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The use of BDNF to enhance the patency rate of small-diameter tissue-engineered blood vessels through stem cell homing mechanisms

Wen Zeng [a], Can Wen [a], Yangxiao Wu [a], Li Li [a], Zhenhua Zhou [b], Jianhong Mi [a], Wen Chen [a], Mingcan Yang [a], Chunli Hou [a], Jiansen Sun [a], Chuhong Zhu [a,*]

[a] Department of Anatomy, Key Lab for Biomechanics and Tissue Engineering of Chongqing, Third Military Medical University, Gao Tan Yan Street, Shaping Ba District, Chongqing 400038, China
[b] Department of Neurology, Southwest Hospital, Third Military Medical University, Chongqing 400038, China

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ABSTRACT

The patency rate of small-diameter tissue-engineered blood vessels is the determinant for their application in coronary artery bypass grafting. The coronary artery is innervated by vagus nerves. The origin of vagus nerves is rich in brain-derived neurotrophic factors (BDNF). We have investigated whether BDNF could improve the patency rate of small-diameter tissue-engineered blood vessels through promoting stem cell homing and paracrine activity. In vitro, we isolated early and late endothelial progenitor cells (EPCs) and found BDNF could promote single clone formation and paracrine function of EPCs, and could also induce the proliferation, migration and differentiation of late EPCs. BDNF could enhance the capturing of EPCs in parallel-plate flow chamber. Flow cytometric analysis and laser-scanning confocal microscope showed BDNF could enhance the mobilization and homing of C57BL/6 mouse EPCs after wire injury. Based on it, BDNF was coupled to the lumen surface of the blood vessel matrix material incubated with collagen through SPDP to construct BDNF-modified small-diameter tissue-engineered blood vessel. The blood vessel patency rate was significantly higher than that of control group 8 weeks after implantation in rats and the endothelialization level was superior to control. These results demonstrate that BDNF can effectively improve patency of small-diameter tissue-engineered blood vessels through stem cell homing and paracrine, and it is expected to play an important role in the construction of substitutes for coronary artery bypass grafting.

1. Introduction

Recently, global cardiovascular disease morbidity and mortality have been steadily increasing. The leading causes of non-communicable diseases (NCD) deaths in 2008 were cardiovascular diseases (17 million deaths or 48% of all NCD deaths). The development of tissue-engineered blood vessels (TEBV) has broad and important applications in coronary artery bypass grafting. Niklason et al. developed functional tissue-engineered blood vessels [1]. The development and application of TEBV have provided new avenues for substitute treatments for cardiovascular diseases [2,3]. Coronary artery bypass grafting requires small-diameter blood vessels; however, small-diameter TEBV (<6 mm) often fail due to early thrombosis after grafting. Therefore, thrombosis and intimal hyperplasia are limiting factors for the development of TEBV [4,5]. Because endothelial cells play important roles in antithrombosis, inhibition of platelet aggregation and pathological proliferation of smooth muscle cells, the rapid endothelialization of TEBV effectively prevents thrombosis and intimal hyperplasia and improves the blood vessel patency rate [6–8].

The endothelial progenitor cells (EPCs) are precursor cells that directly differentiate into vascular endothelial cells. Blood vessel injury is directly repaired by increasing the EPCs content in the blood, decreasing intimal hyperplasia and thrombosis and reducing the vascular restenosis rate [9,10]. Homing EPCs secrete multiple factors via paracrine signaling. These factors act on normal endothelial cells surrounding the lesion, promoting their differentiation and proliferation to repair damaged endothelium [11,12]. Currently, EPCs have an increasingly apparent role in TEBV construction as adult stem cells. Our previous studies have shown that the mobilization and homing of EPCs have an important role in promoting the endothelialization of TEBV and enhancing their long-term patency rate [13].

Coronary heart disease and some brain ischemic diseases are primarily caused by coronary atherosclerosis and carotid artery

* Corresponding author.
E-mail address: zhuch99@yahoo.com (C. Zhu).

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intimal hyperplasia [14–16]. It is important to understand the physiological structure of these arteries to build biomimetic small-diameter TEBV. Physiologically, blood vessels and nerves are intimately associated. The coronary arteries and the internal and external carotid arteries are innervated by the vagus nerve, which is the branch of the 10th pair of cranial nerves. The vagus nerve originates from the central sulcus of medullary olives in the brain, and studies have shown that the medulla is enriched in brain-derived neurotrophic factors (BDNF) [17]. BDNF also acts on the heart, skeletal muscle, large arteries and other specific organs and tissues during blood vessel formation [18,19]. BDNF overexpression in the midgestational mouse heart increases capillary density, suggesting the essential regulatory role of BDNF in cardiac microvascular endothelial cells during development [20]. BDNF promotes endothelial cells survival and induces neoangiogenesis in ischemic tissues. Unlike many vascular growth factors that act on many vascular beds, BDNF activity is relatively restricted to central arteries and the vessels of the cardiac and skeletal muscle and the skin. In addition, BDNF activity induces the formation of new blood vessels in human tissues and organs [21–23]. However, it is still unclear whether BDNF has any effect on the patency rate of small-diameter TEBV that are used as substitutes for the coronary and carotid arteries.

