ProBDNF Acts as an Angiogenesis Inhibitor

Hua Li[1], Fangfang Bi[2], Andrew Beck[1], Larisa Bobrovskaya[1], Xinfu Zhou[1*]

[1]Clinical and Health Sciences, University of South Australia, Adelaide, Australia
[2]Department of Neurology, Xiangya Hospital, Central South University, Changsha, China
Email: *Xin-Fu.Zhou@unisa.edu.au

Abstract

Brain-derived neurotrophic factor (BDNF) has been considered a new angiogenesis mediator. ProBDNF, the precursor of BDNF, plays opposite neuronal functions to BDNF, but the role of proBDNF on angiogenesis remains unknown. We found human umbilical vein endothelial cells (HUVEC) expressing BDNF, proBDNF, p75^{NTR}, Sortilin and TrkB. ProBDNF significantly decreased HUVEC viability in MTT assay, and this inhibition was neutralized by anti-proBDNF. Endothelial cell tube formation assay showed that proBDNF significantly inhibits HUVEC angiogenesis *in vitro*. Matrigel plug assay disclosed that proBDNF also impeded angiogenesis *in vivo*, while anti-proBDNF greatly facilitated angiogenesis. Immunostaining of CD31 and α-SMA in Matrigel plugs confirmed the inhibitive effect of proBDNF on angiogenesis. In conclusion, proBDNF can act as an angiogenesis inhibitor. It added more evidence to the "Yin-Yang" theory by showing mBDNF is a mediator of angiogenesis as "Yang" and proBDNF works as an angiogenesis inhibitor as "Yin".

Keywords

mBDNF, proBDNF, Endothelial Cells, Angiogenesis, Cancer

1. Introduction

BDNF is a member of the neurotrophin family that preferentially binds to TrkB, leading to neuronal survival, long term potentiation (LTP) and synaptic plasticity. The precursor of BDNF (proBDNF) is cleaved by either intracellular or extracellular enzymes to form mature BDNF (mBDNF). proBDNF can also release and bind to p75^{NTR} with high affinity, leading to neuronal death and long term depression (LTD) [1] [2] [3]. Researchers have analogized the opposing effects of proBDNF and mature BDNF on neurotrophic function with ancient Chinese "Yin and Yang" theory [4]. The philosophy of Yin (阴) and Yang (阳) formed in

ancient China describes a complementary, interconnected, and interdependent relationship between two opposites [5]. The balance of proBDNF and mBDNF is maintained through proteolytic processing of proBDNF. Neurotrophins have been widely studied for their functions in the nervous system. However, it is becoming increasingly clear that neurotrophins also play crucial roles in the vascular system. Recent studies indicated that nerve growth factor (NGF) induced HUVEC proliferation *in vitro* [6]. In a Chorioallantoic Membrane (CAM) assay, NGF increased angiogenesis in a dose-dependent manner [7]. In Matrigel plug assay, NGF also promoted endothelial cell invasion and cord formation [8]. In recent work, NGF has been shown to increase cell proliferation and migration of the human endothelial cell line [9]. Similarly, BDNF displayed a direct role in angiogenesis. BDNF promoted endothelial cell survival and induced angiogenesis through the TrkB receptor [10]. BDNF increased angiogenic tube formation in HUVEC [11]. Overexpression of BDNF in a mouse endothelial cell line can promote cell proliferation, migration, and invasion [12]. NGF and BDNF also play an indirect role in angiogenesis by inducing vascular endothelial growth factors (VEGF) expression in different cell types [13] [14] [15].

