Original Research

Tβ4 contributes to survival and microvessels formation of endothelial progenitor cells via MAPK/ERK pathway

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Abstract

Endothelial progenitor cells (EPCs) are involved in microvessels formation and can be used as a potential therapeutic option for cardiovascular repair and regeneration. However, the therapeutic efficiency affected by the survival and microvessels formation capacity of EPCs limits their clinical application. In this study, we assessed the facilitative effect of Thymosin Beta 4 (Tβ4) by examining the status of EPCs treated with different concentrations of Tβ4, and injected Tβ4-treated EPCs into the mouse myocardium to detect their effect. We found that the effect of Tβ4 on EPCs was dose-dependent and promoted EPCs proliferation, migration and microvessels formation in vitro and in vivo. Furthermore, the expression of (Mitogen-activated protein kinase, MAPK)/ (Extracellular regulated protein kinases 1/2, ERK) signaling pathway-related factors was increased after Tβ4 treatment, whereas the promoting effect of Tβ4 was attenuated after treatment with a MAPK/ERK pathway inhibitor (PD98059). This study suggests that Tβ4 promotes survival and microvessels formation of EPCs via the ERK MAPK pathway, which could help explore therapeutic options for EPCs use in cardiovascular repair and regeneration.

Keywords: EPCs; Tβ4; MAPK/ERK pathway

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Introduction

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality worldwide, such as atherosclerosis (AS) and myocardial infarction (MI), and closely associated with endothelial dysfunction [1, 2]. Therapeutic angiogenesis is one promising approach aimed at forming new blood vessels or maturing existing vasculature to bypass blocked arteries and maintain organ perfusion [3], while microvessels sparse or dysfunction hinders the treatment of ischemic heart disease by reperfusion. Promoting microvessels formation (angiogenesis and arteriogenesis) has the potential to rescue ischemic myocardium.

Circulating EPCs contribute to microvessels formation and the maintenance of intravascular homeostasis [4], and a number of preclinical and clinical studies targeting the therapeutic potential of EPCs in ischemic heart diseases have been performed [5]. For example, in animal models of limb ischemia and myocardial infarction, autologous EPCs transplantation and drug mobilization have been used to promote vascular repair and neovascularization. EPCs are bone marrow-derived cells involved in angiogenesis [6], when blood vessels are damaged, endothelial cells (ECs) secrete vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1) to activate EPCs [7], which are spindle-shaped and mainly expressed CD34, a highly glycosylated transmembrane protein, vascular endothelial growth factor receptor 2 (VEGFR2) and CD133, glycosylated transmembrane peptides binding cholesterol [8, 9]. Proliferation and differentiation of EPCs directs growth of the vascular system and repair of injured endothelium [10]. EPCs has emerged as an important new cell therapy strategy for CVDs.

Thymosin Beta 4 (Tβ4) is the most expressed protein of the thymosin family in mammals [11], which is widely expressed in a variety of tissues and cells other than red blood cells, and is abundantly expressed in EPCs [10]. Tβ4 is a pleiotropic factor involved in a variety of physiological and pathological processes. Tβ4 is essential for cardiac development and adult heart, it has anti-inflammatory properties and promotes endothelial cell differentiation, migration and angiogenesis [12, 13, 14], thus Tβ4 has a protective and repairing effect on myocardial ischemia or ischemia-reperfusion [15, 16, 17]. Numerous studies have shown that Tβ4 increased EPCs migration in vitro [18], and Tβ4-treated EPCs transplantation increased capillary density [19], and that Tβ4 treatment also led to a substantial increase in cardiac angiogenesis and wound healing after MI [20, 21]. Tβ4 play a beneficial role in the treatment of heart disease.

This study combines in vitro and in vivo experiments to explore the signaling pathways by which Tβ4 regulates EPCs survival and angiogenesis. We believe our findings will contribute to the development of new EPCs therapeutic strategies for CVDs.

Materials and Methods

Ethics statement

Adult male C58BL/6 mice (6 weeks age), weighing 20±2 g obtained from Charles River (Beijing, China). Mice were bred and kept in special-pathogen-free (SPF) condition, with free access of food and water. All animal experiments were performed in accordance with institutional guidelines for animal experiments.

