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Thymosin β4 promotes glucose-impaired endothelial progenitor cell function via Akt/endothelial nitric oxide synthesis signaling pathway

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Abstract. Circulating endothelial progenitor cells (EPCs) are a subtype of hematopoietic stem cells, which can differentiate into endothelial cells and restore endothelial function. However, high glucose decreases the number and impairs the function of EPCs. A previous study showed that thymosin β4 (Tβ4), a pleiotropic peptide beneficial for multiple functions of various types of cells, could promote EPC migration and dose-dependently upregulate the phosphorylation of Akt and endothelial nitric oxide synthesis signaling (eNOS). In present study, the hypothesis that Tβ4 can improve glucose-suppressed EPC functions via the Akt/eNOS signaling pathway and restores the production of nitric oxide (NO) is investigated. EPCs were isolated from the peripheral blood of healthy volunteers and formed a cobblestone shape after 3-4 weeks of cultivation. Then, EPCs were treated with high concentrations of glucose (25 mM) for 4 days and administrated with Tβ4 for further study. Transwell migration and tube formation assays were performed to access the migratory and angiogenic ability of EPCs. In addition, the quantity of Akt, eNOS and the concentration of nitric oxide (NO) was investigated. Functional studies showed that high concentrations of glucose significantly suppressed EPC function, while this adverse effect was reversed by the administration of Tβ4. In addition, Akt small interfering (si)RNA and eNOS siRNA were demonstrated to reduce the protective effect of Tβ4 against glucose-impaired EPC functions. These findings suggest that Tβ4 improves glucose-impaired EPC functions via the Akt/eNOS signaling pathway.

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Introduction

Diabetes mellitus is associated with an increased risk of ischemic cardiovascular diseases, including coronary heart disease and stroke, which seriously jeopardize human health (1). The mechanism of this association is complicated. Vascular injury, occlusion or degradation caused by endothelial dysfunction in hyperglycemia may serve an important role. Accumulating evidence suggests that circulating endothelial progenitor cells (EPCs) can promote reendothelialization and restore endothelial function (2,3). Nevertheless, the number of EPCs is decreased in type I and type II diabetes (4,5). In addition, EPC functions, such as proliferation, migration and angiogenesis, are severely suppressed in diabetes (6,7). Therefore, ameliorating the impaired functions of EPCs is urgent and critical for EPC-based cell therapy, particularly with regard to ischemic cardiovascular disease associated with diabetes.

Thymosin β4 (Tβ4) is a pleiotropic peptide serving various roles in cell migration (8,9), angiogenesis (10), apoptosis (11) and inflammation (12). Recent studies have suggested that Tβ4 can exert cardiovascular protective effects by enhancing the proliferation, migration and angiogenesis of a variety of stem or progenitor cells (9,13-15). Previous studies by the authors of the present study demonstrated that Tβ4 could promote EPC migration, prevent EPCs from serum deprivation-induced apoptosis and reduce the senescence of EPCs *in vitro* (11,16,17). However, whether Tβ4 can improve glucose-impaired EPC function, and the underlying mechanism associated with this, remain unclear. In there present study, the effect of Tβ4 on glucose-impaired EPC function is investigated, and the potential signaling pathway involved is discussed.

Materials and methods

Cell culture. Mononuclear cells were isolated from the peripheral blood of healthy donors by density-gradient centrifugation using Ficoll separating solution (Cedarlane Laboratories, Ltd., Hornby, Canada). Then, $1x10^7$ mononuclear cells were plated on fibronectin (Chemicon, Temecula, CA, USA)-coated plates

and incubated with endothelial growth medium-2 (EGM-2 MV; Clonetics, Walkersville, MD, USA). After 3 days of culturing, non-adherent cells were removed by washing with phosphate buffered saline (PBS; 0.2 M, pH 7.4) and new medium was applied. A number of cells grew into EPCs which exhibited a 'cobblestone' shape after 3-4 weeks. The EPCs were detached by trypsin and collected for the following experiments.

