Chimeric Vessel Tissue Engineering Driven by Endothelialized Modules in Immunosuppressed Sprague-Dawley Rats

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Modular tissue engineering is a means of building functional, vascularized tissues using small (~1 mm long 0.5 mm diameter) components. While this approach is being explored for its utility in adipose and cardiac tissue engineering and in islet transplantation, the initial question in this study was to assess the fate of the endothelial cells (EC) after transplantation delivered on the surface of modules, without an embedded cell. Rat aortic EC-covered collagen gel modules were transplanted into the omental pouch of allogeneic (outbred) Sprague-Dawley rats with and without immunosuppressive drug treatment (atorvastatin and tacrolimus) for 3–60 days. There was a significant increase in vessel density at all time points in the drug treated rats as compared to untreated rats. Green fluorescent protein (GFP)-positive donor rat aortic EC migrated from the surface of the modules and formed primitive vessels by day 7. In the untreated rats, the GFP-positive cells were not seen after day 7. In drug-treated rats, GFP-positive vessels matured over time, accumulated erythrocytes, were supported by host smooth muscle cells, and formed chimeric vessels that survived until day 60. This resulted in the formation of a densely vascularized, perfusable network by day 60. To our knowledge, this is the first study that demonstrates that primary unmodified EC, without the addition of supporting cells, form a chimeric and stable vascular bed in allogeneic, although drug-treated, animals.

Introduction

A SIGNIFICANT CHALLENGE in tissue engineering is the development of an adequate blood supply for the survival of an engineered tissue construct. Without an internal vascular system, tissue survival is limited by diffusion of essential nutrients and oxygen. Hence, only cells within a diffusion distance of 150–200 μm of the host blood vessels are adequately perfused and there can be significant cell loss at the core of implanted engineered tissues without an adequate vascular network.^{1,2} Another challenge is the immune response and rejection of engineered tissues, which leads to most *in vivo* tissue engineering being done in immune-compromised animals to circumvent or at least minimize these issues. The use of immune-compromised models also decreases the inflammatory response that in part drives wound healing, angiogenesis, and tissue remodeling, critical aspects of the ultimate integration of the tissue construct with the host.

Modular tissue engineering is a means of assembling blood perfusable constructs with benefits that include uniform cell density, scalability, mixed cell populations, and vascularization.³ Functional cells are embedded in submillimeter-sized

collagen rods and the outside surface is coated with endothelial cells (EC). These units are referred to as modules and form microtissues when there are cells embedded in them. By varying the type of embedded cells in the individual microtissue, it is expected that complex modular tissue-engineered constructs can be produced for different applications; HepG2 cells⁴ and smooth muscle cells⁵ have been incorporated into modular constructs to date. The microtissues are used to fill a tissue cavity and the randomly packed modules create channels by the virtue of the spaces among the modules (Fig. 1A). Human umbilical vein EC (HUVEC)-coated modules implanted into an omental pouch in nude rats formed primitive vessels within the spaces between modules until 7 days, but survival was limited due to the severe macrophage response.⁶ Because we were ultimately interested in creating a vasculature around a transplanted functional tissue, such as pancreatic islets, we chose to implant rat aortic EC (RAEC) isolated from outbred Sprague-Dawley rats into outbred Sprague-Dawley rats with the use of immunosuppressive drugs to study the survival of the implanted EC and the formation of the resulting blood vessels, as analyzed by histology and microcomputed tomography (CT).

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FIG. 1. Transplanted endothelial cells (EC) participate in vessel formation. **(A)** Schematic showing randomly assembled modules. **(B)** The average number of vessels that contain at least one green fluorescent protein (GFP)-positive cell in the drug-treated group. There were significantly more GFP-positive vessels at day 14 than at days 21 or 60 (* $p < 0.05$). Average values $\pm$ standard error of the mean (SEM). At days 7 and 14 $n = 2$, at day 21 $n = 4$, and at day 60 $n = 5$. **(C)** Transplanted GFP rat aortic EC (RAEC) around endothelialized modules (dashed outline) were detected with an anti-GFP antibody (brown stain) in drug-treated animals. At day 3, transplanted GFP-RAEC were primarily on the surface of modules **(a)** and at day 7, GFP-RAEC migrated off the modules to form vessels **(b)**. By day 14 **(c)**, a number of GFP-RAEC were incorporated into vessels (arrows) of various sizes and these could be seen out to day 60 **(d, e)**. Scale bar = 100 μ m. Color images available online at www.liebertonline.com/ten.

