Recombinant TAT-Thymosin β-4 Promotes Angiogenesis by Activating VEGFR2 Signaling Pathway

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Research Article

Keywords: Recombinant TAT-Thymosin, Angiogenesis , VEGFR2 Signaling Pathway

Posted Date: September 27th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-913761/v1

Abstract

Thymosin β-4 is a 43-amino acid intracellular polypeptide that was originally isolated from bovine thymus. Of the 16 known thymosin families, thymosin β-4 is the most common type found in all tissues. Thymosin β-4 regulates angiogenesis, cell differentiation, morphogenesis, migration, and organogenesis and is linked to a dynamic equilibrium between G-actin and F-actin. In particular, thymosin β-4 is well-known for its angiogenic and anti-apoptotic functions. In this study, we synthesized thymosin β-4 linked with the well-known cell-penetrating peptide TAT (YGRKKRRRQRRR). TAT-thymosin β-4 promotes angiogenesis and cell migration *in vitro* via the VEGFR2 signaling pathway and reduces apoptosis. To examine angiogenic potential *in vivo*, a Matrigel Plus assay was conducted that revealed the angiogenic effect of TAT-thymosin β-4. In conclusion, TAT-thymosin β-4 promotes blood vessels and is expected to be applicable in regenerative medicine for all organs requiring blood vessels.

1. Introduction

Angiogenesis is the process of forming blood vessels from the pre-existing vasculature[1]. It is necessary in the process of development, but is also crucial for the recovery of damaged or pathological tissue[1–3]. However, abnormal angiogenesis is associated with several diseases, including cancer, cardiovascular disease, diabetes, and age-related macular degeneration[1]. Thus, modulators of angiogenesis, such as vascular endothelial growth factor and its related molecules (inhibitors such as pegaptanib sodium, ranibizumab, bevacizumab, and antibodies) have been approved for treating diseases described above[1]. Other growth factors such as platelet-derived growth factor, fibroblast growth factor, angiopoietins, and thymosin-β4 have also been shown to be modulators of angiogenesis[1–4].

Beta-thymosins (thymosin-β) comprise a structurally related family that is highly conserved in the animal kingdom[4,5]. There are 16 types of beta-thymosins, but only 3 types are found in humans (thymosin-β4, thymosin-β10, and thymosin-β15)[6,7]. Although highly homologous in genetic structure, these three proteins are derived from different genes and thus, are distinct products. NMR studies have revealed that thymosin-β4 is mostly unstructured in aqueous solutions with some preferential α-helix structures[8,9]. Thymosin β-4 controls the assembly and disassembly of actin filaments through its initial interaction with G-actin[8,9]. Thus, the mechanism by which thymosin-β4 influences angiogenesis, cell differentiation, morphogenesis, cell migration, and organogenesis is assumed to be linked to the maintenance of a dynamic equilibrium between G-actin and F-actin[8–10].

As such, thymosin β-4 is a promising drug candidate for many diseases. However, unlike other angiogenic modulators, thymosin-β4 does not act through receptors on the cell surface but acts directly on cells[11]. This is a major obstacle to the use of thymosin-β4 as a drug target. For this reason, we combined the well-known cell-penetrating peptide TAT and thymosin-β4 for intracellular translocation from the extracellular area[12,13].

The recombinant protein produced in E. coli by molecular cloning techniques revealed the angiogenic effects of thymosin-β4 *in vitro* and *in vivo*. Here, we provide first experimental evidence that recombinant TAT- thymosin-β4 is an angiogenic modulator of the VEGFR2 signaling pathway.

2. Results

2.1. Cell penetration of TAT-Thymosin ß4

To confirm the cell penetration of TAT-thymosin β-4, HUVECs treated with TAT-thymosin β-4 were observed. In immunocytochemical staining images, it was confirmed that TAT-thymosin β-4 translocated to the cytosol more than naïve thymosin β-4 (Fig. 1).

2.2. In vitro angiogenic effect of TAT-Thymosin ß-4

To investigate the angiogenic effect of TAT-thymosin β-4, a tube formation assay was performed. At 0–10 μg/mL, the angiogenic effect of TAT-thymosin β-4 increased proportionally (Fig. 2a–f). However, at 15–50 μg/mL concentration, no angiogenic effect was observed; rather an unhealthy condition of the cells was observed (Fig. 2g–j). We considered a concentration of 10 μg/mL to be the optimal concentration for angiogenesis. VEGF treated group was used as a positive control for angiogenesis (Fig. 2k)

In addition, cell migration, an important factor in angiogenesis, was examined using a wound healing assay. As in the tube formation assay, 10 μg/mL was ideal for cell migration assay (Fig. 3a, b), and cell migration increased proportionally at a concentration of 0–10 μg/mL.

2.3. TAT-Thymosin ß4 activates VEGFR2 signaling pathway

To investigate the intracellular mechanism of TAT-thymosin β-4-mediated angiogenesis in HUVECs, western blotting was conducted. First, VEGFR2 and phospho-VEGFR2 protein levels were measured. While the expression level of VEGFR2 showed no significant change at a concentration of 0, 5, or 10 μg/mL TAT-thymosin β-4, the level of p-VEGFR2 increased at concentrations of 0, 5, and 10 μg/mL. At concentrations of 15 and 20 μg/mL, both VEGFR2 and p-VEGFR2 levels were reduced (Fig. 4a, b). Likewise, endothelial markers (Angiopoietin 1, Angiopoietin 2) showed similar changes to p-VEGFR2 (Fig. 4a, b). These data strongly suggest that TAT-thymosin β-4 activates the VEGFR2 signaling pathway in HUVECs and induces angiogenesis.

