ORIGINAL ARTICLE

Spontaneous Formation of Extensive Vessel-Like Structures in Murine Engineered Heart Tissue

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Engineered heart tissue (EHT) from primary heart cells contains endothelial cells (ECs), but the extent to which ECs organize into vessel-like structures or even functional vessels remains unknown and is difficult to study by conventional methods. In this study, we generated fibrin-based mini-EHTs from a transgenic mouse line (Cdh5-CreERT2 × Rosa26-LacZ), in which ECs were specifically and inducibly labeled by applying tamoxifen (EC^{iLacZ}). EHTs were generated from an unpurified cell mix of newborn mouse hearts and were cultured under standard serum-containing conditions. Cre expression in 15-day-old EHTs was induced by addition of *o*-hydroxytamoxifen to the culture medium for 48 h, and ECs were visualized by X-gal staining. EC^{iLacZ} EHTs showed a dense X-gal-positive vessel-like network with distinct tubular structures. Immunofluorescence revealed that ECs were mainly associated with cardiomyocytes within the EHT. EC^{iLacZ} EHT developed spontaneous and regular contractility with forces up to 0.1 mN. Coherent contractility and the presence of an extensive vessel-like network were both dependent on the presence of animal sera in the culture medium. Contractile EC^{iLacZ} EHTs successfully served as grafts in implantation studies onto the hearts of immunodeficient mice. Four weeks after implantation, EHTs showed X-gal-positive lumen-forming vessel structures connected to the host myocardium circulation as they contained erythrocytes on a regular basis. Taken together, genetic labeling of ECs revealed the extensive formation of vessel-like structures in EHTs *in vitro*. The EC^{iLacZ} EHT model could help simultaneously study biological effects of compounds on cardiomyocyte function and tissue vascularization.

Introduction

RECENT PROGRESS in tissue engineering and stem cell technologies has encouraged attempts to bioengineer 3D heart muscle equivalents for cardiac regeneration. Since cardiomyocytes are terminally differentiated, the loss of cardiomyocytes after myocardial infarction cannot be compensated by endogenous mechanisms. Cell-based therapies are generally performed by the use of two different approaches. Cells are either injected directly into the myocardium or used to construct cardiomyocyte patches *in vitro*, which are surgically attached to the myocardium for the replacement of impaired tissue. The retention rate of injected cells is low due to various factors such as cell washout or hypoxia. Bioengineered cardiomyocyte patches could overcome these obstacles. In successful studies, hydrogel-based patches were vascularized after

implantation into the host and the muscle-like structure was preserved *in vivo*, accompanied by newly formed blood vessels.¹⁻³ Blood-perfused vessels in the graft contained graft-derived vascular cells, indicating that pre-existing vascular structures participated in the process of *in vivo* vascularization.⁴ Similar conclusions were drawn from studies using 3D cell sheet grafts without⁵ or with the addition of endothelial cells (ECs) to the cardiomyocyte cultures.⁶ The extent to which ECs form vascular structures in 3D cardiac constructs *in vitro* and whether such structures form lumina have not been systematically studied and are hampered by methodological limitations. Immunohistochemistry or immunofluorescence staining of EC markers such as lectin or CD31 does not discriminate between graft- and host-derived cells, and GFP labeling of ECs⁶ requires genetic marking and supplementation of the native cell mix with exogenous cells.

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It was the goal of the present study to develop an engineered heart tissue (EHT)-based model, which allows simple and unambiguous identification of native ECs, to test angiogenic factors in this system and to see whether graft-derived ECs participate in vascularization of EHTs after implantation on hearts. We used a genetic approach and crossed an inducible and endothelial-specific Cre-transgenic strain (Cdh5-CreERT2)⁷ into a β -galactosidase reporter strain, in which the lacZ gene is irreversibly induced in a Cre-dependent manner (EC^{iLacZ}).

Materials and Methods

See the online Supplemental file for a detailed description of Materials and Methods, including histological, molecular biological, and statistical analyses.