Materials and Methods

Cells

Sprague-Dawley RAEC were purchased from VEC Technologies (Rensselaer, NY) and maintained on 0.2% gelatin-coated flasks in MCDB-131 complete medium with 10% fetal bovine serum at 37°C and 5% CO₂. Cells were used between passages 3 and 5. In some cases, RAEC were stably transduced with HIV-1-based recombinant lentivirus (LV) encoded for enhanced green fluorescent protein (GFP). Briefly, vesicular stomatitis virus glycoprotein-pseudotyped LV including the pHR'-cPPT-EF-GW-SIN plasmid (containing GFP) were generated by transient transfection of 293T cells as described before.^{7,8} For cell transduction, RAEC were infected with recombinant LV-GFP at a multiplicity of infection (MOI) of 10 in the presence of 8 μ g/mL protamine sulfate. Sixteen to 18 h postinfection, the supernatant was removed and GFP-RAEC were cultured with a fresh medium for at least 2 days before use. The transduction efficiency of the GFP was measured by flow cytometry and was >95% in all cases.

Module fabrication

Type 1 bovine collagen (3.1 mg/mL; Cohesion Technologies, Palo Alto, CA) modules (~2 mm long $\times$ 0.6 mm diameter) were prepared as before.³⁻⁵ RAEC (2.5×10^6) were seeded

dynamically onto the surface of 1 mL of modules (produced using 2.5 m of tubing) for 45 min on a low-speed shaker and incubated for 7 days before implantation. RAEC contracted collagen modules to ~1 mm long $\times$ 0.5 mm diameter. Collagen-only modules were prepared as above but without EC seeding.

Module transplants

Adult female Sprague-Dawley rats (7 weeks of age, 250–300 g; Charles River, Wilmington, MA) were individually housed and fed *ad libitum*. Animals were divided into two groups: untreated and drug (tacrolimus and atorvastatin) treated. Tacrolimus (Astellas, Markham, ON, Canada) was administered intramuscularly daily (days 1–6: 0.3 mg/kg, days 7–14: 0.2 mg/kg, and days 15–60: 0.1 mg/kg) in saline, and atorvastatin (Pfizer, Kirkland, QC, Canada) was administered daily from the day before surgery until 21 days after surgery via oral gavage at a dose of 0.5 mg/kg in sterile water. Approximately 500 modules, suspended in 0.5 mL of phosphate-buffered saline, were implanted in an omental pouch created as described before.⁶ For both untreated and drug-treated groups, animals were transplanted with endothelialized modules for 3, 7, 14, 21, and 60 days ($n = 5$). Collagen-only modules (without RAEC) were also transplanted in untreated and treated animals for 3 or 7 days

($n=2$). The study was approved by the University of Toronto animal care committee.

Perfusion studies

Animals were heparinized (500 U; LEO Pharma Inc., Thornhill, ON, Canada) 5 min before the procedure by subcutaneous injection. Following a published protocol,⁹ the descending aorta was cannulated and heparinized phosphate-buffered saline (5 U/mL) was perfused at a constant pressure of 100 mmHg until the blood was flushed from the vascular system. Animals were then perfused with 25 mL of Microfil[®] solution (MV-122; Flow-Tech, Carver, MA). The Microfil solution was allowed to polymerize for at least 90 min and then the omental tissue was excised into 4% formalin (Sigma Aldrich, Oakville, ON, Canada) and embedded in 1% agar solution, and images were obtained with a General Electric Medical Systems MS8 microCT.