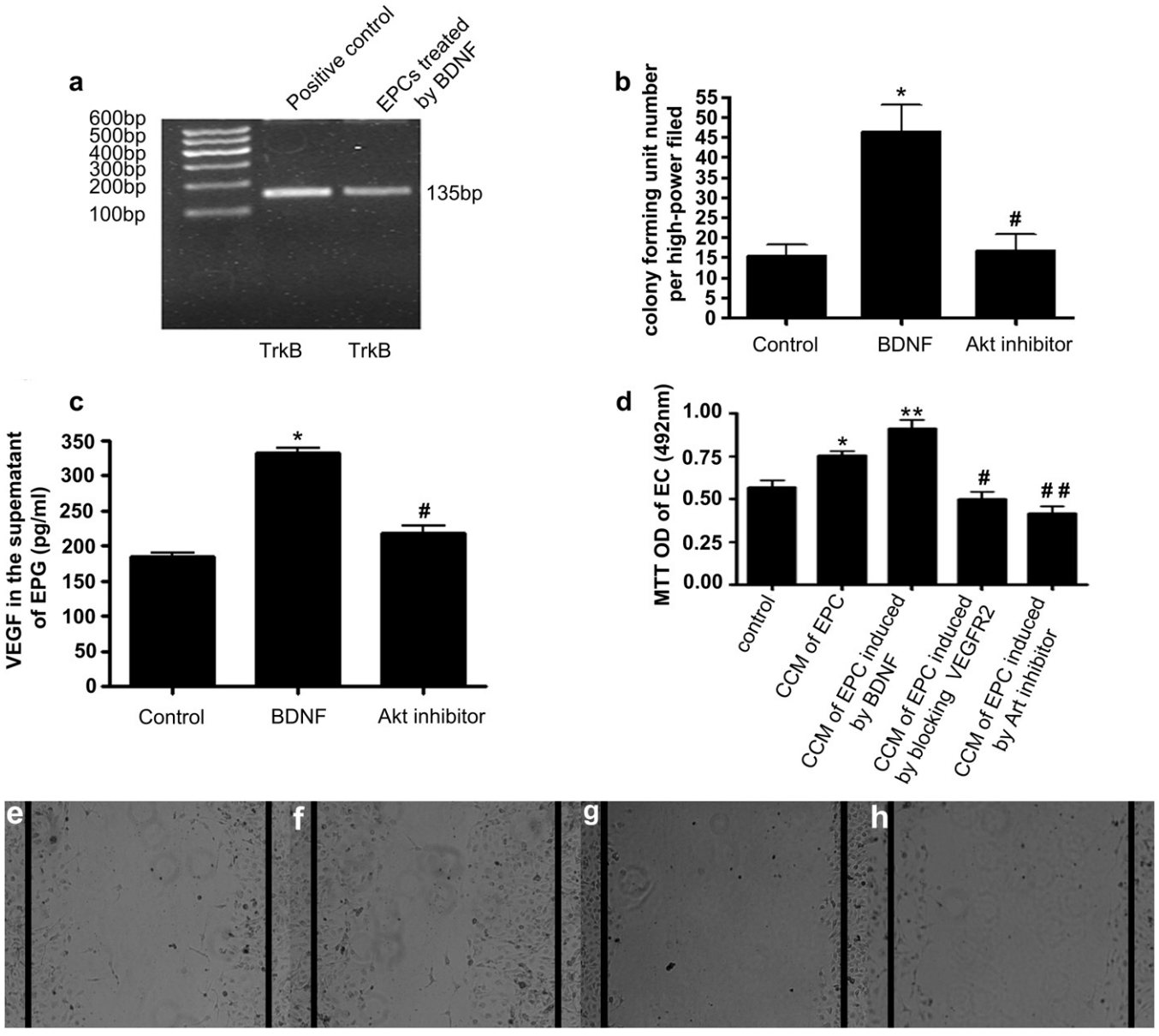

Fig. 1. BDNF promoted early EPCs to form colony-forming units and the role of paracrine. (a) RT-PCR showed EPCs treated by BDNF express TrkB. (b) The average number of colony forming-units per high-power field in methylcellulose semi-solid medium ($^{*}p < 0.05$ $n = 10$) versus control. $\#p < 0.05$ ($n = 10$) versus condition medium of EPCs induced by BDNF group. Values are mean ± SE. (c) The VEGF concentration in the supernatant of different group EPCs was determined by ELISA. $^{*}p < 0.05$ ($n = 10$) versus control group, $\#p < 0.05$ ($n = 10$) versus condition medium of EPCs induced by BDNF group. Values are mean ± SE. (d) MTT assay showing the OD value (492 nm) of EC cultured by condition medium of EPCs. $^{*}p < 0.05$ ($n = 10$) versus control group, $^{**}p < 0.05$ ($n = 10$) versus condition medium of EPC group, $\#p < 0.05$ ($n = 10$) versus condition medium of EPCs induced by BDNF group, $\#\#p < 0.05$ ($n = 10$) versus condition medium of EPCs induced by BDNF group. Values are mean ± SE. (e–h) Scratch test showed the endothelial cell migration induced by condition medium of EPCs, condition medium of EPCs induced by BDNF, condition medium of EPCs induced by BDNF and anti-VEGFR-2 antibody and condition medium of EPCs induced by BDNF and Akt inhibitor.

2. Materials and methods

2.1. Isolation of EPCs and cell culture

Mononuclear cells (MNCs) were isolated using density-gradient centrifugation (Lymphoprep; Axis-Shield) from the peripheral blood (PB) of healthy human volunteers. The MNCs were seeded into 24-well plates (Costar) and cultured in M199 medium (Hyclone Laboratories) that was supplemented with 10% FBS, 10 ng/ml vascular endothelial growth factor (VEGF; R&D Systems, Minneapolis, MN, USA), 3 ng/ml basic fibroblast growth factor (bFGF; Roche Applied Science, Indianapolis, IN, USA), and heparin (90 mg/ml; Sigma–Aldrich, USA) in the presence of penicillin (100 units/ml), streptomycin (100 mg/ml), and Fungizone (0.25 mg/ml) (all purchased from Sangon Biotech, Shanghai, China) as basic medium, which has been used in our previous study [13,24]. All cells were maintained at 37 °C with 5% CO_2 in a humidified incubator for 14 days. PE-anti-CD34 (eBioscience), APC-anti-VEGFR-2 (VEGFR-2; R&D Systems, Minneapolis, MN, USA) and anti-vWF (R&D Systems, Minneapolis, MN, USA) were used as markers to identify cultured EPCs by immunostaining.

2.2. RT-PCR assay

Human EPCs were cultured in the basic medium with 100 ng/ml BDNF (R&D Systems, Minneapolis, MN, USA) for 14 days [25]. Total RNA was extracted using the TRIzol (Roche) reagent, and the quality of RNA was examined using the Experion™ RNA StdSens Analysis system (Bio-Rad). For TrkB, we used the forward primer 5′-CCTGGCATCGTGGCATTTC-3′ and the reverse primer 5′TCTCAGTCCCACATAAGC-TTCAACA-3′. Reverse transcription was performed using ReverTra Ace reverse transcriptase (Toyobo). The amount of mRNA was normalized to that of β-actin, which was used as an internal control.

2.3. EPCs colony-forming units

Methylcellulose semi-solid medium was used to observe EPCs colony formation. $CD133^+$ early endothelial progenitor cells were purified from PB MNCs by positive selection with anti-$CD133^+$ microbeads using a magnetic cell sorter device (Miltenyi Biotec). The early endothelial progenitor cells were added to methylcellulose medium at a 1:10 dilution. A total of 1.1 ml of cell suspension was plated into 35-mm culture dishes with 100 ng/ml BDNF with or without 5 μM the Akt inhibitor triciribine (Kangchen.com, Shanghai). The cells were incubated at 37 °C and 5% CO_2 in a humidified incubator. The cultured cells were photographed with an inverted microscope (Olympus IX51) after 7 days.

2.4. Differentiation of EPCs that were plated onto electrospun collagen material

EPCs that were cultured for 14 days were harvested using 0.25% trypsin–EDTA (Hyclone Laboratories) and labeled using 20 ng/ml calcein (Invitrogen). The labeled EPCs were plated onto the electrospun collagen material and incubated with 100 ng/ml BDNF in basic medium. Labeled EPCs in basic medium alone were used as controls. The cells were incubated at 37 °C and 5% CO_2. After 7 days, the EPCs that were plated onto electrospun collagen material were fixed using 4% paraformaldehyde. A polyclonal anti-CD31 antibody (R&D Systems, Minneapolis, MN, USA) and a secondary antibody TRITC-IgG (Beyotime) were used to incubate EPCs for 1 h as previously described. A laser-scanning confocal microscope (LSCM) was used to analyze EPCs growth and differentiation on electrospun collagen material.

2.5. The proliferation assay and migration assay of EPCs

EPCs that were cultured at 37 °C and 5% CO_2 in an incubator for 14 days were harvested using 0.25% trypsin-EDTA and plated onto a 96-well plate (Costar) with basic medium, basic medium containing 100 ng/ml BDNF or basic medium containing 100 ng/ml BDNF and 5 μM the Akt inhibitor triciribine. The cells were incubated at 37 °C and 5% CO_2 for 72 h. We added 0.5 μl of 50 μM EdU (RiboBio Co., Ltd.) into each well containing 500 μl of medium for 4 h. The cells were fixed using 4% paraformaldehyde and incubated with 2 mg/ml aminoacetic acid for 5 min with oscillation. The cells were incubated with 100 μl of the penetrant into each well with 10 min of oscillation followed by 100 μl of 1× EdU solution for 30 min. DAPI were used to stain cell nuclei.

EPCs that were cultured at 37 °C and 5% CO_2 for 14 days were harvested with 0.25% trypsin–EDTA and plated into the upper chamber of a 24-well transwell migration insert (pore size: 5 μm). The lower chamber contained basic medium with or without BDNF (100 ng/ml). In another transwell, the Akt inhibitor triciribine (5 μM) and 100 ng/ml BDNF were added to the lower chamber. After 24 h, the cells on the upper side of the membrane were removed, and the cells on the lower side of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. Light microscopy was immediately used to observe the cell morphology, and the number of cells that migrated into the lower chamber was determined.

2.6. The paracrine function of endothelial progenitor cells

After 7 days of culture, 100 ng/ml BDNF with or without 5 μM Akt inhibitor or 0.3 μg/ml VEGFR-2 antibody (R&D Systems, Minneapolis, MN) were added to endothelial cells. The media were replaced with fresh media after 12 h incubation, and the culture was incubated for another two days. The cell culture supernatant from each group was aspirated, and the VEGF levels in the supernatant were determined using ELISA. Endothelial progenitor cell culture supernatant was incubated with endothelial

