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Functional Small Diameter Neovessels using Endothelial Progenitor Cells Expanded *Ex Vivo*

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Abstract

Arterial conduits are increasingly preferred for surgical bypass because of inherent functional properties conferred by arterial endothelial cells, especially nitric oxide production in response to physiologic stimuli. We tested whether endothelial progenitor cells can replace arterial endothelial cells and promote patency of tissue-engineered small diameter blood vessels (4 mm). To address this, endothelial progenitor cells were isolated non-invasively from peripheral blood of sheep, expanded *ex vivo*, and subsequently seeded onto decellularized porcine iliac vessels. Endothelial progenitor cell-seeded grafts remained patent for 130 days as a carotid interposition graft in sheep, while non-seeded grafts occluded within 15 days. Importantly, the endothelial progenitor cell-explanted grafts exhibited contractile activity and nitric oxide-mediated vascular relaxation that was similar to native carotid arteries but which has not been reported previously for tissue-engineered small diameter grafts. These results suggest that endothelial progenitor cells can function similarly to arterial endothelial cells and thereby confer longer vascular graft survival. Due to their unique properties, endothelial progenitor cells may have more general applications for other tissue-engineered structures and also in treating vascular diseases.

Introduction

Cardiovascular disease, including coronary artery and peripheral vascular disease, is the leading cause of mortality in the United States¹. Surgical replacement or bypass surgery is the most common intervention for coronary and peripheral atherosclerotic diseases with 550,000 bypass cases performed per year¹. Autologous vessels are the preferred replacement grafts for diseased segments smaller than 5 mm in diameter, but many patients do not have suitable vessels². An alternative approach for small diameter vessels is to tissue-engineer vessels by using scaffolds that are based either on a biodegradable or on a decellularized matrix. Previously, *in vitro* culture of smooth muscle and endothelial cells on biodegradable or biological scaffold material produced a cellularized vessel with strong mechanical properties,

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but these grafts thrombosed shortly after implantation *in vivo*³⁻⁵. An alternative approach for small diameter vessels is to allow repopulation of the tissue-engineered graft with host cells *in vivo*. A decellularized matrix, based either on intestinal collagen or native vessels, remodeled *in vivo* with infiltration of host smooth muscle cells and endothelial cells into the matrix and demonstrated good patency *in vivo*⁶⁻⁸. One limitation of these studies is that the re-populated endothelial cells were not functional in endothelial-dependent relaxation assays performed on the explanted grafts, which may effect long-term patency rates. Furthermore, these results are difficult to extrapolate to humans because endothelialization of vascular grafts occurs more readily in the canine and rabbit models used in the studies⁹.

Endothelial cells are a crucial component of the normal vascular wall, providing an interface between the bloodstream and surrounding tissue of the blood vessel wall. Endothelial cells also play an important role in physiological events including angiogenesis, inflammation, and prevention of thrombosis. In addition to the endothelial cells that comprise the vasculature, there is evidence for non-hematologic, endothelial progenitor cells (EPCs) circulating in the blood¹⁰⁻¹⁴. EPCs migrate to regions of the circulation that have pathologic conditions affecting the injured endothelium, including traumatic and ischemic injury¹⁵⁻²³. In normal adults, the concentration of EPCs in peripheral blood is 2-3 cells per milliliter, while levels are 3.5 fold higher in cord blood^{14,23-24}. Obtained from peripheral blood, EPCs provide a novel and attractive endothelial cell source yet to be explored for tissue-engineering cardiovascular structures that require a functional endothelium.

