Tissue Engineering of Vascularized Cardiac Muscle From Human Embryonic Stem Cells

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Abstract—Transplantation of a tissue-engineered heart muscle represents a novel experimental therapeutic paradigm for myocardial diseases. However, this strategy has been hampered by the lack of sources for human cardiomyocytes and by the scarce vasculature in the ischemic area limiting the engraftment and survival of the transplanted muscle. Beyond the necessity of endothelial capillaries for the delivery of oxygen and nutrients to the grafted muscle tissue, interactions between endothelial and cardiomyocyte cells may also play a key role in promoting cell survival and proliferation. In the present study, we describe the formation of synchronously contracting engineered human cardiac tissue derived from human embryonic stem cells containing endothelial vessel networks. The 3D muscle consisted of cardiomyocytes, endothelial cells (ECs), and embryonic fibroblasts (EmFs). The formed vessels were further stabilized by the presence of mural cells originating from the EmFs. The presence of EmFs decreased EC death and increased EC proliferation. Moreover, the presence of endothelial capillaries augmented cardiomyocyte proliferation and did not hamper cardiomyocyte orientation and alignment. Immunostaining, ultrastructural analysis (using transmission electron microscopy), RT-PCR, pharmacological, and confocal laser calcium imaging studies demonstrated the presence of cardiac-specific molecular, ultrastructural, and functional properties of the generated tissue constructs with synchronous activity mediated by action potential propagation through gap junctions. In summary, this is the first report of the construction of 3D vascularized human cardiac tissue that may have unique applications for studies of cardiac development, function, and tissue replacement therapy. (Circ Res. 2007;100:263-272.)

Key Words: embryonic stem cells • tissue engineering • angiogenesis

The adult mammalian heart has limited regenerative capacity and therefore any significant myocardial cell loss is mostly irreversible and may lead to progressive loss of ventricular function and heart failure development. Despite the improvements in several pharmacological, interventional, and surgical therapeutic measures, the prognosis for heart failure patients remains poor. An attractive experimental solution to this significant medical problem may be to repopulate the damaged heart with new myogenic cells. Consequently, myocardial cell replacement therapy has emerged as a novel experimental therapeutic paradigm aiming to improve the function of the failing heart. In general, 2 principal strategies were suggested: the first focused on direct transplantation of isolated cells into the dysfunctional myocardial areas, whereas the second attempted to combine ex vivo cells with polymeric scaffolds generating a tissue-engineered muscle construct, followed by in vivo engraftment of the engineered muscle.

Despite the encouraging results in several animal studies, clinical translation of these approaches have been hampered by the lack of sources for human cardiomyocytes and by the significant cell death following cell transplantation into the hostile ischemic myocardium.1 The latter problem may be even aggravated following the transplantation of clinically relevant, thick tissue–engineered muscle. Insufficient graft vascularization is considered among the main factors responsible for this limited graft survival.2–4 Although engraftment of myogenic cells within the heart results in an angiogenic reaction, this host-derived graft vascularization usually does not provide the transplanted myocytes with the abundant capillary network that normally exists in the heart.

It is therefore postulated that enrichment of the degree of graft vascularization may significantly improve the survival of the transplanted myocytes. Furthermore, beyond the necessity of endothelial capillaries for the delivery of oxygen and nutrients to the grafted cardiomyocytes, endothelial–car-
diomyocyte interactions may also play a key role in enhancing cardiomyocyte and endothelial development, proliferation, maturation, and organization,\textsuperscript{6,7} which may therefore further enhance graft function and survival.

The ability to generate an engineered vascularized muscle tissue was recently demonstrated by us using the skeletal muscle model.\textsuperscript{8} We have shown that such prevascularization of the engineered skeletal muscle construct promoted survival, vascularization, and perfusion of the implant. In this study, we hypothesized that a triple-cell-based culture of cardiomyocytes, endothelial cells (ECs), and embryonic fibroblasts (EmFs) will result in the generation of highly vascularized cardiac tissue in vitro.

Given the attractive potential of human embryonic stem cell (hESC)-derived cardiomyocytes (hESC-CMs) in future cell therapy strategies for heart failure, we evaluated the ability to form engineered cardiac tissue using these cells. To this end, the hESC-CMs were seeded on 3D biodegradable, highly porous, polymeric scaffolds. To promote in vitro tissue vascularization, we constructed multicellular scaffolds in which hESC-CMs were combined with hESC-derived ECs (hESC-ECs) or human umbilical vein ECs (HUVECs) with or without EmFs. We demonstrate that this multicellular tissue engineering strategy enables, for the first time, the generation of highly vascularized human engineered cardiac tissue with cardiac-specific ultrastructural, molecular, and functional properties.

Materials and Methods
An expanded Materials and Methods section is provided in the online data supplement at http://circres.ahajournals.org.