EPC migration assay. EPC migration was assessed using a transwell migration assay (3422; Costar, Cambridge, MA, USA). A total of $5x10^4$ cells were re-suspended in 100 μl serum-free endothelial basal medium-2 (EBM-2; Clonetics) and placed in the upper chambers of 24-well transwell plates with 8 μm-pore membranes. Then, 600 μl EGM-2 supplemented with 10% bovine serum albumin (Amresco LLC, Solon, OH, USA) was placed in lower chambers. After 6 h of incubation at 37˚C, the upper side of membrane was wiped gently with cotton swabs to remove the cells that had not migrated. The migrated cells were fixed in 4% paraformaldehyde and stained using 4'6-diamidino-2-phenylindole (Roche Applied Science, Indianapolis, IN, USA). Migrated cells were counted with a fluorescence microscope at x400 magnification and 5 random fields were picked for counting. The average cell number of these 5 fields was considered as the cell migration number for each group.

EPC tube formation assay. Tube formation was performed with Matrigel matrix basement membrane (BD Biosciences, Franklin Lakes, NJ, USA) to evaluate the ability of EPC angiogenesis. Matrigel solution was thawed at 4˚C overnight, diluted with EBM-2 and added in a 96-well plate at 37˚C for 1 h to solidify. EPCs were collected with trypsin and re-suspended with EGM-2 MV medium, then $2x10^4$ EPCs were placed on the matrix for further incubation at 37˚C for 6-8 h. Tube formation was observed with a light microscope (magnification, x100) and 5 random fields were chosen for each assay. The average tube formation counted by branch point numbers was compared in different groups by Image Pro Plus version 6.0 (Media Cybernetics, Warrendale, PA, USA).

Small interfering RNA (siRNA) transfection. siRNA against Akt and eNOS were synthesized by GenePharma Co., Ltd. (Shanghai, China) and a nonsense sequence was designed as a negative control. The effective Akt siRNA sequence was as follows: Sense, 5'-GCACUUUCGGCAAGGUGAUTT-3' and antisense, 5'-AUCACCUUGCCGAAAGUGCTT-3'. The effective eNOS siRNA sequence was as follows: Sense, 5'-CAGUACUACAGCUCCAUUATT-3' and antisense, 5'-UAAUGGAGCUGUAGUACUGTT-3'. Then, $4x10^5$ EPCs were placed in 6-well plates, and when cells reached 60-80% confluence, they were transfected with Akt and eNOS siRNA and cultured for 24 h, then incubated with a high concentration of glucose (25 mM) for 4 days (except for the control group), and with or without Tβ4 (ProSpec-Tany TechnoGene, Ltd., Rehovot, Israel) for the last 24 h. The effectiveness of Akt and eNOS knock-down was determined by western blotting.

Western blots analysis and measurement of nitric oxide (NO). Total protein from EPCs was extracted with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentration was measured using a BCA assay kit (P0012; Beyotime Institute of Biotechnology). Equal quantities of protein (20 μg) were run on a 10% tris-glycine gradient gel, then transferred to polyvinylidene difluoride membranes and blocked with 5% non-fat milk in Tris-buffered solution containing 0.1% Tween 20 (TBST) for 1 h at 25˚C. Membranes were then incubated with primary antibodies at 4˚C overnight and with secondary antibodies at room temperature for 1-2 h. Membranes were washed three times with TBST for 15 min before and after the incubations. Finally, proteins were visualized with enhanced chemiluminescence reagent (Lianke Biotechnology, Hangzhou, China) and exposed to Image Quant LAS-4000 (Fujifilm, Tokyo, Japan). Grayscale of the bands were measured by Multi-Gauge 3.0 image analysis software (Fujifilm). Antibodies including anti-phospho-Akt-Ser^{473} (1:1,000; 3787), anti-Akt (1:1,000; 9272), anti-phospho-endothelial nitric oxide synthase (eNOS)-Ser^{1177} (1:1,000; 9571), anti-eNOS (1:1,000; 9572) and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; 7074) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). After incubation of EPCs with or without Tβ4 in glucose for 4 days, the conditioned medium was examined for NO using Griess regent (Beyotime Institute of Biotechnology).

