Thymosin β4 promotes endothelial progenitor cell angiogenesis via a vascular endothelial growth factor-dependent mechanism

YANBO ZHAO*, JIALE SONG*, XUKUN BI*, JING GAO, ZHIDA SHEN, JUNHUI ZHU and GUOSHENG FU

Department of Cardiology, Biomedical Research (Therapy) Center, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310016, P.R. China

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Abstract. Endothelial progenitor cells (EPCs) are a promising cell source for tissue repair and regeneration, predominantly through angiogenesis promotion. Paracrine functions serve a pivotal role in EPC-mediated angiogenesis, which may be impaired by various cardiovascular risk factors. Therefore, it is important to identify a solution that optimizes the paracrine function of EPCs. Thymosin β4 (Tβ4) is a peptide with the potential to promote tissue regeneration and wound healing. A previous study demonstrated that Tβ4 enhances the EPC-mediated angiogenesis of the ischemic myocardium. In the present study, whether Tβ4 improved angiogenesis by enhancing the paracrine effects of EPCs was investigated. A tube formation assay was used to assess the effect of angiogenesis, and the paracrine effects were measured using an ELISA kit. The results indicated that Tβ4 improved the paracrine effects of EPCs, evidenced by an increase in the expression of vascular endothelial growth factor (VEGF). EPC-conditioned medium (EPC-CM) significantly promoted human umbilical vein endothelial cell angiogenesis *in vitro*, which was further enhanced by pretreatment with Tβ4. The effect of Tβ4 pretreated EPC-CM on angiogenesis was abolished by VEGF neutralizing antibody *in vitro*, indicating that increased VEGF secretion had a pivotal role in Tβ4-mediated EPC angiogenesis. Furthermore, transplantation of EPCs pretreated with Tβ4 into infarcted rat hearts resulted in significantly higher VEGF expression in the border zone, compared with EPC transplantation alone. To further investigate whether the Akt/eNOS pathway was involved in Tβ4-induced VEGF secretion in EPCs, the expression levels of VEGF in EPC-CM were significantly decreased following knockdown of Akt or eNOS by small interfering RNA transfection. In conclusion, Tβ4 significantly increased angiogenesis by enhancing the paracrine effects of EPCs, evidenced by the increased expression of VEGF. The RAC-α serine/threonine-protein kinase/endothelial nitric oxide synthase signal transduction pathway was involved in the regulation of Tβ4-induced VEGF secretion in EPCs. Further studies are required to investigate the long-term prognosis of patients with coronary heart disease following Tβ4-pretreated EPC transplantation.

Correspondence to: Dr Junhui Zhu or Dr Guosheng Fu, Department of Cardiology, Biomedical Research (Therapy) Center, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, 3 East Qingchun Road, Hangzhou, Zhejiang 310016, P.R. China
E-mail: zhujhsrrsh@zju.edu.cn
E-mail: fugs@zju.edu.cn

*Contributed equally

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Introduction

Human endothelial progenitor cells (EPCs) serve an important role in angiogenesis and endothelial repair (1,2). However, it is difficult to obtain an adequate number of EPCs for transplantation from one single donation. The function of EPCs is impaired in the presence of various cardiovascular disease risk factors, including age, hypertension and diabetes (3,4). Therefore, increasing the number of EPCs obtained from donations and improving their function is critical for EPC transplantation optimization.

In addition to direct differentiation into mature endothelial cells to repair endothelial injury, EPCs are capable of secreting protective paracrine factors to accelerate repair, which is another critical mechanism of EPC transplantation (5). There is increasing evidence to indicate that EPCs secrete various paracrine factors, including stromal cell-derived factor (SDF)-1, vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), to improve endothelial cell function and repair various types of wounds in different animal models (6). EPC-conditioned medium (EPC-CM) promotes angiogenesis in rat artery loops, inhibits apoptosis in endothelial cells and increases the capillary density of an ischemic limb (7). Kim *et al* (8) reported that an injection of EPC-CM alone improves the recovery of diabetic wounds, and even requires a smaller number of cells compared with EPC transplantation.

Thymosin β4 (Tβ4) is a small protein widely distributed in numerous cells and tissues, which mediates multiple biological reactions, including vessel formation and wound healing (9). Previous studies indicated that Tβ4 promotes EPC proliferation, migration and adhesion, and inhibits apoptosis

and senescence *in vitro* (10-12). Since these actions may all contribute to angiogenesis, there has been an increased interest in evaluating the role of Tβ4 in EPC angiogenesis.