Cell Culture and Isolation
hESCs (H9/2 clone, passages 30 to 60) were grown and induced to differentiate as previously described.\textsuperscript{1,2} For cardiomyocyte isolation, spontaneously beating areas were microdissected and dissociated from plated embryoid bodies (EBs) after 25 to 30 days of differentiation, as previously described.\textsuperscript{1} hESC-derived ECs were isolated from 13- to 15-old-day EBs using CD31 antibodies as described.\textsuperscript{2} HUVECs (Clonetics) were grown in EGM-2 medium. EmFs were cultured in DMEM supplemented with 10% FBS.

Scaffolds
Porous sponges composed of 50% poly-L-lactic acid (PLLA) (Polysciences) and 50% polyactic-glycolic acid (PLGA) (Boehringer-Ingeheim) were fabricated as previously described,\textsuperscript{3} with pore sizes of 212 to 600 μm and 93% porosity. The procedure of cell seeding on the scaffolds is detailed in the online data supplement.

Immunostaining
Immunostaining of 5-μm sections was performed using the Biocare Medical Universal horseradish peroxidase–diaminobenzidine kit. A detailed list of the antibodies used, the dilutions, and the staining procedure can be found in the online data supplement.

Cell Viability Assay
Before cell seeding, ECs were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/mL) for 45 minutes in 37°C. The percentage of DAPI-stained cells, stained with calcein and ethidium-1 homodimer, was calculated from images of dispersed single cells, taken in ×200 magnification.

RT-PCR Studies
For RT-PCR analysis, scaffolds were incubated with Tryspin 0.5% (Gibco) for 8 minutes in the presence of 1U/μL RNase inhibitor (RNAsin; Promega) to allow cell dissociation. RNA was isolated from the dispersed cells using the High Pure RNA isolation kit (Roche), and reverse transcription of the isolated RNA into cDNA was conducted using Reverse-IT 1st Strand Synthesis Kit (ABgene) according to the instructions of the manufacturer. Real-time PCR and RT-PCR for the various genes was performed using the primers and conditions detailed in the online data supplement.

Laser Scanning Confocal Ca Imaging
Scaffolds were loaded with 5 μmol/L fluo-4 Ca\textsuperscript{2+} AM (Molecular Probes) indicator to visualize free Ca\textsuperscript{2+} levels (according to the instructions of the manufacturer). Intracellular calcium transients were imaged with a confocal imaging system (Olympus Fluoview) mounted on an upright BX51WI Olympus microscope equipped with a ×60 (0.9 NA; Olympus) water objective.

Statistical Analysis
All results are expressed as mean±SEM. When comparing more than 2 groups, ANOVA was used, followed by a post hoc Bonferroni, Student’s t test or Mann–Whitney rank sum test was used to compare between two groups. We considered a probability value of ≤0.05 to be statistically significant.

Results
Generation of the Engineered Human Cardiac Tissue
To explore the ability to establish a 3D supportive environment for generation of a vascularized cardiac tissue, we used PLLA (50%)/PLGA (50%) biodegradable scaffolds. The PLGA was selected to allow relatively fast degradation (≈3 weeks) to facilitate cellular ingrowths, whereas the PLLA was chosen to provide mechanical support for the 3D structure. We evaluated 3 cell culture combinations: (1) scaffolds seeded with hESC-CMs (4×10⁵ cells) alone; (2) cocultures comprised of hESC-CMs (4×10⁵ cells) and HUVECs or hESC-derived ECs (hESC-ECs\textsuperscript{8}) (4×10⁵ cells); and (3) a triple-cell culture comprised of hESC-CMs, HUVECs, or hESC-ECs, supplemented with EmFs (2×10⁵ to 4×10⁶ cells).

The cells were seeded into the scaffolds together with Matrigel to facilitate cell seeding and to keep the cells on the scaffolds. Based on our previous studies,\textsuperscript{8} we hypothesized that the ECs would be able to organize into 3D vascular structures within the cardiac muscle construct. EmFs were added in attempts to stabilize the vessels and improve the vascularization of the engineered tissue, based on their potential to differentiate to smooth muscle cells when cultured in the presence of ECs.

The scaffolds were monitored microscopically for the appearance of spontaneous contraction every day following cell seeding. Synchronous contraction appeared initially after 4 days in the cardiomyocytes constructs (n=4). The regional contractions gradually spread until the entire scaffold was beating synchronously (Video in the online data supplement). A similar pattern of initiation of contraction (4 to 6 days) was also found in the scaffolds containing cocultures of hESC-CM+HUVEC (n=6) and in the triple-cell culture of hESC-CM+HUVEC+EmF.
The engineered cardiac tissue constructs were observed for 2 weeks, after which they were fixated and used for detailed histological examination. Histological analysis of the tissue-engineered constructs showed that the seeded cells lined both the inner and the outer surfaces of the scaffolds and that the hESC-CMs could be identified in all scaffolds studied in all three groups (Figure 1).

