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Thymosin β4 regulates endothelial cell function via activating the AKT pathway.

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Key words: thymosin β4; HUVECs; proliferation; migration; apoptosis; AKT signaling.

Abstract. The vascular eendothelial cells are highly heterogeneous and associated with numerous diseases. Thymosin β4 (Tβ4) plays pleiotropic roles in endothelial cell differentiation, migration and angiogenesis. However, the underlying mechanisms played by Tβ4 in the regulation of endothelial cells have not yet been well investigated. In the present study, Tβ4 -GFP adenovirus, transfected into human umbilical vein endothelial cells (HUVECs), and cell morphology were analyzed by fluorescence microscopy. ELISA was used to determine the concentration of Tβ4 expression. Furthermore, the effects of Tβ4 overexpression on HUVECs proliferation, apoptosis and migration were investigated. Real-time quantitative PCR and western blot were conducted to examine mRNA and protein expression in HUVECs with Tβ4 overexpression. Moreover, the underlying molecular mechanism of Tβ4 in HUVECs function was tested through treatment with LY294002, a PI3K/AKT inhibitor. Overexpression of Tβ4 increased the cell ability of HUVECs, and up-regulated the expression of the proliferation markers PCNA and Cyclin D1. In addition, overexpression of Tβ4 reduced HUVECs apoptosis, both under normoxic and hypoxic conditions. Moreover, overexpression of Tβ4 increased the ability of HUVECs to migrate through the membrane and up-regulated levels of MMP-2 and MMP-9. The use of LY294002 decreased the p-AKT (Ser473) level, which was induced by Tβ4 overexpression. Importantly, LY294002 reduced Tβ4-induced HUVECs proliferation and migration. In conclusion, our results suggest that Tβ4 is a major regulator of HUVECs function by activating the AKT signaling pathway.

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La timosina β4 regula la función de las células endoteliales, activando la vía AKT.

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Palabras clave: timosina β4; HUVEC; proliferación; migración; apoptosis; señalización de AKT.

Resumen. Las células endoteliales vasculares son muy heterogéneas y están asociadas con numerosas enfermedades. La Tβ4 desempeña papeles pleiotrópicos en la diferenciación, migración y angiogénesis de células endoteliales. Sin embargo, los mecanismos fundamentales que realiza la Tβ4 en la regulación de las células endoteliales aún no han sido bien investigados. En el presente estudio se analizaron el adenovirus Tβ4 –GFP transfectado en las células endoteliales de vena umbilical humana (HUVEC) y la morfología celular, mediante microscopía de fluorescencia. ELISA se utilizó para determinar la concentración de la expresión de Tβ4. También, se investigaron los efectos de la sobrexpresión de la Tβ4, sobre la proliferación de las HUVEC, la apoptosis y la migración. Se realizaron la PCR cuantitativa en tiempo real y el Western blot para examinar el mRNA y la expresión de proteínas en las HUVEC con sobreexpresión de Tβ4. Además, se probó el mecanismo molecular subyacente de Tβ4 sobre la función de las HUVEC mediante el tratamiento con LY294002, un inhibidor de la PI3K/AKT. La sobreexpresión de Tβ4 aumentó la capacidad celular de las HUVEC, regulando al alza la expresión de los marcadores de proliferación PCNA y Ciclina D1. Además, la sobreexpresión de Tβ4 redujo la apoptosis de las HUVEC, tanto en condiciones normóxicas como hipóxicas. Por otra parte, la sobreexpresión de Tβ4 aumentó la capacidad de las HUVEC a migrar a través de la membrana y reguló hacia arriba los niveles de MMP-2 y MMP-9. El uso del LY294002 disminuyó el nivel de p-AKT (Ser473), que fue inducido por la sobreexpresión de Tβ4. Es importante destacar que LY294002 bajó la proliferación y la migración de las HUVEC inducidas por Tβ4. En conclusión, nuestros resultados sugieren que Tβ4 es un regulador principal de la función de las HUVEC mediante la activación de la vía de señalización de AKT.

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INTRODUCTION

The vascular endothelial cells are highly heterogeneous and associated with numerous diseases, such as atherosclerosis (1). Atherosclerosis causes stroke and coronary heart disease (2), aging induced cardiovascular disease (3), diabetes mellitus (4), vascular injuries induced by limb ischemia and reperfusion (5), etc. However, the underlying mechanism of endothelial cell proliferation, apoptosis and migration has not been fully elucidated.