Histology and immunostaining

Animals were sacrificed and the omental pouch was excised into 4% neutral buffered formalin and fixed for 48 h. Tissue samples were embedded in paraffin and 4 μ m sections were cut at three levels that were 100 μ m apart. Sections were processed and stained for hematoxylin and eosin (Fisher, Ottawa, ON, Canada), Masson's trichrome (Fisher), TUNEL (Promega, Nepean, ON, Canada), and various antibodies:

Bandeiraea Simplicifolia Lectin I (BS-1; 1:300 dilution; Vector Laboratories, Burlingame, CA), GFP (1:8000, AB6556; Abcam, Cambridge, MA), CD68 (1:600 dilution; MCA341; AbD Serotec, Raleigh, NC), TCR $\alpha\beta$ (1:600 dilution; MCA453; AbD Serotec), Von Willebrand factor (vWF; 1:5000 dilution; CL20176A-R; Cedarlane, Burlington, ON, Canada), and smooth muscle α -actin (SMA; 1:1000 dilution; AB5694; Abcam). Sections were viewed with a Zeiss Axiovert light microscope equipped with a CCD camera; fluorescent images were taken with an Olympus Upright microscope equipped with a Photometrics CoolSNAP camera.

Histology quantification

BS-1, a microvascular EC marker,¹⁰ or vWF (both micro- and macrovascular EC)-positive cells were counted using Chalkley and microvessel density (MVD) methods by a blinded observer as described before.⁶ Briefly, a 25-point Chalkley eyepiece graticule was applied and the eyepiece was rotated to align the maximum number of dots with BS-1-positive cells in three representative areas at 200 $\times$ magnification. The dots that overlaid BS-1-positive structures were counted and the mean of the three counts were represented semi-quantitatively where a mean of eight or greater was counted as high (+) and a mean of less than eight was counted as low (-); a separate score ($\pm$) was reported for each animal. The MVD method was applied to count BS-1 or vWF-positive vessels (with a defined lumen) at 400 $\times$

FIG. 2. Trichrome staining shows the remodeling of transplanted modules (thin arrows) with and without drug treatment. Collagen-only modules collapsed together and degraded over 7 days (**a, b**). At day 3, a hematoma (RBC) formed in the untreated (**c**) and treated (**h**) rats transplanted with endothelialized modules but not with collagen-only modules (**a**). At day 7, host cells infiltrated the region around endothelialized modules in both treatment conditions (**d, i**). In the untreated group, infiltrating cells persisted until day 60 (**g**) but not in the treated group (**l**). There was a formation of a collagen deposit (CD) around the modules (**d–g, i–l**). This CD contained a number of blood vessels (thick arrows), which increased with time, in both the treated (**j–l**) and untreated (**e–g**) animals. Magnification is 50 $\times$. Color images available online at www.liebertonline.com/ten.

magnification in three representative areas and the average of the three MVD counts was used for statistical analysis. Macrophages (CD68 positive) were counted in five representative sections at 400 $\times$ magnification and the average of these counts was used for statistical analysis. GFP vessels were counted in one section of the whole omentum tissue. The whole microscope slide (stained with GFP) was digitized using the Aperio ScanScope XT and cross-sectional diameter of each GFP vessel was measured manually in the Aperio ImageScope software (Vista, CA). Vessels were then binned according to size ranges as either: capillaries (1–9 μ m), small arterioles/venules (9–15 μ m), large arterioles/venules (15–75 μ m), or abnormal (>75 μ m).

Statistical analysis

A one-way analysis of variance with least significant difference (LSD) *post hoc* analysis was applied to compare means between multiple groups. Data were considered statistically significant at $p < 0.05$. All analysis was done with Statistica Version 5.1 software (Statsoft, Tulsa, OK).

Results

Endothelialized module remodeling and RAEC survival

When endothelialized modules were transplanted into the omental pouch of rats, chimeric vessels containing both host and donor EC were produced. The transplanted RAEC were presumed to contribute both indirectly and directly to aspects of the remodeling that occurred upon implantation of the modules to form vessels: RAEC-secreted factors that recruited host cells and migrated from the modules to participate in vessel formation. RAEC transduced with GFP (GFP-RAEC, Fig. 1C) remained mostly on the surface of the modules at day 3. At day 7, some GFP-positive vessels

(with defined lumens) were seen near the modules; in untreated rats, no GFP-positive cells were seen after day 7. GFP-RAEC vessel maturation began at day 7, at which time vessels started to form and by day 14 accumulated erythrocytes in their lumens, presumably reflecting anastomoses to the host circulation. GFP-RAEC were seen in vessels of varying sizes near the modules at days 14, 21, and 60. The number of GFP-positive blood vessels decreased from 14 to 60 days (Fig. 1B). This decrease in GFP-positive blood vessel density was attributed to the pruning of unneeded vessels¹¹ or perhaps the replacement of donor EC by host cells as the graft stabilized.

