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The promotion of endothelial progenitor cells recruitment by nerve growth factors in tissue-engineered blood vessels☆

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ABSTRACT

Endothelial progenitor cells (EPCs) mobilization and homing are critical to the development of an anti-thrombosis and anti-stenosis tissue-engineered blood vessel. The growth and activation of blood vessels are supported by nerves. We investigated whether nerve growth factors (NGF) can promote EPCs mobilization and endothelialization of tissue-engineered blood vessels. In vitro, NGF promoted EPCs to form more colonies, stimulated human EPCs to differentiate into endothelial cells, and significantly enhanced EPCs migration. Flow cytometric analysis revealed that NGF treatment increased the number of EPCs in the peripheral circulation of C57BL/6 mice. Furthermore, the treatment of human EPCs with NGF facilitated their homing into wire-injured carotid arteries after injection into mice. Decellularized rat blood vessel matrix was incubated with EDC cross-linked collagen and bound to NGF protein using the bifunctional coupling agent N-succinmidyl3-(2-pyridyldit-hio) propionate (SPDP). The NGF-bound tissue-engineered blood vessel was implanted into rat carotid artery for 1 week and 1 month. NGF-bound blood vessels possessed significantly higher levels of endothelialization and patency than controls did. These results demonstrated that NGF can markedly increase EPCs mobilization and homing to vascular grafts. Neurotrophic factors such as NGF have a therapeutic potential for the construction of tissue-engineered blood vessels in vivo.

1. Introduction

Tissue-engineered blood vessels (TEBV) have extensive and important applications in blood vessel replacement therapies associated with vascular bypass or the repair of acute vascular injury [1,2]. However, transplantation leads to high occlusion rates of small diameter (≤6 mm) vascular grafts because of thrombosis and neointimal proliferation in small caliber vascular grafts that lack endothelium [3–5]. Endothelialization of the vascular graft can solve this problem because the formation of the endothelial cell layer of the vascular surface can prevent thrombosis and restenosis [6]. The ideal surface for vascular grafts remains the native endothelium [5]. A tissue-engineered blood vessel can be created by seeding autologous endothelial cells in vitro [7], but this requires lengthy periods of cell culture. Therefore, in vivo endothelialization of vascular grafts after implantation inside the body is a good choice [8,9]. It has been proven that endothelialization of damaged vessels can be promoted by the mobilization, migration and differentiation of endothelial progenitor cells (EPCs) [10]. Following endothelial insult or ischemia, EPCs mobilize from the bone marrow and migrate to sites of damage where they differentiate into cells that replace the apoptotic endothelial cells. This process may promote vascular re-endothelialization and angiogenesis while reducing neointimal proliferation and thrombosis, as well as lowering the possibility of blood vessel stenosis [11]. Thus, factors that induce the mobilization and homing of autologous EPCs in vivo will provide a new opportunity for promoting autologous endothelialization of TEBV and artificial vascular [12,13].

Because the vessels and nerves of living creatures are always concomitant and interdependent, their growth patterns are

☆ This paper is one of a newly instituted series of scientific articles that provide evidence-based scientific opinions on topical and important issues in biomaterials science. They have some features of an invited editorial but are based on scientific facts, and some features of a review paper, without attempting to be comprehensive. These papers have been commissioned by the Editor-in-Chief and reviewed for factual, scientific content by referees.

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similar and they follow the same migration routes. Importantly, vascular activity and nutrition are regulated by nerves. Recent studies indicate that the nervous system plays an important role in the development of the embryonic cardiovascular system through paracrine activity involving neurotrophins such as nerve growth factors (NGF). For example, sensory nerves determine the pattern of arterial differentiation and blood vessel branching [14,15]. However, neurotrophins have not been tested in the engineering of vascular tissue. Therefore, the roles of neurotrophins in constructing complete, functional TEBV are unknown. Neurotrophic factors are important in maintaining the normal function of vascular endothelium. Nerve growth factors (NGF), the most representative of the neurotrophic factors, is a multifunctional polypeptide that can combine with TrkA on endothelial cells to trigger proliferation and migration of endothelial cells, as well as increase the expression of adhesion molecules that promote angiogenesis [16]. It is not clear, however, if NGF can promote EPCs mobilization, homing and endothelialization of TEBV in vivo.

EPCs mobilization and homing is a key event in the development of an anti-thrombotic and anti-stenotic tissue-engineered blood vessel. NGF can repair both blood vessels and nerves. We investigated whether NGF can promote EPCs mobilization and endothelialization of tissue-engineered blood vessels. In order to confirm our hypothesis, we demonstrated the effects of NGF on EPCs mobilization, migration and differentiation in vitro and vivo. Furtherly, we constructed NGF-bound TEBV which was implanted into rat carotid artery to investigate the function of NGF on endothelialization of TEBV. This work may be important for the construction of neural control anti-thrombotic and anti-stenotic TEVB in the future.

2. Materials and methods

2.1. Human cell isolation and culture

Peripheral blood (PB) was obtained from healthy human volunteers. Mononuclear cells (MNCs) were isolated by density-gradient centrifugation (Lymphoprep; Axis Shield) from PB. The MNCs were cultured in M199 medium (Hyclone Laboratories) supplemented with 10% FBS, 10 ng/ml vascular endothelial growth factor (VEGF; R&D Systems, Minneapolis, MN), 3 ng/ml basic fibroblast growth factor (bFGF; Roche Applied Science, Indianapolis, IN, USA), and heparin (90 mg/ml; Sigma, USA) in the presence of penicillin (100 Units/ml), streptomycin (100 mg/ml), and fungizone (0.25 mg/ml) (all purchased from Sangon Com, Shanghai, China) as basic medium, which was similar in our previous study [17]. Nerve growth factor (NGF; 10 μM) (R&D Systems, Minneapolis, MN) [18] was added to the basic medium in the NGF treatment group. The Akt blocking group was treated with 5 μM of the Akt inhibitor triciribine (Kangchen.com, Shanghai) [19] and 10 μM NGF in basic medium. Control cells were cultured in basic medium without supplementation. Non-adherent cells were removed after 24 h, and the adherent cells were maintained in culture. All cells were maintained at 37 °C in a humidified incubator at 5% CO_2 for 14 days. The identity of cultured EPCs was similar to our previous study (data not shown) [17].

2.2. Real-time analysis

Human EPCs were cultured in the three media formulations described above for 14 days. Total RNA was extracted with Trizol (Roche) reagent and the quality of RNA was examined by the Experion™ RNA StdSens Analysis system (Bio-Rad). The forward primer for CD31 was 5′-GACGTGCAGTACACGGAAGTTCA-3′ and the reverse primer was 5′-GACGTGCAGTACACGGAAGTCA-3′. The forward primer for VE-Cadherin was 5′-GCGACTACCAGGACGCTTTCA-3′ and the reverse primer was 5′-CATGTATCGGAGGTCGATGGTG-3′. The forward primer for TrkA was 5′-AGAGTCT TGTTCAGGTCAACGTC-3′ and the reverse primer was 5′-AGAGGCTCT TCAGGTCAACGTC-3′. The forward primer for P75 was 5′-TCAGTGGCATGGCTCCAGTC-3′ and the reverse primer was 5′-GCAGTATCCAGTCTCAGCCCAAG-3′. Reverse transcription was performed with ReverTra Ace reverse transcriptase (Toyobo). Gene expression was studied using the iQ™5 Real Time PCR Detection System (Bio-Rad) with the SYBR Green I detection method. The amount of mRNA was normalized to an internal control (β-actin). The results were analyzed using the Bio-Rad iQ™5 software.