Animals

The investigation conforms to the guide for care and use of laboratory animals published by the NIH (Publication No. 85-23, revised 1985). Cdh5-CreERT2 $\times$ Rosa26-LacZ mice were described previously⁷ and maintained on a C57/BL6 background. Heterozygous Cre-positive mice were crossed with homozygous Rosa26-LacZ reporter mice. Cre expression was induced by tamoxifen injection (*in vivo*; 2 mg/day, i.p. for 5 days) or *o*-hydroxytamoxifen added to EHT culture medium on day 15 (OHT, *in vitro*; 1 nM for 48 h), resulting in LacZ gene expression.

Generation, culture, and measurements of EHTs

EHTs were generated according to our 24-well EHT silicone postmethod as previously described for neonatal rat and mouse heart cells.^{8,9} Freshly isolated, unpurified heart cells from 0- to 1-day-old mice were resuspended in culture medium at a final density of 6.8×10^6 cells/mL culture medium (composition below). Agarose casting molds were generated by pipetting liquid 2% agarose in 24-well plates and adding Teflon spacers. After agarose solidification (10 min), Teflon spacers were removed and silicone racks with four pairs of elastic silicone posts each were placed onto the 24-well plate (six racks per plate) in a way that pairs of posts reached from above into the agarose casting molds. Human fibrinogen was prewarmed to 37°C to reduce viscosity and added to the resuspended cell mix at a final concentration of 2 mg/mL. To avoid the formation of fibrinogen clots, the mixture was pipetted up and down immediately after the addition of fibrinogen. Other compounds in the mix were aprotinin (2.5 μ g/mL) and 10% Matrigel (BD Bioscience 356235). Thrombin (3 μ L/EHT, 100 U/mL; Sigma T7513) was added to this mix (97 μ L/EHT) and the suspension was pipetted into rectangular agarose casting molds (L $\times$ W $\times$ D 12 $\times$ 3 $\times$ 4 mm) in a 24-well plate. Fibrin polymerization (37°C, 7% CO₂, 2 h) led to a rectangular fibrin cell-gel formation around the tips of the silicone posts. The racks were then transferred to fresh 24-well plates containing different culture media and kept in cell culture (21% oxygen) for 15–17 days. Serum culture medium included DMEM (Biocrom F0415), 10% horse serum (Gibco 26050), 2% chick embryo extract, 1% penicillin/streptomycin (Gibco 15140), insulin (10 μ g/mL; Sigma I9278), and aprotinin (33 μ g/mL; Sigma A1153). Growth factor-supplemented

serum-free culture contained hydrocortisone (50 ng/mL), T₃ (0.5 ng/mL), bovine serum albumin (0.5 mg/mL), M199 20%, IGF-1 (20 ng/mL), epidermal growth factor (EGF; 10 ng/mL), CT-1 (10 ng/mL; Sigma SRP4011), angiotensin II (1 μ g/mL), IL-1 β (10 ng/mL; Sigma SRP3083), basic fibroblast growth factor (bFGF; 10 ng/mL), and TGF- β 1 (20 ng/mL).

Spontaneous contractility of EC^{iLacZ} EHT was recorded over time by a video optical recording system and automatically evaluated by customized software (CTMV) as published before.⁸ Contractility parameters analyzed were frequency, force, contraction, and relaxation times determined from 20% of peak maximum to peak maximum.

EHT lengths were measured with images taken from the video optical recording. Taking the length of the scale bar into account (no. of pixels per mm) the length of an EHT from center to center between the two silicone posts can be measured.

EC^{iLacZ} EHT as a graft for transplantation studies

Immunodeficient NMRI nu/nu mice served for EHT transplantation studies to avoid graft rejection and the need for pharmacological immunosuppression. As an anesthetic, xylazine/ketamine (Rompun®; Bayer: 12 mg/mL ketamine, 1.6 mg/mL xylazine in 0.9% NaCl; 10 mL/kg body weight) was injected subcutaneously and mice were intubated during surgery (mouse ventilator TOPO™ Dual Mode Ventilator; Kent Scientific); 0.5–0.8 L/min oxygen was connected to the inflow of the ventilator and 2.5% isoflurane served as inhalational anesthetic. After opening the chest cavity, the EHT was washed in phosphate-buffered saline for a few minutes, removed from the silicone rack, and threaded using a 6-0 suture with the help of a needle on its two edges onto the left ventricle of the heart. Double knots were made on each side of the EHT to keep it fixed onto the heart. After cardiac surgery, mice received an analgesic (buprenorphine hydrochloride [Sigma], 0.1 mg/kg, s.c.). After recovery from cardiac surgery, the mouse was placed in a clean cage with a sterile filter on top. Metamizole sodium solution (Novaminsulfon-ratiopharm®, 500 mg/mL metamizole sodium; Ratiopharm) was added to the drinking water (1.3 mg/mL). Hearts were harvested for analyses 4 weeks after EHT implantation, fixed, and X-gal stained as described in the online supplement.