Thymosin beta 4 (Tβ4) was first isolated from calf thymus and it is a highly conserved G-actin-sequestering peptide (6). It has been

reported that Tβ4 is expressed in various cell types, such as endothelial cells, and plays pleiotropic roles in endothelial cell differentiation, migration, and angiogenesis (7-9). Smart *et al.* (10) found that Tβ4 knockdown reduced the coronary vasculogenesis and angiogenesis by a significant reduction in the pro-angiogenic cleavage product N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) in mice heart. Tβ4, which was secreted from the myocardium, promoted epicardium-derived cells inward migration and differentiation into endothelial cells, and further to form the coronary vasculature (11). Tβ4 could activate mast cells to produce angiogenesis associated factors, such as VEGF, and stimulate endothelial cell migration and differentiation (12). Although the pieces of evidence mentioned above demonstrated beneficial roles of Tβ4 for cardiac disease treatment by regulating endothelial cell function, the underlying mechanisms played by Tβ4 have not yet been well considered. In this study, we aimed to investigate the role of Tβ4 in the regulation of HUVECs proliferation, apoptosis, and migration through the AKT signaling pathway.

MATERIALS AND METHODS

Cell culture

Human HUVECs were obtained from Nanjing KeyGen Biotech Co. Ltd. (Nanjing, China) and cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), streptomycin and penicillin (Nanjing KeyGen Biotech Co. Ltd.) at 37°C under a humidified 5% CO_2 atmosphere. The hypoxic condition was made in a sealed chamber with a gas mixture containing 5% CO_2, 92% N_2, and 3% O_2.

Cell transfection

TB4-GFP adenovirus (Ad-Tβ4) and its negative control (Ad-NC) were obtained from GenePharma Co., Ltd. (Shanghai, China). Logarithmic growth phase HUVECs were seeded into 6-well plates (8×10^3 cells/well) and then infected with Ad-Tβ4 or Ad-NC at an MOI of 25. The efficiency of infection was assessed by green fluorescence protein (GFP), and expression of GFP in HUVECs was confirmed by fluorescence microscopy.

The enzyme-linked immunosorbent assay (ELISA)

The concentration of Tβ4 in supernatant fluid of cultured HUVECs were analyzed by a human Tβ4 ELISA kit (Jingkang Bioengineering Co., Ltd., Shanghai, China) according to the manufacturer's instructions.

Cell proliferation assay

To investigate the effect of Tβ4 up-regulation on HUVECs, an MTT assay was designed. In brief, 5×10^4 HUVECs were seeded into 96-well plates and were infected with Ad-Tβ4 or Ad-NC for 24 h. Then 20 μL of 5 mg/mL [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)] (Amresco, Washington, USA) in PBS was added and incubated at 37°C for 4 h. Subsequently, 150 μL dimethylsulfoxide (DMSO; Sigma, USA) was added to each well to dissolve the formazan product. The absorbance was measured at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell apoptosis assay

To explore the effect of Tβ4 on HUVECs apoptosis, 1×10^6 HUVECs cells were collected, washed with PBS, and resuspended in 100 μL binding buffer, containing 5 μL Annexin V-APC and 5 μL 7-AAD (BD Biosciences, Franklin Lakes, NJ, USA) at room temperature. Then, the cell apoptosis was tested using a flow cytometer (BD FACSCalibur, BD Biosciences, CA, USA). Four subpopulations were divided in figure from flow-cytometric analysis: normal cells (Annexin V-APC−/7-AAD−), necrotic cells (Annexin V-APC−/7-AAD+), early apoptotic (Annexin V-APC+/7-AAD−) and late apoptotic (Annexin V-APC+/7-AAD+). Apoptosis index was the total rates of early apoptotic and late apoptotic cells.

Cell migration assay

For cell migration assay, the Transwell cell culture chambers with 8-μm pore polycarbonate membrane filters (Millipore, Billerica, MA, USA) were used. Approximately 5×10^5 HUVECs, which mixed in FBS-free RPMI-1640, were seeded into the upper chamber, whereas the lower chamber was filled with RPMI-1640 containing 5% FBS. After 24 h, non-migrated cells from the upper chamber were removed; and migrated cells to the bottom side of the membrane were fixed with 90% alcohol and stained with crystal violet. The migrated cells were counted under a light microscope with 200-fold magnification.

RNA isolation and real-time quantitative PCR

Total RNA from cultured HUVECs was extracted using a miRNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed into cDNA by MuLV reverse transcriptase (NEB, USA). Real-time quantitative PCR (RT-qPCR) analysis was performed using Fast SYBR Green (Applied Biosystems, Forster City, CA, USA). Details of the specific RT-qPCR primers to determine relative levels of gene expression are shown in Table I.

