 to 5×10^6 iESCs or media at 10 sites along the ischemia border via a microsyringe. The chest was closed by a standard operative technique, and monkeys were monitored in an intensive care facility until full recovery.

Hemodynamic Measurements and Infarct Size

Cardiac contractility and relaxation were monitored via a high-fidelity transducer-tipped pressure catheter (SPR 839; Millar Instruments, Houston, TX). Signals were digitized by use of a data translation series analog digital converter and then stored and analyzed on a Millar PVAN data acquisition and analysis system. Values derived from pressure traces were averaged over no less than 20 beats. To determine infarct size, mice were euthanized 30 days after acute myocardial infarction (AMI) to harvest the heart. Trichrome-stained mouse heart sections were photographed, and infarct area was measured using computer software. Percent infarct was determined as the ratio of infarct area to total area. Primate infarct size was determined via magnetic resonance imaging (MRI) using a 1.5-T Siemens Magneton Symphony Syngo and Espree scanner (Siemens AG, Erlangen, Germany). To calculate scar mass, 0.2 mmol/kg of Gd-DTPA (Magnevist; Bayer) was administrated at a rate of 0.004 mmol/kg per minute, and left ventricle short-axis segmented TurboFLASH images (repetition time, 700 ms; echo time, 4.18 ms; flip angle, 25°; slice thickness, 5.5 mm; field of view, 200–250 mm; and matrix, 123×256) were acquired ≈10 minutes after

Table. Top 20 Most Upregulated and Downregulated Cell Signal Pathways by iESC Treatment of Infarcted Heart

GO Identification	GO Terms	*P* Value	Fold Change
Upregulated cell signal pathways			
GO:0044260	Cellular macromolecular metabolic process	2.78e-44	163
GO:0006139	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	7.62e-41	129
GO:0006259	DNA metabolic process	1.9e-40	53
GO:0044249	Cellular biosynthetic process	1.08e-39	132
GO:0006260	DNA replication	6.38e-38	35
GO:0022403	Cell cycle phase	1.46e-37	48
GO:0000279	M phase	3.68e-35	44
GO:0000278	Mitotic cell cycle	3.16e-32	40
GO:0034645	Cellular macromolecule biosynthetic process	9.16e-32	108
GO:0009059	Macromolecule biosynthetic process	3.29e-31	108
GO:0007067	Mitosis	1.87e-25	31
GO:0000280	Nuclear division	1.87e-25	31
GO:0000087	M phase of mitotic cell cycle	2.52e-25	31
GO:0048285	Organelle fission	3.39e-25	31
GO:0006974	Response to DNA damage stimulus	1.24e-22	32
GO:0010457	Gene expression	5.94e-22	97
GO:0051276	Chromosome organization	1.4e-21	36
GO:0060255	Regulation of macromolecule metabolic process	1.58e-19	80
GO:0080090	Regulation of primary metabolic process	3.57e-19	79
GO:0051171	Regulation of nitrogen compound metabolic process	6.22e-19	73
Downregulated cell signal pathways			
GO: 0006952	Defense response	13.81e-10	11
GO: 0009615	Response to virus	1.31e-7	5
GO: 0006954	Inflammatory response	3.43e-6	6
GO: 0002694	Regulation of leukocyte activation	5.61e-6	5
GO: 0050865	Regulation of cell activation	5.99–6	5
GO: 0008228	Opsonization	8.55e-6	2
GO: 0032020	ISG-15 protein conjugation	1.71e-5	2
GO: 0009611	Response to wounding	3.33e-5	6
GO: 0006935	Chemotaxis	4.07e-5	4
GO: 0001932	Regulation of protein amino acid protein phosphorylation	4.67e-5	4
GO: 0042325	Regulation of phosphorylation	6.24e-5	5
GO: 0019220	Regulation of phosphate metabolic process	7e-5	5
GO: 0051174	Regulation of phosphorus metabolic process	7e-5	5
GO: 0051249	Regulation of lymphocyte activation	9.43e-5	4
GO: 0050829	Defense response to gram negative bacterium	000102	2
GO: 0001934	Positive regulation of protein amino acid phosphorylation	000115	3
GO: 0031399	Regulation of protein modification process	000117	4
GO: 0010562	Positive regulation of phosphorus metabolic process	000128	3
GO: 0042327	Positive regulation of phosphorylation	000128	3
GO: 0045937	Positive regulation of phosphate regulation process	000128	3

GO indicates gene ontogeny; iESCs, irradiated embryonic stem cells.

the contrast administration. All MRIs were postprocessed at a workstation (Leonardo, Siemens).

Fluorescent In Situ Hybridization Analysis

Single-label (whole Y chromosome) fluorescent in situ hybridization analysis was performed according to the manufacturer's protocol (Cambio, Cambridge, UK). Serial sections from paraffin-embedded whole heart blocks were cut to 4-μm thickness. Tissue sections on coated slides were dewaxed, rehydrated, treated with sodium thiocyanate solution and pepsin, fixed, and dehydrated. After air drying, 15 to 20 μL of denatured nucleotide probe was added to each slide, covered by coverslip, and sealed. After overnight hybridization at 37°C, the coverslip was removed and serial washings were performed. Nuclei were counterstained with 4',6-diamidino-2-phenylindole Slides were examined by fluorescent microscope (Olympus BX-41) with an appropriate filter system.

Bromodeoxyuridine Assay

One hour after the surgery, mice were administered BrdU intraperitoneal injection daily (75 mg/kg; Sigma) for 5 days. Mice hearts were harvested 1 month after surgery and fixed in 4% formaldehyde. The heart sections were embedded and cut into 5-μm-thick sections. BrdU antibody (Roche) was used to target BrdU-positive cells according to the manufacturer's protocol. Myocytes were identified by sarcomeric actin labeling. The percentage of BrdU-positive myocyte nuclei was determined by confocal fluorescent microscopy.

Isolation of Cardiac Total RNA

Total RNA was isolated from murine heart tissue per the RNeasy Mini kit (Qiagen, Venlo, the Netherlands). Mouse hearts were placed in liquid nitrogen and ground with a mortar and pestle. Tissue powder and liquid nitrogen were decanted into RNase-free liquid nitrogen–cooled 2-mL microcentrifuge tubes with Buffer RLT (Qiagen). The lysate was pipetted directly into a QIAshredder spin column (Qiagen) placed in a 2-mL collection tube, centrifuged for 2 minutes at full speed, and supernatant was removed by pipetting and transferred to a new microcentrifuge tube. One volume of 70% ethanol was added, mixed immediately by pipetting, and 700 μL of sample was transferred to an RNeasy spin column placed in a 2-mL collection tube and centrifuged for 15 seconds at 8000*g*. Buffer RW1 (700 μL; Qiagen) was added to the RNeasy spin column and centrifuged for 15 seconds at 8000*g* to wash the spin column membrane. The spin column membrane was washed twice by adding 500 Buffer RPE (Qiagen) to the RNeasy spin column and centrifuged for 15 seconds at 10 000*g*. The RNeasy spin column was placed in a new 1.5-mL collection tube, and 30 to 50 μL RNase-free water was added directly to the spin column membrane and centrifuged for 1 minute at 8000*g* to elute the RNA.

Illumina Expression Analysis

Biotin-labeled complementary ribonucleic acid (cRNA) was generated from high-quality total RNA using the Illumina TotalPrep RNA Amplification kit (Ambion). Signals were developed with Streptavidin-Cy3 and scanned with an Illumina iScan System.

Microarray Data Analysis

RNA expression analysis was performed using the Illumina MouseRef-8 BeadChip, which provides coverage of ≈25 700 genes and expressed sequence tags. Mice were euthanized and heart tissue RNA was isolated at 12 hours, 24 hours, 3 days, 7 days, and 28 days after AMI and injection of either iESC or media. Raw signal intensities of each probe were obtained using data analysis software (Beadstudio; Illumina) and imported to the Lumi package of Bioconductor for data analysis before transformation and normalization. For each time point, differentially expressed genes were identified using an ANOVA model with empirical Bayesian variance estimation. Initially, genes were identified as being differentially expressed on the basis of a statistically significant (raw $P<0.01$) and 2-fold change (up or down) in expression level in ESC-treated RNA samples compared with controls.