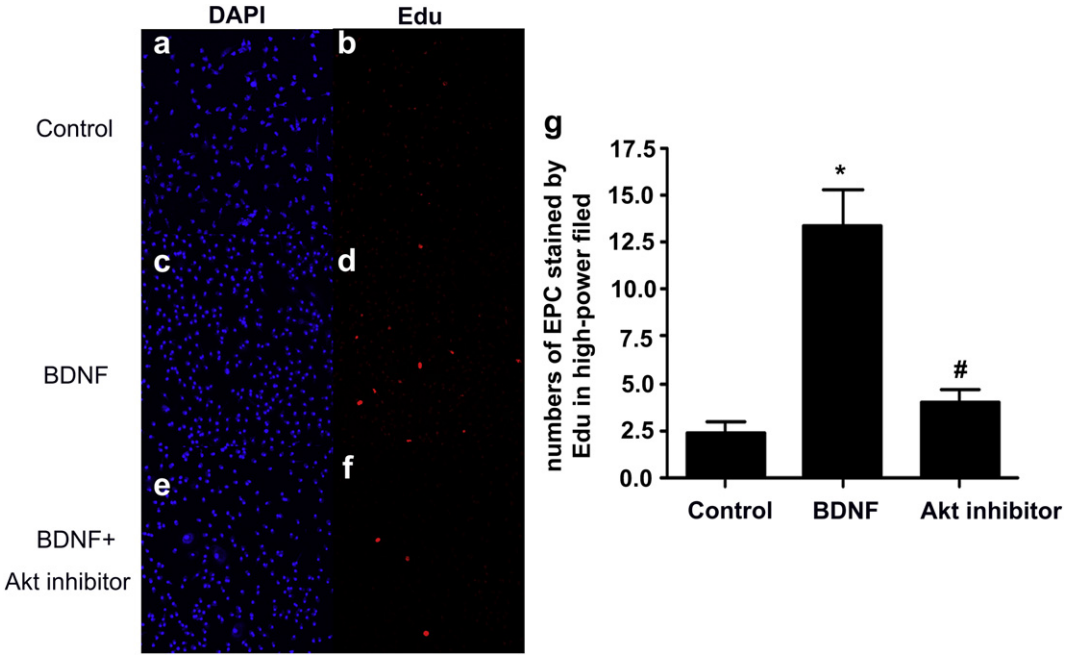

Fig. 2. Edu incorporation reflected the proliferation of EPCs cultured for 14 days under the activation of BDNF. (a, b) DAPI (blue) was used to stain nucleus and Edu (red) was incorporated into EPCs which were proliferating in control group (orig. mag. × 100). (c, d) DAPI (blue) was used to stain nucleus and Edu (red) was incorporated into EPCs which were proliferating in BDNF group (orig. mag. × 100). (e, f) DAPI (blue) was used to stain nucleus and Edu (red) was incorporated into EPCs which were proliferating in BDNF and Akt inhibitor group (orig. mag. × 100). (g) The number of EPCs stained by Edu in per high-power filed in each group. $^*p < 0.05$ ($n = 10$) versus control, $\#p < 0.05$ ($n = 10$) versus BDNF-treated EPCs. Values are mean ± SE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cells that had been pre-seeded into a 96-well plate and incubated at 37 °C and 5% CO_2 for 72 h. The proliferation status of the endothelial cells was determined using the MTT method. A scratch test was used to observe endothelial cell migration that was induced by the supernatant containing EPCs-derived paracrine factors.

2.7. The mobilization of C57BL/6 mouse endothelial progenitor cells after carotid artery wire injury

Wire injury in the carotid artery was performed after anesthesia in 8-week-old C57BL/6 mice. The day after injury, each mouse of the experimental group was intraperitoneally injected with 25 μg/kg BDNF each day for 3 days [26]. In the control group, the same volume of physiological saline was injected. On day four, the peripheral blood was isolated from each mouse and the mononuclear cell layer was extracted. PE-CD34 (eBioscience) and APC-VEGFR-2 (VEGFR-2; R&D Systems, Minneapolis, MN) were used to label cells. The number of PE-CD34/APC-VEGFR-2-positive cells was determined using flow cytometry. Serum VEGF, SDF, and IL-8 levels in injured mice were determined using a VEGF-ELISA kit (R&D Systems, Minneapolis, MN), SDF-1-ELISA kit (Uscn Life Science & Technology Company, USA) and IL-8-ELISA kit (Jingmei Biotech), respectively.

2.8. Endothelial progenitor cell homing

$CD133^+$ cells that were selected using $CD133^+$ magnetic beads were incubated with 100 ng/ml BDNF for 18 h before they were labeled with 20 ng/ml CM-Gil

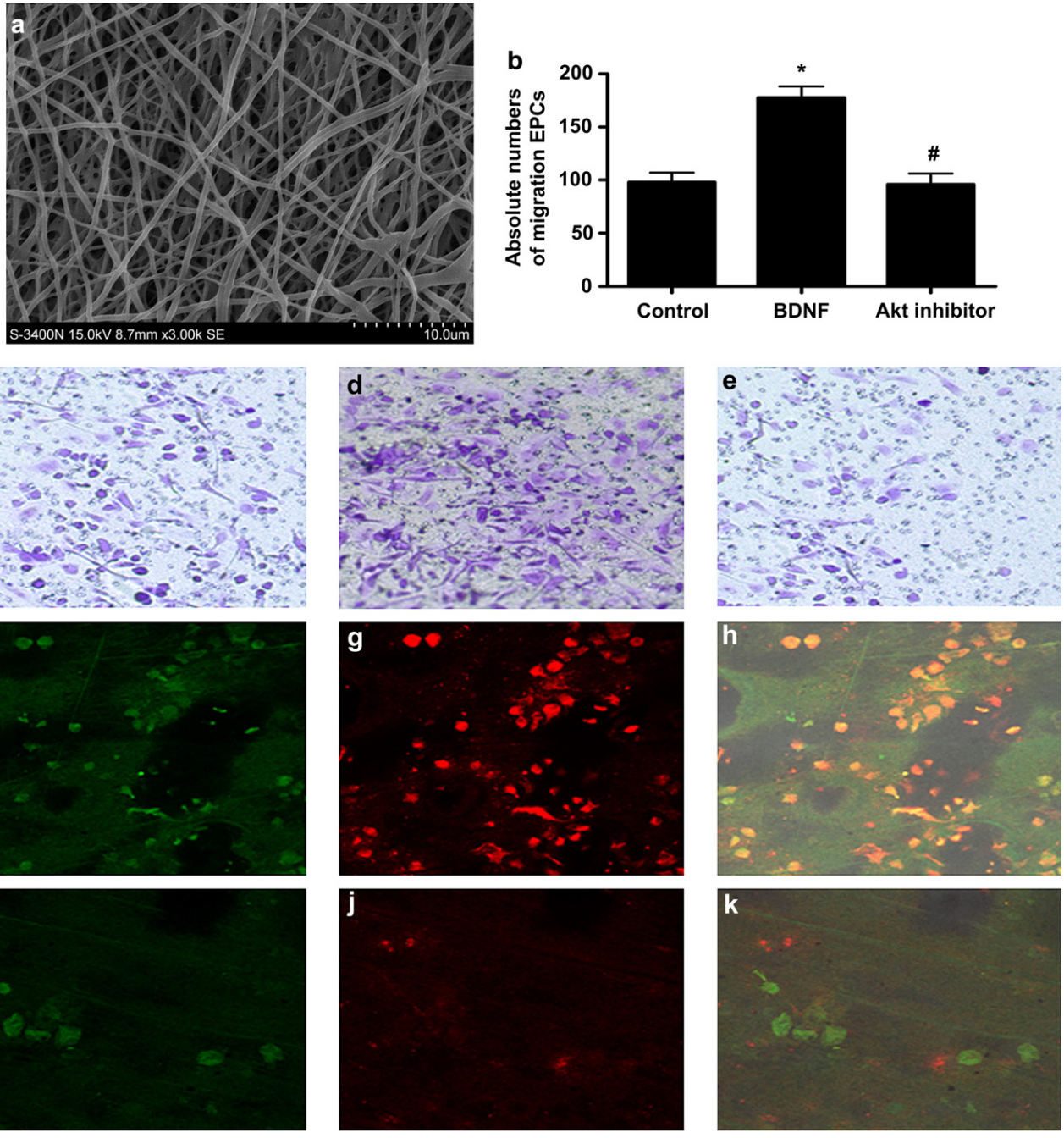

Fig. 3. BDNF-induced EPCs proliferation, migration and differentiation on material. (a) SME showed the structure of electrospun collagen material. (b) The absolute number of EPCs migrating to the lower chamber in each group. $^*p < 0.05$ ($n = 10$) versus control, $\#p < 0.05$ ($n = 10$) versus BDNF-treated EPCs. (c–e) The medium in the lower chamber contained BDNF or BDNF and Akt inhibitor. After 24 h, EPCs migrating from the upper chamber to the lower chamber were stained by crystal violet in each group (orig. mag. × 100). (f–h) 4×10^3 EPCs cultured for 14 days were digested and labeled by calcein (green) and plant on collagen electrospinning material. After 7 days with BDNF inducing, EPCs immunostained for CD31 (red) (orig. mag. × 100). (i–k) EPCs on collagen electrospinning material without BDNF inducing as control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Invitrogen) and 20 ng/ml calcein (Invitrogen). The cells were injected into the tail veins of C57BL/6 mice following wire injury for 3 days. The mice were divided into a BDNF group and a control group with 10 mice in each group. At the same time, cyclosporin was peritoneally injected at a concentration of 4 mg/kg (Novartis Pharma Stein AG). Two days later, the mice were sacrificed. The injured carotid artery was excised, fixed, mounted and observed under the LSCM to examine the endothelial progenitor cells.

