Myogenic Endothelial Cells Purified From Human Skeletal Muscle Improve Cardiac Function After Transplantation Into Infarcted Myocardium

Masaho Okada, MD,*‡ Thomas R. Payne, Ph.D,*‡ Bo Zheng, MD,*‡ Hideki Oshima, MD, Ph.D,*‡ Nobuo Momo, MD,† Kimimasa Tobita, MD,† Bradley B. Keller, MD,† Julie A. Phillippi, Ph.D,*¶ Bruno PÉault, Ph.D,*‡ Johnny Huard, Ph.D*‡§

Pittsburgh, Pennsylvania

Objectives
The aim of this study was to evaluate the therapeutic potential of human skeletal muscle-derived myoendothelial cells for myocardial infarct repair.

Background
We have recently identified and purified a novel population of myoendothelial cells from human skeletal muscle. These cells coexpress myogenic and endothelial cell markers and produce robust muscle regeneration when injected into cardiotoxin-injured skeletal muscle.

Methods
Myoendothelial cells were isolated from biopsies of human skeletal muscle using a fluorescence-activated cell sorter along with populations of regular myoblasts and endothelial cells. Acute myocardial infarction was induced in male immune-deficient mice, and cells were directly injected into the ischemic area. Cardiac function was assessed by echocardiography, and donor cell engraftment, angiogenesis, scar tissue, endogenous cardiomyocyte proliferation, and apoptosis were all evaluated by immunohistochemistry.

Results
A greater improvement in left ventricular function was observed after intramyocardial injection of myoendothelial cells when compared with that seen in hearts injected with myoblast or endothelial cells. Transplanted myoendothelial cells generated robust engraftments within the infarcted myocardium, and also stimulated angiogenesis, attenuation of scar tissue, and proliferation and survival of endogenous cardiomyocytes more effectively than transplanted myoblasts or endothelial cells.

Conclusions
Our findings suggest that myoendothelial cells represent a novel cell population from human skeletal muscle that may hold promise for cardiac repair. (J Am Coll Cardiol 2008;52:1869–80) © 2008 by the American College of Cardiology Foundation

The number of patients suffering from severe heart failure after myocardial infarction (MI) is increasing in Western countries. As an alternative to heart transplantation, cellular cardiomyoplasty is a promising new approach for preventing advanced heart failure and restoring cardiac function in injured hearts. Researchers have investigated a variety of cell sources for cardiac repair (1–6). Initial studies in cell therapy for cardiac repair used committed skeletal myoblasts (SkMs), largely because they can be readily harvested and easily expanded in vitro before autologous transplantation. However, high cell death rates were reported shortly after SkMs injection in both cardiac and skeletal muscles (7–11), which has hindered the therapeutic usefulness of SkMs. Many subsequent attempts have been made to identify and isolate alternative stem cells or progenitors from skeletal muscle that could have a greater capacity for post-transplantation cell survival and muscle regeneration (12).

We have previously isolated a population of skeletal muscle-derived stem cells (MDSCs) that adhere more slowly to culture flasks than committed SkMs after enzy-
matic dissociation of murine skeletal muscle (13). When injected into the myocardium of infarcted hearts, MDSCs displayed excellent survival and engraftment, induced angiogenesis, and improved left ventricular (LV) function in a more effective manner when compared with that seen with the transplantation of SkMs (14). This distinct difference between mouse SkMs and MDSCs for MI repair indicated that skeletal muscle-derived progenitor cells are heterogeneous in therapeutic efficacy.

Although murine MDSCs display many characteristics associated with the myogenic lineage, these cells also have attributes typically associated with the endothelial lineage. Upon isolation, MDSCs expressed CD34, a marker characteristic of endothelial progenitor cells. In addition, these cells differentiated into endothelial cells and promoted angiogenesis either upon stimulation with vascular endothelial growth factor (VEGF) in culture or after transplantation into skeletal and cardiac muscles (13–15). Studies published by other investigators have documented the presence of myogenic progenitor cells that coexpress markers associated with the myogenic and endothelial lineages in the interstitial spaces of adult mouse skeletal muscle and in the dorsal aorta of mouse embryos (16,17). Together, these findings led us to the recent identification and isolation of progenitor cells residing within adult human skeletal muscle that also coexpress myogenic and endothelial cell markers (18). Transplantation of these myoendothelial cells into the injured skeletal muscles of immunodeficient mice resulted in considerable improvement of their functional ability compared with that seen with the injection of conventional myogenic and endothelial cells (18).

Here, we investigated the therapeutic potential of myoendothelial cells for cardiac repair using an acute MI model created in immunodeficient mice. We show that myoendothelial cells provided greater therapeutic benefit after MI than did endothelial cells, myogenic cells, and unsorted myoblasts. This therapeutic advantage may be collectively attributed to the high regenerative, angiogenic, antifibrotic, and cardio-protective and –stimulatory effects of myoendothelial cells.