2.3. EPCs colony forming units in methylcellulose semisolid medium

Methylcellulose semisolid medium was divided into three groups. NGF (10 μM) was added to the first group. NGF (10 μM) and the Akt inhibitor triciribine (5 μM) were added to the second group. The third group was left unsupplemented as a control. $CD133^+$ progenitor cells were purified by positive selection with anti-$CD133^+$ microbeads from PB MNCs using a magnetic cell sorter device (Miltenyi Biotec). $CD133^+$ progenitor cells were added to methylcellulose medium at a 1:10 dilution, and 1.1 ml was dispensed into 35-mm culture dishes in triplicate. The cultures were incubated at 37 °C and 5% CO_2 in a humidified incubator without disturbance. Cultured cells were photographed with an inverted microscope (Olympus IX51) after 7 days.

2.4. EPCs migration in transwell chambers

EPCs cultured for 14 days were harvested with 0.25% Trypsin-EDTA (Hyclone Laboratories) and 10^5 cells were loaded into the upper chamber of a 24-well transwell migration insert (pore size: 5 μm). The lower chamber contained basic medium with or without NGF (10 μM). After 24 h, the cells on the upper side of the membrane were wiped away, while cells on the lower side of the membrane were fixed with 4% paraformaldehyde, and stained with crystal violet. In another transwell, the Akt inhibitor triciribine (5 μM) and 10 μM NGF were added to the lower chamber. Cell morphology was immediately determined by light microscopy, and the number of cells migrating into the lower chamber was determined.

2.5. MTT assay

EPCs cultured for 14 days were harvested with 0.25% Trypsin-EDTA and transferred to a 96-well plate. EPCs were cultured in M199 for 3 days. MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] was dissolved in PBS at 5 mg/ml and filter sterilized through a 0.2 μM filter and stored at 2–8 °C. 4 h before the end of the incubation, 20 μl of the MTT solution was added to each well, and the plates were incubated at 37 °C for 4 h. The medium was removed and 150 μl of DMSO was added to each well. After 15 min of oscillation, the plate was placed in an incubator for 5 min to eliminate air bubbles. On a plate reader, the absorbance at a wavelength of 492 nm was measured for each sample.

2.6. Vascular injury, NGF administration, flow cytometry, and ELISA assay

Carotid arterial injury was performed in 8-week-old male C57BL/6 mice. A straight spring wire (0.25 mm diameter) was inserted into the carotid artery and placed there for 3 min [13,20]. This wire injury is reported to remove completely the arterial endothelium. Human NGF (10 nmol/kg/ml) [21], VEGF (10 nmol/kg/ml), NGF and VEGF, or saline were injected intraperitoneally just after arterial injury and once daily for the following 3 days. There were 10 mice in each group. PE-conjugated anti-CD34 (eBioscience) and APC-conjugated anti-VEGF receptor-2 (VEGFR-2; R&D Systems, Minneapolis, MN) monoclonal antibodies were used to analyze the quantity of EPCs in peripheral blood (PB) with a FACSCaliber flow cytometer (Becton Dickinson). The concentrations of stromal cell-derived factor 1α (SDF-1α) and VEGF in the serum of injured C57BL/6 mice were determined using an SDF-1α ELISA Kit (Uscn life science & technology company, USA) and a VEGF ELISA Kit (Jingmei Biotech).

2.7. EPCs homing and differentiation in vivo

$CD133^+$ progenitor cells were purified by positive selection with anti-CD133 microbeads from PB MNCs using a magnetic cell sorter device (Miltenyi Biotec) [13]. $CD133^+$ progenitor cells were incubated with NGF (10 μM) for 18 h and then labeled using 20 ng/ml CM-dil (Invitrogen) and 20 ng/ml calcein (Invitrogen). 5×10^6 labeled EPCs were injected i.v. into C57BL/6 mice 3 days after wire injury. C57BL/6 mice injected with untreated EPCs served as the control group. There were 10 mice in each group. Ciclosporin (4 mg/kg; Novartis Pharma Stein AG) was injected intraperitoneally every day. Recipients were sacrificed 2 days after EPC injection for analysis of cell homing [22]. Injured arteries were harvested and the luminal surface of arteries were exposed and fixed with 4% paraformaldehyde. In a second cohort of treated and control mice, we harvested the injured arteries 14 days after wire injury, and fixed the samples with 4% paraformaldehyde overnight. Fixed samples were then treated with a polyclonal anti-CD31 antibody (10 mg/ml) (R&D Systems, Minneapolis, MN) followed by 10 mg/ml Cy5-conjugated secondary antibody (Beyotime) for 2 h at room temperature. Laser scanning confocal microscope (LSCM) was used to analyze the level of EPCs homing and differentiation.

2.8. Tissue-engineered blood vessel preparation

Fresh adult carotid arteries were obtained from Wistar rats and were digested to rupture the native cells and partially extract cytoplasmic elements and soluble extracellular matrix (ECM). Vascular tissues were then treated repeatedly with enzyme-based solutions to digest nucleic acids and cellular membranes. This multistep process removes nucleic acids, lipids, cellular membranes, cytoplasmic

components, and soluble matrix molecules while retaining the insoluble fibrillar collagen and elastin ECM that is suitable for recellularization [10,11]. The decellularized rat arteries were incubated with 5 mM EDC (Sigma; St. Louis, MO) and cross-linked with comminuted bovine hide 4 mg/ml collagen (Kensey Nash; Exton, PA) for 24 h. The efficiency of collagen incubation was determined by SME. Next, rat arteries were incubated in 10 mg/ml DTT (Beyotime) for 30 min, and then treated with the bifunctional coupling agent N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, 2 mg/ml; Pierce) for 2 h. Arteries were then incubated with 2% (v/v) 2 mg/ml SPDP-conjugated 0.5 mg/ml NGF protein for 24 h [23]. To detect TEBV-bound NGF on the luminal surface, rat arteries were incubated overnight at 4 °C with 10 mg/ml anti-NGF antibody (R&D Systems, Minneapolis, MN) then treated with a secondary antibody (Beyotime) for 30 min after the bifunctional coupling agent N-succinimidyl 3-(2-pyridyldithio) propionate for 1 h at 37°C.

