

Cardiovascular, Pulmonary and Renal Pathology

Formation of Human Myocardium in the Rat Heart from Human Embryonic Stem Cells

Michael A. Laflamme,* Joseph Gold,[†]
Chunhui Xu,[†] Mohammad Hassanipour,[†]
Elen Rosler,[†] Shailaja Police,[†] Veronica Muskheli,*
and Charles E. Murry*

From the Department of Pathology,* University of Washington, Seattle, Washington; and Geron Corporation,[†] Menlo Park, California

Human embryonic stem cells (hESCs) offer the opportunity to replenish cells lost in the postinfarct heart. We explored whether human myocardium could be generated in rat hearts by injecting differentiated cardiac-enriched hESC progeny into the left ventricular wall of athymic rats. Although initial grafts were predominantly epithelial, noncardiac elements were lost over time, and grafts consisted predominantly of cardiomyocytes by 4 weeks. No teratomatous elements were observed. Engrafted cardiomyocytes were glycogen-rich and expressed expected cardiac markers including β -myosin heavy chain, myosin light chain 2v, and atrial natriuretic factor. Heat-shock treatment improved graft size approximately threefold. The cardiac implants exhibited substantial angiogenesis, both recipient and graft derived. Importantly, there was greater proliferation in human cardiomyocytes than previously seen in rodent-derived cardiomyocytes: 14.4% of graft cardiomyocytes expressed the proliferation marker Ki-67, and 2.7% incorporated the thymidine analog BrdU 4 weeks after transplantation. This proliferation was associated with a sevenfold increase in graft size over the 4-week interval. Thus, hESCs can form human myocardium in the rat heart, permitting studies of human myocardial development and physiology and supporting the feasibility of their use in myocardial repair. (*Am J Pathol* 2005, 167:663–671)

Because the adult human heart has little regenerative capacity, irreversible injury to the myocardium, such as by infarction, typically results in the formation of a non-contractile scar and often initiates progressive heart failure. Given the limited supply of transplant hearts in the

face of a large population of patients with end-stage heart disease, much attention has recently been directed at cell transplantation strategies as an alternative strategy to ameliorate cardiac injury.^{1–3} As currently envisioned for human therapeutic application, a suitable myogenic cell type from either an autologous or appropriately matched allogeneic source is delivered to the infarcted zone in an attempt to repopulate the lost myocardium. A number of different cell types have been considered for such therapies, including skeletal myoblasts,^{4–6} bone marrow-derived hematopoietic stem cells,^{7–10} mesenchymal stem cells,^{11–14} intrinsic cardiac stem cells,^{15,16} and embryonic stem cells (ESCs).^{17–22}

ESCs, pluripotent cells derived from the inner cell mass of pre-implantation-stage embryos, have a number of potential advantages over some of these other cell types for cardiac repair applications. First, there are well-defined protocols for the isolation and maintenance of ESCs, and they have a tremendous capacity for *in vitro* expansion, making them likely scalable for human applications.²³ Second, ESCs have an unquestioned ability to differentiate into functional cardiomyocytes *in vitro*,^{17,18,21,24,25} which stands in contrast to a number of other candidate cell sources for which this capacity remains controversial.^{8,26} Furthermore, human ESC (hESC)-derived cardiomyocytes possess the cellular elements required for electromechanical coupling with the host myocardium (eg, gap and adherens junctions^{17,18}), and it is therefore expected that, when transplanted, these cells could electrically integrate and contribute to systolic function. This property represents a significant advantage over other cell types, such as skeletal muscle, which might yield meaningful function improvement but should do so predominantly through modulation of dia-

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Address reprint requests to Charles E. Murry, MD, PhD, Center for Cardiovascular Biology and Regenerative Medicine, Department of Pathology, 815 Mercer Street, Room 453, University of Washington, Seattle, WA 98109. E-mail: murry@u.washington.edu.

stolic function (eg, by passively ameliorating postinfarct ventricular dilatation^{5,27,28}).

In the current study, we sought to form human myocardium by implanting cardiomyocytes from hESCs into the hearts of immunodeficient, "nude" rats. We report that this strategy resulted in the formation of substantial human myocardial tissue, and furthermore, that these cells proliferated at high rates for at least 1 month after implantation. Grafts showed associated angiogenesis, and the size of the resultant graft was enhanced by preceding heat-shock treatment of the implanted cells.

Materials and Methods

Cell Culture

Undifferentiated H1 (male) and H7 female hESCs²⁹ were maintained under feeder-free conditions as previously described³⁰ and detailed in the supplemental material (<http://ajp.amjpathol.org>). These were differentiated *in vitro* as embryoid bodies for 3 weeks and then enriched to ~15% cardiomyocytes, using Percoll (Amersham Biosciences, Piscataway, NJ) gradient centrifugation¹⁸ (supplemental material at <http://ajp.amjpathol.org>). Unless otherwise stated, embryoid body outgrowths were heat-shocked 24 hours before implantation to improve survival (via 30-minute incubation at 43°C).