We propose that EPCs may be an ideal source of autologous endothelial cells for seeding small diameter grafts since no sacrifice of a native vessel is necessary to obtain the cells. By pre-seeding EPCs onto a vascular graft, a non-thrombogenic barrier for the vascular graft could be created, thereby improving the patency rate of the vascular graft *in vivo*. We tested the feasibility of this approach in a sheep model, an established model to study cardiovascular structures. In addition, we selected a decellularized graft since these grafts have well preserved extra-cellular matrix and mechanical properties resembling native vessels. The decellularized matrix thrombosed at high frequency when placed into the carotid position of sheep, consistent with previous vascular graft studies in the sheep and the need to have endothelial cells seeded on the graft prior to implantation²⁵.

Results

To test the feasibility of using EPCs to endothelialize tissue-engineered small diameter vascular grafts, venipuncture-derived blood samples (15cc) were obtained from one to two week old lambs without prior stimuli to mobilize EPCs from the bone marrow. EPCs were partially purified using a histopaque density gradient followed by plating the leukocyte fraction, which contained EPCs, onto fibronectin-coated plates as previously described¹². Serial transfer of the cell suspension to new fibronectin-coated plates was performed to remove rapidly adherent hematopoietic cells. After 21 to 28 days in culture, the number of outgrowth colonies ranged between 2 to 3 per 1×10^7 total input cells. If allowed to continue growing, the outgrowth cells expanded exponentially and reached 1.6×10^9 cells by 3 weeks.

The outgrowth cells exhibited a cobblestone morphology that is characteristic of endothelial cells (Fig.1a). The endothelial phenotype was confirmed by immunostaining with specific antibodies directed against endothelial markers. A representative EPC preparation uniformly expressed CD31 on the cell-cell membrane borders (Fig.1b) and expressed von Willebrand factor (vWF) in cytoplasmic granules similar to Weibel-Palade bodies (Fig.1c). In addition, the EPCs incorporated acetylated low density lipoprotein (aLDL) (Fig.1d). E-selectin was minimally expressed in unstimulated cells (Fig.1e), but upon stimulation with lipopolysaccharide (LPS), was significantly upregulated in the EPCs (Fig.1f). To further

document the endothelial phenotype, whole cell extracts were analyzed by immunoblot with specific antibodies to KDR/Flk-1, the vascular endothelial growth factor (VEGF) receptor-2 and Tie-2, the angiopoietin receptor (Fig.1g and h). The EPCs expressed equivalent amounts of Tie-2 and KDR when compared to human dermal microvascular endothelial cells (HDMEC). EPC response to VEGF and basic fibroblast growth factor (bFGF) was determined in cellular proliferation assays (Fig.1 i and j). Increasing VEGF and bFGF from 0 to 9 ng/ml resulted in a 4-fold and 8-fold induction of cell number, respectively. The proliferative effect of VEGF and bFGF was relatively similar to that seen with HDMEC when using the same experimental assay²⁶. Consistent with the endothelial phenotype, the EPCs also formed capillary tubes within 6-12 hours when plated on Matrigel (data not shown). The expression of multiple endothelial markers detected by two different methods and the functional response to physiological stimuli unequivocally confirmed the endothelial identity of the outgrowth cells. Importantly for tissue-engineering, the endothelial phenotype remained constant for 20 passages, demonstrating the stability and feasibility of EPCs for clinical application.