Statistical Analysis

All statistical comparisons of parameters between different animal groups were performed by Student 2-tailed *t* test, and $P<0.05$ was considered statistically significant.

Results

Intramyocardial Injection of Mouse iESCs Improves Cardiac Function and Decreases Infarct Size in Mice

ICR female mice were exposed to occlusion of the left coronary artery for 1 hour. Before reperfusion, animals were subdivided into 2 control groups and 2 treatment groups. Control groups received either no injection or injection of media from ESC culture (conditioned media) or injection of murine fibroblasts (mouse embryonic fibroblasts). The treatment groups received intramyocardial injection of either male ESCs or male iESCs treated with radiation doses between 20 and 100 Gy. Injections were performed at the border zone of the infarct between contracting and noncontracting ischemic myocardium (Figure 1).

One month later, a high-fidelity transducer-tipped pressure–volume catheter was introduced via the carotid artery into the left ventricle to monitor blood pressure, cardiac contractility, and cardiac relaxation. In comparison with control (untreated or media-treated or mouse embryonic fibroblasts), hearts treated with ESCs or iESCs showed a significant improvement in cardiac function (Figure 1). In hearts exposed to iESCs, systemic blood pressure, cardiac contractility, and cardiac relaxation were markedly enhanced ($P<0.01$) compared with untreated or media-treated controls (Figure 1).

There was no noticeable radiation dose–response curve in that cardiac function improved similarly with nonirradiated ESCs (n=10) and iESCs (n=23) irradiated with 20 to 25 Gy (n=6), 30 Gy (n=8), 50 to 75 Gy (n=5), and 100 Gy (n=4) (Figure 1). All mice were treated with intramyocardial injection of 10^6 to 5×10^6 ESCs or iESCs. There was no apparent dose–response curve detected based on the range of cells injected. The percent infarct to total heart area was significantly less ($P<0.05$) in mice receiving iESCs (Figure 1, Online Figure I).

Intramyocardial Injection of Irradiated Human ESCs Improves Cardiac Function and Decreases Infarct Size in Rhesus Macaque Monkeys

One million to 5 million human iESCs (100 Gy single fraction dosimetry; 4 monkeys) or conditioned media (4 monkeys) were injected via microsyringe directly into myocardium at the penumbra between ischemic and nonischemic tissues of rhesus macaque monkeys after 50 minutes of occlusion and release of the left anterior descending coronary artery. Before ischemia and 30 days after treatment, a high-fidelity transducer-tipped pressure–volume catheter was introduced under fluoroscopic guidance via the femoral artery into the left ventricle to monitor cardiac contractility and relaxation (Figure 2).

There was no baseline difference in cardiac function between the 2 groups of monkeys before AMI (Figure 2). One month after AMI and treatment, myocardial contractility and relaxation of human iESC-treated monkeys were significantly improved compared with control-treated (conditioned media) monkeys ($P=0.02$ and $P=0.01$, respectively).

Cardiac MRI 30 days after acute AMI and treatment demonstrated significant differences (Figure 2) in myocardial infarct area (Online Figure II), percent scar, and scar mass between human iESC-treated hearts and control-treated (conditioned media) hearts ($P<0.01$). Compared with preinfarct cardiac MRI, 1 month after AMI, media-treated hearts had a significant decline in left ventricular ejection fraction by 9.5% compared with an increase of 3.6% in iESC-treated monkeys.

Safety of iESCs

Irradiation inhibited ex vivo ESC proliferation and prevented ESC viability. When exposed to irradiation in culture, neither mouse nor human ESC proliferation was detectable after 72 hours at single-fraction doses >50 Gy (Figure 3A), and viability was markedly reduced and subsequently extinguished by 7 days after irradiation (Figure 3B).

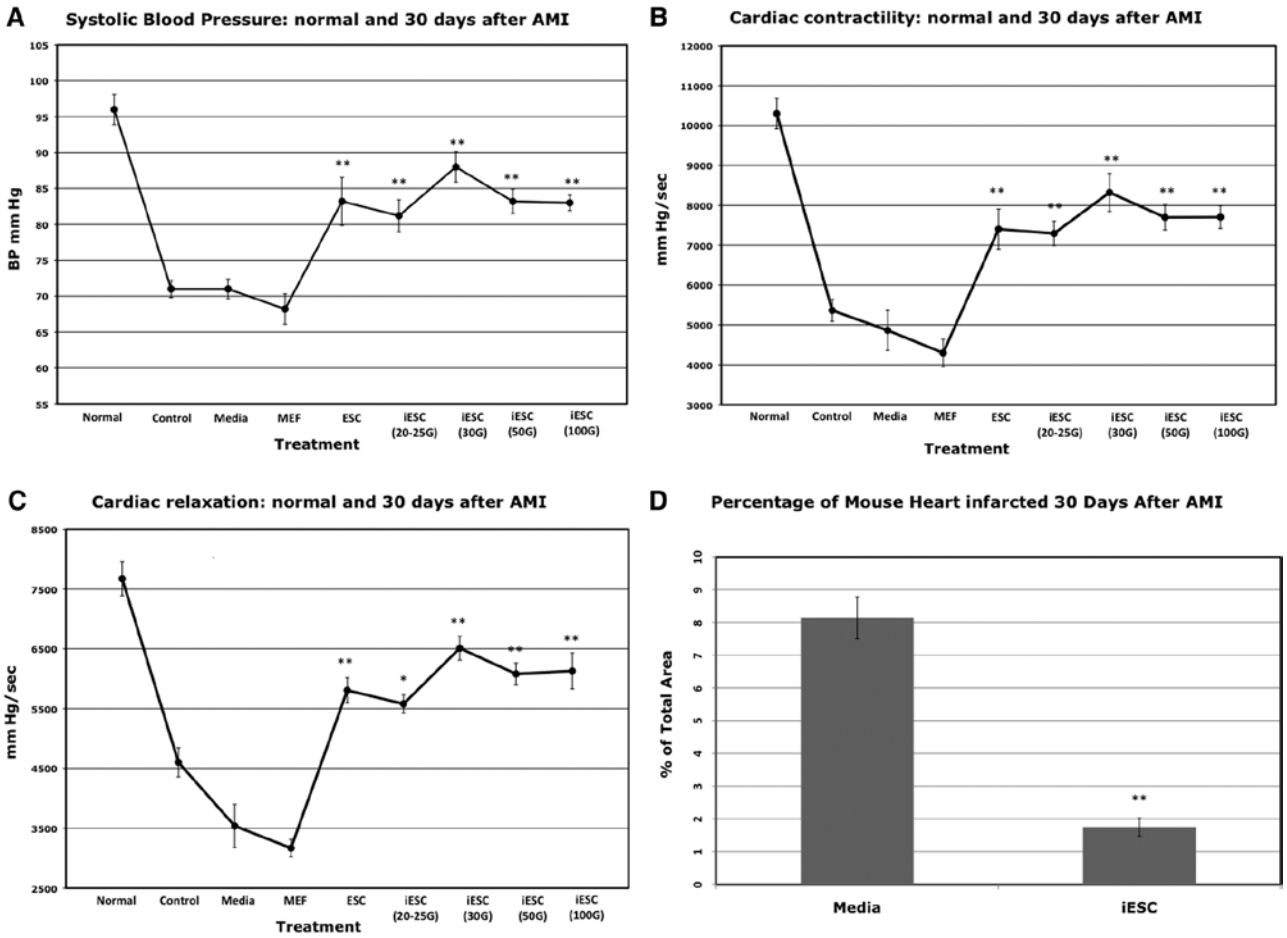

Figure 1. A–D, Blood pressure, cardiac contractility, and relaxation in normal murine hearts and 30 days after acute myocardial infarction (control) or 30 days after acute myocardial infarction and injection of conditioned media (media), mouse embryonic fibroblasts (MEF), embryonic stem cells (ESCs), or irradiated ESCs (iESCs) treated with 20 to 25 Gy, 30 Gy, 50–75 Gy, or 100 Gy irradiation. **P*<0.05, ***P*<0.01 compared with media control. All values are presented as mean±SEM. AMI indicates acute myocardial infarct, BP, blood pressure.

