Del-1 Overexpression in Endothelial Cells Increases Vascular Density in Tissue-Engineered Implants Containing Endothelial Cells and Adipose-Derived Mesenchymal Stromal Cells

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We used a combination of strategies to stimulate the vascularization of tissue-engineered constructs *in vivo* including a modular approach to build larger tissues from individual building blocks (“modules”) mixed together. Each building block included vascular cells by design; modules were submillimeter-sized collagen gels with an outer layer of endothelial cells (ECs), and with embedded adipose-derived mesenchymal stromal cells (adMSCs) to support EC survival and blood vessel maturation *in vivo*. We transduced the ECs that coat the modules with a lentiviral construct to overexpress the angiogenic extracellular matrix (ECM) protein Developmental endothelial locus-1 (Del-1). Upon injection of modules in a subcutaneous SCID/Bg mouse model, there was an increase in the number of blood vessels for implants with ECs transduced to overexpress Del-1 compared with control implants (with enhanced green fluorescent protein [eGFP]–transduced ECs) over the 21-day duration of the study. The greatest difference between Del-1 and eGFP implants and the highest number of blood vessels were observed 7 days after transplantation. The day-7 Del-1 implants also had increased SMA+ staining compared with control, suggesting increased blood vessel maturation through recruitment of SMA+ smooth muscle cells or pericytes to stabilize the newly formed blood vessels. Perfusion studies (microcomputed tomography, ultrasound imaging, and systemic injection of fluorescent UEA-1 or dextran) showed that some of the newly formed blood vessels (both donor derived and host derived, in both Del-1 and eGFP implants) were perfused and connected to the host vasculature as early as 7 days after transplantation, and at later time points as well. Nevertheless, perfusion of the implants was limited in some cases, suggesting that further improvements are necessary to normalize the vasculature at the implant site.

Introduction

ONE OF THE KEY ISSUES in tissue engineering is the lack of an internal vascular network and the need for a rapid connection to the host vasculature upon transplantation of tissue-engineered constructs. Here, we approach this issue using a combination of strategies: (1) a modular strategy to build endothelialized tissue constructs^{1,2}; (2) Developmental endothelial locus-1 (Del-1), an angiogenic extracellular matrix (ECM) matrix protein, as a means of tipping the angiogenic balance in the transplanted ECs from quiescent to proangiogenic³; and (3) adipose-derived mesenchymal stromal cells (adMSCs) as vascular support cells to aid the survival of endothelial cells (ECs) upon transplantation and to stabilize the newly formed blood vessels perhaps by adopting a pericyte role.^{4,5}

The modular approach consists in fabricating small tissue constructs (“modules”), and packing the modules together to form a larger tissue.^{1,2} Each module is typically made of

collagen, with the outer surface of each module seeded with ECs, and with either functional cells (such as cardiomyocytes⁶ and islets⁷) or vascular support cells (such as MSCs^{4,8}) embedded inside the modules. The modular approach has several advantages. First, the tissue constructs include a vascular component by design, with the ECs seeded on the surface of the modules. Second, the modules have a uniform cell distribution. This method of tissue fabrication is also scalable. Finally, the modular approach is minimally invasive as the modules are simply injected using a syringe and needle.

In a previous study, we transduced ECs to overexpress Del-1 using a lentiviral system, and we showed that the ECs overexpressing Del-1 formed more sprouts *in vitro*, and differentially expressed a number of genes known to be involved in angiogenesis.³ However, the number of blood vessels formed *in vivo* was still limited, in a severe combined immunodeficient/beige (SCID/Bg), subcutaneous implant model, presumably due to extensive loss of ECs through apoptosis

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after implantation. In a different study using the same animal model and the modular approach (without Del-1), adMSCs embedded inside the modules prevented apoptosis of ECs.⁴ Donor-derived vessels formed at the implant site; some of these vessels persisted for at least 90 days, and their connection to the host vasculature was confirmed by microcomputed tomography (microCT) 21 days after implantation.

Here, the goal was to combine these strategies to further improve the tissue vascularization outcome *in vivo*. Specifically, this study aimed to (1) explore whether the combination of ECs overexpressing Del-1 and adMSCs in the context of modular tissue engineering enhances the vascularization of the tissue constructs compared with cotransplantation of control ECs and adMSCs, in terms of blood vessel density, as well as blood vessel maturation and function (i.e., connection to host vasculature), and (2) expand our understanding of the remodeling process and fate of transplanted cells. Further, a variety of imaging methods were used to assess the quality of perfusion in the new vessels.

Materials and Methods

Cells

Primary human umbilical vein endothelial cells (HUVECs; Lonza) were transduced and cultured as described elsewhere,³ with HIV-1-based recombinant lentivirus encoding for either Del-1-IRES-eGFP (Del-1 HUVECs), or eGFP alone (eGFP HUVECs, as control). The lentiviruses were prepared and designed by Dr. J. Medin's lab, University Health Network, Toronto. Mouse Del-1 major cDNA was a kind gift from Dr. T. Quertermous' lab, Stanford University. Human adMSCs (Lonza) were maintained in DMEM (Sigma) with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin/streptomycin (Life Sciences Corporation), with medium changes every 3–4 days. The adMSCs were used for implants at passage 3.

Implants

Implants containing HUVECs and adMSCs were prepared using methods described elsewhere.^{2–4} Briefly, the adMSCs were first mixed with a neutralized solution of collagen (Pure-Col, Inamed Biomaterials; 3.1 mg/mL type-I bovine dermal collagen; 1×10^6 adMSCs/mL of neutralized collagen solution). The collagen with adMSC solution was then gelled inside polyethylene tubing (Intramedic™ PE60; Becton Dickinson), followed by cutting of the tubing into small pieces using an automatic tube cutter (FCS Technology, Inc.). Finally, the cut tubing was vortexed to separate the gelled collagen pieces (modules, initial size ~2-mm long and 0.6 mm in diameter) from the tubing.

All the modules obtained after gelling of ~1.5 mL collagen solution with adMSCs inside 3 m of tubing were seeded with either Del-1 HUVECs or eGFP HUVECs overnight ($\sim 4 \times 10^6$ HUVECs), in a 50:50 mixture of HUVEC and adMSC culture medium (EGM-2/DMEM with 10%FBS). The next day, ~0.1 mL of contracted, settled modules (i.e., all modules available from one pack of 3 m tubing) in phosphate-buffered saline (PBS) was injected subcutaneously in the dorsum of SCID/Bg mice (6–7 weeks of age, male; Charles River Laboratories), as described previously.³ For all experiments, animals were separated in two groups: group 1, Del-1; group 2, eGFP. For immunohistochemistry analysis,

we used $N=5$ animals per group for day-3 analysis (with all implant surgeries for day-3 analysis done in one single batch), and $N=5$ animals per group per time point for day-7, -14, and -21 analysis (totally 30 animals for two groups and three time points; implant surgeries were done in two to three separate batches, with similar numbers of Del-1 and eGFP animals in each batch). Separate surgeries were done for imaging experiments. After surgery, mice were individually housed in sterile cages and provided free access to sterilized food and water under the approval of the University of Toronto animal care committee.