Once a consistent procedure for culturing EPCs from peripheral blood of sheep ($n=20$) was established, we seeded them onto vascular grafts. We choose decellularized vascular vessels that were prepared from 4-mm diameter porcine iliac arteries of 4 to 5 cm in length. After harvesting, the iliac vessels were incubated with trypsin, and then processed by a Triton X-100 detergent and ammonium hydroxide extraction technique (G. E. A., *et. al.*, manuscript in preparation). The resultant vessel graft appeared devoid of cellular content, as seen by histological analysis (Fig.2a), by the lack of RNA detected in a reverse transcription-polymerase chain reaction assay (data not shown), and by the lack of an immune response after implantation of 3mm² pieces of decellularized matrix into the peritoneal cavity of mice (data not shown). Furthermore, Movat staining demonstrated that the graft had a well-preserved extracellular matrix architecture, including internal and external elastic lamella (Fig.2b). After seeding a confluent monolayer of EPCs on the lumen of the graft, we next optimized preconditioning with variations in imposed shear stress to yield the maximal retention of EPCs prior to graft implantation *in vivo*. Previous studies demonstrate that up to 80% of non-preconditioned endothelial cells on ePTFE grafts are dislodged after exposure to shear stress *in vitro* or after implantation *in vivo*, and underscore the importance of pre-conditioning the endothelial cells on vascular grafts²⁷⁻²⁸. We simulated *in vivo* arterial conditions by subjecting the graft to high shear stress (25 dynes/cm²). Pretreatment of the seeded graft under conditions of low shear stress (1 dyne/cm²) for two days resulted in a cell density of 30 cells/mm (Fig. 2c, open bar left). Subjecting the graft to high shear stress (25 dynes/cm²) for another two days resulted in a cell density of 13 cells/mm, a reduction of nearly 50% (Fig.2c, black bar left). In contrast, gradually increasing the shear stress from low (1 dyne/cm²) to high (25 dynes/cm²) over two days (Fig.2c, open bar right) followed by two additional days of high shear stress resulted in the same cell density of about 32 cells/mm (Fig.2c, black bar right). Scanning electron microscopy revealed a confluent monolayer of endothelial cells (Fig.2d).

Once the pre-conditioning of the seeded graft was optimized, we assessed function of the EPCs by testing their ability to produce nitric oxide (NO). We assayed for NO because of its central role in normal vascular tissue homeostasis²⁹⁻³⁵. NO is not only a strong vasodilator but also an inhibitor of platelet aggregation, vascular smooth muscle proliferation, and leukocyte adhesion, all of which are strong anti-thrombotic and anti-atherogenic properties²⁹⁻³⁵. We tested NO production by the EPC in a bio-assay using organ chamber methodology (Fig.2e). Endothelial-denuded segments of guinea pig aorta were contracted with U46619, a thromboxane analog, and then exposed to increasing concentrations of the calcium ionophore A23187 that was perfused through the EPC-seeded graft in the same organ chamber. Dose-dependent relaxation of the aorta segments was observed (Fig.2e, ▲). This dose-dependent relaxation was significantly attenuated by adding 10⁻³ M of the endothelial NO-synthase inhibitor L-NAME (77.5 % vs. 58.5 %, $P<.001$, Fig.2e, ■). When A23187 was perfused

through the grafts that were not seeded with EPCs, the aortic segments remained fully contracted (data not shown). Exposing the aortic segments to sodium nitroprusside (SNP), an endothelial-independent NO donor, resulted in a dose-dependent relaxation, indicating normal smooth muscle function in the guinea pig aortic segments (Fig.2e, ●). Taken together, these results showed that after pre-conditioning, the EPCs on the graft produced NO.

To assess the efficacy of EPC-seeded grafts *in vivo*, we performed implantation studies in sheep. Autologous EPCs were isolated from one to two week old lambs, expanded in culture, and seeded onto a decellularized grafts. The grafts were then preconditioned over two days, gradually increasing shear stress from low to high. Seeded ($n=7$) and unseeded ($n=4$) grafts, 4 to 5 cm in length, were implanted to replace a segment of the carotid artery using two end-to-end anastomoses. The only anti-coagulant administered was daily aspirin (81 mg) for 7 days post-operatively. All grafts demonstrated unobstructed flow at the time of implantation. We performed ultrasound Doppler flow studies daily and carotid duplex studies two weeks postoperatively to assess patency, flow, and thrombus formation. Three out of four control, non-seeded grafts occluded from thrombus formation within 5 days of implantation (Fig.3). One control graft was still patent when harvested at 15 days, but 50% of the lumen was obstructed by thrombus upon carotid duplex imaging and histologic analysis. All the grafts seeded with the EPCs were fully patent at the time of harvest, either 15 or 130 days after implantation (Fig.3). A representative arteriogram of a 130-day EPC-seeded explant demonstrated a smooth luminal surface with no signs of thrombus, obstruction, or aneurysmal dilation (Fig.4a). A macroscopic view of the 130 day EPC-seeded explant shown in Fig. 4e confirms the the smooth surface of the vessel lumen.

