Human Engineered Heart Muscles Engraft and Survive Long Term in a Rodent Myocardial Infarction Model


Rationale: Tissue engineering approaches may improve survival and functional benefits from human embryonic stem cell–derived cardiomyocyte transplantation, thereby potentially preventing dilative remodeling and progression to heart failure.

Objective: Assessment of transport stability, long-term survival, structural organization, functional benefits, and teratoma risk of engineered heart muscle (EHM) in a chronic myocardial infarction model.

Methods and Results: We constructed EHMs from human embryonic stem cell–derived cardiomyocytes and released them for transatlantic shipping following predefined quality control criteria. Two days of shipment did not lead to adverse effects on cell viability or contractile performance of EHMs (n=3, P=0.83, P=0.87). One month after ischemia/reperfusion injury, EHMs were implanted onto immunocompromised rat hearts to simulate chronic ischemia. Bioluminescence imaging showed stable engraftment with no significant cell loss between week 2 and 12 (n=6, P=0.67), preserving ≤25% of the transplanted cells. Despite high engraftment rates and attenuated disease progression (change in ejection fraction for EHMs, −6.7±1.4% versus control, −10.9±1.5%; n=12; P=0.05), we observed no difference between EHMs containing viable and nonviable human cardiomyocytes in this chronic xenotransplantation model (n>12; P=0.41). Grafted cardiomyocytes showed enhanced sarcomere alignment and increased connexin 43 expression at 220 days after transplantation. No teratomas or tumors were found in any of the animals (n=14) used for long-term monitoring.

Conclusions: EHM transplantation led to high engraftment rates, long-term survival, and progressive maturation of human cardiomyocytes. However, cell engraftment was not correlated with functional improvements in this chronic myocardial infarction model. Most importantly, the safety of this approach was demonstrated by the lack of tumor or teratoma formation. (Circ Res. 2015;117:720-730. DOI: 10.1161/CIRCRESAHA.115.306985.)

Key Words: cardiac function tests ■ cardiac MRI ■ cell survival ■ myocardial infarction ■ myocardial ischemia ■ tissue engineering ■ transplantation

Myocardial infarction (MI) leads to a substantial loss of cardiomyocytes without significant endogenous regeneration in humans under the present state-of-the-art clinical practice. Reduced force generation and increased stiffness of the infarct scar induce dilative remodeling, which can ultimately result in heart failure and death. The advent of efficient differentiation protocols that allow for the generation of billions of cardiomyocytes from pluripotent stem cells, including both embryonic stem cells (ESCs) and induced pluripotent stem cells, has raised the prospect of cell therapies that aim to replenish lost cardiomyocytes with exogenously generated ones.

Preclinical and clinical studies on cell-based heart regeneration provide evidence that cardiac cell therapies may be realized via implantation of cell suspensions into or around the infarcted scar tissue. Alternatively, tissue engineered heart
muscle (EHM) has been engrafted in allogeneic animal models with chronic and acute MI, providing evidence for enhanced cell retention and heart repair. Clinical translation of direct injection of autologous cell suspensions was relatively straightforward because of the adaptability of, for example, bone marrow cell processing procedures and availability of minimal invasive techniques for cell delivery. However, except for one study showing significant remuscularization of infarcted left ventricle in a nonprimate model, most literature reports limited cell retention and survival, typically <5% viability at 4-6 weeks. Whereas, in acute or subacute cardiac disease, <5% might not be critical, especially if beneficial effects are primarily expected to be because of paracrine signaling. For chronic disease with extensive ventricular scarring and with the goal to remuscularize the failing heart, however, cardiomyocyte retention is essential for maximum therapeutic impact and may be difficult to achieve by cell injections alone. Tissue-engineered constructs help to retain cells at the site of implantation and may, in addition, provide immediate mechanical support to the recipient heart. To date, a range of different cell types and biomaterials have been used to make such mechanical constructs. In vivo studies have mostly been performed in rats because of the availability of primary cardiomyocytes for allogeneic implantation of tissue-engineered grafts. More recent studies have used fibrin or collagen hydrogels comprising human ESC-derived cardiomyocytes (ESC-CMs) or scaffold-free approaches. Cell sheets made from ESC-derived cardiac progenitors have been tested in humans, and sheets made from induced pluripotent stem cell–derived cardiomyocytes have also been tested recently in preclinical models. A challenge to the field is the construction of tissues of a critical thickness to provide mechanical assistance and a sustained transplant retention.

To address these challenges, we constructed macroscale EHM from human ESC-CMs by adapting a technique that has previously shown promising results with rat primary cells in a rat MI model. We generated EHM loops using cell sources and a tissue engineering process compatible with good manufacturing practice. These loops were implanted onto chronically infarcted rat recipient hearts. Cell survival was tracked for ≤220 days using noninvasive imaging and histological characterization of graft size and composition. We quantified changes in infarct size, systolic function, and dilative remodeling using magnetic resonance imaging (MRI), as well as diastolic function, using ultrasound. Finally, we demonstrated the feasibility of a decentralized EHM production and allocation facilitating clinical translation.

### Methods

An expanded Methods section is available in the Online Data Supplement.

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### Cultivation of Human ESCs and Differentiation to Cardiomyocytes

Human H7 ESC line obtained from WiCell (Madison, WI) was expanded in a suspension culture system as previously described to approximately passage 70. Cardiac differentiation was induced with small molecules CHIR99021 and IWP4. Cells were harvested at day 18 post induction. Cell viability, percentage of cardiac troponin T, and CD90 positive cells were assessed using fluorescence–activated cell sorting.

### Generation of EHM

Human ESC-CMs (2.5x10^6) were first mixed carefully on ice with collagen type I and serum-free EHM medium and then cast into custom-made molds according to a previously published protocol. After condensation (5 days in casting molds), EHM were transferred onto mechanical stretchers for functional maturation for an additional 12 to 14 days. EHM media was changed every other day. After quality control (force of contraction >0.1 mN/EHM loop measured by isometric force measurements), EHM were shipped at room temperature with a temperature logger to record ambient temperature in 50 mL polypropylene tubes with 50 mL fresh media. Shipping conditions were established by testing EHM survival and function after 72 hours of mock shipments (EHM immersed in culture medium at an ambient temperature of 21°C). For the xenograft survival studies that relied on bioluminescence imaging (BLI), EHM were constructed from ESC-CMs expressing firefly luciferase and tdTomato red fluorescent protein using the above outlined procedure.

### Force Generation and Viability of EHM

Active force generation of EHM was measured in organ baths before shipment in Göttingen, Germany, and after receipt at Stanford in Tyrode’s solution containing 1.8 mmol/L calcium under 1.5 Hz field stimulation for 1 to 4 EHM loops from each production lot. Cell viability and cardiomyocyte content were assessed before and after shipping using terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling and cardiac troponin T staining.

### MI and EHM Transplantation

MI was induced in 8- to 10-week-old male nude athymic rats (n=74, Charles River, Wilmington, MA) by occluding the left anterior descending coronary artery for 1 hour. This was immediately followed by reperfusion. Surgery was performed aseptically under 1.5% to 2% inhaled isoflurane anesthesia. One month later, MRI and ultrasound imaging were performed, and rats were assigned to the different treatment groups (control, n=14; EHM, n=18; irradiated [not viable] EHM, n=12; and long-term survival experiments, n=14). After baseline imaging (1 month after MI), a second thoracotomy was performed, and EHM were attached to the left ventricular free wall using 8 to 12 stitches (7-0 Prolene). Rats received 2 EHM loops or stitches only for the control group. To evaluate a potential immune suppression regimen for EHM after xenogeneic transplantation, immunocompetent male Sprague Dawley rats (n=7, Charles River) underwent the same procedure but received Tacrolimus (8 mg/kg/d; Astellas Pharma, Northbrook, IL) twice daily for 30 days. Rats were performed by an experienced microsurgeon (M.W.). For survival studies which relied on BLI, EHM made from firefly luciferase and tdTomato reporter line cardiomyocytes were implanted.