To determine whether injected iESCs survived within the myocardium of recipient mice, we injected male (Y chromosome–positive) mouse iESCs (100 Gy) into the peri-infarct myocardium of female mice. One month later, hearts were studied for the remaining Y chromosome via fluorescent in situ hybridization by a blinded investigator (P.A.). The Y chromosome was detectable in <0.000025% (1 in 40000) of cardiac cells (Figure 4), a level of stem cell DNA persistence previously attributable in the literature to the clinically insignificant and rare occurrence of cell fusion between donor stem cells and cardiomyocytes.[11]

On autopsy, despite immunocompetent mice receiving no immune suppression and receiving major histocompatibility disparate nonirradiated ESCs, 3 of the 10 mice that received nonirradiated ESCs had development of intracardiac teratomas. No immunocompetent mouse treated with iESCs (n=23) demonstrated teratomas in any organ system. In addition, 24 immunodeficient NOG mice that were unable to reject human tissue were injected with 10^6 to 10^7 iESCs (100 Gy) directly into the myocardium under ultrasonic guidance and followed-up for 18 months with no subsequent pathology or teratoma formation. In comparison, 14 of 23 NOG mice that underwent nonirradiated ESC injection into the myocardium under ultrasound guidance had development of teratomas either within the heart or at distal sites. Because of the ethics of primate research,[21] no monkey was euthanized for analysis of cardiac tissue; instead, cardiac MRI was repeated 10 to 12 months after treatment. No monkey demonstrated abnormal cardiac growths or pathology.

Irradiated ESCs Promote Ex Vivo Somatic Cell Proliferation and Survival

To determine whether irradiated ESCs may function as an ex vivo feeder layer to stimulate proliferation of somatic cells, we cocultured mouse iESCs with primary cultures of either murine splenocytes or cardiomyocytes. When freshly isolated mouse splenocytes were cocultured with iESCs, splenocyte numbers proliferated exponentially compared with non cocultured cells, even when iESCs were pretreated with up to 150 Gy (Online Figure III). To test the effect of iESCs on cultured cardiomyocytes, primary mouse cardiomyocytes isolated from adult hearts were cocultured in transwells with iESCs without direct contact for 48 hours. iESC-cocultured cardiomyocyte survival was significantly higher compared with non cocultured cells (Online Figure IV).

In Vivo Cardiomyocyte DNA Synthesis Is Increased in iESC-Treated Hearts

To determine whether iESCs promoted cardiomyocyte DNA synthesis in vivo, mice were injected beginning on the day of

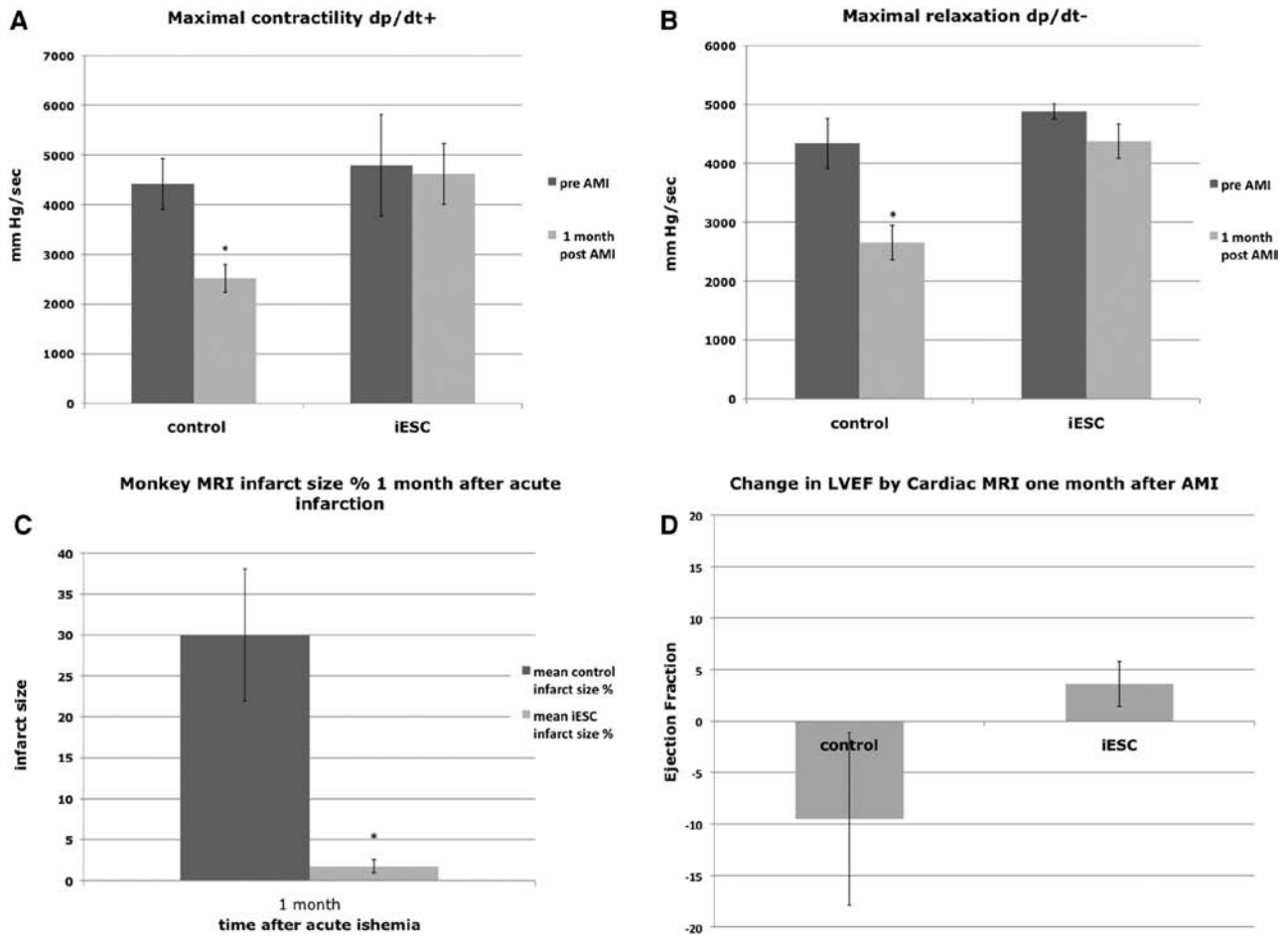

Figure 2. A–D, Rhesus macaque monkey cardiac contractility (dp/dt+) and relaxation (dp/dt–) and infarct size by magnetic resonance imaging (MRI) in normal hearts and 30 days after acute myocardial infarction (AMI) and injection of either conditioned media (control) or irradiated (100 Gy, single fraction) human embryonic stem cells (iESCs). *P<0.05 compared with baseline (preinfarct) value. All values are presented as mean±SEM. MEF indicates mouse embryonic fibroblasts; LVEF, left ventricular ejection fraction.

myocardial infarction once per day for 5 consecutive days with BrdU. BrdU is a synthetic nucleoside analog of thymidine that incorporates into DNA (but not RNA) during DNA synthesis. One month later, mice were euthanized and myocardium was analyzed for BrdU by a blinded investigator (P.A.). Streaks of BrdU-positive cardiomyocytes were identified within the