Results

Generation of EC^{iLacZ} EHT in a strip format

To evaluate whether fibrin-based EHTs support the spontaneous formation of primitive vessel networks, we used an unpurified mix of heart cells enzymatically isolated from neonatal (day 0–1) Cdh5-CreERT2-Rosa26-LacZ mice for EHT generation (Fig. 1A, B). In these mice, ECs are specifically and conditionally labeled in the presence of tamoxifen. This results in activation of a genetically modified Cre recombinase, which is expressed under the control of the EC-specific Cdh5 promoter.⁷ Generation and culture of transgenic murine EHT were performed in a 24-well format on silicone racks as previously described for rat and murine wild-type EHT (Fig. 1C).⁹ Using this protocol, EC^{iLacZ} EHT could be subjected to a short fixation and to the X-gal staining protocol in a whole-mount format (Fig. 1D). After 24 h of

FIG. 1. Schematic illustration of methodic workflow for the morphological analysis of vasculature in EC^{iLacZ} engineered heart tissue (EHT). **(A)** A $Cdh5$ -CreERT2 $\times$ Rosa26-LacZ mouse model was created for the purpose of endothelial cell (EC)-specific labeling (EC^{iLacZ}). Cre expression was kept under the control of a *Cdh5* promoter and Cre recombinase was fused to a tamoxifen-inducible estrogen receptor construct (ERT2). These mice served for neonatal heart supply. **(B)** The heart cells were isolated from neonatal hearts by a trypsin-based digestion. **(C)** Neonatal heart cells served for the generation of strip formatted EHT in a 24-well plate. **(D)** EHT was cultured for 15 days and expression of Cre was induced by the addition of *o*-hydroxytamoxifen (OHT) to the culture medium for 48 h. On day 17 of culture, EHT underwent a short fixation (45 s) and subsequent X-gal staining for 24 h, which resulted in the visualization of extensive LacZ-positive vascular structures **(E)**. Color images available online at www.liebertpub.com/tea

X-gal staining, EC structures were identified by the specific blue color indicating LacZ gene expression (Fig. 1E).

EC^{iLacZ} EHTs develop spontaneous contractility

EC^{iLacZ} EHT developed macroscopically similar to EHT from wild-type mice, started to beat spontaneously and showed a cardiac tissue structure as previously described.⁹ In the presence of animal sera, the fibrin matrix was remodeled over time, and coherent contractions with deflection of the silicone posts were observed after ~ 6 days of culture. Under growth factor-supplemented serum-free conditions, contractile parameters such as force and frequency could not be recorded due to a lack of coherent contraction (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). Spontaneous beating of EC^{iLacZ} EHT was monitored over a culture period of 17 days with a setup for video optical recording as previously described (Fig. 2A, B for representative recordings). Between days 6 and 15, force significantly increased ($53 \mu N$ on day 6 and $94 \mu N$ on day 15, $p < 0.001$; Fig. 2C), which was accompanied by an increase in vessel-like structures (Supplementary Fig. S2). The rate did not significantly differ over the culture period and ranged from ~ 148 to 230 bpm, similar to WT.⁹ Contraction (T1 20%) and relaxation (T2 20%) times did not differ between early and late time points of culture, indicating a stable contractile behavior of EHT over the culturing time without arrhythmia (Fig. 2D).

