Online Data Supplement

Methods Supplement

Cell Culture

Two of the originally derived human ESC lines (the male H1 and female H7 lines)[1] were maintained in the undifferentiated state at Geron Corporation using previously detailed feeder-free conditions.[2] Differentiation was achieved by methods previously shown to result in substantial cardiomyogenesis.[3] In brief, embryoid bodies were formed by allowing small clumps of undifferentiated human ESCs to grow in suspension culture, and differentiation was induced by withdrawal of mouse embryonic fibroblast-conditioned medium. After 4 days in suspension culture, embyroid bodies were plated onto gelatin-coated substrates, and the adherent outgrowths were fed every 1-2 days with differentiation medium containing 20% fetal bovine serum (Hyclone). Consistent with prior experience, beating foci were first noticeable after 8-10 days in culture, with maximal numbers of beating clusters observed approximately 3 weeks from the initiation of differentiation. Implantation studies were therefore performed with cells after 21-25 days of differentiation conditions.

Four to seven days prior to implantation, ~15-40 T225 flasks containing the embryoid body outgrowths were filled with medium and were shipped by overnight courier to the University of Washington. Unless stated otherwise, cultures were heat-shocked to improve survival with a 30 minute transition to 43°C one day prior to engraftment.[4] This intervention was performed because of preceding experience by our laboratory with related cell types had indicated it to greatly promote graft survival. Its efficacy on hESC-derived grafts is validated in the present study.

On the day of implantation, embryoid body outgrowths were enzymatically dispersed with application of Blendzyme IV (Roche, prepared at 0.56 U/ml in phosphate-buffered saline) for 30 minutes at 37°C and enriched for cardiomyocytes by separation over a discontinuous Percoll gradient, using previously detailed methods.[3] A subset of the cardiomyocyte-enriched cell preparation (e.g. the densest "fraction 4" layer[3]) was routinely plated-out and immunostained for cardiac markers including sarcomeric actin and/or myosins after 24 hours in culture. This analysis revealed the enriched cells to be 6.8-23.4 % cardiomyocytes. Of note, as detailed in a prior report, equivalently prepared cells have been exhaustively examined for the presence of skeletal muscle cells and have been found uniformly negative.[3] For this reason, immunohistochemistry with striated muscle markers, including sarcomeric actin and myosins, can be confidently performed on the human ESC-derived cells to identify cardiomyocytes.

Cell Implantation

All studies were approved by the University of Washington Animal Care and Use Committee and were conducted in accordance with federal guidelines. The protocol for cell implantation has been detailed in multiple previous reports by our group.[4-8] We chose the athymic nude rat as a host because of its well-established history in xenotransplantation[9] and the comparative ease of eventual physiologic studies in the rat. Thus, 200-300 gram male nude (Rh-rnu/rnu) rats were obtained from Harlan (Indianapolis) and anesthetized with ketamine-xylazine. Rats were intubated, mechanically ventilated, and their chests were aseptically opened. Cells ($0.5\text{-}10.0 \times 10^6$, generally 5.0×10^6) were suspended in 70 μl serum-free differentiation medium and injected into the anterior left ventricular myocardium using a Hamilton syringe with a 30-gauge needle. The chest was closed and the rat allowed to recover for the specified interval.

One hour prior to sacrifice, some of the rats received an intraperitoneal pulse of 5-bromodeoxyuridine (BrdU, 1.0 ml of a 10 mg/ml solution, prepared in sterile phosphate-buffered saline) to mark cells synthesizing DNA. At 1, 3, 7, 14, and 28 days post-engraftment, rats were euthanized with pentobarbital, and their hearts were fixed overnight with either methyl Carnoy's solution or 4% formaldehyde. The fixed hearts were then vibratome-sectioned to 500 micron thickness to ensure equivalent sampling, and these uniform transverse sections were routinely processed and paraffin-embedded for histology.

Online Supplement Table 1 (below) details our overall experience with hESC-derived cardiomyocyte grafting, including the various cell preparations employed, the number of cells implanted, heat-shock history, and graft outcome.

