

In Situ Blood Vessel Regeneration Using SP (Substance P) and SDF (Stromal Cell–Derived Factor)-1 α Peptide Eluting Vascular Grafts

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Objective—The objective of this study was to develop small-diameter vascular grafts capable of eluting SDF (stromal cell–derived factor)-1 α -derived peptide and SP (substance P) for in situ vascular regeneration.

Approach and Results—Polycaprolactone (PCL)/collagen grafts containing SP or SDF-1 α -derived peptide were fabricated by electrospinning. SP and SDF-1 α peptide-loaded grafts recruited significantly higher numbers of mesenchymal stem cells than that of the control group. The in vivo potential of PCL/collagen, SDF-1, and SP grafts was assessed by implanting them in a rat abdominal aorta for up to 4 weeks. All grafts remained patent as observed using color Doppler and stereomicroscope. Host cells infiltrated into the graft wall and the neointima was formed in peptides-eluting grafts. The lumen of the SP grafts was covered by the endothelial cells with cobblestone-like morphology, which were elongated in the direction of the blood flow, as discerned using scanning electron microscopy. Moreover, SDF-1 α and SP grafts led to the formation of a confluent endothelium as evaluated using immunofluorescence staining with von Willebrand factor antibody. SP and SDF-1 α grafts also promoted smooth muscle cell regeneration, endogenous stem cell recruitment, and blood vessel formation, which was the most prominent in the SP grafts. Evaluation of inflammatory response showed that 3 groups did not significantly differ in terms of the numbers of proinflammatory macrophages, whereas SP grafts showed significantly higher numbers of proremodeling macrophages than that of the control and SDF-1 α grafts.

Conclusions—SDF-1 α and SP grafts can be potential candidates for in situ vascular regeneration and are worthy for future investigations.



Visual Overview—An online [visual overview](#) is available for this article. (*Arterioscler Thromb Vasc Biol.* 2018;38:e117-e134. DOI: 10.1161/ATVBAHA.118.310934.)

Key Words: endothelial cells ■ regeneration ■ stem cells ■ substance P ■ tissue engineering

Cardiovascular disease is the major cause of death worldwide, which is responsible for >17.3 million deaths annually.^{1,2} Autologous vessels, such as saphenous veins and radial or left internal mammary arteries are gold standard replacement options for cardiovascular disease; however, donor-site-associated infection risks, shortage of suitable donors, and high long-term failure necessitate the alternative options. Artificial blood vessels including expanded polytetrafluoroethylene (ePTFE), poly (ethylene terephthalate; Dacron), and polyurethane have shown good potential as alternatives to the autologous grafts; however, they often fail because of thrombosis, intimal hyperplasia, atherosclerosis, calcification, and infection when used as

substitutes for small-caliber arteries (inner diameter \leq 6 mm).³ In coronary artery bypass grafting, the use of PTFE conduits resulted in 1-year patency rate of \approx 60%, which declined to 32% after 2 years.⁴ Patency of ePTFE prostheses is 40% to 50% when used to bypass the proximal popliteal artery at 5 years and 20% when used for infrapopliteal bypass at 3 years.^{5,6} In above the knee fem-pop bypass, results have shown PTFE graft patency rates of \approx 59% at 5 years.⁷ Klinkert et al⁵ reviewed extensive data on above-knee bypass grafting using saphenous vein and PTFE and reported that the later types of grafts exhibited poor primary and secondary patency in comparison to the vein grafts after 2 and 5 years. Last, Greenwald et al⁸ reviewed a substantial data

Received on: September 25, 2017; final version accepted on: May 16, 2018.

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The online-only Data Supplement is available with this article at <http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.118.310934/-/DC1>.

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Arterioscler Thromb Vasc Biol is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.118.310934

Nonstandard Abbreviations and Acronyms

α-SMA	α -smooth muscle actin
BM	bone marrow
ECs	endothelial cells
EPCs	endothelial progenitor cells
ePTFE	expanded polytetrafluoroethylene
hpf	high power field
MSCs	mesenchymal stem cells
PCL	polycaprolactone
Sca-1	stem cells antigen-1
SDF-1α	stromal cell–derived factor-1 α
SDVGs	small-diameter vascular grafts
SMCs	smooth muscle cells
SP	substance P
TE	tissue engineering
vWF	von Willebrand factor

of the implanted ePTFE-based small-diameter vascular grafts (SDVGs) and concluded that most of the vascular grafts failed because of the intimal hyperplasia. In addition, these grafts lack growth potential and require reoperation, particularly in pediatric patients. Therefore, it is pertinent to find an appropriate biodegradable polymer for the fabrication of SDVGs.⁹ Tissue engineering (TE) has been proposed to overcome these challenges by delivering tissue-engineered blood vessels. However, conventional TE approaches comprise lengthy and costly in vitro procedures.

An emerging avenue is to design cell-free grafts which can activate host's own regenerative capabilities, orchestrate cell response, and develop extracellular matrix.¹⁰ By avoiding the time-consuming ex vivo cell manipulation procedures, this method can deliver cost-effective, yet off-the-shelf available grafts. Recent reports after in situ tissue regeneration have documented remodeling of cell-free grafts into functional arteries.^{11–14} An inflammation-mediated response has been suggested, comprising an initial rapid recruitment of immune cells followed by the influx of vascular cell types.^{15,16} Besides, a variety of bioactive molecules such as SDF (stromal cell–derived factor)-1 α , VEGF (vascular endothelial growth factor), BDNF (brain-derived neurotrophic factor), and G-CSF (granulocyte colony stimulating factor) have been proposed to improve the cellularization, remodeling, and the patency of prosthetic materials.^{17–22} Despite obvious advantages of the abovementioned chemokines, they can be easily degraded by the proteolytic microenvironment and cannot be easily synthesized or incorporated into scaffold materials, which limit their wide applicability. Therefore, alternative candidates such as, cationic peptides, bioactive lipids, and short peptide sequences have gathered attention of the research community.^{19,20,23}

Recent reports have documented the potential of an endogenous neuropeptide, SP (substance P) in the mobilization of stromal-like cells and progenitor cells.^{24–26} Moreover, SP has been shown to enhance the neovascularization, modulate the host's inflammatory response, and stimulate the production of a variety of cytokines and growth factors.^{27,28} We and others have previously documented that SP promotes tissue repair

in various injury models.^{20,25,26} In view of the multifunctional nature of both angiogenic and chemotactic activities, the use of SP seems of great potential for TE. We think that the cellular and immunomodulatory nature of SP could be very useful for the remodeling of vascular grafts through host cell recruitment, immunomodulation, cell proliferation, and differentiation. Similarly, SDF-1 α has been shown to enhance endogenous cell homing, neovascularization, and tissue repair. It is an important chemoattractant of progenitor cells, lymphocytes, and monocytes and also plays an important role in the retention of bone marrow (BM)-resident stem cells.¹⁹ SDF-1 α is a homing beacon attached to the extracellular matrix and encourages the recruitment of resident BM cell.²⁹ Despite its significant role in embryogenesis, cardiogenesis, and neovascularization, SDF-1 α can undergo proteolytic degradation by metalloproteinases, dipeptidylpeptidase-4, neutrophil elastase, or cathepsin G and its activity may be diminished (half-life of SDF-1 α in vivo: 25.8 minutes).^{30,31} To avoid the limitations associated with the degradation of SDF-1 α by metalloproteinases and dipeptidylpeptidase-4, SDF-1 α variants have been engineered and documented to enhance tissue repair and regeneration in injured tissues and synthetic grafts.^{19,32,33} However, only few reports documented the in situ blood vessel regeneration using SDF-1 α peptide.¹⁹ Furthermore, whereas SP and SDF-1 α peptides have been shown to induce in situ tissue repair and regeneration, it is yet unclear which of these biomolecules is more effective for in situ blood vessel regeneration.

The objective of this study was, therefore, to evaluate the potential SP and SDF-1 α peptide to induce in situ tissue regeneration in synthetic SDVGs. Our study presents the development and application of a platform for the fully noncellular biodegradable scaffolds for vascular TE, which is demonstrated in vivo. Polycaprolactone (PCL) was selected as a base polymer, which is a Food and Drug Administration-approved biodegradable polymer with wide applications in TE. We and others have documented good patency and healing characteristics of PCL-based SDVGs.^{12,34,35} However, inherently being a hydrophobic polymer, PCL possesses limited biocompatibility and needs biofunctionalization. We introduced microfibrillar PCL-based SDVGs, which showed higher cellularization, vascularization, and remodeling than that of the nanofibrillar grafts.³⁶ We hypothesized that the incorporation of stem cell-inducing/recruiting factors will further enhance the potential of PCL for TE applications. We used co-electrospinning from 2 separate spinnerets to fabricate vascular grafts and assessed the biocompatibility of vascular grafts after implantation in an abdominal aorta in rats for up to 4 weeks.

Materials and Methods

The authors declare that all supporting data and methods used are available within the article and in the [online-only Data Supplement](#).

Materials

PCL pellets (Mn=80000 Da) were purchased from Sigma Aldrich (St Louis, catalog number, 440744). 1,1,1,3,3,3-fluoro-2-propanol (HFIP) was purchased from Tokyo Chemical Industry (Tokyo, Japan, catalog number, H0424, purity=99%). Methanol (catalog number, 288306, anhydrous \geq 99.0%), chloroform (catalog number, 34860, purity \geq 99.9%), and ethyl alcohol (catalog number, 459836, purity \geq 99.5%) were obtained from Sigma Aldrich (St Louis). Triton X-100 was procured from Thermo Fisher Korea Ltd (Catalog number,

A16046). Collagen type 1 was a gift from the Saining Biotechnology Company (Tianjin, China). SP (RPKPQQFFGLM; purity $\geq 95.0\%$) and SDF-1 α peptides (SKPVVLSYR; purity $\geq 95.0\%$) were custom synthesized by Pepton (Daejeon, Korea).

Fabrication of Fibrous Vascular Grafts

The hybrid PCL/collagen type 1 (PCL/Col) grafts were prepared by co-electrospinning from 2 separate spinnerets. A 25% w/v solution of PCL was prepared in a 5:1 (v/v) mixture of chloroform and methanol by stirring overnight. Collagen type 1 was dissolved in HFIP at room temperature for 24 hours to obtain 8% w/v solution. SP or SDF-1 α peptides were dissolved in deionized water at room temperature for 24 hours to obtain 1.0 mg/mL solution. Collagen and peptide solutions were mixed (4:1 v/v) and stirred for another 12 hours. Two 10-mL syringes were filled with PCL or collagen solution with or without peptides and connected to a 21-G blunt-ended needle that served as the charged spinneret. The apparatus consists of a syringe pump (Cole Parmer, Vernon Hills, IL), a high-voltage generator (DWP503-1AC, Dong-Wen High Voltage power supply factory, Tianjin, China), and a rotating steel mandrel (2.0 mm in diameter) as a collector. The flow rate of PCL solution was adjusted at 8 mL/h. The flow rate of collagen solution was set at 0.6 mL/h to fabricate hybrid PCL/Col grafts. The voltage between the needle tip and the rotating mandrel was set at 11 kV for PCL and 12 kV for Col. The distance between the spinneret and the collector was set at 10 cm for PCL and Col solutions. The obtained electrospun grafts were vacuum dried for 48 hours before further treatment.

For cell proliferation, in vitro Transwell migration assay, and release studies, electrospun membranes were fabricated. A 10% w/v solution of PCL was prepared in a 5:1 (v/v) mixture of chloroform and methanol by stirring overnight. Collagen solution containing SP or SDF-1 α peptide was prepared as mentioned earlier. A rotating aluminum mandrel was used as a collector, and aluminum foil was wrapped around the mandrel to collect the fibers. The flow rate of PCL and collagen solution was adjusted at 2 mL/h and 0.6 mL/h, respectively. The voltage between the needle tip and the rotating mandrel was set at 15 kV for PCL and 12 kV for Col. The distance between the spinneret and the collector was set at 15 cm for PCL and Col solutions. The obtained electrospun membranes were vacuum dried for 48 hours before further treatment.