VEGF is an important paracrine factor secreted by progenitor cells to promote angiogenesis. Barcelos *et al* (13) reported that prominin 1 $(CD133)^+$ progenitor cells and the cell-conditioned medium (CCM) of $CD133^+$ cells contain high levels of VEGF-A and interleukin (IL)-8, and application of these cells or $CD133^+$ CCM accelerates the healing process of diabetic ulcers in the lower limbs of rats. In addition, neutralizing antibodies against VEGF-A or IL-8 inhibit the healing effect of $CD133^+$ CCM (13). Wnt family member 7A expression is increased in $CD133^+$ cell-treated wounds compared with either $CD133^-$ cell- or collagen-treated wounds. Secreted frizzled-related proteins are Wnt antagonists, and their application abolishes the facilitation of wound closure and reparative angiogenesis by $CD133^+$ CCM (13). These findings indicate that the promotion of angiogenesis is primarily mediated by paracrine factors secreted by progenitor cells. However, whether Tβ4 stimulates EPCs to secrete an increased amount of VEGF to improve endothelial function requires further investigation. In the present study, it was examined whether pre-treatment of Tβ4 was able to augment the volume of VEGF mRNA and secretion of serum VEGF in EPC-CM. Additionally, the present study aimed to determine whether the increased amount of VEGF was able to further promote the angiogenesis of endothelial cells, which may improve the efficacy of therapeutic EPC transplantation.

Materials and methods

Cell culture. EPCs were isolated, cultured and characterized according to previously described techniques (10-12). The present study was approved by the Ethics Committee of Sir Run Run Shaw Hospital of Zheijang University (Hangzhou, China) and written informed consent was obtained from 30 healthy individuals (20-55 years old; 50:50 male:female). The date range of the recruitment was from September 2014 to December 2014. All the samples were collected in the biomedical research center of Sir Run Run Shaw Hospital. To obtain EPCs, mononuclear cells were isolated from human peripheral blood via density-gradient centrifugation (400 x g; 35 min; room temperature) with Ficoll separating solution (Cedarlane Laboratories, Burlington, ON, Canada). The mononuclear cells were subsequently placed on fibronectin (Merck KGaA, Darmstadt, Germany)-coated plates and incubated with endothelial growth medium-2 (EGM-2MV; Lonza Group, Ltd., Basel, Switzerland). After 7 days of incubation at 37˚C and 5% CO_2, 10^6 of cells grew into EPCs, which exhibited a spindle shape. The EPCs were detached using trypsin and subsequently collected for further experiments. Human umbilical vein endothelial cells (HUVECs) were obtained from ScienCell Research Laboratories, Inc. (San Diego, CA, USA) and cultured in endothelial cell medium supplemented with 10% fetal bovine serum (both ScienCell Research Laboratories, Inc.). For experimental treatments, cells (passages 3-8) were grown to 70-90% confluence.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from EPCs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA synthesis was performed with 1 μg total RNA using a PrimeScriptTM RT Master Mix (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. The reaction mixture was incubated under the following condition: RT, 37˚C for 15 min; and inactivation of RT with heat treatment, 85˚C for 5 sec. RT-qPCR was conducted using the SsoFastTM EvaGreen Supermix with Low ROX (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The reaction mixture was incubated under the following thermocycling condition: Polymerase activation, 95˚C for 30 sec; denaturation, 95˚C for 5 sec; annealing/extension, 55˚C for 30 sec; melt curve analysis, 65-95˚C in 0.5˚C increments for 2 sec. Data analysis was performed with an ABI 7500 cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the $2^{-\Delta\Delta Cq}$ method, as previously described (14). GAPDH was used as the endogenous control for mRNA expression. The primers used were as follows: GAPDH forward, 5'-GGGTGT GAACCATGAGAAGT-3' and reverse, 5'-GACTGTGGTCAT GAGTCCT-3'; VEGF forward, 5'-TCTTGGGTGCATTGG AGCCT-3' and reverse, 5'-AGCTCATCTCTCCTATGTGC-3'; and IL-8 forward, 5'-CCTGATTTCTGCAGCTCTGT-3' and reverse, 5'-AACTTCTCCACAACCCTCTG-3.

Small interfering (si)RNA transfection. RAC-α serine/threonine-protein kinase (Akt) and endothelial nitric oxide synthase (eNOS) siRNA, in addition to the scramble sequence negative control siRNA, were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). When 60-80% confluence was reached, cells were transfected with 150 pmol Akt siRNA, eNOS siRNA or negative control siRNA with HiPerFect Transfection Reagent (Qiagen GmbH, Hilden, Germany) incubated for 24 h (15-25˚C). The effectiveness of Akt and eNOS knockdown was confirmed with western blotting. The Akt siRNA sequence was as follows: Forward, 5'-GCACUU UCGGCAAGGUGAUTT-3' and reverse, 5'-AUCACCUUG CCGAAAGUGCTT-3'. The eNOS siRNA sequence was as follows: Forward, 5'-CAGUACUACAGCUCCAUUATT-3' and reverse, 5'-UAAUGGAGCUGUAGUACUGTT-3. The scramble siRNA sequence was as follows: Forward, 5'-GAC CCAAAUUUGACAUGAUTT-3' and reverse, 5'- AUCAUG UCAAAUUUGGGUCTT-3'.

ELISA. A density of $4x10^5$ transfected EPCs were seeded in 6-well plates and treated with 0, 10, 100 and 1,000 ng/ml Tβ4 (ProSpec-Tany TechnoGene, Ltd., East Brunswick, NJ, USA) for 37˚C 24 h. Conditioned media were subsequently collected for the VEGF ELISA (cat. no. DVE00; Abcam, Cambridge, UK).