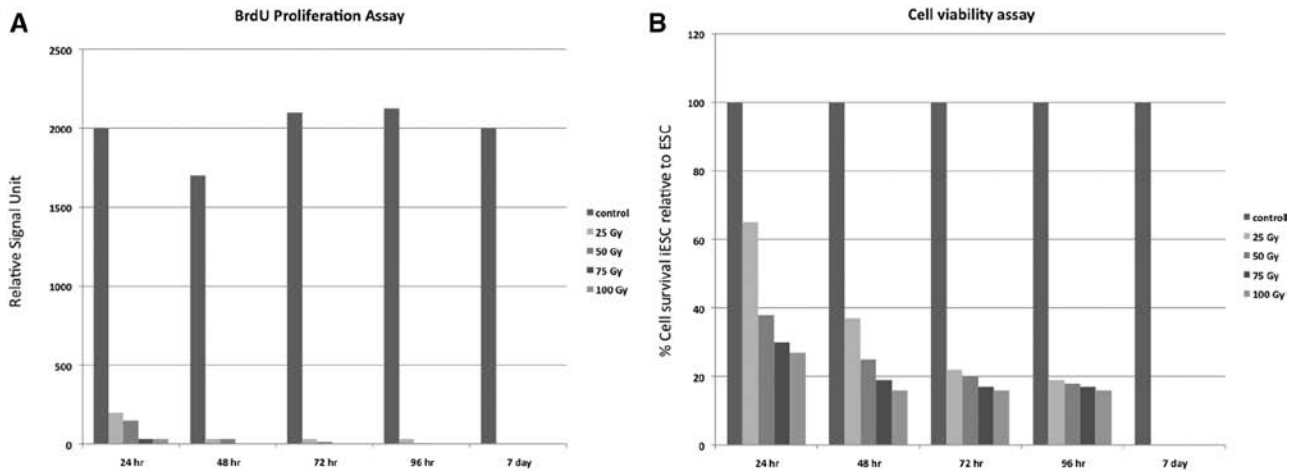

Figure 3. A, Proliferative ability of embryonic stem cells (ESCs) and irradiated embryonic stem cells (iESCs). Proliferation ability of mouse ESCs treated with different dosage of irradiation (0, 20, 25, 30, 35 Gy) and followed-up for different time intervals (24, 48, 72, 96 hours, 7 days). **B**, ESC and iESC survival after irradiation. The ESCs were then irradiated at different doses (0, 25, 50, 75, 100 Gy) and then cultured for 24, 48, 72, and 96 hours and 7 days, respectively, after irradiation. BrdU, 5-Bromo-2′-deoxyuridine.

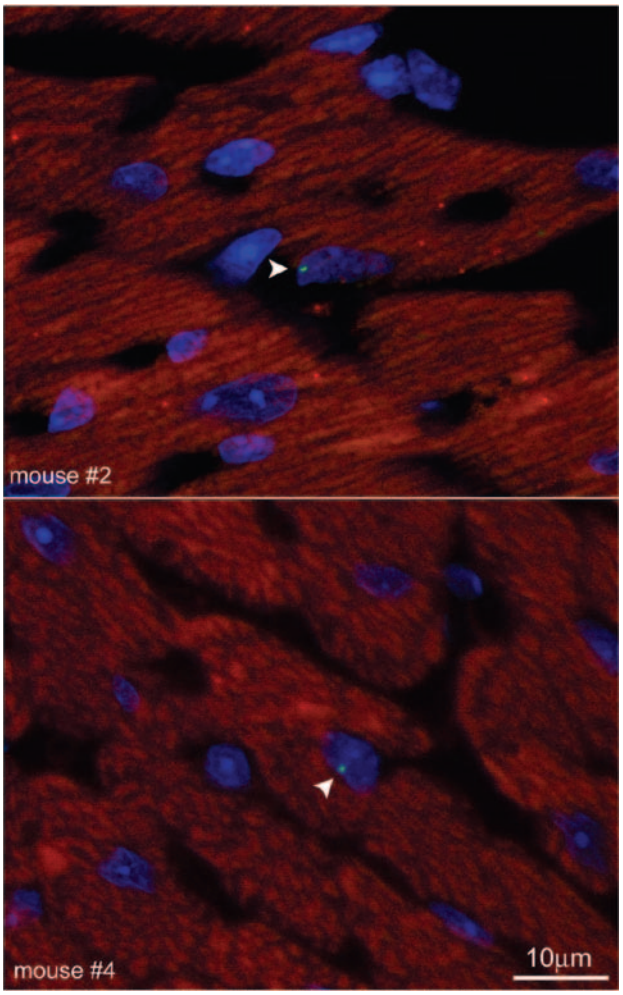

Figure 4. Fluorescent in situ hybridization (FISH) of Y chromosome in female hearts treated with male irradiated embryonic stem cells (iESCs). FISH reveals 1 in 40 000 myocyte nuclei positive for ESC Y-chromosome DNA 1 month after ischemia-induced myocardial infarct in female mice and intramyocardial injection of male iESCs.

injured left ventricle; an average of 12% cardiomyocytes were labeled by the halogenated nucleotide in the region bordering the infarct (Figure 5). The corresponding value in the remote myocardium was 1.7%. In control untreated infarcted mice, BrdU-labeled myocytes were 2% and 0.5% in the area adjacent to and distant from the scarred myocardium, respectively. Peri-infarct recipient cardiomyocyte DNA synthesis for iESC-treated mice (12%) was significantly greater than the rare occurrence of Y-chromosome iESC DNA (0.000025%).

Gene Expression Profile of iESC-Treated Hearts: Upregulation of Proliferation, Chromosome Remodeling, and Metabolic Pathways and Downregulation of Inflammatory and Profibrotic Pathways

Gene expression microarray profiling was performed to evaluate differences in gene expression between iESC-treated hearts and conditioned media–treated hearts at 12 hours and 1, 3, 7, and 28 days after AMI (Figure 6). Between iESC- and media-treated hearts, 539 genes were expressed differentially (>2-fold increase or decrease) at 12 hours, 39 genes were expressed at 24 hours, and 7 genes were expressed at 3 days after AMI. There was no difference in gene expression profile between conditioned media–treated and iESC-treated mice at 7 and 28 days after treatment. By 28 days after treatment, the gene expression profile of iESC-treated mice was no different from normal hearts.

Differences between highly expressed genes (Figure 6, red) and lower expression levels (Figure 6, green) abated over time and were not different in expression profile from normal (noninfarcted) hearts by 28 days after myocardial infarction (Figure 6). The 20 most upregulated and downregulated cell signal pathways in iESC-treated hearts after AMI are listed in the Table. After acute myocardial ischemia and iESC injection, microarray cardiac expression gene profiling demonstrated upregulation of genes involved in cell cycle, chromosome remodeling, and metabolism pathways, whereas inflammatory pathways were downregulated. Time difference in the gene expression of 12 cell cycle–dependent genes and 7 inflammation-related genes confirmed by reverse-transcriptase polymerase chain reaction between iESC-treated and media-treated hearts at 12 hours and at 1, 3, 7, and 28 days after AMI validated microarray results (Online Figure V).

iESC Effect on Postinfarct Inflammation

T-cell infiltration within peri-infarct tissue tended to be lower in iESC-treated hearts compared with controls at 3 days (55 mm^2 vs 41 mm^2; P=0.16) and 6 days (P=0.44) after AMI, but the difference was not statistically significant (Online Figure VI). There also was no significant difference in endothelial cell (CD31) content at 3 days (1820 mm^2 vs 1744 mm^2; P=0.34) or 6 days (P=0.41; Online Figure VII).

Electron Micrographs of Cultured iESCs

Membrane bridges, tunneling nanotubules, and microvesicles function as communication networks for exchange of cellular information. Therefore, we performed electron micrographs of cultured murine and human iESCs exposed to irradiation doses between 25 and 100 Gy. Membrane bridges (Figure 7A) and microvesicles usually between 100 and 500 nm (Figure 7B) were commonly located between cellular interfaces.

Discussion

Many types of stem cells have been used to treat ischemic heart disease, including mesenchymal stem cells derived from various tissues, bone marrow stem cells, peripheral blood stem cells, and $CD34^+$ or $CD133^+$ purified HSCs. These adult stem cells have been used in clinical trials, with modest benefit in some studies[2] and no benefit in other trials.[22] Despite numerous preclinical animal models and clinical trials, the mechanism of how these diverse types of cells benefit cardiac function is debatable.