Cell Implantation and Histological Analysis

All studies were approved by the University of Washington Animal Care and Use Committee and were conducted in accordance with federal guidelines. Using surgical techniques previously reported by our group^{6,28,31-33} and further detailed in the supplemental materials (<http://ajp.amjpathol.org>), 0.5 to 10 million Percoll-enriched hESC-derived cardiomyocytes were directly injected into the uninjured left ventricular walls of 200- to 300-g male nude rats (Harlan, Indianapolis, IN). At 1 to 28 days after engraftment, rats received a 1-hour pulse of 5-bromodeoxyuridine (BrdU, 10 mg), followed by euthanasia with pentobarbital. Engrafted hearts were fixed and vibratome-sectioned at 500- μ m thickness to ensure equivalent sampling. These uniform transverse sections were routinely processed and paraffin-embedded for histology. Sections were analyzed histologically, phenotyping the implanted cells with the histochemical and immunohistochemical markers detailed in the supplemental materials (<http://ajp.amjpathol.org>). Graft lineage was traced using *in situ* hybridization with human-specific probes (including Y-chromosome, Alu repeat, and pan-centromeric targets), as is also detailed in the supplemental materials (<http://ajp.amjpathol.org>).

Statistics

Values are expressed as mean \pm SEM, and statistical testing was performed using *t*-test with $\alpha = 0.05$ for significance (Excel Data Analysis Toolpak; Microsoft, Redmond, WA).

Results

Time Course of Graft Composition

An early and important goal was to examine the time course of differentiation by the hESC-derived myocardial grafts after implantation in the heart of the nude rat recipient. One day after transient heat shock to improve survivability, human ESC-derived cardiomyocytes were harvested and enriched by Percoll centrifugation. Parallel *in vitro* experiments indicated that, before implantation, approximately 10 to 15% of the used cells were positive for cardiac markers such as sarcomeric myosin heavy chain and/or actin. For this time course study, 5×10^6 cells were then implanted into the uninjured left ventricular myocardium of 10 nude rats. Recipient animals were sacrificed at 1, 7, 14, and 28 days after engraftment ($n = 2-4$ /group), and hearts underwent histological analysis.

Representative images from this series are contained in Figure 1, and, for this study, *in situ* hybridization with a human-specific Y chromosome sequence was used to follow the lineage of the (male H1-derived) graft cells. One day after implantation, the grafts consisted of confluent masses of generally poorly differentiated cells, interspersed with strands of host myocardium. Graft cells had high nuclear-to-cytoplasmic ratios, and a relatively small population of cells with a clear cytoplasm was noted. There was extensive cell death in the grafts, as evidenced by the intense granulocytic infiltration, nuclear condensation, and Y-positive karyorrhectic debris noted among graft cells. Not surprisingly, evaluation of DNA fragmentation using terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling staining confirmed the extensive cell death at the 1-day time point (not shown). In occasional areas, the adjacent host myocardium had undergone contraction band necrosis, likely from the trauma of injection. These findings were similar to the appearance of early grafts with other cell types in the heart, including neonatal rat cardiomyocytes.³¹

Immunohistochemistry on the 1-day-old grafts revealed them to be polymorphous in composition: the vast majority of cells were epithelial (pan-cytokeratin+), but there were small discrete islands of cardiac (sarcomeric myosin heavy chain) and endodermal (α -fetoprotein+; not shown) cells also present.

As illustrated in Figure 1, the composition of the hESC-derived grafts changed importantly over time. In contrast to the initial grafts, both 7 and 14 days after transplantation, there were only comparatively rare cytokeratin+ or α -fetoprotein+ elements, and the clustered sarcomeric myosin heavy chain+ cardiac cells appeared expanded. (Note that, as would be expected, the graft cells at all later time points were present on a highly fibrotic background.) Most remarkably, after 28 days, grafts were found to be composed predominantly of sarcomeric myosin heavy chain-positive human myocardium; all other cytokeratin+ or α -fetoprotein+ elements had been eliminated from the heart. (As is quantified in Figure 1B, there was a more than sevenfold increase in the cross-sec-