Statistical analysis. All experiments were performed for at least three individual experiments. Statistical analysis was performed using SPSS version 19.0 software (IBM SPSS, Amronk, NY, USA), using one-way analysis of variance nad post-hoc least standard difference analysis. Results are presented as the mean ± standard error. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Tβ4 ameliorates glucose-impaired EPC migration and angiogenesis. It was investigated whether the administration of Tβ4 could improve glucose-impaired EPC functions. As shown in Fig. 1A, incubation of EPCs with medium with a high concentration of glucose (25 mM) for 4 days could significantly suppress EPC migration ($P<0.01$ vs. the control group). However, treatment with Tβ4 for 24 h after the exposure of EPCs to high glucose conditions could improve glucose-suppressed EPC migration in a concentration-dependent manner; the maximal pro-migratory dose was observed at 1,000 ng/ml ($P<0.01$ vs. the glucose group; Fig. 1A). In addition, high concentrations of glucose significantly impaired EPC tube formation ability ($P<0.01$ vs. the control group; Fig. 1B). Treatment with Tβ4 for 24 h after glucose exposure ameliorated glucose-suppressed EPC tube formation ability in a concentration-dependent manner; the maximal pro-angiogenic dose was observed at 1,000 ng/ml ($P<0.01$ vs. the glucose group; Fig. 1B).

Tβ4 restores glucose-inhibited Akt/eNOS phosphorylation and NO production. High concentrations of glucose can inhibit Akt and eNOS activation and reduce NO production in EPCs (18). A previous study indicated that Tβ4 may activate the phosphorylation of Akt and eNOS (16,17). Therefore, the