Western Blot

HUVECs were lysed with the RIPA lysis buffer (Sigma, USA) according to the manufacturer's instruction. Equal protein was separated by using the 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). The membranes were blocked with 5% skim milk, and probed overnight at 4°C using the following primary antibodies: anti-PCNA (Abcam, MA, USA), anti-Cyclin D1 (Abcam, MA, USA), anti-MMP-2 (Cell Signaling Technology, Beverly, MA, USA), anti-MMP-9 (Cell Signaling Technology, Beverly, MA, USA), anti-cleaved caspase-3 (Abcam, MA, USA), anti-AKT (Cell Signaling Technology, Beverly, MA, USA), anti-p-AKT (Ser473) (Cell Signaling Technology, Beverly, MA, USA), and GAPDH (Abcam, MA, USA). Then, the membrane was incubated with HRP-labelled secondary antibody (Abcam, MA, USA) and visualized using an enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis

The data in this study were analyzed by Graphpad Prism 5.0 software and displayed as mean ± standard deviation (SD). The Stu-

TABLE I
PRIMER SEQUENCES USED IN RT-qPCR EXPERIMENTS.

Gene name	Primer sequence (5'→3')
PCNA	Forward: CCTGCTGGGATATTAGCTCCA
	Reverse: CAGCGGTAGGTGTCGAAGC
Cyclin D1	Forward: GCTGCGAAGTGGAAACCATC
	Reverse: CCTCCTTCTGCACACATTTGAA
MMP-2	Forward: TGACTTTCTTGGATCGGGTCG
	Reverse: AAGCACCACATCAGATGACTG
MMP-9	Forward: TGTACCGCTATGGTTACACTCG
	Reverse: GGCAGGGACAGTTGCTTCT
GAPDH	Forward: GGAGCGAGATCCCTCCAAAAT
	Reverse: GGCTGTTGTCATACTTCTCATGG

dent's t-test was used to compare the means between the two groups. A value of $p<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Tβ4 overexpression promotes HUVECs proliferation

To explore the role of Tβ4 in the regulation of HUVECs function, we infected HUVECs with Ad-Tβ4 or Ad-NC (Fig. 1A). An ELISA analysis was conducted to verify the transfection efficiency, and the results showed that Ad-Tβ4 significantly elevated Tβ4 expression in HUVECs (Fig. 1B).

The overexpression of Tβ4 significantly promoted HUVECs growth (Fig. 2A). Moreover, RT-qPCR and western blot analysis indicated that the mRNA and protein expression of PCNA and Cyclin D1, which are cell proliferation markers, significantly increased in HUVECs infected with Ad-Tβ4 (Fig. 2B-D). These data confirmed that Tβ4 promoted the growth of HUVECs.

Tβ4 overexpression reduces HUVECs apoptosis

To investigate whether Tβ4 could affect HUVECs apoptosis, HUVECs were stained with Annexin V, and followed by flow cytometry. As shown in Fig. 3A, HUVECs infected with Ad-Tβ4 showed less rate of cell apoptosis compared with cells infected with Ad-NC. The level of cleaved caspase-3 in HUVECs infected with Ad-Tβ4 was down-regulated when compared with HUVECs infected with Ad-NC (Fig. 3B). Also, the effect of Tβ4 on HUVECs apoptosis under hypoxic conditions was examined by flow cytometry. The apoptosis results showed that hypoxia significantly increased the rate of HUVECs apoptosis, whereas overexpression of Tβ4 decreased HUVECs apoptosis after exposure to hypoxic conditions (Fig. 3C). Moreover, overexpression of Tβ4 reduced the level of cleaved cas-