Immunohistochemistry

***In vivo* samples.** The modules were explanted 3, 7, 14, and 21 days after the implant, fixed in 10% neutral buffered formalin, and processed for immunohistochemistry and analyzed as before.³ A CD45 antibody (BD Pharmingen™ No. 550539, 1:100, rat monoclonal antibody, detects mouse CD45, also called leukocyte common antigen) was also used to evaluate leukocyte infiltration at the implant site.

***In vivo* blood vessel counts:** The number and diameter of donor-derived (GFP+) blood vessels (with defined lumen), as well as the total number (and diameter) of blood vessels (donor+host, CD31+) present at the implant site were manually counted and measured, as previously described.³ Digitized SMA-stained histology slides were analyzed using the Positive Pixel Count Algorithm available with the Aperio ImageScope software (Aperio Technologies; version 11) to determine the SMA density at the implant site ($N=5$).

***In vivo* cell counts:** Proliferating cells (Ki67+; human), apoptotic cells (caspase-3 cleaved+; mouse and human), and leukocytes (CD45+; mouse) present at the implant site were manually counted in five hot-spots within each implant. A Zeiss Axiovert light microscope with a 20× objective lens and equipped with a CCD camera was used to take pictures of the implants, and ImageJ software (ImageJ 1.45; NIH) was used to manually count the cells. The average of the counts per sample was used for statistical analysis ($N=5$; $n=5$).

***In vitro* samples.** Some modules were collected immediately after coating with HUVECs ($N=4$) or were cultured *in vitro* for an additional 7 days under standard cell culture conditions using a 50:50 mix of EGM-2 medium and DMEM with serum ($N=4$). Samples were processed for histology as previously described.³ Antibodies for desmin (Dako No. M0760; 1:200, mouse monoclonal antibody), PDGFRβ (Abcam No. ab32570; 1:400, rabbit monoclonal antibody), collagen IV (Dako No. M0785; 1:100, mouse monoclonal antibody), laminin (Sigma No. L9393; 1:100, rabbit polyclonal antibody), and fibronectin (BD Transduction Laboratories No. 610078; 1:2000, mouse monoclonal antibody) were also used. The histology slides were imaged using an Olympus BX61 light microscope with a 20× objective lens and equipped with an Olympus DP70 camera, or digitized using the ScanScope XT brightfield scanner with a 20× objective lens. Ki67+ cells were manually counted as previously described.³

Microfil® injection and microCT imaging

For microCT analysis, we used $N=3$ animals per group for analysis at day 7 postsurgery. Mice were heparinized (100 units heparin, LEO Pharma, Inc.; subcutaneous injection

5 min before the surgery), and then anesthetized with isoflurane. A small incision was made below the rib cage, the diaphragm was cut open, and a 24G catheter was inserted into the left ventricle of the heart. Mice were then perfused with ~100 mL of PBS with 5 U/mL heparin—warmed to 37°C—at a rate of 15 mL/min using a peristaltic pump. PBS perfusion was followed by perfusion with ~30 mL of Microfil Silicone Rubber solution (MV-122; Flow-tech), obtained by mixing the Microfil compound, the diluent, and the curing agent in a 8:20:2 ratio by volume. The Microfil solution was then allowed to polymerize for 2 h at room temperature. The tissue of interest was explanted and fixed in 10% formalin for 24–48 h, and then stored in PBS until analyzed ($N=3$; day 7 analysis). Before analysis, tissue samples were carefully dissected to remove extra skin and hair, embedded in 1% agar, and then scanned at 14 μ m resolution for 2 h with a microCT scanner (GE eXplore Locus SP; GE Healthcare) at the Mouse Imaging Centre, Toronto Centre for Phenogenomics. A total of 720 views were acquired through 360° rotation with the X-ray source at 80 kVp and 80 μ A. Three-dimensional microCT data were reconstructed at 14 μ m resolution using the Feldkamp algorithm.

Injection of Rhodamine-labeled UEA-1 lectin or Texas Red®-conjugated dextran

Separate groups of mice were analyzed at day 7 post-surgery for UEA-1 lectin or dextran perfusion. Additional animals were tested at day 22, after ultrasound imaging. Mice received tail vein injections of Rhodamine-conjugated UEA-1 (Vector Laboratories No. RL-1062; 50 μ g Rhodamine-labeled UEA-1 diluted in 100 μ L saline with 1 mM CaCl_2 per mouse) 10 min before sacrifice (similar to Leung and Sefton⁹). Other mice received tail vein injections of Texas Red®-conjugated dextran, 70,000 MW, lysine fixable (Life Technologies, Inc., No. D1864; 250 μ L of a 25 mg/mL solution per mouse) 30 min before sacrifice (adapted from Enis *et al.*¹⁰). The tissues of interest were explanted and fixed in 10% formalin overnight at 4°C, and then kept for another overnight in 30% sucrose solution at 4°C (UEA-1 study) or fixed in 4% paraformaldehyde overnight at 4°C (dextran study). The fixed tissues were embedded in Tissue-Tek® OCT compound (Sakura Finetek) and snap frozen in liquid nitrogen. Thick frozen sections (10–12 μ m) were then cut and imaged using an Olympus BX50 upright fluorescence microscope with automated whole-slide tiling capabilities, 10 $\times$ objective lens, CoolSNAP HQ² (Photometrics) CCD camera, and the MetaMorph® (Molecular Devices) image analysis software ($N=6$ for UEA-1 lectin day 7; $N=2$ –3 for UEA-1 lectin day 22; $N=3$ for dextran day 7; and $N=3$ for dextran day 22).

High-frequency ultrasound imaging

For ultrasound imaging experiments, we used $N=5$ –6 animals per group per time point at days 7, 14, and 21 postsurgery. The same animals that were analyzed at day 7 were reanalyzed at day 14 (minus one animal that died before day 14), and again at day 21 (minus one animal due to insufficient contrast agent). All implant surgeries for ultrasound analysis were done in one single batch.

High-frequency ultrasound imaging was performed using the Vevo®2100 Imaging System with a solid-state array transducer (MS-250; VisualSonics, Inc.) at a center frequency

of 18 MHz. Dual-mode presentation of a grayscale image side-by-side with a contrast-enhanced image facilitated the selection of the region of interest (ROI). The following settings were kept consistent throughout the study: 4% power, wide beam width, 38 dB contrast gain, and 35 dB dynamic range. In preparation for the imaging, mice were anesthetized with 2% isoflurane (Abbott Laboratories), shaved, and placed prone on an imaging stage. For contrast-mode imaging, each mouse received a bolus of 50 μ L (2×10^9 microbubbles/mL) of the Vevo MicroMarker® Contrast Agent (VisualSonics, Inc.) via tail vein injection, at a rate of 600 μ L/min, using a syringe pump. Two-dimensional image series were collected to measure ultrasound signal at the implant site before, during, and after microbubble injection. After imaging, mice were individually housed in sterile cages and provided free access to sterilized food and water under the approval of Sunnybrook Research Institute animal care committee. The same mice were used for imaging at days 7, 14, and 21 after implantation of the modules ($N=5$ –6). At the end of the ultrasound analysis study, the same animals were used for either dextran or UEA-1 perfusion studies on day 22. Ultrasound imaging was done at a separate location and animals were transferred on day 6 and returned on day 22 for UEA-1 and dextran perfusion studies on the same day.