Vascularization of the Engineered Human Cardiac Tissue

Scaffolds consisting of just hESC-CMs contained only few von Willebrand factor–positive (vWF+) or CD-31+ cells (Figures 1A and 2A). The addition of HUVECs resulted in a significant increase in the quantity of the ECs when compared with the scaffolds containing only hESC-CMs (Figures 1B and 2B). Despite the increase in EC density, the ECs did not organize into blood vessels and were mainly present as compact cell clusters (Figures 1B, 2B, and 3A).

We next studied the effects of adding EmFs to the constructs containing hESC-CMs and ECs. Examination of these tri-culture 3D scaffolds revealed that the addition of EmFs resulted in the generation of highly vascularized engineered cardiac muscle (Figures 1C and 2C). Both immunohistochemical and immunofluorescent stainings demonstrated the organization of the ECs into a dense network of vessels that was present within and in some cases also closely adjacent to the cardiac tissue (Figures 1C and 2C).

To further analyze the effects of the EmFs on cardiac muscle vascularization, we performed quantitative immunostaining analysis using anti-vWF antibodies. Three parameters of tissue vascularization and vessel organization were assessed: (1) the number of lumens per millimeter squared; (2) the lumen area density; and (3) the EC area density. Tri-culture scaffolds containing hESC-CM+HUVEC+EmF were characterized by a significantly higher number of vessels and displayed an increased lumen area density when compared with the cocultures, which did not contain the EmFs (Figures 3, 4A, and 4B). We also found a higher EC density (stained positively for vWF) in the tri-culture scaffolds (Figure 4C). Comparison between the tri-culture scaffolds containing HUVECs to EmFs at a ratio of 1:1 and 2:1 revealed no significant difference in the degree of vascularization based on the above mentioned parameters (Figures 3B, 3C, 4). The supporting effects of EmFs on the organization of the ECs into vessel networks were also sustained when the HUVECs were replaced with hESC-ECs using similar cell ratios (Figure 3D).
Expression of Angiogenic and Vasculogenic Factors

To evaluate the expression of key angiogenic and vasculogenic factors in the 3D vascularized cardiac tissue, we assessed the expression of vascular endothelial growth factor (VEGF)-A, platelet-derived growth factor (PDGF)-B, angiopoietin 1 (Ang-1), and basic fibroblast growth factor at the mRNA level. Similar to the histological quantification of the vascularization process, the RT-PCR analysis revealed increased gene expression of the angiogenic factors VEGF-A, PDGF-B, and Ang-1 in the tri-culture cardiac tissue (on addition of EmFs) (Figure 4D). We did not note an increase in the basic fibroblast growth factor mRNA levels in the tri-culture.

A major factor known to contribute to EC organization is the presence of pericytes or smooth muscle cells.8,10–14 We therefore assessed whether the EmFs in the tri-cultures differentiated into smooth muscle cells. Immunostainings for α-smooth muscle actin (SMA) demonstrated the presence of SMA⁺ cells within the engineered cardiac tissue (Figure 3E). In many cases, these SMA⁺ cells were demonstrated to integrate into the formed blood vessels and were localized adjacent to vWF⁺ cells (Figure 3E).

Temporal Assessment of EC Viability and Proliferation

Because the presence of EmFs affected not only the degree of EC organization but also the EC density, we hypothesized that EmFs may also influence EC viability and proliferation. We therefore assessed the degree of EC viability at several time points following cell seeding (1 hour, 24 hours, 72 hours, and 1 week). The ECs were prelabeled with DAPI, and cell viability was evaluated using calcein AM (staining viable cells) and ethidium homodimer 1 (staining dead cells) (Figure 5A). At 1 hour and 24 hours following cell seeding, there were no significant differences in cell viability between the scaffolds with and without EmFs. However, at 72 hours and 1 week, EC viability was significantly higher in scaffolds containing EmFs (Figure 5B).

An alternative explanation to the higher number of ECs in the tri-culture compared with the coculture scaffolds may be related to alteration of the proliferative capacity of the ECs. To quantify this aspect, we performed double immunostainings for human Ki67 (a marker for cycling cells) and vWF (Figure 5C). We found that the percentage of proliferating ECs within the tri-culture (10.7±1%) was significantly higher (P<0.01) than those of the coculture (4.4±1.5%) (Figure 5D).

Cardiomyocyte Structural Organization and Proliferation

We next continued characterizing the cardiomyocyte tissue within the scaffold. As can be seen in the immunohistochemistry images in Figure 1, the cardiomyocytes were arranged in aggregates, some of which consisted of relatively small hESC-CMs being isotropically arranged, whereas others were comprised of longitudinally oriented cell bundles containing more structurally mature cardiomyocytes. The latter areas were mainly located at the periphery of the scaffolds.