EPCs isolation and culture

The male mice were sacrificed by cervical dislocation and immersed in 75% alcohol for 8 minutes for sterilization. EPCs were derived from the bone marrow of mice. Briefly, mononuclear cells were separated from the tibia and femur of mouse bone marrow using density gradient centrifugation. Cells were incubated with EGM-2MV (Lonza, Basel, Switzerland) in 37℃ and 5% CO_2. After 4 days of culture, non-adherent cells were removed by washing with phosphate-buffered saline (PBS), and adherent cells continue incubated in fresh media. Cells of 2-3 weeks were

used for identification and subsequent experiments.

EPCs characterization and treatment

The morphology of EPCs was observed by inverted phase contrast microscope (Leica, Germany). EPCs were also characterized by EPC surface markers CD34, CD133 and VEGFR2. The antibodies used for flow cytometry were CD34Cy5 (BD Pharminogen, San Diego, CA, USA), CD133PE (Miltenyi Biotech, China) and VEGFR2-2CFS (R&D Systems, China) to assess the expression of CD34, CD133 and VEGFR2. To explore the impact of Tβ4 on EPCs, different concentrations of Tβ4 (0, 0.05, 0.1 and 0.2 μM) were added to the EPC culture medium for 24 hours.

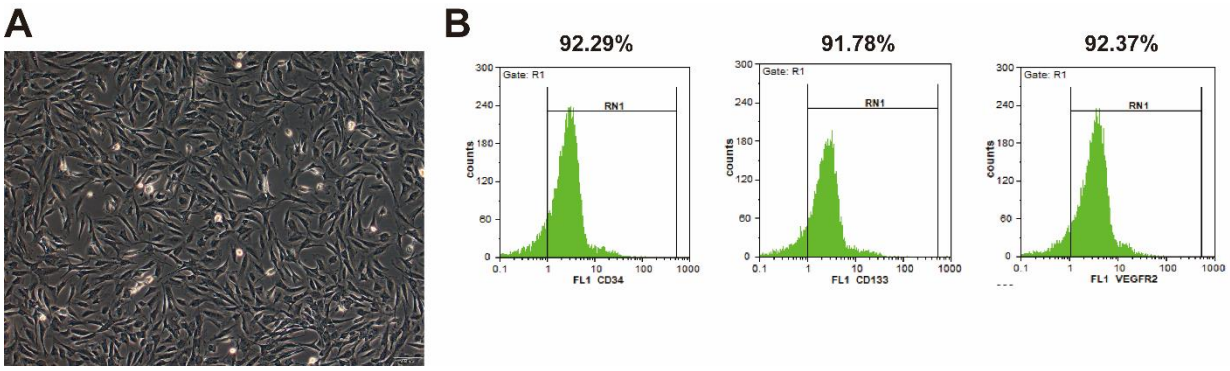

Figure 1 Identification of endothelial progenitor cells (EPCs) derived from mouse bone marrow. (A) Typical cell morphology following 7 days of culture (magnification, ×200). (B)The flow cytometry analysis shows that EPCs positively express CD34, CD133, and VEGFR2.

Cell viability assay

Cell viability was assayed using the CCK-8 (Dojindo Co., Kumamoto, Japan) according to the manufacturer's protocol. EPCs were collected and adjusted to 1×10^4 cells/well, each well added with CCK-8 solution (10 μL). After cultured 4 hours in 37°C, optical density (OD) values at 450 nm in each group were detected using the Multiskan LUX microplate reader (Thermo Fisher Scientific, China). The cell proliferation curves of EPCs were plotted using GraphPad Prism 9 (GraphPad Software, Inc.).

Plate colony formation assay

Single-cell suspensions of adherent cells were prepared and seeded at 1×10^4 cells per well in 96-well plates, and cultured for 24 hours. Added different concentrations of Tβ4 (0, 0.05, 0.1 and 0.2 μM) into each group, respectively. After cultured for 2 weeks, the supernatant was discarded, fixed in 4% paraformaldehyde (Beyotime Biotechnology, China) and stained with Giemsa Stain solution (Solarbio, China) for 30 minutes, washed off the stain slowly with running water and air dry to determine the colony formation efficacy of cell in the four groups.

Cell migration assay

Transwell chambers (BD Biosciences, China) without Matrigel were used for the experiment, according to the manufacturer's instructions. Cells migrating to the lower side of the upper compartment were stained with 1% crystal violet. Cells in different regions were photographed under DM1000 microscope (Leica, Germany).