Mechanical Testing

Longitudinal mechanical properties of as-spun and explanted vascular grafts (n=5 per group) were determined using a tensile-testing machine with a load capacity of 100 N (Instron-3345, Norwood, MA) at room temperature. Vascular grafts (gauge length, 1.0 cm) were pulled longitudinally at a strain rate of 10 mm/min until rupture. Tensile strength and an ultimate elongation at break were measured. Young modulus was calculated from the initial linear region (up to 5% strain) of the stress-strain curve.

Morphological Analysis

The grafts were mounted on an aluminum stub and sputter-coated with gold and palladium. Scanning electron microscope (HITACHI, X-650, Japan) at an accelerating voltage of 15 kV was used to observe the morphology of graft surface. Based on the scanning electron microscope images, the average diameter of fibers was measured by using Image-Pro Plus software. The diameter of at least 100 fibers was measured and averaged.

Cell Proliferation Assay

Cell proliferation was evaluated by Cell Counting Kit-8 (catalog number, CK04; Dojindo Molecular Technologies, Rockville, MD). The human mesenchymal stem cells (MSCs; passage 8, Center for Regenerative Medicine, Texas A&M University, College station, TX) were expanded at 37°C, 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM; catalog number, SH30244.01; Hyclone, UT) supplemented with fetal bovine serum (FBS; catalog number,

SH30084.03, Hyclone, UT), and penicillin/streptomycin (PS/SM, catalog number, 15140-22, Gibco, Life Technologies Corporation, NY) and passaged at 70% confluence using 0.05% Trypsin-EDTA (catalog number, 25300-054, Gibco, Life Technologies Corporation, NY). PCL/Col, SDF-1, and SP samples (diameter, 10.0 mm) were punched from electrospun membranes and sterilized using ethylene oxide gas at 1.0 bar and 35°C (E.O Gas Sterilizer, PERSON-E035/50, PERSON Medical, Korea). About 40 μ L of cell suspension (1.893×10^3 cells) was added on the sample, and the plate was incubated at 37°C with 5% CO₂ for 2.5 hours. Afterward, 300 μ L of the medium was added, and the plates were again incubated at 37°C with 5% CO₂ for specified period. At day 3 and 6, cell proliferation was measured following manufacturer's instructions. Briefly, 30 μ L of cell counting kit-8 solution was added into each well and the plate was incubated for 2.5 hours. Aliquots from each sample (150 μ L) were transferred into a 96-well plate and measured for the absorbance at a wavelength of 450 nm with UV spectrophotometer (752 Ultraviolet Grating Spectrophotometer, Shanghai, China).

In Vitro Transwell Migration Assay

The migratory response of rat BM MSCs (passage 7) toward PCL/Col, SDF-1, and SP grafts was analyzed using a transwell migration assay containing a polycarbonate membrane (pore size=8 μ m; catalog number, 35224; SPL Life Sciences, Gyeonggi-do, South Korea) according to a previously published protocol.²⁰ BM MSCs were isolated in our laboratory and the detailed procedure of isolation was described previously.²⁰ Cells were cultured in DMEM (Life Technologies, Waltham, MA) supplemented with FBS (10%) and PS/SM (1%). Collagen type 1 (catalog number, 354236; Rat Tail, 3.57 mg/mL; Corning Korea Company Ltd, Seoul, Korea; diluted to 0.1 mg/mL in acetic acid) was added in the inserts and those were incubated at 37°C with 5% CO₂ for 2 hours. After washing with Dulbecco's Phosphate Buffered Saline (DPBS, catalog number, LB 201-02; WELGENE, Daegu, Korea) thrice, serum-free medium (500 μ L) was added in the inserts and cell suspension (3.0×10^4 per insert) was added into the inserts. Cells were homogenized for up to 48 hours in an incubator. Afterward, 750 μ L of the serum-free medium was added into the wells of the transwell plate. Control, SDF-1, and SP grafts (length=1.5 cm, diameter, 2.0 mm) were sterilized under ultraviolet light for 30 minutes and placed in the wells. Wells containing medium only served as negative controls. Inserts were washed with PBS and the fresh medium was added. The transwell plate was incubated at 37°C with 5% CO₂ for 48 hours. After washing with DPBS, 500 μ L of 4% paraformaldehyde (PFA, catalog number, P2031; Biosesang, Gyeonggi-do, Korea) was added in the inserts and wells, respectively for 20 minutes. PFA was removed, and 500 μ L of cold ethanol (100%) was added into the inserts and wells for 20 minutes. Afterward, 500 μ L of crystal violet was added into the inserts and wells for 15 minutes. Crystal violet (catalog number, HT90132-1L; Sigma Aldrich) was removed from the wells, and the upper part of the membranes was carefully wiped with a cotton-tipped swab. The migrated cells were observed using an inverted microscope (Nikon Eclipse TE2000-U Kanagawa, Japan).

Evaluation of the Release of Peptides

Release studies of SDF-1 α and SP peptides from electrospun membranes were performed by using high-performance liquid chromatography (Agilent Technologies, 1200 Series) with YMC-Pack Pro C18 Column (I.D, 250 \times 4.6 mm, S-5 μ m, 12 nm). The flow rate was adjusted at 1.0 mL/min. Gradient conditions were used as follows: 3% to 20% B in 2 minutes, 20% to 50% B in 10 minutes, 50% to 70% B in 1 minute, and 70% to 3% B in 4 minutes (A, 0.1% TFA in water; B, 0.1% TFA acetonitrile) for SP and 3% to 10% B in 2 minutes, 10% to 40% B in 10 minutes, 40% to 60% B in 1 minute, and 60% to 3% B in 4 minutes (A, 0.1% TFA in water; B, 0.1% TFA acetonitrile) for SDF-1 α peptide. SDF-1 α peptide and SP standards were also characterized, and the released amount of peptides was calculated from the peak area of the standards and the samples.

Transplantation of Vascular Grafts

Sprague Dawley rats (male; age, 10–12 weeks; weight, 280–320 g; Laboratory Animal Center of the Academy of Medical Sciences, Beijing, China) were divided into 3 groups (control, SDF-1, and SP; n=5 for each group). An additional set of 15 animals (n=5 per group) received control, SDF-1, or SP grafts for the evaluation of the mechanical properties and the measurement of patency by using color Doppler at 4 weeks. All animal experiments were performed using the guidelines set by the Tianjin Committee of Use and Care of Laboratory Animals, and the overall project protocols were approved by the Animal Ethics Committee of the Nankai University. Rats were anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg body weight; catalog number, C8383; Sigma Aldrich). Heparin (100 U/kg; Dawa Pharmaceutical Co, Limited, China, Catalog number, HEA751829M4) was administered for anticoagulation by tail vein injection before surgery. A midline laparotomy incision was then performed, and the abdominal aorta was isolated, clamped, and transected. Three types of grafts (n=5 per group), namely PCL/Col (control), PCL/Col+SP (SP group), and PCL/Col+SDF-1 α peptide (SDF-1 group) were transplanted. The tubular grafts (2.0 mm in inner diameter and 1.0 cm in length) were sewed in an end-to-end fashion with 8 to 10 interrupted stitches using 9-0 monofilament nylon sutures (Yuan Hong, Shanghai, China). No anti-coagulation drug was administered to rats after surgery. The duration of the observation was 1 month. Before euthanize, animals were anesthetized with a chloral hydrate. Animals were euthanized by injection of an overdose of chloral hydrate. Grafts were explanted, rinsed with saline, and cut into 2 halves from the middle. One part was fixed with 4% PFA and embedded in an optimal cutting temperature compound (Tissue-Tek, Sakura) for frozen cross-sections. The other part was longitudinally cut into 2 pieces. One piece was first observed by a stereomicroscope and then fixed with 4% paraformaldehyde for up to 4 hours. The other piece was fixed with 2.5% glutaraldehyde overnight and dehydrated with gradient ethanol for scanning electron microscope observations.

Color Doppler Ultrasound

After 1 month, the patency and the blood velocity of the grafts were visualized by high-resolution ultrasound (Vevo 2100 systems, Canada) after the rats were anesthetized with isoflurane.

Characterization of Explanted Vascular Grafts

To prepare samples for scanning electron microscopy, the explants were rinsed with DPBS and fixed with 2.5% glutaraldehyde (catalog number, G5882, Sigma Aldrich) overnight, and dehydrated in ascending series of ethanol. Samples were affixed onto aluminum stubs with carbon tape, sputter-coated with gold, and observed by scanning electron microscopy (Quanta 200, Czech).

For sectioning and staining, explanted grafts were fixed with 4% PFA for up to 4 hours, dehydrated by 30% sucrose solution until the graft sank to the bottom. After embedded in optimal cutting temperature compound (Tissue-Tek O.C.T compound; catalog number, 25608–930; Sakura, Japan), the explants were cryosectioned to 6 μ m thickness. Subsequently, the sections were stained with hematoxylin and eosin (H&E) and Masson trichrome. Images were observed under an inverted microscope (Nikon Eclipse TE2000-U Kanagawa, Japan) and analyzed by Nikon NIS Elements software.

To carry out immunofluorescence staining, sections were fixed in cold acetone for 10 minutes, air-dried, and rinsed once with 0.01 mmol/L PBS. Then slides were incubated with 5% normal goat serum (Zhongshan Golden Bridge Biotechnology, China) for 45 minutes at 4°C. For intracellular antigen staining, 0.1% Triton-PBS was used to permeate the membrane before incubation with serum. Then the sections were incubated with primary antibodies in PBS overnight at 4°C followed by incubation with secondary antibodies in PBS for 2 hours at room temperature. The nuclei were counterstained with 4,6-diamidino-2-phenylindole containing mounting solution (DAPI Fluoromount G; catalog number, 0100-20; Southern Biotech, England).

Endothelial cell (EC) staining was performed using anti-von Willebrand factor (vWF) antibody (Abcam, ab6994, 1:300). Mural cells were stained using anti- α -smooth muscle actin antibody (α -SMA, 1A4; Abcam, ab7817, 1:100). The average thickness of α -SMA⁺ layer was counted by using image J. Briefly, the scale was drawn on the image by using a drawing tool, and its length was selected by using the set scale tool. Afterward, by using the drawing tool, α -SMA⁺ layer was selected and its length was measured. At least 10 high power field (hpf) images were used for the quantitative analysis per sample. For stem cell recruitment, sections were stained with anti-Sca-1/Ly6A/E antibody (D7; ab25195, 1:100 in 0.1 mol/L PBS) overnight at 4°C. For laminin staining, sections were stained with anti-laminin antibody (L9393, Sigma Aldrich, 1:50 in 0.1 mol/L PBS) overnight. For CD68 staining, sections were incubated with anti-CD68 antibody (KP1; Abcam, ab955, 1:100 in 0.1 mol/L PBS) overnight. For CD206 staining, sections were incubated with CD206 (C-20; Santa Cruz Biotechnology, INC, sc-34577, 1:100 dilution) overnight. For collagen type 1 staining, sections were incubated with collagen type 1 antibody (ab34710, Abcam, 1:200). For Sca-1/ α -SMA immunofluorescence staining, sections were incubated with anti-Sca-1/Ly6A/E antibody (D7; ab25195, 1:100 in 0.1 mol/L PBS) and anti- α smooth muscle actin antibody (1A4; Abcam, ab7817, 1:100) antibodies for overnight at 4°C. For ki67 staining, sections were incubated with ki67 antibody (ab15580, Abcam, 1:100 dilution in 0.1 mol/L PBS) for overnight at 4°C. Alexa Flour 488 goat anti-rabbit and goat anti-mouse IgG (1:200, Invitrogen) were used as the secondary antibodies for 2 hours at room temperature. The sections without incubation with primary antibodies were used as negative controls. Slides were observed under a fluorescence microscope (Zeiss Axio Imager Z1, Germany). For quantitative analysis of recruited cells, 10 hpf images were used per sample and 5 samples were used per group. The fields were not chosen manually, rather we followed a regulation for selecting the fields and 10 uniformly distributed fields were selected around a circle of graft wall. Histological and immunohistochemical analysis experiments were performed thrice.