### Bioluminescence Imaging

BLI was performed using the Xenogen in vivo Imaging System (Alameda, CA) as previously described. See Online Data Supplement.

### Magnetic Resonance Imaging

Cardiac function and scar size were assessed 1 day before (ie, 1 month after MI) and 28 days after EHM transplantation or sham surgery (control group) using a 7T MR901 Discovery horizontal bore scanner (Agilent Technologies, Santa Clara, CA) as previously described.
Ultrasound Imaging
On days when rats underwent MRI, pulsed wave Doppler and tissue Doppler ultrasound imaging were also performed to assess the diastolic relaxation of rat hearts using a Vevo 2100 ultrasound system (Visualsonics, Toronto, Canada). Diastolic dysfunction was defined as E′/A′ < 1 after a previously published definition. 26

Immunohistochemistry and Histological Methods
Immunofluorescence and histological analyses were performed using standard protocols. See Online Data Supplement.

Statistical Analysis
Results are shown as mean±SEM. To test whether there was a difference in cell viability, cardiomyocyte content, or force generation because of the transatlantic shipment, 2-tailed Wilcoxon ranked sum tests were used. To test if a linear relation between number of viable cells and radiance as measured by BLI exists, we performed a regression analysis. To verify if the radiance from EHM loops changed between day 14 and 85, a 2-tailed Wilcoxon ranked sum test was used. To test if immune suppression in Sprague Dawley rats was a significant factor that might explain the observed variation in radiance and relative radiance, a linear mixed effects model with fixed effects for design, time, and random effects for individual rats was used (the residuals were tested for normality using a Shapiro-Wilk test). To test for differences in cardiac function, a linear mixed effects model was used with fixed effects for functional parameter, treatment group, time and a random effect for individual rats. If such an effect was found, the Wilcoxon ranked sum tests with Bonferroni corrections of P values were then performed. For the comparisons of relative changes, the difference between day 28 and day –1 was calculated for each animal followed by 1-way ANOVA (residuals were tested for normality using a Shapiro–Wilk test). If such an effect was found, Wilcoxon ranked sum tests with Bonferroni corrections of P values were then performed. Statistical analysis was performed using R software version 2.8.1.

Results
Transatlantic Shipment Did Not Change Cell Viability or Contractility of EHMs
To assess the feasibility of EHM generation and transplantation in a clinically relevant scenario, human ESCs were differentiated to cardiomyocytes in spinner flasks at City of Hope following a good manufacturing practice compatible protocol (Figure 1A in the Online Data Supplement). Production batches yielded ≤2 billion cells consisting of ≤95% cardiomyocytes (Figure 1B and IC in the Online Data Supplement). Cryopreserved cells were shipped from California to Germany. They were then thawed and cast into EHM loops, cultured for 17 to 19 days, and shipped for 2 to 3 days from Göttingen, Germany to Stanford, CA (Figure 1A–1C). After arrival, EHMs were allowed to recover for 1 day in an incubator followed by force measurements and histological examinations. Shipment did not lead to adverse changes in cell viability (n=3, P=0.83), viable cardiomyocyte content (n=3, P=0.86), or active force generation (n=3, P=0.87). Figure 1D–ID in the Online Data Supplement) highlighting the feasibility of decentralized production and shipment to application sites. EHM exhibited an average force of 0.35±0.11 mN/ EHM loop (n=9), which is equivalent to 1.07±0.84 nN/viable EHM-CM. Differences in cardiomyocyte yield of 2 differentiation batches used to make EHMs led to slight differences in contractile force and viable cardiomyocyte content before their implantation (Figure II in the Online Data Supplement).

Interestingly, when we performed terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling staining, we noticed that human nuclear antigen antibody labeled only terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling negative cells, indicating the potential suitability of human nuclear antigen positive cells as surrogates for viable human cells (Figure IIIA–IIIID). Closer examination of EHMs revealed regions of densely packed cardiomyocytes with aligned sarcomeres reminiscent of embryonic cardiac tissue typically on the outer edges of EHM (Figure 1E). The majority of EHM cross sections exhibited cardiomyocytes aligned along the primary strain axis, clustered together in band-like structures (Figure 1F and 1G). Cardiomyocytes in these bands showed organized sarcomeres but exhibited little connexin 43 expression, highlighting these cells immature phenotype.

EHMs Demonstrated Stable Engraftment Following Week 2 After Implantation
To assess long-term survival, EHM were made with cardiomyocytes from a firefly luciferase and tdTomato reporter line.

Figure 1. Human engineered heart muscle (EHM) shows cardiomyocyte (CM) alignment, robustness in viability, and force generation after 2 days of shipment. A and B. Photographs of EHM on individual stretchers for mechanical maturation and on a plate before force testing. C. Fluorescence microscopy image of an EHM illustrating the distribution of CMs (α-sarcomeric actin, α-act) across its cross section with slightly higher concentration along the outer boundaries. D. Two days of shipment did not change cell viability (n=3, P=0.83), CM content (n=3, P=0.86), or active force generation (n=3, P=0.87). E. Cardiac-like tissue organization of CMs (human cardiac troponin T, cTnT) was found in some areas of EHMs particular toward the outer boundaries. F. Most of the EHM cross sections showed CM (β-myosin heavy chain, MyHC) alignment in band-like structures along the principle strain axis with low levels of connexin 43 (Connexin 43) expression. G. Band-like cell arrangement is further illustrated by a maximum intensity projection of a 250-μm thick imaging volume from the center of an EHM loop. Scale bars: 5 mm (B); 1 mm (C); 50 μm (E and F); and 100 μm (G).
Differentiation of reporter line ESCs to cardiomyocytes using a monolayer version of the small molecule protocol yielded between 80% and 90% cardiomyocyte purity (Figure IVA and IVB in the Online Data Supplement). EHMs made from these cells generated a strong bioluminescent signal before and after transplantation onto infarcted hearts of nude rats (Figure 2A). The BLI signal of implanted grafts declined for the first 2 weeks after implantation. However, no significant decline was found between week 2 and 12 (n=6, P=0.33), indicating stable long-term engraftment and survival of ≤25% in individual rats with an average of 10±4% (Figure 2B and 2C). We used relative radiance (radiance normalized to an initial maximum) as a surrogate for cell viability and engraftment because we observed a strong linear correlation between viable cells and measured average radiance (Figure IVC–IVE in the Online Data Supplement, intercept: P=0.30, slope: P=2.2E−16).

**EHM Graft Survival in Immunocompetent Rats With Immune Suppression Is Comparable With Nude Rats**

Further development of EHMs as a potential therapy for heart failure will require functional assessments in larger animal models for which no immunocompromised strains exist to date. Thus, we also decided to test the suitability of Tacrolimus to prevent EHM rejection in immunocompetent Sprague Dawley rats. As expected, transplantation of EHMs without immune suppression led to rejection and almost complete cell loss after 1 week (n=2). In contrast, twice daily oral administration of Tacrolimus enabled cell survival similar to implantations in nude rats illustrating the suitability of this approach (Figure 2D and 2E, n=5, SD+Tacrolimus; n=6, nude rats; P=0.88).