TUNEL assay

The TUNEL assay was done using the TUNEL assay kit obtained from Abcam, Cambridge, UK. Using 1% paraformaldehyde, the cells were fixed for 15 minutes, and after fixation the cells were rinsed with PBS. Then the cells were treated with 70% ethanol and incubated for 30 minutes on ice, and after incubation the cells were washed with buffer, and 50 μL of DNA labeling solution was added to the cells, and incubated for 1 hour at 37°C. The cells were then washed with buffer

after incubation and suspended again in propidium iodide (PI) solution (Yeasen Biotechnology, China) for 30 minutes and incubated in dark. The cells were then observed under fluorescent microscope (Olympus Corporation, Japan).

Tube formation assay

The tube formation assay is a useful indicator of angiogenesis potential. EPCs were seeded onto a 96-well plate pre-coated with Matrigel (50 μL) and cultured at 37℃ in a 5% CO_2 incubator. The formed network of tubes was visualized at ×100 magnification by DM1000 microscopy (Leica, Germany).

Western blot assay

Western blot was performed using routine protocols. Tissues and cells were lysed in RIPA buffer (Beyotime, China) supplemented with 0.1% protease inhibitors, after centrifugation (14, 000 rpm, 30 minutes), the supernatant fraction was collected and the protein concentration was quantitated by BCA Protein Assay Kit (Beyotime, China). Equal amount of protein was separated on SDS-PAGE gel and electrotransferred to PVDF membrane, and blocked. The membranes were incubated overnight at 4℃ with following primary antibodies (1:1000): c-caspase-3 (cat. No. 9661; Cell Signaling Technology, Danvers, MA, USA), Bax (cat. No. 2772; Cell Signaling Technology, Danvers, MA, USA), Bcl-2 (cat. No. 3498; Cell Signaling Technology, Danvers, MA, USA), VEGF (cat. No. 65373; Cell Signaling Technology, Danvers, MA, USA), p-p38 (cat. No. 4511; Cell Signaling Technology, Danvers, MA, USA), p38 (cat. No. 8690; Cell Signaling Technology, Danvers, MA, USA), p-ERK1/2 (cat. No. 9101; Cell Signaling Technology, Danvers, MA, USA), ERK1/2 (cat. No. 4695; Cell Signaling Technology, Danvers, MA, USA) or GAPDH (cat. No. 5174; Cell Signaling Technology, Danvers, MA, USA). Then the appropriated HRP conjugated goat anti-rabbit secondary antibody (1:3000; cat. No. 7074; Cell Signaling Technology, Danvers, MA, USA) was applied. Protein bands were scanned after luminesced and developed, and their gray values were analyzed by Image J software v1.53i (National Institutes of Health) with GAPDH used as the internal reference.

Animal study

Mice were divided into three groups: intramyocardial injection of PBS, 4×10^6 0.2 μM Tβ4-treated EPCs, 4×10^6 0.2 μM Tβ4-treated EPCs with PD98059. Mice were euthanized after 3 days, and hearts were rapidly excised after PBS perfusion. Cardiac tissues of mice were embedded in OCT (Biosharp Life Sciences, China) for 12 hours at room temperature, then cut into 6-μm sections using CM1950 Frozen Slice (Leica, Germany) for analysis.

Ki-67 immunohistochemistry

Cell proliferation was assessed by Ki-67 immunohistochemistry. The anti-mouse Ki-67 antibody (cat. No. 9449; Cell Signaling Technology, Danvers, MA, USA) was 1:500 diluted and immunostaining was done using the Vectastain ABC kit (Vector Labs, CA, USA) and the DAB substrate kit (Vector Labs, CA, USA) according with the manufacturer's protocol. Strong brown nuclear immunoreactivity was considered as positive staining.