Statistical Analysis

Origin Pro 9 Software Version (MA) was used for statistical analysis. Shapiro-Wilk test and Levene tests were used to assess the normal distribution of data and the homogeneity of variance, respectively by using origin software. Multiple comparisons were performed using a 1-way ANOVA followed by Tukey post hoc analysis for cellular infiltration into graft wall, smooth muscle regeneration, and blood vessel regeneration by using origin pro 9 software or Kruskal-Wallis test followed by Dunn test for multiple comparisons for in vitro cell migration, in vivo stem cell recruitment, collagen type 1 staining, SMC layer regeneration, and macrophages recruitment by using GraphPad Prism 7. Significance level was accepted a *P* values <0.05. Data are expressed as the mean \pm SEM.

Results

Characterization of Electrospun Grafts

Co-electrospinning has been widely used to fabricate scaffold materials because of its unique capacity for integrating the advantages of different polymer components into a graft. This study evaluated the potential of SP or SDF-1 α peptides releasing grafts for in situ vascular tissue regeneration. Collagen was used as a mean to provide the sustained release of SP and SDF-1 α -derived peptide. The processing parameters of PCL fibers have been optimized to fabricate macroporous and microfibrillar grafts to facilitate cellularization and remodeling, which has been documented by us before.²⁸ Our group has also previously optimized the electrospinning conditions to fabricate collagen fibers (data not shown), which were used to encapsulate SP and SDF-1 α -derived peptides

in this study. We envision that collagen nanofibers will degrade in vivo resulting in the release of SP and SDF-1 α peptides, which can enhance endogenous stem and progenitor cell mobilization and recruitment. Vascular grafts (inner diameter, 2.0 mm, wall thickness, 400–500 μm) were fabricated by electrospinning, and the morphology was assessed by scanning electron microscopy. Uniform, continuous, and smooth fibers without bead defects and exhibiting a well-defined morphology were formed (Figure 1). The average diameter of microfibers was measured by using image J and found to be $7.069\pm 0.6159\ \mu\text{m}$, $8.1607\pm 0.5871\ \mu\text{m}$, and $8.9246\pm 1.031\ \mu\text{m}$ in control, SDF-1, and SP grafts, respectively (Figure 1G).

We evaluated the in vitro release of SP and SDF-1 α peptides from electrospun membranes (n=5 per group) by using high-performance liquid chromatography, and the cumulative released amount of SP and SDF-1 α peptides was found to be 57.79 ± 9.96 and $68.75\pm 12.20\%$, respectively for up to 5 days. The release profile of SP and SDF-1 α peptides has been shown in Figure I in the [online-only Data Supplement](#). We did not observe the released amount of the peptides from electrospun membranes beyond this time point by using high-performance liquid chromatography, which may be caused by the detection limit of high-performance liquid chromatography (<0.1 ppm).

Mechanical properties of control, SDF-1, and SP grafts including tensile strength, elongation at break, and Young modulus were determined and summarized in Figure II in the [online-only Data Supplement](#) (n=5 grafts per group). Tensile strength values were found to be 1.9129 ± 0.1759 , 1.8143 ± 0.1149 , and 1.8059 ± 0.1994 MPa for control, SDF-1, and SP grafts, respectively. Young modulus values were found to be 3.3843 ± 0.6152 , 3.0955 ± 0.2069 , and 3.3390 ± 0.2351 MPa for control, SDF-1, and SP grafts, respectively. On the contrary, elongation at break values was found to be 585.757 ± 75.140 , 585.613 ± 9.274 , and $666.3083\pm 101.669\%$ for control, SDF-1, and SP grafts, respectively. The tensile strength, Young modulus, and elongation at break values of the native rat abdominal aorta were also measured and found to be 1.4941 ± 0.061 MPa, 1.4167 ± 0.145 , and $160.507\pm 24.44\%$, respectively (Figure II in the [online-only Data Supplement](#)). These results indicate that the control, SDF-1, and SP grafts did not significantly differ in terms of the mechanical properties.

Cell Proliferation Assay

Before in vivo implantation of the grafts, we assessed the viability of MSCs after attaching onto electrospun membranes. The cell growth was elucidated by using cell counting kit-8 for up to 6 days. Optical density values were measured at 450 nm and found to be 0.3435 ± 0.0025 , 0.3635 ± 0.0045 , and 0.3840 ± 0.000 for PCL/Col, SP, and SDF-1 groups, respectively at day 3 (Figure 1H). On the contrary, cell growth was slightly increased at day 6, and the optical density values were found to be 0.3650 ± 0.012 , 0.4530 ± 0.0525 , and 0.4230 ± 0.019 for PCL/Col, SP, and SDF-1 groups, respectively at day 6 (Figure 1H). These data demonstrate good cell viability on electrospun membranes.

In Vitro Cell Migration Assay

Since SP and SDF-1 α peptides have been shown to act as mitogens, which enhance stem and progenitor cell mobilization in vitro and in vivo, an in vitro Transwell migration assay was performed to evaluate the role of vascular grafts with or without SP and SDF-1 α peptides (n=5) in terms of the cell migration. Wells containing medium only served as negative controls. The representative images are shown in Figure 2. As can be seen from the figure, only a few cells migrated toward the negative control and PCL/Col groups. On the contrary, many cells migrated toward the SDF-1 and SP groups. These results were also quantified by using Image J, and the cell density was found to be 4508.86 ± 576.54 (n=5), 28966.78 ± 2999.39 (n=5), 157889.74 ± 21188.30 (n=5), and 135415.88 ± 14519.59 (n=5) μm^2 per mm^2 for the negative control, PCL/Col, SDF-1, and SP groups, respectively (Figure 2E). These data indicate that both SDF-1 and SP may significantly enhance the recruitment of MSCs in vitro (SDF-1 versus negative group, $P=0.0065$, SP versus negative control, $P=0.0283$, SDF-1 versus control, $P=0.0292$ and SP versus control, $P=0.0103$), which may be beneficial to orchestrate endogenous stem and progenitor cells in vivo.

Patency of Grafts

PCL/Col (control), SDF-1, and SP grafts (total, n=30) were implanted into the rat abdominal aorta to replace a segment of the native aorta for up to 4 weeks. All of the animals survived and were healthy. Before explanation of vascular grafts at 4 weeks, the patency of the grafts was assessed by the color Doppler, which showed that all grafts were patent and the blood flow was normal (Figure 3A). The blood velocity was measured at the proximal, middle, and distal sites of the implanted grafts. Control, SDF-1, and SP grafts did not significantly differ in terms of the blood velocity at the proximal (control, 858.966 ± 129.283 ; SDF-1, 882.195 ± 139.345 ; and SP, 965.7769 ± 63.906 mm/s), middle (control, 950.331 ± 127.77 ; SDF-1, 869.061 ± 85.959 ; and SP, 892.8634 ± 146.726 mm/s), and distal sites (control, 912.009 ± 112.54 ; SDF-1, 859.916 ± 75.028 ; SP, 863.563 ± 83.245 ; Figure 3C). The grafts were explanted and evaluated for the patency and occlusion. Grafts were cut from the middle and observed using a stereomicroscope. All grafts were patent in the 3 groups without any aneurysm and bleeding. The inner surface of grafts, observed under a stereomicroscope, was smooth and free of thrombi or platelet aggregation (Figure 3B). Meanwhile, the grafts integrated well with the adjacent blood vessels, as demonstrated in Figure 3. H&E staining also revealed that grafts were patent with little neotissue formation (Figure 4).

Evaluation of Mechanical Properties of Explanted Grafts

The mechanical properties of explanted vascular grafts at 4 weeks were also measured and the obtained results are summarized in Figure II in the [online-only Data Supplement](#) (n=5 per group). Tensile strength values were found to be 1.6543 ± 0.105 , 1.7085 ± 0.034 , and 1.6686 ± 0.0715 MPa for control, SDF-1, and SP grafts, respectively. Young modulus values were found to be 2.0549 ± 0.1437 , 1.9323 ± 0.0328 ,

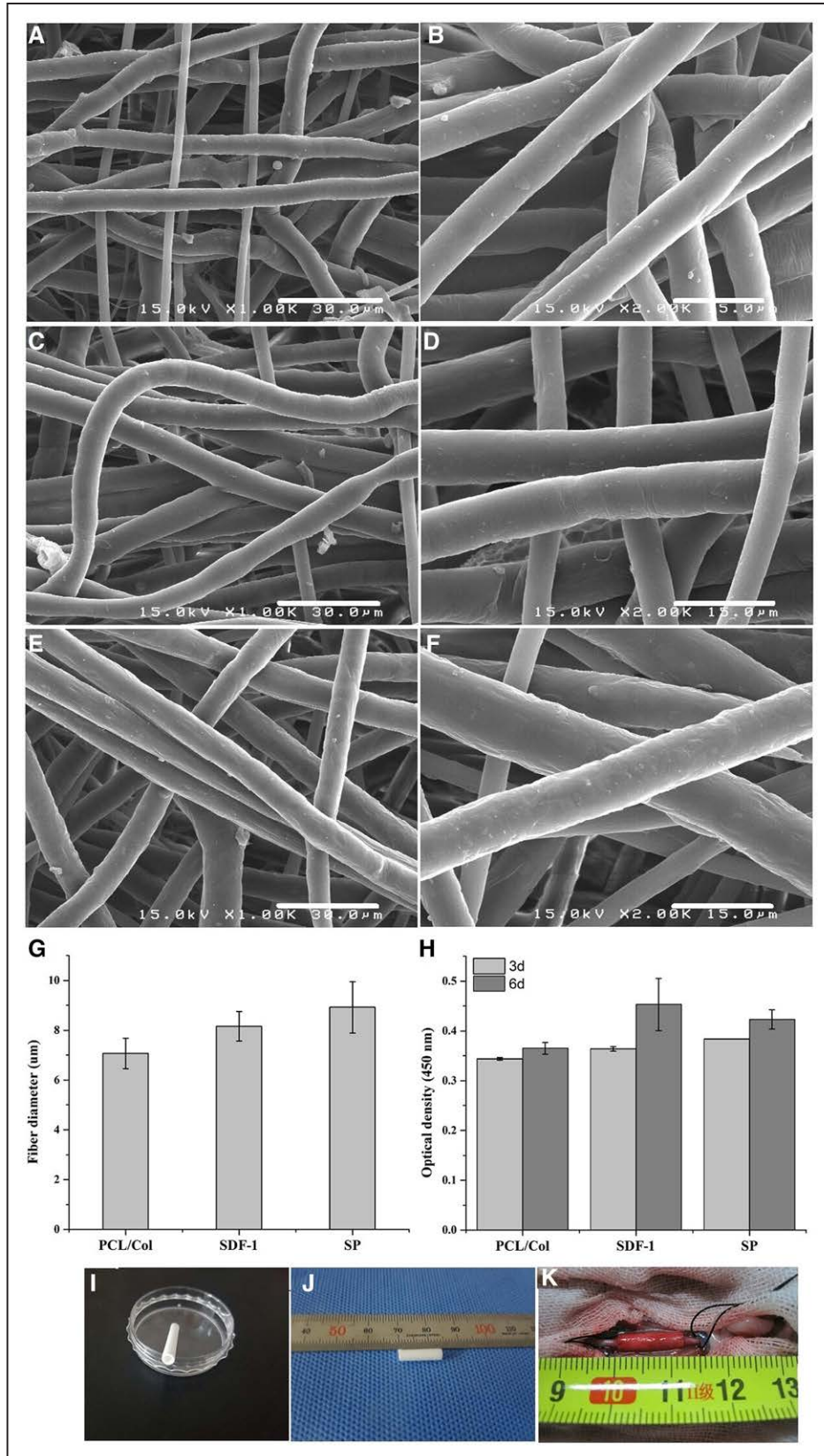


Figure 1. Scanning electron microscope (SEM) micrographs of vascular grafts. Control (A, B), SDF (stromal cell–derived factor)-1 α (C, D), and SP (substance P; E, F). Scale bar, 30 μ m (A, C, E) and 15 μ m (B, D, F). The fiber size was measured by using at least 100 fibers per groups. The average diameter of micro-fibers was found to be 7.069 \pm 0.6159 μ m, 8.1607 \pm 0.5871 μ m, and 8.9246 \pm 1.031 μ m in control, SDF-1, and SP grafts, respectively (G). H, Cell proliferation in vitro. Data are shown as mean \pm SD (n=5 per group) and evaluated by 1-way ANOVA followed by Tukey post hoc analysis. I, J, Pictures of vascular grafts before implantation. K, Picture of an implanted graft. Col indicates collagen; and PCL, polycaprolactone.