EPC tube formation assay. The tube formation assay was performed using a Matrigel matrix™ basement membrane (BD Biosciences, Franklin Lakes, NJ, USA) to investigate HUVEC angiogenesis under treatment with different types of EPC-CM (8). Matrigel solution was thawed at 4˚C overnight and subsequently diluted with EBM-2 (Clonetics™; Lonza Group, Ltd., Basel, Switzerland) to solidify in a 96-well plate at 37˚C for 1 h. A total of $2x10^4$ HUVECs were seeded on the matrix for further incubation with either 2 ml EPC-CM, Tβ4-EPC-CM or Tβ4-EPC-CM with 0.4 μg/ml

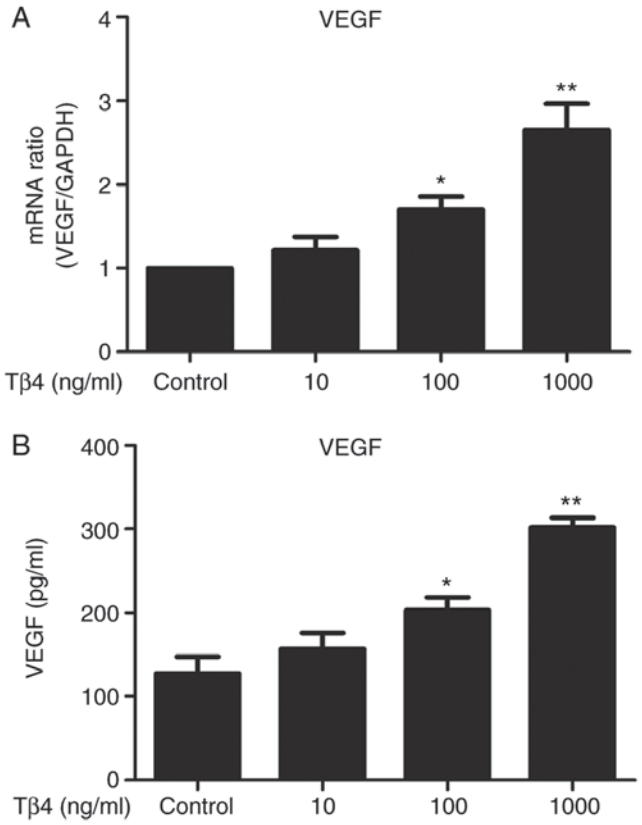

Figure 1. Tβ4 increases VEGF secretion in human EPCs. (A) Treatment with Tβ4 increased the mRNA and (B) protein expression of VEGF in EPCs. n=4. *P<0.05, **P<0.01 vs. control group. Tβ4, thymosin β4; VEGF, vascular endothelial growth factor; EPCs, endothelial progenitor cells.

VEGF antibody (cat. no. MAB293; R&D Systems, Inc., Minneapolis, MN, USA) at 37˚C for 6 h. The CCM was the supernatant of different group of EPCs obtained following centrifugation at 150 x g for 5 min. Tube formation was assessed using a light microscope (magnification, x100) and five random fields were selected for each assay. The average branch point numbers that indicated tube formation were compared using Image Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Experimental animal model. Animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA; 8th Edition, 2011) and were approved by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China). The rat myocardial infarction (MI) model was established as previously described (10). A total of 40 male Sprague-Dawley rats (8 weeks; weighing 200-250 g) were purchased from the Experimental Animal Center of Zhejiang University (Hangzhou, China). Experimental rats were anesthetized and intubated for artificial ventilation. A left thoracotomy was subsequently performed. The left anterior descending coronary artery (LAD) was ligated with a 6-0 suture with the fourth intercostal space open. Sham-operated (sham) rats received surgery without LAD ligation. Rats in the EPC group (EPCs) were intramyocardially injected with $1x10^6$ human EPCs re-suspended in 150 μl EGM-2, while rats in the Tβ4-EPC group (Tβ4-EPCs) received the same amount of Tβ4 pretreated human EPCs (EPCs were pretreated with 1,000 ng/ml Tβ4 for 24 h). Rats receiving 150 μl blank EGM-2 without cells were set as the control group. A total of three sites in the border zone of the infarcted heart received three injections. The rats were sacrificed 4 weeks following this procedure.

Immunohistochemical staining. Immunohistochemical analysis of VEGF (1:100; cat. no. ab46154; Abcam) staining was performed, in which 5 μm fresh frozen sections (-20˚C) of rat cardiac tissue were incubated with the primary antibody at 37˚C for 2 h. The process was followed by incubation with specific horseradish peroxidase (HRP)-conjugated secondary antibodies at 37˚C for 10 min (1:100; cat. no. SPN-9001; OriGene Technologies, Inc., Beijing, China) and DAB at 37˚C for 2 h. Sections were rinsed in PBS and subsequently counterstained with 0.5% hematoxylin (cat. no. ZLI-9608; OriGene Technologies, Inc.) at room temperature for 1 min, dehydrated and mounted. A total of five random fields were selected for assessment, and the area of myocardial fibrosis and the expression of VEGF were quantified using Image-Pro Plus 6.0.