Methods

Human skeletal muscle cell isolation. The procurement of human skeletal muscle biopsies (male subject, 21 and 23 years old, female subject, 26 years old) from The National Disease Research Interchange was approved by the Institutional Review Board at the University of Pittsburgh Medical Center. The human skeletal muscle biopsies were placed in solution with Hank’s balanced salt solution and transported to the laboratory on ice.

The human skeletal muscle biopsy was finely minced, and then digested for 60 minutes at 37°C with type I and type IV collagenase (100 μg/ml) and dispase (1.2 μg/ml; all from GIBCO, Invitrogen Corp., Carlsbad, California). The digested tissue was pelleted and resuspended in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO) and 1% (v/v) penicillin/streptomycin. The pellet was then enzymatically dissociated, and passed through a 40-μm filter to obtain a single-cell suspension. Approximately 7 × 10⁵ cells were recovered per gram of tissue.

We used fluorescence-activated cell sorting (FACS) to isolate various cell fractions based on their expression of the cell surface markers CD56, CD34, and CD144, as previously described (18). We also used an unpurified (unsorted) cell population as a control experiment. Briefly, the cell pellet was suspended in DMEM that was supplemented with 2% FBS. The cell solution was then incubated on ice with allophycocyanin (APC)-Cy7-conjugated mouse anti-human CD45, APC-conjugated mouse anti-human CD34, phycoerythrin (PE)-Cy7-conjugated mouse anti-human CD56 (all from BD Biosciences, San Jose, California), and PE-conjugated mouse anti-human CD144 (Beckman Coulter, Fullerton, California). Isotype control antibodies were APC-Cy7-, APC-, PE-Cy7-, and PE-conjugated mouse IgG1 (all from BD Biosciences); 7-aminactinomycin D (7-AAD, ViaProbe; BD Pharmingen, San Jose, California) was added to each tube for dead cell exclusion. Background staining was evaluated with isotype-matched control antibodies and a CompBeads set (Becton-Dickinson, San Jose, California) was used to optimize fluorescence compensation settings for multicolor analyses and sorts. A minimum of 1 × 10⁵ live cell events were analyzed using a FACScalibur flow-cytometer (Becton-Dickinson) and the CellQuest software (Becton-Dickinson). Cell sorting was performed on a FACSaria dual-laser fluorescence cell sorter (Becton-Dickinson). Sorted cells were reanalyzed in all experiments. For this study, we collected the following populations: CD56⁺ CD34⁻ CD144⁻, CD56⁺ CD34⁺ CD144⁻, and CD56⁺ CD34⁺ CD144⁺. The 3 sorted cell populations were plated in collagen-coated 96-well plates at a density of 500 cells per well in proliferation medium consisting of DMEM, 10% (v/v); FBS, 10% (v/v); horse serum, 1% (v/v); penicillin/streptomycin, 1% (v/v); and chick embryo extract (GIBCO-BRL, Carlsbad, California) and incubated at 37°C in a 5%
CO₂ atmosphere. At 70% confluence, cells were passage dissociated with trypsin/EDTA (GIBCO-BRL), replated at a cell density of 1.0 to 2.5 × 10³ cells/cm² and cultured for 3 to 4 weeks before intracardiac transplantation. To track donor cell fate after injection, we transduced myoendothelial cells with a retrovirus encoding for a nuclear LacZ (nLacZ) reporter gene as previously described (15,18).

**Intramyocardial cell transplantation into an acute MI.** The Institutional Animal Care and Use Committee, Children’s Hospital of Pittsburgh, approved the animal and surgical procedures performed in this study (Protocol 37/04). A total of 74 male nonobese diabetic severe combined immunodeficiency (NOD/SCID) mice (The Jackson Laboratory, Bar Harbor, Maine) were used for this study. Overall, 5 animals died during surgery, resulting in a 6.8% operative mortality. One animal was excluded from this study due to the presence of a thymic lymphoma, which is known to naturally occur on occasion in this mouse strain (19). Infarcted mice were randomly allocated between the types of treatment (saline, CD56⁻CD34⁻CD144⁻, CD56⁻CD34⁺CD144⁺, and CD56⁺CD34⁺CD144⁺ cells) for each donor. The 3 sorted cell populations and unsorted cells were injected (3 × 10⁵ cells in a solution of 30 μl of phosphate-buffered saline [PBS] per heart) into the hearts of mice immediately after inducing MI as previously described (14). In control mice, 30 μl of PBS alone was injected. The investigator creating the infarction injury and injecting the cells was blinded to the contents of the injectant. Histological and functional studies were performed at 5 days and 2 and 6 weeks post-transplantation (n = 14 animals sacrificed at 5 days, 25 animals sacrificed at 2 weeks, and 29 animals sacrificed at 6 weeks). Echocardiography was performed by a blinded investigator to assess the heart function of anesthetized mice, as previously described (14).