To confirm the relationship between TAT-thymosin β-4 and VEGFR2, VEGF antagonists (bevacizumab and sorafenib) were treated in TAT-thymosin β-4-treated HUVECs. VEGFA secreted in the cell culture medium was increased in the TAT-thymosin β-4 treatment group but no change was observed in the group treated with both TAT-thymosin β-4 and VEGF antagonist (Fig. 5a, c). Western blotting of the cell lysates showed that the amount of VEGFA was similar to the change in the cell culture medium (Fig. 5b, c). The p-VEGFR2 level was increased in the TAT-thymosin β-4 treatment group, but reduced in the TAT-thymosin β-4 and

antagonist co-treatment groups (Fig. 5b, c). Similar trends of increase and decrease were observed for p-Akt and p-ERK (Fig. 5b, c).

2.4. TAT-Thymosin ß-4 suppresses apoptosis

To investigate the relationship between apoptosis and TAT-thymosin β-4 expression, western blotting was performed (Fig. 6) using an apoptosis western blot cocktail (Table 1). The positive control group included hydrogen peroxide (50 μM)-treated HUVECs[14]. The level of cleaved PARP was decreased in HUVECs treated with low concentrations of TAT-thymosin β-4 (5, 10, 15 μg/mL) but increased in 20 μg/mL TAT-thymosin β-4-treated HUVECs compared to that in untreated HUVECs. The level of Procaspase-3 did not change at low concentrations of TAT-thymosin β-4 but decreased in 20 μg/mL TAT-thymosin β-4-treated HUVECs. The level of cleaved caspase-3 was not changed in 5 μg/mL TAT-thymosin β-4-treated HUVECs but decreased at 10 μg/mL and increased at 15, 20 μg/mL similar to the VEGF treatment group or positive control group (Fig. 6).

Table 1
Antibodies list used for immunocytochemistry or western blotting.

	Manufacturer	Cat#
Anti-thymosin β4	Abcam	ab14335
Anti-F-actin	Abcam	ab205
Anti-VEGFA	Santacruz	sc-7269
Anti-VEGFR2	Sigma	SAB4501645
Anti-phospho VEGFR2	Merk	PS1013
Anti-Angiopoietin1	Millipore	AB3120
Anti-Angiopoietin2	Millipore	AB3121
Anti-phospho AKT	CST	9271
Anti-AKT	CST	9272
Anti phospho-ERK	CST	9102
Anti-ERK	CST	9101
Anti-CD31	Abcam	ab28364
Apoptosis Western blot Cocktail	Abcam	ab136812
Anti-β-actin	Sigma	A5441

2.5. String database analysis

The protein group interacting with VEGF and the protein group interacting with thymosin β-4 were largely identical to each other (Fig. 7a). As a result of analyzing only highly correlated proteins, thymosin β-4 was detected upstream of VEGF (Fig. 7b). This result is consistent with the results of western blotting using VEGF inhibitors (Fig. 5). These data strongly suggest that TAT-thymosin β-4 activates the VEGFR2 signaling pathway through VEGF, thereby exhibiting an angiogenic effect.

2.6. In vivo angiogenic effect of TAT-Thymosin ß4

The Matrigel plug, which was dissected from the animal 2 weeks after injection, showed newly formed blood vessels in the TAT-thymosin β-4-treated group and the VEGF-treated group, while the non-treated group showed no blood vessels (Fig. 8). The hemoglobin content of the Matrigel plug was measured using the Drabkin's reagent. A total of 5.25 ± 0.64 ug/mg of hemoglobin was detected in VEGF-containing Matrigel, and 5.84 ± 0.82 ug/mg of hemoglobin was detected in TAT-thymosin β-4-containing Matrigel. In the Matrigel plug of control group, only 1.07 ± 0.24 ug/mg of hemoglobin was detected (Fig. 8b, c). These data strongly suggest that TAT-thymosin β-4 has as much angiogenic capacity as VEGF *in vivo*.

3. Discussion

Thymosin β-4 is capable of angiogenesis similar to VEGF[12,13]. In the case of VEGF, receptors on the cell surface interact with VEGF, so it is possible to target it using a drug without cell penetration[15,16]. Thymosin β-4 has many clinical benefits such as angiogenesis, cell migration, cell adhesion, differentiation, prevention of apoptosis, cell survival, and tissue regeneration. It is a promising new drug candidate, if it is easy to transfect into cells.

To achieve this, we used molecular cloning techniques. We combined TAT, a well-known transmembrane molecule, and thymosin β-4 at the DNA level to produce a fusion protein in E. coli. When HUVECs were treated with the fusion protein, an increase in the cytoplasmic fusion protein was confirmed compared to that of the control. HUVECs expressing the cytosolic fusion protein exhibited increased angiogenesis and migration ability and prevented apoptosis *in vitro*. In addition, angiogenic effects have been observed *in vivo*. We found that cytosolic thymosin β-4 promoted VEGFA secretion. Secreted VEGFA binds to VEGFR2 in autocrine or paracrine regulation, which activates the VEGFR2 signaling pathway.

Recently, dermal wound healing and regeneration effects of thymosin β-4 have been demonstrated in a full-thickness excisional rat model and impaired-healing model[17–19]. Furthermore, based on these regenerative properties, RGN-137 and RGN-259 have been clinically studied for several diseases such as pressure ulcers, venous stasis ulcers, and corneal wounds[4].

However, compared to VEGF, the efficacy of angiogenesis is poor and studies on the topic are scarce. Although the molecular weight of thymosin β-4 is small, it is partially introduced into the cytosol. This explains why the number of studies on thymosin β-4 is small. Translocation to the cytosol of thymosin β-4 has numerous potential applications.