Immunofluorescence

Samples were soaked in PBS for 10 minutes to remove OCT and washed three times in PBS with 0.5% Triton X-100 and incubated in primary antibodies CD31 (cat. No. 3528; Cell Signaling Technology, Danvers, MA, USA) and α-SMA (cat. No. 19245; Cell Signaling Technology, Danvers, MA, USA) overnight at 4℃. Washed three times in PBS, and incubated in secondary antibodies conjugated with Alexa Fluor 594 (cat. No. 75261; Cell Signaling Technology, Danvers, MA, USA) and Alexa Fluor-488 (cat. No. 34105; Cell Signaling Technology, Danvers, MA, USA). Double positive labeling of CD31 and α-SMA were examined by laser scanning confocal

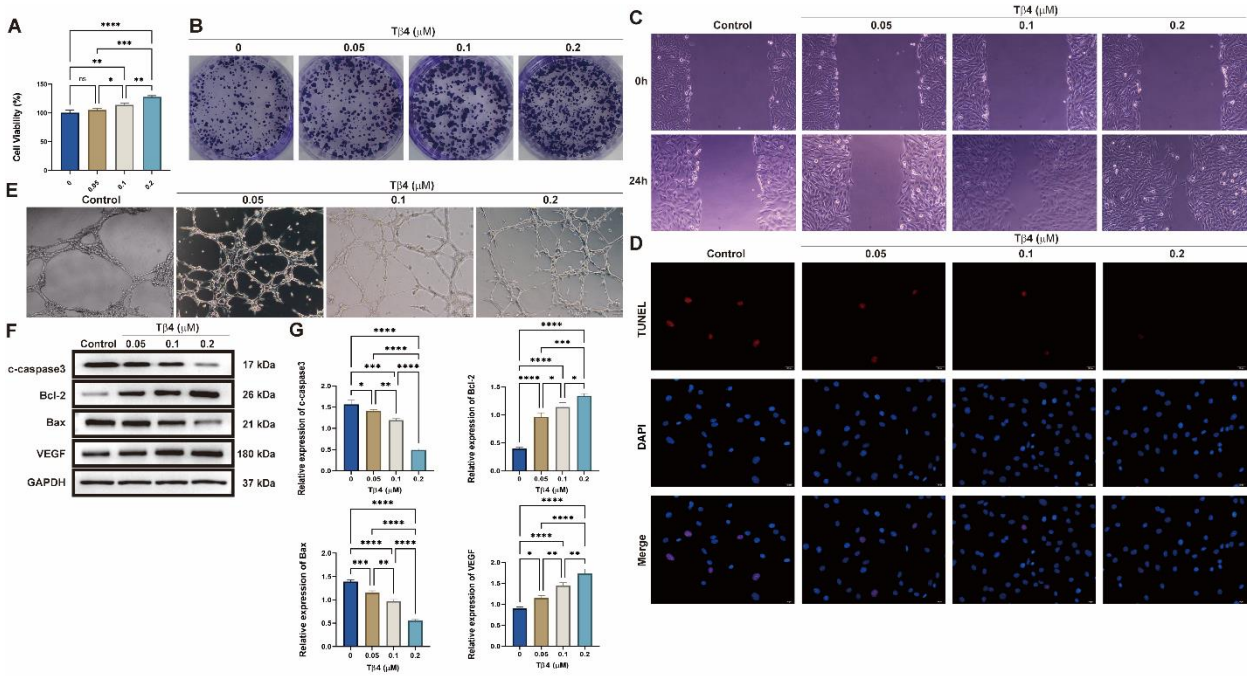

Figure 2 Evaluation of EPCs proliferation, migration, apoptosis and microvessels formation. (A)Cell viability of 0, 0.05, 0.1, 0.2 μM Tβ4 treated EPCs tested by CCK-8. (B)Colony formation assay for 0, 0.05, 0.1, 0.2 μM of Tβ4 treated EPCs. (C)Differences in the effect of different Tβ4 concentrations (0, 0.05, 0.1, 0.2 μM) by transwell assay on the migration capacity of EPCs. (D)Tunel assay was used to analysis cell apoptosis for EPCs after stimulation with increasing (0, 0.05, 0.1, 0.2 μM) concentration of Tβ4. (E)Tube formation assay for 0, 0.05, 0.1, 0.2 μM of Tβ4 treated EPCs. (F)-(G)Western blot was used to detected c-caspase3, Bcl-2, Bax and VEGF expressions after stimulation with increasing (0, 0.05, 0.1, 0.2 μM) concentration of Tβ4. **P*<0.05, ***P*<0.01, ****P*<0.001, **** *P*<0.0001.

microscopy (FV300, Olympus, Japan).