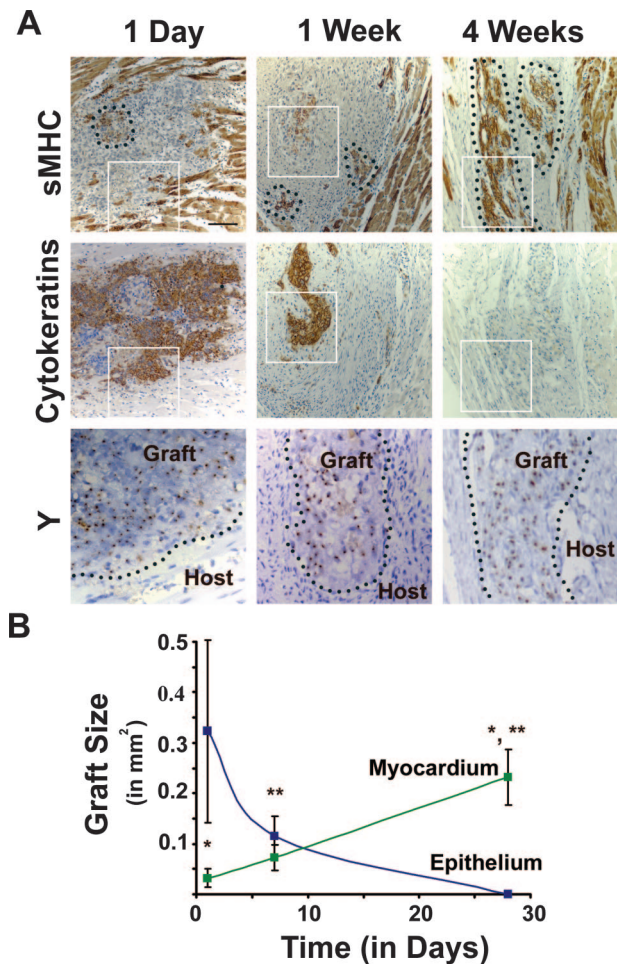


Figure 1. Time course study of human ESC cardiomyocyte grafts. **A:** The three top panels show immunostaining (brown deposit) for sarcomeric myosin heavy chain (sMHC) at 1 day, 1 week, and 4 weeks after transplantation and identify host and graft cardiomyocytes. The three middle panels show immunostaining for epithelial cells (pan-cytokeratins) on adjacent sections. Note that, over time, the tightly clustered human myocardial implants (enclosed within **dotted lines**) appear to have expanded, whereas epithelial elements decrease in extent and are totally absent by 4 weeks. The lineage of the engrafted human cells is confirmed in the three bottom panels, in which adjacent sections have undergone *in situ* hybridization with a human-specific probe against the Y-chromosome (punctate intranuclear dots), these from fields corresponding to the boxes in the above panels. Scale bar = 50 μ m; counterstain is hematoxylin. Y *in situ* images are magnified an additional 2.5-fold. **B:** The plot shows the changing composition of the graft versus time ($n = 4$ to 10 hearts per time point). Whereas the mean total epithelial (ie, pan-cytokeratin+; **blue line**) graft cross-sectional area declines with time and is totally absent by 4 weeks, the mean total cardiac graft area shows a sevenfold increase from the 1-day to 4-week time points. Note that the mean cardiac graft area at 4 weeks shows a statistically significant difference from that at both the 1-day (*) and 1-week (**) time points by Student's *t*-test ($P < 0.05$).

tional area of the human cardiac implants between 1 and 28 days after transplantation.) A small number of graft-derived endothelial cells were retained at 4 weeks (as is detailed subsequently), but otherwise the human-specific Y chromosome *in situ* hybridization identified only graft-derived cardiomyocytes or extraordinarily rare fibroblastic cells. No heterologous or teratomatous elements were found after 28 days despite exhaustive searching ($n = 10$ rats receiving H1-derived cells).

Dose-Response Studies and Analysis of Graft Differentiation

Multiple additional grafting experiments have been performed, variably using cells derived from the H1 or H7 ESC line and evaluating grafts at time points up to 4 weeks (Supplemental Table 1 at <http://ajp.amjpathol.org>). Dose-response studies were performed to identify an optimal number of cells to implant into the heart. Rats ($n = 4-6$ /group) received doses of 0.5, 1, 5, or 10 million hESC-derived cardiomyocytes, and their hearts were examined histologically 4 weeks after implantation. The resultant graft size varied considerably, even with a constant dose of cells. This variability likely is due in part to varying successful cell delivery and survival^{33,34} and could also be influenced by differential immunorecognition by the nude rat (which has been maintained on an outbred genetic background). No grafts were detected in rats receiving 0.5 million cells, and only a single small graft was observed in the four rats receiving 1 million cells. In contrast, grafts were present in the vast majority of hearts receiving 5 or 10 million cells, but no clear increase in graft size was seen at the highest dose. Therefore, for all described studies, unless stated otherwise, a "standard" dose of 5 million cells was implanted.

Extensive additional immunophenotyping has been performed on the numerous hESC-derived myocardial grafts (Table 1). No obvious differences have been observed between grafts derived from undifferentiated cultures that have varied in their number of passages *in vitro* or in their parental line of origin (ie, H1 or H7). The hESC-derived cardiac implants expressed numerous cardiac markers at all time points, including sarcomeric actin (Figure 2A) and myosin (Figure 2F), smooth muscle α -actin, myosin light chain 2v (Figure 2E), and atrial natriuretic peptide (Supplemental Figure 1 at <http://ajp.amjpathol.org>). As a general trend, grafts that were implanted for increasing lengths of time showed increasing maturation, such that 4-week-old transplants typically exhibited clear sarcomeric banding, generally in alignment with adjacent host cardiomyocytes (Figure 2F). The predominant isoform of sarcomeric myosin heavy chain in developing and adult human ventricular myocardium is the β -isoform,^{35,36} and consistent with this, the human cardiac grafts showed immunoreactivity for the β -isoform and not for the α -isoform (Figures 3A; 4, B and C; and 5, A and B). (In contrast, as expected for the mature rat heart,³⁷ the host myocardium immunostained strongly for the α -myosin heavy chain and did not express the β -isoform, thereby serving as another means to identify the also morphologically distinct hESC-derived cardiac implants.)

A surprising result was that, despite the preceding observation of immunoreactivity for the gap junction protein connexin-43 by the hESC-derived cardiomyocytes *in vitro*^{17,18} (and their clear-cut synchronous spontaneous contraction in culture), the engrafted human cardiomyocytes were found consistently negative for connexin-43 (not shown). At present, it is uncertain whether this failure to immunostain for connexin-43 reflects an *in vivo* switch

Table 1. Changing Composition and Immunophenotype of Human ESC-Derived Intracardiac Grafts versus Time

	1 day	1 week	2 weeks	4 weeks
Sarcomeric actin	+ / + + +	+ + / + + +	+ + / + + +	+ + + / + + +
β -Myosin heavy chain	+ / + + +	+ + / + + +	+ + / + + +	+ + + / + + +
α -Myosin heavy chain	–	–	–	–
Smooth muscle α -actin	+ / + +	+ + / + + +	+ + / + +	+ + + / + +
N-cadherin	+ / +	+ + / + + +	+ + / + + +	+ + + / + + +
Atrial natriuretic peptide	+ / +	+ + / + +	+ + / + +	+ + + / + +
Myosin light chain 2v	NE	+ + / + +	+ + / + + +	+ + + / + + +
Connexin-43	–	–	–	–
Pan-cytokeratins	+ + + / + + +	+ / + + +	+ / + + +	–
α -Fetoprotein	+ / + + +	–	–	–
β III-Tubulin	–	–	–	–
S-100 protein	–	–	–	–
Fast skeletal myosin heavy chain	–	–	–	–