The unorganized, smaller, hESC-CMs with higher nuclear to cytoplasmatic ratio (Figure 6A, arrows), usually denote a less mature stage of cardiomyocyte development.15 Double-immunostaining studies with anti–troponin I and anti-human Ki67 antibodies revealed that these areas of small cardiomyocytes contained many proliferating cardiomyocytes (Figure 6A, arrows). In contrast, positively stained Ki67 cardiomyocytes were rarely found in the more structurally organized areas and the cardiomyocytes in these regions were larger and displayed a more mature structural phenotype (Figure 6A).

We next assessed the effects of EC presence in the engineered cardiac tissue on the level of cardiomyocyte proliferation. Interestingly, as shown in Figure 6A and 6B, the percentage of the proliferating cardiomyocytes (Ki67⁺) was significantly higher in the scaffolds containing the ECs, both in the presence and absence of EmFs, when compared with scaffolds containing only cardiomyocytes.
Expression of Cardiac Differentiation Markers in the Engineered Cardiac Tissue

To assess the effect of the co-/tri-culture system on the differentiation and maturation of the hESC-CMs we performed semiquantitative RT-PCR (Figure 6C) and quantitative real-time RT-PCR (Figure 6D) studies evaluating both markers of early-immature cardiomyocytes (atrial natriuretic factor, Nkx2.5, myocyte enhancer factor 2C, and α-skeletal actin) and markers of more mature, differentiated, cardiomyocytes (myosin light chain-2V, α-myosin light chain, α-cardiac actin, and troponin I). Gene expression analysis revealed upregulation in the expression of markers of cardiomyocyte maturation such as myosin light chain-2V, troponin I, and α-cardiac actin. However, the levels of α-myosin heavy chain were only mildly effected by the cell combination used (Figure 6C and 6D). Surprisingly, the upregulation of cardiomyocyte maturation markers was not accompanied by downregulation of gene markers of early and immature cardiomyocytes. One possible explanation to the latter phenomenon may be the presence of areas containing highly dividing immature cardiomyocytes also within the co- and tri-cultures scaffolds (Figure 6A, arrows).

Ultrastructural Characterization of the Engineered Cardiac Tissue

Transmission electron microscopy of the scaffolds demonstrated the presence of cardiomyocytes in both the early-immature and more mature stages of development. The
immature cardiomyocytes were fewer and were characterized by the presence of relatively disorganized myofibrils (Figure 7A), which, in some cases, were associated with a distinct electron dense material (the developing Z-bodies). In contrast, myofibrils were more abundant in the relatively mature cells. They were organized in similar directions and were confined to parallel Z bands forming the typical sarcomeric pattern (Figure 7B). The cardiomyocytes contained mitochondria that were packed around the sarcomeres (Figure 7B). Beyond the presence of mitochondria and sarcomeric organization, the hallmarks of more mature cardiomyocytes are the presence of T-tubules and sarcoplasmic reticulum and the formation of gap junctions. In some cells, the presence of developing T-tubules associated with sarcoplasmic reticulum (Dyads) could be noted (Figure 7B), which were located around the sarcomeric structures. In addition, we could also detect the presence of specialized junctional structures responsible for electromechanical coupling between neighbor-

Figure 5. Temporal assessment of endothelial cell viability and proliferation. A and B, Endothelial cell viability was evaluated at several time points following cell seeding (1 hour, 24 hours, 72 hours, and 1 week) in the presence and in the absence of EmFs using calcein AM (green-staining viable cells) and ethidium homodimer-1 (red-staining dead cells). Images in A represents viability assessed following 1 week. C, Double-immunofluorescent staining using anti–human Ki67 (red) and anti-vWF (green) antibodies for the identification of proliferating endothelial cells (indicated by arrows). D, Quantitative analysis of EC proliferation. In the tri-culture constructs, the percentage of cycling ECs was significantly higher (10.7±1%) than in the coculture (4.4±1.5%) (P<0.01).
ing cardiomyocytes. These included the presence of intercalated discs containing desmosomes and gap junctions (Figure 7C and 7D). Similarly, immunofluorescent stainings demonstrated the formation of gap junctions comprised of connexin 43 between the human cardiomyocytes (Figure 7E and 7F).

Impulse Propagation
The engineered cardiac tissue demonstrated spontaneous synchronous contractions of the cardiomyocytes within and between scaffold pores (supplemental Video). Coupling of cardiomyocyte contraction and electrical excitation is known to be mediated via transmembrane Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) release. To evaluate the presence of synchronous Ca\(^{2+}\) transients within the engineered cardiac tissue, we performed laser confocal Ca\(^{2+}\) imaging studies using the free Ca\(^{2+}\) binding dye fluo-4. Figure 8A depicts a typical line scan image through 6 cells. Note the synchronous surges of intracellular Ca\(^{2+}\) levels within the contracting hESC-CMs.

Because the ultrastructural characterization clearly indicated the presence of gap junctions between adjacent hESC-CMs (Figure 7D), we sought to determine whether these formed gap junctions mediate impulse conduction between the hESC-CMs and therefore allow synchronous contraction. The gap junction uncoupler 1-heptanol (1 mmol/L) was applied to the spontaneously contracting engineered cardiac tissue. As expected, administration of 1-heptanol resulted in complete inhibition of impulse propagation, as identified by the calcium imaging studies (Figure 8B).