4. Materials And Methods

4.1. Recombinant protein and cell penetration

We used an advanced TAT sequence (YGRKKRRRQRRR). Its tricodon DNA sequences (33 bp) were ligated with PCR-amplified TMSB4X sequences (136 bp) in the pBluescript Sk2 + plasmid multiple cloning site using T4 ligase. Thereafter, the plasmids were transformed into E. coli (RBC Bioscience, New Taipei City, Taipei) for protein production. Recombinant proteins were isolated using a Sepharose FF column (Merk-Millipore, Burlington, VT, USA).

HUVECs were treated with isolated TAT-thymosin β-4 to confirm transfection. Trypsinized HUVECs (1×10^4 cells per well) were plated in a 24-well culture plate and treated with 10 μg/mL of TAT-thymosin β-4 for 1 h. The control group was treated with normal thymosin β-4. Thereafter, the cells were fixed with 4% paraformaldehyde and incubated with anti-thymosin β-4 (Table. 1). Next, anti-rabbit-IgG Alexa Fluor 488 and DAPI were used for visualization.

4.2. Tube formation and wound healing assay

Pre-chilled growth factor-reduced Matrigel was added to a 24-well culture plate. The cells were incubated at 37°C for 30 min to solidify the Matrigel. HUVECs (7.5×10^4 cells per well) were trypsinized and added to Matrigel-coated 24-well culture plates. TAT-thymosin β-4 was added according to the group composition (0, 2, 4, 6, 8, 10, 15, 20 μg/mL concentration). Four hours later, tube formation images were obtained, processed, and calculated using ImageJ software.

Poly-L-lysine (50 μg/mL) was coated onto a 60-mm cell culture dish. Fibroblasts (8×10^5 cells per well) were seeded to create a confluent monolayer. The scratch was created with a p200 tip and incubated for 16 h. Wound healing images were obtained, processed, and calculated using ImageJ software.

4.3. Protein analysis

Total cellular protein samples were collected from each group. Western blotting was performed according to the guidelines supplied by Bio-Rad (Hercules, CA, USA). Primary antibodies (Table 1) were diluted 1:1000 in 3% bovine serum albumin (BD Biosciences Franklin Lakes, NJ, USA) and visualized by chemiluminescence imaging. Band density was measured using ImageJ software. We performed analysis of protein-protein interactions for proteins related to TAT-thymosin β-4 and VEGF using a string-database platform (http://string-db.org).

4.4 Statistical analysis

SPSS software, v20.0 (SPSS, Inc., Chicago, IL, USA) was used. Values are reported as mean ± standard deviation (SD). Statistical differences were assessed using a non-parametric one-way Kruskal–Wallis test. Statistical significance was set at $P < 0.05$.

4.5. Matrigel plug assay

To investigate the angiogenic properties of TAT-thymosin β-4, an *in vivo* Matrigel plug angiogenic assay was performed. Six-week-old BALB/c nude mice were purchased from Orient Bio (Seongnam, Gyeonggi-do, Korea). All experiments in mice were performed according to the guidelines of the Korea Polytechnic University Institutional Animal Care and Use Committee (KPU-2020-0001, 1 May 2020). All animals were maintained in a 12 h light/dark cycle at 23 ± 1°C and 50 ± 10% relative humidity with ad libitum feeding. One hundred micrograms of Matrigel (BD Biosciences, San Jose, CA, USA) containing 60 U/mL heparin (Sigma-Aldrich, CA, USA) mixed with TAT-thymosin β-4 (n = 3) or VEGFA (n = 3) was injected subcutaneously into BALB/c nude mice. Two weeks later, the Matrigel plugs were removed from the animals after euthanasia. Thereafter, vessel formation in Matrigel plug was quantified by measuring the hemoglobin using Drabkin's reagent (Sigma-Aldrich, CA. USA) [20] .

5. Conclusions

Several previous studies reported the angiogenic effect of thymosin β-4. However, it is functional only when it is located in the cytoplasm. For this reason, we prepared a fusion protein by combining thymosin β-4 with the cell-penetrating peptide TAT for cytosolic translocation. Translocated TAT-thymosin β-4 is located in the cytoplasm, promotes angiogenesis, and reduces the apoptosis of HUVECs. Furthermore, TAT-thymosin β-4 also promotes angiogenesis *in vivo*.

In this study, we confirmed angiogenesis *in vivo*. However, we did not confirm whether angiogenic effects were maintained under pathological conditions. In future studies, we plan on employing a foot ulcer animal model to check whether thymosin β-4 can be developed as a therapeutic agent by inducing angiogenesis under pathological conditions.

Declarations

Author Contributions

Chul Min Kim, Yun-Mi Jeong, Seokhwan Yun: Writing Original Draft, *in vivo* investigation, Funding

Jae-Hun Kim, GuoLong Jin, Hyeongkwon Oh, Jung Seok Kim, and Dal Yong Park: *In vitro* investigation

Jin-Hyung Shim, and Won-soo Yun: Review, Editing, and Funding

Songwan Jin: Writing Original Draft, Conceptualization

All authors reviewed the manuscript.

Acknowledgements:

This work was funded by the Ministry of Education [NRF-2017R1A6A1A03015562, NRF-2020R1I1A1A01068566, NRF-2020R1I1A1A01054595, NRF-2019R1I1A1A0106325713] and the Korea

Institute for Advancement of Technology [P0002007] funded by the Korean Government.

Institutional Review Board Statement:

The animal study was performed at the Laboratory Animal Resource Center of Korea Polytechnic University and approved by the Korea Polytechnic University Institutional Animal Care and Use Committee (KPU-2020-0001, 1 May 2020). All experimental protocols were carried out in compliance with the ARRIVE Guidelines.

Informed Consent Statement:

Not applicable.

Data Availability Statement:

All results generated or analyzed during the present study are included in this published article. Data and materials will be made available upon request via email to the corresponding author (yuntobi@kpu.ac.kr).