**Histological analysis.** The mice were sacrificed at 5 days, 2 weeks, and 6 weeks after cell transplantation for histological evaluation of the heart tissue. Hearts were flash frozen in 2-methylbutane (Sigma-Aldrich, St. Louis, Missouri) that was pre-cooled in liquid nitrogen. For immunohistochemical assays, some excised hearts were fixed in 10% formalin (Vector Laboratories) to stain collagen deposition on frozen paraffin. For immunofluorescent human VEGF staining, cryosections were incubated with Xgal substrate for 2 h and incubated overnight at 4°C with a goat antihuman VEGF (1:100, R&D Systems, Minneapolis, Minnesota), followed by incubation with a donkey antigoat IgG 555 (1:200, Molecular Probes).

To determine whether donor cells expressed cardiac cell markers, tissue samples were incubated with Xgal substrate for 2 h. Then the sections were incubated with mouse antihuman cardiac troponin T (1:50, Abcam, Cambridge, Massachusetts), followed by donkey-antimouse IgG 488 (1:400, Molecular Probes). Alternatively, Xgal-stained slides were incubated with antiacardiomyosin I at room temperature for 2 h followed by the addition of donkey antigoat IgG 555 (1:200, Molecular Probes). To determine capillary density within the infarct zone, we performed immunostaining of CD31 on cryosections and measured capillary density within the infarct region of each heart, as previously described (14).

For Ki-67 immunohistochemical staining, deparaffinized sections were immersed in preheated sodium citrate buffer and were incubated overnight at 4°C with a rat monoclonal antimouse Ki-67 antibody (1:50, DAKO, Carpinteria, California) that does not cross-react with human Ki-67. Positive reactivity was determined with Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, California) and visualized as purple with the VIP (violet-colored precipitate) substrate kit (Vector Laboratories). To determine the number of proliferating myocytes, tissues were counterstained with antiacardiomyosin I (cTnI) (1:20,000, Scripps, San Diego, California), processed with Vectastain Elite ABC Kit (Vector Laboratories), and then visualized as brown through the DAB (3,3’-diaminobenzidine) substrate kit (Vector Laboratories). The number of mitotically active endogenous cardiomyocytes within the peri-infarct region was measured in 8 high-power fields (400× magnification).

For terminal dUPT nick end-labeling (TUNEL) staining, the deparaffinized sections were digested with proteinase K according to the protocols provided by manufacturer (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit; Chemicon, Temecula, California). The TUNEL stain was visualized with a substrate system that stained purple (VIP substrate kit; Vector Laboratories). The samples were also lightly counterstained with anti-c-TnI as described in the preceding text with the Ki-67 stain. The number of apoptotic endogenous cardiomyocytes within the peri-infarct region was measured in 4 high-power fields (400× magnification).

For human-specific VEGF immunohistochemical staining, deparaffinized sections were immersed in preheated sodium citrate buffer and were incubated overnight at 4°C with a goat antihuman VEGF (1:50, R&D Systems) and were incubated overnight at 4°C with a goat antihuman VEGF (1:50, R&D Systems). The slides were then processed with Vectastain Elite ABC Kit (Vector Laboratories). A positive stain was visualized as brown using the DAB substrate kit (Vector Laboratories). These sections were counterstained with eosin.

We used Masson’s trichrome staining kit (IMEB, San Marcos, California) to stain collagen deposition on frozen
cryosections of the heart. The area of infarct scar and the area of the entire cardiac muscle section were measured using a digital image analyzer (Image J, National Institutes of Health, Bethesda, Maryland). The scar area fraction was calculated as the ratio of scar area to the entire cardiac muscle area and was averaged from 7 sections per heart.

**Hypoxia assay.** We cultured all 3 cell populations under hypoxic conditions (2.5% O2) in vitro and measured the amount of VEGF that was secreted into the cell culture supernatant by enzyme-linked immunoadsorbent assay (ELISA) (R&D Systems, Minneapolis, Minnesota), as previously described (21). Total RNA (n = 3) was extracted from cell lysates using an RNeasy kit with DNase I digestion (Qiagen, Valencia, California). After RNA extraction, quantitative real-time polymerase chain reaction (qPCR) was carried out as described previously (22,23) using TaqMan One-Step RT-PCR Master Mix (Applied Biosystems Inc., Foster City, California) and 1 μl total RNA as the template. qPCR assays were carried out in triplicate on an ABI Prism 7900HT sequence detection system in the core facility of the Genomics and Proteomics Core Laboratories of the University of Pittsburgh. Sequences for target gene primers and probes (IDT-Integrated DNA Technologies, Coralville, Iowa) were as follows:

- Human Vegf165 (NM 001033756): forward: 5ʹ CATGCA-GATTATGCGGATCAA 3ʹ; reverse: 5ʹ TTTGTTGTGCTGATTAGAAGCTCAT 3ʹ; and TaqMan probe: 5ʹ CCTCACCAGGGCCAGCACATTAGGAGA 3ʹ
- Human Igf-I (X57025): forward: 5ʹ TGTATTGCGCA-CCCCTCA3ʹ; reverse: 5ʹ TGCCTTCTTCAAATGACTTCTT3ʹ; and TaqMan probe: 5ʹ CAGCTCGCTCTGTCCGTA-CCC 3ʹ
- Human Hgf (X 16323): forward: GAGGCCATGGTGC-TATACTTTG; reverse: TCAGCGCATGT TT TA TTGCA; and TaqMan probe: CCCTCACACCGCTGGGAGTA (5ʹ 6FAM and 3ʹ TAMRA-Sp)
- Human b-Fgf (NM 002006): forward: GCCTGAT- CATGTGGTCTCTAGA; reverse: TTATGGCTCACTGCAACCTTTGA; and TaqMan probe: AAGTGATCAA CCCCCCACC-TTAGGCCCT (5ʹ 6FAM 3ʹ TAMRA-Sp)

All target genes were normalized to the reference housekeeping gene 18S (Applied Biosystems Inc.). Gene expression levels were analyzed using SDS 2.2 Software (Applied Biosystems Inc.) and were calculated as total amount RNA (in arbitrary fluorescence units) compared with negative control levels (normal O2) for each time point based on the comparative ΔCt method (separate tubes) (24).

**Statistical analysis.** All measured data are presented as mean ± standard error. Kaplan-Meier survival curve estimation with log-rank test was performed to compare the survival rate among experimental groups. For the analysis of the echocardiographic data, the scar tissue area data, the endogenous cardiomyocyte proliferation data, and the VEGF qPCR and ELISA data, we applied the 2-way analysis of variance (ANOVA) test and the Tukey multiple comparison test. The 1-way ANOVA and the Tukey post-hoc tests were performed to analyze the number of fskMyHC-expressing myocytes and capillary density data in all groups. For the endogenous cardiomyocyte apoptosis data, we applied the Kruskal-Walls and the Dunn’s multiple comparison tests at each time point. The Student t test was performed for the Vegf and Hgf gene expression data in myoendothelial cells cultured under normoxic and hypoxic culture conditions. Statistical significance was set at a value of p < 0.05. All statistical tests were performed using SigmaStat (Systat Software Inc., Point Richmond, California).

**Results**

**Cell isolation by FACS.** Cells were isolated from 3 samples of adult human skeletal muscle (first subject, male, age 21 years; second subject, male, age 23 years; third subject, female, age 26 years) by FACS on the basis of their differential expression of the myogenic (CD56) and endothelial cell (CD34 and CD144) surface markers (18). CD45 was used to exclude hematopoietic cells (18). The fraction of myoendothelial cells (CD56+ CD34+CD144+CD45−) represented 1.8% of the total cell population, while endothelial cells (CD56− CD34+CD144−CD45−) and myogenic cells (CD56+ CD34−CD144−CD45−) accounted for 9.0% and 2.6%, respectively. The mean numbers of viable sorted cells that were recovered per experiment were 9.6 × 10⁴ CD56+CD34−CD144−CD45− cells, 45.8 × 10⁴ CD56+CD34−CD144−CD45− cells, and 8.8 × 10⁴ CD56+CD34+CD144+CD45− cells. Purities of these 3 sorted cell populations were, respectively, 89.1%, 92.8%, and 81.1%, as confirmed by flow reanalysis. The myogenic, endothelial, and myoendothelial cell isolates were expanded in culture before intramyocardial transplantation, using a protocol previously described (18).

We have previously evaluated the myogenic and endothelial cell phenotype of sorted cells after cultivation using the myogenic marker CD56 and the endothelial cell markers CD146 and Ulex europeaus agglutinin 1 receptor (18). The phenotype of the sorted endothelial cells (CD56+ CD34−CD144−) and myoendothelial cells (CD56+ CD34+ CD144+) changed after expansion in tissue culture flasks. Myogenic cells (CD56+ CD34−CD144−) retained a high level of CD56 expression, but a portion of cells also expressed CD146 after cultivation. Interestingly, 65% of cultured endothelial cells expressed CD56 even though this
population was 92.8% pure (CD56-CD34+CD144+) after FACS isolation. A large portion of cultured myoendothelial cells retained a myogenic and endothelial cell phenotype. Expression of Ulex europaeus agglutinin 1 receptor was low in all cell types.