Spontaneous vascular network formation in EC^{iLacZ} EHT

To test the functionality of the genetic mouse model, adult $Cdh5$ -CreERT2-Rosa26-LacZ mice were treated with tamoxifen for 5 days; another 5 days later, the hearts were harvested and stained with X-gal. Blue blood vessels were seen throughout the heart, but eosin-stained sections revealed

a patchy pattern, indicating that activation of the ROSA26 locus under the chosen *in vivo* conditions was incomplete (Fig. 3A–C). In contrast, X-gal-stained 18-day-old EC^{iLacZ} EHT showed a dense interconnected network of ECs (Fig. 3D–J). The network was aligned along the force lines, ranging from one silicone post to the other. Eosin-stained paraffin cross and longitudinal sections of X-gal-stained EHT showed frequent lumina, indicating the formation of a primitive vascular network (Fig. 3G–I). Immunofluorescence analysis for a cardiac cell marker α -actinin and X-gal showed that the endothelial structures were mainly associated with cardiomyocyte bundles within the EHT (Fig. 3J).

We further aimed at quantifying vessel-like structures in EHT, which were observed in the whole-mount tissues (Fig. 4A). On average, per 1000 μm length of an EHT, a total of 60 ± 3 vessel-like structures with 43 ± 7 branching points were discernible (Fig. 4B). The total length of vessel-like structures amounted to $10,500 \pm 350 \mu m$ ($n = 5$). Thus, given an average EHT diameter of 500–700 μm , the predominantly longitudinally aligned vessel-like structures should be an estimated 40–80 μm apart in transverse direction (taking the cylindrical shape of an EHT into account). However, as a 2D projection of a 3D tissue strip might lead to an overestimation of vessel density and branching might be partially a projection artifact, we prepared thin longitudinal X-Gal- and eosin-stained sections. This analysis not only revealed that vessel-like structures were indeed branched but also formed lumina that were interconnected (Fig. 4C). Diameters of lumina were 6–8 μm and intervessel distances ranged from 20 to 60 μm (Fig. 4D).

Vessel formation in EC^{iLacZ} EHT depends on serum

To evaluate whether culture conditions affect vessel formation, EHTs were generated in the presence (Fig. 5A) or

FIG. 2. Spontaneous contractile activity of EC^{iLacZ} EHT, measured with video optical recording over a culturing period of 17 days. **(A)** A contour recognition mode automatically recognizes both ends of the EHT and follows them over time (gray squares). **(B)** Original recordings of spontaneous activity show the force development in an EC^{iLacZ} EHT at different time points of culture (days 6, 10, and 17). Note that force increases over time. EHT was recorded over a time period of 60 s; here a time window of 10 s is shown. **(C)** Contractile parameters such as force and frequency in beats per minute were measured over a culturing period of 17 days. **(D)** Contraction time (T1=time from 20% force to peak force) and relaxation time (T2=time from peak force to 20% force) were monitored over time. $N=7$ EHTs per group. Data are mean $\pm$ SEM. *** $p < 0.001$ versus day 6 (one-way ANOVA plus Bonferroni post-tests).

in the absence of 10% horse serum (standard condition, Fig. 5B). This analysis revealed a marked reduction in EC density under serum-free conditions, amounting to $\sim 84\%$ as quantified by software recognizing the blue areas within EHT cross sections (Fig. 5C, D). This was accompanied by greater shortening of EHT (final length $\sim 30\%$ compared with serum-cultured EHTs, Fig. 5E) and less reduction in diameter (final diameter $+27.4\%$; Fig. 5F). Transcript analysis showed markedly lower levels of *Pecam1* (CD31), *Kdr* (VEGFR2), and *Myh6* (alpha-myosin heavy chain) and higher levels of *Myh7* (beta-myosin heavy chain) and the apoptosis marker, *Bax*, in EHTs cultured for 15 days under serum-free conditions (Fig. 5G–K). Western blot analysis of CD31, X-gal, and β -MHC confirmed the mRNA analysis (Fig. 5L).