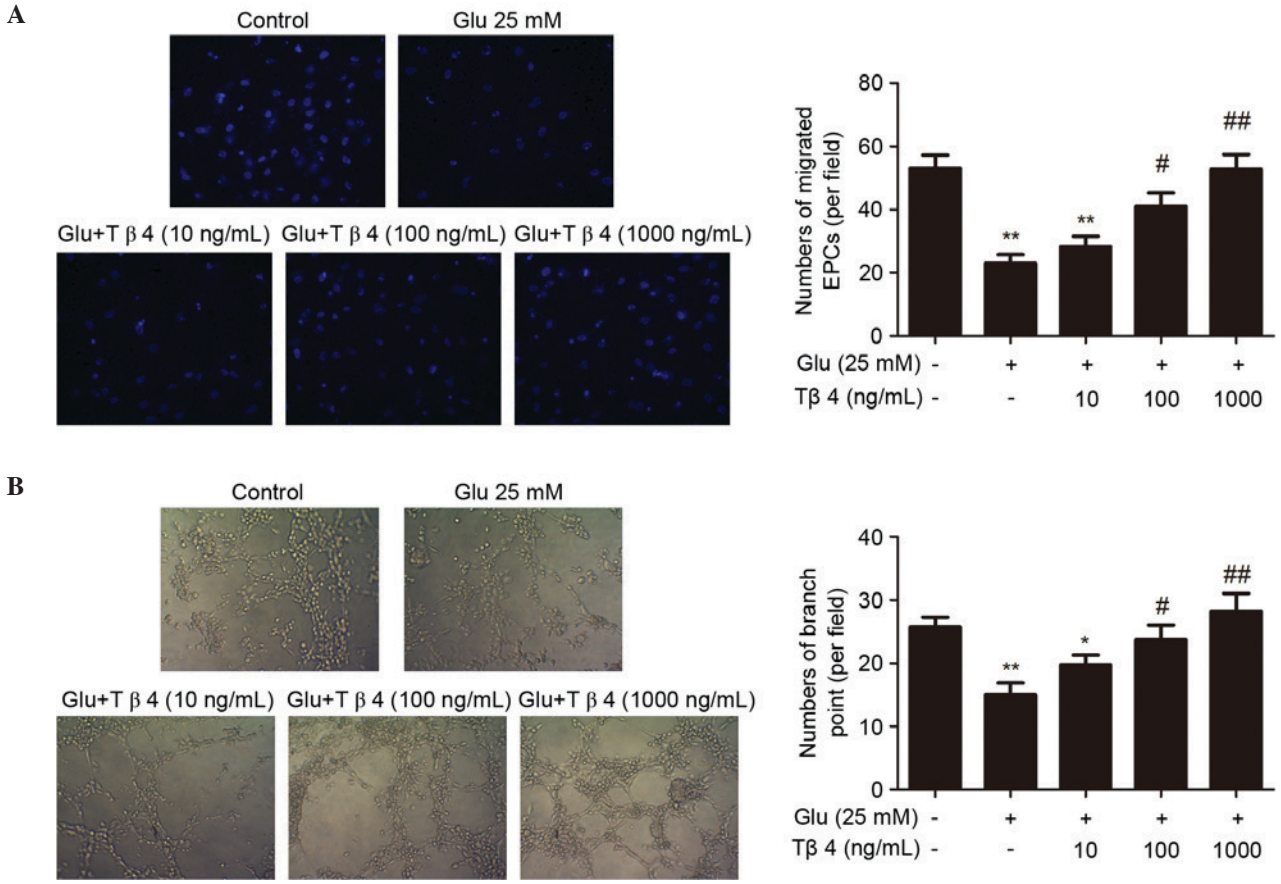

Figure 1. Tβ4 restores glucose-suppressed EPC migration and angiogenesis. Following treatment of EPCs with various concentrations of Tβ4 (10 ng/ml, 100 ng/ml and 1000 ng/ml) for 24 h, cultured EPCs were evaluated by using functional assays following incubation with high glucose medium for 4 days. (A) A transwell migration assay was used to assess the effect of Tβ4 on EPC migration in high concentrations of Glu. 4'6-diamidino-2-phenylindole staining was performed to determine the number of migrated EPCs (magnification, x400). (B) An *in vitro* tube formation assay was performed to investigate the effect of Tβ4 on EPC neovascularization in high concentrations of Glu. Number of branch points was counted to evaluate the effect of Tβ4 on EPC angiogenesis in high-glucose medium. (n=4). *P<0.05 and **P<0.01 vs. the control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the high-glucose group. Tβ4, thymosin β4; EPC, endothelial progenitor cell; Glu, glucose.

effects of Tβ4 on glucose-exposed EPCs were investigated in order to determine whether Tβ4 can restore glucose-inhibited Akt and eNOS phosphorylation in EPCs. As shown in Fig. 2A, high concentrations of glucose (25 mM) dramatically down-regulated the phosphorylation of Akt at Ser^{473} and eNOS at Ser^{1177} in EPCs, while there was no significant change in total Akt and eNOS expression. The inhibition of eNOS phosphorylation led to reduced NO production of EPCs (Fig. 2B). However, administration of Tβ4 in the glucose medium for 24 h concentration-dependently upregulated the phosphorylation of Akt and eNOS, and augmented NO production (Fig. 2).

Akt/eNOS signaling pathway is involved in the amelioration of glucose-impaired EPC functions by Tβ4. To identify the role of the Akt/eNOS signaling pathway in the beneficial effect of Tβ4 on glucose-impaired EPC functions, EPCs were transfected with Akt siRNA and eNOS siRNA to decrease Akt and eNOS activity, respectively. As shown in Fig. 3, administration of Akt and eNOS siRNA (50 nM) markedly inhibited the benefit of Tβ4 in improving EPC migration and tube formation, suggesting that the Akt/eNOS pathway accounts for Tβ4-induced improvement of glucose-suppressed EPC functions.

Discussion

Previous studies performed by the authors of the present study have demonstrated that Tβ4 may exert an important role in enhancing EPC functions, including promoting migration, inhibiting apoptosis and senescence (11,16,17). However, the capacity of Tβ4 to reverse impaired EPC functions in some pathological conditions remains unclear. The present study demonstrated that Tβ4 could reverse glucose-impaired EPC functions in a dose-dependent manner and elucidated that the beneficial effect of Tβ4 may be associated with the Akt/eNOS signaling pathway and NO-related mechanisms.