2.9. NGF-treated tissue-engineered blood vessel in vivo

Initially, we used NGF-treated TEBV and control TEBV to transplant into rats, with 10 animals used in each group, for each time period. Wistar rats (8 weeks old, 200 g) were obtained from the Third Military Medical University animal facility. The rats were anesthetized with 1% pentobarbital sodium (Sigma) and placed in a supine position. The left common carotid artery (CCA) was exposed and clamped, and then ligated, and the control graft was placed end-to-end and sutured with 11-0 interrupted stitches under a surgical microscope. The NGF-treated graft was inosculansed into the right common carotid artery of rats. All procedures were approved by the Institutional Animal Care and Use Committee and Institutional Review Board Service at the Third Military Medical University, Chongqing. Experimental animals were followed for up to 1 week and 1 month, after which time the

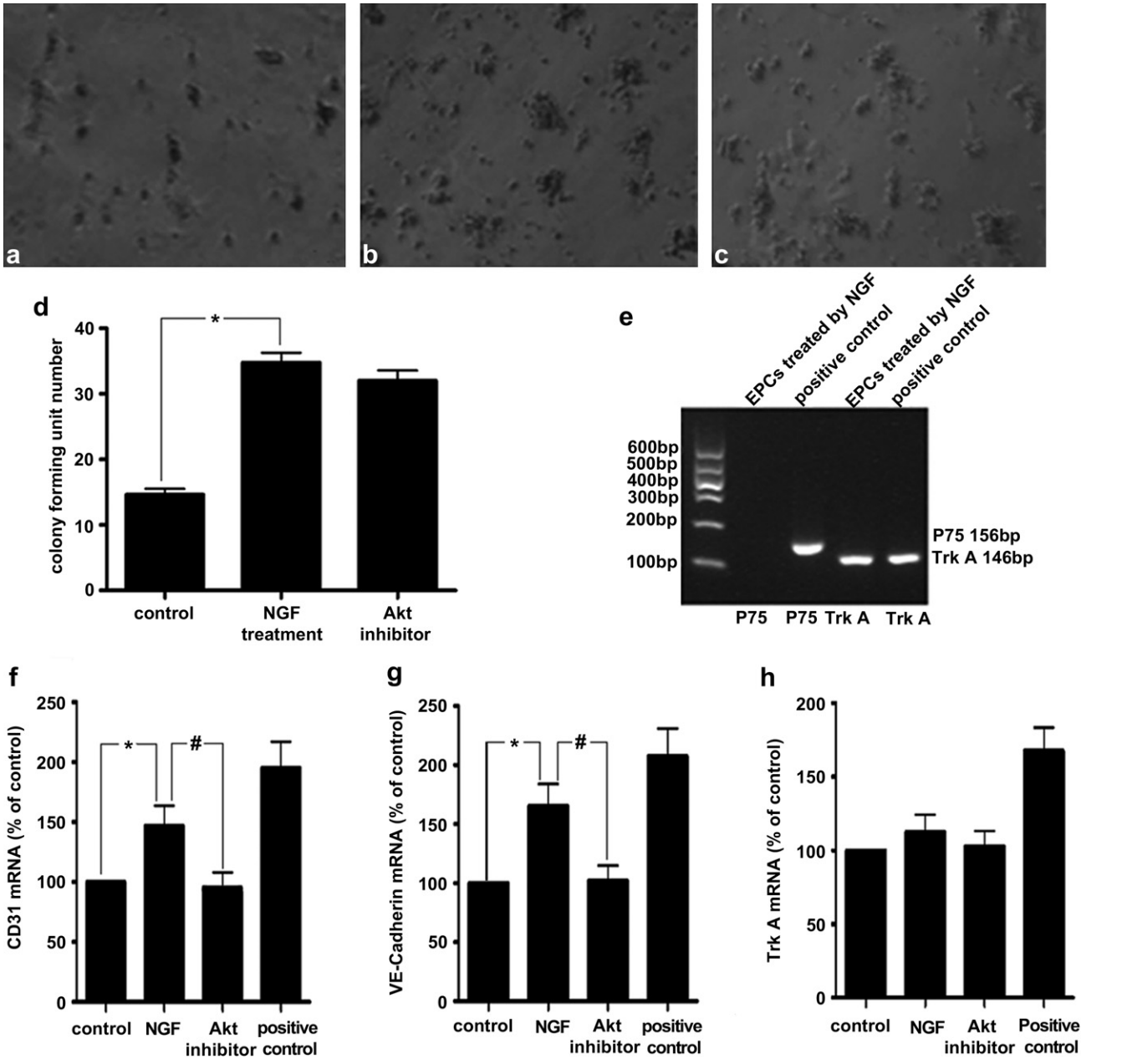

Fig. 1. NGF promoted EPCs to form colony forming units and to express endothelial cell specific markers. (a–c) $CD133^+$ progenitor cells purified by positive selection with anti-$CD133^+$ microbeads from PB MNCs formed colony forming units in methylcellulose semisolid medium on day 7 in each group (orig. mag. ×100). (d) The average number of colony forming units per high-power field in methylcellulose semisolid medium ($^*p < 0.05$ $n = 10$) versus control. Values are mean ± SE. (e) RT-PCR showed that EPCs treated by NGF expressed Trk A not P75. (f) Quantitative real-time PCR data showing the expression of CD31 in EPCs cultured for 14 days in each group. CD31 expression in endothelial cells served as the positive control. $^*p < 0.05$ ($n = 10$) versus control, $\#p < 0.05$ ($n = 10$) versus NGF-treated EPCs. Values are mean ± SE. (g) Quantitative real-time PCR data showing the expression of VE-Cadherin in EPCs cultured for 14 days in each group. VE-Cadherin expression in endothelial cells served as a positive control. $^*p < 0.05$ ($n = 10$) versus control, $\#p < 0.05$ ($n = 10$) versus NGF-treated EPCs. Values are mean ± SE. $^*p < 0.05$ ($n = 10$). (h) Quantitative real-time PCR data showing the expression of TrkA in EPCs cultured for 14 days in each group. There were no significant differences among the three groups.

grafts were explanted for the assessment of histology and function, as assessed by a Doppler TS420 transit time perivascular flowmeter (Transonic Systems Inc, New York, USA). Cryosections were prepared at 10 mm thickness, and a polyclonal anti-CD31 antibody and a secondary antibody TRITC-IgG (Beyotime) were used to incubate endothelial cells as previously described. The level of vessel endothelialization was detected by immunofluorescence of endothelial cells growing on the luminal surface of TEBV. Polyclonal anti-CD31 antibody and murine monoclonal anti-KDR (10 mg/ml; R&D Systems, Minneapolis, MN) antibody along with FITC-IgG and TRITC-IgG secondary antibodies (Beyotime) were used as detailed above. HE staining was made in the frozen sections of NGF-treated TEBV and control TEBV harvested 1 month after transplanting into rats and the degree of endothelialization was determined by SME.

2.10. Statistics

All experimental data are expressed as means ± SE. Each experiment was repeated at least three times. Statistical significance of the differences between two groups was determined using Student's *t* test. All p values were two-tailed, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. NGF on EPCs colony forming units and differentiation

To investigate the effect of NGF on EPCs colony formation, we conducted colony forming unit assays in methylcellulose semisolid medium. There were more colony forming units in the NGF treatment group after 7 days than in the control group (Fig. 1a–c) and the average number of colony forming units per high-power field was 2-fold of that in the control group. However, the Akt inhibitor tribicibine did not affect the number of EPC colony forming units induced by NGF (Fig. 1d), indicating that NGF can promote EPC colony formation, but it does not act through the Akt pathway.

RT-PCR showed that at the 14th day of EPC culture, EPCs treated with NGF expressed TrkA receptor but not the P75 receptor (Fig. 1e). To confirm that NGF stimulates EPCs to differentiate into endothelial cells in an Akt independent manner, we measured the expression of CD31 and VE-Cadherin in EPCs by real-time PCR assay. The expression of CD31 and VE-Cadherin in the NGF-treated group was higher than in controls, whereas cells treated with the Akt inhibitor exhibited decreased expression of the two specific endothelial cells markers. We took the expression in endothelial cells as the positive control. The expression of these markers in the control group was baseline (100%). The expression of CD31 in the NGF-treated group was 147% of the control group, while in the Akt blocking group it was 63% of the NGF-treatment group (Fig. 1f). The expression of VE-Cadherin in the NGF group was 165% of controls, but inhibition of Akt resulted in a reduction to 62% of the NGF group (Fig. 1g). This indicated that NGF can promote EPCs to differentiate into endothelial cells and express specific endothelial surface markers. However, the expression of TrkA in the NGF group was 112% of controls, and Akt inhibition decreased expression to 92% of the NGF group, although there were no significant differences among the three groups (Fig. 1h). Thus, NGF does not influence the expression of TrkA receptor on EPCs.