Semiquantitative description of the changing immunophenotype of the hESC-derived cardiomyocyte grafts over time. Entries refer to the extent (first parameter) and intensity (second parameter) of the immunostaining for the listed antigens. –, no immunoreactivity. When present, the extent of immunoreactivity was characterized as rare or focal (+), multifocal but limited (++), and extensive (+++). The intensity of the immunoreactivity was graded as weak (+), moderate (++), or strong (+++).

to another connexin isoform or merely results from a level of expression below the threshold of immunohistochemical detection. (We have attempted immunohistochemistry for other connexin isoforms, including connexin-40 and -45, but have not identified antibodies with sufficient specificity on control sections.) It should be noted that the engrafted cardiomyocytes did show strong immunoreactivity, with a diffuse membranous pattern, with a pan-cadherin antibody (Figure 2H; Supplemental Figure 1, A and B, at <http://ajp.amjpathol.org>). Because N-cadherin is an important structural element of the intercalated disk, present in both the developing and adult heart,³⁸ this staining pattern suggests that these cells may be at an early stage in the formation of these sites of specialized electromechanical cell contact. Similar membranous staining for pan-cadherin and absent staining for connexin-43 was observed in early-stage rat cardiomyocyte grafts, although the rat cell had formed mature intercalated disks by 4 weeks.³¹

Also as expected for embryonic human myocardium,³⁹ the cardiac grafts were found to be heavily glycogen laden, as evidenced by strong reactivity for the histochemical periodic acid-Schiff reaction (PAS stain; Figure 2, C and D) that was entirely amylase digestible. Of note, despite fairly extensive searching by immunohistochemistry, grafts were negative at all time points for markers of skeletal muscle (fast skeletal myosin heavy chain), neuronal (β III-tubulin), and glial (S-100 protein) cell types.

Recipient- and Graft-Derived Angiogenesis of Human Cardiac Implants

We tested whether the human myocardial grafts elicited an angiogenic response from either host- or donor-derived cells, using rat- and human-specific endothelial probes. The human cardiac implants showed a substantial degree of rat vessel ingrowth, this determined by immunohistochemistry for rat endothelial antigen-1 (RECA-1). In separate studies (not shown), we confirmed previously reported results that this antibody reacts uniformly and exclusively with rat (and not human) endothe-

lial cells.⁴⁰ Morphometric quantification of the RECA-1+, rat-derived graft angiogenesis was then undertaken, using adjacent sections immunostained for β -myosin heavy chain to identify the human cardiac implants of interest. In so doing, we observed a mean vascular density of 305.0 ± 46.2 rat vessels/mm², representing $6.0 \pm 1.3\%$ of the cardiac graft cross-sectional area ($n = 10$ engrafted hearts) (Figure 3, A and C).

An unexpected finding was the roughly comparable contribution of human graft-derived angiogenesis within the cardiac implants. Two human-specific markers were used to identify human endothelial cells within the cardiac grafts, namely a human-specific antibody against CD31/PECAM and *Ulex europaeus* agglutinin I.⁴¹ Again, both markers were shown to react uniquely with human but not rodent endothelium in separate studies. Remarkably, *Ulex* identified a substantial number of human vessels within the β -myosin heavy chain+ cardiac implants: a mean density of 176.5 ± 42.4 human vessels/mm², occupying a mean of $4.6 \pm 1.2\%$ of the cardiac graft cross-sectional area ($n = 9$ engrafted hearts). Qualitatively similar results were obtained with human-specific CD31/PECAM immunostaining (Figure 3, A, B, and D). Interestingly, when summed together, the density of host- and graft-derived vessels is reasonably close to that of the distant native myocardium, which we quantified as 1476.3 ± 187.3 vessels/mm² or a mean of $15.0 \pm 0.7\%$ of the myocardial cross-sectional area ($n = 11$ hearts).

Improved Cardiac Graft Size with Preceding Heat-Shock Treatment

Our group had previously shown that heat shock significantly reduces death of neonatal cardiomyocytes after grafting.³³ We therefore examined the effect of heat shock (by incubation at 43°C for 30 minutes) on hESC-derived cardiomyocytes. Heat-shocked cells showed a marked induction of Hsp-60, Hsp-70, and Hsp-90 (Figure 4A), and these cells showed significant protection against hydrogen peroxide induced injury *in vitro* (Figure 4B). Heat shock had no effect on the proliferation of