Statistical analysis

Data are expressed as the mean ± standard deviation. Differences between groups were compared using one-way ANOVA. Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, Inc.). Comparisons between two groups were performed using unpaired two-tailed Student's t-test. P < 0.05 was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

Identification and characterization of EPCs

EPCs were isolated form mouse bone marrow and cultured in fibronectin-coated medium, and the cells were observed to be shuttle-shaped and covered with protrusions under the microscope (Figure 1A). Furthermore, greater than 90% expression levels of EPC surface markers CD34, CD133 and VEGFR2 detected by flow cytometry were 92.29%, 91.78% and 92.37, respectively (Figure 1B).

Tβ4 promotes EPCs proliferation, migration, microvessels formation and reduces apoptosis in a dose-dependent manner

After being identified and characterized, EPCs isolated form mouse bone marrow stimulated with different concentrations of Tβ4 (0, 0.05, 0.1 and 0.2 μM). CCK-8 assay and plate clone formation assay were performed to examine cell proliferation and clone with different Tβ4 concentrations, respectively (Figure 2A, B). It demonstrated that cell viability increased with augmenting Tβ4 concentration. The Transwell assay showed an improved mobility of EPCs with increasing Tβ4 (Figure 2C). Apoptosis decreased

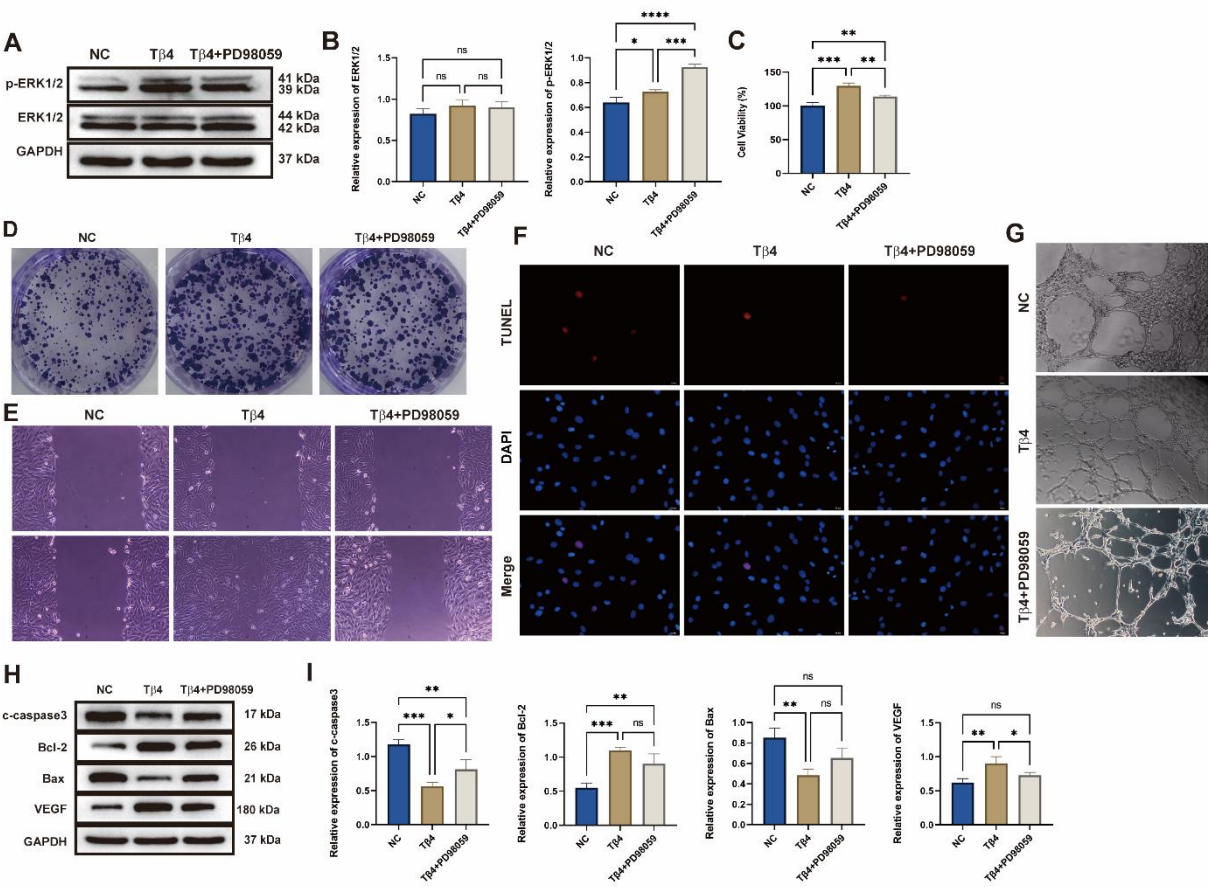

Figure 3 Tβ4 effected EPCs proliferation, migration, apoptosis and microvessels formation via MAPK/ERK signaling pathway. (A)-(B) Western blot assay for p-ERK1/2 and ERK1/2 expression of 0.2 μM Tβ4 treated or 0.2 μM Tβ4 with MAPK/ERK pathway inhibitors co-treated EPCs. (C)Cell viability of 0.2 μM Tβ4 treated and MAPK/ERK