At present, diabetes mellitus has become a common diseases worldwide, which is associated with poor clinical outcomes and premature morality due to its cardiovascular complications (19,20). Several studies have suggested that the imbalance between endothelial injury and repair is a key process of diabetes-associated cardiovascular complications (21,22). Endothelial function is impaired in diabetes and the complication of vascular occlusion in diabetic patients has been attributed to compromised collateral vessel formation due to diminished capacity of mature endothelial cells (1). EPCs have proliferating potential, differentiating into endothelial cells and promoting regeneration and angiogenesis of

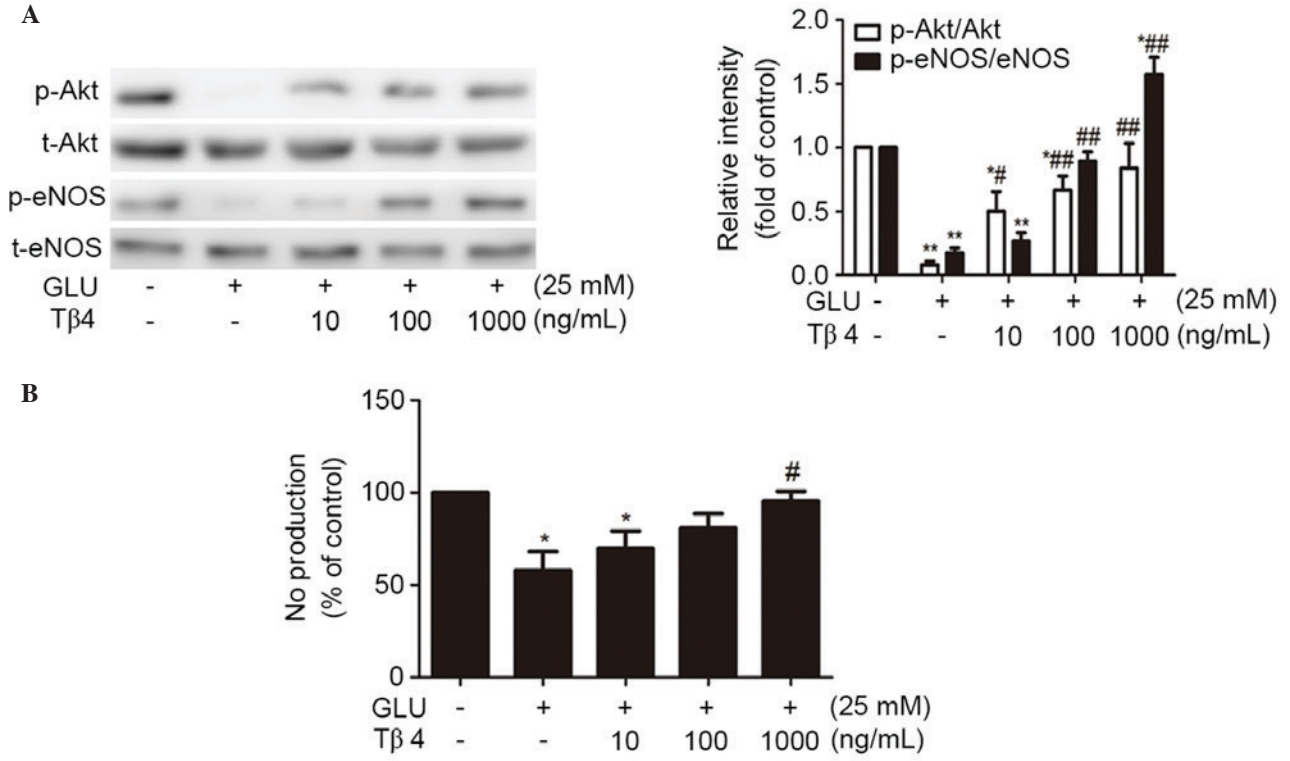

Figure 2. Tβ4 recovers glucose-inhibited EPC Akt/eNOS activation and NO production. (A) Effects of Tβ4 on the expression and phosphorylation of Akt and eNOS, examined in EPCs by western blotting. (B) Nitrate production was measured by Griess reagent. (n=4). *P<0.05 and **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the high-glucose group. Tβ4, thymosin β4; EPC, endothelial progenitor cell; Glu, glucose; NO, nitric oxide; eNOS, endothelial NO synthesis; p-eNOS, phosphorylated eNOS t-eNOS, total eNOS; p-Akt, phosphorylated-Akt.

endothelial cells, which serves an important role in maintaining endothelial function and integrity, as well as post-natal neovascularization (23). Interestingly, emerging evidence has showed that post-natal neovascularization in adults may be involved with EPCs directly (24). Hence, EPCs could be a potential method for cardiovascular therapy. However, it has been revealed that the number and function of EPCs may be reduced by several cardiovascular risk factors, such as hypertension, hyperglycemia and hypercholesterolemia (25). Reduced numbers and suppressed function of EPCs can be associated with endothelial dysfunction, impaired angiogenesis and decreased compensational collateralization in occlusive vascular diseases (26). Type I and type II diabetes are both associated with decreased numbers and impaired function of EPCs. Therefore, strategies to improve the suppressed functions of EPCs is critical and beneficial for EPC-based cell therapy, particularly in treating diabetic patients.