Chronotropic Response of the Engineered Cardiac Tissue
The beating frequency of the spontaneously contracting cardiac tissue was evaluated following application of different pharmacological agents. Appropriate positive and negative chronotropic responses were observed following application of the \(\beta\)-agonist isoproterenol (1 \(\mu\)mol/L) and the muscarinic agonist carbachol (1 \(\mu\)mol/L). Thus, isoproterenol increased the beating frequency of the engineered cardiac issue from 1.6±0.3 to 2.1±0.3 Hz (\(P<0.05\), \(n=4\)),
HUVECs or hESC-ECs and EmFs. This was manifested by the addition of chronotropic agents. Importantly, vascularization of the cardiac tissue was strongly promoted by the addition of vascularization of the cardiac tissue revealed the presence of both cardiomyocytes in early and more mature stages of development. A, Early developing cells showing disorganized myofibrils that in some cases were associated with a distinct electron dense material (Z-bodies). B, Relatively mature cells showing myofibrils organized in similar direction and confined to parallel Z bands (Zb) forming a typical sarcomeric pattern. Beyond the presence of mitochondria (Mi), the existence of T-tubules (TT), and sarcoplasmic reticulum (SR) indicates a more developed maturation stage. C and D, Presence of the specialized cell–cell junctions, including the intercalated disc containing desmosomes (shown as a letter D) and gap junctions (GJ). E and F, Double-immunofluorescent staining using anti–troponin I and Cox43 antibodies revealed the presence of gap junctions composed of connexin 43.

Figure 7. Ultrastructural characterization of the engineered cardiac tissue. Transmission electron microscopy demonstrating the presence of both cardiomyocytes and EmFs. This was manifested by increased density of vessels within the cardiac tissue accompanied by the generation of stabilized vessels containing smooth muscle cells. Gene expression analysis revealed that upregulation of key angiogenic and vasculogenic markers occurred in the tri-culture engineered tissue. Intriguingly, we found that the addition of EmFs significantly augmented EC density within the engineered tissue, possibly through the augmentation of both EC survival and proliferation capacity. Similarly, the presence of ECs significantly increased the proliferative capacity of the cardiomyocytes in the co-/tri-culture conditions. Finally, the vascularization of the cardiac vessel network within the engineered tissue resulted in upregulation of both early and late markers of cardiomyocyte differentiation and maturation.

Replacement of defective myocardial areas by functional cardiomyocytes undoubtedly depends on the ability of the grafted tissue to survive within the hostile ischemic environment. Previous studies, using various cell sources, revealed that transplantation of single cells results in significant cell death. An alternative approach to transplantation of single cells may be replacement of diseased myocardial areas by in vitro–designed cardiomyocytes forming a synchronously contracting and well-coupled muscle network. However, a major limitation of this approach is the maximal size of the constructed tissue. This is mainly attributable to the high metabolic demands, inherent intolerance of anaerobic metabolism, and the compact nature of the cardiac muscle strands. Consequently, the maximum size of engineered cardiac muscle is confined by the maximum diffusion distance of oxygen and nutrients (≈100 μm).

Thus, construction of clinically relevant cardiac tissues must allow full thickness perfusion of the preformed engineered cardiac tissue. This issue is even of greater importance when considering the scarce vascularization of the myocardial scar. The spontaneous development of primitive capillaries within cardiac tissue constructs that have been reported in studies using primary cultures of neonatal rat ventricular cardiomyocytes probably stems from the mixed population of cells present in the rat ventricle. This vasculature has provided a partial solution to this key limiting problem and probably promoted the survival of implanted cardiac scaffolds in subsequent experiments. However, construction of an engineered cardiac tissue from the potential clinically relevant cell source of hESC-CMs did not result in the generation of significant capillary network when used alone.

We therefore hypothesized that to induce vascularization of the engineered cardiac tissue, additional cells should be used. Recent studies have demonstrated that generation of organized and stabilized blood vessels require not only the presence of ECs but also pericytes and smooth muscle cells. The latter provide prosurvival signals, inhibit EC apoptosis, structurally support newly formed capillaries, promote vessel structural integrity, and encourage the generation of basement membrane. The positive interaction between the endothelial and mural cells is mediated through a number of molecular signals including VEGF-A, PDGF-B, Ang-1, and transforming growth factor-β.