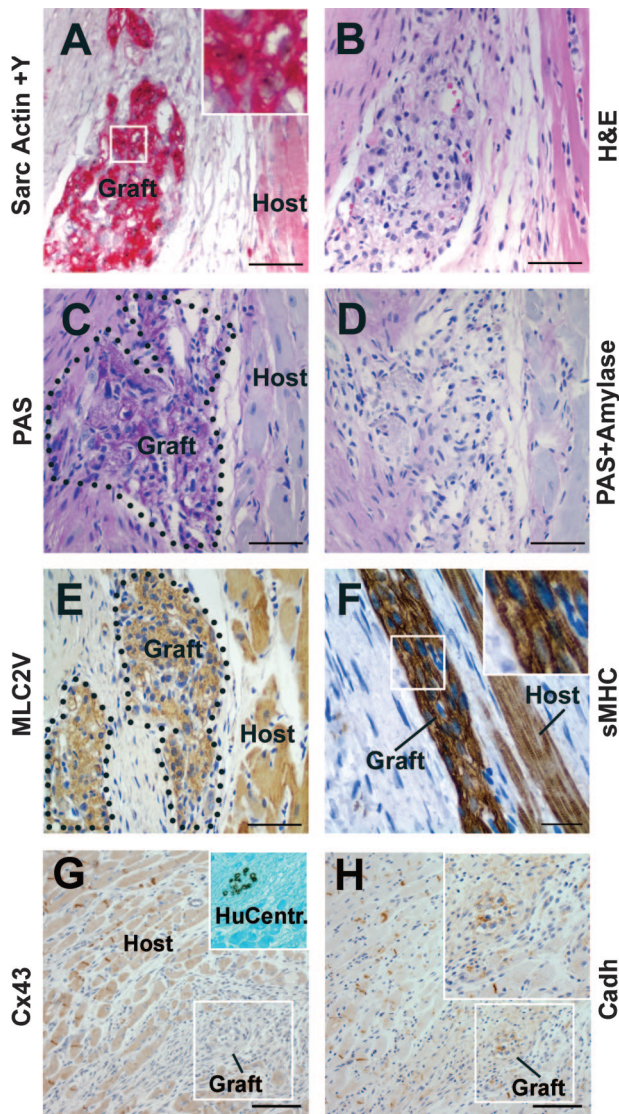


Figure 2. Additional phenotyping of human ESC cardiomyocyte grafts. All images depict 4-week-old grafts. **A:** Clustered human ESC-derived cardiomyocyte graft cells are positive for sarcomeric actin (sarc Actin, immunostain indicated by red deposit) and are identified by their reactivity for the human-specific Y-chromosome probe by *in situ* hybridization (Y, punctate brown intranuclear signal, best seen in **inset** magnified an additional threefold). **B:** The peculiar appearance of the engrafted cardiac cells by routine H&E stain in a serial section, by which they show a distinctly vacuolated appearance. This pattern is likely accounted for by the results depicted **C**, wherein engrafted cardiomyocytes were found to be intensely PAS reactive. This PAS reaction is completely removed by preceding amylase digestion (PAS+Amylase), as depicted in **D**, indicating engrafted cardiomyocytes are heavily glycogen-laden. **E:** The cardiomyocyte graft cells stained positive for the ventricular-specific marker myosin light chain 2v (MLC2V). As depicted by sarcomeric myosin heavy chain (sMHC) immunostain in **F**, the engrafted cardiomyocytes assume sarcomeric banding and alignment with host fibers (**inset** magnified an additional twofold). **G** and **H:** Adjacent sections immunostained with antibodies against connexin-43 (Cx43) and pan-cadherins (Cadh), respectively. The **inset** in **G** shows *in situ* hybridization with the human-specific pan-centromeric probe (on adjacent section), thus identifying a clump of human ESC-derived cardiomyocytes residing within the indicated box. This clump of cells showed the expected strong reactivity with β -myosin heavy chain (not shown). Note the total absence of connexin-43 reactivity, despite the expected intercalated disk staining pattern on the surrounding host myocardium. In contrast, this same field does show definite diffuse membranous staining of the human cardiac implant with anti-pan-cadherins. Scale bars = 50 μ m.

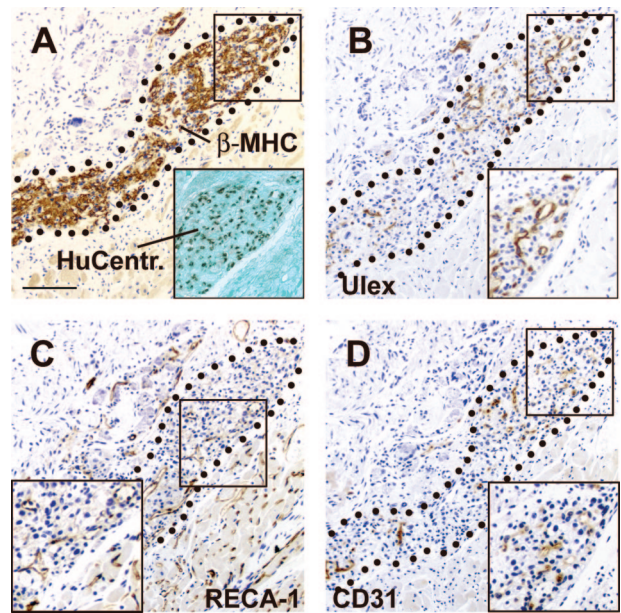


Figure 3. Human ESC-derived cardiac grafts show both recipient- and graft-derived angiogenesis. **A:** A typical cardiac implant 4 weeks after transplantation; the graft myocardium is readily distinguished from that of the host by its strong immunoreactivity for β -myosin heavy chain (β -MHC) and by *in situ* hybridization on an adjacent section with a human-specific pan-centromeric probe (HC, within **inset**). The ingrowth of both rat-derived and human-derived (ie, graft) vessels into this cardiac implant is demonstrated in **B** through **D**, which contain adjacent tissue sections stained with species-specific endothelial markers. **B** and **D:** Human endothelial cells, as detected by staining with *U. europaeus* agglutinin I and a human-specific anti-CD31 antibody, respectively. **C:** Rat endothelial cells, as defined by the rat-specific pan-endothelial antibody RECA-1, which marks the microvasculature of both the cardiac implant and the surrounding host myocardium. Scale bar = 100 μ m; **insets** magnified an additional 1.5-fold. Sections are counterstained with hematoxylin, except for the **inset** of **A**, which is counterstained with Fast Green.