Graft-derived vascular structures participate in graft vascularization in vivo

To evaluate whether EC^{iLacZ} EHT can be utilized as grafts to study vessel ingrowth and EC survival on a murine heart *in vivo*, we implanted EHT onto the left ventricle of NMRI nu/nu mouse hearts. Histological analyses were performed 4 weeks after EHT engraftment. Before heart extraction, Cre expression was induced *in vivo* by tamoxifen application (2 mg/mouse per day by intraperitoneal injection for 5 days). Mice were housed in the cage for

another 4 days and hearts with EHT grafts were harvested and underwent short fixation and the X-gal staining protocol. Murine EHTs were visible as blue–white structures covering the left ventricle of the heart (Fig. 6A). Cells in the EHT survived on the surface of the heart *in vivo* as detected in hematoxylin/eosin-stained paraffin sections, but exhibited a low density compared with native myocardium (Fig. 6B, C). Eosin staining of cells in the EHT region near the myocardium was more intense than staining of cells in the periphery (Fig. 6B, C). ECs originating from the EC^{iLacZ} EHT could be identified by the intense blue color after X-gal staining (Fig. 6D–F) and were mainly found incorporated into blood vessels of varying caliber and clear lumina (Fig. 6E, F). The density of X-gal-positive blood vessels with lumina tended to be higher in regions close to host myocardium, indicating connection to the host circulation.

Vascular structures in transplanted EHT are connected to host circulation

To answer whether the vascular structures in EC^{iLacZ} EHT were structurally intact and coupled to the host myocardium, paraffin sections of EHTs transplanted onto NMRI nu/nu mouse hearts were probed with an antibody against TER-119 to visualize erythrocytes. Vascular structures in EHT regularly showed TER-119-positive erythrocytes (Fig. 7A–D).

FIG. 3. Morphological analysis of EC^{iLacZ} adult murine heart and EC^{iLacZ} EHT after X-gal staining. (A) Whole-mount X-gal staining of EC^{iLacZ} heart was performed after 5 days of i.p. injection of tamoxifen and 5 days of sitting time in the cage. Extensive vascular structures are visualized in blue color (A, B). (C) In eosin-stained paraffin sections of X-gal-stained heart vessel, structures can be seen in blue and tissue is colored in pink. Light microscope images of X-gal-stained EC^{iLacZ} EHT on day 18 of culture in 2.5 $\times$ (D) and in 10 $\times$ (E, F) magnification, depicting the area near the silicone post (E) and the middle part of the EHT (F). Eosin-stained paraffin cross sections of EC^{iLacZ} EHT (G, H) and a longitudinal section (I). (J) Immunofluorescence images show alpha-actinin in green visualizing sarcomeres in cardiomyocytes, nuclei in blue (DRAQ5 staining), and X-gal-positive structures indicating vessels in red. Antibodies used were directed against cardiac alpha-actinin (green) and against LacZ (X-gal, red).

FIG. 4. Quantification of X-gal-positive vessel-like structures in EHT. **(A)** Whole-mount microscopic image (10 $\times$). *White arrows* indicate some of many potential branching sites of vessels. **(B)** All vessel structures in a 1000 μ m longitudinal section of the EHT were marked in *white* by quantification software. **(C)** Longitudinal X-gal and eosin-stained sections of an EHT. **(D)** X-gal staining intensity and distance determinations in a vessel-dense area of an EHT. Color images available online at www.liebertpub.com/tea

Discussion

In the present study, we have fabricated spontaneously contracting murine EHTs from a transgenic mouse model, in which ECs can be labeled specifically by induction with tamoxifen EC^{iLacZ} . EHTs were functionally and morphologically similar to normal mouse EHT⁹ and demonstrated a vessel-like network *in vitro*, which was dependent on the presence of serum in the culture medium. The longitudinally orientated and interconnected vessel-like network in EHT *in vitro* showed clear lumen formation and a density approaching that of native heart tissue. After transplantation, vessels connected to the host circulation. Together with the possibility to exactly time Cre induction, this system is well suited for further studies of vessel formation in 3D heart muscle constructs and to proceed with the goal of transplanting EHT to diseased hearts in the future.

EC^{iLacZ} EHTs showed an extensive and continuous network of EC-positive vascular structures, indicating complete or almost complete labeling of ECs with the protocol used *in vitro*. This contrasts with the patchy *in vivo* results and can likely be explained by the simpler access of tamoxifen to the cells when given as the active metabolite directly in the culture medium. Problems with the tamoxifen-mediated induction of recombination have been reported previously, which include toxicity of tamoxifen and that the system may be somewhat leaky, resulting in constitutive expression of Cre.¹⁰ Another study described a lack of protein loss after Cre-mediated recombination.¹¹ The limitations of the system can often be overcome by repeated applications and/or higher doses of tamoxifen.^{12,13} The extent of vascular network formation was remarkable and, to the best of our knowledge, better than any former results. Reasons may include that this is the first study in which endogenous ECs were genetically labeled rather than genetically labeled ex-