Discussion
The present study describes, for the first time, the generation of a 3D, engineered, vascularized, human cardiac tissue that is based on the use of hESCs. Ultrastructural characterization of the engineered cardiac tissue revealed the presence of differentiating cardiomyocytes with a typical sarcomeric pattern, formation of gap-junctions, and dyads containing T-tubules and sarcoplasmic reticulum. Calcium imaging studies demonstrated that the generated cardiac tissue contracted spontaneously and synchronously with gap junctions mediating impulse propagation between the beating cardiomyocytes. The 3D tissue also responded to both positive and negative chronotropic agents. Importantly, vascularization of the cardiac tissue was strongly promoted by the addition of HUVECs or hESC-ECs and EmFs. This was manifested by increased density of vessels within the cardiac tissue accompanied by the generation of stabilized vessels containing smooth muscle cells. Gene expression analysis revealed that upregulation of key angiogenic and vasculogenic markers occurred in the tri-culture engineered tissue. Intriguingly, we found that the addition of EmFs significantly augmented EC density within the engineered tissue, possibly through the augmentation of both EC survival and proliferation capacity. Similarly, the presence of ECs significantly increased the proliferative capacity of the cardiomyocytes in the co-/tri-culture conditions. Finally, the vascularization of the cardiac vessel network within the engineered tissue resulted in upregulation of both early and late markers of cardiomyocyte differentiation and maturation.
revealed that mesenchymal stem cells and EmFs can differentiate into mural cells in the presence of ECs. Based on this information, we added to the human cardiomyocytes in the 3D scaffolds both ECs and EmFs. The tri-culture system resulted in the generation of highly vascularized 3D cardiac tissue accompanied by the integration of smooth muscle cells to the newly formed capillaries.

The positive effect of the EmFs on tissue vascularization was also evident by an increase in the density of ECs. Two possible mechanisms were suggested to explain this finding. First, temporal assessment of EC viability revealed that the presence of EmFs inhibited EC death. As described above, the presence of mural cells is known to promote EC survival. VEGF, secreted from the mural cells, has a pivotal role in effecting EC survival, mainly through upregulation of apoptosis inhibitors such as Bcl-2 and X-linked inhibitor of apoptosis protein. Hence, the upregulation of VEGF in the tri-culture system provides a reasonable explanation for the increase in EC viability. This finding is further supported by recent work indicating that fibroblasts inhibit HUVEC apoptosis. The second mechanism for the increased EC density in the tri-culture is the significant increase of EC proliferation. This effect may also be attributed to endothelial mitogenic factors such as VEGF-A, PDGF-B, and Ang-1 (upregulated in the tri-cultures and known to be secreted from EmFs).

An important finding of this study was the relatively high degree of cardiomyocyte proliferation when ECs were added, when compared with scaffolds containing only hESC-CMs (in which the proliferation rate was similar to that previously reported in similar-stage cultured EBs). Because the quantity of cycling cardiomyocytes was augmented in both the co- and tri-culture conditions, we speculate that cell-cycle activation was the result of the interaction between hESC-CMs and the ECs. Although limited data exist on the modulating role of ECs on early differentiating cardiomyocytes, a well-characterized example of endothelial–cardiomyocyte interaction is the neuregulin-erbB signaling pathway. Neuregulin-1, secreted from ECs during cardiac development, has been demonstrated to play a key role in promoting cardiomyocyte proliferation and survival. Additional modulators of cardiomyocyte proliferation, known to be expressed in ECs (including HUVECs), are members of the insulin-like growth factor (IGF) family of proteins. Recently, Medvetz et al reported that both IGF-1 and IGF-2 augment the proliferation rate of hESC-CMs in a dose-dependent manner. Other possible modulators of cardiomyocyte proliferation may include endothelial secretion of PDGF-B and neurofibromatosis-1.

In summary, we described a novel approach for the establishment of a vascularized human cardiac tissue in vitro. The engineered 3D tissue construct exhibited typical structural and functional properties of early-cardiac tissue. This established system may provide a powerful tool for assessing the interactions among cardiomyocytes, ECs, and mural cells during embryonic heart development. It may also be used as a unique in vitro 3D model of human cardiac tissue for several pathophysiological and pharmacological studies. Finally, we believe that engineering vascularized cardiac tissue before implantation to the infarcted myocardium will enable improved graft survival and may result in an increased functional benefit.

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Disclosures

None.

References


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Expanded Materials and Methods

Propagation of the hESC lines and in vitro cardiomyocyte differentiation

Pluripotent hESC of the H9.2 clone (passage 30-60) were grown in the undifferentiated state on top of mouse embryonic fibroblast feeder layer as previously described\(^1\), \(^2\). The culture medium consisted of 20% FBS (HyClone), 79% knockout DMEM supplemented with 1mM L-glutamine, 0.1 mM mercaptoethanol, and 1% nonessential amino acids (all from Life Technologies).

To induce differentiation, the hESC were dispersed to small clumps using collagenase IV (1 mg/mL, Life Technologies). Then they were transferred to plastic petri-dishes and cultured in suspension for 7-10 days where they formed embryoid bodies (EBs). Hereupon the EBs were plated on gelatin-coated culture dishes. Spontaneously beating areas were noted in some of the EBs after 5-20 days of plating. The contracting areas within the EBs were microdissected with a curved 23G needle after 25-30 days of in vitro differentiation. The contracting areas were then dissociated into small cell clusters by incubation with 1mg/ml of collagenase B (Roche) for 45 minutes as previously described\(^1\).