The clinical application of viable ESCs for heart disease is limited by the formation of in vivo teratomas. Irradiation, depending on dose, would inhibit cell division and would prevent both long-term viability and formation of teratomas. It is common laboratory practice to culture cells over irradiated cells that are themselves unable to proliferate but are capable of maintaining survival and expansion of other nonirradiated cells. In fact, the irradiated cells are termed feeder layers in acknowledgment of their ability to maintain viability of nonirradiated cells. We reasoned that for a multicellular organism

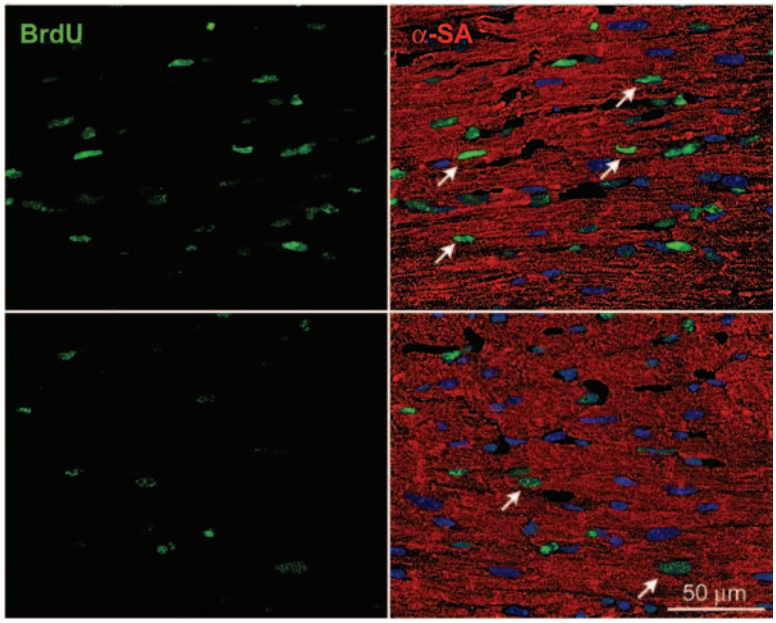

Figure 5. 5-Bromo-2′-deoxyuridine (BrdU) incorporation into nuclei within peri-infarct zone. Immunofluorescent staining for 5-bromo-2-deoxyuridine (green) 1 month after ischemia-induced myocardial infarct in female mice, with (**top row**) or without (**bottom row**) intramyocardial injection of male irradiated embryonic stem cells (iESCs). Sarcomeric actin labeling demonstrated on the right. Twelve percent of peri-infarct myocytes were positive for BrdU in mice receiving iESCs vs 2% in mice not treated with iESCs.

to develop, and as confirmed by ex vivo feeder layer technology, cells communicate with other cells, and that because of the lack of a basement membrane and requirement of coordination with other cells for successful embryogenesis, ESCs would be most likely to interact with neighboring cells.

It previously has been suggested (the dead cell hypothesis) that transplanted apoptotic adult stem cells may improve postmyocardial cardiac function by modulating (suppressing) the local inflammatory response.[23] To our knowledge, no investigator until now has injected either apoptotic or mitotically inactivated but viable cells into ischemic myocardium. In our RNA microarray profiling, hearts treated with iESCs demonstrated a broad decrease in inflammatory and profibrotic genes. However, staining and quantification of T-cell infiltration revealed no difference between iESC-injected hearts and controls. In addition, injection of fibroblasts pretreated with 40 Gy of irradiation did not improve cardiac function. In contrast, both nonirradiated ESCs and iESCs treated with

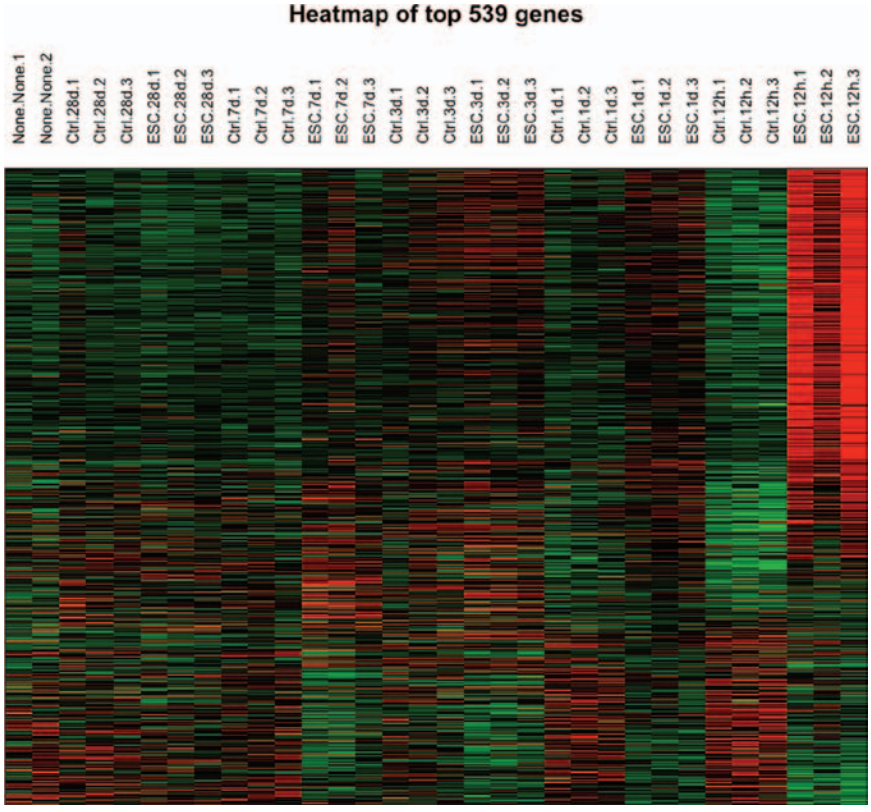

Figure 6. Heat map of genes differentially expressed between irradiated embryonic stem cell (iESC)-treated and control (Ctrl) media-treated hearts at different time points after acute myocardial infarction.

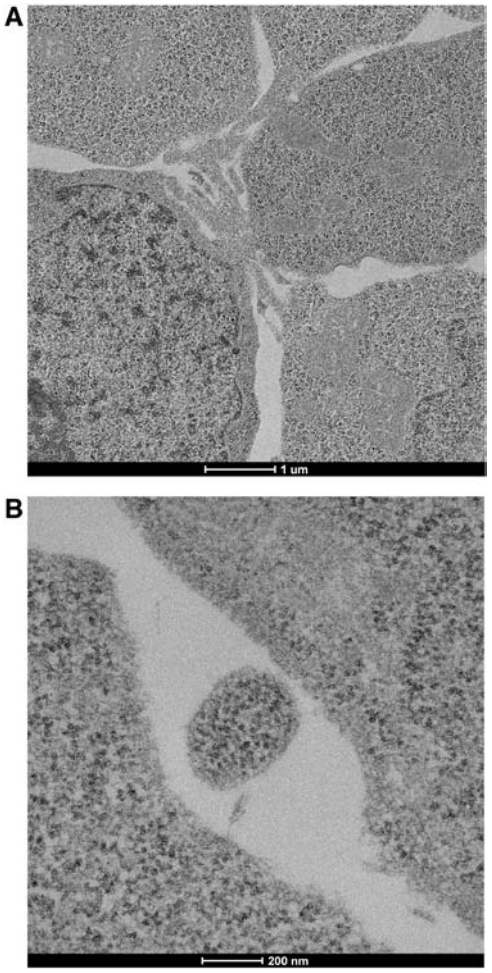

Figure 7. A, Electron micrograph of membrane bridges between irradiated embryonic stem cells (iESCs) in culture. B, Electron micrograph of microvesicles between iESCs in culture.

doses of irradiation from 20 to 100 Gy improved cardiac function equally. Perhaps more important than immune suppression and as demonstrated by microarray gene profiling is the increased expression in iESC-treated hearts of numerous survival genes involved in DNA proliferation, chromatin remodeling, and metabolism.

Because the irradiated cells are not viable and will be apoptotic within days, no immune suppression is necessary. If long-term survival is not necessary for effectiveness, immune suppression is not necessary and not desired for prolongation of in vivo survival. When irradiated cells are used as feeder layers in the laboratory, they are routinely used across species. For example, a common cell line used to maintain survival of human cells in culture is an irradiated mouse NIH3T3 cell line. Therefore, it should not be surprising that in vivo–irradiated ESCs are major histocompatibility independent and are effective across species.