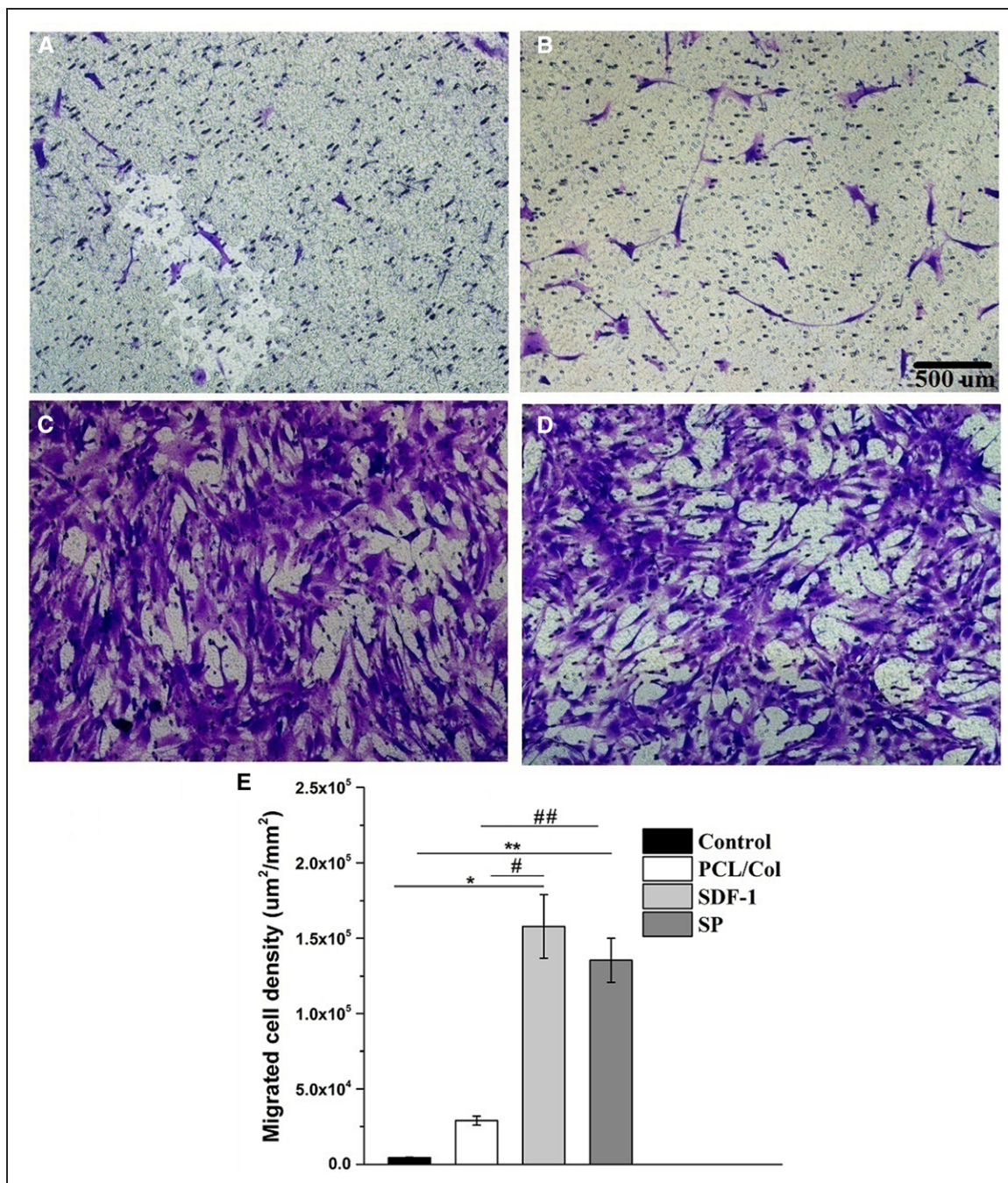


Figure 2. In vitro cell migration assay. Negative control (A), PCL/Col (B), SDF-1 (C), and SP (D). Only a few cells were recruited toward negative and positive control groups (migrated cell density per hpf, negative control, 4508.86 ± 576.54 , $n=5$ and positive control, $28966.78 \pm 2999.39 \mu\text{m}^2/\text{mm}^2$, $n=5$; E). On the contrary, SDF-1 and SP groups recruited higher numbers of MSCs (migrated cell density per hpf, μm^2 per mm^2 , SDF-1, $157889.74 \pm 21188.30 \mu\text{m}^2/\text{mm}^2$, $n=5$ and SP, $135415.88 \pm 14519.59 \mu\text{m}^2/\text{mm}^2$, $n=5$). Data are shown as mean \pm SEM and evaluated by Kruskal-Wallis test followed by Dunn multiple comparisons test. * $P=0.0065$, ** $P=0.0283$, # $P=0.0292$, and ## $P=0.0103$. Scale bar, 500 μm . COL indicates collagen; hpf, high power field; MSC, mesenchymal stem cell; PCL, polycaprolactone; SDF, stromal cell-derived factor; and SP, substance P.

and 1.7937 ± 0.0854 MPa for control, SDF-1, and SP grafts, respectively. On the contrary, elongation at break values were 402.656 ± 60.846 , 357.952 ± 35.125 , and $459.81 \pm 48.83\%$ for control, SDF-1, and SP grafts, respectively. These results indicate that the mechanical properties of explanted control, SDF-1, and SP grafts did not appreciably differ. Satisfactorily, the mechanical properties of the 3 kinds of explanted vascular grafts were more close to that of the native abdominal aorta

as compared with that of the as-spun grafts (native abdominal aorta, tensile strength, 1.4941 ± 0.061 MPa; Young modulus, 1.4167 ± 0.145 ; and elongation at break, $160.507 \pm 24.44\%$, respectively).

Vascular Tissue Regeneration and Remodeling

Cellular infiltration and neotissue formation in the implanted grafts was evaluated by H&E staining and immunofluorescence

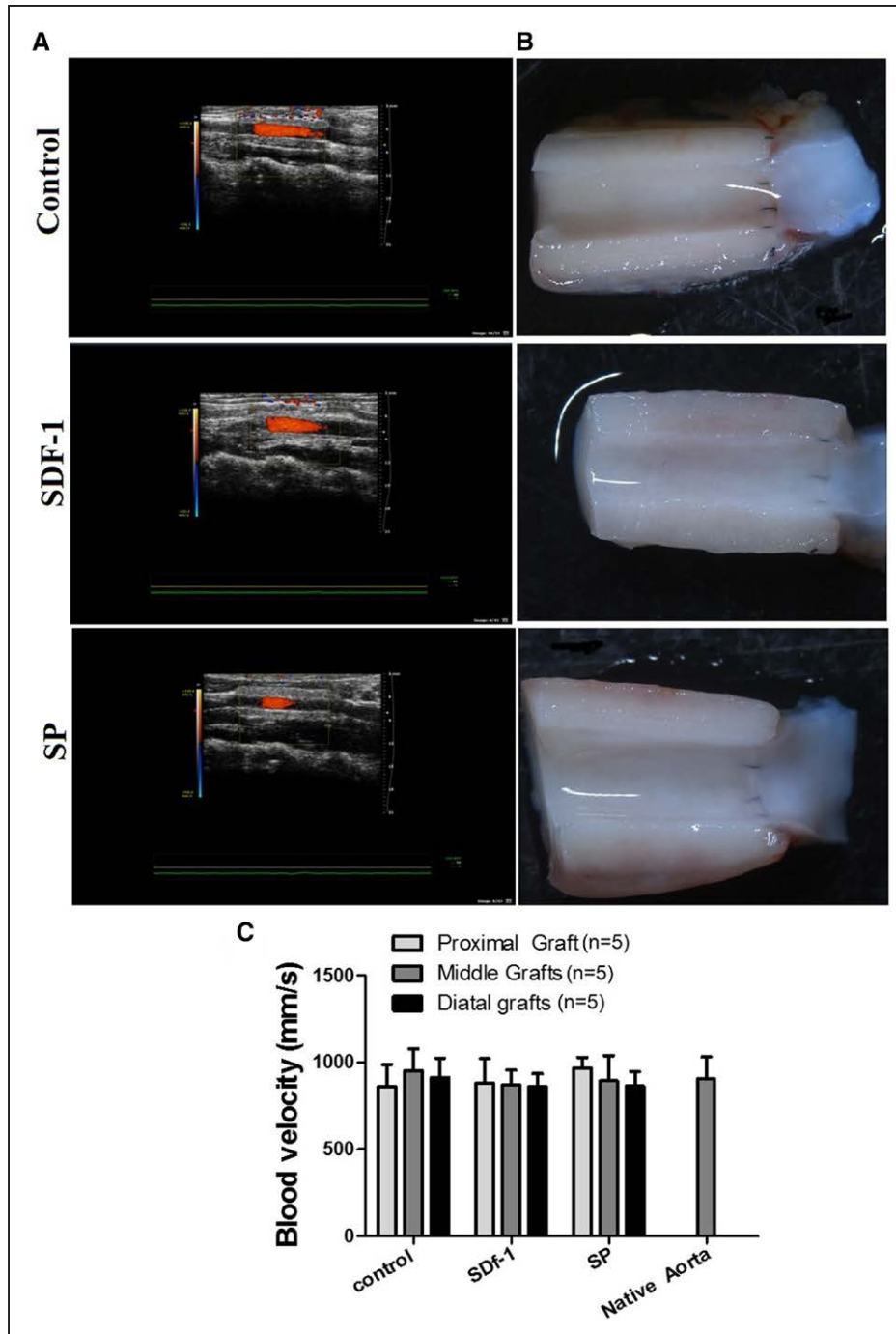


Figure 3. Patency of vascular grafts at 4 wk. **A**, Color Doppler images of vascular grafts were captured before the explantation of grafts (n=5 per group). **B**, Stereomicrographs of grafts retrieved 4 wk after implantation. The luminal surface of the explanted grafts was smooth and free of platelet aggregates or thrombi. Data are shown as mean±SEM (n=5 per group) and evaluated by 1-way ANOVA followed by Tukey post hoc analysis (**C**). SDF indicates stromal cell-derived factor; and SP, substance P.

staining. Cross-sections of explanted grafts were stained with H&E to identify the neointimal formation, cellular infiltration, and neotissue formation. As can be observed from the Figure 4, host cells infiltrated into the control, SDF-1, and SP grafts and new tissues were formed. At 4 weeks, the macroporous morphology of the grafts allowed extensive cell penetration into the whole graft wall. Four weeks after implantation, the lumen surface of SDF-1 and SP grafts was almost covered by the neointima (Figure 4E, 4F, 4H, and 4I; dotted lines

indicate the formation of neointima). Conversely, the control grafts showed the incomplete neointimal coverage (Figure 4D and 4G). Cell infiltration was qualitatively and quantitatively analyzed based on the DAPI-stained sections and the representative pictures are shown in Figure 4M through 4O and Figure III in the [online-only Data Supplement](#). For quantitative analysis of the cell density, 10 hpf images were used per sample and 5 samples were used per group. In the control grafts, the cell density was higher in the adventitial side, whereas the