The collected ultrasound data were analyzed using the Vevo2100 software (version 1.4.1.4510 with VevoCQ version 1.3.8.0). The implant region (ROI) was manually selected on the acquired ultrasound images, the imaging clip was corrected for movement during imaging using the built-in software function, and the change in ultrasound signal in the implant area following microbubble injection was quantified using the VevoCQ software algorithm. The peak enhancement (PE) and wash-in rate (WiR) results were used for comparison purposes between Del-1 and eGFP implants. PE in contrast mode represents the increase in nonlinear ultrasound signal (produced by microbubbles) after microbubble injection, with a high PE value corresponding to a high level of perfusion. Parametric images that show PE value distribution within the implant (heat maps) were also generated using the VevoCQ software. WiR is a measure of the rate of “filling” of the implant with microbubbles after microbubble injection, with high WiR values suggesting higher rate of perfusion.

Statistical analysis

A one-way analysis of variance with LSD *post hoc* was used to compare means between multiple groups. Differences between means were considered statistically significant at $p < 0.05$. All statistical analyses were performed with the SPSS Statistics software (IBM Corp.; version 20).

Results

Module characterization before implantation

The effect of Del-1 on EC sprouting and mRNA expression *in vitro* was shown in a previous article³; Del-1 HUVECs expressed 20 times more Del-1 on average than eGFP HUVECs when cultured on TCPS. Immunohistochemistry was used to confirm eGFP expression before implantation (Fig. 1); all HUVECs (UEA-1+ and CD31+) also expressed eGFP (GFP+), in both Del-1 and eGFP modules. Additionally, the

FIG. 1. Histology images of modules *in vitro* (day 0 postfabrication). The samples were serially cut; the same modules are seen with the different histology stains. All the endothelial cells (ECs) (UEA-1+ and CD31+) appeared to express eGFP (GFP+). Good EC coverage of the modules was observed for both Del-1 and eGFP modules. Scale bar=50 μ m. Color images available online at www.liebertpub.com/tea

modules were evenly coated with HUVECs in both cases. The embedded adMSCs did not express SMA (a marker of pericyte differentiation) prior to implantation. All modules with adMSCs and HUVECs were similar in shape and size (ellipsoids, ~ 0.3 -mm long and 0.25 mm in diameter); without adMSCs the modules were ellipsoids of ~ 0.4 -mm long and 0.3 mm in diameter.³

In vivo vascular density

Unlike what was seen without adMSCs,³ cotransplantation of Del-1 HUVECs with adMSCs led to significant improvement in vascularization compared with cotransplantation of eGFP HUVECs with adMSCs, with a higher number of blood vessels formed for Del-1 implants compared with eGFP implants. Figure 2 shows representative images of the visual appearance of the tissues at explant. Blood vessels were visible within all Del-1 and eGFP explants, with Del-1 day-7 explants having a relatively more vascularized appearance, consistent with the significantly higher vessel density results at day 7.

Implanted HUVECs migrated off the surface of the modules and started to form blood vessels in the area between the modules as early as day 3 (the earliest time point included in this study), in both Del-1 and eGFP implants.

Both donor (GFP+ /UEA-1+ /CD31+) and host-derived (CD31+ /UEA-1- /GFP-) blood vessels were present at the implant site, with the majority of the donor-derived blood vessels formed in the area between the modules, and the majority of host-derived blood vessels formed in the fibrous tissue surrounding the modules (Fig. 3). Red blood cells were visible inside the lumen of many of these donor-derived blood vessels, as early as day 3 and even more prominently at later time points, suggesting that they were connected to the host vasculature and perfused (Fig. 3).

The blood vessel density was consistently higher in Del-1 compared with eGFP implants, with the greatest overall difference (number of vessels formed and presence of SMA staining) observed 7 days after implantation. For Del-1 implants, the density of blood vessels increased significantly from day 3 to 7, and then decreased and remained relatively constant from day 14 to 21 (Fig. 4A, B). Many of the EC-lined vessels in the implant area were also surrounded by an SMA+ layer (presumably pericytes or smooth muscle cells that wrap around the blood vessels as part of the process of vessel maturation) as early as day 3, but most prominently at day 7 and later time points (as seen in Fig. 4). The level of SMA+ staining was higher for Del-1 implants relative to eGFP implants at days 7 and 14, and similar at day 21.

FIG. 2. Photographs of representative tissues at explant. Blood vessels were visible within all Del-1 and eGFP explants, with Del-1 day-7 explants having a relatively more vascularized appearance. Arrows indicate the location of implants; insets focus on the implant only. Color images available online at www.liebertpub.com/tea

The average size of the blood vessels (both donor derived and total) increased from day 3 to 7 for both Del-1 and eGFP implants, and remained relatively unchanged from day 7 to 21 (Table 1), with the majority of the blood vessels having the size characteristics of capillaries, small arterioles, and small venules, as expected. Slightly larger donor-derived blood vessels were observed for Del-1 compared with eGFP implants at day 7, with more donor-derived vessels in the 9–15 μm range, and fewer vessels smaller than 9 μm .

MicroCT imaging of perfused vessels

Connection of the implant blood vessels to the host vasculature was evaluated 7 days after implantation using microCT imaging. The images in Figure 5 show a view of the entire implant region. We found many large blood vessels that were perfused with the Microfil contrast agent 7 days post-transplantation. Qualitatively, it appeared that there were more blood vessels that were perfused and not leaky within Del-1 implants compared with eGFP implants; large pools of contrast agent accumulated at the implant site in the eGFP case, but not Del-1.

Perfusion with Texas Red-labeled dextran

We observed at both day 7 (Fig. 6A) and day 22 (Fig. 6B), and in both Del-1 and eGFP cases, that some of the blood vessels at the implant site (donor derived and host derived) were perfused with the fluorescent dextran. The dextran filled the lumen of some of the donor-derived (GFP+) blood vessels and it did not leak into the surrounding tissue, suggesting that the vessels that were perfused had functional cell–cell junctions, restricting the leakage of their content into the surrounding tissue. Nevertheless, we also observed many donor-derived blood vessels that were not perfused

with the dextran at day 7, as well as day 22 (the low-magnification images in Fig. 6 show the whole implant region on the histology slide). In general, it appeared that the larger donor-derived blood vessels were preferentially perfused relative to the smaller vessels. Some host-derived blood vessels formed at the implant site were also filled with dextran (dextran-filled circular structures that are not GFP+ in Fig. 6; some examples of host-derived blood vessels are shown with arrows). In general, the perfused host-derived blood vessels were seen at the edge of the implants, in both Del-1 and eGFP cases, and at both day 7 and 22.