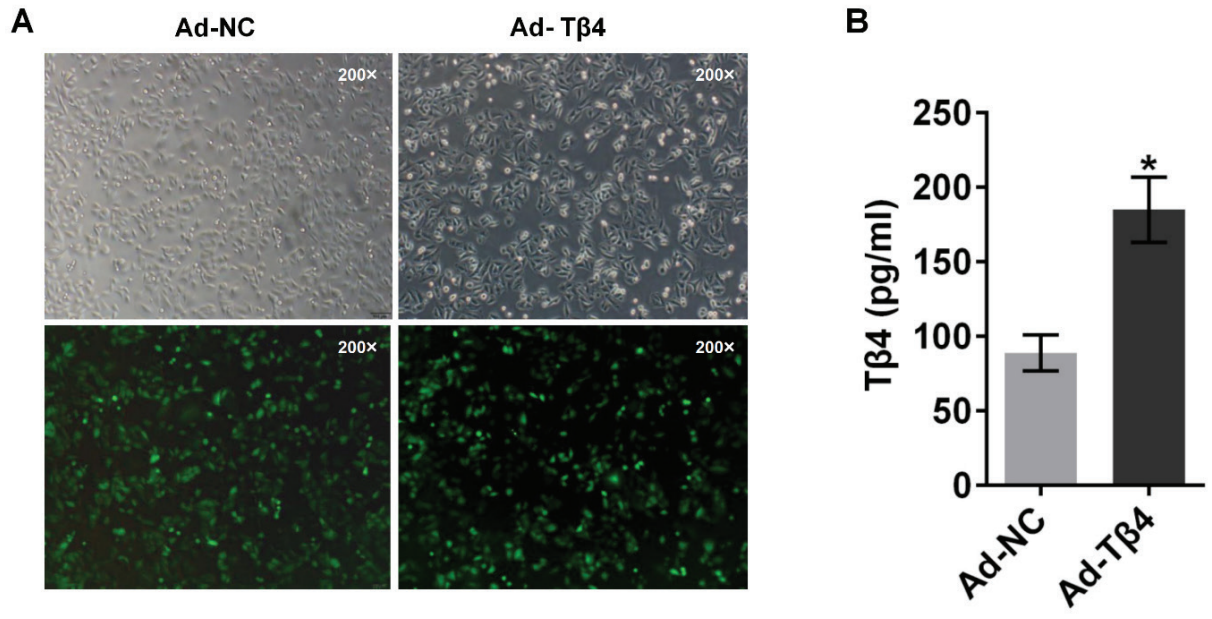

Fig.1. The level of Tβ4 was increased in HUVECs infected with Ad-Tβ4. (A) Morphology of HUVECs observed under a fluorescence microscope upper panel, bright field, 200×; lower panel, fluorescence field, 200×). (B) Tβ4 concentration determined by ELISA. Data are presented as means ± S.D. from 3 independent experiments. *$p<0.05$.

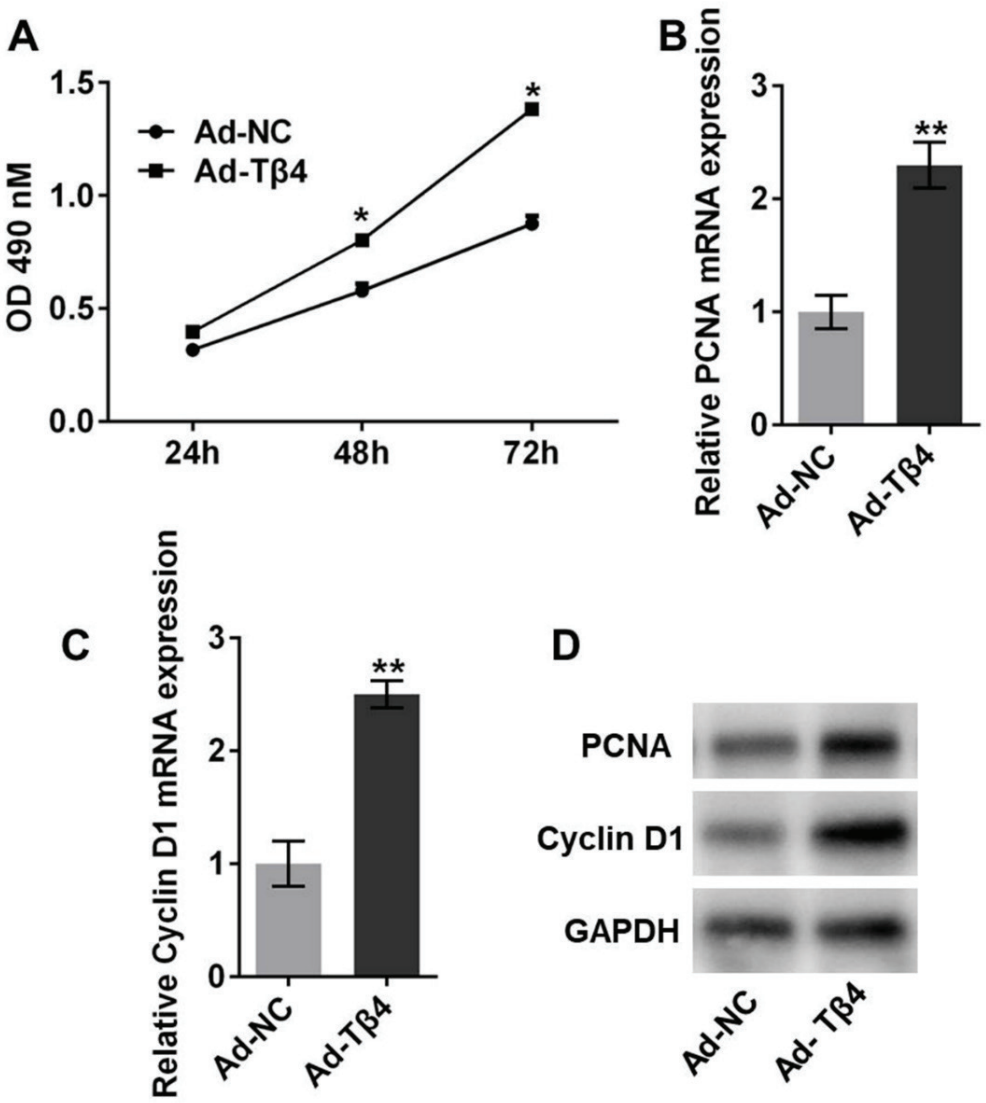

Fig. 2. Tβ4 overexpression promoted HUVECs proliferation. (A) CCK-8 analysis of the cell proliferation in HUVECs infected with Ad-NC or Ad-Tβ4. (B, C) The relative mRNA level of PCNA (B) and Cyclin D (C) in HUVECs infected with Ad-NC or Ad-Tβ4. (D) The protein level of PCNA and Cyclin D in HUVECs infected with Ad-NC or Ad-Tβ4. Data are presented as means ± S.D. from 3 independent experiments. $^{*}p<0.05$, $^{**}p<0.01$.