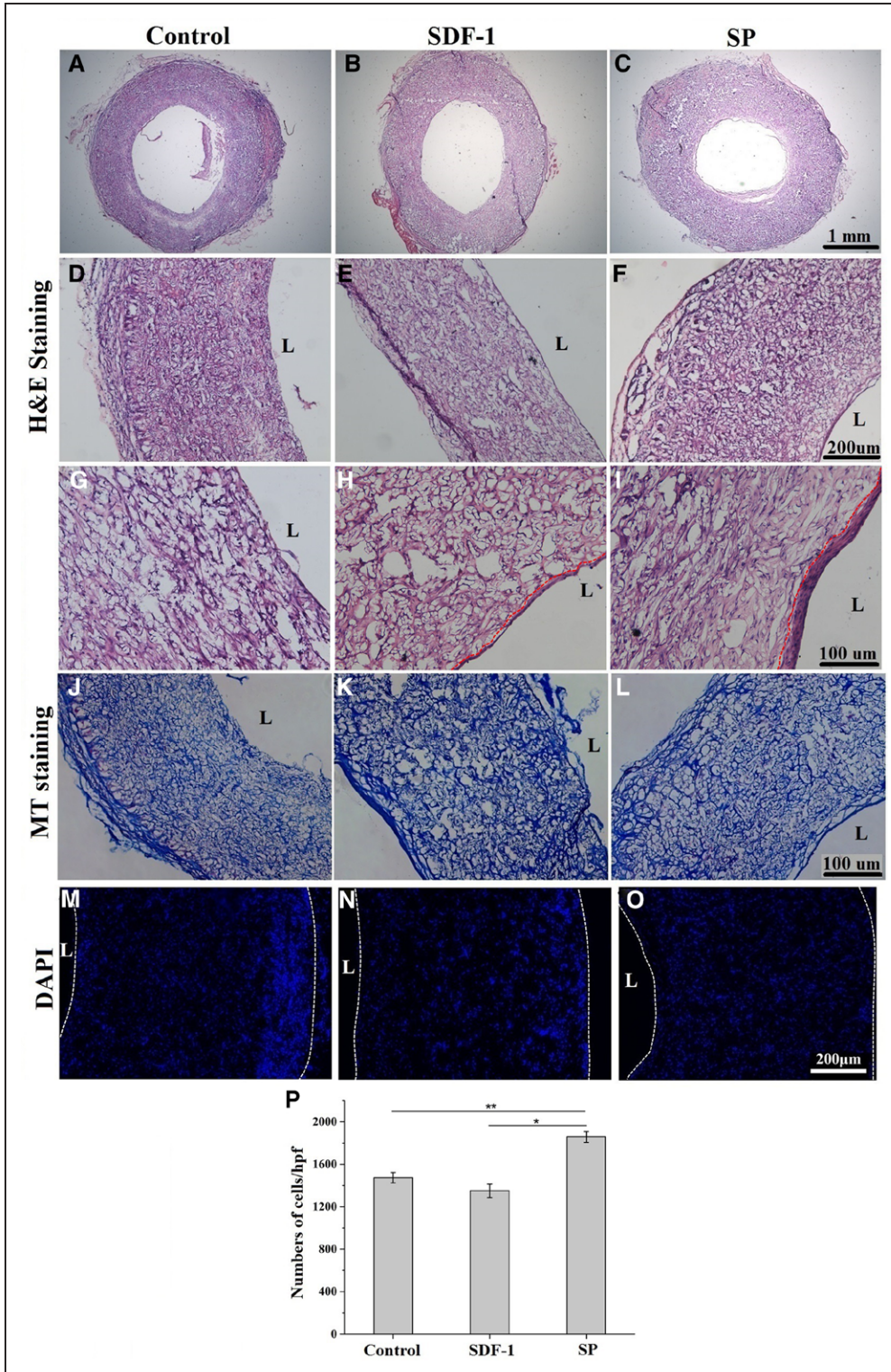


Figure 4. Histological analysis of vascular grafts (n=5 per group) explanted 4 wk after implantation. **A–I**, H&E staining, **(J–L)** Masson trichrome staining, and **(M–O)**, DAPI staining. Grafts were well-integrated with the surrounding tissues as demonstrated using H&E staining. SP and SDF-1 grafts showed neointimal formation on the luminal side (**E, F, H, I**). MT staining showed the regeneration of collagen in the neointima and the graft wall. Cross-sections were also stained with DAPI to discern cellularization. Control (**M**), SDF-1 (**N**), and SP (**O**). Cell infiltration was qualitatively and quantitatively analyzed based on the DAPI-stained sections. For quantitative analysis of cell density, 10 high power field (hpf) images were used per sample and 5 samples were used per group. Control and SDF-1 grafts did not significantly differ in terms of the cell density per hpf (control, 1473.83±49.69; SDF-1, 1350.22±63.36), whereas SP grafts showed significantly higher cell density per hpf than that of the control and SDF-1 grafts (1857.86±50.18; **P**). Data are presented as mean±SEM and evaluated by 1-way ANOVA followed by Tukey post hoc analysis. **P*=0.00001081 and ***P*=0.00086931. SDF indicates stromal cell-derived factor; and SP, substance P.

cell distribution was homogenous in the SDF-1 and SP grafts (Figure 4M through 4O and Figure III in the [online-only Data Supplement](#)). Statistical analysis confirmed that the cell density is similar for the control and SDF-1 grafts at 4 weeks (control, 1473.83 ± 49.69 and SDF-1, 1350.22 ± 63.36 cells per hpf), whereas the cell density of SP grafts (1857.86 ± 50.18 cells per hpf) is significantly higher than that of control and SDF-1 grafts (SP versus control, $P=0.0008693$; and SP versus SDF-1, $P=0.00001081$), which indicated that SP facilitated more cell infiltration (Figure 4P). We envision that the different categories of host cells can invade scaffold materials including but not limited to the monocyte/macrophages, vascular cells types such as smooth muscle cells (SMCs) and ECs, myofibroblasts, and peripheral blood- and BM-derived cells.

In the current study, Masson trichrome staining was performed to detect collagen deposition in the vascular grafts. All grafts showed successful regeneration of collagen in the graft wall (Figure 4J through 4L). Moreover, SDF-1 and SP grafts also showed the regeneration of collagen in the neointima. We also performed collagen type 1 immunofluorescence staining and found that the collagen type 1 density was significantly higher in SP grafts than that of the control and SDF-1 grafts (control, 15892.27 ± 2589.17 ; SDF-1, 25125.0 ± 2809.45 ; and SP, $53039.08 \pm 7198.05 \mu\text{m}^2/\text{mm}^2$; SP versus control, $P=0.0024$ and SP versus SDF-1, $P=0.0358$; Kruskal-Wallis test followed by Dunn multiple comparisons test; Figure IV in the [online-only Data Supplement](#)). Though all vascular grafts contain collagen and we did not cross-link vascular grafts before implantation, it is expected that the incorporated collagen would have been degraded by the 4 weeks. Rahmanian-Schwarz et al³⁷ reported the complete biodegradation of noncrosslinked collagen type 1 42 days after subcutaneous implantation in rats. Similarly, Kuo et al³⁸ reported >90% mass loss of rat tail collagen type 1 implanted for 1 week subcutaneously.

Rapid Endothelialization

The lumen of the native blood vessels is lined with a layer of cobblestone-like ECs, the endothelium, which plays a crucial role in maintaining vascular homeostasis, including antithrombosis and prevention of restenosis, resulted by SMCs over proliferation (Figure V in the [online-only Data Supplement](#)).³⁹ Therefore, rapid endothelialization is of considerable importance to guarantee good performance of artificial SDVGs after in situ implantation. In this study, the endothelium regeneration was discerned by scanning electron microscopy and immunofluorescence staining. The lumen of the explanted grafts was observed by scanning electron microscopy to analyze the endothelialization (Figure 5A). At 1 month, the suture sites of all grafts were covered by ECs with cobblestone-like morphology, which is in agreement with the previous reports.^{39,40} In scanning electron microscope micrographs, some bare fibers could be observed on the luminal side of the control grafts, whereas, bare fibers were not evident in SDF-1 and SP grafts, which suggest better endothelialization. Interestingly, the luminal surface of the SP grafts demonstrated the presence of ECs with a cobblestone-like morphology which were elongated in the direction of the blood flow, which is similar

to the normal morphological features of the endothelium of the native abdominal aorta (Figure 5A; Figure V in the [online-only Data Supplement](#)).^{39,40} Endothelialization was further discerned by immunostaining using vWF antibody. At 4 weeks, control grafts show only few areas positive for vWF, whereas SDF-1 and SP grafts revealed the formation of a vWF-positive monolayer on the luminal side (Figure 5B). Such a difference reveals that SP and SDF-1 α could promote endothelialization in cell-free grafts. We also observed few vWF-positive area in the SDF-1 α grafts, which may be caused by the formation of capillaries and blood vessels in the graft wall.

Regeneration of SMCs

The mechanical properties of regenerated arteries rely on the successful regeneration of extracellular matrix components (ie, collagen and elastin) and SMCs. Therefore, we examined the effect of SP and SDF-1 peptides on SMCs regeneration by immunofluorescence staining with α -SMA antibody. As shown in Figure 6, the SMCs regeneration was better in SDF-1 and SP grafts compared with the control grafts. The α -SMA⁺ cells have been indicated by the arrows. The numbers of cells with DAPI and α -SMA overlapping were counted and found to be 8.6 ± 1.47 , 19.67 ± 0.88 , and 24.17 ± 1.93 per hpf in the control, SDF-1, and SP grafts, respectively (SDF-1 versus control, $P=0.00172$ and SP versus control, $P=0.00367$; Figure 6J). This suggests that SDF-1 and SP promote SMCs regeneration in cell-free grafts. The thickness of the α -SMA⁺ layer was measured by using image J and found to be $34.88 \pm 1.63 \mu\text{m}$, $53.38 \pm 1.19 \mu\text{m}$, and $85.94 \pm 4.85 \mu\text{m}$ for control, SDF-1, and SP grafts, respectively. SDF-1 and SP grafts showed significantly higher SMC content than that of the control grafts (SDF-1 versus control, $P=0.0212$, SP versus control, $P \leq 0.0001$, and SDF-1 versus SP, $P=0.0026$, Kruskal-Wallis test followed by Dunn multiple comparisons test).

Stem Cell Recruitment

Previous studies have documented that SDF-1 α could promote stem cell homing.³⁰⁻³³ Meanwhile, SP could mobilize endogenous stem/progenitor cells, which are then recruited toward the injury site. Therefore, we evaluated the recruitment of endogenous stem cells by using Sca-1 (stem cell antigen-1) antibody. As can be observed from Figure 7, control grafts hardly show the presence of Sca-1⁺ cells 4 weeks after implantation. On the contrary, SDF-1 and SP grafts recruited many Sca-1⁺ cells. We also performed quantitative analysis and counted Sca-1⁺ cells in 10 hpf images per sample. The numbers of Sca-1⁺ cells were found to be 1.5 ± 0.5 , 7.0 ± 1.53 , and 14.0 ± 1.52 per hpf in the control, SDF-1, and SP grafts, respectively (Figure 7J). Statistical analysis indicates that SP grafts recruited significantly more numbers of stem cells than that of the control group (SP versus control, $P=0.0338$), which is in agreement with our in vitro observations. We also performed Sca-1/ α -SMA costaining to evaluate the possible differentiation of stem cells into vascular cell types. Indeed, we found a few Sca-1⁺ α -SMA⁺ cells in the graft wall, which indicate that the recruited cells may differentiate into other vascular cell types (Figure VI in the [online-only Data Supplement](#)). We also stained explanted grafts with ki67 antibody to detect