**EHM Grafts Consist Primarily of Cardiomyocytes 1 Month After Implantation**

Human EHMs could be delineated on hematoxylin and eosin or Masson’s trichrome–stained sections via a small layer of fibrotic tissue separating them from the host myocardium (Figure 3A–3D). Although EHMs contained a substantial number of cell debris, and cardiomyocytes were primarily aligned in band-like structures prior to implantation, 1 month after implantation, we observed human grafts consisting of densely packed cardiomyocytes. We did not observe any fibroblasts in these grafts regardless of the fibroblast content in cell batches used to make EHM. Human grafts were found in all analyzed animals that received EHM (5/5 rats; Figure 3E and 3F). Cells staining positive for human markers were almost exclusively cardiomyocytes interspersed with host-derived vessels (Figure 3E). Cardiomyocytes themselves were not aligned along the major strain axis and they expressed...
little connexin 43 (Figure 3F). In line with this early embryonic phenotype, sarcomere width was small, not spanning the width of cells (Figure 3G).

EHM grafts contained few rat macrophages dispersed in the graft, indicating stable engraftment, corroborating our BLI data (Figure 3H). Graft thickness was typically 300 to 400 µm leading to a graft size of 0.5±0.1 mm³ (n=5) at 1 month after implantation (Figure VA and VB in the Online Data Supplement). Only a small number of human nuclei were in an active state of the cell cycle as evidenced by Ki67 labeling (0.95±0.15%, Figure VC and VD in the Online Data Supplement).

**High Cardiomyocyte Engraftment Rates Did Not Translate into Significant Functional Improvements**

It is unlikely that a cardiac patch or cardiomyocyte cell suspensions could be implanted shortly after MI in humans because this would require manufacturing immune-matched patches or cells in advance and might interfere with other acute treatment strategies. Hence, we focused on whether EHM implantations would have any effects on recipient hearts in a chronic MI model. For that, implantation of EHM (n=14), irradiated EHM (n=9, a control for mechanical effects and dead cells), or control surgery (n=12, sham) were performed 1 month after MI. Systolic function and scar size (assessed by MRI), as well as diastolic relaxation (assessed by ultrasound), were measured 1 day before and 1 month after implantation or sham surgery (Figure 4A–4C, Figure VI in the Online Data Supplement). End-diastolic and end-systolic volumes increased over time in all groups (Figure 4D and 4E). Although increases in end-diastolic volumes were not significantly different (P=0.35), end-systolic volumes increased faster in the control group than in the EHM group (control n=12 versus EHM n=14; P=0.04). This difference was also reflected in a more pronounced decline in ejection fraction for the control group compared with EHM and irradiated EHM groups (P=0.03, Figure 4F and 4G). Notably, changes in ejection fraction were not different between viable and nonviable (lethally irradiated) EHM (P=0.19). This finding is consistent with the anticipated lack of electric integration of human xenografts in rat hearts, but highlights the possibility that cell-independent effects (eg, activation of immune cells, mechanical stabilization) could also elicit therapeutic effects. EHM implantations did not lead to any changes in scar size either (P=0.12; Figure 4H–4J). Finally, we reasoned that EHM transplantation may affect diastolic function because of left ventricular stiffening. We did not observe any significant changes in diastolic function because of EHM transplantations (control treatment, n=9; EHM, n=12; irradiated EHM, n=8; P=0.12; Figure 4K). Morphologically, irradiated EHM did contain high numbers of fibroblasts/myofibroblasts and macrophages (Figure VII in the Online Data Supplement). A summary of all parameters measured via MRI and ultrasound can be found in Tables I and II in the Online Data Supplement.

**Long-Term Engraftment Led to More Mature Cardiomyocyte Organization**

Tumor or teratoma formation is the primary safety concern for all cell therapies derived from pluripotent cells. In
addition, regenerative therapies need to demonstrate functional integration of transplanted cells into the host tissues. To address these concerns, EHM grafts were analyzed at 110 (Figure 5A–5D) and 220 days (Figure 5E–5H) after implantation. No tumors or teratomas were found in any of these rats at 110 or 220 days (n=9 and n=5, respectively). Human grafts contained a high number of host-derived blood vessels, almost comparable with normal myocardium (Figure 5A and 5E, Figure IXA and IXB in the Online Data Supplement). Cardiomyocyte alignment and connexin 43 expression increased from day 110 to day 220 reaching a level similar to later fetal development stages in humans (Figure 5B and 5F). Cardiomyocytes exhibited well-organized sarcomeres which frequently spanned the width of cells and aligned with the circumferential strain axis (Figure 5C and 5G). Grafts contained a small number of human cells which were in an active state of the cell cycle (0.93±0.18%, Figure 5D and 5H).

Figure 4. Engineered heart muscle (EHM) implantation reduces dilative remodeling but this effect is not because of human cardiomyocyte (CM) engraftment. A–C. The top row shows representative 2-chamber long-axis views at end-diastole (ED) 1 day before and 28 days after EHM implantation or sham surgery (control group). The middle row shows the same hearts at end-systole (ES), whereas the bottom row shows corresponding late gadolinium enhancement images (LGE) at midventricular level. D and E, ED volumes increased in all groups but were not significantly different (P=0.35). ES volumes also increased in all groups, but the increase in the control group was most pronounced compared with all other groups (control, n=12 vs EHM, n=14; P=0.05). F and G. Ejection fractions declined in all groups. The control group showed the highest relative decline compared with EHM implantation groups (P=0.03); however, there was no difference between EHM and irradiated EHM grafts that did not contain viable CMs (P=0.19), indicating that beneficial effects on remodeling may not be directly mediated by grafted cells. H–J. No significant difference was observed for changes in scar size over time (P=0.32). K. There was a trend toward increase in diastolic dysfunction (E/A′<1) for hearts receiving irradiated EHM loops, whereas a small decline was observed for the other groups; however, this did not reach statistical significance (P=0.12). Scale bars: 5 mm (A–C).
maturation, we also found occasional areas in grafts consisting of clusters of glycogen-rich cells exhibiting a hamartoma-like phenotype (n=4 of 4 rats tested), characterized by large spider cells containing cleared areas and intervening strands of cytoplasm (Figure 5I). Fewer and smaller cleared cytoplasmic areas were found at day 110 compared with day 220, but cells were rich in glycogen, indicated by Periodic acid–Schiff staining (Figure IXC–IXF in the Online Data Supplement).

Hamartoma-like cells not only exhibited smooth muscle actin expression but also contained sarcomeric proteins which sometimes arranged into myofibrils (Figure 5J–5K, Figure IXG and IXI in the Online Data Supplement). Similar to clinical observations of naturally occurring hamartomas, we did not find any indication of a proliferative phenotype of hamartoma-like cells, as we detected no cells staining positive for the proliferation marker Ki67 (Figure 5L; Figure IXJ in the Online Data Supplement).

**Discussion**

Preclinical development of potential cell therapies should be performed using clinically relevant cell production protocols and suitable animal models. This will involve production of cells in large batches followed by cryopreservation and direct delivery of cryopreserved cells after thaw as has been recently demonstrated or processing them into an implantable product, such as cardiac patches or EHM. As this will require good manufacturing practice facilities that are not widely available, cardiac patches or EHM will have to be robust enough to be transported to an application site. We have demonstrated that EHM can be generated from human ESC-CMs in sufficient numbers for preclinical experiments and survive 2 to 3 days of shipment without impact on EHM function. Transport stability should facilitate the potential clinical translation of this approach.