Histology and Immunohistochemical Studies

Five-micron sections were stained with hematoxylin and eosin, or subjected to immunohistochemistry as previously described.[5,6,8,10] Unless stated otherwise, hematoxylin was used as the nuclear counterstain in immunohistochemical studies. Immunostaining was performed with antibodies directed against the following muscle antigens: sarcomeric myosin heavy chain (clone MF-20, Developmental Studies Hybridoma Bank), α- and β-myosin heavy chain isoforms (clones BA-G5 and A4.951, respectively; both from American Type Culture Collection), sarcomeric actin (clone 5C5, Sigma), myosin light chain 2V (rabbit polyclonal, a gift from Dr. K. Chien), atrial natriuretic factor (a gift from Dr. J. Gutkowska), pan-cadherin (rabbit polyclonal, Sigma), connexin43 (Chemicon), smooth muscle α-actin (clone IB4, Dako) and fast skeletal myosin heavy chain (clone MY-32, Sigma). Endothelial cells were identified by species-specific probes: anti- pan rat endothelium (RECA-1, Abcam) and anti-CD31/PECAM

(Dako) and the lectin *Ulex europaeus* (biotin-conjugated, Vector) for human endothelium. Also employed were antibodies recognizing the following non-cardiac cell types: neurons (βIII-tubulin, Sigma), oligodendrocytes and astrocytes (S-100 protein, Dako), endoderm (α-fetoprotein, Dako), and epithelium (pan-cytokeratin cocktail (AE1/AE3, Dako). Cell proliferation was studied by immunostaining for BrdU as previously described[6,8], as well as with a human-specific antibody against the nuclear proliferation marker Ki-67 (MIB-1 clone, Dako). In both instances, the percentage of graft cells double-positive for β-myosin heavy chain (Vector Red chromagen) and BrdU (brown DAB precipitate) was determined by blinded analysis. For the subset of experiments quantitatively examining graft size and associated fibrosis, a blue-black chromagen (Vector SG) highlighting the immunostain of interest was combined with a Sirius Red counterstain for fibrous content. TUNEL staining was performed using the In Situ Cell Death kit (Roche). Histochemical staining for the periodic acid Schiff reaction (PAS) was performed using a commercially available kit (MasterTech), with preceding amylase digestion (50mg/ml type VI-B amylase, Sigma) performed on slides at 37°C for 30 minutes, as appropriate.

In Situ Hybridization Studies

Graft lineage was followed by in situ hybridization for the human Y chromosome[11] for cells derived from the male H1 hESC line or, more generally, with commercially-available, digoxigenin-labeled, human-specific probes directed against pan-centromeric (Cytocell) or Alu repeat sequences (PanPath). All evaluated sections were either double-labeled with one of these human markers or a contiguous section was so-labeled. (When performing the aforementioned double-labeling, immunostaining for the cell type of interest preceded in situ hybridization,

which was performed as we have detailed previously.[11]) That said, the human ESC-derived cardiac implants were also quite distinct morphologically and could also be readily distinguished from the host on the basis of their exclusive immunoreactivity for β-myosin heavy chain, as opposed to the host ventricular myocardium which expressed predominantly α-myosin heavy chain and only trace, if any, β-chain.

Morphometric Analysis

Morphometry was performed on a subset of engrafted hearts by collecting digital photomicrographs of fields of interest (Spot Diagnostic Instruments) as visualized with a 10X or 20X objective (Olympus BX41 microscope). Recall that all hearts were vibratome-sectioned and therefore uniformly sampled, allowing extrapolation of graft volume from the measured cross-sectional area. Montage images were assembled (Photoshop Elements, Adobe) for this purpose, and parameters including graft cross-sectional area, graft-associated fibrosis, and vessel number/density were quantitated by a blinded reviewer.

Analysis of heat-shock effects on human ESC-derived cardiomyocytes in vitro

For in vitro studies into the effects of heat shock on cell survival, two preparations of H7 hESC-derived embryoid body outgrowths were subjected to heat shock (by incubation at 43 °C for 30 minutes) 24 hours before Percoll fractionation and analysis. Induction of heat shock proteins was determined by Western blot, using commercially-available antibodies against heat shock protein isoforms Hsp60, Hsp70, and Hsp90 (Stressgen Biotechnologies). Protein loading was controlled for by immunoblotting for β-actin (Sigma). The quantity of viable heat-shocked and non-heat-shocked cells after hydrogen peroxide challenge was determined by MTS [3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] colorimetric assay (Cell Titer 96 assay, Promega)[12] using 490 nm absorbance (Wallac Victor2 plate reader, Perkin Elmer).