Both ESCs and ESCs differentiated into cardiomyocytes have been used to treat ischemic heart disease in animal models.[16–20] Peri-infarct myocardium injected with nonirradiated ESC has been reported to result in improved myocardial function,[16–18] but, as confirmed in our study, injection of nonirradiated ESCs is complicated by the formation of teratomas and cannot be applied safely in clinical trials. ESCs can also be differentiated into cardiomyocytes.[20] ESC-derived cardiomyocytes are a form of cell replacement therapy,[20] whereas iESC is a therapy designed to facilitate endogenous repair. Thus, the future clinical use of iESC-derived versus ESC-derived cardiomyocytes will likely be different.

After AMI, animals treated with intramyocardial injection of iESCs had significant improvement in systemic blood pressure, left ventricle contractility, left ventricular relaxation, and, on Masson trichrome staining, decreased infarct size. Intramyocardial injection of conditioned media did not improve left ventricular parameters, and postinfarct parameters were similar to the natural history of untreated hearts after AMI. To determine whether iESCs had persisted within treated hearts 1 month after intracardiac injection of iESCs, mice were euthanized and fluorescent in situ hybridization analysis of the Y chromosome in female recipient hearts was performed under blinded conditions. Only 1 in 40 000 counted myocardial nuclei demonstrated the ESC-derived Y chromosome, indicating that intramyocardial persistence of ESC DNA is a rare event and occurs at a similar low and clinically insignificant frequency as cell fusion between stem cells and cardiomyocytes demonstrated in other studies.[10–12] BrdU, a thymidine analog that is incorporated into cells replicating DNA, was positive within sections of left ventricular myocardium at a frequency of 12%. In comparison, control hearts undergoing ischemic ligation without iESC injection demonstrated a level of 2% BrdU-positive nuclei. The 6-fold increase in myocyte nucleotide synthesis in the border zone after the delivery of iESC could be consistent with either activation of resident cardiac progenitor cells[24–28] with generation of new cardiomyocyte progeny or synthesis of new DNA within resident mature cardiomyocytes.

New DNA synthesis within at-risk cardiomyocytes may occur by means of the following: (1) DNA repair; (2) karyokinesis (nuclear division); (3) cytokinesis (cell division); or (4) increased DNA ploidy (DNA content per cell). Volume-stressed hearts have been reported to compensate by nuclear remodeling and increased DNA ploidy.[29] The nonuniform nuclear fluorescent intensity in BrdU-labeled myocytes (Figure 5) may be construed to suggest variation in DNA synthesis consistent with ploidy and DNA repair, because myocyte regeneration from progenitor cells might be anticipated to demonstrate uniform fluorescence within daughter nuclei. However, BrdU labeling is not considered sensitive enough to detect DNA repair,[30] whereas repair and replacement of BrdU with its normal analog (thymidine) also may result in nonuniform fluorescence.

It recently has been reported that after acute injury in the adult heart, de novo cardiomyocytes differentiate from cardiac progenitor cells.[31] Accentuation of BrdU labeling by injection of iESCs is consistent with enhanced de novo cardiomyocyte differentiation from resident cardiac progenitor cells. Whether new DNA synthesis is the result of ploidy, DNA repair, karyokinesis, cytokinesis, or a combination of these events in a mature cardiomyocyte or cardiac progenitor cell, the disproportionate increase in BrdU-positive nuclei compared with Y-chromosome-positive nuclei indicates that injection of iESC into ischemic myocardium supports recipient cardiomyocyte DNA synthesis and survival.

Consistent with in vivo results, iESCs also enhanced ex vivo proliferation and survival of cells, such as lymphocytes (splenocytes) and cardiomyocytes, and may well enhance ex vivo proliferation of numerous cells including fibroblasts. However, we found no in vivo evidence that iESCs preferentially enhanced fibroblasts proliferation over cardiomyocyte regeneration. In fact, microarray analysis demonstrated a decrease in profibrotic gene expression, and histology and cardiac MRI revealed decreased infarct size and decreased fibrosis (decreased gadolinium enhancement) in mice and monkeys, respectively. Conversely, we also found no evidence that injection of fibroblasts (mouse embryonic fibroblasts) improved cardiac function.

Exchange of information between cells may occur by secretion of molecules (paracrine), gap junctions, or bridging between cells via membrane continuity and shipping of cargo via microvesicles or exosomes. It seems implausible that a single paracrine factor secreted by iESCs could so profoundly alter DNA proliferation, metabolism, chromatin remodeling, inflammatory, profibrotic, and survival pathways. Media injected from iESC-cultured cells had no beneficial effect, also indicating absence of a secreted paracrine molecule. Cellular bridging via membrane projections and microvesicles has been established as a method of both proximal and more distal cell-cell communication,[32–40] has been demonstrated to facilitate intercellular exchange of ribonucleic acids, cytoplasmic proteins, and organelles such as mitochondria, and has been implicated in repair between somatic cells.[38,39] Electron micrographs of ESCs show that intercellular microvesicles and membrane bridging are common in vitro. The beneficial effects of iESCs on stressed cardiomyocytes in vivo similarly may be secondary to membrane bridging and transfer of iESC cytoplasm, including proteins, ribonucleic acids, nucleic acids such as ATP, the energy currency of a failing heart,[41] and organelles to recipient cells.

It is probable that there is more than a single mechanism of action because cell-cell exchange by membrane bridging between iESCs and neighboring host cardiomyocytes, paracrine factors, and scaffolding effects may be involved. This may be viewed as an opportunity rather than a handicap because beginning in the last half of the 20th century, the use of feeder layers in the laboratory allowed for significant advances in cellular therapy, despite the fact that the exact factors required to replace the feeder layer remain, in many circumstances, unknown. Similar to ex vivo application of an irradiated feeder layer, this technique, applied in vivo, also may contribute significantly to the future of cellular therapy. Our data raise questions about previous postulated mechanisms for cellular therapy of heart disease, because irradiated cells were not used as a control. Finally, the use of iESCs may be a practical, relatively inexpensive, and safe clinical therapy that has, to this date, been entirely overlooked and that may be used on its own or to supplement other cellular therapies.

To our knowledge, no previous study of any type of stem cell–mediated repair of damaged myocardium included nonviable (lethally irradiated) stem cells for either treatment or as a control. Herein, we demonstrate that iESCs may provide an in vivo feeder or chaperone effect for stressed cardiomyocytes. Surprisingly, when viewed in retrospect, it has not been previously recognized that one can reverse-engineer ex vivo standard tissue culture feeder cell methodology for in vivo purposes (ie, injection into the host of an irradiated feeder cell). This chaperone effect does not require differentiation, permanent integration, or persistence of iESCs, and it is major histocompatibility-independent. Clinical application of iESCs would allow elimination of expensive and time-consuming ex vivo differentiation of ESC-derived cardiomyocytes.[20] However, clinical use of iESCs would require Food and Drug Administration–approved xenogeneic-free ESC culture conditions before irradiation and in vivo injection.

Sources of Funding

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Disclosures

Dr Burt retains stock in Genani. The other authors have no conflicts to report.

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Novelty and Significance

What Is Known?

- Multipotent adult stem cells, such as mesenchymal (connective tissue) stem cells or hematopoietic (blood) stem cells, have been reported to improve cardiac function after intracoronary or intramyocardial injection.
- Pleuripotent embryonic stem cells cannot be safely injected into the heart because they have a high propensity to form a teratoma.
- Embryonic stem cells are considered potentially useful for cardiac cell therapy only after laboratory differentiation into cardiomyocytes.