2.9. The preparation and grafting of BDNF-coupled tissue-engineered blood vessels

Under sterile conditions, the carotid artery of a Wistar rat (200 g) was dissected and washed with physiological saline to remove the blood. The artery was digested using 0.05% trypsin for 40 min at 37 °C with 5% CO_2. Nucleic acids and fatty acids were removed using treatments with RNase, DNase and lipase, respectively, to obtain acellular and extracellular matrix-depleted blood vessel matrix material that retained vascular collagen and elastic fibers [27]. The prepared vascular matrix material was incubated with 4 mg/ml collagen (Kensey Nash; Exton, PA, USA) solution for 24 h, and the collagen-incubated vascular matrix material was coupled with 5 mM EDC (Sigma–Aldrich, St. Louis, MO, USA) for 24 h. The vascular matrix material was washed 3–5 times with PBS, and the collagen incubation status of the blood vessel inner surface was examined using scanning electron microscopy. Using the methods of a previous study [25], the collagen-coupled vascular matrix material was incubated with 2 mg/ml of N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP, Pierce) solution for 2 h and washed three times with PBS. The vascular matrix material was incubated in 10 mg/ml DTT (Beyotime) solution for 30 min followed by three washes with PBS. The vascular matrix material was incubated in a solution containing 2% (v/v) SPDP (2 mg/ml) and BDNF (500 ng/ml) for 24 h and washed three times with PBS [28]. A stretched preparation of TEBV was fixed and incubated with 10 mg/ml BDNF antibody (Biosynthesis Biotechnology Co., Ltd.) at 4 °C overnight. The following day, the stretched preparation was washed three times with PBS. The secondary antibody was added to the stretched preparation and incubated for 30 min at 37 °C. The sections were washed three times with PBS, and the sections were mounted.

The constructed BDNF-coupled tissue-engineered blood vessels and control blood vessels were grafted to the carotid arteries of 200 g Wistar rats that were anesthetized with 1% pentobarbital sodium (Sigma). The rats were divided into two groups containing ten rats each. 8 weeks after surgery, the rats were anesthetized using 0.3% pentobarbital sodium. The blood flow in the carotid artery with grafted tissue-engineered blood vessels or the healthy carotid artery on the other side of the same rat was measured using a Doppler flowmeter. HE staining was performed on frozen sections of BDNF-coupled TEBV and control TEBV. Weigert staining was used to stain the elastic fibers of TEBV. The grafted tissue-engineered blood vessels were dissected and sectioned for slides. Anti-VEGFR-2 and anti-CD31 antibodies were used to fluorescently label the cells, which were observed using LSCM.

2.10. Shear stress experiments in the parallel-plate flow chamber

The parallel-plate flow chamber that was used in the present study has been previously described by others [29] and by us [30]. The following chamber dimensions were used: width, $w = 16$ mm and height, $h = 0.5$ mm. The volume flow rate was $Q = 0.103$ ml/s, the circulating fluid viscosity was $\mu = 1.3$ m Pa s, and the wall shear stress was $\tau = 6 \times Q \times \mu/(w \times h^2) = 0.2$ Pa. Collagen that was coupled with BDNF via SPDP and collagen itself were used to coat slides and cross-linked under UV for 4 h. The slides were placed into the parallel-plate flow chamber. The circulating fluid comprised the EPC suspension. The EPC suspension flowed over the collagen slides at 0.01 ml/min. The length of time for EPCs that rolled on the slides in the same high-power field and the number of EPCs that adhered to slides were determined using an inverted microscope (Olympus IX51). The EPC suspension was pre-incubated with 10 μg/ml TrkB-blocking peptide (BioVision) for 2 h before flowing over slides.

2.11. Statistical methods

The homogeneity of the variance test and t test of the experimental data were performed using SPSS12.0 software. $p < 0.05$ was considered statistically significant. The experimental data were expressed as the mean ± standard deviation ($m \pm s$).

3. Results

3.1. BDNF-induced colony-forming units and the paracrine function of EPCs

Gel electrophoresis showed that the EPCs that were cultured in the medium containing BDNF for 14 days expressed TrkB (Fig. 1a). We used a methylcellulose semi-solid culture method to observe the effects of BDNF on single clone formation of early EPCs. The

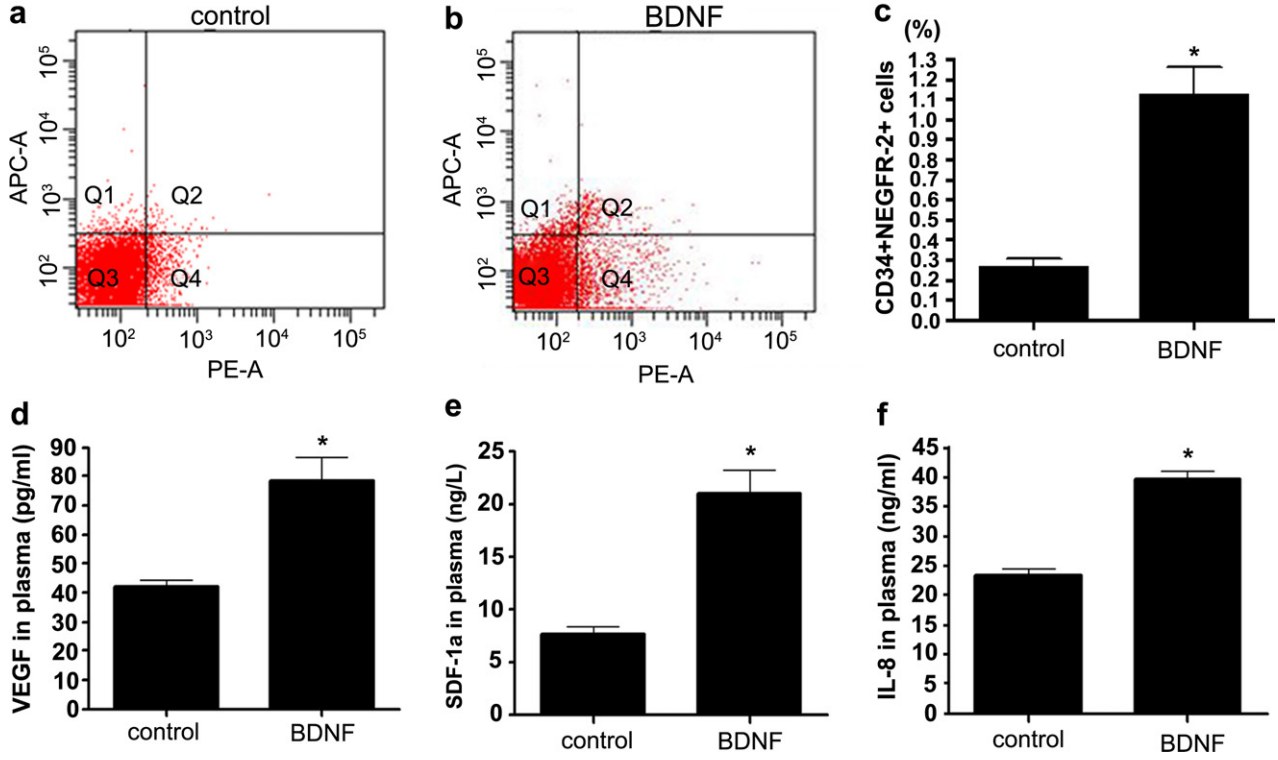

Fig. 4. BDNF-stimulated EPCs mobilization in C57BL/6 mice and the upregulation of three important molecules in serum. (a, b) C57BL/6 mice PB was collected on day 3 after injury and injections of saline, BDNF, and subjected to FACS analysis. EPCs were labeled with PE-CD34/APC-VEGFR-2. (c) The frequencies of $CD34^+/VEGFR\text{-}2^+$ EPCs in each group. $^*p < 0.05$ ($n = 10$) versus control group. Values are mean ± SE. (d) The VEGF concentration in the serum of C57BL/6 mice injured by wire and injection of BDNF for 3 days was determined by ELISA. $^*p < 0.05$ ($n = 10$) versus control. Values are mean ± SE. (e) The SDF-1 concentration in the serum of C57BL/6 mice injured by wire and injection of BDNF for 3 days was determined by ELISA. $^*p < 0.05$ ($n = 10$) versus control. Values are mean ± SE. (f) The IL-8 concentration in the serum of C57BL/6 mice injured by wire and injection of BDNF for 3 days was determined by ELISA. $^*p < 0.05$ ($n = 10$) versus control. Values are mean ± SE.

results show that seven days after culture, the number of colony-forming units of EPCs in the BDNF-treated group is significantly higher than that in the control group (Fig. 1b). The concomitant treatment of cells with an Akt inhibitor inhibited BDNF-induced EPCs colony formation. These results indicate that BDNF promotes colony formation of early EPCs via the Akt pathway.