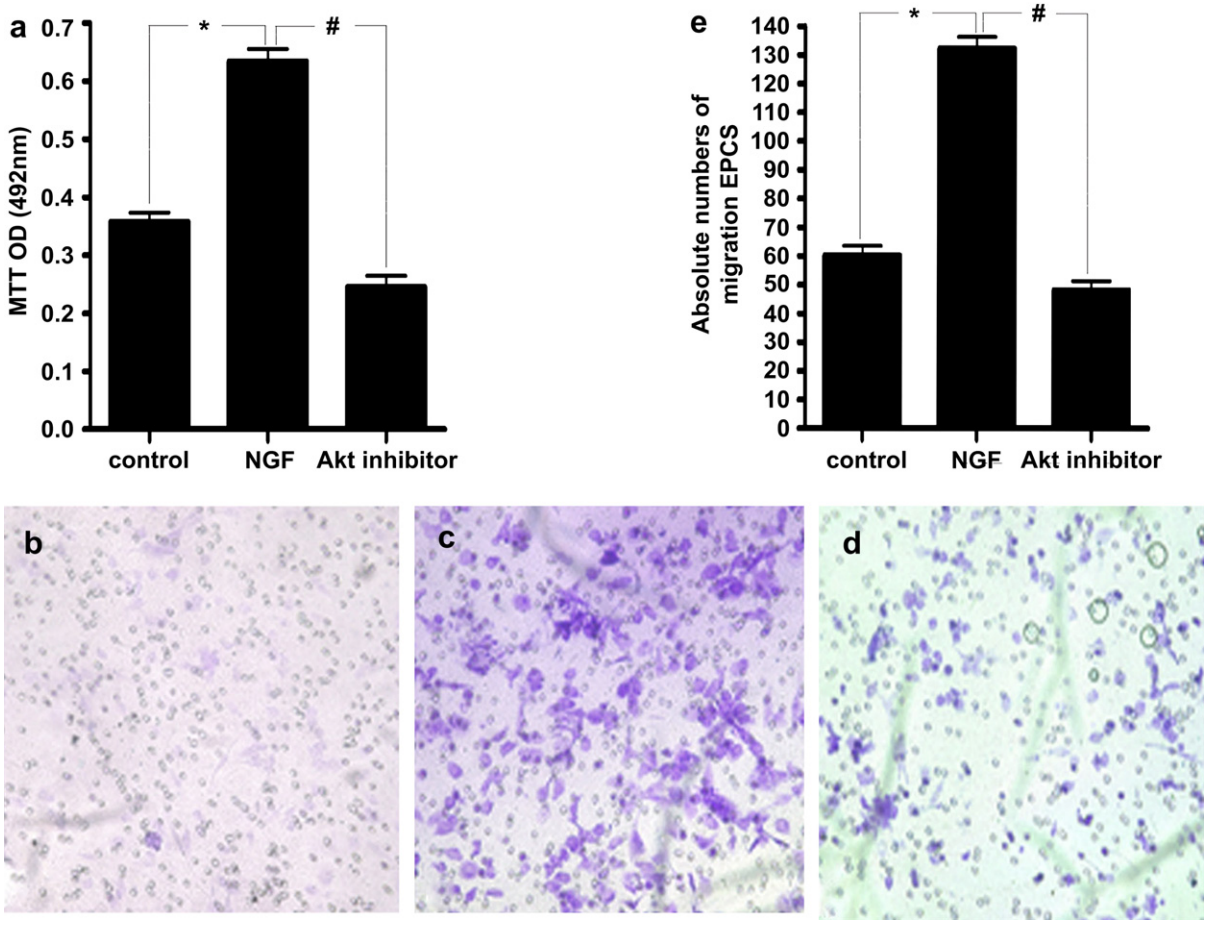

Fig. 2. NGF induced EPC proliferation, migration and tube formation. (a) MTT assay showing the OD value (492 nm) of EPCs cultured for 14 days in each group. *$p < 0.05$ ($n = 10$) versus control, #$p < 0.05$ ($n = 10$) versus NGF-treated EPCs. Values are mean ± SE. (b–d) 10^5 EPCs were placed in the upper chamber of a 24-well transwell migration insert. The medium in the lower chamber contained NGF or NGF and Akt inhibitor (NGF&Akt). After 24 h, EPCs migrated from the upper chamber to the lower chamber in each group (orig. mag. ×100). (e) 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 NGF-treated EPCs.

3.2. The effect of NGF on EPCs proliferation and migration

In a MTT assay, the OD value (492 nm) of the NGF treatment group was 2-fold of that in the control group; in the Akt blocking group, the OD value was approximately 1/3 of the NGF group (Fig. 2a). Therefore, NGF can protect EPC viability and promote their proliferation, but this effect is inhibited by the Akt inhibitor tricirribine, demonstrating that NGF upregulates EPCs proliferation through the Akt pathway.

To determine if NGF can induce EPCs migration, transwell assays were performed. Violet cells represented EPCs that migrated from the upper chamber into the low chamber (Fig. 2b–d). NGF treatment resulted in a 2-fold increase in the number of migrating EPCs compared to controls, but the number of migrating EPCs was reduced to 1/3 by addition of the Akt inhibitor (Fig. 2e). Akt plays a crucial role in NGF-induced migration of EPCs.

3.3. The function of NGF to EPCs mobilization

Early EPCs possess high expression of CD34, but expression declines as EPCs mature and begin to express endothelial cells markers such as VEGFR-2. To confirm that NGF stimulates EPCs mobilization from the bone marrow into the peripheral circulation, C57BL/6 mice were treated with NGF and VEGF or VEGF alone, and EPCs were enumerated in the blood by PE-CD34 and APC-VEGFR-2 staining (Fig. 3a–d). Flow cytometric analysis revealed that the number of $CD34^+VEGFR\text{-}2^+$ EPCs in the peripheral blood of NGF + VEGF-treated mice was approximately 2-fold that in mice treated with VEGF alone. However, treatment with VEGF alone elicited 2-fold more blood EPCs than treatment with NGF alone, although mice in both treatment groups exhibited significantly more blood EPCs than untreated control mice (Fig. 3e). Thus, NGF promotes EPCs mobilization from bone marrow to peripheral blood, but not as robustly as VEGF. Moreover, NGF synergizes with VEGF to promote the mobilization of EPCs.

In addition, we measured the concentrations of SDF-1 and VEGF in the serum of wire-injured mice that were injected with NGF or saline for 3 days. The serum concentration of SDF-1 in NGF-treated mice was approximately 2-fold higher than in controls (Fig. 3f). Similarly, the concentration of VEGF in the serum of the NGF treatment group was also about 2-fold more than controls (Fig. 3g).