**Cardiac repair.** The therapeutic effect of myogenic, endothelial, and myoendothelial cells for cardiac repair was assessed in an immunodeficient mouse model of an acute MI. Over the course of this study, we did not observe a significant difference in survival rates among the groups (p = 0.13; Kaplan-Meier survival curve) (Fig. 1A). In uninjured NOD/SCID mice (age 12 weeks, weight 27.2 ± 1.4 g), we have measured baseline LV dimensions and function by echocardiography. Baseline end-diastolic area (EDA) of uninjured mice (n = 5 mice) was 9.3 ± 0.3 mm² (Fig. 1B), and baseline fractional area change (FAC) was 46.4 ± 1.5% (n = 5) (Fig. 1C). The hearts in all 3 cell groups had smaller LV dimensions, as assessed by EDA (Fig. 1B) and end-systolic area (data not shown), when compared with those in the control group, which consisted of PBS injections into the heart. Compared with baseline uninjured hearts and control PBS-injected hearts (12.2 ± 1.5 mm²), hearts that received intracardiac injections of myoendothelial cells (9.6 ± 0.8 mm²) tended to preserve LV dimensions at 2 weeks after cell transplantation. However, by 6 weeks, the LV expanded in hearts injected with myoendothelial cells (12.6 ± 0.8 mm²), and was only slightly less than PBS-injected hearts in size (13.8 ± 1.7 mm²) (Fig. 1B).

LV contractility, as determined by FAC values, improved 20% and 17% at 2 and 6 weeks after MI, respectively, in the myogenic group (2 weeks: FAC 19.6 ± 2.9%, 6 weeks: 18.5 ± 2.4%) compared with that seen in the control PBS group (2 weeks: 16.4 ± 2.6%, 6 weeks: 15.9 ± 3.1%) (Fig. 1C). Transplanted endothelial cells (2 weeks: 23.4 ± 1.4%, 6 weeks: 19.0 ± 1.9%) improved systolic function by 47% and 25% at 2 and 6 weeks, respectively, compared with control injections of PBS (Fig. 1C). In comparison to the other 2 cell groups, LV contractility was the strongest in the myoendothelial group (2 weeks: 30.0 ± 3.0%, 6 weeks: 27.8 ± 3.3%) resulting in FAC values that were 84% and 75% greater than those in the control PBS group at 2 and 6 week time points, respectively (Fig. 1C) (p < 0.05, myoendothelial cells vs. myogenic cells and PBS).

We performed injections using unsorted human myoblasts isolated from the same donor (Figs. 1D and 1E). No difference in LV dimensions was observed between the unsorted myoblasts (EDA: 11.7 ± 0.6 mm²) and sorted CD56+ myogenic cells (11.5 ± 0.5 mm²) (Fig. 1D). Hearts injected with unsorted human myoblasts displayed poor LV contractility (FAC: 18.3 ± 2.1%) at 2 weeks after MI, which was comparable to hearts injected with the sorted CD56+ myogenic cell population (CD56+CD34−CD144−, FAC 19.6 ± 2.9%) (Fig. 1E).

Injection of all 3 sorted cell populations was repeated using cell isolates from another donor (male, 21 years old). Again, hearts injected with myoendothelial cells (FAC: 37.4 ± 1.9%) displayed the strongest LV contraction compared with that in the other groups at 2 weeks after MI (p < 0.05, myoendothelial cells vs. myogenic cells, endothelial cells, and control PBS). In comparison to the injection of PBS only (23.0 ± 1.9%), cardiac contractility improved 62% with the injection of myoendothelial cells, 28% with the injection of endothelial cells (28.7 ± 2.8%), and 7% with the injection of myogenic cells (24.6 ± 1.3%).

**Scar tissue formation.** The extent of scar tissue formation within the infarcted area was evaluated using Masson’s trichrome stain (Fig. 2A). At both time points (2 weeks and 6 weeks), LV infarct scar area was smaller, as determined by scar area fraction values, in all 3 cell groups when compared with the control PBS group, with the most notable improvement occurring at the 6 week time point (Fig. 2B). Hearts injected with myoendothelial cells exhibited less scar tissue than those injected with PBS 2 and 6 weeks after infarction (p < 0.05, myoendothelial cells vs. PBS) (Fig. 2B).

**Engraftment.** Cell engraftment was assessed by immunohistochemical analysis of the skeletal muscle-specific marker fskMyHC (Fig. 3B, green). The majority of engrafted human cell-derived myocytes had aligned with the host myocardial fibers and along the ischemic border zone and scar area (Figs. 3A and 3B). Interestingly, the CD56+ myogenic cells regenerated less fskMyHC+ myofibers when compared with the CD34+CD144+ endothelial cells (Fig. 3C). Compared with both of these groups, the CD56+CD34+CD144+ myoendothelial cells regenerated significantly more skeletal myofibers (p < 0.05, myoendothelial cells vs. myogenic cells, endothelial cells) (Fig. 3C), indicating that these cells have an efficient transplantation capacity, as observed in skeletal muscle (18). Heart sections were also co-stained with antibodies directed against human-specific PCNA and fskMyHC (Fig. 3D). A few nuclei (blue) in the fskMyHC engraftment region (green) expressed PCNA (red). This finding confirms the presence of human cells within the fskMyHC+ engraftment region, and suggests that a fraction of myoendothelial cells are proliferative for up to 2 weeks after implantation.