Western blot analysis. Rat cardiac tissue was harvested for western blot analysis. The protein was extracted using radioimmunoprecipitation buffer (Thermo Fisher Scientific, Inc.) and protein concentration was determined with a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology, Haimen, China). A total of 50 μg protein sample was loaded per lane on a 10% Tris-glycine gradient gel, and subsequently transferred onto polyvinylidene difluoride membranes and blocked with 5% non-fat milk for 1 h at room temperature. Following this, membranes were incubated with the following primary antibodies (1:1,000) overnight at 4˚C: VEGF (cat. no. Ab46154; Abcam), eNOS [cat. no. 32027; Cell Signaling Technology (CST), Inc., Danvers, MA, USA], Akt (cat. no. 2920; CST, Inc.) and GAPDH (cat. no. 5174; CST, Inc.). The membrane was subsequently incubated with goat anti-rabbit IgG-HRP secondary antibodies (1:5,000; cat. no. 7074; CST, Inc.) at room temperature for h. Proteins were visualized with enhanced chemiluminescence reagent (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and Image Quant LAS-4000 (GE Healthcare Life Sciences, Shanghai, China). The grayscale values of the bands were examined using Image Multi-Gauge software 2.0 (Fujifilm, Tokyo, Japan).

Statistical analysis. Data are presented as the mean ± standard error of the mean of least three independent experiments. Statistical analyses between groups were performed using SPSS software version 19.0 (IBM Corp., Armonk, NY, USA) using one-way analysis of variance with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Tβ4 increases the secretion of VEGF from human EPCs. The present study investigated the effect of Tβ4 on the expression of VEGF in EPCs. Treatment with Tβ4 significantly increased the expression of VEGF in EPCs at the transcriptional (Fig. 1A) and protein (Fig. 1B) levels. This pro-secretory effect was dose-dependent, the highest VEGF mRNA/protein

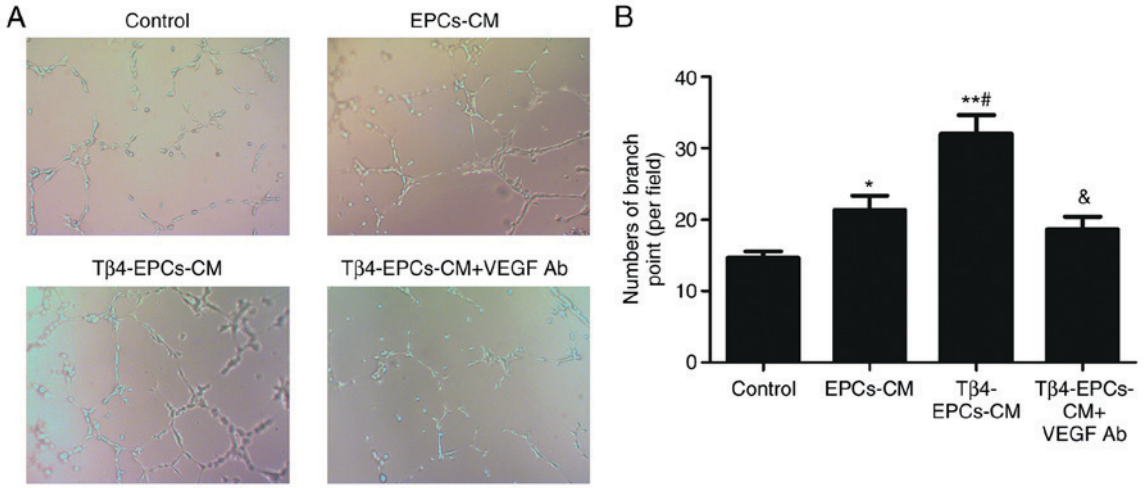

Figure 2. VEGF is a critical mediator of Tβ4-enhanced angiogenesis. (A) EPCs-CM, Tβ4-EPCs-CM and Tβ4-EPCs-CM with VEGF Ab was added to test the angiogenic ability of HUVECs *in vitro* at the concentration of 1,000 ng/ml for 24 h. Compared with the control group, EPCs-CM enhanced the angiogenic effect. This effect was further promoted in the 1,000 ng/ml Tβ4-EPC-CM-treated group. VEGF Ab reversed the pro-angiogenic effect of Tβ4-EPC-CM. (B) Angiogenesis was quantified by determining the number of branch points in five random fields of view. Magnification, x100. n=6. *P<0.05, **P<0.01 vs. control group. $^{\#}$P<0.05 vs. EPCs-CM treated group. $^{\&}$P<0.05 vs. Tβ4-EPC-CM group. EPCs, endothelial progenitor cells; Tβ4, thymosin β4; VEGF, vascular endothelial growth factor; CM, conditioned medium; Ab, antibody.

concentrations were observed at 1,000 ng/ml Tβ4 for 24 h. At the transcriptional and protein levels, the fold changes in expression were 2.65 and 2.54-fold, respectively, compared with the control group.