hESC-derived cardiomyocytes *in vitro* (supplemental materials at <http://ajp.amjpathol.org>). We then compared graft size using heat-shocked or naïve hESC-derived cardiomyocytes in rats sacrificed 1 week after implanting 5 million cells. Figure 4, C and D, shows representative fields with islands of human myocardium on a fibrous background, taken from the median-sized grafts from the recipients of control and heat-shocked cells, respectively. Overall, heat-shock treatment produced an approximately threefold increase in cardiac graft cross-sectional area, with the mean total graft area increasing to 0.135 ± 0.043 mm² for the heat-shocked cells versus 0.044 ± 0.016 mm² for the control cells ($P < 0.05$). In contrast, no difference in total graft-associated fibrosis was observed between the two sets (4.9 ± 1.2 versus 4.7 ± 0.5 mm² for heat shocked and non-heat shocked, respectively).

Proliferation of Human ESC-Derived Cardiac Implants

The progressive increase in cardiomyocyte graft size over time suggested that these cells might have significant *in vivo* cell cycle activity. To test this, rats received an intraperitoneal bolus of the thymidine analog BrdU 1 hour before euthanasia. The incorporation of BrdU, which

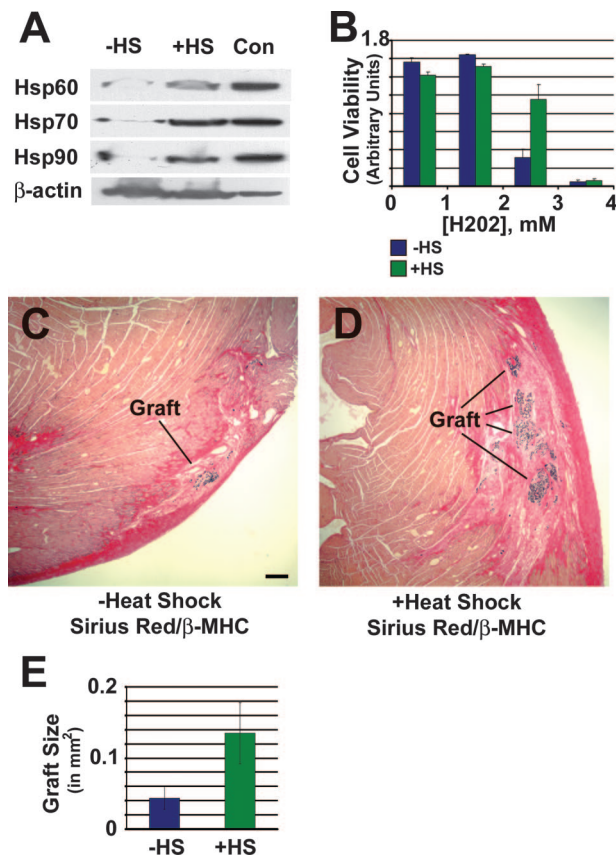


Figure 4. Heat-shock pretreatment is cytoprotective and yields large grafts. **A:** The expression of heat-shock proteins in H7 hESC-derived, Percoll-fractionated cardiomyocytes, as determined by Western blot analysis with antibodies against Hsp60, Hsp70, and Hsp90. Note the induction of all three isoforms in heat-shocked cells (lane +HS) over untreated controls (lane -HS). Heat-shocked HeLa cell lysate (lane Con) was included as positive control. Expression of β -actin was measured to control for protein loading. **B:** The protective effect of heat shock on hESC-derived cardiomyocytes is examined by quantifying (by MTS assay) the survival of control (blue) and heat-shocked (green) Percoll-fractionated cells in the face of varying concentrations of hydrogen peroxide ($n = 2$ preparations per condition). Note the enhanced cell survival in response to heat shock at the intermediate peroxide concentration. **C and D:** Human ESC-derived cardiac implants and associated fibrosis in hearts receiving control and heat-shocked cells, respectively. Fields contain representative islands of β -myosin heavy chain+ human cardiomyocytes (blue/black deposit), each taken from the heart with the median-sized graft from its corresponding experimental group. As was typical, cardiac grafts were present on a background of Sirius Red-positive scar tissue (intense red staining), with staining specificity subsequently confirmed under polarized light). **E:** Heat-shock treatment (+HS) yielded a statistically significant, ≈ 3 -fold difference in cardiac graft volume over non-heat-shocked cells (-HS). Scale bar = 200 μ m.

marks cells in the S phase of the cell cycle, was detected immunohistochemically in sections from 1- and 4-week grafts. These sections were also immunostained with an antibody against β -myosin heavy chain to facilitate identification of the human cardiac implants, and the fraction of BrdU-positive cardiac graft cells was quantitated in a blinded fashion. This analysis revealed a remarkably high degree of proliferative activity, with $6.4 \pm 0.8\%$ ($n = 5$) of cardiac graft nuclei double-positive 1 week after transplantation and $2.7 \pm 0.3\%$ ($n = 7$) after 4 weeks (Figure 5). Analogous results were obtained by immunohistochemical studies with a human-specific monoclonal antibody recognizing the nuclear proliferative marker Ki-67, known to identify cells in all active phases of the cell cycle