Conflicts of Interest:

Jung Seok Kim and Dal Yong Park are shareholders of the Union Korea life science. The other authors declare no conflict of interest.

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Figures

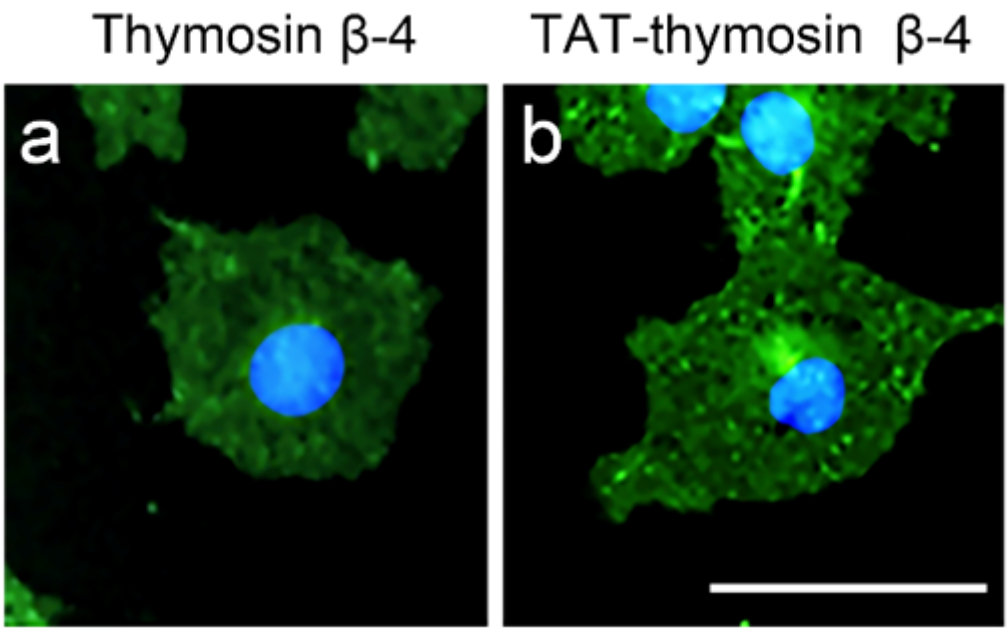

Figure 1

Immunocytochemistry of transfected fusion protein. a HUVECs treated with thymosin β-4. b HUVECs treated with TAT-thymosin β-4. TAT-thymosin β-4 treatment group showed increased cell penetration of thymosin β-4 than did naïve thymosin β-4 group. Scale bar indicates 10 μm.