We used 2 spinner flask differentiation batches and 2 monolayer differentiation batches to generate all the cardiomyocytes and reporter line cardiomyocytes required for this study. The cardiomyocyte yield varied from 70% to 95%, EHM made from these batches showed small differences in cardiomyocyte content and contractile forces. In contrast to monolayer cultures where cells that died after thawing and plating were discarded with media changes,
tissue-engineered constructs retain cells that died during the formation. In this context, it is important to note that plating efficiency of cryopreserved cardiomyocytes is generally similar (50%–80%) in classical monolayer and EHM culture. Thus, a high count for nuclear fragmentation of apoptotic cells in EHM leads to an overestimation of cell death because of 3-dimensional culture. Likewise, dead cardiomyocytes do not stain positive for cardiac markers and can hence not be separated from noncardiomyocytes. These differences need to be considered when EHM (typical thickness, 0.8–1 mm) are compared against thin tissue-engineered constructs or single cell sheets with thicknesses <70 µm. Given the different techniques and shapes used to make cardiac tissues, comparing contractile forces on a force per cell basis may be a more suitable comparison.

Our EHM loops generated 1.07±0.84 nN force/viable cardiomyocyte, which is similar to previous reports. 27,28 The microstructure of tissue-engineered constructs is dependent on the geometry and production process. In line with previous publications, EHMs exhibited cardiomyocyte alignment along the major strain axis and low expression levels of the gap junction protein connexin 43 as is anticipated for embryonic cardiomyocytes. 17,27,28

Although transport stability is an important parameter, long-term survival and functional integration are arguably the most important aims for regenerative cell therapies. Previous studies have primarily relied on histological assessments performed 1 month or earlier after implantation. 8,13,14,19,29 Although instructive, assessments of graft size and survival kinetics are difficult to perform histologically. BLI of genetically modified reporter cell lines allows for the noninvasive tracking of cell survival over time. Using this approach, we found that cell death was limited to the first 2 weeks following implantation with no significant cell loss afterward ≤85 days. Cell survival/engraftment did not change after the second week following transplantation. This indicates that engrafted cells should survive for the entire lifespan of the animal. EHM implantation led to long-term engraftment rates of 25% in some animals, exceeding previously reported engraftment rates of <5% for single-cell suspensions. 7,10,11 The factors responsible for cell death observed during the first 2 weeks are difficult to parse out. It is likely that diffusion-limited supply of nutrients and oxygen is an important component, particularly because EHMs are not immediately perfused via a preformed vasculature. In contrast, EHMs are vascularized via an ingrowth of capillaries. 15,30 Previous studies have indicated that including endothelial cells in tissue-engineered constructs can lead to improved engraftment rates. 29,31 However, it is difficult to attribute this better engraftment to faster revascularization because timescales for that have not been assessed. Moreover, the presence of endothelial cells or even preformed capillaries per se does not resolve the issue of lack of immediately communicating vasculature. A potential limitation of our approach is the use of a constitutively active reporter ESC cell line where the expression of luciferase is not coupled to the expression of cardiac genes. Nonetheless, with a cardiomyocyte purity of ≥90% following differentiation of the reporter line and grafts consisting almost exclusively of human cardiomyocytes, it is likely that the survival and engraftment estimates accurately track the behavior of human cardiomyocytes.

Composition and cellular phenotype of grafts are important considerations when assessing the ability of implanted structures to integrate into the host myocardium. One month after implantation, EHMs contained a host-derived vascular network similar to previous reports for cell injections 8 and patch transplantations. 29,32 Although EHMs contained dead cells, cardiomyocytes aligned in band-like structures, and fibroblasts in vitro, 1 month after implantation, grafts consisted of densely packed living human cardiomyocytes that showed limited sarcomere alignment, similar to previous reports for cell injections 4 or patch transplantations. 15

The lack of fibroblasts in grafts indicates a preferential survival of human cardiomyocytes because EHMs were made from cell batches containing ≤30% of fibroblasts. Grafts contained few macrophages, indicating that grafts had stabilized at this time point in concordance with our BLI data. Connexin 43 expression was low and poorly organized in these grafts, indicating the immature phenotype of these cardiomyocytes, consistent with what, has been demonstrated previously. 32,33

EHMs demonstrated good engraftment and survival by longitudinal BLI and postmortem histology, we therefore decided to assess functional changes in the recipient hearts in a clinically relevant chronic rat MI model because there are already many available treatment options for acute MI. Standard of care treatment for acute MI in humans includes the use of antithrombotic drugs, antiplatelet therapies, and rapid reperfusion of the culprit vessel. These measures alleviate myocardial ischemia and reduce scar sizes. New developments, such as drug eluting stents, modulation of inflammation, inhibition of apoptosis pathways, post conditioning, and blocking of mitochondrial transmembrane pore opening, are currently under investigation. 30 In addition to these pharmacological approaches, adult stem cell therapies are currently being tested as a potential treatment option for acute and subacute MI. 1 Although improvements in the treatment in acute MI have been made, a substantial number of patients will progress to heart failure. This patient population with chronic MI would benefit most from regenerative therapies that can reduce adverse remodeling and restore contractility.

In our study, we used a 1-hour ischemia/reperfusion model which led to moderate rat heart infarct sizes (≈10% of LVM) and performed therapeutic interventions 1 month after MI. Cardiac MRI illustrated continued dilative remodeling in all groups, but significantly faster increases in end-systolic volumes were observed for the control group. EHM implantation significantly reduced the decline in ejection fraction compared with the control group. However, there were no significant differences between EHMs and irradiated EHMs which did not contain any viable human cells, indicating that the observed effect is not be mediated by living cells, but likely mechanical and immune cell related. Our data imply that despite high engraftment rates and stable long-term survival of human ESC-CMs, recipient rat hearts did not show any functional improvements attributable to cell transplantation in this
chronic MI model. Although this may be surprising in light of previous published studies that found functional improvements, however, those studies were primarily observations in acute and subacute models with cell delivery shortly after MI.\textsuperscript{5,7,10,35,36} Paracrine factors may have a limited impact on chronic scar tissue,\textsuperscript{7} and mechanical contributions are unlikely from human cardiomyocytes which cannot contract at the rate of the recipient rat heart (≈400 bpm). A similar observation has previously been made for direct injection of human ESC-CMs into a chronic rat MI model, which also failed to find functional improvements.\textsuperscript{37} There is limited data available assessing functional improvements in rats after transplantation of tissue-engineered constructs or cell sheets containing human cardiomyocytes and none of them have used a chronic MI model.\textsuperscript{19} It is also possible that longer observation periods are required to observe beneficial effects from human cardiomyocyte survival in chronic rodent MI models. We observed a high number of fibroblasts or myofibroblasts in irradiated EHMs, but we did not observe any changes in diastolic function for control or EHM groups. Any material or tissue-engineered construct transplanted onto the heart might affect diastolic function. More detailed studies are needed to systematically address the risk of clinically relevant diastolic dysfunction associated with tissue-engineered heart repair.