We cultured EPCs for seven days and treated them with BDNF for 12 h. The paracrine function of endothelial progenitor cells was examined using ELISA. We found that the concentration of VEGF in BDNF-treated culture supernatant was increased by 2-fold compared to that in the control supernatant. The addition of an Akt inhibitor with BDNF significantly decreased the amount of VEGF (Fig. 1c). When the BDNF-stimulated EPCs culture supernatant was used to culture endothelial cells, the proliferation of endothelial cells was significantly increased. However, this effect disappeared after blocking VEGFR-2 (Fig. 1d). The scratch test showed that BDNF induced the migration of endothelial cells in an Akt-dependent manner (Fig. 1e–h).

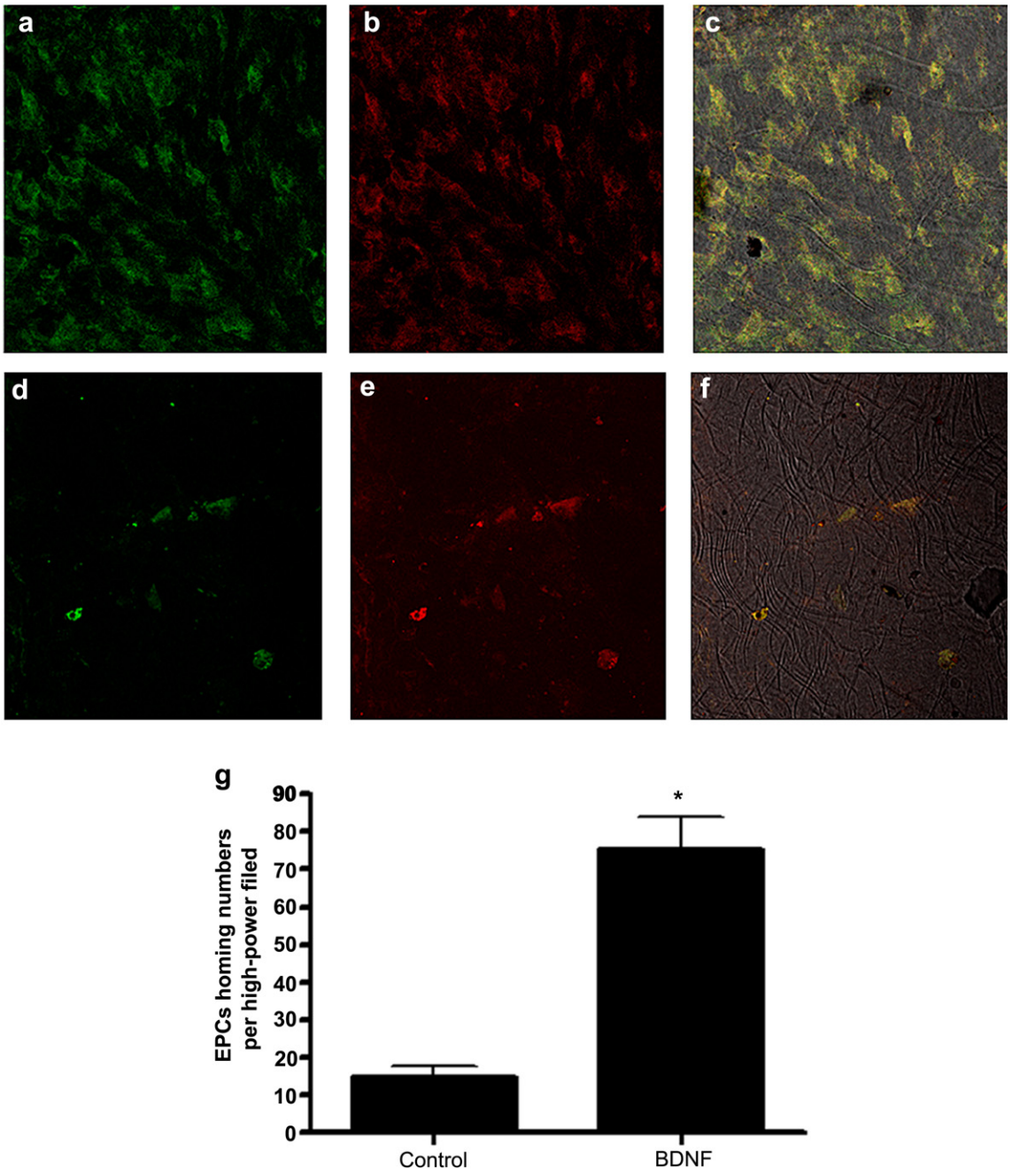

Fig. 5. BDNF facilitated EPCs homing to carotid arterial injury by wire. $CD133^+$ progenitor cells purified by positive selection with anti-$CD133^+$ microbeads from PB MNCs were incubated with BDNF or not for 18 h at 37 °C and labeled with CM-Dil (red) and calcein (green) before injection into C57BL/6 mice. EPC homing to carotid arteries were determined by LSCM analysis. (a, b) Images showing positive staining for CM-Dil (red) and calcein (green), respectively, in the BDNF-treated group (orig. mag. × 200). (c) Image showing merge of (a) and (b) (orig. mag. × 200). (d, e) Images showing positive staining for CM-Dil (red) and calcein (green), respectively, in the control group (orig. mag. × 200). (f) Image showing merge of (d) and (e) (orig. mag. × 200). (g) The number of EPCs homing to carotid arteries per high-power field in each group. $^*p < 0.05$ ($n = 10$) versus control. Values are mean ± SE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. The effect of BDNF on the proliferation, migration and differentiation of late endothelial progenitor cells

EdU incorporation reflects the proliferation of EPCs that were cultured for 14 days of BDNF stimulation. We found that the proliferation of EPCs that were cultured in the presence of BDNF for 14 days was significantly increased, whereas the proliferation was decreased following inhibition of the Akt pathway (Fig. 2). Similarly, transwell experiments confirmed that BDNF-stimulated EPCs exhibited increased migration that was 1.8-fold higher compared to the control EPCs. However, after the addition of an Akt inhibitor, BDNF-induced EPCs migration was significantly reduced (Fig. 3b–e).

We labeled 14-day cultured endothelial progenitor cells with calcein, plated them onto electrospun collagen material and incubated the cells with BDNF to induce their differentiation. Seven days later, significantly more BDNF-stimulated EPCs had differentiated into endothelial cells compared to the control group (Fig. 3f–k).

3.3. BDNF-induced mobilization of EPCs after wire injury in C57BL/6 mice

Flow cytometry analysis demonstrated that the mobilization of C57BL/6 mouse EPCs increased after BDNF treatment. The numbers of $CD34^{+}$/VEGFR-2^{+} cells in the BDNF group were approximately 5-fold greater than those in the control group (Fig. 4a–c). This result indicates that BDNF promotes endothelial progenitor cells to mobilize from the bone marrow to the peripheral blood in C57BL/6 mice following wire injury.

We examined plasma VEGF, SDF-1 and IL-8 levels in C57BL/6 mice after wire injury in the BDNF and control groups. We demonstrated that VEGF, SDF-1 and IL-8 levels were significantly higher in the BDNF group compared to those in the control group (Fig. 4d–f).

3.4. BDNF-induced endothelial progenitor cell homing

To observe the effect of BDNF on EPCs homing, we used BDNF to treat EPCs that had been labeled with CM-Dil (red) and calcein (green) before injecting them into wire-injured C57BL/6 mice through the tail vein. Three days later, the attachment of EPCs in the injured carotid artery was observed using LSCM. We found that the number of EPCs in the BDNF group was 5-fold higher than that in the control group (Fig. 5). This result indicates that BDNF promotes endothelial progenitor cell homing in wire-injured C57BL/6 mice.