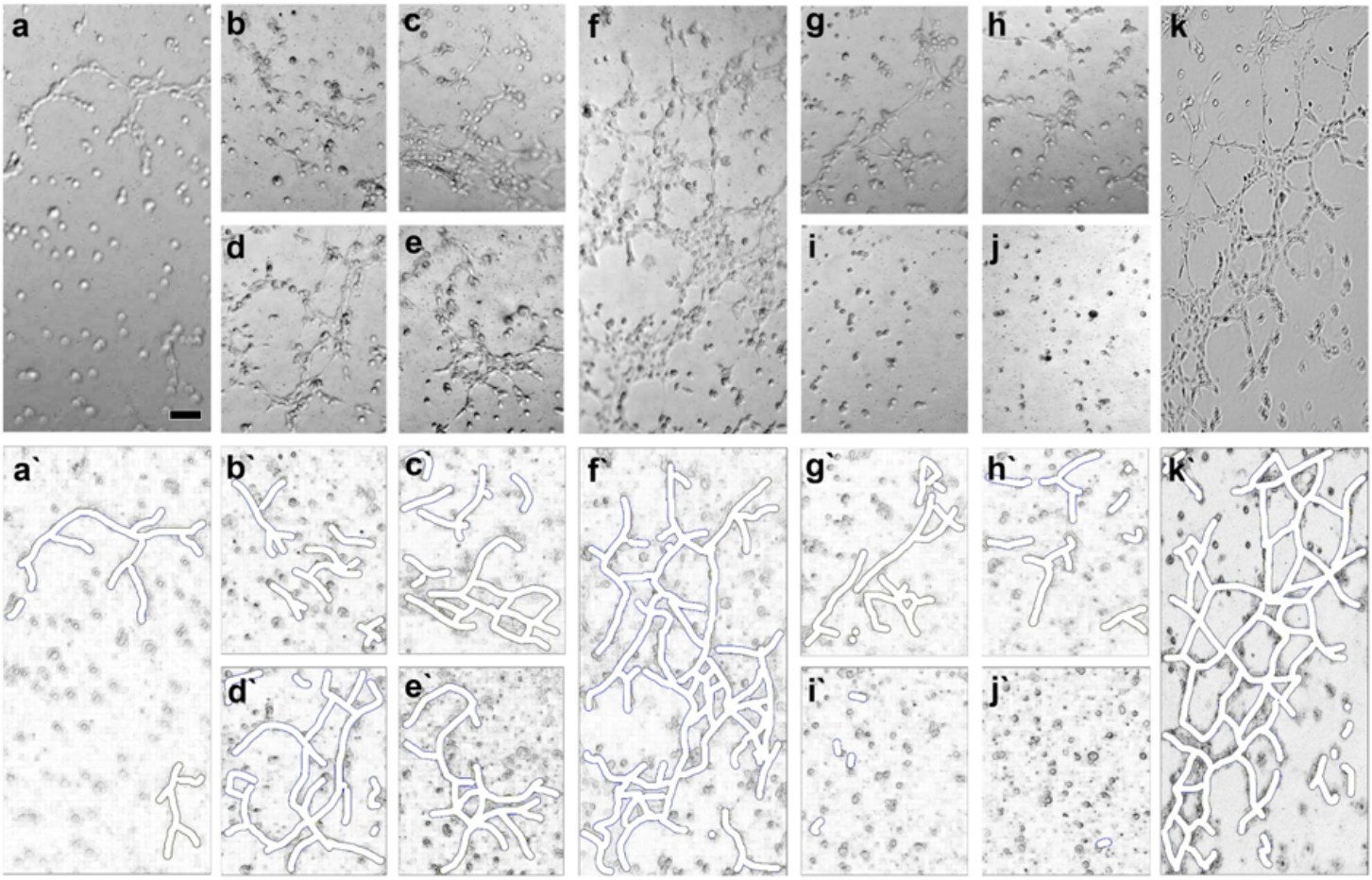

Figure 2

Tube formation assay. TAT-thymosin β-4 of concentrations a 0 μg/mL, b 2 μg/mL, c 4 μg/mL, d 6 μg/mL, e 8 μg/mL, f 10 μg/mL, g 15 μg/mL, h 20 μg/mL, i 30 μg/mL, and j 50 μg/mL was treated. k VEGF-treated positive control. a–f Tube formation ability was increased in proportion to the TAT-thymosin β-4 concentration. f,k The 10 μg/mL treatment group showed similar tube formation ability to the VEGF treated group. g–j the tube formation ability was lost. a— k Images were processed sequentially using ImageJ software from original images (a-k). Scale bar indicates 50 μm.

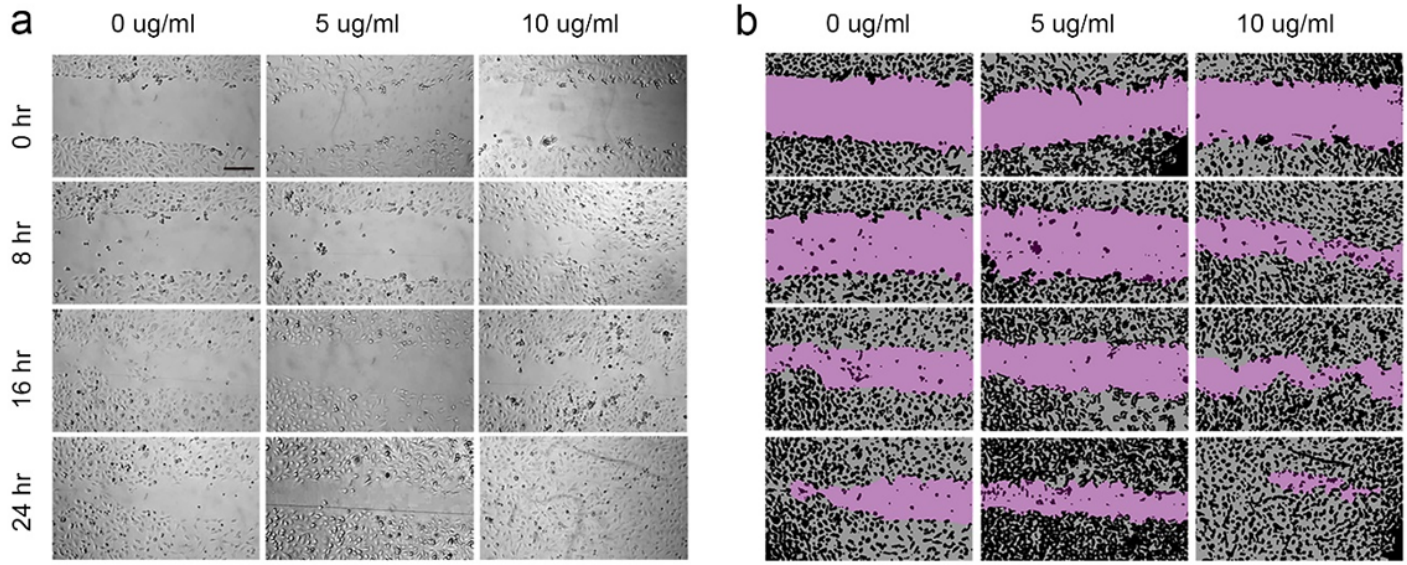

Figure 3

Cell migration assay. a TAT-thymosin β-4 treated to HUVEC cells in concentration of 0, 5, 10 μg/mL. b Images were processed using ImageJ software from original images to (a) highlight the areas where cells have not migrated. Scale bar indicated 50 μm.