**Evaluation of cardiac differentiation.** In hearts injected with nLacZ–transduced myoendothelial cells, we evaluated whether any donor cells colocalized with the cardiac-specific isoforms of troponin I and troponin T (cTnT) and the gap junction protein connexin43, a protein essential for intercellular electrical connections between cardiomyocytes (14,15). We observed only a few cells containing nLacZ+ nuclei that colocalized with cTnl (Figs. 3E to 3H) and cTnT (Figs. 3I to 3L). However, the vast majority of nLacZ+ cells did not colocalize with these cardiac markers. In addition, we did not observe expression of connexin43 at the interface of the graft and host myocardium, suggesting that entrapped cells were electrically isolated from host cardiomyocytes. Connexin43 gap junction proteins were not expressed by donor cells within the graft.
Neoangiogenesis. Capillary density within the infarct tissue was assessed by CD31 (platelet endothelial cell adhesion molecule) immunostaining (Fig. 4A, red). Intramyocardial transplantation of all 3 cell populations resulted in an increase in the number of CD31+ capillaries when compared with that seen with the transplantation of PBS only (p < 0.05, myoendothelial cells, myogenic cells, and endothelial cells vs. PBS) (Fig. 4B). Of the 3 cell types, the
myoendothelial cells induced the greatest increase of capillaries in the infarct area (p < 0.05, myoendothelial cells vs. myogenic cells and endothelial cells) (Fig. 4B), which was followed by endothelial cells and myogenic cells, respectively. These findings suggest that each transplanted cell population had an angiogenic effect; however, myoendothelial cells were the most effective at stimulating angiogenesis.

**Endogenous cardiomyocyte proliferation and apoptosis.** We have assessed host cardiomyocyte proliferation and apoptosis at 5 days and 6 weeks after MI. Five days after MI, we observed numerous proliferating endogenous cells located at the infarct border zone. We observed no difference among the groups for the total number of Ki-67-positive cells at the peri-infarct zone (myoendothelial cells 68 ± 10 Ki-67+ cells, n = 3 hearts; endothelial cells 63 ± 16, n = 4; myogenic cells 63 ± 12, n = 3; saline control 60 ± 5, n = 4; p = 0.859). We also measured the number of cells that coexpressed both Ki-67 and cTnI within the peri-infarct region (Figs. 5A to 5C). Five days after cell transplantation, hearts injected with myoendothelial cells had more proliferating Ki-67+/cTnI(+) cardiomyocytes (13 ± 4 cardiomyocytes) within the infarct border zone than hearts injected with either endothelial cells (3 ± 1), myogenic cells (2 ± 1), or saline (3 ± 1) (p < 0.05, myoendothelial cells vs. myogenic cells, endothelial cells, and PBS) (Fig. 5C). Six weeks after cell transplantation, in hearts injected with CD56<sup>+</sup> myogenic cells (1 ± 1) and CD34<sup>+</sup>CD144<sup>+</sup> endothelial cells (2 ± 1), the number of proliferating endogenous cTnI<sup>+</sup> cardiomyocytes was comparable to control hearts injected with PBS (1 ± 1) 6 weeks after MI (Fig. 5C). However, endogenous cardiomyocyte proliferation was the highest in hearts injected with myoendothelial cells (6 ± 1) (p < 0.05, myoendothelial cells vs. myogenic cells, endothelial cells, and PBS) (Fig. 5C).

We performed multilabel staining for DNA end-labeling by TUNEL and cTnI to determine the effect of cell therapy on endogenous cardiomyocyte apoptosis in the peri-infarct regions. At 5 days after MI, the occurrence of apoptotic cardiomyocytes within the peri-infarct regions was less frequent in hearts injected with myoendothelial cells (7 ± 1 TUNEL+ cardiomyocytes) when compared with the transplantation of PBS (27 ± 6), myogenic cells (20 ± 3), and endothelial cells (14 ± 2) (p < 0.05, myoendothelial cells vs. PBS) (Fig. 5D). By 6 weeks after cell transplantation, the number of apoptotic cardiomyocytes decreased considerably. Hearts injected with all 3 cell types had slightly fewer apoptotic cardiomyocytes in the peri-infarct regions when compared with hearts receiving transplantation of PBS only (p < 0.05 myoendothelial cells vs. PBS) (Fig. 5D). Taken together, these results suggest that the transplantation of myoendothelial cells had a beneficial effect on endogenous cardiomyocyte proliferation and apoptosis, with the most marked effect occurring shortly after implantation.