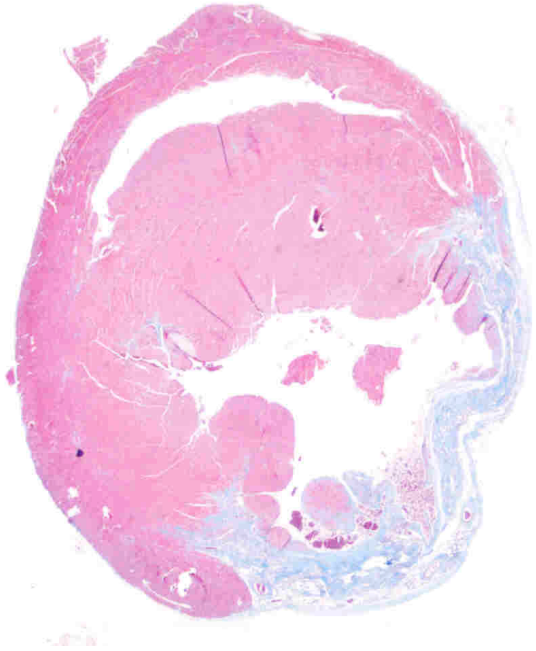
What New Information Does This Article Contribute?

- Irradiated embryonic stem cells cannot divide, exist transiently, and cannot form teratomas.
- Irradiated embryonic stem cells injected into the pneumbra of ischemic myocardium after acute ischemia improve myocardial function and decrease infarct size.
- Irradiated embryonic stem cells induce endogenous myocardial tissue regeneration.

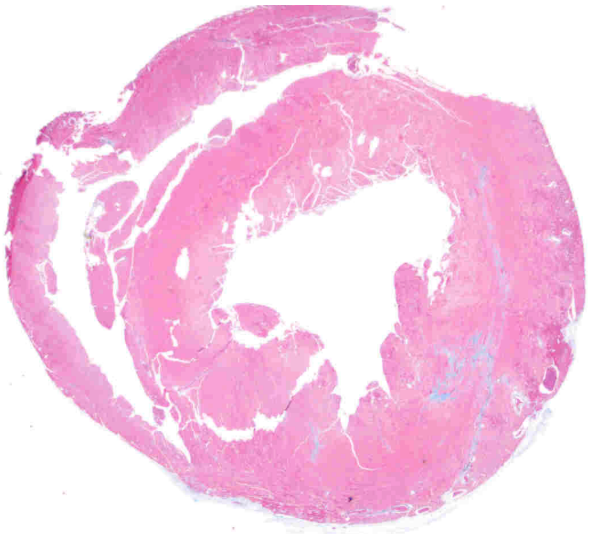
Because current cell therapy is based on the rationale of replacing a damaged cell with a healthy cell, previous studies of cell therapy for heart disease used viable and mitotically active cells. For the first time, we demonstrate that mitotically inactivated embryonic stem cells (ie, cells that cannot proliferate or divide) can repair damage myocardium after acute ischemia. Irradiated embryonic stem cells exist only transiently; they do not integrate or persist in the host tissue and are major histocompatibility and immunologically independent They also do not require immune suppression for efficacy because irradiated embryonic stem cells induce endogenous tissue repair without permanent integration into the recipient's myocardium. This advances the cell therapy paradigm from using cells to replace damaged tissue to using cells to induce endogenous tissue regeneration.

Supplemental Material

Supplement figure I - Example of mouse infarct size by trichrome stained heart sections

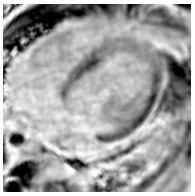


mouse 30 days post AMI

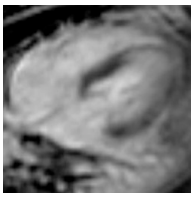


mouse 30 days post AMI treated with iESC

Supplement figure II – Example of Rhesus monkey infarct size by cardiac MRI

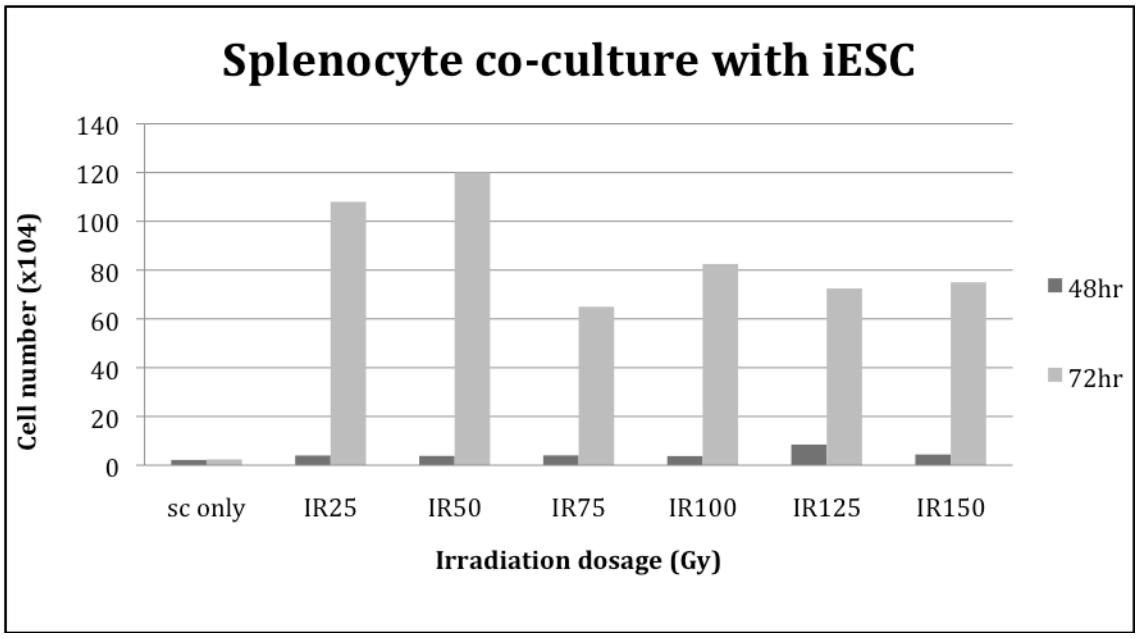


monkey MRI 30 days post AMI



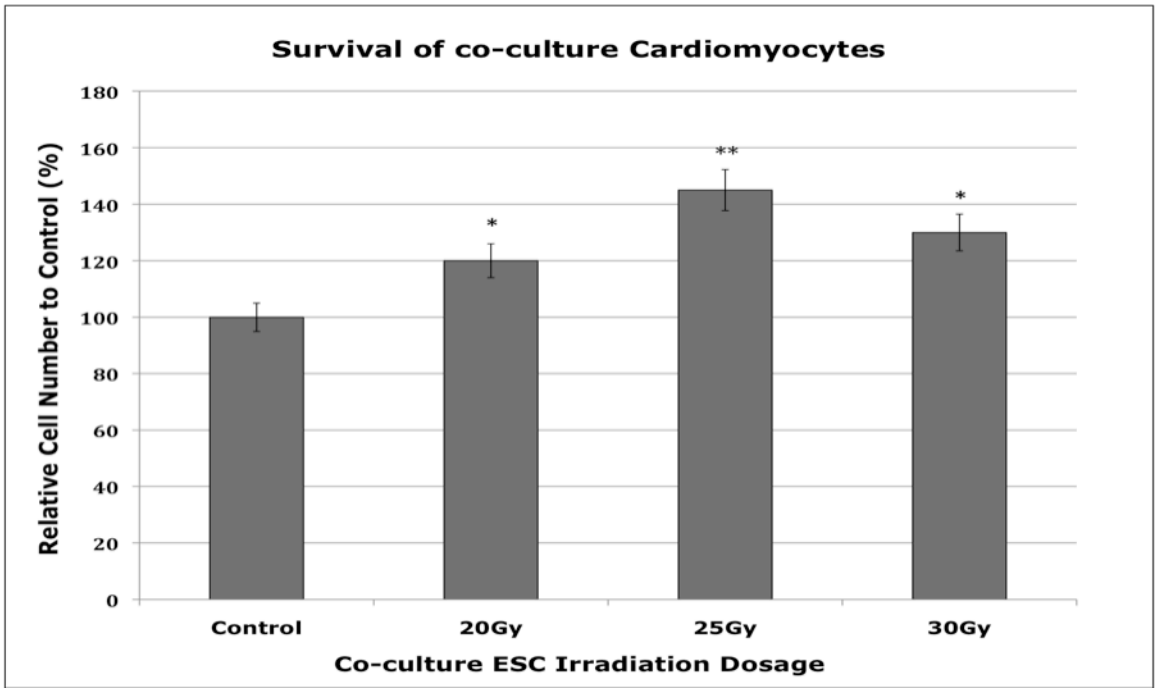
monkey MRI 30 days post AMI treated with iESC