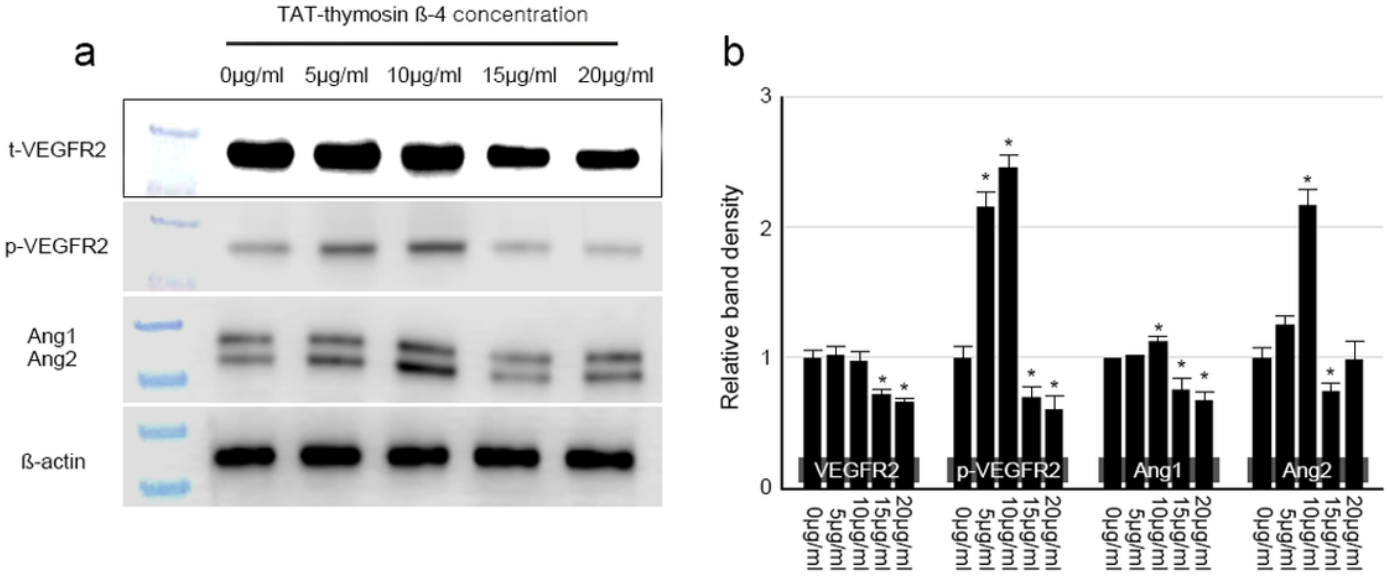

Figure 4

Activation of VEGFR2 signaling pathway according to TAT-thymosin β-4 concentration. a VEGFR2 and p-VEGFR2 indicate VEGFR2 signaling pathway. Angiopoietin 1 and Angiopoietin 2 showed vascular endothelial differentiation through VEGFR2 signaling pathway. b Relative band densities compared to β-actin and were measured at least three times for statistical analysis and all data were expressed as mean ± standard deviation. Asterisk indicates significant difference ($p<0.05$) compared to control group (0 μg/mL).

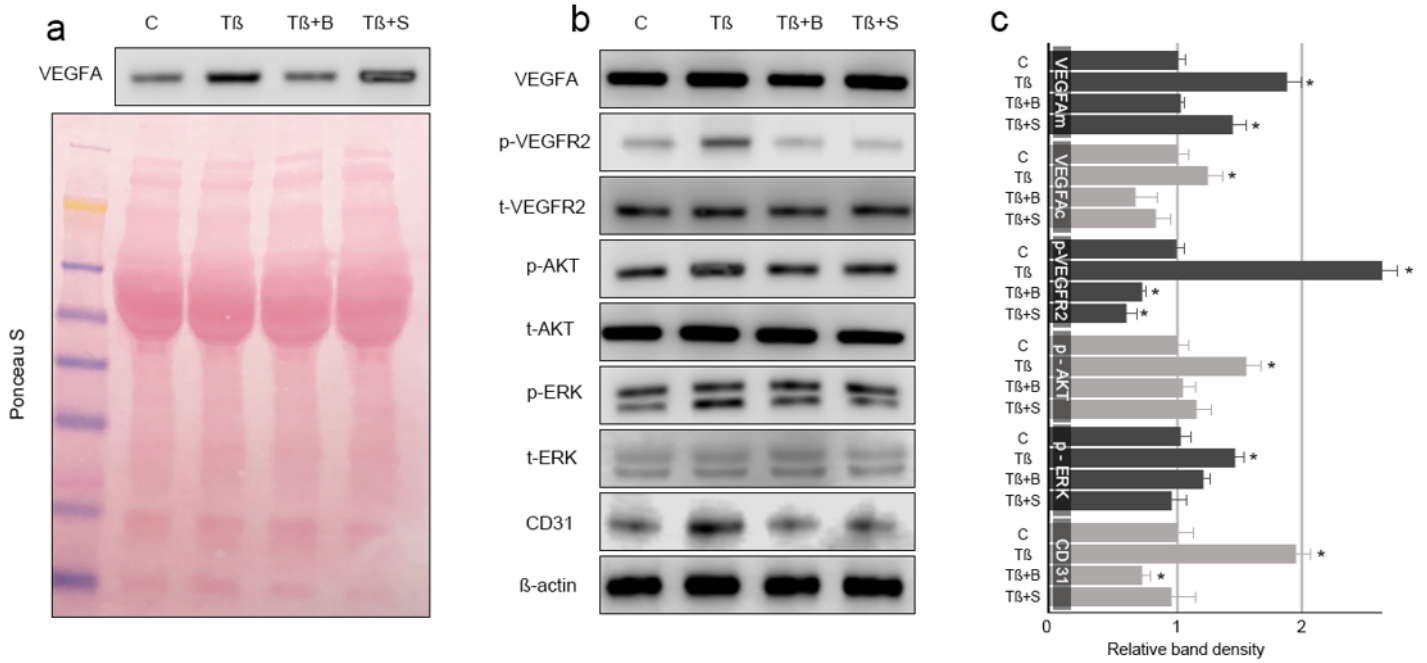

Figure 5

Loss-of-function study using a VEGF antagonist. a Western blotting of cell culture medium for VEGF. PonceauS was used as standard because there was no β-actin in cell culture medium. b Western blotting of cell lysates for VEGF, p-VEGFR2, t-VEGFR2, p-AKT, t-AKT, p-ERK, t-ERK, CD31. c Relative band density compared to Ponceau S or β-actin and were measured at least three times for statistical analysis and all data were expressed as mean ± standard deviation. Asterisk indicates significant difference ($p<0.05$) compared to control group (0 μg/mL).