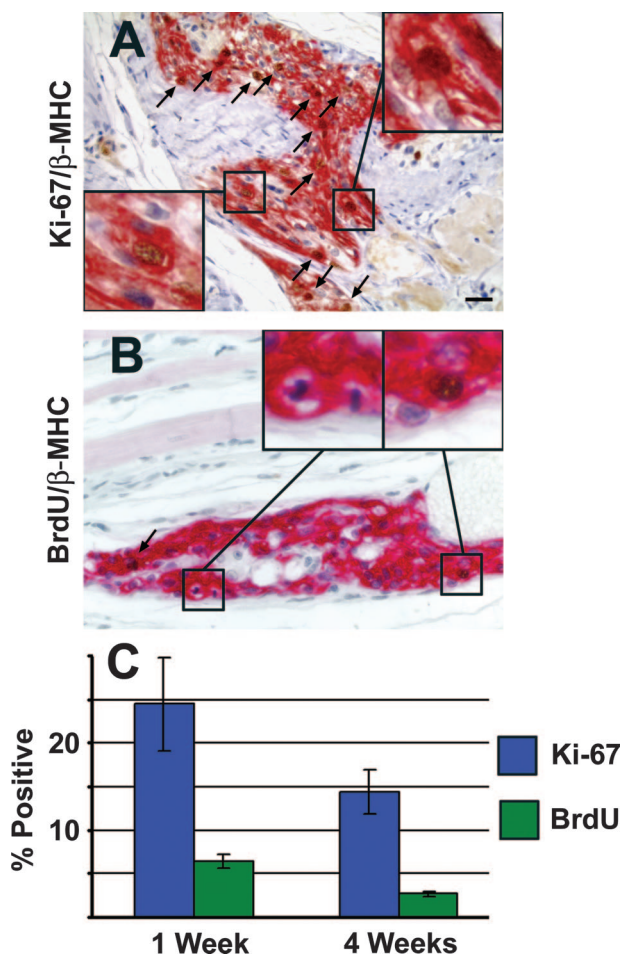


Figure 5. Human ESC-derived cardiac grafts are highly proliferative. **A:** A 4-week-old cardiac implant is double-immunostained for β -myosin heavy chain (red deposit), the predominant myosin isoform in the human cardiomyocytes, and a human-specific antibody against the proliferative marker Ki-67 (brown intranuclear staining). Note that the host myocardium is β -myosin heavy chain negative. Representative Ki-67+ cardiomyocyte nuclei are indicated by the insets (magnified an additional threefold) and arrows. **B:** Another 4-week-old implant is double-immunostained for β -myosin heavy chain (red deposit) and anti-BrdU (brown intranuclear staining). Two clear-cut BrdU+ and β -myosin heavy chain+ human cardiomyocytes are indicated by the arrow and right inset. In addition, occasional mitotic figures were noted within the human cardiomyocytes, as illustrated by the left inset. Magnification, $\times 400$ (insets magnified an additional threefold). **C:** 1 week after transplantation, $28.3 \pm 5.4\%$ of β -myosin heavy chain-positive graft cells were Ki-67+ and $6.4 \pm 0.8\%$ were BrdU+. As long as 4 weeks after transplantation, a still robust $15.8 \pm 3.4\%$ of graft cardiomyocytes were Ki-67+ and $2.7 \pm 0.3\%$ were BrdU+. Scale bar = 100 μ m.

(eg, all cells outside of G₀).⁴² In this instance, $24.5 \pm 5.4\%$ ($n = 4$) of cardiac graft nuclei after 1 week and $14.4 \pm 2.6\%$ ($n = 6$) after 4 weeks were double positive for Ki-67 and β -myosin heavy chain. Further evidence of the substantial proliferative capacity of the implanted human cardiomyocytes included the observation of infrequent but readily detectable mitotic figures (Figure 5B). The high proliferative rates of these human cardiomyocytes stand in stark contrast with experience with rodent embryonic, fetal, or neonatal cardiomyocytes, all of which have been found to rapidly exit the cell cycle on grafting, if not in their preceding *in vitro* preparation.^{31,43-45}

Discussion

In the present study, we successfully demonstrated the capacity of the differentiated progeny of human ESCs to form stable grafts of human myocardium within the rat heart. In so doing, we have shown that these cells form reasonably sized and expanding grafts that, at least by 4 weeks, are composed predominantly of cardiomyocytes. The grafts expressed many cardiac markers (Figures 1 to 5; Table 1), including sarcomeric actins and myosins, ventricular myosin light chain, atrial natriuretic peptide, and others. Interestingly, the human cells expressed β -myosin heavy chain, the major motor protein of slow heart rate species (including humans), whereas the surrounding rat myocardium expressed the α -isoform. This indicates that the rat heart environment does not override the normal human pattern of myosin expression. The human cardiac implants clearly matured over time and responded to the host environment, as evidenced by the formation of graft fiber strands aligned in parallel with the host myocardium, as well as appropriately aligned sarcomeres (Figure 2F).