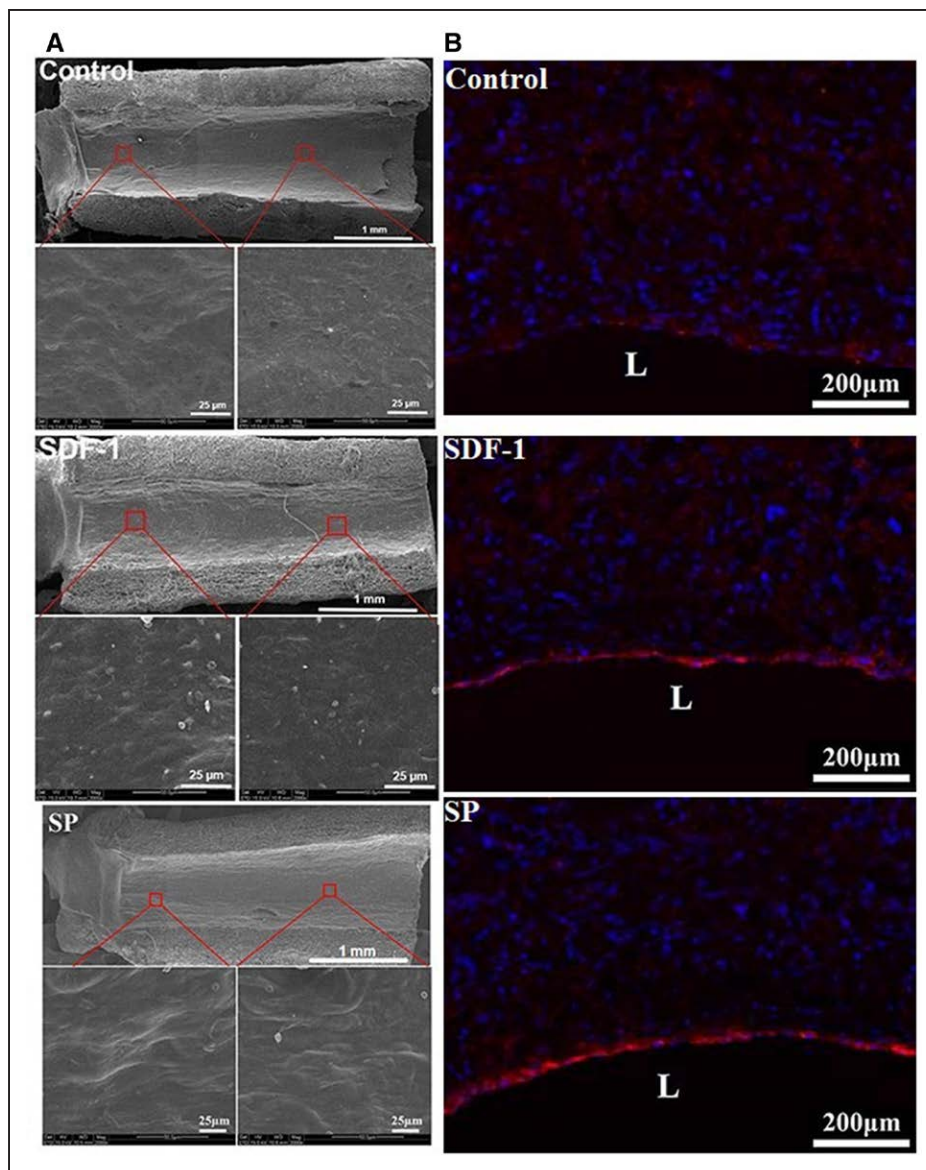


Figure 5. Endothelialization of vascular grafts explanted after 4 wk ($n=5$ per group). Explanted grafts were observed with scanning electron microscope (SEM) and also stained with vWF (von Willebrand factor). **A**, Scanning electron micrographs of explanted grafts and **B** immunofluorescence staining of vascular grafts with vWF antibody. SP grafts revealed the formation of endothelial cells (ECs) with cobblestone-like morphology on the luminal side. Scale bars have been shown on images. SP and SDF-1 grafts also showed a confluent layer of vWF-positive ECs on the luminal side. Scale bar, 200 μm . SDF indicates stromal cell-derived factor; and SP, substance P.

proliferating cells and found a few ki67^+ cells in the graft wall of control, SDF-1, and SP grafts (Figure VII in the [online-only Data Supplement](#)). Whereas control and SP grafts showed almost similar numbers of ki67^+ cells, SDF-1 grafts showed slightly higher numbers of ki67^+ cells.

Evaluation of Angiogenesis in Grafts

We performed immunostaining for laminin to delineate the regeneration of blood vessels in the explanted grafts and the representative images are shown in Figure 8.²⁶ Control grafts exhibit only a few numbers of laminin⁺ blood vessels. On the contrary, SDF-1 and SP grafts showed significantly higher numbers of laminin⁺ blood vessels than that of the control group. The laminin⁺ area was calculated by using image J and was found to be $1300.5 \pm 181.5 \mu\text{m}^2$, $5587.62 \pm 369.99 \mu\text{m}^2$, and

$6985.0 \pm 580.08 \mu\text{m}^2$ per hpf in control, SDF-1, and SP grafts, respectively (SDF-1 versus control, $P=0.00205$ and SP versus control, $P=0.000167031$; Figure 8J). These data indicate that SP and SDF-1 α could promote vascularization in cell-free vascular grafts. Ko et al²⁶ also reported higher regeneration of laminin-positive blood vessels in poly (L-lactide)/gelatin sponges eluting SDF-1 and SP.

Evaluation of Inflammatory Response in Grafts

It has been well-documented that host's inflammatory cells populate implanted scaffold materials and they can either facilitate or prevent tissue regeneration. Macrophages are one type of inflammatory cells, which have been further divided into different subtypes such as, classically activated M1 macrophages and alternatively activated

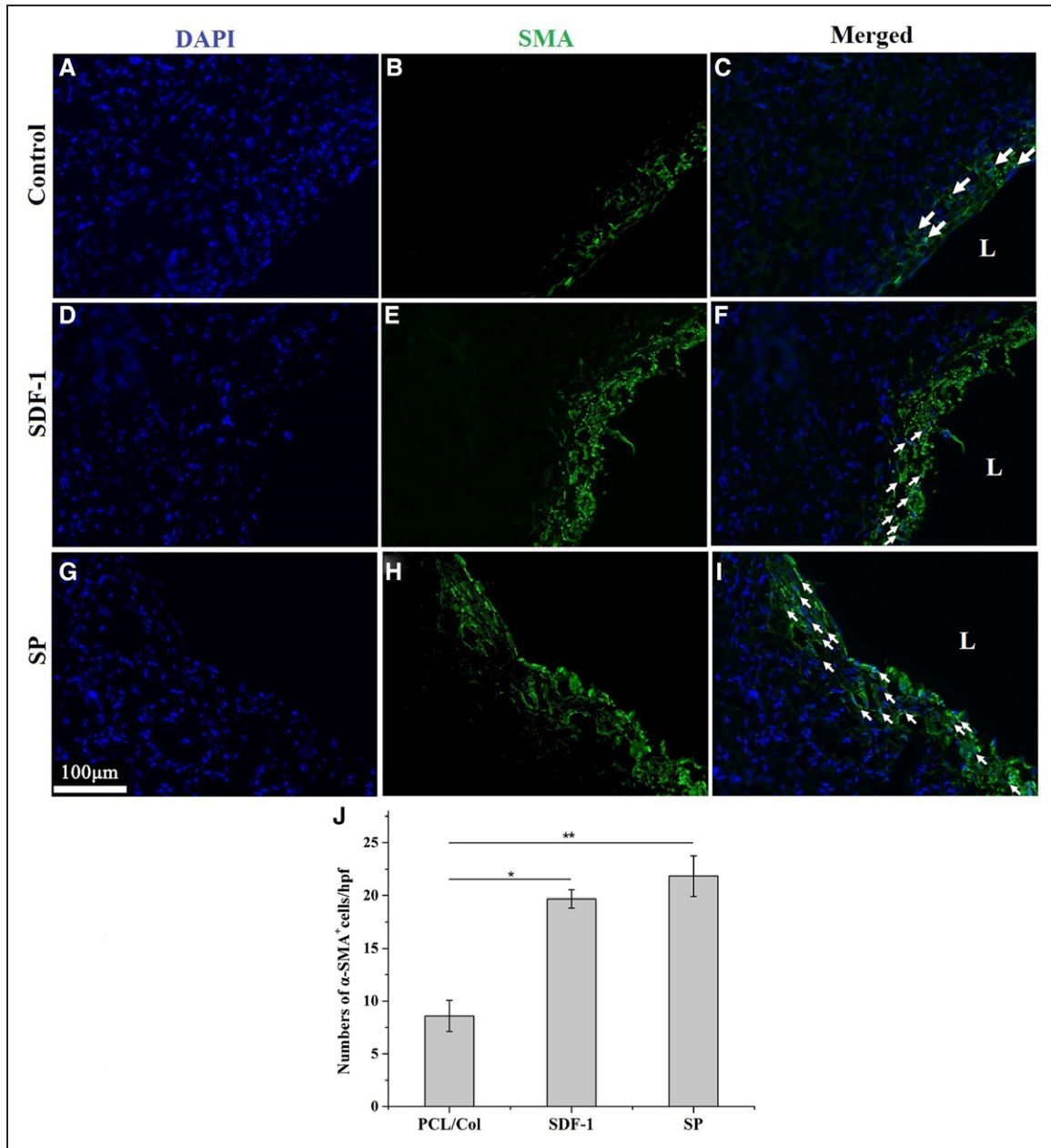


Figure 6. Smooth muscle cells (SMCs) regeneration in vascular grafts retrieved at 4 wk (n=5 per group). Explanted grafts were stained with α -smooth muscle actin (α -SMA) antibody. The α -SMA⁺ cells have been indicated by arrows (A–I). The numbers of α -SMA⁺ cells were counted and found to be 8.6 ± 1.47 , 19.67 ± 0.88 , and 24.17 ± 1.93 per hpf in control, SDF-1, and SP grafts, respectively (J). SDF-1 and SP grafts showed significantly higher numbers of α -SMA⁺ cells than that of the control grafts (SDF-1 vs control, $P=0.00172$ and SP vs control, $P=0.00517$; E, F, H, I). Scale bar, 100 μ m. Data are shown as mean \pm SEM and evaluated by 1-way ANOVA followed by Tukey post hoc analysis. * $P=0.00172$ and ** $P=0.00517$. COL indicates collagen; hpf, high power field; PCL, polycaprolactone; SDF, stromal cell-derived factor; and SP, substance P.

M2 macrophages.^{41–44} We performed immunofluorescence staining to discern M1 and M2 macrophages in the implanted grafts and the representative images are shown in Figure 9. Arrows indicate the CD68⁺ and CD206⁺ macrophages. As can be observed from the Figure 9, control, SDF-1, and SP grafts did not significantly differ in terms of CD68⁺ (M1) macrophages. On the contrary, SP grafts showed higher numbers of CD206⁺ macrophages in comparison to control and SDF-1 grafts. The numbers of CD68⁺ and CD206⁺ macrophages were also quantified by using 10 hpf images per sample and found to be 4.86 ± 0.83 ,

5.64 ± 0.54 , and 4.17 ± 0.56 per hpf in control, SDF-1, and SP grafts, respectively 4 weeks after implantation (Figure 9G). On the contrary, control and SDF-1 grafts showed significantly less numbers of CD206⁺ (M2) macrophages than that of the SP grafts (SP versus control, $P=0.0020$ and SP versus SDF-1, $P \leq 0.0001$). The numbers of CD206⁺ (M2) macrophages were found to be 8.45 ± 1.11 , 5.12 ± 0.45 , and 22.21 ± 1.18 per hpf in control, SDF-1, and SP grafts, respectively (Figure 9H). These data indicate that SP facilitates macrophages polarization, which may be beneficial for TE applications.