3.4. The contribution of NGF to EPCs homing

Wire-induced injury to the mouse carotid artery causes complete removal of endothelium. NGF-treated EPCs and control EPCs were labeled and injected into injured mice. The homing of transferred EPCs to sites of wire injury was observed by LSCM. Cultured EPCs were labeled with CM-Dil (red) and calcein (green) and injected into mice. The number of labeled EPCs treated with NGF per high-power field in sites of wire injury was more than 2-fold higher than controls (Fig. 4), indicating that NGF treatment can facilitate EPCs homing to injured sites. In another two groups harvested at 14 days after injection, immunostaining with anti-CD31 antibody on the luminal surface revealed that there was a significant increase in the number of endothelial cells derived from NGF-treated EPCs than control EPCs (Fig. 5).

3.5. The role of NGF on TEBV endothelialization

We used decellularized rat arteries incubated with collagen and cross-linked with EDC to generate TEBV. SME revealed that collagen had accumulated on the luminal surface of the TEBV (Fig. 6a and b). NGF-bound TEBV and control TEBV were transplanted into rat

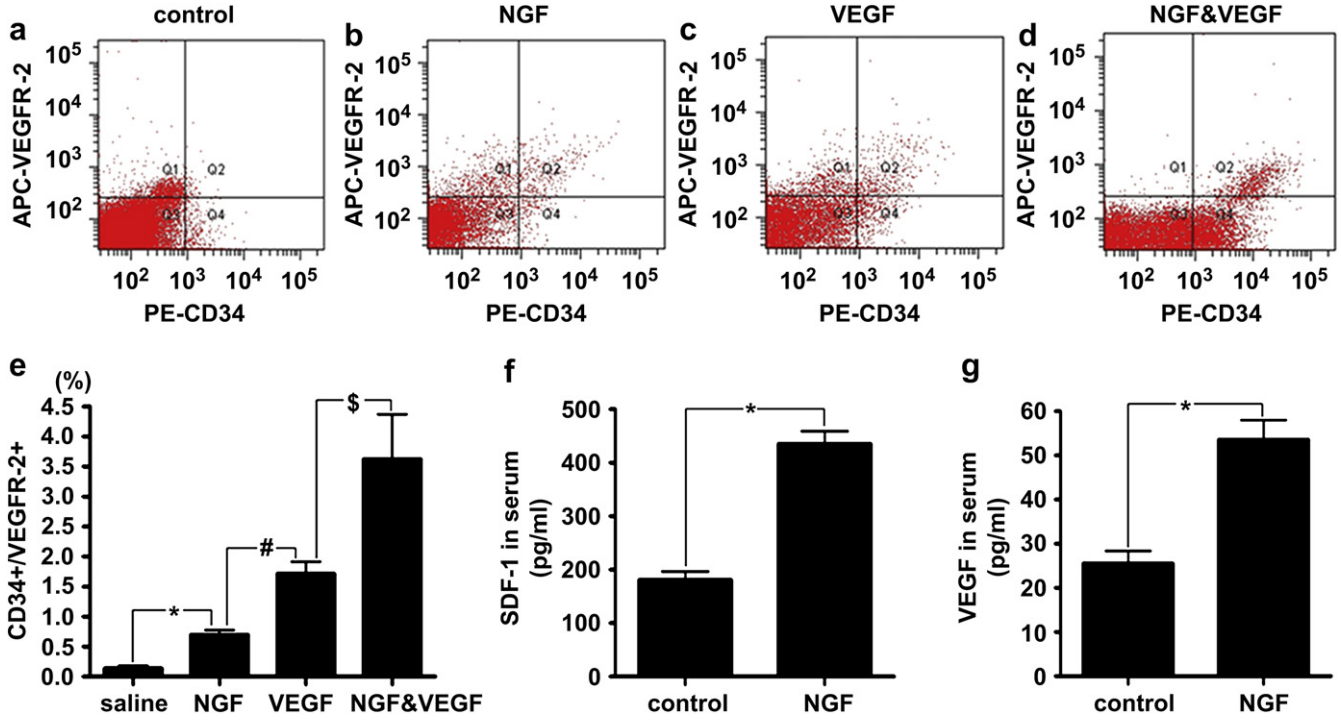

Fig. 3. NGF stimulated EPC mobilization in vivo and the upregulation of two important molecules in serum. (a–d) C57BL/6 mice PB was collected on day 3 after injury and injections of saline, NGF, VEGF, or NGF&VEGF, and subjected to FACS analysis. EPCs were labeled with PE-CD34 and APC-VEGFR-2. (e) The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