The host response remodeled the transplanted modules and surrounding tissue over time as shown in the Masson trichrome images (Fig. 2). By day 7, collagen-only modules (no EC) were largely resorbed into the surrounding tissue (Fig. 2a, b), as has been detailed before.⁶ Endothelialized modules elicited a more complex host response. Early (within 3 days) in the remodeling process, there was the formation of a hematoma around the largely intact endothelialized modules (Fig. 2c, h) in both treated and untreated animals. At the same time, there was also a collagen-rich region that formed around these modules; this collagen rich area continued to increase in size as the hematoma resolved (Fig. 2d–g, i–l). This collagen deposit was morphologically different from the transplanted endothelialized modules, which retained their roughly cylindrical shape even after the EC migrated off their surface, and might reflect collagen produced by the infiltrating cells or degraded modules. Such a hematoma did not form without endothelialized modules or in sham surgeries, suggesting that the transplanted EC were a causative agent. As time progressed (by day 21), the hematoma was largely resolved and reabsorbed (Fig. 2f, k). Modules assembled both in the interior and exterior of the hematoma and intact modules were seen until day 60 (Fig. 2g, l).

FIG. 3. Serial sections show examples of apoptotic (TUNEL), proliferating (Ki67), or smooth muscle α -actin (SMA) cells (brown staining, arrows) around endothelialized modules (dashed outline) in untreated and treated animals at day 7. A few apoptotic cells were detected on the surface of the modules (a, c), while several proliferating cells were in the tissue surrounding the modules (b, d). Particularly in treated rats, some of the infiltrating cells were positive for SMA (e). Scale bar = 100 μ m. Color images available online at www.liebertonline.com/ten.

The cellular response to endothelialized modules was characterized, at day 7, as an influx of cells surrounding the modules in the exterior of the hematoma (Fig. 2d, i). Many of these cells were proliferative (Fig. 3b, d) as noted by positive staining with Ki67. While some of the infiltrating cells were likely monocytes, particularly in the treated animals, a large number of the cells stained positive for SMA and were presumably myofibroblasts or perhaps pericytes (Fig. 3e). On the surface of the modules in both treated and untreated rats, a number of apoptotic cells (TUNEL) were also observed (Fig. 3a, c); at least some of these apoptotic cells were likely the transplanted EC. Interestingly, at day 7, a number of microvascular blood vessels (BS-1 positive, Fig. 4) begin to appear in the collagen deposit region surrounding the modules.¹⁰ The number of these blood vessels increased over time in both the treated and untreated groups. These vessels are characterized further below.

Tissue vascularization and impact of inflammation and immune responses

Rat EC can initiate both humoral and cellular immune responses in an allotransplant setting.¹² Anticipating similar responses with endothelialized modules, we used tacrolimus, an immune-suppressant, and atorvastatin to enhance allograft survival. Atorvastatin may directly decrease the immune response to EC as demonstrated by decreased proliferation of T cells exposed to xenogeneic EC *in vitro*,¹³ improved EC survival,¹⁴ increased NO production,^{15,16} and increased number of circulating endothelial progenitor cells.¹⁷ With tacrolimus and atorvastatin treatment, inflammatory cell numbers (CD68⁺) were significantly reduced at day 21 (Fig. 5A). Also, while T cells were detected near endothelialized modules in untreated rats, there was a complete absence of T cells in treated rats (Fig. 5B). As a result,