**Analysis of paracrine factors.** For all 3 populations, we used qPCR to measure the expression of secretable factor genes, including vascular endothelial growth factor (Vegf<sub>165</sub>), hepatocyte growth factor (Hgf) (25–27), insulin-like growth factor-I (Igf-I), and basic fibroblast growth factor (b-Fgf). All of these factors are known to act as therapeutic agents in the heart (28,29). Under normal culture conditions, we observed expression of Vegf<sub>165</sub>, Hgf, and Igf-I genes from myoendothelial cells of first donor sample. There was no detectable expression of the b-Fgf gene (Fig. 6A). Interestingly, while the Hgf gene was expressed in CD56<sup>+</sup>CD34<sup>+</sup>CD144<sup>+</sup> cells (myoendothelial) (Fig. 6A), we did not observe measurable expression in CD56<sup>+</sup>CD34<sup>−</sup>CD144<sup>−</sup> (myogenic) and CD56<sup>−</sup>CD34<sup>−</sup>CD144<sup>−</sup> (endothelial) cells (data not shown). Hypoxic conditions stimulated a 4.4-fold increase in Vegf<sub>165</sub> gene expression by myoendothelial cells. In contrast, the expression of Hgf and Igf-I genes decreased upon exposure to hypoxia, and b-Fgf gene expression remained undetectable (Fig. 6A). Of note, Vegf<sub>165</sub> gene expression was considerably higher when compared with other analyzed factors (Fig. 6A), suggesting that VEGF may represent a major factor secreted by myoendothelial cells, as previously observed with murine MDSCs (21). All 3 populations increased Vegf<sub>165</sub> expression when cultured under hypoxic conditions.
gene expression under hypoxic conditions relative to normoxia (p < 0.01, normoxia vs. hypoxia) (Fig. 6B). The Vegf165 mRNA results were further confirmed by ELISA where all tested cell types had at least a 4-fold increase in VEGF protein secretion under hypoxic conditions when compared with that in control normoxic conditions (p < 0.05, normoxia vs. hypoxia) (Fig. 6C). Although only a few factors were analyzed here, our findings suggest that myoendothelial cells secrete factors, especially when subjected to ischemic conditions, which may have therapeutic effect in vivo.

**In vivo VEGF expression.** We evaluated whether human VEGF was expressed in vivo. Using an antibody that is specific for human VEGF, we observed human VEGF expression within cell-injected hearts 5 days after MI (data not shown). Engrafted nLacZ-tranduced donor cells were found to colocalize with human VEGF (data not shown).

**Discussion**

Satellite cells, which are myogenic progenitor cells located beneath the basal lamina of skeletal myofibers, are known to reside in close proximity to capillaries (30). A developmental relationship between satellite cells and endothelial cells, besides their spatially close proximity in skeletal muscle, has been suggested by Tamaki et al. (16), who have documented the presence of myogenic progenitor cells that coexpress markers associated with the myogenic and endothelial lineages within the interstitial spaces of murine skeletal muscle. In addition, the identification of skeletal muscle...
progenitors originating from the embryonic dorsal aorta has led to the notion that a subset of satellite cells may have a vascular origin (17). Our research has resulted in the identification and isolation of progenitor cells residing within adult human skeletal muscle that coexpress myogenic and endothelial cell markers (18). These myoendothelial cells also demonstrated a potent regenerative capacity when transplanted into injured skeletal muscles (18), indicating that they may yield a better therapeutic promise than myoblasts.

The main finding in this study is that transplantation of human myoendothelial cells attenuated LV dysfunction more effectively than either myogenic or endothelial cells in an acute MI. At the 6-week time point, cardiac contractility in the myoendothelial cell-injected hearts was 40% and 50% greater compared with that in hearts injected with either endothelial cells or myogenic cells, respectively. The improved therapeutic benefit elicited by the injection of myoendothelial cells could be at least partially explained by the reduced levels of scar tissue and apoptotic cardiomyocytes and the increased levels of engraftment, angiogenesis, and endogenous cardiomyocyte proliferation when compared with that observed with myogenic and endothelial cells.

A key goal of cardiac cell transplantation for myocardial infarct repair is to modify LV remodeling in order to increase functional repair. Despite the fact that all 3 cell types did not prevent LV dilation compared with PBS control, myoendothelial cells significantly reduced LV infarct size and sustained LV contractile function better than the other 2 cell types and PBS. It remains to be investigated if the beneficial effects of myoendothelial cells will reduce LV dilation at longer time points.

Our histological analysis demonstrated a higher level of engraftment based on the number of regenerated myofibers in myoendothelial cell-injected hearts when compared with the other cell-injected hearts. Because there is a correlation between the magnitude of functional improvement and donor cell engraftment (31–33), the large degree of engraftment of myoendothelial (CD56+CD34+/CD144+) cells is a good indicator of their enhanced ability to improve cardiac function. This superior engraftment is likely related to the superior survival characteristics of myoendothelial cells, as observed in skeletal muscle (18), which could be due to their enhanced ability to resist oxidative stress compared with myogenic and endothelial cells. This same enhanced survival characteristic was also observed with murine MDSCs, which were found to be more resistant to oxidative stress than SkMs (14).