ogenous ECs being added as previously described.^{6,14,15} X-Gal staining may provide a more complete visualization of the complex vascular structures than fluorescence analyses of immuno- or genetically stained ECs. Additionally, in contrast to a traditional transgenic model, the LacZ gene expression can be induced by tamoxifen specifically in ECs at any time point. For the current study, it was crucial that LacZ gene expression was induced in the mouse after EHT transplantation and shortly before analysis as ECs can undergo endothelial—mesenchymal transition and acquire a fibroblast-like phenotype and lose their EC characteristics,¹⁶ which in our case would make it impossible to distinguish between an LacZ signal coming from a fibroblast or an EC. The good degree of vessel formation compared to previous reports could also indicate that fibrin EHT provide a better growth environment than other systems. An important limitation of the LacZ staining is the washout of the blue signal during immunohistochemistry. In fact, this limitation prohibited the approach of a double staining of the EC marker CD31 and LacZ in EHTs. Additionally, in the fibrin EHT environment, antibody-based staining procedures (e.g., for CD31 or von Willebrand factor) were more challenging than in native heart samples. On this background, we started the genetic labeling project, which is presented here. Based on our results, we think that the genetic model faithfully detects ECs and is convincing. The mouse model, which served for EHT generation, was previously described by Benedetto *et al.* for EC signal specificity⁷; furthermore, the morphology of the LacZ+ structures in EHTs, both in the whole mount and the sections, exhibited very typical aspects of blood vessel capillaries: thin, longitudinally oriented branched structures with lumina of 6–8 μ m.

The formation of EC-positive vascular networks in EHT was strongly dependent on the presence of serum, likely explained by the fact that animal sera contain large amounts of

FIG. 5. Extensive vasculature structures in EC^{iLacZ} EHT are dependent on the presence of animal sera in the culture medium. Examples of light microscopic images of X-gal-stained EC^{iLacZ} EHT cultured under serum (A) and serum deprivation (B) after 15 days of culture in 10× magnification, and an example of EC area quantification of an eosin-stained paraffin section (C). The green marked areas indicate structures recognized as X-gal positive. EC area quantification (D), EHT length (E), and diameter (F) measured in EHT cultured in the presence or absence of animal sera. $N = 7-13$ EHT per group. (G–K) Real-time quantitative PCR analysis for *Pecam1* (CD31) (G), *Kdr* (VEGFR2) (H), *Myh6* (alpha-myosin heavy chain) (I), *Myh7* (beta-myosin heavy chain) (J), and *Bax* (BCL2-Associated X-Protein) (K) mRNA levels was performed after 17 days of EC^{iLacZ} EHT culture in the absence or presence of serum. (L) Western blot analysis for β-MHC, β-Gal, CD31, and total ERK after 17 days of culture. $N = 3-7$ EHTs per group. Data are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus serum-free conditions (student's t -test). Color images available online at www.liebertpub.com/tea

angiogenesis-modifying growth factors.¹⁷ In fact, serum deprivation during cell culture led to apoptosis of vascular ECs.¹⁸ Polypeptide growth factors present in animal sera can activate transmembrane receptors and induce intracellular signaling pathways that promote cell survival. An example is the ser-

ine-threonine protein kinase Akt, which has antiapoptotic effects¹⁹ and is activated by insulin-like growth factor (IGF) analogs or platelet-derived growth factor (PDGF) BB. Under serum-free conditions without supplementation of growth factors, vessel-like structures were completely absent, and