FIG. 4. Vessel formation is enhanced with tacrolimus and atorvastatin treatment. BS-1 lectin stain was used to identify microvascular endothelial cells of both host and donor origin. **(A)** Average Chalkley count of the BS-1 lectin-positive cells. Drug-treated animals had more BS-1-positive cells than the untreated animals up to day 14. Each animal was scored by the number of BS-1 cells that fall on the Chalkley grid for three 400 $\times$ views and a mean score of eight or higher is positive (+) and less than eight is negative (–), five animals/group. **(B)** Average microvessel density count of BS-1-positive blood vessels. Drug-treated animals (gray bars) had significantly more BS-1-positive vessels than untreated animals (white bars) at days 7, 14, and 21 ($*p < 0.05$). Average of five animals/group $\pm$ SEM. **(C)** Average microvessel density counts of Von Willebrand factor (vWF)-positive vessels; drug-treated animals (gray bars) had significantly more blood vessels than untreated animals (white bars) at days 21 and 60 ($*p < 0.05$); five animals/group $\pm$ SEM. **(D)** There were isolated BS-1-positive cells (arrows) around endothelialized modules (highlighted with a dashed line) at day 3; in some cases, BS-1-positive cells were on the surface of the modules (**a**, **e**). At day 7, there was a mixture of isolated BS-1-positive cells and a few vessels surrounding endothelialized modules (**b**, **f**). By days 14 and 21, there were many BS-1 vessels near modules (**c**, **d**, **g**, **h**) and the vascular density was more pronounced with drug treatment (**g**, **h**). Scale bar = 100 μ m. Color images available online at www.liebertonline.com/ten.

FIG. 5. Inflammatory and immune cells near endothelialized modules. **(A)** Average counts of CD68 (macrophages)-positive cells. In untreated animals (white bars), the number of CD68-positive cells significantly increased from days 3 to 7 ($*p < 0.05$) and remained high until 21 days. With drug treatment (gray bars), macrophages peaked at day 14 and were significantly reduced by 21 days. When compared to control animals, there were significantly ($*p < 0.05$) fewer macrophages at days 7 and 21. Data shown are the average of five sections/animals $\times$ five animals/group $\pm$ SEM. **(B)** T cells ($\alpha\beta$ T cell receptor, brown stain, arrows) were seen near endothelialized modules (dashed line) at day 14 **(a)** in untreated animals but not with immunosuppressant treatment **(b)**. Scale bar = 100 μ m. Color images available online at www.liebertonline.com/ten.

both transplanted EC survival and overall vessel density (donor and host derived) was greater with treatment.

Microvessel EC were identified by BS-1 lectin staining and there was a high number of individual microvascular EC (BS-1 positive) around the modules at day 3 with treatment as measured by Chalkley counts (Fig. 4A). Over time, the EC formed vessels near modules resulting in lower Chalkley counts at later times. The Chalkley method counts individual EC that may or may not be in vessels, as well as those in microvessels with a lumen. Over time, the number of isolated EC decreases as they die or as they become incorporated into vessels. Thus, a drop in Chalkley count is consistent with the corresponding BS-1 positive MVD increase, which were significantly higher in all the treated animals after day 7 (Fig. 4B). Both micro- and macrovessel EC were identified by vWF staining and the total vessel density counts was higher with drug treatment at 21 and 60 days (Fig. 4C).

RAEC-driven vascularization

Over time, there were changes in the number (Fig. 1C) and maturity (Fig. 6) of blood vessels. The ratio of large arterioles and venules to capillaries (based on vessel diameter) increased from 7 to 21 days, continuing out to 60 days, suggesting that the capillaries were maturing as part of the normal angiogenic process (Fig. 6A). The vessel maturation was also evident by the recruitment of smooth muscle cells (SMA positive). At day 14, there were many GFP-positive vessels but little incorporation of SMA-positive cells in the walls of these vessels (Fig. 6B a, 6B e). The vessels matured over time and by day 60, three types of GFP-RAEC-derived vessels could be distinguished: small-diameter vessels without SMA (capillary-like vessels; Fig. 6B b, 6B f, 6B i), larger-

diameter vessels with a thin layer of SMA (venule-like vessels; Fig. 6B c, 6B g, 6B j), or large-diameter vessels surrounded by a thicker ordered ring of host SMA-positive cells (arteriole-like vessels; Fig. 6B d, 6B h, 6B k). GFP-RAEC-derived vessels were commonly seen at 60 days and some of these vessels were chimeric as they also included host EC (vWF positive; GFP negative).