At 1 day after implantation, the grafts were composed predominantly of epithelial cells, with only much smaller populations of cardiomyocytes and α -fetoprotein+ endodermal cells. By 4 weeks, however, these heterologous elements were no longer present, such that the grafts were almost exclusively cardiac, otherwise containing only endothelial cells or very rare fibroblastic cells (total $n = 36$ hearts, including recipients of H1 and H7 cells, both heat shocked and untreated). The mechanisms by which the implanted noncardiac cells are cleared from the rat heart are presently unknown but are the subject of ongoing investigation by our group. Importantly, although we are encouraged by this eventual clearance of noncardiac elements by the nude rat heart and by the absence of teratomatous and/or heterologous elements at 28 days (despite exhaustive histological evaluation of each heart), we recognize the definite need for additional preclinical safety studies to exclude such tumor formation. Because of the obvious unacceptability of even rare or small teratomata within the heart, such studies will need to examine longer time points, as well as distant organs.

Furthermore, the present study demonstrates that the total volume of cardiac graft produced with implantation of hESC-derived cardiomyocytes can be significantly increased by preceding heat-shock treatment (Figure 4). In preceding cell grafting studies by our group with rat neonatal cardiomyocytes, we showed that cell death at early time points was greatly reduced by heat-shock pretreatment.³³ We speculate that a similar cytoprotective effect is occurring with the present cell type. (We also compared proliferation—obviously another means to a larger graft—in heat-shocked and untreated hESC-derived cardiomyocytes and found it to have a negligible effect.) Although the precise molecular events underlying this cytoprotection remain incompletely elucidated, heat-shock treatment of hESC-derived cardiomyocytes produced the expected induction in heat-shock proteins (including Hsp60, Hsp70, and Hsp90). The cardioprotective

action of Hsp70 has been particularly well-demonstrated by numerous groups,^{46–49} and this and other heat-shock-induced effectors may limit cell death by acting as “molecular chaperones” for damaged proteins,⁵⁰ inhibiting pro-apoptotic signaling,⁵¹ or opposing oxidative stress.⁵²

The presence of both host (rat) and graft (human) vessels within the hESC-derived cardiac implants is also intriguing. Although the present histological study cannot address whether the human-derived vessels are in fact interconnected with the rest of the rodent vasculature, their extensive ingrowth invites speculation that they may be expanding to support the growth of the cardiac implants and that deliberate “doping” of the implanted hESC-derived cardiomyocytes with hESC-derived endothelial cells might further promote graft survival.

A final unexpected and important finding in the present study was the tremendous proliferative capacity of the engrafted hESC-derived cardiomyocytes. BrdU marking studies showed that 6.4% of these cells were synthesizing DNA at 1 week after transplantation, as well as a still-respectable 2.7% after 4 weeks (Figure 5). Because S phase is only a fraction of the cell cycle, the total percentage of cycling graft cells is likely severalfold higher. Consistent with this, the proliferation marker Ki-67, which identifies cells in all active phases of the cell cycle (G_1 , S, G_2 , and mitosis), identified 24.5% of engrafted cardiac cells after 1 week and 14.4% after 4 weeks. The persistent proliferative activity of this cell type after transplantation distinguishes them from rodent embryonic,^{43–45} fetal, and neonatal cardiomyocytes,³¹ each of which has been shown to very rapidly exit the cell cycle on culture or engraftment. We speculate that this species difference may reflect the 13-fold greater gestation period of humans relative to mice or rats. In any case, this substantial increase in cardiomyocyte proliferation may offer significant benefits for cardiac repair.

Although we have taken pains to carefully follow the lineage of the graft cells using human-specific *in situ* hybridization probes (as well as human-specific sarcomeric and proliferation markers), it is worth noting that recent studies have demonstrated the occurrence of rare but definite, spontaneous cell fusion events both *in vitro*⁵³ and *in vivo*.^{15,54,55} Although very infrequent, cell fusion can lead to misattribution in the phenotype of implanted cells, because it can result in a single cell exhibiting both donor and host markers. With regard to the present studies, although we are open to the possibility of rare human-rat fusion events, this phenomenon seems highly unlikely to account for the substantial quantities of human myocardium formed in the present studies. (Cell fusion events have typically involved $\ll 1\%$ of input cells in related experiments, even when using cells with a “fusiogenic” phenotype.⁵⁵) Moreover, it is worth reemphasizing that we have here used a previously differentiated and cardiac-enriched cell preparation with a well-established cardiac phenotype¹⁸ and that the proportion and characteristics of these cells histologically at initial time points are entirely reflective of the input population. We thus conclude that hESC-derived cardiomyocytes can be used to

form substantial and proliferating implants of developing human myocardium within the uninjured rat heart.

While this manuscript was under review, two groups also reported the successful formation of stable graft of hESC-derived cardiomyocytes in pharmacologically immunosuppressed pigs⁵⁶ and guinea pigs.⁵⁷ Importantly, these independent studies by the Gepstein and Li groups unambiguously demonstrated electromechanical integration of the cardiac implants with the host myocardium, and they provide exciting proof-of-concept for this cell type for applications as a biological pacemaker. Their cell preparation differed from our own in that they physically dissected spontaneous beating foci from differentiated embryoid body outgrowths, an approach not readily scalable to human cell therapeutic applications. On the other hand, because an important contribution of the present study is that hESC-derived cardiomyocytes have sustained proliferative capacity after implantation, input cell number requirements may be less limiting than intuitively expected. Given the gradual *in situ* expansion of the graft, one may not have to implant the full quantity of cells required for a therapeutic effect, and angiogenesis (perhaps both host- and graft-derived) may be able to match its increasing metabolic requirements. Finally, in addition to having implications for potential cell transplantation therapies, we expect this model to permit unique, previously inaccessible *in vivo* studies into the developmental biology of early human myocardium, in essence, using the rat heart as a “bioreactor” for the differentiating human cardiomyocytes.

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