A xenogeneic cell transplantation model in rats can only give limited information on functional coupling of contractile human cardiomyocytes. Electric coupling of human cardiomyocytes with rat hearts has never been demonstrated likely because of inherent heart rate differences (60–120 versus 400 bpm). In contrast, electric coupling of human cardiomyocytes has been demonstrated in a guinea-pig model with heart rates of 200 to 250 bpm.\textsuperscript{38} Although electric coupling is a prerequisite for mechanical force contribution from transplanted cells, the lack of immune-deficient guinea-pig or larger animal models renders these models unsuitable for long-term engraftment, cell maturation, vascularization, and tumor/teratoma risk assessment. Accordingly, immunocompromised rat xenograft models are of high regulatory relevance (according to FDA and the Paul-Ehrlich-Institute). However, the lack of electric integration reduces the use of rodent models to assess arrhythmia risk which could be high because of the immature phenotype of cardiomyocytes at the time of transplantation. A recent study observed substantial albeit nonlife threatening arrhythmia after injection of 1×10^9 cardiomyocytes into the myocardium of nonhuman primates.\textsuperscript{6} Large animal studies will be required to assess the arrhythmia risk from EHM transplantation. In contrast to grafts at 1 month after implantation, grafts that had been implanted for >3 months showed a higher vascular density. Long-term engraftment also improved sarcomere structure and alignment, as well as connexin 43 expression patterns.\textsuperscript{1} This was particularly evident for transplants that had been engrafted for 220 days, where connexin 43 expression exhibited a pattern typical for early postnatal stages in humans,\textsuperscript{39} which has not been reported for ESC-CMs thus far.\textsuperscript{5,6,37} This data indicate that transplanted human cardiomyocytes will mature in the host even in a xenogeneic setting. Prolonged survival of cardiomyocytes exposed to cyclic stress and electric stimulation is likely important for improved maturation. However, additional research will be required to assess whether other factors present in the local environment play a role in this process as well.

Although this is encouraging, we also noticed the appearance of foci of glycogen-rich cardiomyocytes in grafts that had been implanted for >3 months. These nonproliferating collections of cardiomyocytes expressed cardiac markers, but had overly abundant glycogen exhibiting a hamartoma-like phenotype.\textsuperscript{30,41} The exact histogenesis of these foci is not known, but reports in pediatric cardiac hamartomas have indicated that they may regress over time.\textsuperscript{32} Interestingly, a hamartoma-like cardiomyocyte phenotype has not been described previously for ESC-CMs, which might be because of differences in the differentiation protocols, EHM manufacture, and the longer engraftment period assessed in this study.\textsuperscript{4,6,29,32,37}

Potential safety implications of hamartoma-like cells will require further characterization, particularly the reversibility of this cardiomyocyte phenotype. No disconcerting evidence for other safety parameters was found: we did not observe any animal death during our studies, indicating that lethal arrhythmias did not occur nor did we observe any tumors or teratomas in any of the animals used for this study.

In summary, this study demonstrates that implantation of EHM made from human ESC-CMs leads to long-term engraftment of implanted cells in a chronic MI model. We observed progressive maturation of human grafts with sarcomere alignment after 3 months and enhanced connexin distribution supporting the notion that ESC-CMs can mature in vivo in a xenogeneic setting. Advancing tissue engineered heart repair into relevant large animal models will be a key step to demonstrate both safety and preliminary efficacy, in order to enable clinical translation.

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Disclosures

None.

References


Novelty and Significance

**What is Known?**
- Cardiomyocyte loss after myocardial infarction can lead to deleterious remodeling of the heart culminating in heart failure.
- Transplantation of human embryonic stem cell-derived cardiomyocytes (ESC-CMs) shortly after experimentally induced myocardial infarction improves cardiac function in animal models.
- Survival of ESC-CMs after transplantation is low, potentially limiting the efficacy of this approach.

**What New Information Does This Article Contribute?**
- Transplantation of ESC-CMs as macroscopic tissue-engineered structures leads to high cell engraftment rates and stable long-term survival.
- ESC-CM engraftment did not correlate with functional improvements in a rodent model where cells were transplanted 1 month after myocardial infarction.
- ESC-CM transplantation was safe as no tumors or teratomas were detected during long-term follow-up.

Cardiac regeneration requires the replacement of cardiomyocytes lost during periods of oxygen undersupply, such as myocardial infarction. This may be achieved by delivering ESC-CMs. However, survival of transplanted cells in the heart is low, limiting the feasibility of this approach. In this study, we provide evidence that tissue engineering approaches can improve cell retention and lead to long-term engraftment and survival of transplanted cells. Furthermore, progressive structural maturation of transplanted human cardiomyocytes was observed. Although engraftment is a prerequisite for functional benefits from active force generation by transplanted cells, we did not observe such benefits in a chronic myocardial infarction model. This indicates that functional benefits from human cardiomyocytes may not be accurately estimated in rodent myocardial infarction models. However, rodent models are suitable to assess long-term cell survival and tumor or teratoma risk, which are difficult to assess in larger animals where immunocompromised strains do not exist. Our study shows that transplantation of ESC-CMs did not lead to tumor or teratoma formation, which is the primary safety concern for therapies using cells derived from ESCs. These results encourage further translational research in large animal models to estimate the functional benefits from such a therapy.
Human Engineered Heart Muscles Engraft and Survive Long Term in a Rodent Myocardial Infarction Model


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SUPPLEMENTAL MATERIAL

Human engineered heart muscles engraft and survive long term in a rodent myocardial infarction model

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SUPPLEMENTAL METHODS

**Cultivation of human ESCs and differentiation to cardiomyocytes.** Undifferentiated H7 human ESCs obtained from WiCell (Madison, WI) were maintained in aggregates in suspension culture as previously described\(^1\). Briefly, suspension-adapted ESCs were seeded as single cells at a density of 2.5-3x10^5 cells/mL in spinner flasks (Corning) containing culture medium StemPro hESC SFM (Life Technologies) with 40 ng/mL bFGF (Life Technologies) and 10 \(\mu\)M Y27632 (EMD Millipore). Medium was changed daily by demi-depletion with fresh medium without Y27632. Cells were single cell-dissociated with Accutase (Millipore) and passaged every 3-4 days. Cell suspension cultures were maintained in 5% CO\(_2\) with 95% relative humidity at 37°C.

For cardiac differentiation, undifferentiated ESC suspension culture was expanded to a scale sufficient to seed in 1L spinner flasks. Passage 70 had been reached at the time of differentiation. The cell aggregates in suspension cultures were directly induced with 18 \(\mu\)M CHIR99021 (Stemgent) in RPMI 1640 medium (Life Technologies) with 1x B27\(^\circ\) Supplement minus insulin (Life Technologies) for 24 hr. On day 3 of differentiation, 5 \(\mu\)M IWP-4 (Stemgent) was added for 2 days to further induce cardiac differentiation. From day 7, RPMI 1640 with 1x B27\(^\circ\) Supplement (Life Technologies) was used as basal medium. Thereafter, 60-80% of medium was changed every 2-3 days until cell harvest around day 18-21. At cell harvest, differentiated cell aggregates were treated with Liberase TH (Roche) at 37°C for 20-30 min and then were dissociated into single cells with TrypLE (Life Technologies) at 37°C for 5-10 min. Cells at 1-3x10^7 cells/mL were cryopreserved with CryoStor CS10 (Biolife Solutions, Inc.) supplemented with 10 \(\mu\)M Y27632 in liquid nitrogen.
**Generation, cultivation and differentiation of tdTomato and Luciferase positive hESCs.**

**Generation of firefly luciferase-tdTomato (Fluc-tdT) reporter cell line:** Human ESCs (H7) were transduced with a lentiviral vector carrying an EF1 promoter driving firefly luciferase-T2A-tdTomato red fluorescent protein reporter gene. Cells expressing tdTomato were selected post-transduction using fluorescent activated cell sorting (FACS; BD Aria II, BD Bioscience, San Jose, CA).