The vascularized bed matured over time as confirmed by microCT imaging (Fig. 7). Whole tissue microCT imaging at day 21 showed a higher overall vessel density around implanted modules in the drug treated animals (Fig. 7). These newly formed vessels integrated with the host vasculature but were presumable leaky at day 21 as shown by the leaking of the microfil (albeit under high pressure as needed for perfusion) from the vessels. By 60 days, there was a decrease in the leakiness in the vascular bed and an increase in the perfusable vascularization of the tissue implanted with modules (Fig. 7).

Discussion

Reports of allogeneic EC transplantation are scarce. RAEC were transplanted into a myocardial scar in Sprague-Dawley rats with cyclosporine treatment and were shown to increase vascular density in the infarct region.¹⁸ A recent report describes the use of the rat omentum to prevascularize a cardiac patch, although no mention was made of immune suppression, and there was no tracking of the transplanted cells.¹⁹ There are also several reports of EC transplantation in immune-compromised animals^{20–22} (see below). Here we report that native, unmodified EC develop over 21–60 days into mature blood vessels in an allogeneic transplant model. The transplanted EC formed capillaries as early as 7 days that matured into arteriole-like and venule-like structures by

FIG. 6. Chimeric vessels mature over time. **(A)** At each time point, the percent of GFP vessels with diameter in the range of capillaries, small arterioles/venules, large arterioles/venules, or abnormal. Over time, there was a decrease in the numbers of capillaries and an increase in the numbers of large arterioles/venules. **(B)** Serial sections show examples of GFP-RAEC (anti-GFP antibody, **a–d**) in vessels around endothelialized modules (dashed line) with corresponding SMA-positive cells (**e–h**) and vWF-positive cells (**i–k**) at days 14 and 60. Transplanted cells formed small vessels that contained erythrocytes at day 14 (**a**) and some supporting smooth muscle cells (**e**). These vessels matured further over time and by day 60 formed chimeric vessels that were either capillaries (**b**) with little or no supporting SMA-positive cells (**f**), venules (**c**) with a small layer of SMA-positive cells (**g**), or arterioles (**d**) with a thick layer of SMA-positive cells (**h**). In all cases, GFP vessels were positive for rat vWF (**i–k**). Scale bar = 25 μm . Color images available online at www.liebertonline.com/ten.

60 days. Since it is necessary to test tissue engineering strategies in such animals, this milestone is important to the future of tissue engineering. The host inflammatory and immune response is a critical determinant of the success of tissue construct integration by altering, for example, cytokine and matrix metalloproteinase-driven remodeling and transplanted cell survival. In this context one needs to be mindful of the significant differences between the immunoreactivity of rat and human EC²³ (e.g., MHC II expression) so that translation to humans from rats still requires considerable effort. Also, many cells are sensitive to the immune suppressants in clinical practice (e.g., tacrolimus is toxic to pancreatic islets²⁴) so that there is great interest in devising an alternative, safer immune modulation protocol. That a clinically relevant therapeutic strategy was used here is an important consideration, nonetheless.

Several groups have shown that transplantation of primary EC, particularly HUVEC, in immune-compromised rodents can produce mature vessels under specific circumstances. Transplanted HUVEC transfected with an anti-apoptotic gene, *Bcl-2*,^{21,25} or HUVEC mixed with mesenchymal precursor cells²⁰ were shown to form stable blood vessels in SCID mice. Also, HUVEC-derived vessels in SCID mice were shown to increase overall tissue perfusion in an ischemic hindlimb model²⁰ and to effectively vascularize tissues, including skin substitutes²⁶ and three-dimensional skeletal muscle constructs.²⁷ This work has been extended recently to the problem of prevascularizing a cardiac patch with HUVEC in a nude rat with cyclosporine,²⁸ or Sprague-Dawley rats with immune suppression.²⁹ Combined, these studies suggest that primary EC transplantation can drive vascularization *in vivo*. However, long-term HUVEC survival

FIG. 7. The new vasculature has a leaky core that resolves partially over time. Microcomputed tomography images of whole omental pouch containing endothelialized modules. Drug-treated animals had a greater overall vessel density than nontreated animals. At day 21, there was a large leaky core detected in both untreated (**a**) and treated animals (**c**). At day 60 there was a greater vessel density in the treated animal and the leaky core had decreased in size (**b**, **d**). Magnified images of day 21 (**e**) and day 60 (**f**) treated animals showing the blood vessel density and architecture.