Perfusion with Rhodamine-labeled UEA-1 lectin

For both Del-1 and eGFP implants, we found that many of the blood vessels of donor origin (GFP+) present at the implant site were connected to the host vasculature by day 22, since many of these vessels were also labeled with the fluorescent UEA-1 lectin injected systemically (Fig. 7C, D; the low-magnification images show the whole implant region on the histology slide). On the contrary, only very few (if any) UEA-1-positive vessels were visible at the implant site at day 7 (Fig. 7A, B).

High-frequency ultrasound imaging of perfused vessels

Ultrasound imaging with microbubble contrast agent injection showed that the microbubbles reached the implant site after systemic injection, as the ultrasound nonlinear signal at the implant site (generated by the microbubbles) increased soon after injecting the microbubbles into the tail vein. This suggests that the implant blood vessels were indeed connected to the host vasculature. Heat maps of PE of ultrasound signal after microbubble injection show a high content of microbubble at the implant site, particularly at day

FIG. 3. Histology sections of serially cut tissue samples at **(A)** day 3, **(B)** day 7, **(C)** day 14, and **(D)** day 21 after implantation. Implanted human umbilical vein endothelial cells (HUVECs) migrated off the surface of the modules and formed many blood vessels in the area between the modules (GFP+ and UEA-1+ for donor-derived vessels; CD31+ for donor- or host-derived vessels), in both Del-1 and eGFP implants. Examples of donor- or host-derived blood vessels are indicated with arrows. Examples of host-derived blood vessels are indicated with (*); the extra row of images in Figure 3B (bottom row) was added to show an example of host-derived blood vessels at day 7. Examples of vessels invested with a smooth muscle cell layer (SMA+) are indicated with arrows. Erythrocytes were visible (see insets and examples indicated with arrows) in the lumen of many of these blood vessels (both donor and host derived; in both Del-1 and eGFP implants) as well, suggestive of connection to the host vasculature. Scale bar is 100 μ m for lower magnification images and 50 μ m for higher magnification. Squares indicate the areas that are shown in the higher magnification images. Circle shows an individual module. Color images available online at www.liebertpub.com/tea

FIG. 4. Density of blood vessels and SMA staining at the implant site. Cotransplantation of Del-1 HUVECs and adipose-derived mesenchymal stromal cells (adMSCs) (black bars) led to an increase in the number of donor-derived blood vessels (GFP+; **A**), as well as total number of blood vessels (donor+host, CD31+; **B**), and increased SMA staining (**C**) compared to eGFP HUVECs and adMSCs (patterned bars). These differences were most noticeable at day 7. All vessels (with defined lumen) were counted and normalized to the area occupied by the implant on the whole histological section. The Aperio ImageScope Positive Pixel Count Algorithm was used for SMA staining. Graphs show average $\pm$ SEM; $N=5$; analysis of variance (ANOVA) with LSD *post hoc* and $p<0.05$ (*) considered significant.

7, in both Del-1 and eGFP implants. The heat maps presented as example in Figure 8A show the same Del-1 and eGFP implants, at days 7, 14, and 21 (longitudinal study on the same animals). By day 21, the microbubble content of the implant (and the associated ultrasound signal) decreased, and more extended areas with low microbubble content are seen within the implants compared with day 7. Quantification of PE using the VevoCQ image analysis algorithms showed high microbubble content for both eGFP and Del-1 implants at days 7 and 14, and a decrease in microbubble content at day 21. eGFP implants had more microbubble contrast agent than Del-1 at days 7 and 14, although these differences were not statistically significant (Fig. 8B). Quantification of the WiR of microbubbles into the implant using the VevoCQ image analysis algorithms showed a small decrease over time from day 7 to 21 for both Del-1 and eGFP implants, and similar values for Del-1 and eGFP implants (Fig. 8C).

In vivo proliferation and apoptosis of implanted donor cells

Quantitative analysis of proliferating donor-derived (human) vascular cells *in vivo* showed that the number of proliferating cells was more than twofold higher for Del-1 relative to eGFP implants at day 7 ($p<0.05$), and was increased by 35% at day 3 (Fig. 9A). Additionally, the number of proliferating cells was increased at day 7 relative to day 3 for both Del-1 and eGFP implants. Proliferating cells observed in the implant area had migrated off the modules and were mostly located in the area between the modules, either as individual cells or, less frequently, associated with blood vessels presumably undergoing angiogenic remodeling. In general, Ki67+ cells were found in areas where both ECs (GFP+) and pericytes (SMA+) were colocalized. For this reason, however, the identity of Ki67+ cells (whether EC or of adMSC origin) was also difficult to distinguish on serially cut sections. Nevertheless, it is likely that some of the Ki67+ proliferating cells were of EC origin, others were of adMSC origin and were expressing SMA+ pericyte markers, while others were likely of adMSC origin, but not expressing SMA.

We observed fewer apoptotic cells for Del-1 implants; the number of apoptotic cells in Del-1 relative to eGFP implants decreased by $\sim 35\%$ at both days 3 and 7 (Fig. 10A). Also, the number of apoptotic cells decreased by $\sim 60\%$ at day 7 relative to day 3 in both eGFP and Del-1 implants, and with the level of apoptosis in Del-1 implants already lower than eGFP implants at day 3 (Fig. 10A). Some of the apoptotic cells were ECs, and some were part of regressing blood vessels.

In vivo leukocyte infiltration at the implant site

At day 3, there was extensive leukocyte infiltration among the modules that subsided somewhat by day 7 (Fig. 11C). We quantified the CD45+ staining in two regions of the implants: (1) between the modules (Fig. 11A) and (2) in the thin fibrous tissue formed around the implant (Fig. 11B). In the area between the modules, we observed no difference at day 3, and $\sim 20\%$ fewer leukocytes in Del-1 implants relative to eGFP implants at day 7 (although this difference at day 7 was not statistically significant) (Fig. 11A). In general, we observed a decrease in the number of leukocytes from day 3

TABLE 1. BLOOD VESSEL SIZE DISTRIBUTION FOR HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS + ADIPOSE-DERIVED MESENCHYMAL STROMAL CELLS IMPLANTS

Size range	Day 3		Day 7		Day 14		Day 21	
	Del-1	eGFP	Del-1	eGFP	Del-1	eGFP	Del-1	eGFP
Size distribution of GFP+ vessels								
Capillaries (<9 μ m)	90%	91%	43%	59%	48%	46%	41%	38%
Small arterioles or venules (9–15 μ m)	9%	7%	42%	29%	41%	41%	44%	47%
Large arterioles or venules (15–75 μ m)	1%	2%	15%	11%	11%	13%	15%	15%
Other (abnormal, $\geq$ 75 μ m)	0%	0%	0%	0%	0%	0%	0%	0%
Average size, μ m (SEM, μ m)	5.4 (0.3)	5.7 (0.1)	10.6 (0.1)	9.3 (0.5)	10.0 (0.5)	10.2 (0.3)	10.8 (0.2)	11.0 (0.8)
Size distribution of CD31+ vessels								
Capillaries (<9 μ m)	80%	73%	41%	48%	45%	52%	40%	33%
Small arterioles or venules (9–15 μ m)	14%	18%	41%	34%	36%	31%	41%	47%
Large arterioles or venules (15–75 μ m)	6%	9%	18%	18%	19%	17%	19%	20%
Other (abnormal, $\geq$ 75 μ m)	0%	0%	0%	0%	0%	0%	0%	0%
Average size, μ m (SEM, μ m)	6.9 (0.8)	8.0 (0.6)	11.2 (0.2)	10.9 (0.7)	10.9 (0.5)	10.2 (0.4)	11.4 (0.5)	11.82 (0.2)