Angiogenesis is the process of new blood vessels growing from the pre-existing vasculature. Angiogenesis is a critical physiological process during embryonic development and into adult life and also plays vital roles in the pathophysiological processes, such as tumour progression, proliferative retinopathy, cardiovascular disease, etc. [16]. The process of angiogenesis is regulated by the balance between pro-angiogenic and anti-angiogenic factors [17]. When the pro-angiogenic regulators exceed the effects of angiostatic regulators, angiogenesis occurs. For example, in response to pathological conditions such as low pO_2, injury, tumour or cerebral ischemia, the "angiogenic switch" will be activated and endothelial proliferation increases dramatically [18]. Endogenous modulators such as VEGFs, angiotensin II, fibroblast growth factors (FGFs), or the chemokine are released, and endothelial cells start to loosen their junctions. The endothelial cell layer becomes more permeable, and plasma proteins extravasate to form the extracellular matrix (ECM) scaffold. Then endothelial cells migrate to this ECM surface, where they arrange and form capillary structures [19] [20]. Research shows that angiogenesis is the crucial step for recovery after cerebral ischemic injury to meet the oxygenation requirements of local tissues. Cerebral vasculature-based therapy with neuroprotection (neuro-restorative therapies) has been combined for a better method for future clinical ischemic stroke therapies [21].

Angiogenesis intervention is also an evolving strategy for treating cancer by working as a powerful adjunct to traditional radiotherapy and chemotherapy [22]. Unlike conventional cancer therapies, anti-angiogenic therapies target tumour-associated endothelial cells and angiogenesis, blocking the nutrition line for tumour cell growth. Tumour-related angiogenesis is initiated by a cascade of pro-angiogenic factors and involves close contact between tumour cells and host tissues. Inhibiting the pro-angiogenic process effectively and precisely has been a significant challenge for clinicians to develop optimised anti-angiogenic drugs

for cancer. Recent studies in both tumour and endothelial cells have presented a wide variety of molecular targets for developing angiogenesis inhibitors against cancer and other angiogenic-related diseases [20] [23]. Several VEGF blockers have been used in the clinic for treating cancer and eye disease. However, low response rates, rapid vascular regrowth, and the abnormality of tumour vessels made anti-angiogenic therapy more difficult than expected [24] [25]. Optimization and combination of different anti-angiogenesis molecules are future directions for improving the efficacy and efficiency of cancer treatments.

The precursors of neurotrophins usually play opposite roles to mature neurotrophins in the nervous system. However, the role of pro-neurotrophin in angiogenesis remains unclear. In the present study, the effects of proBDNF on endothelial cells and blood vessel formation have been investigated to provide another molecular target for angiogenesis intervention and broaden the choices for treating angiogenesis-related diseases.

2. Material and Methods

1) HUVEC Culture

Human Umbilical Vein Endothelial Cells (HUVEC), Gibco Medium 200 and Large Vessel Endothelial Supplement (LVES) were supplied by ThermoFisher Scientific. LVES is optimized for use with Medium 200 to deliver optimal HUVEC growth and enable superior cell performance. Cryopreserved HUVEC were rapidly thawed in a 37˚C water bath and cultured in the complete media. HUVEC were seeded at a density of 2.5×10^3 cells/cm^2 and incubated at 37˚C in a humidified atmosphere of 5% CO_2. The cell culture medium was replaced at 24 to 36 hours after seeding and every other day until the culture reached 80% confluence (4 to 6 days).

2) Immunocytochemistry

HUVECs were seeded on sterile glass coverslips in 24-well plates and were grown until they reached confluency. The cells were fixed in situ with 4% paraformaldehyde (PFA) and rinsed with phosphate-buffered saline (PBS). The cells were then permeabilized with 0.1% Triton X-100 and incubated in 10% Normal Goat Serum for blocking. The cells were immuno-stained with different primary antibodies: sheep anti-proBDNF (made by our lab), sheep anti-mBDNF (made by our lab), goat anti-p75^{NTR} (Santa Cruz), rabbit anti-sortilin (Abcam), goat anti-TrkB (R & D systems), and *vWF* (Abcam) for 45 min at room temperature. Goat IgG was used for negative immunofluorescent control. After washing, different fluorescent secondary antibodies (Alexa-cy3-conjugated donkey anti-goat antibody or Alexa-488-conjugated sheep anti-rabbit antibody, Invitrogen, Inc.) were added and incubated for 40 min in the dark at room temperature. After washing, nuclei were counterstained with 4',6-Diamidino-2-phenylindole DAPI (Invitrogen, Inc.). Double staining of proBDNF with p75^{NTR} and proBDNF with Sortilin were performed by using mixed primary antibodies from different species (sheep-anti-ProBDNF with rabbit-anti-p75^{NTR} or sheep-anti-proBDNF with

rabbit-anti-Sortilin). Corresponding mixed secondary antibodies were applied after washing. The samples were mounted with glycerine and imaged using confocal microscopy (Zeiss LSM 710, Oberkochen, Germany) at different magnifications.