Histological examination of the seeded grafts at 15 days revealed a confluent monolayer of endothelial cells (data not shown), however there were minimal areas of the lumen that had mild thrombus with cells concentrated at the thrombus-graft interface (Fig. 4b). The graft media was acellular. Histological examination of the entire length of the seeded grafts explanted at 130 days showed cellular intimal thickening as possible pannus from the adjacent arterial segments, infiltration of cells into the graft media, minimal adventitial inflammatory response, and a confluent flattened cell monolayer lining the luminal surface of the graft (Fig. 4c). Staining with the Movat stain demonstrated preservation of the original anatomic features of extracellular matrix, especially elastin (Fig. 4d). The cells within the intimal layer stained positively for smooth muscle alpha-actin, a characteristic of smooth muscle cells (Fig. 4f), while those lining the luminal surface stained for vWF, a characteristic of endothelial cells (Fig 4g).

To determine if the seeded EPCs were still present on the graft at 130 days, we labeled the EPCs prior to seeding with a fluorescent lipophilic carbocyanine tracer that incorporates into cell membranes. Examination of graft tissue sections prior to implantation demonstrated a confluent labeled endothelial cell layer (Fig.4h). Examination of the explanted grafts showed the presence of fluorescent EPC cells that covered approximately 80% and 10% of the graft lumen, at 15 and 130 days respectively (Fig.4i and j). The low percentage of fluorescently labeled EPCs at 130 days may be due to dilution of the membrane-incorporated dye with every cell division, which would suggest a high proliferation rate of the EPCs on the graft. Alternatively, some of the fluorescently labeled EPCs may have detached from the graft *in vivo* or the tracer dye may have diminished fluorescence at 130 days *in vivo*.

Since smooth muscle cells were present in the grafts, we evaluated the explanted graft for contraction and relaxation reactivity (Fig.5). The mid-segments of the 130 day explanted grafts were suspended in an organ chamber and contractility was measured in response to norepinephrine and serotonin. The 130 day explanted grafts contracted in a dose-dependent manner to norepinephrine and serotonin with a maximal response shown in Fig.5a. In

comparison to the explanted grafts, matched native carotid segments generated two-fold more force in response to serotonin and eight-fold more force contraction in response to norepinephrine. These results indicate that the vascular smooth muscle cells observed in the grafts are indeed functional and respond to two physiologically important vasoconstrictors.

Increased endothelial NO production is associated with higher patency rates of coronary artery bypass arterial conduits, for instance radial artery or internal mammary artery³⁶⁻³⁷. In a previous set of experiments we demonstrated the capacity of the EPC-seeded graft to produce NO *in vitro* (Fig. 2e). To further assess the endothelial function of EPC-seeded grafts in an attempt to evaluate their long-term patency, we compared the endothelial-dependent relaxation of explanted 130 day EPC-seeded grafts (◆) to that of native sheep carotid artery (■) and saphenous vein (▲). Segments of each vessel were pre-contracted with serotonin and exposed to increasing doses of acetylcholine inducing receptor-mediated NO production. Acetylcholine induced a dose-dependent relaxation in all three vessels (Fig.5b). Maximal relaxation of the EPC-seeded graft to acetylcholine was significantly greater than saphenous vein segments ($68\% \pm 10\%$ vs $22 \pm 6\%$, $P<.001$), but similar to carotid artery ($68\% \pm 10\%$ vs $81 \pm 8\%$, $P=.088$) (Fig.5b). The acetylcholine induced dose-dependent relaxation of the explanted grafts was attenuated in the presence of the endothelial NO-synthase inhibitor L-NAME ($68\% \pm 10\%$ vs $22 \pm 6\%$, $P<.001$) (Fig.5b, x). The three vessels responded equally to SNP ($P=NS$), an endothelial-independent NO donor, and achieved maximal relaxation, indicating normal vascular smooth muscle cell function of the explanted 130 day EPC-seeded grafts (Fig.5c). Taken together, these results suggest that the endothelial-dependent and endothelial-independent vasomotor properties of the EPC-seeded grafts are very similar to that of native carotid arteries, and, therefore, potentially may have long-term patency rates.