pase-3 in HUVECs after exposure to hypoxic conditions (Fig. 3D). Our data indicated that Tβ4 could inhibit HUVECs apoptosis both under normoxic and hypoxic conditions.

Tβ4 overexpression promotes HUVECs migration

To demonstrate the role of Tβ4 in HUVECs migration, a Transwell migration assay was conducted. As shown in Fig.4.A, overexpression of Tβ4 increased the ability of HUVECs to migrate through the membrane. MMPs are the main proteolytic enzymes that contribute to the degradation of the extracellular matrix and serve critical roles in the invasion process (13). RT-qPCR determined that Tβ4 overexpression up-regulated the mRNA and protein levels of MMP-2 and MMP-

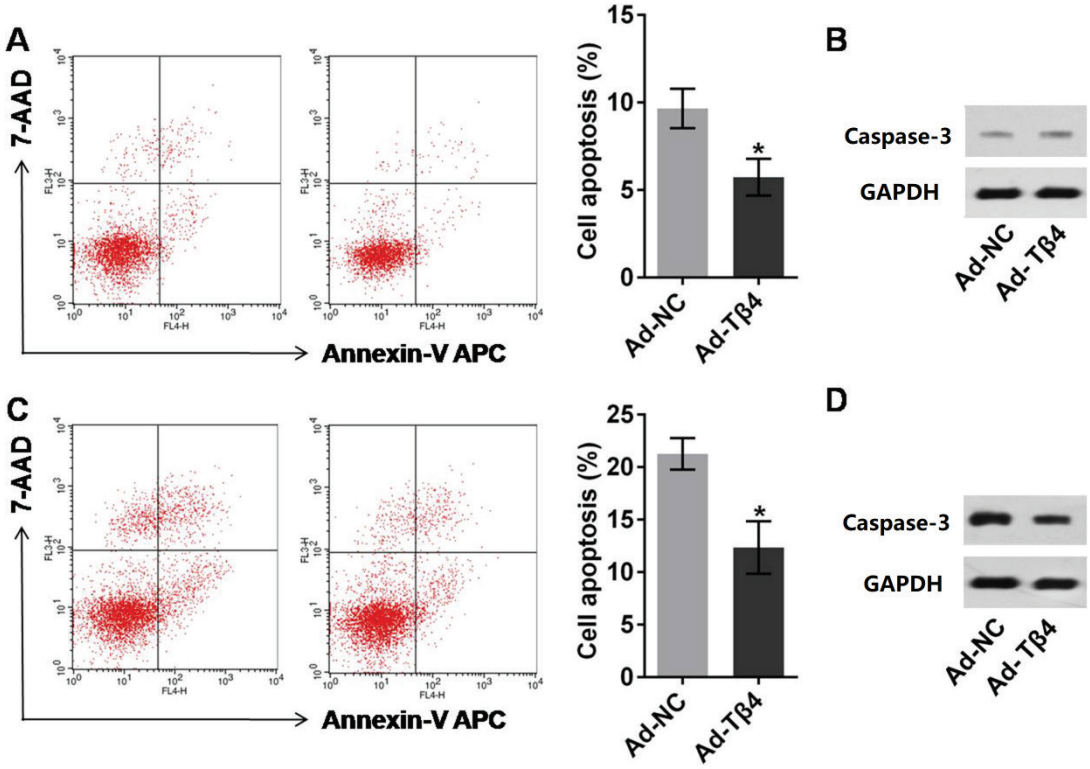

Fig. 3. Tβ4 overexpression reduced HUVECs apoptosis**.** (A) Scatter diagram and Histogram of apoptosis in HUVECs infected with Ad-NC or Ad-Tβ4. (B) The protein level of cleaved caspase-3 in HUVECs infected with Ad-NC or Ad-Tβ4. (C) Scatter diagram and Histogram of apoptosis in HUVECs infected with Ad-NC or Ad-Tβ4 under hypoxic conditions. (D) The protein level of cleaved caspase-3 in HUVECs infected with Ad-NC or Ad-Tβ4 under hypoxic conditions. Data are presented as means ± S.D. from 3 independent experiments. $^{*}p<0.05$.