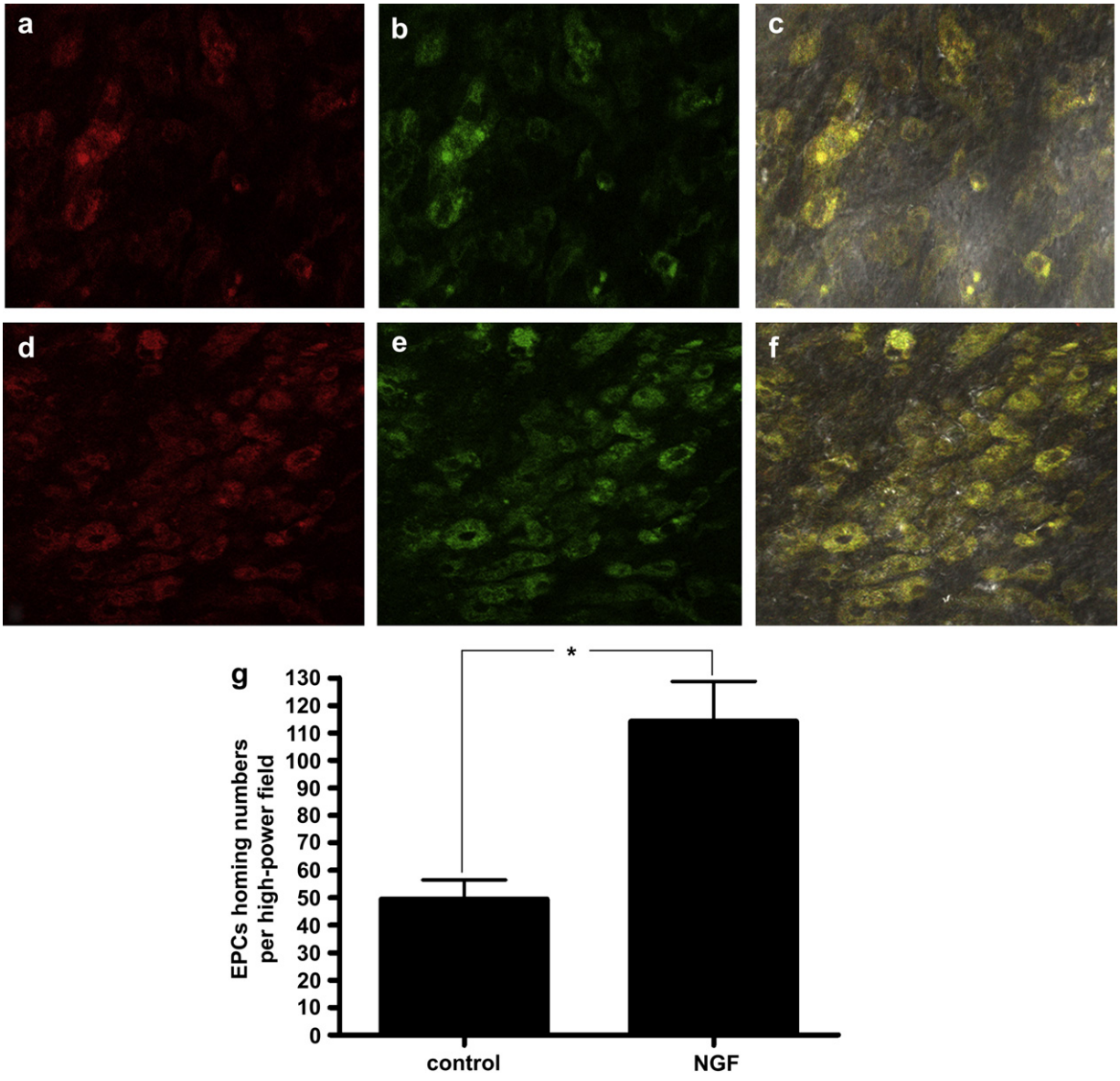

Fig. 4. NGF facilitated EPC homing to carotid arterial injury by wire. $CD133^+$ progenitor cells purified by positive selection with anti-$CD133^+$ microbeads from PB MNCs were incubated with NGF or not for 18 hours at 37°C and labeled with CM-Dil (red) and calcein (green) before injection into C57BL/6 mice. EPCs 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 control 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 NGF-treated 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.

arteries. One week later, the arteries in the NGF-bound group were open and the average blood flow volume was 6.5 ml/min, which approaches the normal blood flow volume of rat arteries. In contrast, the average blood flow volume in the control group was only 3.3 ml/min. LSCM showed that the number of endothelial cells on the luminal surface of TEBV was more than 2-fold greater in the NGF-bound group (Fig. 6c–i). Immunostaining with anti-CD31 antibody in sections revealed that the growth of endothelial cells on the luminal surface of NGF-bound TEBV was greater than that on the luminal surface of control TEBV (Fig. 6 j and k), consistent with the result from the LSCM assay. These observations suggest that NGF can promote endothelialization of TEBV.

In vivo experiment results indicate that all grafts (10 rats in each group) demonstrated good flow at implantation and eight NGF-bound grafts remained open for one months postoperatively as examined by digital camera and assessed by Doppler (6.5 ± 1.46 ml/min) (Fig. 7a). In contrast, one of the grafts (no NGF) remained open for 1 month postoperatively as assessed by Doppler (0.8 ml/min) (Fig. 7c). HE staining showed that NGF-bound TEBV kept patency at 1 month after transplanting into rats and only a little neointimal hyperplasia appeared (Fig. 7b). But control TEBV emerged thrombus and occlusion (Fig. 7d). Furthermore, SME showed that the luminal surface of NGF-bound TEBV was entirely endothelialization at 1 month (Fig. 7e).

4. Discussion

So far, anti-thrombotic modifications of the intima of vascular grafts can inhibit thrombosis, but these anticoagulant effects are temporary, and vascular grafts are meant to last for the life of the patient [4,5]. Since endothelial cells play important roles in thrombosis (inhibiting platelet aggregation, secreting vascular

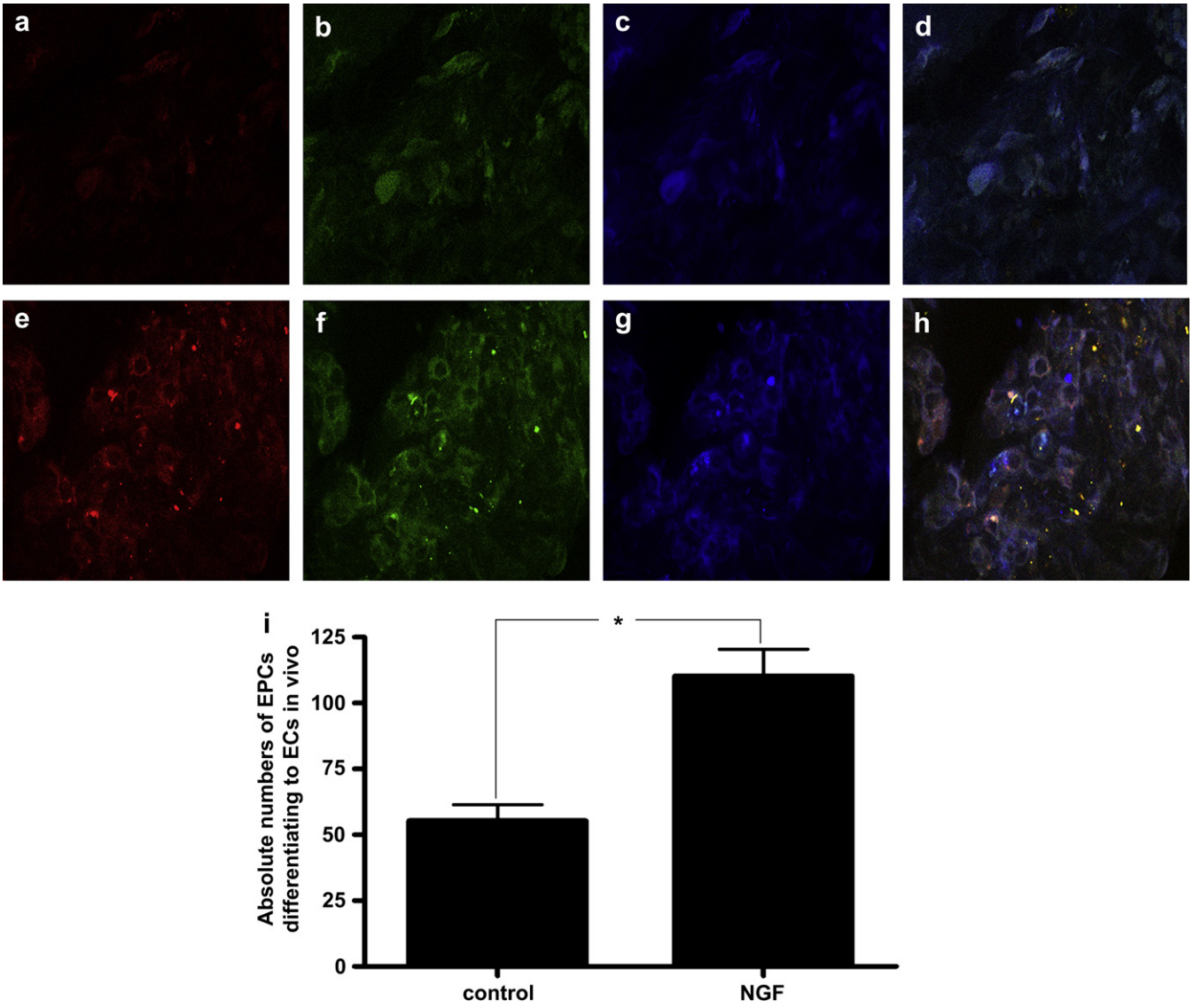

Fig. 5. NGF promoted EPCs to differentiate into ECs after homing to C57BL/6 mice carotid arteries injured by wire. $CD133^{+}$ progenitor cells were incubated with NGF or not for 18 h at 37°C and labeled with CM-Dil (red) and calcein (green) before injection into C57BL/6 mice. The injured carotid arteries were harvested on day 14 after injection and immunostained with anti-CD31 antibody on the luminal surface. (a, b, and c) EPCs in control group were positive for CM-Dil (red), calcein (green), and CD31 (blue), respectively (orig. mag. ×200). (d) Image showing merge of (a), (b) and (c) (orig. mag. ×200). (e, f, g) EPCs in the NGF group were positive for CM-Dil (red), calcein (green) and CD31 (blue), respectively (orig. mag. ×200). (h) Image showing merge of (e), (f) and (g) (orig. mag. ×200). (i) The number of EPCs homing and differentiating to ECs per high-power field. *$p < 0.05$ ($n = 10$) versus control. Values are mean ± SE.