Supplement figure III – iESC stimulated proliferation of co-cultured splenocytes



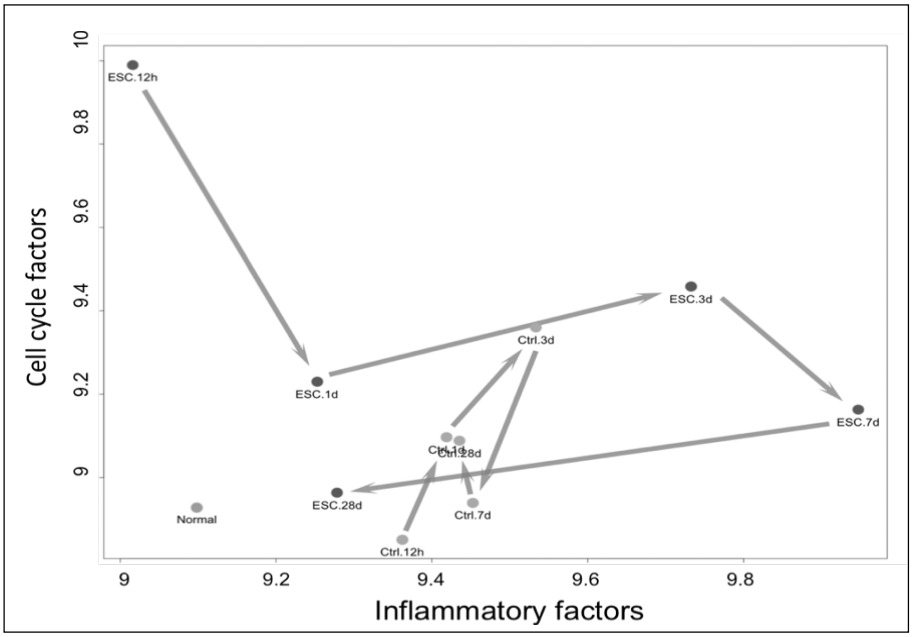
Splenocyte proliferation after co-culture with irradiated (25, 50, 75, 125, and 150 Gy) embryonic stem cells. Gy = gray, IR = irradiation, Sc= splenocytes

Supplement figure IV – iESC prolonged survival of co-cultured cardiomyocytes



Relative cardiomyocyte number compared to control. Primary adult mouse cardiomyocytes were co-cultured with iESC for 48 hours. IESC were treated with different irradiation dosage (20, 25, 30 Gy). * $P < 0.05$, ** $P < 0.01$ versus control All values are presented as mean±S.E.M.

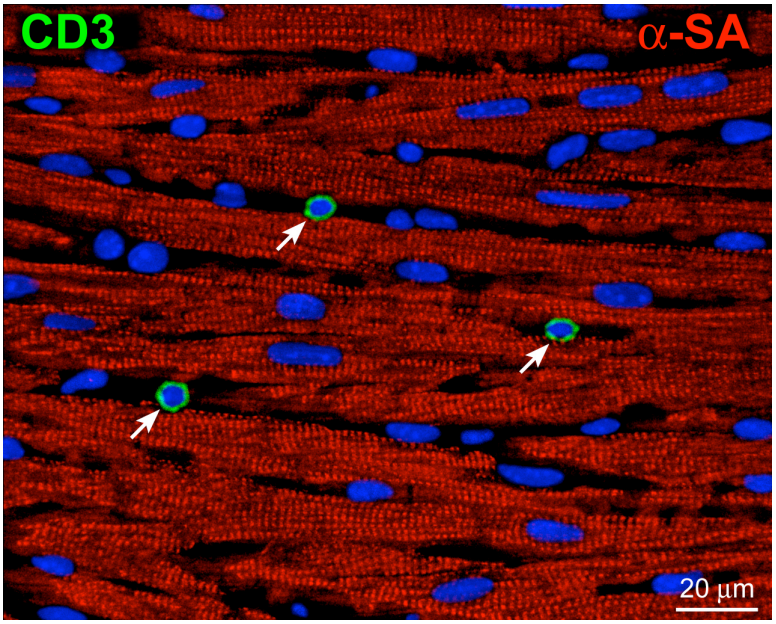
Supplement figure V – Differential expression of 12 cell cycle and 7 inflammatory genes between control and iESC treated mouse hearts after acute myocardial infacrtion



The recovery trajectory iESC-treated vs. control mouse heart after acute myocardial infarction. Each axis is an average of the log2 expression level of 12 cell cycle genes and 7 inflammatory genes, respectively. Details of each gene are as follows:

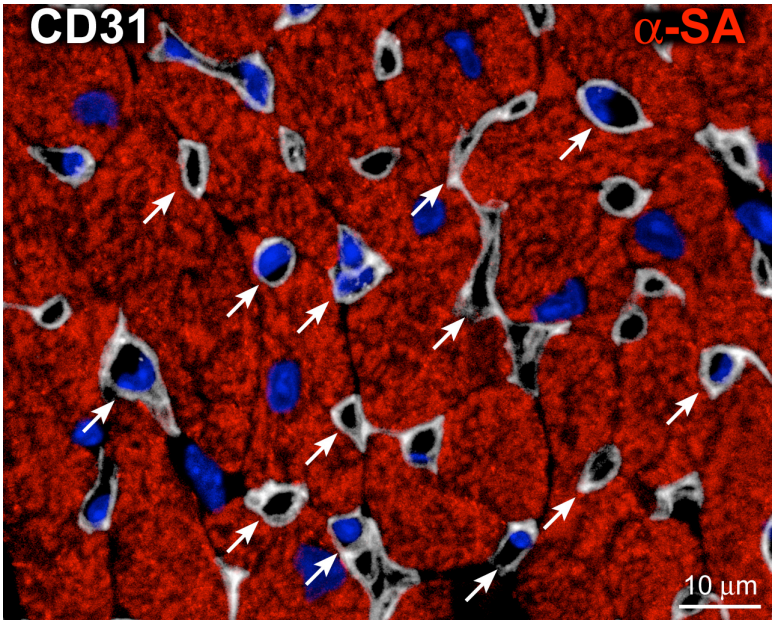
Cell cycle genes
19385" "Ranbp1" Ranbp1 RAN binding protein 1 "56505" "Ruvbl1" Ruvbl1 RuvB-like protein 1 "27401" "Skp2" Skp2 S-phase kinase-associated protein 2 (p45) "66131" "Tipin" Tipin timeless interacting protein "215387" "Ncaph" Ncaph non-SMC condensin I complex, subunit H "67177" "Cdt1" Cdt1 chromatin licensing and DNA replication factor 1 "268697" "Ccnb1" Ccnb1 cyclin B1 "69912" "Nup43" Nup43 nucleoporin 43 "15270" "H2afx" H2afx H2A histone family, member X "20466" "Sin3a" Sin3a transcriptional regulator, SIN3A (yeast) "22092" "Rsph1" Rsph1 radial spoke head 1 homolog (Chlamydomonas) "68298" "Ncapd2" Ncapd2 non-SMC condensin I complex, subunit D2
Inflammation genes
"17087" "Ly96" lymphocyte antigen 96 "14595" "B4galt1" UDP-Gal:betaGlcNAc beta 1,4 galactosyltransferase, polypeptide 1 "93671" "Cd163" CD163 antigen "21937" "Tnfrsf1a" Tnfrsf1a tumor necrosis factor receptor superfamily, member 1a "16889" "Lipa" Lipa lysosomal acid lipase A "22287" "Scgb1a1" Scgb1a1 secretoglobin, family 1A, member 1 (uteroglobin) "11486" "Ada" Ada adenosine deaminase

Supplement figure VI – Example of CD3 staining of mouse heart



mouse heart 6 days after iESC treatment

Supplement figure VII – Example of CD31 staining of mouse heart



mouse heart 6 days after iESC treatment