**Culture of human ESCs:** H7 ESCs were grown as described previously on Matrigel-coated plates (ES qualified, BD Biosciences, San Diego) using chemically defined E8 medium as described previously. The culture medium was changed daily and cells were passaged every four days using Accutase (Global Cell Solutions, Charlottesville, VA).

**Cardiac differentiation:** Human ESCs were grown to 90% confluence and subsequently differentiated into beating cardiomyocytes using a small molecule-based monolayer method adapted after Lian et al. and described by Ebert et al.

**Generation of engineered heart muscle.** Human ESC-CMs (2.5x10^6) were first mixed carefully on ice with collagen type I and serum-free EHM medium and then cast into custom-made molds according to a previously published protocol. Following condensation (5 days in casting molds), EHM were transferred onto mechanical stretchers for functional maturation for an additional 12-14 days. EHM media was changed every second day. Following quality control (force of contraction > 0.1 mN/EHM loop measured by isometric force measurements), EHM were shipped at room temperature with a temperature logger to recorded ambient temperature in 50 ml polypropylene tubes with 50 ml fresh media. Shipping conditions were established by testing
EHM survival and function after 72 hr of mock shipments (EHM immersed in culture medium at an ambient temperature of 21°C).

**Measuring contractile forces of EHM**s. The isometric force test apparatus consisted of a force transducer (Model 724480, Harvard Apparatus, Holliston, MA) with two selectable ranges, 0-0.005 N and 0-0.05 N, and accuracy of 1%; a three-axis motorized positioning system (Model MA4012C-S4-0, Velmex, Bloomfield, NY); and a custom made tissue holder with two 5 mm flexible platinum stimulation electrodes positioned next to two L-shaped stainless steel holders. During tissue stimulation, the test apparatus was immersed in Tyrode’s solution containing 1.8 mM Ca$^{2+}$ (T2397, Sigma, St. Louis, MO). The media was constantly circulated via a variable flow mini-pump (Model 57951-016, VWR, West Chester, PA) and maintained at 37°C. An electrical field stimulator (Model SIU-102, Warner Instruments, Hamden, CT) was used to pace EHM by applying biphasic pulses (1.0 Hz, 10 ms total pulse width, 20V peak-peak) across the platinum electrodes spaced approximately 1-2 cm apart across the tissue. Analog voltage signals from the force transducer were transferred to a USB data acquisition device (USB-6009, National Instruments (NI), Austin, TX) and displayed, analyzed, and recorded with LabView 8.2 software (NI, Austin, TX).

EHMs were mounted around the two L-shaped holders of the force test apparatus. EHM were gradually stretched stepwise, by increments of 125 µm every 15 seconds until total displacement reached 3 mm corresponded to an axial tissue stretch of approximately 15%. Electrical field stimulation of 1 Hz was applied to the tissue at all given stretches, and the resulting isometric forces were sampled to obtain 25 data points per beat. Active and passive force analysis was done using a custom Matlab® script. Twitch force and passive forces are the
maximum and minimum forces produced in each contractile cycle. Active force is the force generated solely during the contraction and was calculated as the difference of twitch and passive forces.

**In vitro BLI of cells and EHM.** Fluc-tdT reporter human ESCs were expanded and differentiated into cardiomyocytes as described above. Cardiomyocytes (differentiation day 25) were detached using trypsin and filtered through a 50 µm pore size filter to avoid cell clumps. The number of viable cells was estimated using Trypan blue exclusion with an automatic cell counter (Countess, Invitrogen, Carlsbad, CA). Cells were diluted and plated in 200 µl media at varying concentrations (1x10^5, 5x10^4, 2.5x10^4, 1.25x10^4, 6.25x10^3, 3.13x10^3, and 1.56x10^3) in a 96-well plate. D-Luciferin (0.05 mg/well, Biosynth International, Itasca, IL) was added and the radiance was measured immediately afterwards using the same bioluminescence imaging (BLI) system used for rats. EHM made from Fluc-tdT cardiomyocytes were removed from their stretchers four hours after arrival in Stanford and placed into 6-well plates with 2 ml of EHM media. D-Luciferin (0.5 mg/well, Biosynth International, Itasca, IL) was added and the radiance was measured every minute until the signal started to decline using the same BLI system used for rats.

**EHM irradiation.** To evaluate the effect of the EHM matrix itself on transplanted hearts, cells in EHM were killed with a high radiation dose. Individual EHMs were placed in 15 ml conical centrifuge tubes, containing culture media, and irradiated with 60 Gray. The irradiator employed in these experiments was a Mark I-68A 137Cs irradiator (JL Shepherd and Associates, San
Fernando, CA). Cell death was verified by TUNEL staining of representative cross sections from irradiated loops.

**Myocardial infarction and EHM transplantation.** Male nude rats (Crl:NIH-Foxn1nu, n=74) and immune competent Sprague Dawley rats (n=7) (Charles River Laboratories, Wilmington, MA) aged 8-10 weeks were used for this study. Anaesthesia was induced with 3-4% isoflurane in oxygen and was maintained at 1.5-2%. Rats were placed on a heating pad with a feedback controller and maintained at 36±1 °C during the surgical procedure. Pre-emptive analgesia in the form of buprenorphine 0.01-0.05 mg/kg or Carprofen subcutaneous and bupivacaine 0.5-2 mg/kg (local infiltration) was provided as well as eye lubrication ointment. After intubation, rats were ventilated and left thoracotomy was performed between the 3rd and 4th ribs. The pericardial sac was opened and the left anterior descending (LAD) coronary artery was occluded for one hour with a 5-0 Prolene suture close to the left atrial appendage. The chest and the incision were closed after LAD reperfusion. An initial MRI scan was performed one month after this surgery to exclude rats with insufficient infarcts (EF >65%, baseline EF before surgery was 73±1%). All rats fulfilling the inclusion criteria (58/74) were randomly assigned to the different treatment groups. Two to four days after the initial MRI and group assignment, a second thoracotomy was performed and EHM s were attached to the left ventricular free wall with 8-12 stitches using a 7-0 Prolene suture. The control group received the same number of stitches without attaching anything to the heart. All surgeries were performed by an experienced microsurgeon (M.W.). Rats received postoperative analgesia for three days and antibiotics via their drinking water (Enrofloxacin, 2.5-4 mg/kg) from the surgery day on until the end of the experiment.
Immune suppression of Sprague Dawley rats. To prevent immune rejection, Tacrolimus (8 mg/kg/day Astellas, Northbrook, IL) was orally administered every 12 hours starting two days before EHM transplantation. Through values were measured to ensure that Tacrolimus levels in the blood were between 10-15 ng/ml. Rats received antibiotics (Sulfamethoxazole / Trimethoprim) via their drinking water throughout the experiment to prevent infections.