VEGF is a critical mediator of Tβ4-enhanced angiogenesis. The present study investigated the effect of EPC-CM on the angiogenesis of HUVECs *in vitro* by performing a tube formation assay (Fig. 2A). It was demonstrated that EPC-CM enhanced the angiogenesis of HUVECs compared with the control group, and pretreatment with 1,000 ng/ml Tβ4 further augmented the effect of EPC-CM. Furthermore, the presence of VEGF antibody reversed the pro-angiogenic effect of Tβ4-pretreated EPC-CM (Fig. 2B).

Akt/eNOS pathway signaling is involved in Tβ4-induced VEGF secretion. To further investigate whether the Akt/eNOS pathway was involved in Tβ4-induced VEGF secretion in EPCs, Akt and eNOS siRNAs were used to knock down the expression of Akt and eNOS. Western blot analysis was performed to confirm that Akt and eNOS expression was effectively reduced compared with the scramble control (Fig. 3A). In addition, the levels of VEGF in EPC-CM were significantly decreased following knockdown of Akt or eNOS, which indicated that the Akt/eNOS pathway was involved in Tβ4-induced VEGF secretion (Fig. 3B).

Tβ4-pretreated EPC transplantation significantly augments VEGF expression in the border zone of infarcted hearts. Immunohistochemical analysis demonstrated that VEGF expression in cardiac tissue was significantly augmented at 4 weeks post-MI compared with the sham group. The expression of VEGF was significantly higher in the EPC transplantation alone group compared with the untreated control group, and it was significantly higher in the Tβ4-EPC-transplanted MI hearts at 4 weeks post-MI compared with the control and EPC groups (Fig. 4A and B). Western blot analysis indicated that VEGF expression was significantly augmented at 4 weeks post-MI. VEGF expression in the EPCs and Tβ4-EPCs groups increased significantly compared with the control group, and the expression of VEGF in the Tβ4-EPCs group was significantly higher compared with the EPCs group (Fig. 4C).

Discussion

Angiogenesis is essential for the survival and functional maintenance of the ischemic myocardium. Numerous known methods of vessel formation have been investigated in normal tissue and tumors, including the recruitment of EPCs that differentiate to endothelial cells, intussusception and sprouting angiogenesis (15). In the present study, it was reported that Tβ4 promoted EPC angiogenesis by increasing the secretion of VEGF, indicating that paracrine functions served an important role in the regulation of angiogenesis. The Akt/eNOS pathway was demonstrated to be involved in the regulation of VEGF expression induced by Tβ4. Furthermore, *in vivo*, VEGF was highly expressed near the border zone of infarcted hearts following transplantation with Tβ4 pretreated EPCs, suggesting that the ischemic myocardium may have benefited from angiogenesis mediated by VEGF expression. Taken together, the experiments of the present study revealed that Tβ4-pretreated EPCs promoted angiogenesis through activation of the Akt/eNOS pathway, and may provide a novel strategy for the effective transplantation of EPCs in MI.

Research has previously been conducted to determine the way in which EPCs home to ischemic areas and become involved in vasculogenesis, thereby increasing the blood flow to these tissues and preserving cardiac function (16,17). By using TEK tyrosine-protein kinase receptor (Tie-2)/LacZ b-galactosidase (β-gal) transgenic mice, Ii *et al* (18) observed that EPCs, from donor mice expressing β-gal driven by the endothelial gene promoter Tie-2, are incorporated into the microvasculature following myocardial ischemia (18). This provides direct evidence that EPCs contribute to neovascularization. Furthermore, EPCs activate angiogenesis