HUVEC cells (Clonetics) (passage 3-6) were grown on regular tissue culture plates in EGM-2 medium supplemented with 2% FBS. Mouse embryonic fibroblasts were cultured in DMEM supplemented with 10% FBS.

Isolation of hESC derived endothelial cells
hESC derived CD31$^+$ cells were isolated as described$^2$. Briefly, differentiating hEBs at days 13–15 were dissociated with 1% trypsin/EDTA (Beit Haemek, Israel) and incubated for 30 min with FITC-labeled anti CD-31 antibodies (BD Pharmingen) on ice. Fluorescent-labeled cells were isolated by using FACSVantge flow cytometry cell sorter (Becton Dickinson) and plated on 1% gelatin-coated plates with endothelial cell growth medium (Clonetics). Cells were passaged by using 0.025% trypsin EDTA (Cambrex Biosciences) and cultured in endothelial cell medium consisting of EGM-2 (Cambrex Biosciences). 

**Engineering vascularized cardiac tissue on 3D biodegradable scaffolds**

Porous sponges composed of 50% poly l-lactic acid (PLLA) (Polysciences, Warrington) and 50% polylactic glycolic acid (Boehringer Ingelheim) were fabricated$^3$ with pore sizes of 212–600 µm and 93% porosity. Briefly, PLLA and PLGA 1:1 were dissolved in chloroform to yield a solution of 5% polymer; 0.24 ml of this solution was loaded into molds packed with 0.4 g of sodium chloride particles. The solvent was allowed to evaporate, and the sponges were subsequently immersed for 8 hrs in distilled water (changed every hour) to leach the salt and create an interconnected pore structure. The sponges were sliced to squares at a volume of ~9 mm$^3$ (3mm*3mm*1mm). Degradation time of the composed sponges is ~6 months. For seeding, the desired number of cells were pooled and resuspended in 8-10 µl of a 1:1 mixture of culture medium and growth factor-reduced Matrigel (BD Biosciences). The suspension was allowed to absorb into the sponges, after which they were incubated for 20 mts at 37°C allowing solidification of the gel. Culture medium was then added. The sponges were detached from the bottom of the plate and incubated at 37 °C on a XYZ shaker. Every other day the medium was
changed. It consisted of 50% EGM-2 medium and 50% standard ES cell culturing medium supplemented with 1% penicillin/streptomycin. Following two weeks of cell culture scaffolds were fixed in 10% formalin and subsequently embedded in paraffin for sectioning.

**Immunocytochemical and immunofluorescence staining**

Transverse sections (5 µm) were placed on slides for Immunostainings. Immunocytochemical staining was carried out using the Biocare Medical Universal HRP-DAB kit (Biocare Medical) according to manufacturer's instructions, with prior heat treatment at 95 °C for 20 mts in ReVeal buffer (Biocare Medical) for epitope recovery. For stainings conducted with anti-vWF antibody deparaffinization and trypsin treatment were conducted for epitope recovery. Primary antibodies used were: monoclonal anti-human: CD31 (1:20); polyclonal rabbit anti-vWF (1:200); monoclonal anti Ki-67 MIB-I (1:30) and anti smooth muscle actin (1:50) (all from Dako); mouse anti-α-sarcomeric alpha actinin (1:100) (Sigma), polyclonal rabbit anti-connexin 43 (1:40), mouse anti troponin I (1:200) (Chemicon). For Immunofluorescent stainings, Secondary antibodies were: Cy3 and Cy2-conjugated anti-mouse IgG (1:100) (Jackson Immunoresearch laboratory, PA); Cy2-conjugated anti rabbit IgG (1:100); AlexaFluor 488 conjugated anti mouse IgG (1:100) (Molecular Probes) or by using Cy3 conjugated anti mouse IgG2b (1:100) (for double monoclonal antibody immunostaining of troponin I). Nuclei were counterstained using DAPI (Sigma). Fluorescence microscopy was performed using a Zeiss microscope (Axiovert 200M) and a CCD Camera (Axiocam, MRc5).

**Endothelial cell, lumen area and lumen number density quantification**
Microscopic pictures were randomly taken at a magnification of 10x and an imaging analysis software (Axiovision 3.1, Carl Zeiss) was used to determine the area of endothelial cells (based on anti-vWF and anti-CD-31 immunostainings), the area of vessels and the lumen formed by the endothelial cells and the total sample area. The number of structures was manually counted. p values were calculated using Student’s t-test.