To examine whether heat-shock might have an effect on the proliferation of hESC-derived cardiomyocytes, H7-derived "fraction 4" cells were isolated and plated-out as described elsewhere (see above and Xu et al.[3]) After 24 hours in culture, cells were either heat-shocked (43° C medium for 30 minutes) or maintained as controls (i.e. medium change without heat shock). After a further 24 hours, cells were pulsed for 24 hours with BrdU (10 μM) and then PBS-rinsed and methanol-fixed. Preparations were then double-immunostained with antibodies against β-myosin heavy chain and BrdU, as detailed above. The percentage of BrdU+-incorporating cardiomyocytes under each condition was then determined by a blinded observer. Heat-shock treatment failed to show a significant effect on the proliferative index of hESC-derived cardiomyocytes: 27.0 ± 1.5% of heat-shocked myocytes were BrdU+ versus 30.0 ± 1.3% of controls (P=0.16; n=8 replicates per condition; 10,663 total nuclei observed).

Analysis of heat-shock effects on human ESC-derived cardiomyocytes in vivo

To determine the efficacy of heat-shock treatment to promote survival of hESC-derived cardiac implants, we engrafted an equal number of rats (n=6 each) with cells both 5 million of either heat-shocked (as above) or naïve cells. As always, an equivalent cohort of cells was plated-down, and the resultant cultures were immunostained for sarcomeric actin in order to verify comparable cardiac purity between the two sets (in this case, 16± 7% of the heat-shocked cells were sarcomeric actin positive versus 19± 4% of the non-heat-shocked cells, n=2 preparations each). Engrafted animals were sacrificed 1 week after transplantation, and the

resultant hearts were subjected to quantitative morphometry in order to determine the total cross-sectional area of both the human ESC-derived cardiac graft (as defined by immunohistochemistry for the human-specific cardiac marker β-myosin heavy chain) and graft-associated fibrosis (as delineated by the Sirius Red histochemical stain).[13] All hearts in this study were uniformly vibratome-sectioned, and this uniform sampling prior to embedding and histologic sectioning permits direct extrapolation of graft and/or scar volume from the totaled cross-sectional area.

Online Supplement Table 1.

Summary of human ESC derived cardiomyocyte graft experience.

Cell source*	Dose (# of implanted cells)	Heat-shock?	Result
H1p41	5.0 x 10^6	Yes	Grafts from 1 day to 4 weeks in 8 of 8 recipient animals; with large grafts (>500 nuclei/histologic section) present after 4 weeks
H1p41	0.5-10.0 x 10^6 +HS	Yes	Small grafts in 3 of 4 recipients of ≥ 5 x 10^6 cells; no grafts in recipients of 0.5 or 1.0 x 10^6 cells (n=2 each)
H1p48	5.0 x 10^6	Yes	Small grafts present in 6 of 6 recipients at 1 and 3 days. Grafts present in 5 of 6 recipients at 1 week (3 large, 2 small), and large grafts present in 3 of 5 recipients at 4 weeks.
H1p59	5.0 x 10^6	Yes	Small graft in 1 of 2 recipients at 4 weeks
H1p60	5.0 x 10^6	Yes	Small grafts in 2 of 2 recipients at 4 weeks
H7p30	2.0 x 10^6	Yes	Large graft at 4 weeks in sole recipient
H7p51	0.5-10.0 x 10^6	Yes	Small grafts in 1 of 2 recipients of 1.0 or 10.0 x 10^6 cells, as well as in 2 of 2 recipients of 5.0 x 10^6 cells. No grafts observed with 0.5 x 10^6 cells (n=2).
H7p51	5.0 x 10^6	Variable†	Variably-sized grafts present in 7 of 8 recipients at 1 week
H7p52	5.0 x 10^6	Variable†	Variably-sized grafts present in 4 of 4 recipients at 1 week

*Cell source refers to the undifferentiated human ESC cultures from which embryoid body outgrowths and eventually cardiac-enriched cell preparations were derived. Undifferentiated cells were either from the H1 or H7 parental line and varied in culture passage number from p30 to p60. For the final two grafting experiments, an equal number of rats were implanted with cells with and without preceding heat-shock treatment.

†For these studies, an equal number of rats were engrafted with heat-shocked or non-heat-shocked cells (used for the analysis depicted in Figure 4).

Online Figure 1. Changing immunophenotype of human ESC cardiomyocyte grafts at 1 and 4 weeks. Left-hand panels (**A, C, E**) depict 1-week old grafts, while right-hand panels (**B, D, F**) are from 4-week old grafts. Panels **A** & **B** show pan-cadherin (Cadh) immunostaining, including staining of regular intercalated disc structures on the surrounding host myocardium and a diffuse membranous pattern on the cardiac graft. The intensity of graft reaction for anti- pan-cadherins is roughly equivalent at both timepoints. Panels **C** & **D** show immunostaining for atrial natriuretic peptide (ANP), and again both timepoints show approximately equivalent, intermediate-intensity reaction. (Note that, while the surrounding host ventricular myocardium shows similar intermediate-intensity reaction, the positive control inset in panel C shows the expected intense, punctuate pattern of reactivity seen on host atrial tissue.) Panels **E** & **F** show immunostaining for smooth muscle α-actin (SMαA), a very early sarcomeric marker in embryonic myocardium. Smooth muscle α-actin immunoreactivity peaks at 1 week and has declined slightly by 4 weeks. Such images were used to generate the semi-quantitative grading summarized in Table 1. Scalebar = 50 μm.