Bioluminescence imaging of rats. Rats were anesthetized with isoflurane, D-Luciferin (170 mg/kg, Biosynth International, Itasca, IL) was injected intravenously via the tail vein catheter, and rats were placed into a BLI system (Xenogen, Alameda, CA). Images were acquired every minute until a stable decline in signal intensity could be observed. Regions of interest (ROI) were placed over the chest centred at the pixel with maximum signal intensity. The ROI with the maximum of the average signal intensity from all the ROIs of one time series belonging to one animal was selected as measurement value for that imaging session. The ROI size was kept constant for all animals and experiments. In order to get a better estimate of the number of transplanted cells, the first BLI time point was always 4 hours after transplantation surgery.

Magnetic resonance imaging (MRI). Imaging was performed 1-4 days prior and 30 days after EHM/Sham implantation, using a preclinical 7T (MR901 Discovery) horizontal bore scanner (Agilent, Santa Clara, CA) with a shielded gradient system (600 mT/m). Rats were anesthetized with isoflurane (3%) and placed onto an animal cradle in prone position. Animals were kept at 37±0.4°C (during image acquisition) via an air heating system while oxygen and anesthetics (1-2% isoflurane) were supplied via a nose cone (0.5 L/min). Data acquisition was performed with a 4-channel phased array receive only surface coil (Rapid MR International, Columbus, OH)
placed around the heart and centred in a decoupled 72 mm transmit/receive volume coil (Agilent). Long- and short-axis scout images were acquired to define the two- and four-chamber long-axis views. The cine long-axis views were used to define the short-axis orientation. A prospectively double gated (ECG and respiration) spoiled gradient echo sequence was used to acquire cine cardiac images with the following parameters for standard cine acquisitions: TE 1.5 ms, TR 6-8 ms, flip angle 15°, slice thickness 1 mm, no slice separation, FOV 50×50 mm², matrix size 192×192, NSA 1 for short-axis and 2 for long-axis. Twenty cine-frames were recorded to cover the cardiac cycle. A single short-axis slice was obtained in approximately 45 seconds, leading to a total scan time of 11-13 min covering the heart from base to apex (14-15 slices). For infarct size measurements, late gadolinium enhancement (LGE) images were acquired 8 min after i.v. infusion of gadolinium (0.8 mmol/kg, Magnevist Bayer, Germany) using an inversion recovery gradient echo sequence with inversion time optimised to null the healthy myocardium. Imaging parameters for these acquisitions were as follows: TE 1.4 ms, TR one breathing interval, TI 280-370 ms, flip angle 90°, slice thickness 1 mm, no slice separation, FOV 40×40 mm², matrix size 192×192, NSA 2, views per segment 2. The acquisition time was roughly 1 min per slice. The imaging protocol for one rat typically required 45-50 min. All images from one animal were combined to a dataset, randomized, and anonymised. Data analysis was performed using the semi-automatic segmentation software Segment (Medviso AB, Sweden) as previously described.

**Ultrasound (US) imaging.** One day prior and 28 days after EHM implantation or control surgery, color Doppler and tissue Doppler US imaging were performed to assess the diastolic function of rat hearts. Rats were anesthetized using 2% isoflurane (Vet One, Meridian, ID, USA)
and imaged in a supine position on a heated platform. Imaging was performed using a real-time microvisualization transducer (MS250) with a frequency of 25 MHz connected to a Vevo 2100 ultrasound system (Visualsonics, Toronto, Canada).

Pulse wave Doppler images were obtained in the apical four-chamber view to record the transmitral flow spectra. The Doppler sample volume was placed in the center of the mitral orifice at the tip level of the mitral leaflets and the peak velocity of early (E) and late (A) transmitral filling was recorded. For tissue Doppler imaging (TDI), the sample volume was placed at the septal side of the mitral annulus to record early (E’) and late (A’) diastolic mitral annulus velocity. Data analysis including calculation of the LV diastolic function indices such as E/A and E’/A’ was performed off-line with the use of a commercially available Vevo Analytic Software by a blinded observer. Diastolic dysfunction was defined as E’/A’ <1 following a previously published definition11.

Immunodetection and histological methods

Heart fixation and section preparation: Rats were anesthetized, cuts into the liver were made and their hearts were perfused with 50 ml cold PBS (4 ºC) containing 0.1 mol/l KCl through a 25G butterfly needle inserted into the left ventricle via the apex. Hearts were cut out and fixed over-night at 4ºC in PBS with 4% PFA. Following fixation, hearts were transferred into 30% sucrose solution and kept at 4ºC until equilibrium was reached. Hearts were embedded in OCT and frozen in hexane containing dry ice. Sections were cut with a cryostat (Leica, Wetzlar, Germany) collected on glass slides, dried and stored at -80ºC.

EHM fixation and section preparation: EHMs were fixed for two hours at 4ºC in PBS with 4% PFA. After fixation, EHMs were processed as described above.
Cell death: TUNEL staining was performed on 25 µm thick cryosections using a commercial kit (Roche, Mannheim, Germany) following the manufacturer’s instructions except for the following modifications. Sections were permeabilized in PBS with 0.5% Triton-X (Sigma) for one hour at room temperature. The TUNEL reaction mixture was diluted 1:4 with TUNEL dilution buffer. After TUNEL staining, sections were washed 3x 10 min with PBS and a standard immunofluorescence staining procedure followed.

Immunofluorescence: Sections were equilibrated to room temperature, washed 3x 10 min with PBS, permeabilized with 0.5% Triton-X (Sigma) in PBS for 60 min at room temperature followed by incubation in blocking solution (5% donkey serum in PBS + 0.1% Tween20, Sigma) for 60 minutes. Sections were incubated with primary antibodies (see Supplemental Table III), and diluted in blocking solution over night at 4ºC in a humid chamber. After washing 3x 15 min with PBS + 0.1% Tween20, sections were incubated with secondary antibodies diluted in blocking solution for 1 hour followed by a final washing step (3x 15 min in PBS + 0.1% Tween20) and covered with cover slips using self-hardening mounting media. Confocal microscopy was performed using a Leica SP8 microscope (Leica, Wetzlar, Germany). A series of images was acquired with a 20x or 63x oil immersion objective. Images were stitched together to generate composite images for further analysis.

Image analysis: Composite images were analysed using Volocity (PerkinElmer, Waltham, MA). Automatic segmentation of nuclei was performed followed by automatic detection of stained objects at respective channels. A region of interest was manually drawn to limit the analysis to the graft. Apoptotic cells were detected by automatic segmentation of TUNEL positive nuclei and co-localization with DAPI positive nuclei. For each EHM, 1,000-2,000 nuclei were analyzed. To estimate the graft size, confocal images were acquired and stitched together to
cover the entire graft. The area staining positive for human beta integrin 1 was estimated using Volocity and multiplied by the number of sections showing a graft and the tackiness of the sections.
**Supplemental Table I: MRI measurements for control and treatment groups**

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<th>Day 1</th>
<th>Day 28</th>
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<tr>
<td></td>
<td>Control (n=12)</td>
<td>EHM (n=14)</td>
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<tr>
<td>Body weight [g]</td>
<td>261 ± 14</td>
<td>258 ± 10</td>
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<tr>
<td>Heart rate [bmp]</td>
<td>393 ± 9</td>
<td>390 ± 5</td>
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<tr>
<td>EDV [µl]</td>
<td>443.4 ± 14.6</td>
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<td>SV [µl]</td>
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<td>56.4 ± 2.6</td>
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<td>CO [ml/min]</td>
<td>96.7 ± 2.7</td>
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<td>LVM [mg]</td>
<td>508.7 ± 8.7</td>
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<td>Scar [µl]</td>
<td>39.1 ± 4.7</td>
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<tr>
<td>Infarct [% of LVM]</td>
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<td>Body weight [g]</td>
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<td>Heart rate [bmp]</td>
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<td>EDV [µl]</td>
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<td>Infarct [% of LVM]</td>
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**Supplemental Table II: Ultrasound measurements for control and treatment groups**