3.5. BDNF-induced enhancement of EPC adhesion on collagen in the parallel-plate flow chamber

The collagen was coupled with BDNF using SPDP. We used the parallel-plate flow chamber to simulate the TEBV lumen captured EPCs in circulation in vivo. The white and round cells in Fig. 6a–c were EPCs that were captured by the collagen following circular flow in the parallel-plate flow chamber. The number of EPCs adhering to the collagen that was coupled with BDNF was approximately 3-fold greater than that on collagen alone. When EPCs were treated with a TrkB-blocking peptide, the number of EPCs that adhered to BDNF-coupled decreased significantly (Fig. 6d). The time of EPCs rolling on the slides through the same

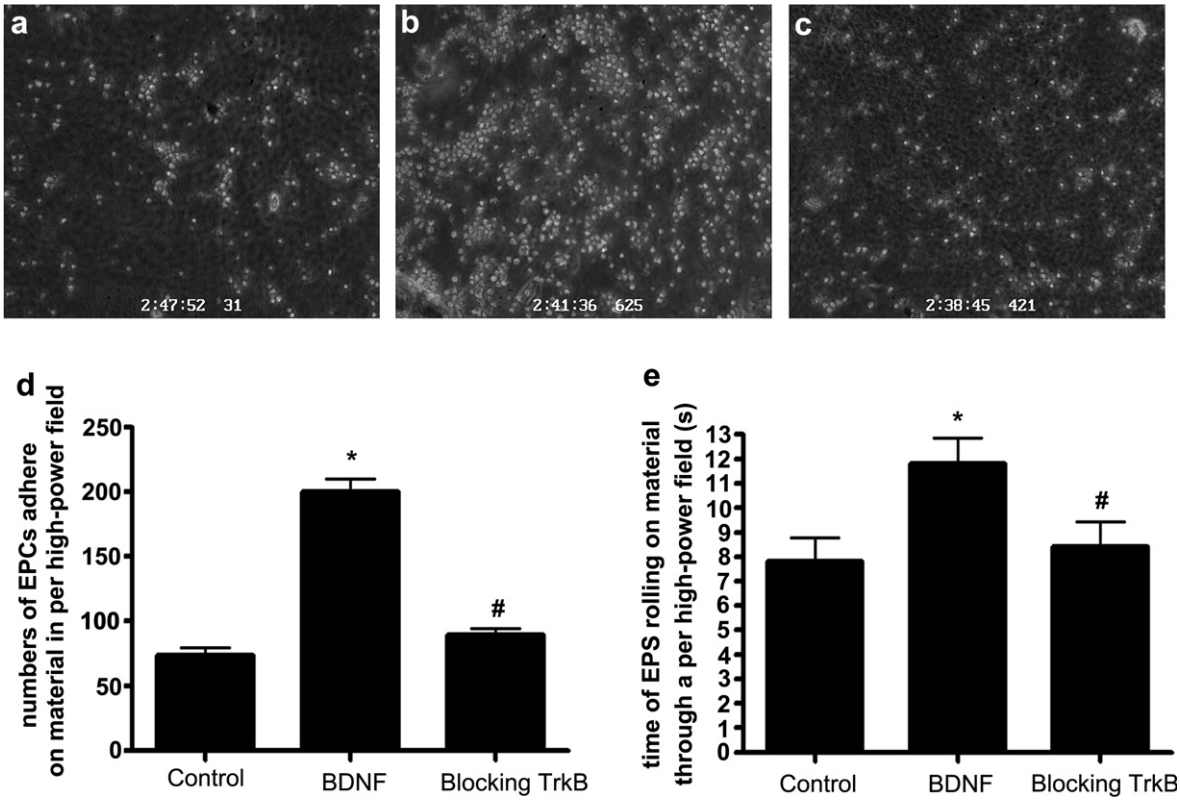

Fig. 6. Collagen coupled with BDNF enhanced EPCs adhesion in parallel-plate flow chamber. (a–c) White and round cells are EPCs captured on collagen itself, collagen coupled with BDNF and collagen coupled with BDNF but EPCs treated by TrkB-blocking peptide respectively. (d) The number of EPCs adhering on the materials. $^{*}p < 0.05$ ($n = 10$) versus control, $\#p < 0.05$ ($n = 10$) versus collagen coupled with BDNF. Values are mean ± SE. (e) The time of EPCs rolling on the materials through a same length in a high-power field. $^{*}p < 0.05$ ($n = 10$) versus control, $\#p < 0.05$ ($n = 10$) versus collagen coupled with BDNF. Values are mean ± SE.

length in the BDNF group was longer than that in the control group. Similarly, the time was shortened when EPCs were treated with the TrkB-blocking peptide (Fig. 6e). The results indicate that TEBV lumen covering the BDNF-coupled collagen captured more EPCs in circulation.

3.6. The positive effect of BDNF on the patency rate of tissue-engineered blood vessels

The prepared vascular matrix material was incubated with collagen after decellularization. SEM showed the vascular matrix material before or after incubation with collagen (Fig. 7a and b). A stretched preparation of TEBV was immunostained for BDNF and observed under the fluorescence microscope to determine the volume of BDNF that was coupled to the TEBV lumen. We observed that BDNF almost completely covered the surface area of TEBV lumen (Fig. 7c and d). We grafted the BDNF-modified TEBV (diameter 1.5 mm) onto the rat carotid artery. Eight weeks later, we found only one unobstructed vessel in the control group, whereas nine vessels were unobstructed in the BDNF-modified group. HE staining showed the average patency rates of each group, and Weigert staining showed that the elastic fibers on BDNF-modified TEBV maintained their integrity (Fig. 8a–h). The Doppler flow-meter analysis showed that the average blood flow of BDNF-modified tissue-engineered blood vessels was 4.98 ml/min, whereas the average blood flow of tissue-engineered blood vessels in the control group was only 0.46 ml/min (Fig. 8i).

Frozen sections from incompletely embolized control TEBV and BDNF-modified TEBV were prepared. The endothelial cells were stained with an anti-CD31 antibody. We found that the endothelialization of BDNF-modified TEBV was higher than that in the control group (Fig. 9a and b). Smooth muscle cells were stained with an anti-SM-actin antibody and showed that BDNF-modified TEBV formed a relatively complete smooth muscle cell layer without pathological proliferation, whereas control TEBV did not exhibit a complete smooth muscle layer (Fig. 9c and d).

Using confocal microscopy, we observed that BDNF-modified TEBV had a significantly higher level of endothelialization compared to control TEBV. The average number of endothelial cells in the BDNF group in a high magnification field was approximately five times greater than that in the control group (Fig. 9e–k). SEM showed the endothelialization of BDNF-modified TEBV by 2