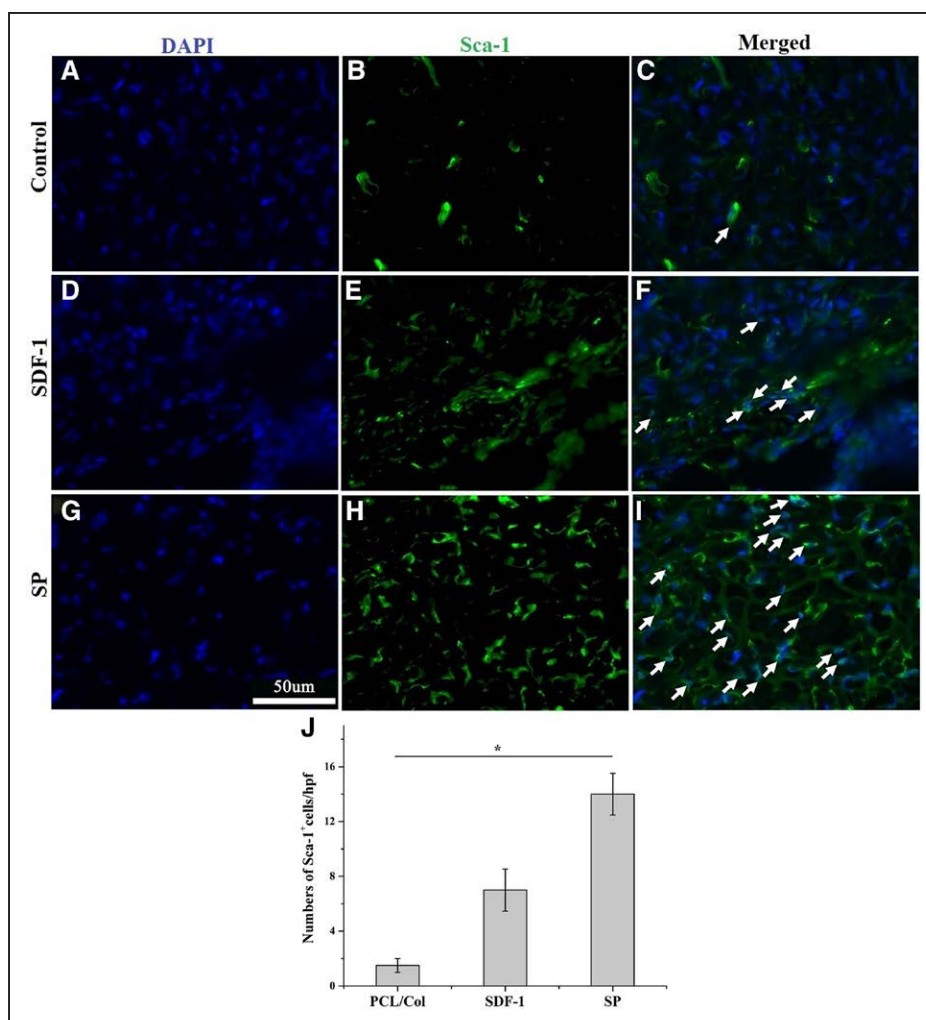


Figure 7. Stem cell recruitment in vascular grafts explanted at 4 wk (n=5 per group). Explanted grafts were stained with anti-Sca-1 antibody. Control grafts show only a few numbers of Sca-1⁺ cells (1.5 ± 0.5 per hpf; **A–C**). On the contrary, SDF-1 and SP grafts showed higher numbers of Sca-1⁺ cells (**D–I**). The numbers of recruited cells were also quantified by using image J and found to be 7.0 ± 1.53 and 14.0 ± 1.52 per hpf in SDF-1 and SP grafts, respectively (**J**). Data are shown as mean \pm SEM and evaluated by Kruskal-Wallis test followed by Dunn multiple comparisons test. * $P=0.0338$. COL indicates collagen; hpf, high power field; PCL, polycaprolactone; Sca, stem cell antigen; SDF, stromal cell-derived factor; and SP, substance P.

Discussion

A plethora of research has been devoted to developing SDVGs, which could remodel *in vivo* and endure blood pressure along with avoiding debilitating complications, such as intimal hyperplasia, thrombosis, and restenosis. This also includes the efforts to identify/select appropriate scaffold materials, processing parameters, and bioactive molecules, which could promote host cell infiltration, endothelialization, and remodeling. Chemokines, such as SDF-1 α have been documented to promote endogenous stem and progenitor cell retention and homing. However, SDF-1 α can be easily degraded by the proteolytic microenvironment. To circumvent this limitation, engineered SDF-1 α variants have been developed and shown to exhibit bioactivity similar to that of the SDF-1 α protein. Additionally, these peptides can be easily synthesized and are economical compared with the whole SDF-1 α protein. Similarly, neuropeptide SP has been shown to enhance neovascularization, tissue regeneration, and tissue repair by mobilizing endogenous stem and progenitor cells from the

BM or peripheral circulation. Our group has developed micro-fibrous PCL grafts, which showed better tissue repair compared with their nanofibrous counterparts.⁴⁰ However, because of the poor biocompatibility of PCL, host cells may not be recruited toward these grafts in a sufficient number to enhance the remodeling *in vivo*.

The objective of this research was therefore to develop SP or SDF-1 α peptides containing vascular grafts which could enhance *in situ* vascular regeneration and to discern which of these peptides has more potential for vascular tissue regeneration. We developed bioactive grafts by loading SP and SDF-1 α peptides. The scaffolds could release these peptides and regulate the tissue repair process. The feasibility of grafts was demonstrated in a rat abdominal artery replacement model, in which the SP and SDF-1 α peptides containing grafts showed rapid endothelialization, enhanced neovascularization, augmented stem cell recruitment, improved SMCs regeneration, and higher content of proremodeling macrophages. Interestingly, SP containing grafts showed slightly higher

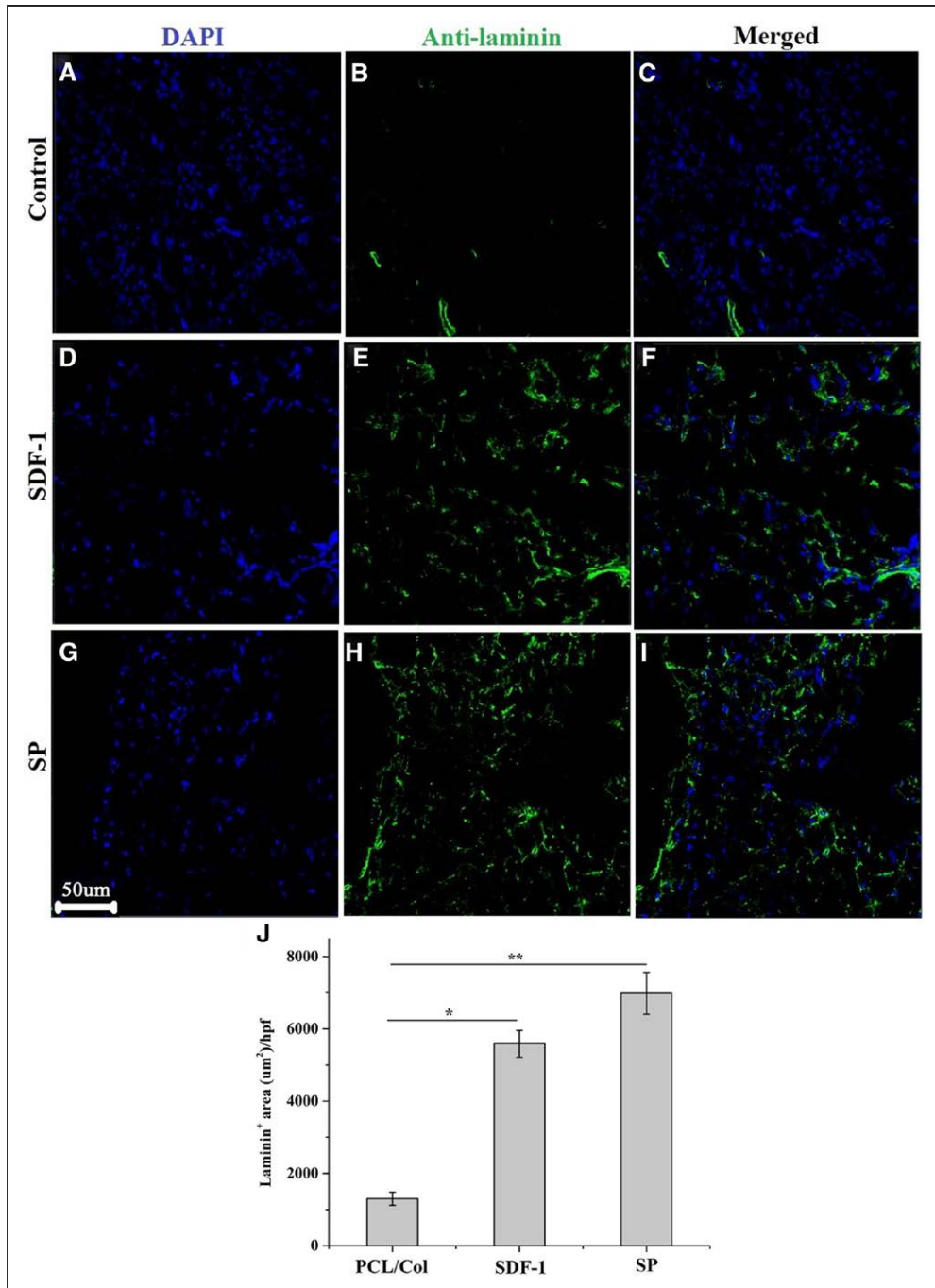


Figure 8. Angiogenesis in the explanted grafts at 4 wk (n=5 per group). Explanted grafts were stained with laminin antibody (A–I). The laminin⁺ area was calculated by using image J and found to be 1300.5±181.5 μm², 5587.62±369.99 μm², and 6985.0±580.08 μm² per hpf in control, SDF-1, and SP grafts, respectively (J). Scale bar, 50 μm. Data are shown as mean±SEM and evaluated by 1-way ANOVA followed by Tukey post hoc analysis. *P=0.00205 and #P=0.000167031. COL indicates collagen; hpf, high power field; PCL, polycaprolactone; Sca, stem cell antigen; SDF, stromal cell-derived factor; and SP, substance P.

cellularization, SMCs regeneration, and M2 macrophages recruitment than that of the SDF-1α grafts.

The endothelium plays a crucial role in the maintenance of vascular tone and the prevention of the cardiovascular diseases. Rapid endothelialization may not only reduce the thrombosis and intimal hyperplasia risks but can also affect the regeneration of SMCs within the graft wall and differentiation of SMCs from synthetic to the contractile phenotype.^{45,46}

Accordingly, various strategies have been put forward to induce rapid endothelialization in artificial blood vessels, such as pre seeding of vascular grafts with endothelial progenitor cells (EPCs), ECs, MSCs, and induced pluripotent cell-derived vascular cell types. Moreover, cell-free grafts loaded with bioactive molecules have been developed to promote the rapid endothelialization of artificial blood vessels or an injured vasculature.^{19,47} Re-endothelialization of graft could occur via