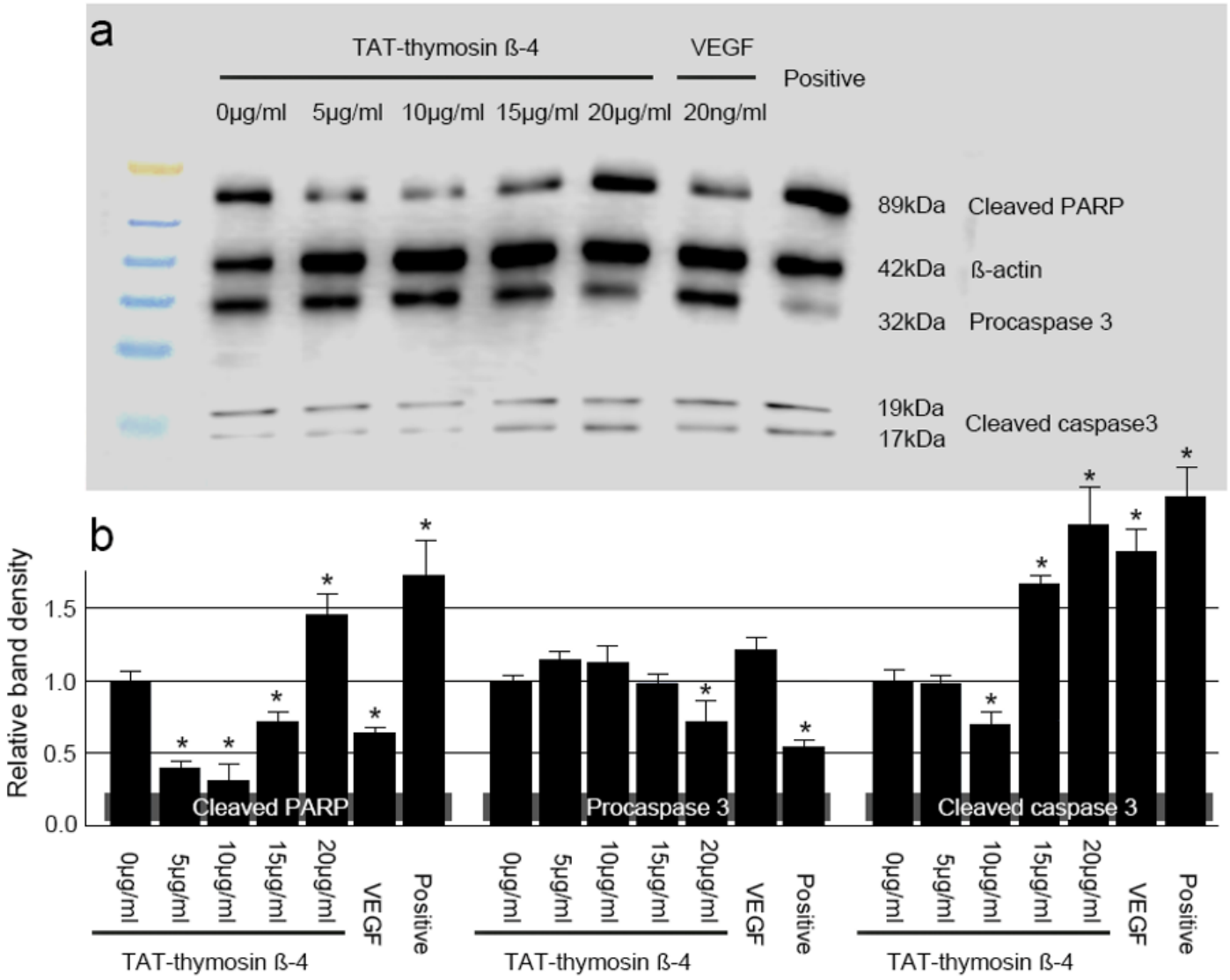

Figure 6

Anti apoptotic effect of TAT-thymosin β-4. a Western blotting of apoptotic markers using apoptosis western blot cocktail consisting of cleaved PARP, β-actin, procaspase 3, and cleaved caspase 3. b Relative band densities compared to β-actin and were measured at least three times for statistical analysis and all data were expressed as mean ± standard deviation. Asterisk indicates significant difference ($p<0.05$) compared to control group (0 μg/mL).