Discussion

Our results demonstrate that EPCs can be isolated from peripheral blood, expanded *ex vivo*, and retained on a decellularized graft. Upon implantation *in vivo*, the EPCs provide an autologous, non-thrombogenic luminal surface that maintained patency for at least 130 days. Furthermore, the EPC-seeded graft remodeled into a neovessel with vasomotor activity to physiological stimuli.

The amount of NO production correlates strongly with patency rates of various vessel conduits used in bypass surgery³⁶⁻³⁷. For instance, the internal mammary artery has greater endothelial-dependent relaxation mediated by NO than the saphenous vein, which may play a role in higher patency rates of internal mammary artery grafts³⁶. We observed an endothelium-dependent relaxation of the 130 day explanted EPC-seeded grafts, which was comparable with native ovine carotid artery, but different from saphenous vein. We believe that this relaxation is dependent on NO production because the addition of the nitric oxide synthase inhibitor L-NAME attenuated the relaxation. Similar studies using the same methodology have shown the internal mammary artery and radial artery to have enhanced NO-mediated relaxation compared to saphenous vein, which may contribute to the long term patency of the internal mammary artery and radial artery grafts³⁷. Furthermore, since some of the seeded EPCs were still present on the graft after 130 days *in vivo*, EPCs may have intrinsic physiological characteristics, including NO production that may explain the patency rate of the EPC-seeded graft. This type of NO-mediated vascular relaxation suggests an important advantage of using EPCs in that this response has not been demonstrated previously in tissue engineered blood vessels.

Further studies are needed to identify the source of the smooth muscle cells in the neo-intima of the vascular graft. One likely source is smooth muscle cell migration across the anastomosis site from the adjacent artery. Examination of sections from the anastomosis site suggests that smooth muscle cells may infiltrate the graft. Another potential source may involve

transdifferentiation of the EPCs into smooth muscle cells. TGF β 1 induces the EPCs to express smooth muscle α -actin *in vitro* (S.K. and J.B., unpublished data) suggesting EPCs are more plastic than mature, differentiated endothelium and may have more potential for remodeling the graft *in vivo*. A third potential source may involve bone-marrow derived mesenchymal stem cells which have the potential to differentiate into smooth muscle-like cells and may be recruited into the graft from the circulation³⁸. Future studies are required to understand the vascular biology of the neo-intima formation on the decellularized graft *in vivo*.

The data presented here further demonstrates the importance of endothelializing vascular grafts for long-term patency. In the past, much effort has focused on endothelializing ePTFE grafts to increase their long-term patency in humans³⁹. Previously, the endothelial source for seeding grafts has relied on obtaining a biopsy from an existing blood vessel. Since EPCs can be obtained non-invasively from relatively small amounts of the patient's peripheral blood, they may eliminate the need to sacrifice healthy vessels for harvesting autologous endothelial cells. In addition, EPCs may have broad clinical applications for tissue-engineering structures that may require an endothelial lining, such as heart valves. Another application of the EPCs may involve engineering the cells *ex vivo* to express specific genes for medical conditions and re-introducing them into selected areas of the body, for instance ischemic myocardium. We have already shown that EPCs are easily infected with a retrovirus and stably express the transgene *in vitro* (S. Kaushal, unpublished observations). Therefore, EPCs may ultimately provide a wide variety of therapeutic options for patients.