In our present study, the functions of circulating EPCs is suppressed in high concentrations of glucose, and Tβ4 was demonstrated to reverse the dysfunction of EPCs. Tβ4 is a polypeptide originally isolated from calf thymus (27). Previous studies have observed that Tβ4 can modulate various physiological and pathological process, such as tissue development, wound healing, cell survival and vessel formation (10,28-30). Previous studies by the authors of the present study have demonstrated that Tβ4 was able to enhance EPC migration, inhibit EPC apoptosis and senescence *in vitro* (11,16). In addition, Tβ4 has been reported to enhance the angiogenesis of endothelial cells (31). Furthermore, Tβ4 exerts excellent therapeutic effects against glucose damage. Tβ4 is able to abolish glucose-suppressed capillary-like tube formation of endothelial cells and promote the recovery of neurological function in diabetic peripheral neuropathy (32). Kim and Kwon found that Tβ4 could ameliorate glucose-injured high-glucose-injured human umbilical vein endothelial cell function through the insulin-like growth factor-1 signaling pathway (33). According to the evidence above, the present study further revealed the beneficial effects of Tβ4 on human EPCs in high concentrations of glucose, which may provide novel insights into its potential application for cardiovascular protection and therapy in clinical cases.

The Akt/eNOS signaling pathway has been reported to be a critical pathway that regulates cell migration and angiogenesis (34). As for EPCs, previous research has demonstrated that exercise and some medications, such as statins, could increase EPC number, proliferation and migration through the activation of the Akt protein (35). The stimulation of Akt protein kinase can further activate eNOS (36), which is essential and pivotal for the mobilization of stem and progenitor cells (37). A number of studies documented that hyperglycemia could affect various functions of different kinds of cells through the Akt/eNOS signaling pathway. Sun *et al* (38) identified that vaspin, a type of adipose factor, could alleviate EPC dysfunction caused by high concentrations of glucose via the PI3K/Akt/eNOS signaling pathway. Chen *et al* (18) indicated that high concentrations of glucose impaired EPC proliferation, migration and angiogenesis by down-regulating the phosphorylation Akt and eNOS, and NO production (18). The authors of the present study have previously revealed that Tβ4 promotes EPC migration and inhibits EPC apoptosis