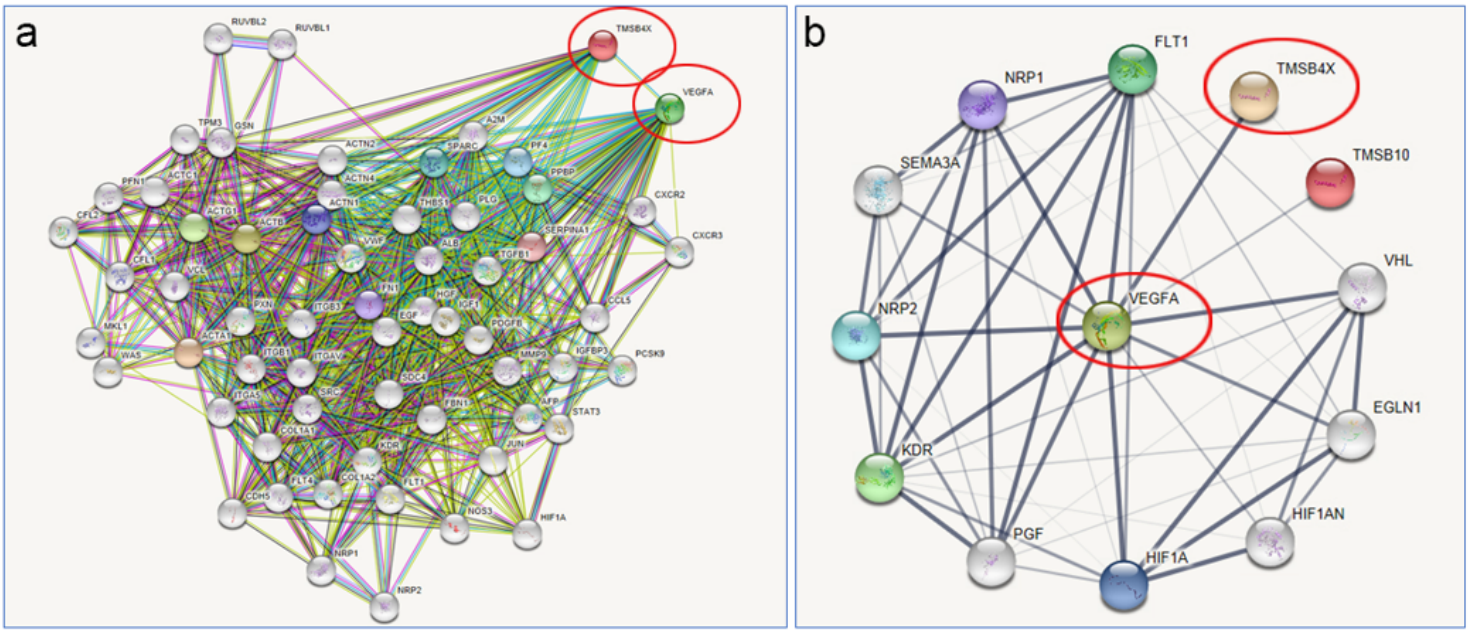

Figure 7

String database analysis using thymosin β-4 (TMSB4X) and VEGF (VEGFA). a Medium confidence 0.400 no more than 20 interactions. b Sorting only those with high relevance.

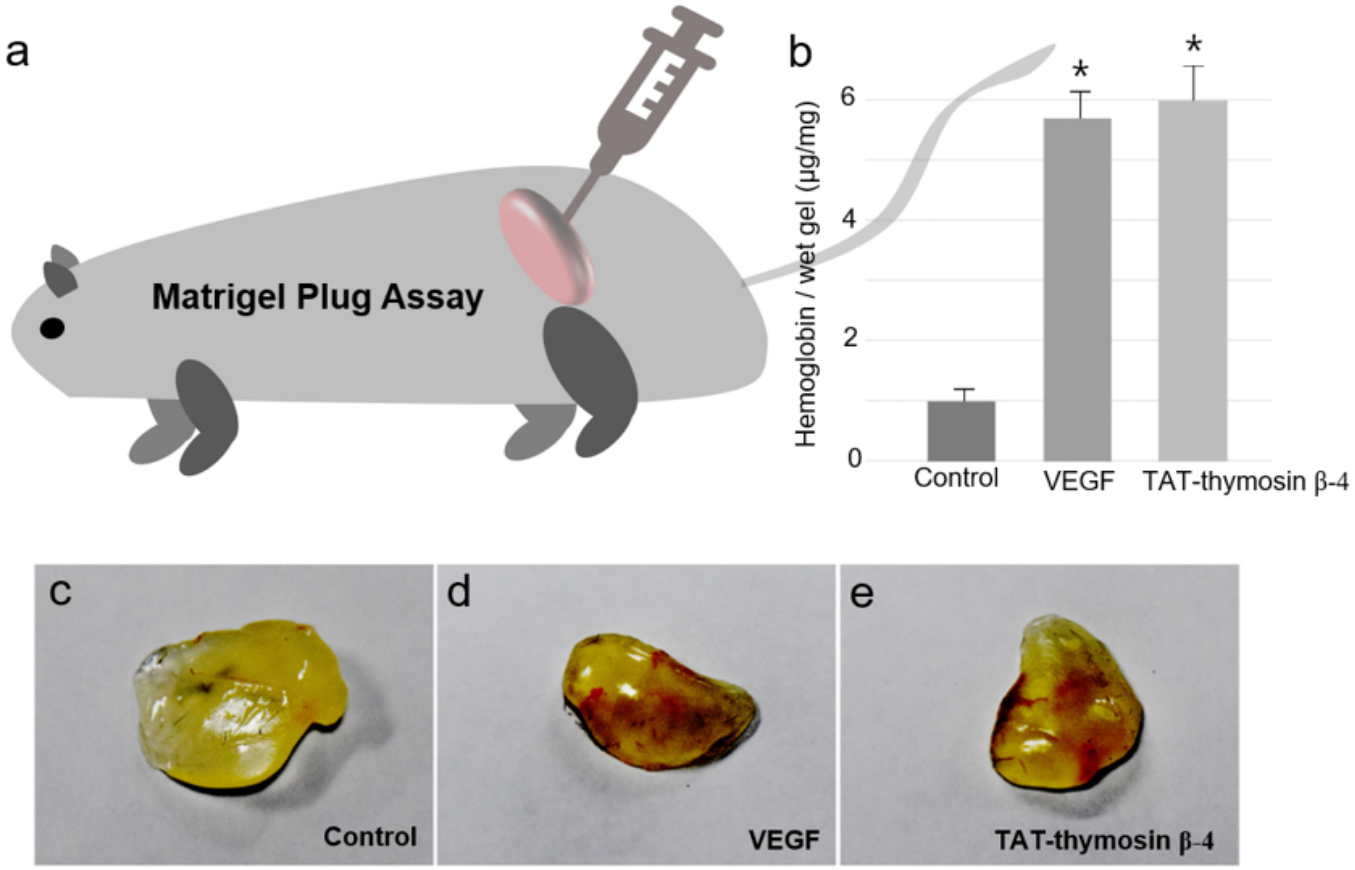

Figure 8

Matrigel plug assay for in vivo angiogenesis. a Schematic illustration of Matrigel plug assay. b Hemoglobin content measurement using Drabkin`s reagent. All data were expressed as mean ± standard deviation. Asterisk indicates significant difference($p<0.05$) compared to control group. c–e Matrigel plug dissected from mice.

Supplementary Files

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