pathway inhibitors treated after 0.2 μM Tβ4 treated EPCs tested by CCK-8. (D)Colony formation assay for 0.2 μM of Tβ4 treated or 0.2 μM Tβ4 with MAPK/ERK pathway inhibitors co-treated EPCs. (E)The different effects of 0.2 μM Tβ4 and 0.2 μM Tβ4 with MAPK/ERK pathway inhibitors by transwell assay on the migration capacity of EPCs. (F)Tunel assay was used to analysis cell apoptosis for EPCs after stimulation with 0.2 μM Tβ4 or 0.2 μM Tβ4 with MAPK/ERK pathway inhibitors. (G)Tube formation assay for 0.2 μM Tβ4 or 0.2 μM Tβ4 with MAPK/ERK pathway inhibitors treated EPCs. (H)-(I) Western blot was used to detected c-caspase3, Bcl-2, Bax and VEGF expressions after stimulation with 0.2 μM Tβ4 or 0.2 μM Tβ4 with MAPK/ERK pathway inhibitors. **P*<0.05、***P*<0.01、****P*<0.001.

as Tβ4 increased evaluated by TUNEL assay (Figure 2D). To determine whether Tβ4 could enhance the microvessels formation behavior of EPCs, a tube formation assay was carried out and showed that Tβ4 promoted angiogenesis (Figure 2E). Western blot was used to analyze the levels of apoptosis-related markers (c-caspase3, Bcl-2, Bax) and vascular endothelial growth factor VEGF (Figure 2F, G). The increase of Tβ4 evoked the upregulation of Bcl-2 (anti-apoptotic protein) and VEGF, and the downregulation of Bax, c-caspase3 (pro-apoptotic proteins). These results suggested that Tβ4 can promote EPCs survival and angiogenesis.

Effect of Tβ4 on proliferation, migration, apoptosis and microvessels formation of EPCs via MAPK/ERK signaling pathway

The MAPKs signaling pathway regulates many cellular activities and is associated with cell proliferation and angiogenesis [34], we therefore investigated the effect of Tβ4 treatment on MAPK expression and activity in EPCs. Cells in the