Temporal assessment of cell viability

To test whether the addition of EmF results in improved endothelial cell survival, we evaluated endothelial cell viability following 1hr, 24hrs, 72hrs and 1week. Prior to cell molding, HUVEC cells were labeled with DAPI (1µg/ml) for 45 minutes in 37ºC. To assess cell viability scaffolds were loaded with Calcein AM (1µM) and Ethidium homodimer-1 (4µM) (Live/Dead ® Viability/Cytotoxicity kit for mammalian cells, Molecular Probes) for 50 minutes at 37ºC on top of a 3D XYZ shaker. Following dye loading scaffolds were washed with PBS (x3) and then dissected to small pieces. To allow cell dissociation from scaffolds, sliced scaffold pieces were incubated with Trypsin-EDTA x2 (Beit Haemek, Israel) for 8 minutes at 37ºC. Pictures of dispersed single cells (isolated using 100µm single cell filter) were taken in X20 magnification using the inverted fluorescence microscope and CCD Camera described above. The percentage of cells stained positive with DAPI and Calcein AM or ethidium-1 homodimer was calculated.

Transmission Electron Microscopy
Scaffolds were fixed in 3% paraformaldehyde, 2% glutaraldehyde and 5mM CaCl$_2$ in 0.1 M cacodylate buffer (pH 7.2) for 90 minutes and room temparture followed by overnight incubation at 4ºC. Following the intial fixation, scaffolds were post fixed 1% O$_3$O$_4$ in 0.1 M cacodylate buffer, 5mM CaCl$_2$, 0.5% Potassium dichromate and 0.5% Potassium hexacyonaferate for 1 h. Scaffolds were stained with 2% aqueous uranyl acetate followed by ethanol dehydration. The scaffolds were then embedded in Epon 812 (Electron Microscopy Sciences). Sections were cut using LEICA Ultracut UCT microtome at a thickness of 70 nm with a diamond knife (Diatome, Biel, Switzerland). Sections were examined with a Tecnai 12 (FEI Company, Eindhoven) transmission electron microscope at an accelerating voltage of 120 kV. Pictures were taken with CCD camera (MegaView III, Soft Imaging System, Germany).

**PCR Studies**

For semi quantitative RT-PCR and real time PCR analysis, scaffolds were frozen in liquid nitrogen 2 weeks following cell molding. Prior to RNA isolation, scaffolds were incubated with Trypsin x 10 (Beit Haemek, Israel) for 8 minutes in the presence of 1U/µL Rnase inhibitor (RNAsin (Promega, U.S.A.)) to allow cell dissociation. RNA was isolated from the dispersed cells using the High pure RNA isolation kit (Roche). Reverse transcription of the isolated RNA into cDNA was conducted using Reverse-iT 1st Strand Synthesis Kit (ABgene) according to manufacture’s instructions. PCR for the various genes was performed using primers and conditions detailed in Table 1. Briefly, each RT-PCR included 30 secs at 95 ºC, 30 secs at 56 ºC and 1 min at 72 ºC using Red Load Taq Master Mix (Larova, Germany). 20ng of cDNA template was used from each scaffold sample.
Taq-man real time PCR studies were performed using assay on demand primers and probes (Applied-Biosystem) in 96-well optical plates in triplicates. The assay on demand primers used were: Troponin I (Hs00165957_m1), MHC (Hs00411908_m1), nkx2.5 (Hs00231763_m1) and ANF (Hs00383231_m1). Samples were cycled for 45 times using an ABI 7700 Sequence Detector (Applied Biosystems). ABI 7700 cycle conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. C_T was calculated under default settings for real-time sequence detection software (Applied Biosystems).

**Laser scanning confocal Ca-imaging**

**Dye loading**

Scaffolds were loaded with fluo-4 Ca AM (Molecular Probes) indicator to visualize free Ca^{2+} levels. To this purpose, 5mM stock solution of fluo-4 AM in DMSO was diluted in standard hESC medium, at dilution of 1:1000 giving a final concentration of 5µM. The non-ionic detergent Pluronic F-127 (Molecular Probes) was used to assist dispersion of the nonpolar fluo-4 AM ester in the aqueous media. Scaffolds were incubated for 45 minutes at 37°C. Following incubation the scaffolds were washed (x3) with indicator-free control tyrode solution removing any dye non-specifically associated with the cell surface. Afterwards the scaffolds were further incubated for 15 minutes allowing complete de-esterification of intracellular AM esters (according to manufacture’s instructions).

Intracellular calcium transients were imaged with a confocal imaging system (Olympus Fluoview) mounted on an upright BX51WI Olympus microscope equipped with a 60x (0.9 n.a.; Olympus) water objective. In addition the mild uncoupler 1-
Heptanol (1mM) was applied to evaluate whether electrical impulse propagation within the cardiac tissue in the scaffolds occur through gap junctions.

**Statistical Analysis**

All results are expressed as mean±SEM. When comparing more than two groups ANOVA was used followed by a post-hoc bonferroni’s. Student’s t-test or Mann whitney rank sum test was used to make a comparison between two groups. We judged a p value of 0.05 or less to be statistically significant.

**REFERENCES**

**Online Table 1** – Primers for the amplification of early and late markers of cardiac differentiation and markers of angiogenesis.

<table>
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