A few of the injected myoendothelial cells were observed to express cardiac cell markers within the infarcted heart. The frequency of this event was quite rare as the vast majority of engrafted donor cells were negative for cTnI and cTnT. It is undetermined from these experiments whether these cardiac marker-expressing donor cells originated from differentiation or fusion with the host cardiomyocyte (14,15,34).
All 3 cell populations had an angiogenic effect upon transplantation, a finding that is consistent with many reports in the field (1,14,20,35–37). Neovascularization of the infarct may help to salvage at-risk myocardium by promoting endogenous cell survival and proliferation, stimulating hibernating myocardium, enhancing scar viability, and attenuating adverse remodeling (20). When we previously reported on the intramyocardial injection of MDSCs,
we observed that most of the new vasculature that formed within the infarct was of host origin. The occurrence of transplanted murine MDSCs differentiating into blood vessels was rare (14,15), indicating that the presence of new vasculature within the infarct may be due to paracrine factors. Likewise, in this study, we found that only a few donor cells had incorporated into the neovascularature, as evidenced by our use of a human-specific CD31 antibody (data not shown), suggesting that the new capillaries are of host-origin and may be induced by a paracrine factor released by the donor cells. In a recent study, we reported that one of the most potent angiogenic factors, VEGF, may be critical for the occurrence of angiogenesis in murine MDSC-treated hearts (21). Overall, the ability of myoendothelial cells to induce the greatest level of angiogenesis within the infarct may be a major factor underlying their therapeutic effect.

Recent reports have demonstrated that a population of myocytes within the myocardium is capable of proliferating after infarction (38,39). Schuster et al. (40) report that myocardial neovascularization results in regeneration and cell cycling of endogenous cardiomyocytes, suggesting that agents that increase myocardial homing of bone marrow angioblasts could induce endogenous cardiomyocytes to enter the cell cycle and improve functional cardiac recovery. We observed that mouse cardiomyocytes located within the peri-infarct region in animals receiving human myoendothelial cells demonstrated much higher mitotic activity than in those receiving PBS at 5 days after cell transplantation. The origin of these proliferating cells remains unknown, but they could originate from resident cardiomyocytes, differentiating cardiac stem cells, or from circulating stem cells that home to the myocardium after infarction (39).

Additionally, TUNEL staining showed reduced levels of apoptosis, suggesting that myoendothelial (CD56⁺CD34⁺CD144⁺) cell transplantation was effective at reducing endogenous cardiomyocyte apoptosis at the initial phase after MI.

Transplanted cells may have a beneficial paracrine effect in the heart by releasing growth factors that promote angiogenesis, cell proliferation, antiapoptosis, antifibrotic effects, and cardioprotective effects (40,41). Although we only evaluated the expression of a few secretable factors, qPCR analysis showed that VEGF and HGF are 2 factors secreted by transplanted human muscle cells in response to the ischemic microenvironment of the infarcted hearts. We observed elevated HGF expression in myoendothelial (CD56⁺CD34⁺CD144⁺) cells but not in the other tested cell populations. It has been previously demonstrated that HGF can greatly increase myogenic regeneration and vascularity while reducing fibrosis inside the graft, which enhances the efficacy of SKM transplantation to infarcted hearts (25). Of all the factors evaluated, VEGF was the only factor to significantly increase upon exposure to hypoxic culture conditions. Since the cells are transplanted into an ischemic environment, VEGF may be highly secreted by the donor cells and be a major factor in the induction of angiogenesis and promotion of endogenous cell survival (1,21,37). In addition, VEGF and HGF may have a synergistic effect resulting in a more robust proliferative, chemotactic, and angiogenic response than either growth factor alone (42,43).

Here, we have shown that human skeletal muscle-derived myoendothelial (CD56⁺CD34⁺CD144⁺) cells represent a unique cell population with a superior ability for myocardial infarct repair when compared with conventional myogenic or endothelial cells, as recently observed in skeletal muscle (18). Based on the results presented here, the beneficial therapeutic outcomes of myoendothelial cell transplantation, including the improvement of cardiac function, attenuation of adverse cardiac remodeling, cardioprotection of at-risk endogenous cardiac cells, and the stimulation of both infarct neovascularization and endogenous cell proliferation, may emanate from the robust engraftment of myoendothelial cells.

Conclusions

Myoendothelial cells represent a unique population of progenitor cells from human skeletal muscle that may be similar to murine MDSCs (14) in regard to the therapeutic repair of skeletal (18) and cardiac muscles.

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Reprint requests and correspondence: Dr. Johnny Huard, Stem Cell Research Center, 4100 Rangos Research Center, 3460 Fifth Avenue, Pittsburgh, Pennsylvania 15213. E-mail: jhuard@pitt.edu.

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