The percentages represent average value for $N=5$.

to 7. In the fibrous tissue layer around the implant, we found that there was a significant increase in leukocytes by $\sim 50\%$ for Del-1 relative to eGFP implants 3 days after implantation (Fig. 11B). By day 7, the number of leukocytes surrounding Del-1 implants decreased and it became $\sim 20\%$ lower relative to eGFP implants (although this difference at day 7 was not statistically significant) (Fig. 11B).

Effect of *in vitro* culture

While most of the effort was devoted to characterization of *in vivo* vascularization, we also characterized some changes in the modules over 7 days of culture *in vitro*. Each individual structure in Figure 12 represents several modules contracted and fused together after *in vitro* culture, and forming a larger "microtissue." The number of proliferating cells was relatively low for both Del-1 HUVEC+adMSC modules and eGFP HUVEC+adMSC modules at day 0, and increased for both Del-1 and eGFP samples from day 0 to 7 (Fig. 12A). The proliferating cells were of both EC and adMSC origin (the latter were GFP $^-$). Based on these similar results for Del-1 versus eGFP modules, we conclude that the

effect of Del-1 on proliferation of either HUVECs or adMSCs is limited in this *in vitro* culture system.

Some adMSCs expressed PDGFR β , but only very few expressed α SMA and desmin after 7 days in *in vitro* coculture with HUVECs in modules (Fig. 13). We did not observe any significant difference between the expression of these pericyte markers in Del-1 compared with eGFP modules in this *in vitro* system. The adMSCs that express pericyte markers appeared to be located in areas with EC present (GFP+ staining identifies ECs in Fig. 13).

Discussion

Del-1 significantly increased the density of blood vessels formed at the implant site

Both the number of donor-derived blood vessels and the total number of blood vessels at the implant site were consistently higher for Del-1 implants compared with eGFP implants over the 21-day duration of the study, with the greatest difference observed 7 days after transplantation (Fig. 4). We speculate that this was due to improved HUVEC survival in the presence of prosurvival factors secreted by adMSCs

FIG. 5. Microfil® perfusion 7 days after implantation. MicroCT images of (A) Del-1 and (B) eGFP subcutaneous implants show that by day 7 after implantation, many of the larger blood vessels formed in the implant area were perfused and connected to the host vasculature. It appears that more vessels were perfused and not leaky in Del-1 implants compared with eGFP ones. Images show a microCT view of the entire implant region. Scale bar is 1 mm; the arrow indicates a perfused blood vessel with a diameter of $\sim 100 \mu$ m.

FIG. 6. Identification of perfused blood vessels (both donor derived and host derived) at the implant site via tail vein injection of Texas Red[®]-labeled dextran. Both at day 7 (**A**) and at day 22 (**B**) we observed that some of the donor-derived blood vessels (GFP+) were filled with fluorescent dextran, and the dextran was contained within the lumen of these blood vessels, and not leaking into the surrounding tissue, for both Del-1 and eGFP implants. Some examples of host-derived blood vessels (not GFP+) are shown with arrows. Scale bar is 200 µm for lower magnification images and 100 µm for higher magnification. The low-magnification images show the whole implant region on the histology slide. Squares indicate the areas that are shown in the higher magnification images. Color images available online at www.liebertpub.com/tea

(VEGF, bFGF, *HGF*, etc.),^{11–13} which then allowed for Del-1 to be produced by the surviving cells. The secreted Del-1 “tipped” the proangiogenic balance in HUVECs further and enabled the formation of a greater number of blood vessels in Del-1 implants compared with eGFP implants. Future studies that focus on the effect of Del-1 on the level of expression of prosurvival and angiogenic factors secreted by the adMSCs may provide further insight into the mechanisms responsible for increased vascularization of the Del-1 implants. The increase in SMA+ cells (Fig. 4) likely also contributed to the increased number of blood vessels in Del-1 HUVEC and adMSC implants, as recruitment of such cells (pericytes and smooth muscle cells) is an important step in the process of maturation of newly formed blood vessels and it is required to prevent blood vessel regression. Presumably at least some of these SMA+ cells were of adMSC origin, which were shown by others to act as pericytes.^{5,14–18} Literature reports showed that Del-1 increased smooth muscle cell proliferation and migration,¹⁹ and it is possible that it had a similar effect on the SMA+ cells present at the implant site, thus contributing to the increase in the number of SMA+ cells that we observed *in vivo* for Del-1 HUVEC and adMSC implants.

In addition, the interaction between adMSCs/pericytes and ECs *in vivo* may have also contributed to the proper assembly of Del-1 in the ECM. It is known that ECs and

pericytes are both required to properly deposit and assemble the basement membrane during blood vessel formation, and poor ECM assembly occurs if pericytes are not present along with the ECs.^{20–23} It is therefore tempting to speculate that improved ECM assembly (with incorporated Del-1) is another mechanism that explains the observed synergistic effect between Del-1 and adMSC cotransplantation with HUVECs *in vivo*. On the other hand, our *in vitro* results were less conclusive. We did not see differences in the expression of pericyte markers by the adMSCs cocultured in modules with either Del-1 or eGFP HUVECs (Fig. 13), nor in the number of proliferating cells (Fig. 12). In fact, while some of the adMSCs expressed PDGFRβ, only very few expressed SMA or desmin after 7 days in culture (Fig. 13). Perhaps the cell culture medium that we chose with a view to facilitate coculture of the two cell types (50/50 mixture of the media typically used for EC and adMSC culture), as well as the static culture conditions (lack of flow) were not favorable for promoting pericyte differentiation.^{24,25} Others in our group found that culture of adMSCs in EGM-2 cell culture medium (medium typically used for HUVEC culture) promoted a proliferative phenotype of adMSCs (unpublished data).