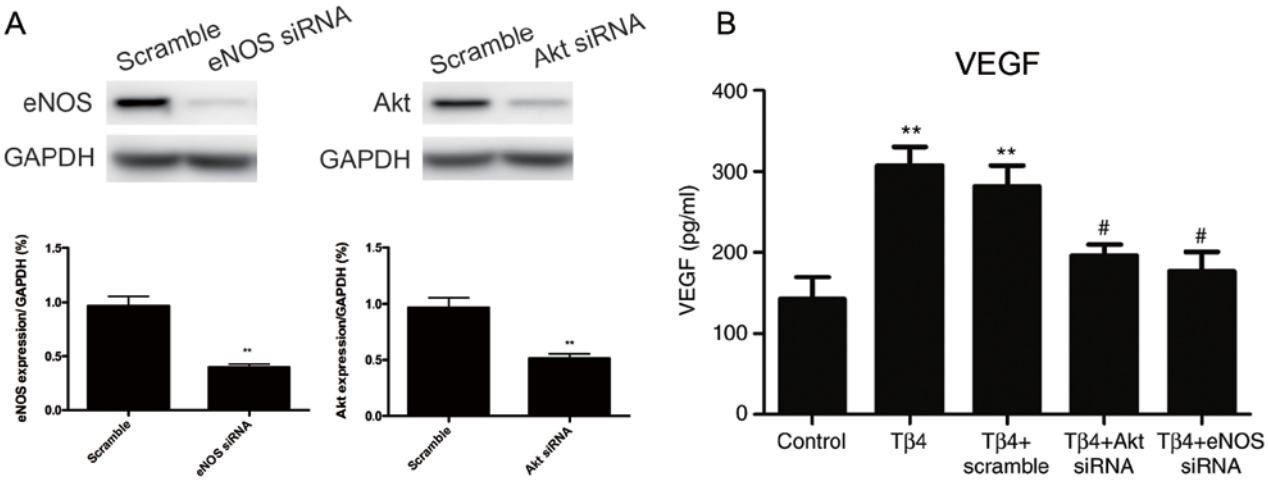

Figure 3. Akt/eNOS pathway signaling is involved in Tβ4-induced VEGF secretion. (A) Successful knockdown of Akt and eNOS expression by siRNA transfection was confirmed by western blot analysis. **P<0.01 vs. scramble siRNA group. (B) VEGF expression in EPC-CM was decreased following knockdown of Akt or eNOS. n=4. **P<0.01 vs. control group. $^{\#}$P<0.05 vs. Tβ4 + scramble group. Akt, RAC-α serine/threonine-protein kinase; eNOS, endothelial nitric oxide synthase; Tβ4, thymosin β4; VEGF, vascular endothelial growth factor; CM, conditioned medium; siRNA, small interfering RNA; EPCs, endothelial progenitor cells.

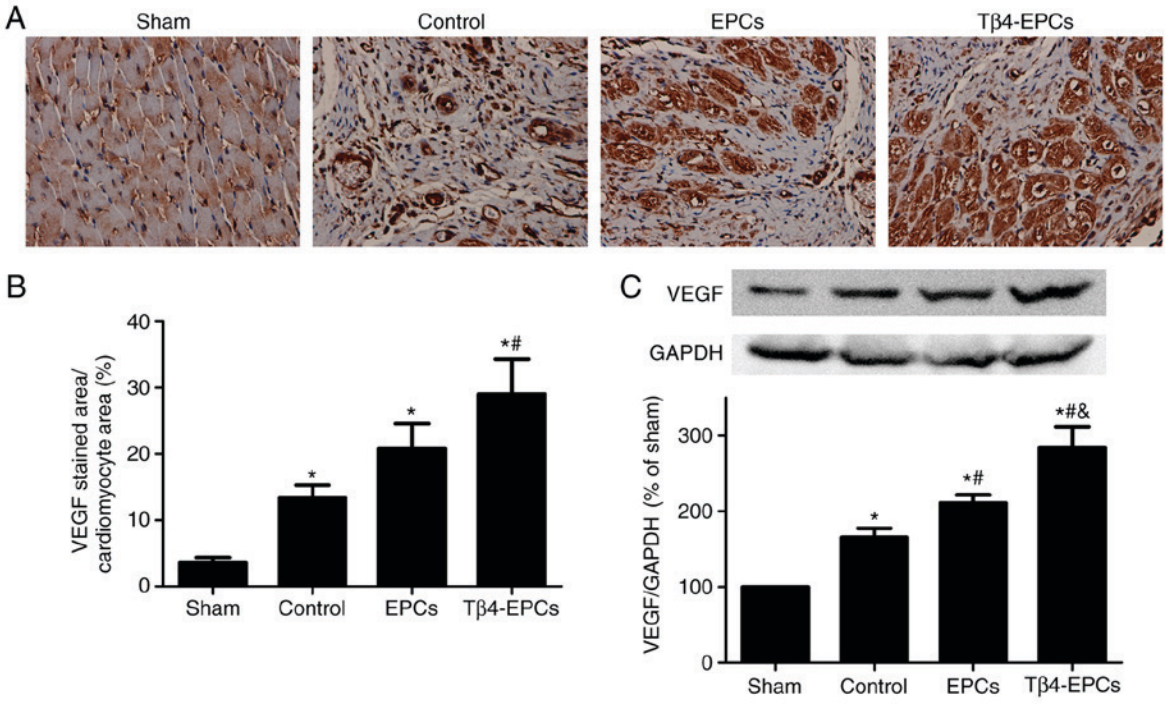

Figure 4. Transplantation of Tβ4-pretreated EPCs significantly augments VEGF expression in the border zone of infarcted hearts. (A) VEGF expression was significantly higher in the EPC group compared with the untreated control group, and further increased in the Tβ4-EPCs group in MI hearts at 4 weeks post-MI, light microscopy was used (magnification, x100). (B) Quantification of the positively stained VEGF area. (C) VEGF expression in the EPC and Tβ4-EPC transplanted group increased significantly compared with the control group, and the expression of VEGF in the Tβ4-EPCs group was significantly higher compared with the EPCs group. n=6. *P<0.05 vs. sham surgery group. $^{\#}$P<0.05 vs. control group with MI surgery. $^{\&}$P<0.05 vs. EPC transplanted group with MI surgery. Tβ4, thymosin β4; EPCs, endothelial progenitor cells; VEGF, vascular endothelial growth factor; MI, myocardial infarction.

through indirect mechanisms, including through the secretion of proangiogenic factors, including VEGF, HGF and SDF-1 (5). The present study demonstrated the ability of EPCs to induce the formation of neovasculature, and the critical role of VEGF in EPC-mediated angiogenesis in infarcted rat hearts, supporting the *in vitro* findings.