In summary, the EPC-seeded grafts develop into neovessels *in vivo* by 130 days and exhibited NO-mediated vascular relaxation. This study is limited by the size of the study groups and the limited number of intervals that were examined *in vivo*. Future long-term studies will determine whether aneurysmal dilation, thrombus formation, hyperplasia of the neo-intima, or other processes might impair the function of the neovessel. Our combined approach of seeding EPCs on a decellularized graft or other vascular grafts may provide an attractive approach for tissue engineering small diameter blood vessels. In addition, due to their physiological properties and easy accessibility, EPCs may have broad applications for treating vascular diseases.

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Appendix

Methods

Isolation of EPCs from peripheral blood

EPCs were harvested from the internal jugular vein of 1 to 2 week old sheep as described¹. After the initial 5 cc of blood was discarded, 15 cc of blood was mixed with 100 units of heparin. The leukocyte fraction was obtained by centrifuging on a histopaque density gradient (Sigma, St. Louis, MO) for 30 minutes at 1000 x g using Accuspin tubes (Sigma, St. Louis, MO). The cell pellet was resuspended in EBM-2 medium (Clonetics, San Diego, CA) with 20% fetal calf serum (Hyclone, Logan, UT) but without hydrocortisone and plated on fibronectin-coated plates. At 24-hour intervals for the next three days, the medium was transferred to new fibronectin-coated plates and thereafter the media was changed every 3 days. An aliquot of cells in the medium on day 3 were counted to determine input cells for final plating.

Indirect Immunofluorescence

Indirect immunofluorescence was performed as previously described using antibodies to CD31 (Santa Cruz Biotechnology, Santa Cruz, CA), vWF (Dako, Carpinteria, CA), and E-selectin²⁶, and imaged for incorporation with fluorescent aLDL (Biomedical Technologies, Inc., Stoughton, MA). Cells were analyzed using a Zeiss Axiphot II fluorescence microscope and photographed using Ektachrome p1600 color slide film.

Western Blot Analysis

Cell lysates were prepared as described⁴¹ and separated by SDS-PAGE and transferred to Immobilon-P membrane. Anti-KDR and anti-Tie-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect these endothelial specific proteins.

Decellularized graft preparation

Freshly harvested porcine iliac blood vessels were placed in distilled water for one hour to remove blood elements. The vessels were then placed into a decellularizing solution containing 1% Triton X-100 and 0.1% ammonium hydroxide (Sigma, St. Louis, MO) and shaken (120-RPM) at 4°C for 72 hours. The solution was changed every 24 hours. After cleaning, the vessels were lyophilized and cold gas sterilized.

Isometric Studies

All isometric tension experiments were performed as previously described³⁷. Guinea pig aortic rings were suspended between 2 tungsten stirrups in an organ chamber bath of oxygenated 37°C Krebs solution. The vessels were increasingly stretched by 0.1 g increments to the optimal resting tension that produced a maximal response to 80 mmol/L KCl and allowed to relax for one hour. EPC-seeded grafts were directly attached to the aortic ring. After U46619 contraction of the aortic ring, relaxation of the aortic ring was induced by stimulating NO production in the EPCs with increasing concentrations of Ca²⁺ ionophore A23187. In addition, the graft response to A23187 and L-NAME at 10⁻³ M was assessed and also in the presence of increasing concentrations of SNP. The EPC-seeded 130-day explant was also tested for vasomotor activity using the same experimental design by sectioning the explanted grafts into 5mm rings. Three rings were tested from each of the four 130-day explants. Response to increasing concentrations of norepinephrine and serotonin was evaluated in the grafts. After maximal contraction with 10⁻⁴ M of serotonin, relaxation responses to increasing concentrations of acetylcholine and SNP were determined in the grafts. Matched segments of ovine carotid artery and saphenous vein were used as the controls.