and vessel formation was only realized after the HUVEC were transfected with antiapoptotic genes (Bcl-2) or co-transplanted with supporting cells (mesenchymal stem cells, embryonic fibroblasts, or perivascular cell precursors) as HUVEC alone did not achieve stable vascularization *in vivo*.^{20,21,25,26} Here, transplanted EC survival was obtained without genetic modification of the EC or without co-transplantation of supporting cells. Presumably, in this model the transplanted EC drive the remodeling process that results in both host EC and supporting cell recruitment required to create a stable vasculature. The reason for the difference between these transplant models and ours is unknown and is currently being investigated by our laboratory. It is tempting to speculate that the difference is in part due to a difference in host response. In normal wound healing, the inflammatory response is important for the recruitment of myofibroblasts and that macrophages and myofibroblasts are involved in the production and remodeling of the extracellular matrix, which is important for angiogenesis.³⁰

At least some of the resulting vasculature is presumed due to infiltration and reorganization of host cells. The hematoma

that forms in the core of the implanted tissue likely reflects the destabilization of the host vessels surrounding the implant due to the secretion of soluble factors from the implanted RAEC. The hematoma is not seen in the collagen-only control implants and is seen early (day 3) with implantation of RAEC, suggesting that it is due to early immature vessel formation. Similar to the initial stage of wound healing, the infiltrating macrophages stimulate the migration of fibroblasts and EC into the wound bed to lay down new extracellular matrix (the collagen deposit seen in Fig. 2e–g, j–l). Many of the infiltrating cells were proliferative (positive for Ki67; Fig. 3b, d) and likely myofibroblastic in nature (positive for SMA; Fig. 3e). These infiltrating cells were presumably responsible for the observed collagen deposit, which supports the neovascularization around endothelialized modules (Fig. 2e–g, j–l). Donor EC also contributed to the neovascularization. Donor EC initially formed immature vessels (Fig. 6B a, 6B e) and by day 60 (longest time point measured) these chimeric vessels matured and represented what appeared morphologically to be all three classes of blood vessels: capillaries, venules, and arterioles

(Fig. 6B). It remains to be seen whether each of these function in like fashion to the native vessel type. This maturation of the blood vessels over time can also be seen with the microCT perfusion, where there is a decrease in the size of the leaky core and an increase in vessel density. Schechner *et al.* have reported a similar maturation process only when donor EC were transduced with an anti-apoptotic gene: Bcl-2-transduced HUVEC suspended in collagen-fibronectin gels initially formed thin walled capillaries in immunodeficient mice and the resulting vessels became arterioles-, venule-, and capillary-like structures 60 days after transplantation.²¹

Since the transplanted EC drive a vascularization process that involves transplanted EC migration and the host response (among other features of tissue remodeling), the nonthrombogenic nature of a quiescent endothelium does not appear to be a consideration in this model. This is contrary to the original premise associated with modular tissue engineering.³ It will be interesting to explore whether the addition of supporting cell types or cytoprotective genes further enhances the vascularization capacity of this model. Studies with supporting mesenchymal stem cells are underway. We are also interested in characterizing whether these vessels can support functional cell types *in vivo* and studies that include islets, cardiomyocytes, or liver or adipose cells are also underway. The premise is that the module-associated vasculature will provide adequate nutrients to enhance engraftment or viability of the transplanted cells or tissue construct. Of particular interest is whether the transplanted cells survive the initial period before vessels become mature enough to be perfuseable under physiologically relevant conditions.

Conclusions

We have demonstrated that it is possible to form a vascular bed by transplantation of endothelialized modules in an allogeneic transplant model with drug therapy. Unlike earlier studies, this vascular bed formed and matured without functional modification to the EC or cotransplantation with accessory cells. This was presumably due to the ability of the transplanted EC to recruit host cells (macrophages, fibroblasts, and EC), which in turn stabilized the developing vessels. This ability to recruit host cells that stabilize the vascularization is an important feature of this animal model and warrants further investigation.

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Disclosure Statement

No competing financial interests exist.

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