A number of studies have reported that two types of EPCs (early and late) may be isolated from peripheral blood (19-21). The tube formation abilities of these two cell types are different. Early EPCs with a spindle shape secrete more angiogenic cytokines, including VEGF, compared with late EPCs, which have a cobblestone shape. However, late EPCs form capillary tubes more efficiently compared with early EPCs. Previous studies demonstrated that following treatment with Tβ4, late EPCs exhibit increased migration (11), and reduced senescence (12) and apoptosis (10). Considering these findings,

the present study used late EPCs to assess angiogenesis under treatment with Tβ4. It was demonstrated that the Akt/eNOS pathway is a critical component in regulating the signaling of multiple biological processes (22,23). Through the activation of this pathway, the migration and anti-apoptotic ability of late EPCs increased, and finally promoted angiogenesis to aid ischemic myocardial survival.

Tβ4 is required in the development of the coronary vasculature. Knocking down Tβ4 expression in the developing heart results in congenital coronary artery anomalies, demonstrating its pivotal role in vasculogenesis (24). By using embryonic EPCs, Kupatt *et al* (25) revealed that Tβ4, prothymosin α and Tβ10 are among the most abundantly secreted factors of early EPCs, suggesting that Tβ4 may be involved in EPC-regulated angiogenesis. The present study provided direct evidence that Tβ4 promoted EPC-mediated angiogenesis by increasing the secretion of VEGF. In the border zone of the infarcted hearts, VEGF expression was highly induced following Tβ4-pretreated EPC transplantation. However, the mechanism underlying Tβ4-induced angiogenesis is not well defined. The upstream kinases targeting Akt include the phosphoinositide 3-kinase (PI3K), phosphorylated (p)-phosphatase and tensin homolog, p-pyruvate dehydrogenase kinase 1 and the p-serine/threonine-protein kinase mTOR pathways (26). Our previous studies demonstrated that Tβ4 predominantly activates PI3K as a principal upstream component of the Akt pathway (11,12). By using siRNAs to target Akt and eNOS, the present study demonstrated that the AKT/eNOS pathway was involved in Tβ4-mediated VEGF expression. Notably, Tβ4 and VEGF are upregulated following the induction of Akt overexpression in bone marrow-derived mesenchymal stem cells (27). This suggests that a positive feedback loop may exist between the Akt pathway and Tβ4. In addition, hypoxia-inducible factor (HIF)-1α protein stability is directly increased by the expression of VEGF induced by Tβ4 (28). It is therefore plausible that under ischemic conditions, Tβ4 induces the expression of VEGF in a HIF-1α-dependent manner.

The secretion of VEGF contributes to endothelial cell differentiation (29). However, the role of VEGF in arterial and venous specification remains unclear and further study is required.

In conclusion, the results of the present study demonstrated that the angiogenesis of EPCs was strongly enhanced following treatment with Tβ4. The Akt/eNOS pathway mediated VEGF expression, which was important for this process. The present study demonstrated a novel way to improve EPC function and may be critical for the optimization of EPC transplantation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ contributed to the study conception and design. JS acquired, analyzed and interpreted the data. XB was involved in drafting the manuscript and interpreting the data. JG and ZS revised the article critically for important intellectual content and interpreted the data. JZ and GF gave the final approval of the version to be published and contributed to the study conception. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Sir Run Run Shaw Hospital of Zheijang University (Hangzhou, China) and written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, *et al*: Evidence for circulating bone marrow-derived endothelial cells. Blood 92: 362-367, 1998.
2. Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM and Asahara T: Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci USA 97: 3422-3427, 2000.
3. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA and Finkel T: Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 348: 593-600, 2003.
4. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM and Dimmeler S: Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 89: E1-E7, 2001.
5. Asahara T, Kawamoto A and Masuda H: Concise review: Circulating endothelial progenitor cells for vascular medicine. Stem Cells 29: 1650-1655, 2011.
6. Urbich C, Aicher A, Heeschen C, Dernbach E, Hofmann WK, Zeiher AM and Dimmeler S: Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 39: 733-742, 2005.
7. Di Santo S, Yang Z, Wyler von Ballmoos M, Voelzmann J, Diehm N, Baumgartner I and Kalka C: Novel cell-free strategy for therapeutic angiogenesis: In vitro generated conditioned medium can replace progenitor cell transplantation. PLos One 4: e5643, 2009.
8. Kim JY, Song SH, Kim KL, Ko JJ, Im JE, Yie SW, Ahn YK, Kim DK and Suh W: Human cord blood-derived endothelial progenitor cells and their conditioned media exhibit therapeutic equivalence for diabetic wound healing. Cell Transplant 19: 1635-1644, 2010.
9. Huff T, Müller CS, Otto AM, Netzker R and Hannappel E: beta-Thymosins, small acidic peptides with multiple functions. Int J Biochem Cell Biol 33: 205-220, 2001.