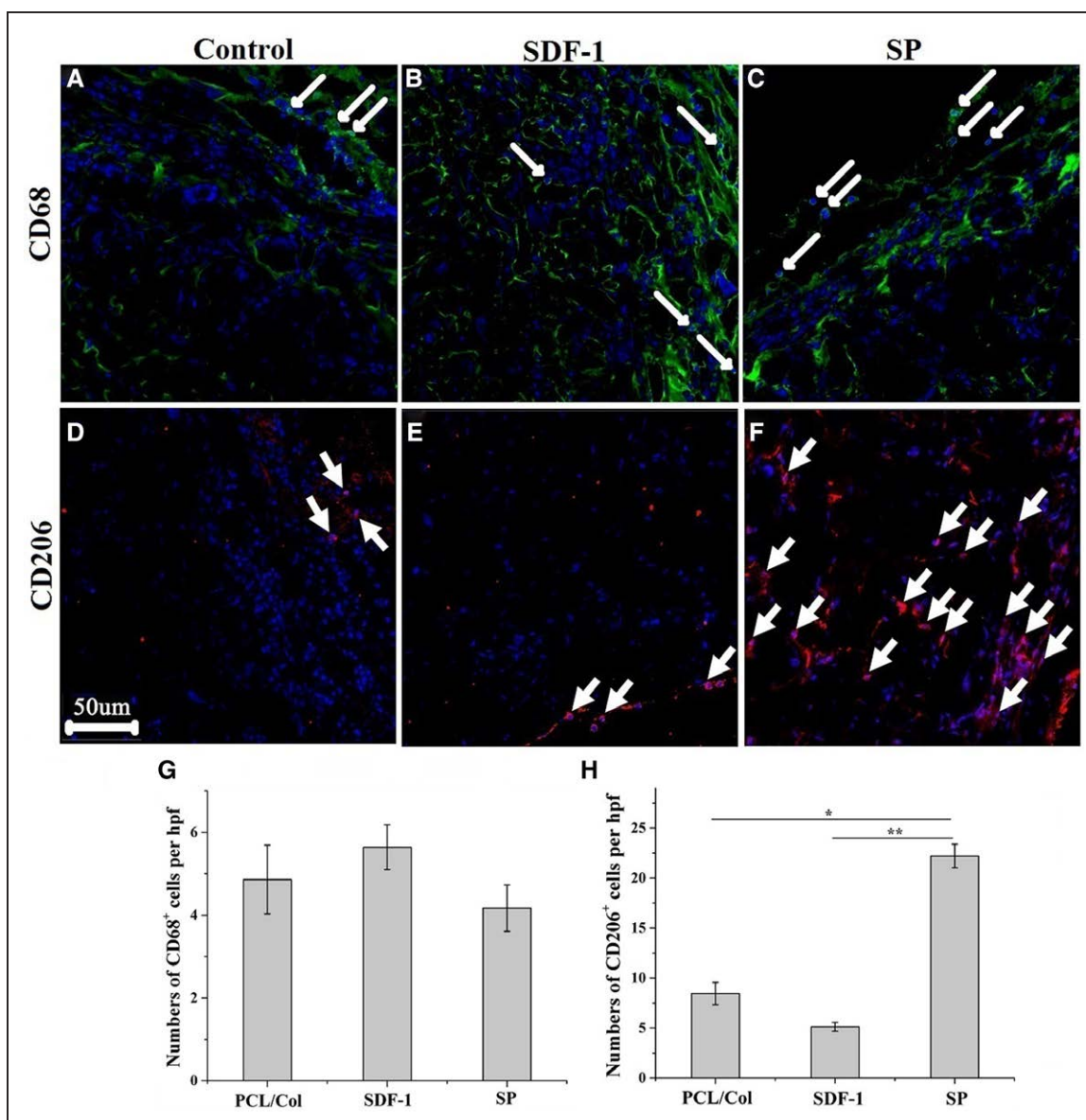


Figure 9. Inflammatory response to explanted grafts at 4 wk (n=5 per group). Explanted grafts were stained with CD68 and CD206 antibodies (A–F). The numbers of CD68⁺ macrophages were counted and found to be 4.86 ± 0.83 , 5.64 ± 0.54 , and 4.17 ± 0.56 per hpf in control, SDF-1, and SP grafts, respectively (G). The numbers of CD206⁺ (M2) macrophages were found to be 8.45 ± 1.11 , 5.12 ± 0.45 , and 22.21 ± 1.18 per hpf in control, SDF-1, and SP grafts, respectively (H). Scale bar, 50 μ m. Data are shown as mean \pm SEM and evaluated by Kruskal-Wallis test followed by Dunn multiple comparisons test. * $P=0.0020$ and ** $P \leq 0.0001$. COL indicates collagen; hpf, high power field; PCL, polycaprolactone; Sca, stem cell antigen; SDF, stromal cell-derived factor; and SP, substance P.

outgrowth of the local ECs from the host vessels adjacent to anastomotic sites or via the recruitment of circulating EPCs as the re-endothelialization of the injured arteries.^{39,40} In this study, we used SP and SDF-1 α peptides to modify vascular grafts, and they demonstrated accelerated endothelialization in rat abdominal artery implantation than that of the control grafts. Interestingly, the luminal surface of SP grafts demonstrated the presence of ECs with cobblestone-like morphology, which were elongated in the direction of the blood flow, which is similar to the normal morphological features of endothelium of the native abdominal aorta.^{39,40}

SDF-1 α has been shown to enhance the blood vessel formation in various injury models and cell-free grafts through the recruitment of stem and progenitor cells.^{32,48,49} Yu et al⁴⁸

documented that SDF-1 α enhanced the recruitment of CD34⁺ cells (EPCs) into the grafts, which later differentiated into ECs. At 4 weeks after implantation, EPCs were not present, suggesting that EPCs, if any, would already have been differentiated into ECs. Similarly, De Visscher et al^{50,51} reported enhanced capture of EPCs and hematopoietic stem cells in fibronectin/SDF-1 α coated grafts, which ultimately improved the patency and the endothelialization of vascular grafts. Noticeably, the fraction of EPCs and hematopoietic stem cells decreased at day 3 than that of day 1. Similarly, Segers et al³² have demonstrated that SDF-1 α peptide enhanced the capillary formation via the recruitment of endogenous stem and progenitor cells. Moreover, recruited stem/progenitor cells may also differentiate into ECs and can lead to enhanced

endothelialization. Taken together, these data support the notion that SDF-1 α peptide can facilitate endothelium regeneration in cell-free vascular grafts.

Similarly, SP has been shown to enhance angiogenesis, which can be because of its direct effect on ECs and its ability to recruit EPCs and stem cells.^{52,53} Amadesi et al⁵⁴ documented that CD34⁺ progenitor cells possess neurokinin-1 receptor and are attracted by the SP gradients.⁵³ These data support the notion that endogenous progenitor cells might be recruited toward bioactive grafts and promote endothelialization. Indeed, this finding was corroborated with our *in vitro* and *in vivo* results, which delineate that SP and SDF-1 α grafts recruited more numbers of MSCs than that of the control group (Figure 2). Nonetheless, we cannot rule out the possibility that the transanastomotic and transmural migration of endogenous vascular cells can also enhance endothelialization in our cell-free vascular grafts.^{55,56}

In addition to the endothelialization, vascular wall remodeling and thus the mechanical properties of grafts are critical for the maturation of artificial blood vessels. Therefore, we determined the recruitment of SMCs to the grafts. At 1 month, SDF-1 and SP grafts recruited more numbers of α -SMA⁺ cells than that of the control group. It has been previously documented that elevated expression of SDF-1 α after mechanical or vascular injury attracts endogenous circulating SMC progenitor cells, which then differentiate into SMCs.^{54,57} Moreover, it has been documented that SDF-1 α attracts Lin⁻/Sca-1⁺ SMC progenitors.⁵⁸ In this study, we found more numbers of Sca-1⁺ cells in SDF-1 and SP grafts than that of the control group, which suggest that smooth muscle progenitor cells might have been recruited to the grafts and differentiated into SMCs. We also observed significantly higher numbers of α -SMA⁺ cells in SP grafts than that of the control grafts. We and others have previously reported that SP induces the recruitment of MSCs, EPCs, pericytes, and α -SMA⁺ cells, which then participate in angiogenesis.^{25,26,52,53} It is thus speculated that SP-induced smooth muscle progenitor cells and MSCs may contribute to SMCs regeneration in vascular grafts. Indeed, we found few Sca-1⁺ α -SMA⁺ cells into our cell-free grafts, which suggest that the recruited stem and progenitor cells may enhance SMCs regeneration (Figure VI in the [online-only Data Supplement](#)). Furthermore, transanastomotic and transmural migration of vascular cell types may also account for SMCs regeneration into cell-free vascular grafts.

Both SP and SDF-1 α have been shown to enhance the mobilization of stem and progenitor cells from BM or peripheral circulation, which may then participate in tissue repair via a direct (through tissue-specific differentiation) or an indirect (through paracrine mechanism) way. We found only a few Sca-1⁺ stem cells in the control grafts, whereas many Sca-1⁺ cells were recruited to the SP and SDF-1 α containing grafts. Schober et al^{57,58} reported that Lin⁻/Sca-1⁺ cells were recruited to the injured vessels by SDF-1 α . Similarly, Amadesi et al⁵⁴ showed that Sca-1⁺ cells possess neurokinin-1 receptors and are attracted by the SP gradient. Because Sca-1⁺ cells are hematopoietic cells, they can differentiate into SMCs and may enhance vascular regeneration. We found significantly higher content of Sca-1⁺ cells in SP grafts compared with the

control groups. Besides, we found a few ki-67⁺ cells in the graft wall, which were higher in SDF-1 grafts in comparison to control and SP grafts (Figure VII in the [online-only Data Supplement](#)).

Vascularization of implanted grafts may also be very helpful for cellularization and neotissue formation. Particularly, cells infiltrated into the thick grafts face nutrient- and metabolic-starvation if there is a paucity of the blood vessels or capillary-like networks in the scaffold materials. Therefore, strategies focused at enhancing angiogenesis and neovascularization are of considerable importance. We found regeneration of more numbers of laminin-positive blood vessels in SDF-1 and SP grafts compared with the control grafts, which can be attributed to SP and SDF-1 α . Ko et al²⁶ reported the more regeneration of laminin-positive blood vessels in poly (L-lactide)/gelatin sponges, which were resulted by the SDF-1 α and SP peptides. In contrast, control grafts exhibited only a few numbers of laminin-positive blood vessels. SDF-1 α has been shown to enhance neovascularization in various injury models and cell-free grafts through the recruitment of stem and progenitor cells. Similarly, SP has been documented to enhance neovascularization and blood perfusion in several injury models and synthetic grafts.^{25,26,52,53,59} Moreover, recruited stem/progenitor cells may also differentiate into ECs and SMCs and lead to more blood vessel formation.

In addition, SP was reported to play a role in inhibiting inflammatory reaction and inducing macrophages polarization.⁶⁰ In accordance, we evaluated if 3 groups differ in terms of the M1 and M2 macrophages. PCL/Col, SDF-1, and SP grafts did not significantly differ in terms of the CD68⁺ (M1) macrophages, whereas SP grafts showed significantly higher numbers of M2 macrophages than that of the control and SDF-1 group. In this study, we did not observe significant difference in term of the M2 macrophages between control and SDF-1 group, which is in agreement with our previous study.²⁷ On the contrary, SP significantly improved the numbers of M2 macrophages. We also found significantly higher numbers of M2 macrophages in SP grafts in our previous study.^{60,61} Similarly, Leal et al⁶² have reported that SP promotes wound healing by modulating the inflammatory response and by enriching the M2 macrophages to the injury site. It has been reported that macrophages secrete cytokines and chemokines, which can attract endogenous cells and enhance tissue regeneration.¹⁶ These data indicate that the enhanced vascular regeneration could be at least partially explained by the beneficial effect of SP in resolving the inflammation. Taken together, SP and SDF-1 grafts showed rapid endothelialization, better SMCs regeneration, enhanced blood vessel formation, and higher endogenous stem/progenitor cell recruitment compared with the control group. Of SDF-1 and SP grafts, SP grafts showed higher cellularization, pre remodeling macrophages phenotype, endogenous stem/progenitor cell recruitment, and SMCs regeneration. These results suggest that the bioactive factors can be a good choice along with an appropriate scaffold material for TE applications.

There are also several limitations in this study. First, we implanted vascular grafts only for up to 4 weeks. Long-term evaluation of these vascular grafts will further elucidate their potential as small-caliber substitutes. Second, we did not

investigate the grafts at earlier time point. Evaluation of cell-free vascular grafts at the earlier time points might be helpful to clearly demonstrate the inflammatory response and the process of cellularization, which is warranted for the future investigations. Third, we did not use a wide panel of antibodies for staining endogenously recruited cells and vascular cell types. Nonetheless, we elucidated that bioactive grafts facilitated endothelialization, SMCs regeneration, stem cell recruitment, and blood vessel regeneration.

Sources of Funding

This study was supported by the National Nature Science Foundation of China (Grant number 81530059, 81772000), KIST Institutional Program, and by the KU-KIST Graduate School of Converging Science and Technology Program.

Disclosures

None.

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Highlights

- Small-diameter vascular grafts capable of eluting SP (substance P) or SDF (stromal cell-derived factor)-1 α peptide were fabricated.
- The in situ blood vessel regeneration potential of grafts was assessed as rat abdominal aorta substitutes.
- SP or SDF-1 α peptide eluting grafts showed significant higher recruitment of stem cells, angiogenesis, endothelialization, and vascular regeneration than that of the control grafts.
- Smooth muscle cell regeneration, vascularization, and stem cell recruitment were prominent in SP grafts.