9 (Fig. 4B and 4C). These data confirmed that Tβ4 remarkably promoted the HUVECs migration.

Tβ4 overexpression activates AKT signaling pathway

It has been reported that Tβ4 could regulate the migratory and proliferative activity of high glucose-treated HUVECs by activation of the AKT signaling pathway (14). Therefore, we hypothesized that overexpression of Tβ4 might regulate HUVECs function via the AKT signaling pathway. As shown in Fig. 5, western blot analysis indicated that the p-AKT (Ser473) level in HUVECs infected with Ad-Tβ4 was significantly increased. To evaluate the possibility that Tβ4 exerts its function through the AKT pathway, HUVECs were treated with LY294002, a PI3K/AKT inhibitor (15-18). After exposure to LY294002 (50 μmol/L (19)), the expression of the p-AKT (Ser473) level was significantly decreased, which was induced by Tβ4 overexpression (Fig. 5).

Inhibition of AKT signaling reduces Tβ4-induced HUVECs proliferation and migration

To demonstrate that the AKT signaling regulated Tβ4-induced proliferation and migration, the Ad-Tβ4 infected HUVECs were treated with LY294002. As shown in Fig. 6A, LY294002 reduced the cell numbers of HUVECs induced by Tβ4 overexpression.

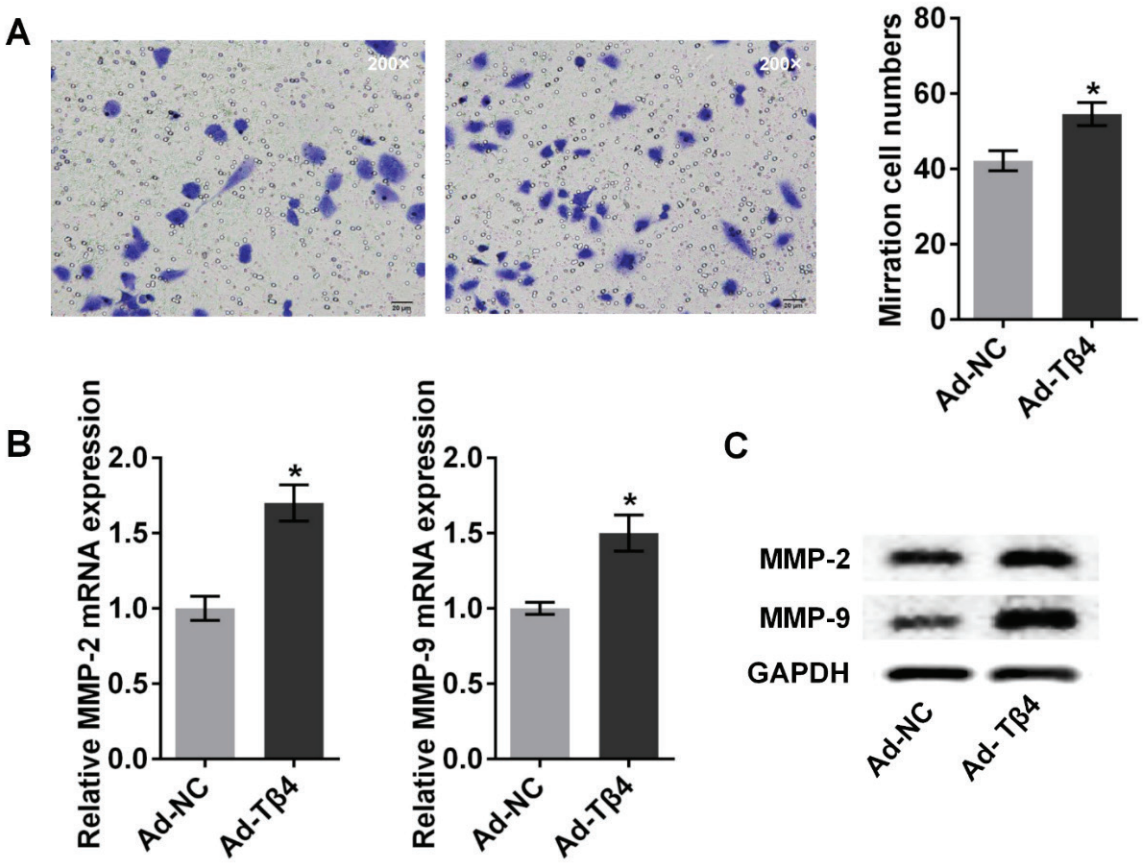

Fig. 4. Tβ4 overexpression promoted HUVECs migration. (A) The cell migratory ability was investigated when HUVECs were infected with Ad-NC or Ad-Tβ4 (200x). (B) The relative mRNA level of MMP-2 and MMP-9 in HUVECs infected with Ad-NC or Ad-Tβ4. (C) The protein level of MMP-2 and MMP-9 in HUVECs infected with Ad-NC or Ad-Tβ4. Data are presented as means ± S.D. from 3 independent experiments. *$p<0.05$.