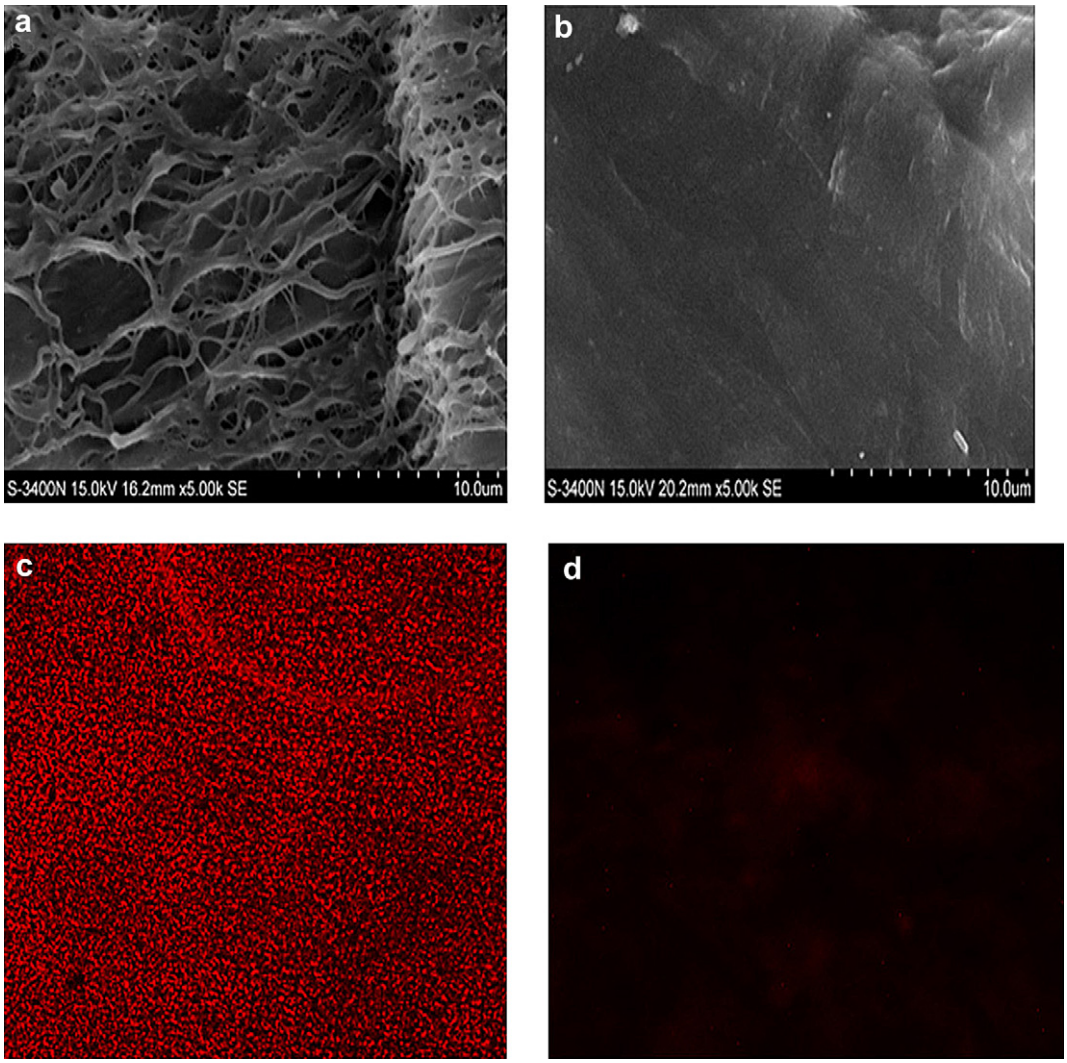

Fig. 7. Construction of TEBV. (a) Wistar rat carotid arteries were obtained and decellularized by enzymatic digestion. No cells on the vessel. (b) Collagen was incubated on the luminal surface of decellularized vessels before cross-linked with EDC and then treated with SPDP and DTT, bound to 2% (v/v) 2 mg/ml SPDP-conjugated 500 ng/ml BDNF protein for 24 h. (c) Stretched preparation of BDNF-modified TEBV was immunostained for BDNF (red). (d) Stretched preparation of control TEBV was also immunostained for BDNF. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

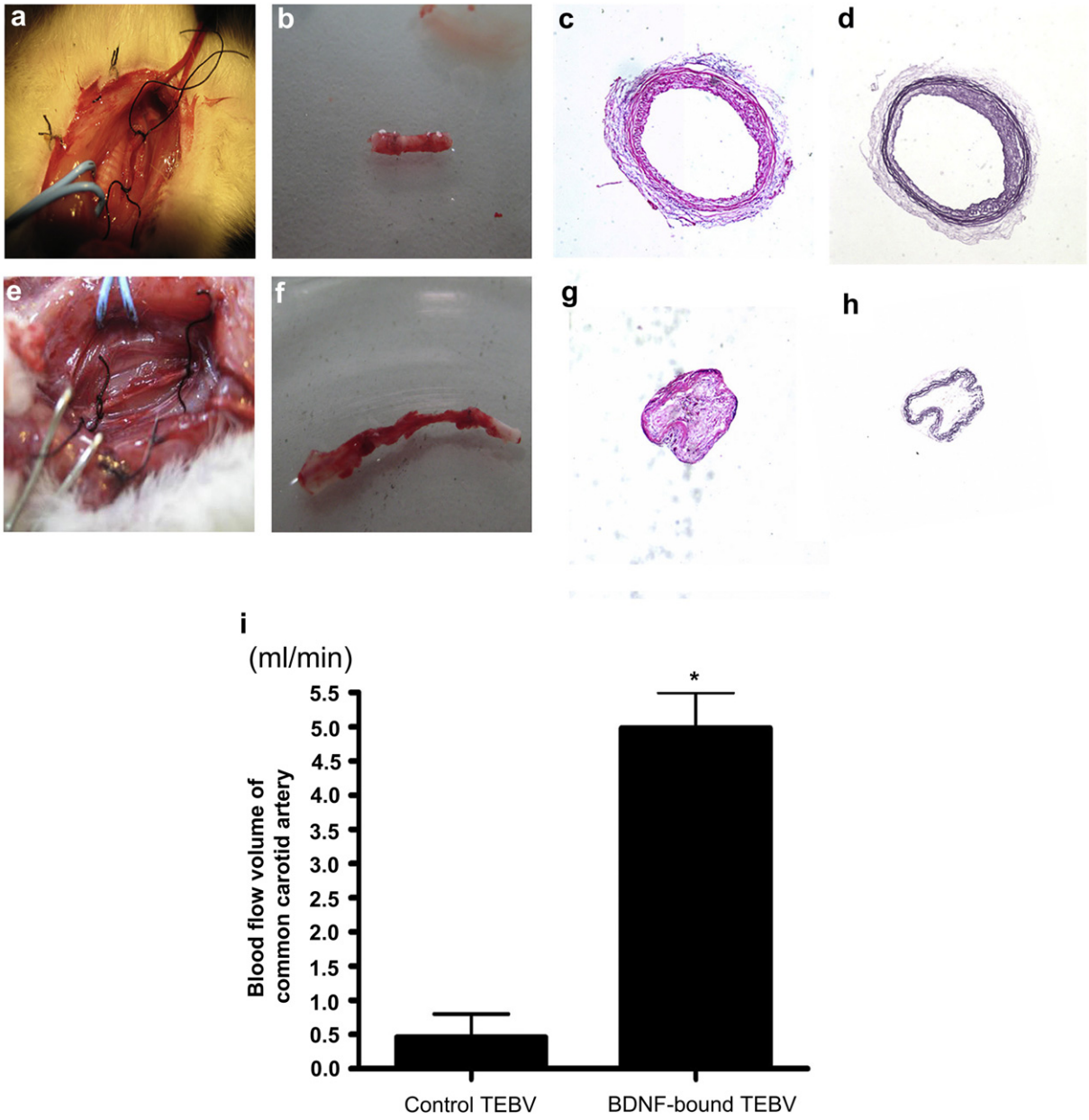

Fig. 8. BDNF up-regulated the patency rate of TEBV in vivo. (a, b) BDNF-bound TEBV graft kept open at 2 month after being transplanted into rat. (c) HE staining of BDNF-bound TEBV graft for 2 month. (d) Weigert staining for elastic fibers on BDNF-bound TEBV. (e, f) Control TEBV graft was occluded at 2 month after being transplanted into rat. (g) HE staining of control TEBV graft for 2 month. It had occluded and emerged thrombus in the luminal. (h) Weigert staining for elastic fibers on control TEBV.(i) Blood flow volume of common carotid artery determined by Doppler at 2 month after being transplanted into rat in control group and BDNF-bound TEBV group. $^{*}p < 0.05$ ($n = 10$) versus control. Values are mean ± SE.

months after implantation (Fig. 9l). Therefore, BDNF-modified TEBV formed a relatively complete endothelial cell layer, and its patency rate was significantly higher than that of the control group.

4. Discussion

Both coronary artery bypass and carotid atherosclerosis substitution treatment require small-diameter TEBV [31,32]. BDNF has a protective effect on central nerve system damage and ischemia. Furthermore, BDNF has an important function in nerve growth and synapse plasticity [25,33]. Studies have shown that BDNF plays a role in maintaining endothelial cell survival and inducing new blood vessel formation in human tissue and organs [21]. BDNF mainly acts on the heart, skeletal muscle, large arteries and other specific organs and tissues during blood vessel formation and mediates the physiological regulation of the cardiovascular system. BDNF promotes endothelial cell survival and induces neoangiogenesis in ischemic tissues. Unlike many vascular growth factors that act on different vascular beds, BDNF activity is relatively restricted to central arteries, vessels of the cardiac and skeletal muscle and the skin [20]. BDNF and its receptor TrkB play an important role in cardiac remodeling after myocardial infarction [34]. Our study showed that normal and BDNF-treated EPCs expressed TrkB [35]. Based on the important function of BDNF in cardiovascular system, we studied its function on EPCs in the current study.