Finally, despite the strong improvement in vascularization with Del-1 implants compared with eGFP implants, we cannot ignore the decrease in the number of blood vessels

FIG. 7. Identification of perfused blood vessels of donor or origin via tail vein injection of Rhodamine-labeled UEA-1 lectin. **(A)** At day 7, very few of the donor-derived blood vessels (GFP+) were also stained with UEA-1 lectin, in both Del-1 and eGFP cases. **(B)** By day 22, many of the donor-derived blood vessels were perfused and labeled with the UEA-1 lectin, in both Del-1 and eGFP cases. Scale bar is 200 μm for lower magnification images and 100 μm for higher magnification. The low-magnification images show the whole implant region on the histology slide. Squares indicate the areas that are shown in the higher magnification images. Color images available online at www.liebertpub.com/tea

over time, still higher for Del-1 implants at day 21, the latest time point included in the study, but statistically not different from eGFP implants (Fig. 4). Nevertheless, by comparison to implants with HUVECs alone (both Del-1 HUVEC and eGFP HUVEC implants) that we explored in a previous study,³ the number of blood vessels [both donor derived and total (host and donor)] was still higher in implants with adMSCs at 21 days post-transplantation, presumably due to the increased HUVEC survival in the presence of adMSCs. We hypothesize that the decrease in blood vessel density in Del-1 HUVEC and adMSC implants was not caused by a loss of Del-1 effect overtime, but rather physiologically justified. Under normal physiological conditions, the blood vessels that are no longer needed are pruned over time.²⁶ In our case, there was no additional functional, metabolically active tissue load within the implant (other than the vascular network itself), and therefore the existence of a rich network of blood vessels was not physiologically required.

Our group has previously shown using primary human microvascular endothelial cells (HMECs) instead of HUVECs (and without Del-1) that the blood vessel density decreased over the initial time frame, but then it was accompanied by an increase in blood vessel size in that case.⁴ We did not observe a similar increase in blood vessel size in the current study (Table 1), perhaps due to the difference in the source of cells

(HUVECs vs. HMECs; adMSCs commercially available vs. isolated in house). The blood vessel size distribution in our implanted tissues was however physiologically consistent, with the majority of vessels falling under the capillary or small arteriole and venule category (average size of HUVEC-derived blood vessels was $<11 \mu\text{m}$ for all time points included in our study). Other differences were also noticeable between HMECs and HUVECs (eGFP or Del-1 HUVECs) cotransplanted with adMSCs. HMECs assembled and formed a greater number of donor-derived blood vessels early after transplantation compared with HUVECs. However by day 7, Del-1 HUVECs had formed more blood vessels (68 vessels/ mm^2 , Fig. 4) and thus surpassed HMEC vessels (<35 vessels/ mm^2), which had begun to regress. Differences between HMECs and HUVECs were also noticeable in terms of host response and vascularization. While host-derived blood vessels mostly infiltrated the edge of the implant in HUVEC + adMSC implants (both Del-1 and eGFP; over 21 days), many host-derived vessels had infiltrated throughout the HMEC + adMSC implants by day 14. We suggest that the difference in vascularization and remodeling as a function of the EC source is a topic that warrants further investigation.

The mechanism of connection of the donor-derived vasculature to the host vessels present at the edge of the implant may be similar to the wrapping and tapping anastomosis

FIG. 8. Peak enhancement of nonlinear ultrasound signal and wash-in rate (WiR) of microbubbles into the implant. **(A)** Heat maps show examples of PE value distribution within the implants after microbubble injection. The regions of interest for heat maps were drawn to include the whole implant region as seen on the two-dimensional ultrasound images; the implants were visible as a “bump” immediately under the skin. The images are from the same Del-1 and eGFP implants, respectively, monitored over time at days 7, 14, and 21. The heat maps show higher signal at day 7, and a decrease over time. **(B)** Quantification of ultrasound signal (PE) confirmed this trend. **(C)** Quantification of the WiR of microbubbles into the implant showed a small decrease over time from day 7 to 21 for both Del-1 and eGFP implants, and similar values for Del-1 and eGFP implants. Graphs show average $\pm$ SEM; $N=5-6$; ANOVA with LSD *post hoc* and $p < 0.05$ (*) considered significant. Color images available online at www.liebertpub.com/tea

mechanism previously described by others.²⁷ In that study, some of the donor ECs wrapped around host vessels present in the vicinity of the implant, destabilizing and remodeling the host vessels through expression of high levels of MMP-9 and MMP-14. HUVECs eventually replaced some of the host-derived ECs, “tapped” into the host vasculature, and diverted blood flow through the developing donor-derived vascular network.²⁷

Blood vessels formed at the implant site were connected to the host vasculature

Histology indicated that modular tissue engineering resulted in vessels but by itself does not say that the vessels are connected to the host vascular system and are perfusable. For all time points included in this study, and as early as day 3, red blood cells were visible inside the lumen of many of

the blood vessels present at the implant site (both donor derived and host derived, Fig. 3), suggesting, but not confirming, that these vessels were perfused and connected to the host vascular supply. We used several perfusion methods to evaluate the quality of the vasculature formed, and to compare between Del-1 and eGFP implants. All perfusion contrast agents were injected at locations separate from the implant site and were able to reach the newly formed vessels (Figs. 5–8). However, the quality of perfusion differed depending on the perfusion method and time point studied. Overall, the perfusion results were encouraging in that they showed that both Del-1 and eGFP implants were connected to the host vasculature as early as day 7 (Figs. 5–8). On the other hand, the effectiveness of perfusion in all of these constructs was significantly impaired compared with normal tissues, suggesting that improvements are still required in order to build a functional vascular tissue. The different

FIG. 9. Proliferating donor-derived cells *in vivo*. **(A)** Quantitative analysis of proliferating donor-derived cells *in vivo*. Significantly more proliferating cells of donor origin were observed for Del-1 compared with eGFP implants at day 7. Proliferating cells were counted in five hot-spots ($20\times$ objective lens) within each implant. Graph shows average $\pm$ SEM; $N=5$ ($n=5$); ANOVA with LSD *post hoc* and $p<0.05$ (*) considered significant. **(B)** Histology images at day 7. Visual analysis of serially cut tissue sections shows that, in general, Ki67+ cells were found in areas where both ECs (GFP+) and pericytes (SMA+) were colocalized. Solid arrows indicate proliferating ECs. Dashed arrows indicate proliferating adMSCs that were also expressing SMA+ pericyte markers. The stars (*) indicate proliferating adMSCs, but not expressing SMA. The circle highlights one individual module. A blood vessel containing proliferating cells is also shown (b.v.). Scale bar is $50\mu\text{m}$. Color images available online at www.liebertpub.com/tea

perfusion studies offered complementary and sometime apparently conflicting information, and generated a more comprehensive image of the quality of perfusion.

MicroCT imaging of the implant vasculature showed that delivery of Del-1 improved the quality of the blood vessels formed, as early as 7 days after transplantation (Fig. 5). The perfused blood vessels in Del-1 implants were visibly less leaky compared with eGFP implants, indicative of enhanced blood vessel maturation. These microCT observations are consistent with the increased SMA+ staining in Del-1 implants compared with eGFP implants, as increased SMA+ staining suggests vessel maturation and stabilization through recruitment of pericytes or smooth muscle cells.