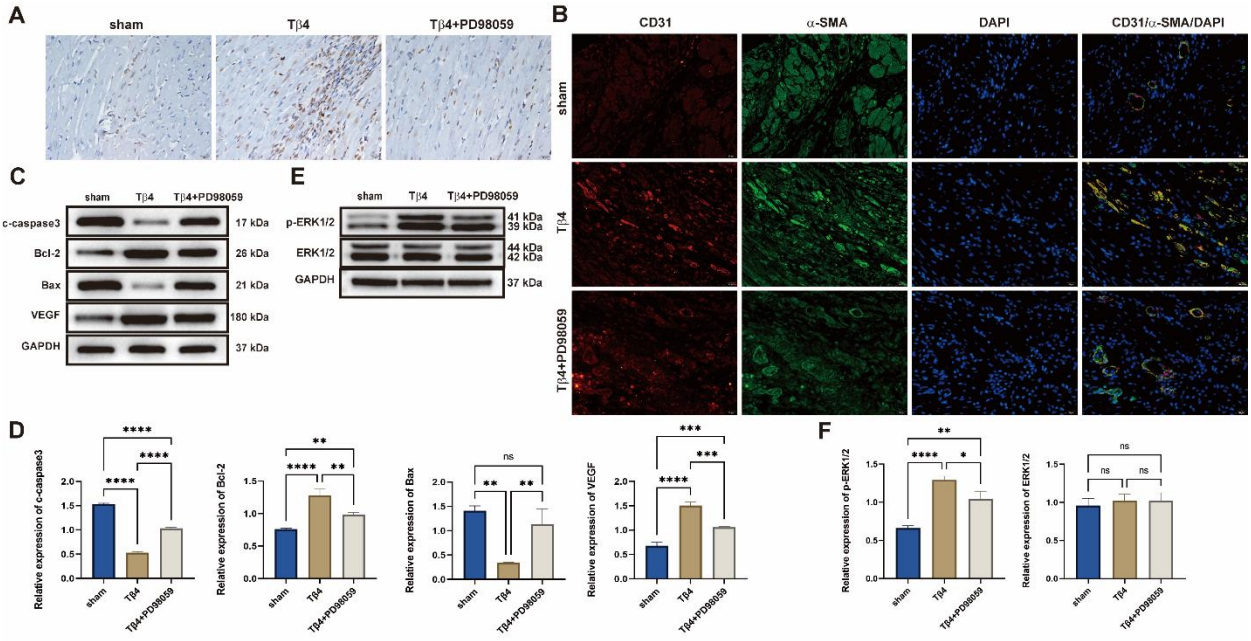

Figure 4 Tβ4 effected EPCs proliferation, migration, apoptosis and microvessels formation via MAPK/ERK signaling pathway in mouse myocardium. (A) Ki-67 immunohistochemical staining was used to evaluated proliferation. (B)Immunofluorescence of CD31 and α-SMA. (C)-(D) Western blot for c-caspase3, Bcl-2, Bax and VEGF expressions. (E)-(F) Western blot for p-ERK1/2 and ERK1/2 expression. **P*<0.05, ***P*<0.01, ****P*<0.001, **** *P*<0.0001.

experimental group were treated with Tβ4 (0.2 μM) and with MAPK/ERK pathway inhibitors PD98059 on the basis of Tβ4 treatment, respectively. Western blot analysis showed that Tβ4 treatment significantly induced MAPK expression in protein levels in EPCs (Figure 3A, B). Phosphorylation of p38 and ERK1/2 are increased with Tβ4 treatment and slightly inhibited by PD98059. Proliferation tested by CCK-8 and clone formation assay (Figure 3C, D), migration detected by Transwell (Figure 3E) and microvessels formation detected by angiogenesis experiment (Figure 3G) all showed the same trend, with Tβ4 treatment significantly increasing proliferation, migration and microvessels formation of EPCs, while Tβ4 treatment followed by PD98059 treatment attenuated the effect of Tβ4. In addition, Tβ4 treatment greatly reduced the level of apoptosis in EPCs, Figure 3F shows a decreased in TUNEL positive cell with Tβ4 treatment, furthermore, the expression of pro-apoptotic protein c-caspase3 and Bax determined by Western blot showed the same downward trend (Figure 3H, I). Expression of both the apoptosis inhibitor Bcl-2 and the vascular endothelial growth factor VEGF increased significantly with Tβ4 treatment, and this increase was attenuated by the effect of PD98059. This suggested that the MAPK/ERK pathway is involved in the regulation of EPCs by Tβ4.

Effect of Tβ4 on proliferation, migration, apoptosis and microvessels formation of EPCs via MAPK/ERK signaling pathway in mouse myocardium

To investigate whether Tβ4 regulation of the MAPK/ERK signaling pathway has the same effect in mouse myocardium as in vitro, we injected Tβ4-treated (0.2 μM) EPCs and PD98059-treated EPCs on the basis of Tβ4 treatment into mouse myocardium respectively. Myocardial tissues from each group of mice injected with different treatments of EPCs for one week were taken. Ki-67 immunohistochemical staining showed an increase in positive cells, indicating high proliferative activity of the Tβ4-treated group (Figure 4A). We provided both CD31 to label endothelial cells and α-SMA staining to label vascular muscle (Figure 4B) and

the results indicating increased microvessels formation in response to Tβ4 treatment. In addition, the WB results also showed an increase in the expression of the apoptosis inhibitor Bcl-2 and the angiogenic factor VEGF (Figure 4C, D), while the expression of the pro-apoptotic protein c-caspase3 and Bax was reduced. These results indicated that proliferation and microvessels formation were promoted while apoptosis was inhibited in myocardial tissues of mice under Tβ4 treatment. These effects of Tβ4 attenuated by MAPK/ERK inhibitor PD98059.