EPC labeling

EPCs were labeled with 1µg/ml of cell tracker CM-DiI (Molecular Probes, Eugene, OR), a fluorescent carbocyanine dye, for 20 min. at 37° and then washed three times with PBS. Subsequently, the labeled EPCs were seeded on the graft.

Pre-conditioning of EPCs on the graft

The grafts were seeded at the density of 1×10⁵ cells/cm² in a rotating dynamic seeder for 6 hours at 12 rpm. EPC-seeded acellular grafts were then placed in a laminar flow bioreactor and shear stress was calculated using Poiseuille's equation: $\tau = 4\eta Q / \pi r^3$, where τ =shear stress, η =fluid viscosity, Q =medium flow rate, and r =radius of the vessel. Shear stress was initiated either at 1 dyne/cm² for two days or gradually increased from 1 dyne/cm² to 25 dyne/cm² by increasing 3 dyne/cm² every 4 hrs over a 2 day period. Subsequently, all the grafts were subjected to 25 dyne/cm² for another two days. Three mid-portion segments of each graft were

analyzed for confluency by examining 5 μ m histological sections and counting the number of nuclei per mm around the circumference at the luminal surface⁴².

Statistical Analysis

All NO results are expressed as mean $\pm$ standard error of the mean (SEM). Statistical significance was evaluated using the unpaired Student's t-test for comparisons between two means.

Surgical Implantation of the grafts

Anesthetized sheep (30 $\pm$ 3 kg) underwent a common carotid artery exposure through a longitudinal incision in the mid neck. Heparin (100 IU/kg; Elkins-Sinn, Cherry Hill, NJ) was administered intravenously prior to arterial clamping, and the graft was placed as an end-to-end anastomosis to the common carotid artery using a 6.0 prolene suture (Ethicon, Somerville, NJ). Arterial flow was re-established and the closure was sutured by layers. Animal care and handling complied with the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health.

Explant Characterization

Tissue sections from the proximal and distal anastomoses and through the midportion of the graft were stained with hematoxylin and eosin. Smooth muscle cells and endothelial cells were identified by immunochemical analysis with antibodies to smooth muscle α -actin (Sigma, St. Louis, MO) and vWF (Dako, Carpinteria, CA), respectively

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Figure 1. EPCs from the peripheral blood exhibit the morphologic and phenotypic properties of endothelial cells

EPCs exhibit typical cobblestone endothelial morphology (a), CD31 (b), von Willebrand factor (c) and incorporate aLDL (d). Unstimulated EPCs were negative for E-selectin expression (e), but LPS stimulated expression of E-selectin (f). g and h, Whole-cell extracts of two different EPC populations, EPC 9-19 and EPC 9-9B, HDMEC, and human fibroblasts were immunoblotted with antibodies shown on the left of each panel. Human fibroblast extracts were the negative control and HDMEC were the positive control. α -tubulin served as a control for protein loading. The three arrows indicate the fully glycosylated mature form of KDR (230 kDa), the partially glycosylated form of KDR (200 kDa), and unglycosylated form of KDR

(150 kDa)⁴⁰. *I* and *j*, Proliferation of EPCs in response to increasing concentrations of VEGF and bFGF. Relative cell number was determined after 72 hours of incubation with the growth factors.

Figure 2. Pre-implantation studies with EPCs seeded on the decellularized matrix

Porcine iliac vessels were harvested and decellularized as described in Methods. *a*, Histology of the decellularized graft by hematoxylin and eosin stain (magnification 20X). *b*, Movat stain showed the internal and external elastin layers (dark brown) (magnification 20X). *c*, EPC density on the luminal surface of the decellularized grafts treated with low (1 dynes/cm²) or gradual (1 to 25 dynes/cm²) shear stress for 48-hours (open bars) and followed by 48 hour treatment with high (25 dynes/cm²) shear stress (black bars). *d*, Scanning electron microscopy of a EPC-seeded graft treated with gradual increases in shear stress for 48 hours and then an additional 48 hours of high shear stress showed a confluent layer of endothelial cells (magnification 880X, bar = 10 μ). *e*, The EPC-seeded graft induced NO production. In an organ