10. Zhao Y, Qiu F, Xu S, Yu L and Fu G: Thymosin beta4 activates integrin-linked kinase and decreases endothelial progenitor cells apoptosis under serum deprivation. J Cell Physiol 226: 2798-2806, 2011.
11. Qiu FY, Song XX, Zheng H, Zhao YB and Fu GS: Thymosin beta4 induces endothelial progenitor cell migration via PI3K/Akt/eNOS signal transduction pathway. J Cardiovasc Pharmacol 53: 209-214, 2009.
12. Li J, Yu L, Zhao Y, Fu G and Zhou B: Thymosin beta4 reduces senescence of endothelial progenitor cells via the PI3K/Akt/eNOS signal transduction pathway. Mol Med Rep 7: 598-602, 2013.
13. Barcelos LS, Duplaa C, Krankel N, Graiani G, Invernici G, Katare R, Siragusa M, Meloni M, Campesi I, Monica M, *et al*: Human CD133+ progenitor cells promote the healing of diabetic ischemic ulcers by paracrine stimulation of angiogenesis and activation of Wnt signaling. Circ Res 104: 1095-1102, 2009.
14. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
15. 15 Carmeliet P and Jain RK: Molecular mechanisms and clinical applications of angiogenesis. Nature 473: 298-307, 2011.
16. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM and Itescu S: Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 7: 430-436, 2001.
17. Kalka C, Tehrani H, Laudenberg B, Vale PR, Isner JM, Asahara T and Symes JF: VEGF gene transfer mobilizes endothelial progenitor cells in patients with inoperable coronary disease. Ann Thorac Surg 70: 829-834, 2000.
18. Ii M, Nishimura H, Iwakura A, Wecker A, Eaton E, Asahara T and Losordo DW: Endothelial progenitor cells are rapidly recruited to myocardium and mediate protective effect of ischemic preconditioning via 'imported' nitric oxide synthase activity. Circulation 111: 1114-1120, 2005.
19. Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, Oh BH, Lee MM and Park YB: Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 24: 288-293, 2004.
20. Tagawa S, Nakanishi C, Mori M, Yoshimuta T, Yoshida S, Shimojima M, Yokawa J, Kawashiri MA, Yamagishi M and Hayashi K: Determination of early and late endothelial progenitor cells in peripheral circulation and their clinical association with coronary artery disease. Int J Vasc Med 2015: 674213, 2015.
21. Cheng CC, Chang SJ, Chueh YN, Huang TS, Huang PH, Cheng SM, Tsai TN, Chen JW and Wang HW: Distinct angiogenesis roles and surface markers of early and late endothelial progenitor cells revealed by functional group analyses. BMC Genomics 14: 182, 2013.
22. Yang X, Zhu W, Zhang P, Chen K, Zhao L, Li J, Wei M and Liu M: Apelin-13 stimulates angiogenesis by promoting cross-talk between amp-activated protein kinase and akt signaling in myocardial microvascular endothelial cells. Mol Med Rep 9: 1590-1596, 2014.
23. Ohashi K, Enomoto T, Joki Y, Shibata R, Ogura Y, Kataoka Y, Shimizu Y, Kambara T, Uemura Y and Yuasa D: Neuron-derived neurotrophic factor functions as a novel modulator that enhances endothelial cell function and revascularization processes. J Biol Chem 289: 14132-14144, 2014.
24. von Kodolitsch Y, Ito WD, Franzen O, Lund GK, Koschyk DH and Meinertz T: Coronary artery anomalies. Part I: Recent insights from molecular embryology. Z Kardiol 93: 929-937, 2004.
25. Kupatt C, Horstkotte J, Vlastos GA, Pfosser A, Lebherz C, Semisch M, Thalgott M, Büttner K, Browarzyk C, Mages J, *et al*: Embryonic endothelial progenitor cells expressing a broad range of proangiogenic and remodeling factors enhance vascularization and tissue recovery in acute and chronic ischemia. FASEB J 19: 1576-1578, 2005.
26. Reyes-Gordillo K, Shah R, Popratiloff A, Fu S, Hindle A, Brody F and Rojkind M: Thymosin-beta4 (Tbeta4) blunts PDGF-dependent phosphorylation and binding of AKT to actin in hepatic stellate cells. Am J Pathol 178: 2100-2108, 2011.
27. Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt RE, Ingwall JS and Dzau VJ: Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 20: 661-669, 2006.
28. Jo JO, Kim SR, Bae MK, Kang YJ, Ock MS, Kleinman HK and Cha HJ: Thymosin beta4 induces the expression of vascular endothelial growth factor (VEGF) in a hypoxia-inducible factor (HIF)-1alpha-dependent manner. Biochim Biophys Acta 1803: 1244-1251, 2010.
29. Carmeliet P and Tessier-Lavigne M: Common mechanisms of nerve and blood vessel wiring. Nature 436: 193-200, 2005.