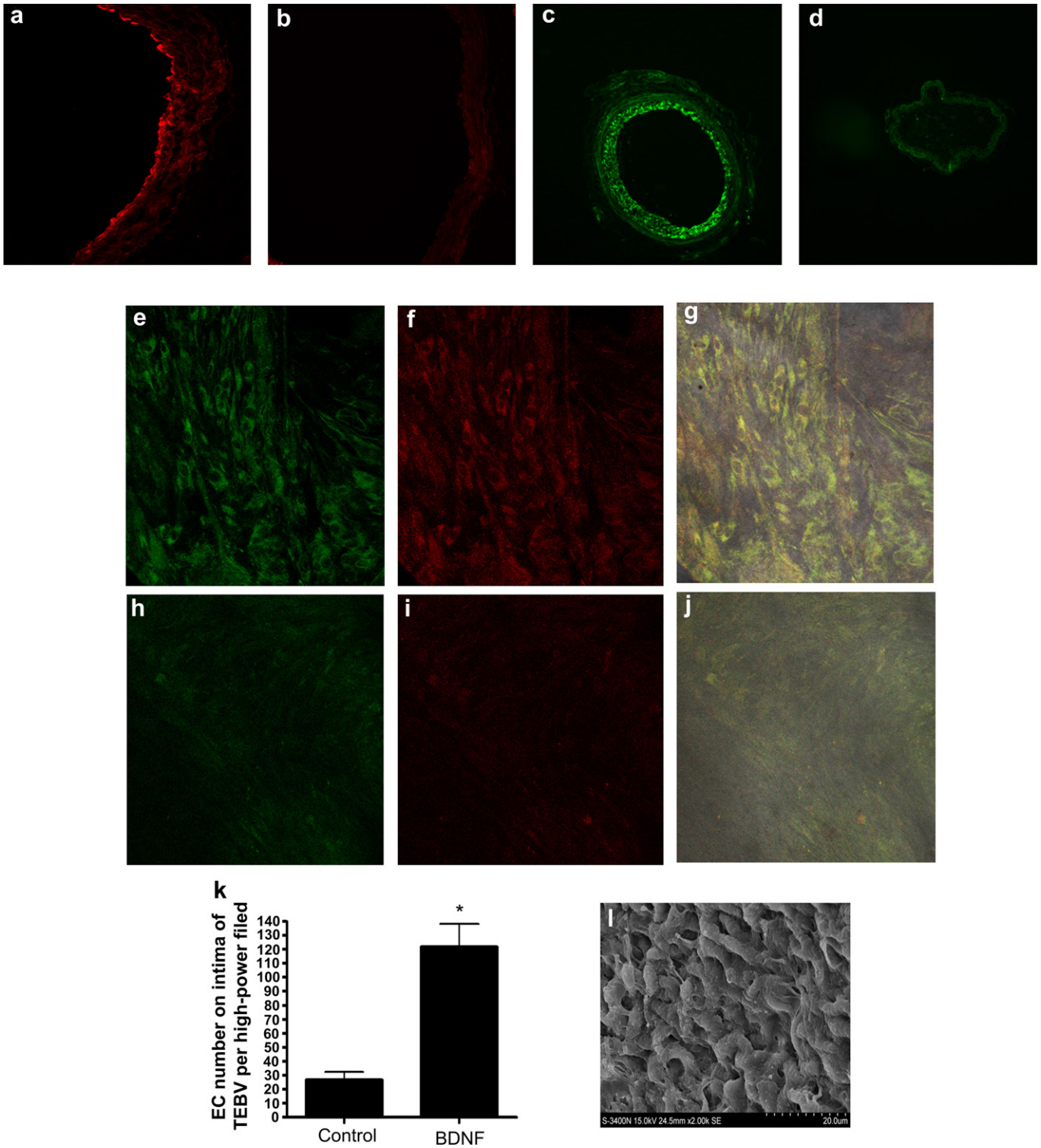

Fig. 9. BDNF promoted endothelialization of TEBV graft transplanted into rats and promoted the formation smooth muscle cells layer. (a) Endothelial cells in cryosections were immunostained for CD31 (red) in TEBV bound with BDNF. (b) Endothelial cells in cryosections were immunostained for CD31 (red) in only one open control TEBV. (c) Smooth muscle cells in cryosections were immunostained for SM-actin (green) in TEBV bound with BDNF.(d) Smooth muscle cells in cryosections were immunostained for SM-actin (green) in only one open control TEBV. (e, f) Endothelial cell growth on the luminal surface of BDNF-bound TEBV 2 month after transplantation. The image shows positive immunostaining for VEGFR-2 (KDR) (green) and CD31 (red), respectively. (g) Image showing merge of (e) and (f). (h, i) Endothelial cell grow on the luminal surface of only one open control TEBV 2 months after transplantation. The image shows positive immunostaining for VEGFR-2 (KDR) (green) and CD31 (red), respectively. (j) Image showing merge of (h) and (i). (k) Endothelialization of BDNF-bound TEBV increased significantly compared with controls ($^*p < 0.05$ $n = 10$). Values are mean ± SE. (l) SME showed the endothelialization of BDNF-bound TEBV 2 months after implantation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

EPCs are divided into early EPCs and late EPCs. Early EPCs are mobilized from bone marrow and homed to the peripheral blood vessel injury position, where they can secrete cytokines, such as VEGF, fibroblast growth factor-4, -9, hepatocyte growth factor and interleukin-8, via paracrine activity. These factors promote the homing of EPCs and act on the endothelial cells at the injury site to induce proliferation, migration and repair of the injured endothelium. Late EPCs continue proliferating and express endothelial cell-specific antigens as they differentiate into endothelial cells. We performed methylcellulose semi-solid culture of EPCs that were isolated from $CD133^+$ magnetic beads. At day 7, we examined single clone formation of early EPCs and showed that BDNF effectively promoted single clone formation of early EPCs. We also found that BDNF promoted the secretion of VEGF from early EPCs, promoting local endothelial cell growth via paracrine functions. This effect was suppressed following inhibition of VEGFR-2.

We used a differential adherent method to culture late EPCs and found that BDNF promoted the proliferation and migration of late endothelial progenitor cells. BDNF also facilitated the differentiation of EPCs into endothelial cells. We used a C57BL/6 wire-injured carotid artery mouse model to mimic clinical balloon injuries after percutaneous transluminal coronary angioplasty. We demonstrated that BDNF increased mouse EPC mobilization after wire injury. Serological ELISA analysis showed that the expression levels of SDF-1, VEGF and IL-8 were increased. In addition, BDNF promoted EPCs homing to mouse vascular injury sites and differentiation into endothelial cells to repair the injured endothelium. This result indicates that BDNF induces early EPCs mobilization and homing and induces paracrine activity at the injury sites. The paracrine function of EPCs promoted the differentiation of homing cells into endothelial cells and proliferation and migration of endothelial cells around the injury site. To simulate TEBV capturing EPCs in circulation in vitro, we used a parallel-plate flow chamber to observe the capture of EPCs in flow by collagen with or without coupled BDNF. The results show that BDNF-coupled collagen captures more EPCs compared to control collagen and that this effect is suppressed after the EPCs were treated with the TrkB-blocking peptide.

Coronary artery bypass with carotid artery substitution is one of the important means of treating coronary heart disease and some cerebral ischemic diseases [36,37] and has created a large demand for small-diameter TEBV [38]. However, intimal hyperplasia and thrombosis often affect its long-term patency, which limits its clinical applications. BDNF promotes the paracrine function of early EPCs and upregulates the proliferation and migration of late EPCs. In addition, BDNF increases the mobilization and homing of EPC in vivo. Therefore, we studied the effect of BDNF on TEBV patency. The EPCs homing to TEBV differentiate into endothelial cells to repair the injured endothelium and promote the proliferation and migration of surrounding endothelial cells via paracrine activity of EPCs. Furthermore, endothelialization of TEBV prevents thrombosis and intimal hyperplasia, which is important to enhance the patency of TEBV after transplantation. We prepared an acellular tissue-engineered vascular stent, and the surface of the treated stent was incubated with collagen. BDNF was coupled to the inner cavity of the tissue-engineered stent using SPDP, and the stent was grafted to a rat carotid artery. We demonstrated that the BDNF-modified TEBV (diameter = 1.5 mm) had significantly less intimal hyperplasia compared to the control group after two months. The blood flow was significantly higher than that of the control group. In addition, the endothelialization on the vascular cavity surface was significantly increased and did not display thrombosis. We showed that BDNF-modified TEBV formed a complete layer of physiological smooth muscle layer at two months after injury.

This result indicates that BDNF facilitates the endothelialization of TEBV by promoting mobilization, homing and local paracrine function of EPCs and inhibiting intimal hyperplasia and thrombosis, which improves the patency of TEBV. Remarkably, this work provides the potential of "off the shelf" tissue engineering approach with regard to future clinical applications and prompts the influence of nerve may be indispensable factor to construction of TEBV.

5. Conclusion

Brain-derived neurotrophic factor (BDNF) promotes stem cell homing to improve small-diameter TEBV patency rate and may play an important role in the construction of coronary artery bypass substitutes.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2011.09.066.

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