Online Supplement References

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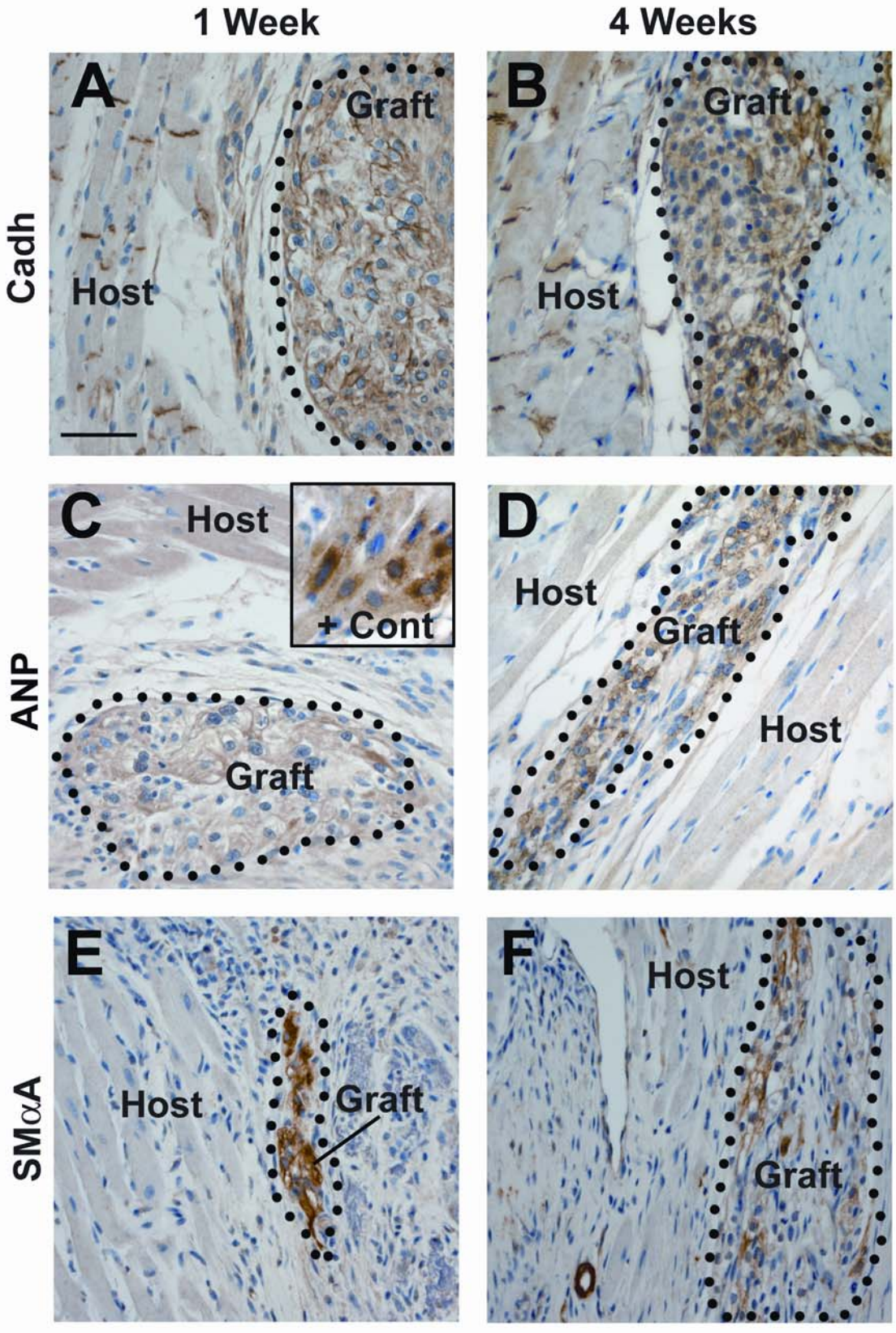
1 Week
4 Weeks
A
Graft
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B
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C
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+ Cont
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