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<td>E [mm/s]</td>
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<td>A [mm/s]</td>
<td>409 ± 31</td>
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<td>E' [mm/s]</td>
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<td>A' [mm/s]</td>
<td>-62 ± 9</td>
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<tr>
<td>E'/A'</td>
<td>1.0 ± 0.2</td>
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<td>Diastolic dysfunction [%]</td>
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Supplemental Table III: List of antibodies

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure I: Differentiation of human ESCs and characterization of EHMs prior to their implantation. (A) Schematic outline of the cell expansion and differentiation process in spinner flasks. (B,C) Cardiomyocyte differentiation efficiencies were 95% and 71% cTnT positive, respectively, for two consecutive production lots. Non-cardiomyocyte fractions for these lots were 16.2% and 30.4% CD90+, respectively. (D-G) Confocal images from an EHM cross section showing cell nuclei (DAPI), apoptotic or dead nuclei (TUNEL), human cardiomyocyte troponin T (h-cTnT), and the overlay of these three channels. (H) Force versus time plot from a human EHM loop. This recording is from an electrically paced EHM where preload was increased stepwise by stretching the EHM. (I) Active force generation measurements were recorded for each EHM contraction and plotted against time. Scale bars D-G: 50 µm.

Supplemental Figure II: EHMs showed small differences in contractile force generation and viable CM content depending on CM purity of cells used to make EHM. (A) Contractile forces of EHMs as well as viable CM content prior to implantation were lower for EHMs made from a differentiation batch with 71% CM yield compared to a batch with 95% CM yield. (B) Confocal microscopy images show the presences of fibroblasts in EHMs prior to implantation. (C,D) FACS plots of digested EHMs showed lower CM content and an increased fraction of fibroblast-like cells prior to transplantation compared to 95% CM content of input cells used to make these EHMs. Scale bar C: 50 µm.

Supplemental Figure III: Staining against human nuclear antigen is a suitable surrogate for cell viability. (A-D) Only nuclei which were TUNEL negative stained positive for human
nuclear antigen (hNA), indicating that hNA staining was a suitable surrogate for viability of human cells. Scale bars A-D: 100 μm.

**Supplemental Figure IV: Characterization of cardiomyocytes from the Fluc-tdT reporter line and EHMs made thereof.** (A,B) FACS plots from two consecutive differentiation lots of the Fluc-tdT reporter line indicated 87% and 91% cardiomyocyte yield (cardiac troponin T, cTnT) respectively. (C,D) Bioluminescence images from EHM loops and cardiomyocyte dilutions made from the same differentiation lot. (E) A regression analysis revealed a strong linear correlation between the number of viable cardiomyocytes and the average radiance of corresponding wells (intercept: P=0.30, slope: P=2.2E-16), indicating that average radiance can be used as a surrogate for viability. (F) Confocal microscopy image from differentiated Fluc-tdTomato reporter cells exhibiting a normal cardiac phenotype with organized sarcomeres. Average radiance expressed as 10^5 photons/second/cm^2/steradian. α-actinin: alpha sarcomeric actinin, td-Tom: tdTomato. Scale bar: 50 μm.

**Supplemental Figure V: One month after EHM implantation, human grafts consisting primarily of cardiomyocytes could be found in all rats examined.** (A,B) EHM implantation yielded grafts with up to 400 μm thickness enveloping a substantial circumference fraction of the recipient heart. Grafts consisted primarily of human cardiomyocytes (beta myosin heavy chain, β-MyHc), but showed no discernible connexin expression (connexin 43, Con43). (C,D) Human grafts (beta 1 integrin, β-Integ) showed a small number of cells (0.95±0.15%) which were in an active cell cycle state (Ki67) at 30 days after implantation. Scale bars: 100 μm.
Supplemental Figure VI: Late gadolinium enhancement images illustrating similar scar sizes before treatment. (A-C) Late gadolinium enhancement images in short-axis orientation, covering the heart from above the LAD occlusion site to the apex acquired prior to EHM implantation or control surgery. A rim of viable myocardium along the endo- and epicardial borders can be seen characteristic for I/R injury. All four groups exhibited similar scar sizes and circumferential extent of the infarct. Scale bars: 5 mm.

Supplemental Figure VII: Irradiated EHM loops contained primarily fibroblasts and macrophages at one month after transplantation. (A,C) Grafts from EHM implantations contained human cells (human nuclear antigen, hNA). Only a small number of macrophages (CD68) and fibroblasts (fibroblast specific protein 1, FSP1) as well as a small amount of smooth muscle positive cells surrounding blood vessels (smooth muscle actin, SMA) could be found in human grafts. (D,F) Control hearts that did not receive any EHM contained some fibroblasts, macrophages, and smooth muscle actin positive cells which might be myofibroblasts in the scar region. (G,I) In contrast to control and EHM, irradiated EHM contained many macrophages, fibroblasts, and smooth muscle active positive cells one month after transplantation. Scale bars: 100 µm.

Supplemental Figure VIII: Implantation of irradiated EHM loops increased diastolic dysfunction. (A,B) There were no significant differences for changes in body weight or heart rate over time between all groups (P=0.83 and P=0.22, respectively; control n=12, EHM n=14, irradiated EHM loops n=9). (C,D) The decline in heart rate was not correlated to the body weight of animals at the time of myocardial infarction or the ejection fraction one day prior to EHM
implantation or control surgery. (E-H) Changes in cardiac output, stroke volume (SV), or heart weight were not significantly different between groups (P=0.13, P=0.18, and P=0.16). (I,J) Pulse wave Doppler and tissue Doppler images from a heart with normal diastolic function illustrated by large early refill velocity (E) and mitral valve annulus displacement velocity (E’) compared to late refilling (A) and annulus displacement (A’). (K,L) Changes in E/A and E’/A’ ratios were not significantly different between groups (P=0.10 and P=0.43), but there was a consistent trend for increased diastolic dysfunction in the group receiving irradiated EHM loops.

**Supplemental Figure IX: Long-term graft survival of transplanted EHMs.** (A,B) Large human grafts (beta 1 integrin, β-Integ) with dense vascular networks (CD31) could be found at 110 and 220 days after EHM implantation. (C,D) Hematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS) staining of 110-day-old EHM grafts exhibiting glycogen rich cells (PAS, dark purple staining) with hamartoma-like phenotype (H&E). Hamartoma-like cells are large cells with cytoplasmic collections of glycogen with intervening strands of cytoplasm radiating from the nuclei. The glycogen collections appear clear with H&E staining and magenta with PAS staining. (E,F) H&E and PAS stainings of normal host myocardium for the same heart as shown in C and D. (G-J) Hamartoma-like cells stained positive for cardiac markers (alpha sarcomeric actin, α-act; cardiac troponin T, cTnT), but did not show any signs of proliferation (Ki67, negative). Some cells still exhibited detectable sarcomeres. Scale bars A,B: 500 µm, C-J: 50 µm.
SUPPLEMENTAL REFERENCES


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Supplemental Figure III
Supplemental Figure IV
Supplemental Figure V
Supplemental Figure VI
Supplemental Figure VII
Supplemental Figure VIII
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