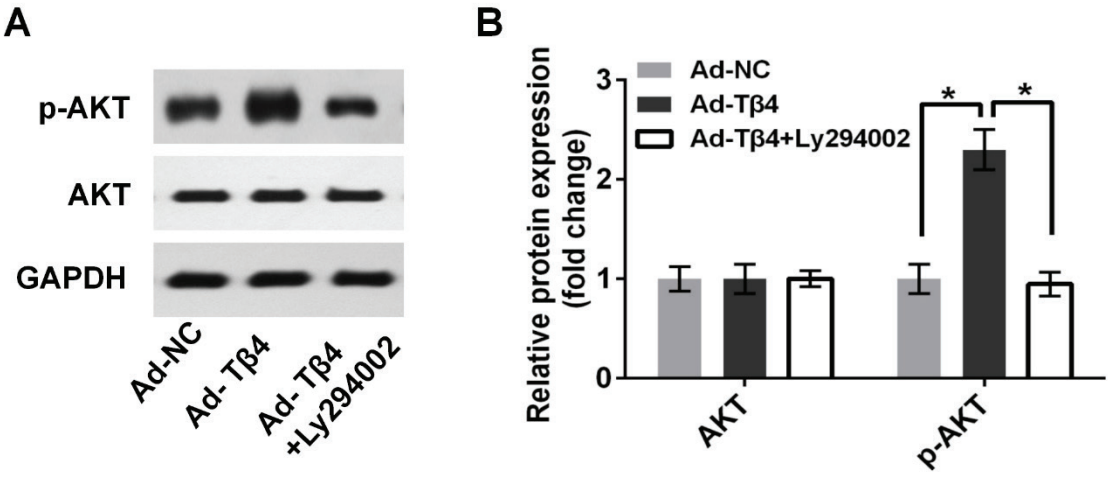

Fig. 5. Tβ4 enhanced AKT signaling. (A) The protein level of p-AKT (Ser473) and AKT in HUVECs infected with Ad-NC or Ad-Tβ4. (B) The density of the western blots bands shown in (A) was quantified using ImageJ software. Data are presented as means ± S.D. from 3 independent experiments. *$p<0.05$.

LY294002 enhanced the apoptosis rate of HUVECs, which was decreased by Tβ4 overexpression (Fig. 6. B). Furthermore, LY294002 could reverse the Tβ4 overexpression-mediated increase in the ability of HUVECs to migrate through the membrane (Fig. 6C).

DISCUSSION

The novelty of our findings was as follows: (1) gene expression modalities of Tβ4 alongside its mechanism of actions in endothelial cells, which clarifies the Tβ4 function in biomolecular levels, (2) the role of Tβ4 in endothelial cells under both, normoxic and hypoxic conditions.

Vascular endothelial cells, which cover the intima of the vascular wall, play an essential role in maintaining vascular wall tension, repairing vascular wall inflammation and promoting vascular proliferation, by secreting a variety of vasoactive substances (20,21). The dysfunction of vascular endothelial cells plays a central role in the pathogenesis of vascular-associated diseases (20). Therefore, controlling endothelial cell function may be a potential novel therapeutic strategy against vascular-associated diseases. The results of this study confirmed that Tβ4 plays an important role in the regulation of HUVECs proliferation, apoptosis, and migration. According to the above findings, this study revealed the beneficial effects of Tβ4 on endothelial cells, which may provide novel insights into the potential application of Tβ4 for vascular protection and therapy in