We also injected a 70 kDa fluorescent dextran and expected it to be retained inside the blood vessels. We found that donor-derived (GFP+) vessels were filled with dextran and not leaky as early as day 7, and at day 22 as well, for both Del-1 and eGFP implants (Fig. 6). Nevertheless, mostly the larger-diameter donor-derived vessels were filled with dextran, and not the smaller vessels. We presume that this is due to the less effective circulation of fluid through the implant by comparison to the rest of the host vasculature. The vasculature at the implant site is highly tortuous and still undergoing remodeling to achieve optimal branching for perfusion. Therefore, blood (or perfusion fluid) encounters

more resistance to flow through the implanted tissue compared with the host vasculature, and preferentially takes the path of least resistance, that is, through the normal tissue. Frequent bypassing of the implant renders the circulation through the implanted tissue less effective compared with normal tissue, similar to what is now recognized about the tumor vasculature.²⁸

UEA-1 lectin perfusion (again via tail vein injection) identified perfused blood vessels of donor origin (UEA-1 lectin binds specifically to the human endothelium, and not the mouse endothelium). We found that day-22 implants had a highly functional vasculature, with the majority of the donor-derived blood vessels (GFP+) also labeled with the UEA-1 lectin (Fig. 7). On the other hand, the day-7 implants showed very few UEA-1 lectin+ vessels (and more, but still relatively few, dextran-filled vessels) (Figs. 6 and 7). The day-7 results were unexpected given the evidently successful perfusion of these vessels with the Microfil compound at day 7, at least in the case of Del-1 implants (Fig. 5). This suggests that although the blood vessels in Del-1 implants were connected to the host vasculature at day 7 and not leaky, the perfusion of these vessels was still not very good. The microCT experiment used both a much higher volume of injected contrast agent and a higher pressure forcing the contrast agent through the vasculature, thus facilitating the

FIG. 10. Apoptotic cells at the implant site. **(A)** Fewer apoptotic cells were observed for Del-1 implants compared with eGFP implants at both time points (days 3 and 7), although these differences were not statistically significant. Apoptotic cells were counted in five hot-spots ($20\times$ objective lens) within each implant. Graph shows average $\pm$ SEM; $N=5$ ($n=5$); ANOVA with LSD *post hoc* and $p<0.05$ (*) considered significant. **(B)** Histology images at day 3. Visual analysis of serially cut tissue sections showed that some of the apoptotic cells were ECs (GFP+), as indicated by the arrows, and some of the apoptotic cells were part of tubular structures that likely were regressing primitive blood vessels (indicated as b.v. in figure). The circle highlights one individual module. Scale bar is 50 μ m. Color images available online at www.liebertpub.com/tea

accumulation of perfusion fluid/contrast agent in the implant vasculature. If a lower perfusion pressure or lower volume of injected contrast agent had been used, then the implant vasculature would have been (at least partially) bypassed (such we believe was the case with day-7 UEA-1 and dextran perfusion studies). The fact that the majority of the donor-derived blood vessels (GFP+) were also labeled with the UEA-1 lectin at day 22, unlike at day 7 (Fig. 7), suggests that the branching and maturity of the implant vasculature was presumably much improved from day 7 to 22. Nevertheless, the implant vasculature at day 22 was presumably still not as effective as the host vasculature, since the fluorescent dextran (which can fill either the implant vasculature or the mouse vasculature at locations separate from the implant) preferentially filled the more mature larger donor-derived vessels at the implant site even at day 22 (Fig. 6).

Finally, ultrasound imaging showed that the microbubble contrast agent injected systemically using a syringe pump accumulated at the implant site in both Del-1 and eGFP implants (Fig. 8). In fact, higher ultrasound signal (PE) was measured at days 7 and 14 compared with day 21 in both Del-1 and eGFP implants. In addition, we saw higher ultrasound signal in eGFP implants compared with Del-1 implants at days 7 and 14, although these differences were not statistically significant. The PE in nonlinear ultrasound signal

is proportional to the amount of microbubbles present in the ROI, and it does not distinguish between microbubbles within the blood vessels and microbubbles leaking out of the vessels and into the surrounding tissue (microbubbles are only 2–3 μ m in size). Therefore, considering that our microCT images at day 7 showed large accumulation of contrast agent outside of leaky vessels for the eGFP implants (Fig. 5), and that blood vessel counts were lower in eGFP implants compared with Del-1 implants (histology data, Fig. 4), it is likely that the higher ultrasound signal in eGFP versus Del-1 implants at day 7 is due to microbubble accumulation in the tissue facilitated by leaky vessels, rather than by an increase in the number of functional vessels. Similarly, the leakiness of the blood vessels at early time points is likely also an important contributor to the increased ultrasound signal that we observed at days 7 and 14 compared with day 21. In this latter case, the decrease in number of vessels by day 21 (Fig. 4) also accounts for the lower ultrasound signal (Fig. 8).

We also quantified the WiR, a measure of the rate of filling of the implant with the microbubbles (perfusion rate, Fig. 8). A high WiR value indicates high rate of perfusion, due to a higher number of blood vessels, or to the presence of larger blood vessels. We found slightly higher WiRs at day 7 and a decrease over 21 days in both Del-1 and eGFP implants (and similar values for Del-1 implants compared

FIG. 11. Leukocyte infiltration *in vivo*. **(A)** Similar number of leukocytes were observed in the area between the modules for Del-1 and eGFP implants at both days 3 and 7, with significantly more leukocytes present at day 3 compared with day 7. Leukocytes (CD45+) were counted in five hot-spots (20 $\times$ objective lens) within each implant. **(B)** More leukocytes (CD45+) were present in the fibrous tissue formed around the implant for Del-1 compared with eGFP implants at day 3, while by day 7 the number of leukocytes in the Del-1 implants decreased and was similar and slightly lower than eGFP implants. Leukocytes (CD45+) were counted in five hot-spots (20 $\times$ objective lens) within each implant. Panels A and B show average $\pm$ SEM; $N=5$ ($n=5$); ANOVA with LSD *post hoc* and $p<0.05$ (*) considered significant. **(C)** Representative histology images of leukocyte infiltration in the implant area at days 3 and 7. Squares in the low-magnification images indicate the areas between the modules and in the fibrous tissue surrounding the implant, which are shown at higher magnification in the row immediately below. The circles highlight individual modules. Scale bar is 500 μ m in high-magnification images, and 50 μ m in low-magnification images. Color images available online at www.liebertpub.com/tea

with eGFP implants), although this decrease over time was small (not statistically significant). We presume that the higher rates of perfusion at day 7 compared with day 21 for both Del-1 and eGFP implants were due to the higher number of blood vessels (donor+host) at day 7 compared with day 21 (as shown by our histology blood vessel count data, Fig. 4), and potentially also due to the accumulation of microbubbles in the tissue from leaky vessels, particularly in the eGFP case. Our histology data showed minimal change in blood vessel size over time (Table 1), so we

speculate that blood vessel size did not play a role in the higher WiR at day 7.