active factors, etc.), endothelialization of the vascular graft is the most effective way to deal with thrombosis and intima hyperplasia [24,25]. Our increasing knowledge of EPCs biology provides new opportunities for vascular graft endothelialization in vivo [26,27]. EPCs are a type of precursor that can differentiate into endothelial cells directly. The high number of circulating EPCs in blood can repair damaged blood vessels by homing to injured vasculature and differentiating into endothelial cells to replace and repair the damaged endothelial cells [28,29]. Based on our previous research, we took peripheral mononuclear cells and isolated the EPCs from the monocytes by changing the culture medium after the first day [17]. Using a combination of surface marker discrimination and adhesion-based EPC isolation, we developed a method to pursue this line of research.

EPCs promote endothelialization of vascular grafts in the body through several important processes, including mobilization of EPCs from bone marrow into peripheral blood, migration to and accumulation in the vascular matrix, and differentiation into endothelial cells [30]. Mobilization from the bone marrow can increase the local number of EPCs; VEGF can mobilize EPCs to promote the formation of new blood vessels. Other stimulating factors that contribute to adjustments in EPC dynamics include SDF-1, bFGF, HGF, angiopoietin-1, G-CSF, EPO, IL-8 and estrogen [31]. Our results show that NGF can improve the number of circulating EPCs effectively. In particular, it can significantly enhance the mobilization function of VEGF, indicating that NGF can promote EPCs mobilization alone and in synergy with VEGF. Our results also show that NGF mobilized EPCs by increasing the levels of SDF-1 and VEGF. However, our study cannot exclude the effects of other EPCs mobilization factors that were not tested. As a result, the specific mechanisms of cross-talk between the cardiovascular and nervous systems in EPCs mobilization deserve further study.

During embryonic and fetal development, there is cross-talk and interdependence between the vascular and neuronal systems [15].

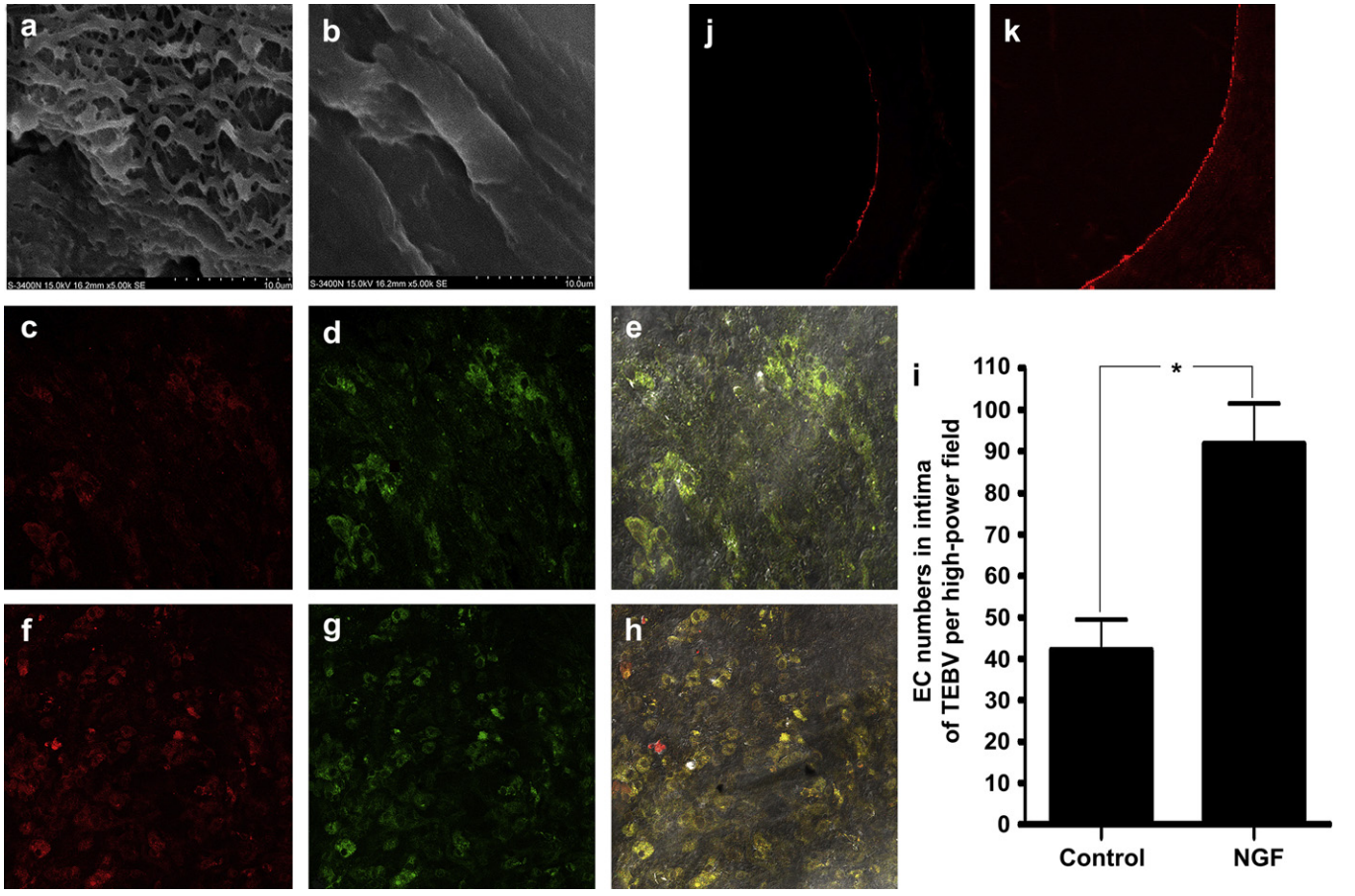

Fig. 6. TEBV treatment and NGF-facilitated endothelialization of TEBV graft transplanted into rats. (a) Fresh adult Wistar rat carotid arteries were obtained and decellularized by enzymatic digestion. (b) Collagen was incubated on the luminal surface of decellularized vessels and cross-linked with EDC. The decellularized vessels incubated with collagen were treated with SPDP and DTT, and then bound to 2% (v/v) 2 mg/ml SPDP-conjugated 0.5 mg/ml NGF protein for 24 h. (c, d) Endothelial cell growth on the luminal surface of control TEBV one week after transplantation. The image shows positive immunostaining for VEGFR-2 (KDR) (green) and CD31 (red), respectively. (e) Image showing merge of (c) and (d). (f, g) Endothelial cell growth on the luminal surface of NGF-bound TEBV one week after transplantation. The image shows positive immunostaining for VEGFR-2 (KDR) (green) and CD31 (red), respectively. (h) Image showing merge of (f) and (g). (i) Endothelialization of TEBV bound with NGF increased significantly compared with controls ($^*p < 0.05$ $n = 10$). Values are mean ± SE. (j) Endothelial cells in cryosections were immunostained for CD31 (red) in control TEBV. (k) Endothelial cells in cryosections were immunostained for CD31 (red) in TEBV bound with NGF.