FIG. 6. Morphological analysis of ECiLacZ EHTs transplanted onto hearts of NMRI nu/nu mice. (A) Whole-mount X-gal staining of a recipient heart with an ECiLacZ EHT on top. Hematoxylin/eosin-stained paraffin sections of the myocardium and EHT on top in 10 $\times$ (B) and 20 $\times$ (C) magnification. *M* indicates myocardium. Eosin-stained paraffin section of the heart with ECiLacZ EHT in a longitudinal view in 5 $\times$ (D) and in 10 $\times$ magnification (E). (F) Unstained paraffin section showing X-gal-stained tubular structures in ECiLacZ EHT on top of the myocardium. Color images available online at www.liebertpub.com/tea

histological analysis revealed large vacuoles within cardiomyocytes in EHT, indicating a high rate of cell death. We therefore used a growth factor-supplemented serum-free medium formerly developed for rat EHT.^{20,21} It contained hydrocortisone (50 ng/mL), T3 (0.5 ng/mL), bovine serum

albumin (0.5 mg/mL), M199 20%, IGF-1 (20 ng/mL), EGF (10 ng/mL), CT-1 (10 ng/mL), angiotensin II (1 μ g/mL), IL-1 β (10 ng/mL), bFGF (10 ng/mL), and TGF- β 1 (20 ng/mL) and was devised to mimic physiological concentrations. This medium reduced the formation of vacuoles and cell death and

FIG. 7. Immunohistochemical analysis with an antibody against the erythrocyte marker, TER-119, in NMRI nu/nu mouse heart with transplanted ECiLacZ EHT. Counterstain hematoxylin. TER-119-stained paraffin sections of the myocardium and EHT on top in 20 $\times$ (A) and in 40 $\times$ (B) magnification. TER-119-stained paraffin sections of another heart and EHT on top in 20 $\times$ magnification (C) and in 100 $\times$ magnification (D), respectively. *M* indicates myocardium. *S* indicates suture. Color images available online at www.liebertpub.com/tea

allowed the generation of spontaneously beating EHT, although at much lower force. CT-1 protects cardiomyocytes in culture during serum-free conditions,²² and IGF-1, EGF, and IL-1 β at low concentrations are known to improve myofibril formation and the development of a physiological form of cardiomyocyte hypertrophy.^{20,22–24} Additionally, IL-1 β has antiproliferative effects on fibroblasts and improves sarcomere formation.²⁵ FGF is known to improve angiogenesis accompanied by an increase in cardiomyocyte size, whereas TGF- β also induces cardiomyocyte hypertrophy and stimulates proliferation of cardiac fibroblasts.²⁶ The latter could explain the shortening of serum-free cultured EHTs. In future approaches, TGF- β might be replaced by soluble vascular endothelial growth factor (VEGF) and PDGF, which could have a beneficial effect on EC survival and proliferation under serum-free conditions. Soluble PDGF in addition to IL-1 β is thought to stimulate VEGF production by smooth muscle cells, synergistically increasing the effect of VEGF.²⁷ However, under serum-free conditions, neither PDGF nor VEGF had a major effect on the vascular network (data not shown), indicating that important factors are still lacking in the above formulation in comparison with serum-containing conditions. Thus, definition of serum-free culture conditions that are optimal for both cardiomyocytes and ECs in 3D constructs requires further work and should be facilitated by the Cdh5 mouse model.

For cardiac regeneration purposes, vascularization of the graft is an important parameter to ensure graft survival and longevity *in vivo*. The present experiments in immunocompromised mice showed that graft-derived (X-gal positive) ECs participate in functional post-transplantation vascularization. This supports earlier observations in infarcted rats⁴ or nude rats.⁶ The highest density of vascular structures was detected in EHT border zones close to the host myocardium, suggesting that ECs in EHT are initially provided with nutrients and oxygen by diffusion from the surface of the host myocardium and later by ingrowth and connection to host blood vessels. Growth factors,^{28,29} gene transfer,^{30,31} and addition of exogenous ECs⁶ have been reported to improve vascular network formation in 3D heart muscle constructs *in vitro* and *in vivo*. This will be particularly important for studies with stem cell-derived cardiomyocytes in which ECs are lacking.³² Further improvements may be achieved by means to perfuse 3D constructs *in vitro*³³ and connect the vascularized constructs directly to the host circulation.^{34,35}

In summary, EC^{iLacZ} EHTs are a useful new tissue culture model to study vessel formation *in vitro* and *in vivo*. Potential applications include testing of compounds with respect to cardiotoxicity and angiogenesis simultaneously. EC^{iLacZ} EHT already served to substantiate an antiangiogenic effect of miRNA-24.³⁶ Such studies will be facilitated by the definition of serum-free culture conditions, which requires further work.

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Disclosure Statement

No competing financial interests exist.

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