While the perfusion methods used in this study suggest that the implanted constructs were indeed connected to the host vasculature and perfused, at least to some extent, as early as day 7, perfusion data by itself do not provide a direct measure of the level of tissue oxygenation or hypoxia. Hence, a future extension of the current ultrasound imaging study is to quantify the oxygen saturation of the blood within the implant by photoacoustic imaging. This technology relies on the

FIG. 12. Proliferation of ECs and adMSCs cocultured in modules *in vitro*. **(A)** Significantly more proliferating cells were observed after 7 days in culture *in vitro* (day 7) compared with the initial time point immediately after fabricating the modules (day 0). Proliferating cells were counted in up to five hot-spots ($20\times$ objective lens) per sample. Graph shows average $\pm$ SEM; $N=4$ ($n\leq 5$); ANOVA with LSD *post hoc* and $p<0.05$ (*) considered significant. **(B)** Identity of proliferating cells at day 7. Images show serially cut tissue sections. Some of the proliferating cells (Ki67+) were ECs (GFP+), indicated with the arrows. Many proliferating cells were not GFP+, and therefore must be of adMSC origin (indicated with the stars). Also, many of the ECs were not proliferating, as they were not Ki67+ (indicated with the dashed capped lines). Scale bar is 50 μ m. Modules contracted and self-assembled into larger micro-tissues. The circle highlights one individual module within the larger micro-tissue. Color images available online at www.liebertpub.com/tea

FIG. 13. Differentiation of adMSCs into pericytes after 7 days *in vitro*. PDGFR β was expressed by some cells, but only very few cells expressed α SMA, and even fewer (if any) desmin. The arrows indicate adMSCs expressing pericyte markers (PDGFR β + and α SMA+) and colocalized with ECs (GFP+). Scale bar is 50 μ m. The circle highlights one individual module within the larger micro-tissue. Color images available online at www.liebertpub.com/tea

different-light-absorption properties of oxygen-bound hemoglobin (oxyhemoglobin) versus deoxyhemoglobin, and it can provide a visual map of the hypoxic versus normoxic areas within the implant.²⁹ Others have used photoacoustic imaging to investigate hypoxia in the tumor vasculature,³⁰ to visualize and quantify the growth of blood vessels into an implanted scaffold over time,³¹ or to examine the brain vasculature.³²

Apoptosis and proliferation in vivo

We analyzed the number of proliferating or apoptotic cells to further assess the effect of Del-1. The higher number of proliferating cells (Ki67+) for Del-1 compared with eGFP implants (Fig. 9) is consistent with the higher number of blood vessels formed within the Del-1 HUVEC+adMSC implants (Fig. 4). Most of these proliferating cells were not associated with any blood vessels, as expected, since the cell layers that form blood vessels are normally quiescent. A fraction of the proliferating cells were however associated with blood vessels, suggestive of the active angiogenic remodeling process that was occurring at the implant site. The proliferating cells were of both EC and adMSC origin, in both Del-1 and eGFP implants (Fig. 9).

We used the activated (cleaved) form of caspase-3 as indicator of cellular apoptosis. Caspase-3 is one of the effector caspases and it is a common effector of both the extrinsic and intrinsic apoptotic pathways. We found fewer apoptotic cells (caspase-3) for Del-1 versus eGFP HUVEC with adMSC implants at both days 3 and 7, suggesting that Del-1 had some antiapoptotic protective effect on the cells in the implant area (Fig. 10).

In our current system, adMSCs were also included in both Del-1 and eGFP implants. Literature reports showed that the antiapoptotic effect of Del-1 on HUVECs was in fact additive to the antiapoptotic effect of VEGF and bFGF³³; both molecules are secreted by adMSCs. Therefore, as expected, we found that many more cells survived and formed blood vessels after transplantation for both Del-1 and eGFP HUVECs cotransplanted with adMSCs, compared to our previous study that involves transplantation of HUVECs alone.

The higher level of apoptosis observed early after transplantation at day 3 compared with day 7 for both Del-1 and eGFP HUVEC with adMSC implants is likely a consequence of both the inflammatory response and of the hypoxic, nutrient-poor environment within the implanted tissue early after implantation. Most apoptotic cells were seen in the area between the modules. We also noticed that some of the apoptotic cells lined the surface of what were likely regressing blood vessels (Fig. 10). Apoptosis is part of the process of eliminating non-functional blood vessels (absence of blood flow) or vessels that fail to mature (lack of recruitment of supporting pericytes or SMCs), as well as part of the normal pruning of blood vessels that are no longer needed if there is no physiological demand from the functional tissue that the blood vessels supply.³⁴

Inflammatory response for Del-1 implants

Although we used an immune-compromised animal model (SCID/Bg), which is T-cell and B-cell deficient and with impaired natural killer cells, all other components of the innate immune system are still active. While leukocytes can be detrimental to the survival of implanted cells, they can also secrete a great number of proangiogenic factors, creating an environment that is favorable for vascularization.^{35,36} We

found that Del-1 induced an altered inflammatory response, and this may have contributed, in part, to the increased vascularization we observed by day 7 post-transplantation.

The higher number of leukocytes for Del-1 versus eGFP implants in the fibrous tissue surrounding the modules at day 3 (Fig. 11) suggests that the Del-1-transduced ECs played a role in either recruiting or retaining these cells at the implant site. Of course, the higher number of apoptotic cells at day 3 compared with day 7 (for both Del-1 and eGFP implants, Fig. 10) also suggests that other than their role in stimulating vascularization, the inflammatory cells also played a role in inducing and clearing the apoptotic cells from the implant site. Further detailing the role of these cells in remodeling has become a goal of current studies.

Literature reports showed that Del-1 interfered with lymphocyte function associated molecule-1 (LFA-1)-mediated leukocyte-EC interactions and acted as endogenous inhibitor of inflammation.³⁷⁻⁴¹ The lower number of leukocytes for Del-1 versus eGFP implants at day 7 (Fig. 11) may therefore be a consequence of the anti-inflammatory properties of the secreted Del-1. Our data suggest that Del-1 has a bimodal effect on inflammatory cell recruitment, depending on the biological context. On one hand, Del-1 played a role in recruiting or retaining more inflammatory cells to the implant site at day 3 (Fig. 11), presumably facilitating the clearance of apoptotic cells and the initiation of tissue vascularization at this early stage after cell transplantation. On the other hand, at day 7, presumably in a different biological context, after the initial tissue repair after implantation has occurred, Del-1 decreased the number of inflammatory cells (Fig. 11). The role of inflammatory cells in the vascularization response is an important topic for further study.

Conclusion

Manipulation of the ECM, here through lentiviral transduction of ECs with Del-1, is a fruitful method to accelerate tissue construct vascularization. Cotransplantation of Del-1 HUVECs and adMSCs in an SCID/Bg animal model increased the number of blood vessels in the implant area compared with cotransplantation of eGFP HUVECs and adMSCs, suggesting a Del-1-induced angiogenic effect. Most importantly, perfusion studies showed that many of these blood vessels were indeed connected to the host vasculature as early as 7 days after transplantation. However, the blood flow through the implant vasculature was still less effective compared with the native host vasculature. Developing new strategies to normalize the implant vasculature and improve perfusion effectiveness is a key next step.

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Disclosure Statement

No competing financial interests exist.

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