NGF promotes proliferation and growth of endothelial cells in a dose-dependent manner. Moreover, NGF promotes the growth of endothelial cells by increasing the release of vascular growth factors such as VEGF, substance P, and NO. There are two types of NGF receptor on endothelial cells, TrkA and P75. The binding of NGF to TrkA on endothelial cells triggers proliferation, induces migration by increasing the expression of adhesion molecules, and promotes angiogenesis [16,32]. We chose an appropriate concentration of NGF treatment based on previous research of functional doses on endothelial cells. Our results indicate that NGF can promote EPCs differentiation into endothelial cells that express the endothelial-specific markers CD31 and VE-calcium. EPCs treated with NGF expressed TrkA receptors instead of P75 receptors, although NGF does not influence the expression of TrkA by EPCs. NGF-induced proliferation, migration and tube formation of EPCs occurs through Akt signaling pathways, but increased colony formation from NGF treatment is Akt independent. Because the function of NGF is very extensive, further studies are necessary to determine the signal mechanisms in different processes.

During the implantation of endovascular stents, percutaneous transluminal coronary angioplasty (PTCA) tends to damage the vascular endothelium [33]. If EPCs can be mobilized from bone marrow to peripheral blood, homing to the damaged site will repair the injured blood vessel [34]. Our results showed that in a wire injury model, NGF can enhance EPCs mobilization and homing for the repair of local damaged vascular endothelium. The wire injury model is the pathologic model of PTCA, and our results suggest that NGF may play an important role in repairing damaged endothelium after PTCA.

NGF can promote EPCs mobilization and homing to wire damaged endothelium in the body; it may also promote endogenous EPC mobilization and homing to promote endothelialization of vascular grafts [35]. To test this concept, we prepared a decellularized blood vessel matrix, incubated collagen on the surface of the blood vessel matrix, coupled NGF to the inner cavity of the TEBV with SPDP, and then transplanted the TEBV in a rat carotid artery model. The results show that the degree of endothelialization in NGF-bound TEBV was significantly higher than the control TEBV group, consistent with the conclusion that NGF promotes the mobilization and homing of EPCs in vivo. Our results also show that NGF can promote the growth and differentiation of EPCs on the surface of artificial vessel matrices in vitro (data not shown). Although NGF can promote the repair of both blood vessels and nerves, it is unclear whether it can promote nerve growth for the development of neural control of the TEVB. The potential value in TEBV deserves further study.

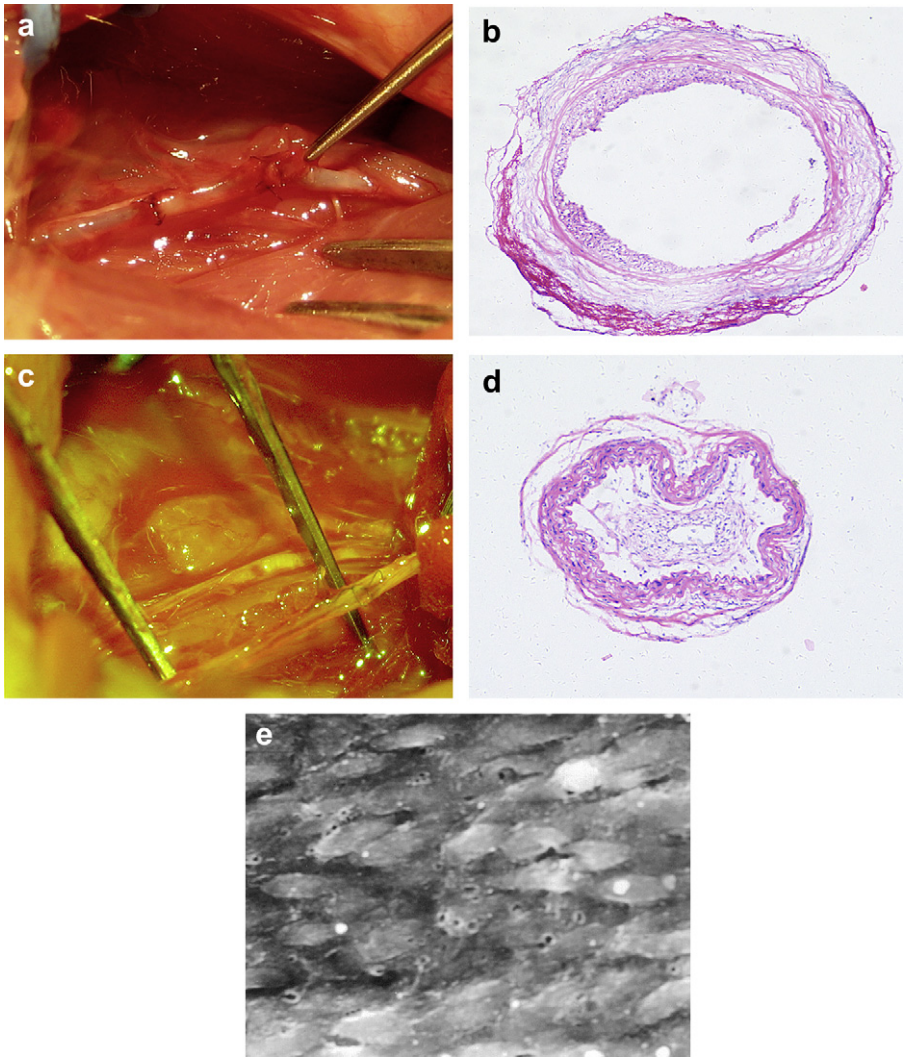

Fig. 7. NGF contributed to TEBV anti-thrombosis in vivo. (a) NGF-bound TEBV graft kept open at 1 month after being transplanted into rat. (b) HE staining of NGF-bound TEBV graft for 1 month. It kept open but there was a little neointimal hyperplasia in the luminal. (c) Control TEBV graft was occluded at 1 month after being transplanted into rat. (d) HE staining of control TEBV graft for 1 month. It had occluded and emerged thrombus in the luminal. (e) SME showed that the growth of endothelial cells on the luminal surface of NGF-bound TEBV was entirely at 1 month.

5. Conclusion

This study shows that neurotrophic factors such as NGF can significantly increase EPC mobilization, homing to injured vessels, and promote TEBV endothelialization in vivo. This finding may have therapeutic potential for the repair of vascular damage after PTCA and the development of tissue-engineered blood vessels in vivo.

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Appendix

Figures with essential colour discrimination. Most of the figures in this article have parts that are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.11.037.

Appendix. Supplementary data

Fig. S1. NGF promoted EPC to grow greater on hybrid electrospinning nano-fiber mesh (a) the photo of hybrid electrospinning nano-fiber mesh. (b) hybrid electrospinning nano-fiber mesh was made by polylactic acid 80%, Silk Protein 10% and collagen 10%. SME showed the diameter, density and arrangement of the fiber. (c–e) Images showing positive staining for CM-Dil (red), calcein (green) and image of white balance, respectively, in the NGF-treated group (orig. mag. ×200). (f) Image showing merge of (c), (d) and (e) (orig. mag. ×200). (g–i) Images showing positive staining for CM-Dil (red), calcein (green) and image of white balance, respectively, in the control group (orig. mag. ×200). (j) Image showing merges of (g), (h) and (i) (orig. mag. ×200).

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biomaterials.2009.11.037.

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