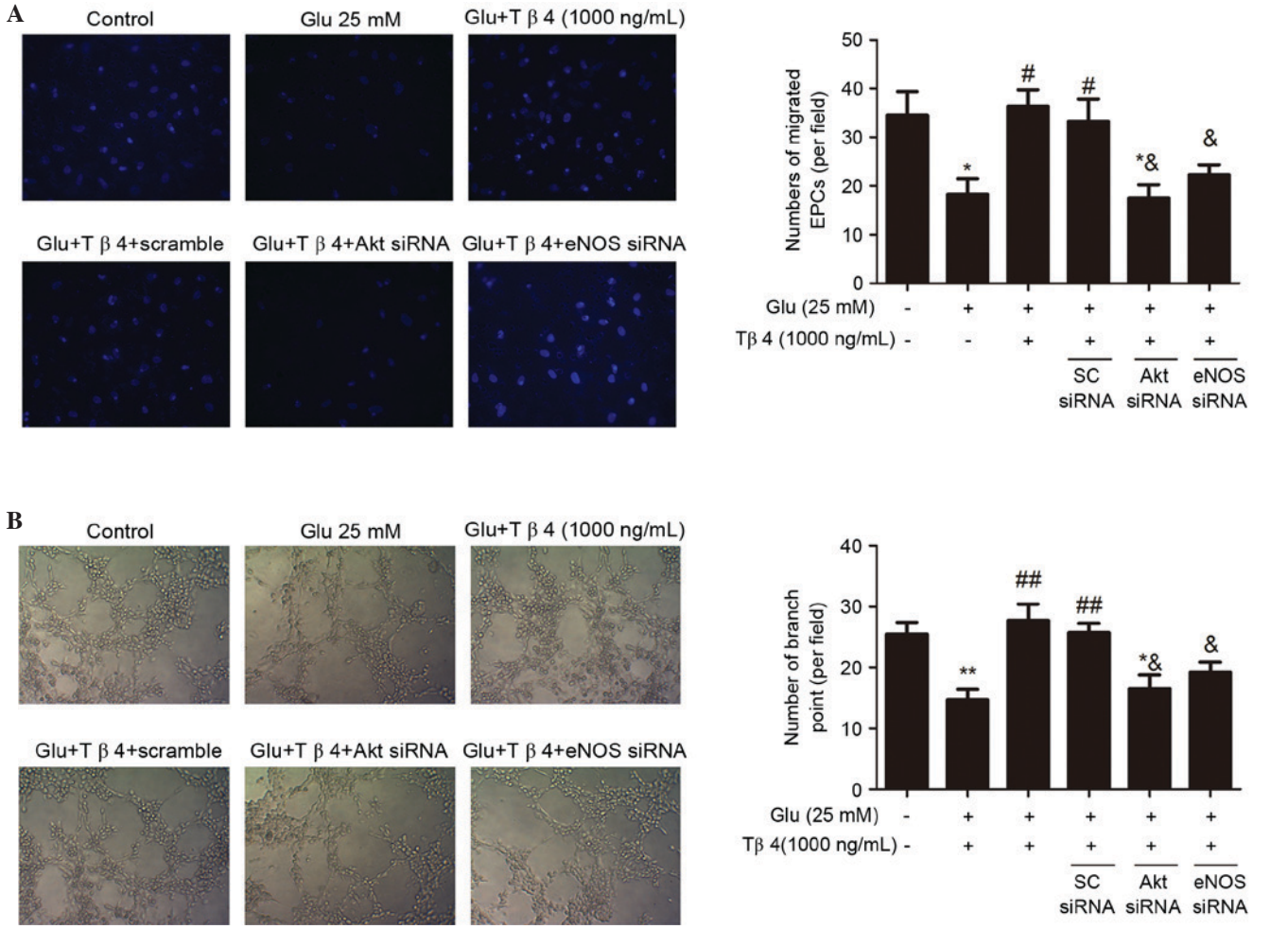

Figure 3. Tβ4 improves glucose-impaired EPC functions via the Akt/eNOS signaling pathway. (A) Tβ4 (1000 ng/ml) was administrated to EPCs 4 days after incubation with high concentrations of glucose. EPCs were pretreated with or without Akt and eNOS siRNA (50 nM) 24 h prior to the administration of Tβ4 (magnification, x400). (B) An *in vitro* angiogenesis assay was performed to determine the effect of Tβ4 on EPC angiogenesis in high concentrations of glucose following the ablation of Akt and eNOS activity by Akt and eNOS siRNA. (n=4; magnification, x100). *P<0.05 and **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the high-glucose group; $^{\&}$P<0.05 vs. the high-glucose + Tβ4 group. Tβ4, thymosin β4; EPC, endothelial progenitor cell; Glu, glucose; eNOS, endothelial NO synthesis; SC, scramble; siRNA, small interfering RNA.

through the PI3K/Akt/eNOS signaling pathway (11,16), therefore, it was postulated that this pathway may account for the Tβ4-mediated improvement of glucose-suppressed EPC functions.

In present study, it was demonstrated that treatment with Tβ4 could provoke Akt and eNOS activity, and enhance the migration and tube formation of EPCs in high concentrations of glucose. These beneficial effects were blocked by the siRNA of Akt and eNOS. These findings suggest that Tβ4 can ameliorate glucose-impaired EPC functions through the Akt/eNOS signaling pathway. According the findings above, Tβ4 may be beneficial for maintaining EPC functions in high concentrations of glucose, which is helpful for understanding the therapeutic potential of Tβ4 for patients with diabetes and cardiovascular complications.

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