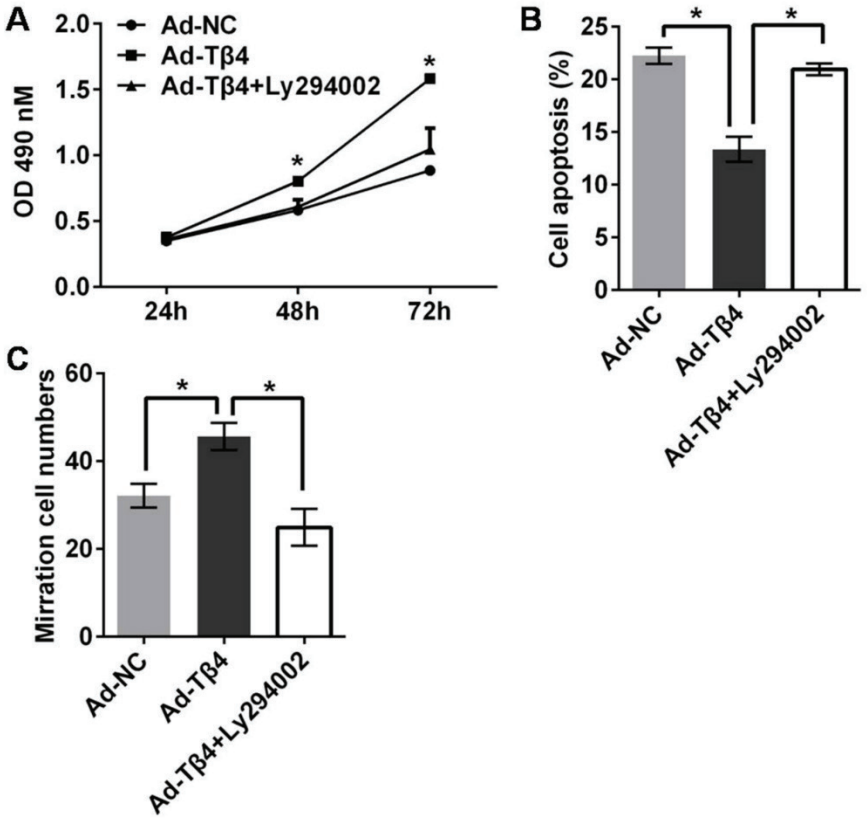

Fig. 6. Tβ4 regulated HUVECs proliferation and migration via AKT signaling (A) Tβ4-induced HUVECs proliferation was abolished by the AKT signaling inhibitor Ly294002. (B) Tβ4-reduced HUVECs apoptosis was abolished by the AKT signaling inhibitor Ly294002. (C) Tβ4-induced HUVECs migration was abolished by the AKT signaling inhibitor Ly294002. Data are presented as means ± S.D. from 3 independent experiments. *$p<0.05$.

vascular-related diseases, including cardiovascular, cerebrovascular, coronary artery, and diabetes diseases.

Tβ4 has been shown to be secreted from embryonic endothelial progenitor cells, endothelial cells, and cardiomyocytes (10,22), suggesting that it may play an important role in endothelial cell function. It has been demonstrated that HUVECs transfected with Tβ4 increased the rate of tube formation on Matrigel while silencing of Tβ4 abrogated tube formation (23). The addition of exogenous Tβ4 can enhance vascular sprouting in cultured HUVECs by inducing several biological responses (24). Ho et al. found that exogenous Tβ4 protected bovine corneal endothelial cells from low-dose ultraviolet-induced oxidative stress and apoptosis (25). Tβ4 also mediated the inhibitory effect on endothelial progenitor cells apoptosis induced by serum deprivation (26). Qiu *et al.* (27) reported that Tβ4 could induce circulating endothelial progenitor cell directional migration, which is essential for re-endothelialization and neovascularization. Moreover, Tβ4 could increase telomerase activity and inhibit the senescence of endothelial progenitor cells (28). In our study, we used Tβ4-expressing adenovirus to demonstrate the effect of Tβ4 on HUVECs proliferation, apoptosis, and migration. Our results showed that Tβ4 overexpression promoted HUVECs proliferation and migration. Furthermore, Tβ4 overexpression could effectively reduce the HUVECs apoptosis under normoxic and hypoxic conditions. Hence, both previous studies and our results indicate that Tβ4 plays an important role in the function of HUVECs.

It has been investigated that the molecular mechanism of Tβ4-regulating endothelial cell function involved various known regulatory pathways (7). It has been reported that Tβ4 could promote the migration and proliferation of embryonic endothelial cells via activating protein kinase C (PKC) activity (29). Moreover, Tβ4 induced the migration of endothelial progenitor cells via the PI3K/AKT/eNOS signaling pathway (14). Lv *et al.* found that Tβ4 significantly reduced VE-cadherin expression levels in HUVECs through the Notch signaling pathway (30). In high-glucose-exposed vascular endothelial cells, Tβ4 protects against hyperglycemia-induced damage of endothelial cells via up-regulating the expression of insulin-like growth factor-1 (IGF-1) (31). In the present study, overexpression of Tβ4 significantly increased the p-AKT (Ser473) level in HUVECs, while LY294002, a PI3K/AKT inhibitor, decreased the p-AKT (Ser473) level, which was induced by Tβ4 overexpression. LY294002 reduced Tβ4-induced HUVECs proliferation and migration. Thus, Tβ4 could regulate the cell function of HUVECs via the AKT signaling pathway. Except for AKT signaling pathway, we couldn't rule out the possibility that Tβ4 regulated HUVECs function by an alternative signaling pathway.

In conclusion, the results of the present study indicate that Tβ4 is a major regulator of HUVECs function. Tβ4 increased proliferation and migration, while reduced apoptosis of HUVECs. The underline mechanism is the increase of p-AKT (Ser473) level by Tβ4, which is demonstrated by the PI3K/AKT inhibitor LY294002. These results provide novel insights into the role of Tβ4 in the pathogenesis of vascular-associated diseases.

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