chamber system, endothelial denuded guinea pig aortic ring was maximally contracted in the presence of U46619 and then relaxation was measured in the presence of increasing concentrations of calcium ionophore A23187 that was perfused directly into the EPC-seeded decellularized graft in the same organ chamber (▲). The relaxation effect mediated by A23187 was also measured in the presence of 10^{-3} M of L-NAME, an endothelial NO-synthase inhibitor (■). Relaxation was also recorded in the presence of increasing concentrations of SNP, an endothelial-independent NO donor (●). Data represent percent reduction in contraction and represent mean $\pm$ SEM derived from 4 grafts. * $P < 0.001$ for dose response of A23187 vs. A23187 and L-NAME.

Figure 3. Long term patency of vascular grafts seeded with EPCs (---) or control vascular grafts with no endothelial cells (—)

Patency was defined by Doppler flow studies and confirmed by carotid duplex studies. The EPC-seeded grafts were harvested at 15 days ($n=3$) or at 130 days ($n=4$). The non-seeded grafts (controls) were harvested at 15 days ($n=4$).

Figure 4. Post-implantation studies of the vascular grafts

a, Arteriogram of the EPC-seeded vascular graft 130 days after implantation. The location of the graft on the arteriogram was determined by staples at the sites of the anastomoses and is indicated by the two arrows. *b*, Seeded graft explanted at 15 days, showing a poorly organized thrombotic deposit overlying acellular graft media (asterisk). *c-f*, Seeded graft explanted at 130 days. *c*, Cellular intimal thickening as pannus from the adjacent arterial segments, infiltration of cells into the graft media, and confluent flattened cell monolayer lining the luminal surface of the graft (arrow). *d*, Section stained for extracellular matrix demonstrates the preservation of the original anatomic features, especially elastin (black). The internal elastic membrane is denoted by a white arrow. *e*, A macroscopic view of the explanted 130 day EPC-seeded vessel, with 1mm scale below. *f*, Staining of the cells within the intimal layer (*i* and inset) for smooth muscle alpha-actin. The medial layer (*m*) is unstained for alpha-actin. *g*, Staining of the cells overlying the intimal layer for vWF, denoted by arrow. Magnification in *B*, *C*, and *F* inset = 200X, in *D* and *F* = 100X, and in *G* = 400X. *h*, The pre-implant graft seeded with endothelial cells labeled with a fluorescent tracer. *i*, The 15 day and *j*, 130 explanted grafts showed the presence of CM-DiI-labeled endothelial cells on the luminal surface of the graft. Panels *g-i* are oriented with the lumen to the left.

Figure 5. Vasomotor responsiveness of explanted grafts at 130 days

a, Three mid-portion segments of each of the 130-day explant graft were tested for contractility activity in an organ chamber. Increasing doses of norepinephrine and serotonin, ranging from 10^{-9} M to 10^{-4} M, contracted the grafts and the maximal responses are shown at 10^{-4} M concentration. *b*, Matched segments of EPC-seeded grafts (◆), carotid artery (■), and saphenous vein (▲) were contracted with 10^{-4} M of serotonin and relaxation was assessed in response to increasing concentrations of acetylcholine. In addition, the EPC-seeded graft was relaxed in the presence of acetylcholine and 10^{-3} M of L-NAME (x). * $P < 0.001$ for EPC-seeded graft vs the L-NAME attenuated response and saphenous vein and $P = .088$ for EPC-seeded graft vs carotid artery. *c*, Matched segments similar to (*b*) were also assessed for

relaxation in the presence of SNP. Data represent percent reduction in contraction and represent mean + SEM ($n=12$).