We further examined the expression of MAPK/ERK pathway-related proteins by Western blot, and the expression of phosphorylated p38 and ERK1/2 were significantly increased in Tβ4-treated group. The role of Tβ4 was similarly diminished by MAPK/ERK inhibitor PD98059.

Discussion

The aim of this study was to investigate the role of Tβ4 treatment in promoting survival and microvessels formation of EPCs. Both in vitro and in vivo experiments were conducted. For the past few years, a growing number of studies have explored endothelial progenitor cells as a possible therapeutic strategy for cardiovascular repair and regeneration, and EPCs from bone marrow or peripheral blood have been used in clinical studies for the treatment of ischemic heart repair and regeneration [22, 23, 24]. Intramyocardial transplantation of EPCs promoted myocardial infarction neovascularization and improved cardiac function, labeling of transplanted EPCs with DILDL-UEA-1 observed that localization of EPCs mainly in the anterolateral wall of cardiac tissue of each dog model, although EPCs had varying degrees of migration [25]. Monsanto MM et al. improved EPCs colonization and angiogenesis in the myocardium by using a 3D microenvironment spontaneously formed by co-culture with EPCs, mesenchymal stem cells (MSCs) and c-Kit$^+$ cardiac interstitial cells [26]. In our study, transplantation of Tβ4-treated EPCs was also found to improve angiogenesis in mouse myocardium, which may contribute to improvement of EPCs angiogenesis in clinical studies. Furthermore, one of the most important roles of EPCs appears to depend on paracrine action. Jia Y et al. found that EPC-Exos stimulated angiogenesis in bone regeneration in vivo [27], and EPC-Exos also enhanced the proliferation, migration and tube formation of endothelial cells in vitro [28]. Therefore, we conducted a further study on the mechanism of Tβ4 on EPCs.

Cell morphogenesis and motility depends on the precise regulation of the dynamics of the actin cytoskeleton, in which the actin-binding protein Tβ4 plays an important role. Many studies have shown that, Tβ4 has protective effects for heart and might be a promising candidate for the treatment of cardiovascular diseases [13]. There have been many explorations of the mechanisms of cardiovascular effects of Tβ4. Yan B et al. found that Tβ4 enhanced cardiomyogenesis by downregulating PTEN and upregulating Akt [29], and Ye L et al. demonstrated that Tβ4 increased the recruitment of endogenous CPCs and thus improved myocardial repair after MI [30]. Our experimental study confirmed that Tβ4-treated EPCs promoted proliferation and microvessels formation of mouse myocardium, which was agreed with other reports. In addition, studies shows that the effect of Tβ4 on EPCs related to Akt/eNOS signaling pathway [31] and JNK MAPK signaling pathway [32].

The MAPKs cascade pathway plays an important role in proliferation, differentiation, apoptosis, stress response and angiogenesis [33], and mainly includes the extracellular signal-regulated kinases ERK1/2, c-Jun amino-terminal kinase/stress-activated protein kinase JNK and the p38 MAPK signaling pathway [34], of which, MAPK/ERK signaling has positive effects on cardiovascular-related diseases [35]. Studies shows that activation of MAPK/ERK signaling enhanced zebrafish cardiac regeneration [36], we therefore explored whether the effect of Tβ4 on EPCs related to ERK/MAPK pathway. In this research, we found that treatment with Tβ4 enhanced the MAPK/ERK pathway in EPCs, whereas treatment with MAPK/ERK pathway

inhibitor weakened the enhancement of Tβ4, indicating that Tβ4 regulates the EPCs function via p38/ERK MAPK pathway.

In conclusion, Tβ4 play a useful role of EPCs proliferation, migration and microvessels formation, which may be attributed to the activation of the MAPK/ERK signaling pathway, and these findings might warrant EPCs-based cytotherapy of CVDs. The direct interaction of Tβ4 in intracellular signaling pathways and its role in cellular processes need to be further explored.

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