3) Western blot analysis

Total protein from HUVEC was obtained to perform western blot analysis. Briefly, HUVEC were gently washed with chilled PBS and then lysed by suspending in ice-cold RIPA buffer with inhibitors (Roche, Castle Hill, NSW, Australia). After sonication, cells were then centrifuged at 16,000 g for 10 min at 4˚C. The supernatants were carefully collected and total protein concentrations were measured by a BCA protein assay kit (Thermo Scientific, Rockford, USA). Protein extracts from HUVEC were separated by 10% SDS-PAGE gels (Bio-Rad, USA) and transferred to a nitrocellulose membrane (GE Healthcare, Life Sciences). The membrane was then blocked with 5% skim milk for one hour at room temperature and then incubated overnight at 4˚C with respective primary antibodies: sheep anti-proBDNF (made by our lab), goat anti-TrkB (R & D system), rabbit anti-Sortilin (Abcam), goat anti-p75^{NTR} (Santa Cruz). After washing with TBS, subsequent secondary antibodies conjugated with HRP (Sigma-Aldrich) were added and incubated at room temperature for one hour. The membrane was washed three times, and ECL reagent (GE Healthcare Life Sciences) was utilized to develop the bands. Image Quant LAS 4000 was used to take the images (GE Healthcare Australia).

4) MTT Assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay is a colorimetric essay, which is commonly used for measuring cell viability, proliferation, and cytotoxicity. MTT, a yellow tetrazolium salt, can be converted to purple formazan by NAD(P)H-dependent oxidoreductase enzymes in living cells. In contrast, dead cells cannot cleave significant amounts of MTT to formazan [26]. The insoluble formazan crystals are then dissolved using a solubilization solution, and the resulting-coloured solution can be quantified by measuring absorbance at 400 to 650 nm. HUVECs were equally seeded in a 96 well plate with complete medium (density: $1 - 5 \times 10^6$ cells per mL) and incubated overnight. The complete medium was then replaced by conditioned culture medium: medium 200 (control group), medium 200 with BSA (100 ng/mL), medium 200 with a series of concentrations of proBDNF (1 ng/mL, 3 ng/mL, 10 ng/mL, 30 ng/mL, and 100 ng/mL) and medium 200 with proBDNF 100 ng/mL and anti-proBDNF (10 μg/mL). After incubating for an hour, 10 μL MTT (3 mg/mL in PBS; Sigma-Aldrich) were added to each well and incubated for 4 hours. After carefully aspirating the MTT medium, 150 μl dimethyl sulfoxide (DMSO) was added to each well. The plate was then shaken at 150 rpm for 5 min on a platform rocker to mix the formazan into the solvent thoroughly. The optical density value was recorded at 490 nm on a Microplate Reader (Bio-Rad, USA). Pentad wells were evaluated for each sample, and the test was repeated

twice.

5) Endothelial cell tube formation assay (*In Vitro* Angiogenesis assay)

Endothelial cell tube formation assay is a well-established *in vitro* angiogenesis assay that utilises the ability of endothelial cells to form capillary-like structures. When reaching 70% - 80% confluence in a T25 flask, HUVECs were starved by depriving serum for 3 - 6 hours prior to performing the tube formation assay [27]. An appropriate volume of Geltrex matrix (ThermoFisher Scientific) was thawed at 4˚C one day before use. Geltrex matrix is a reduced growth factor basement membrane matrix that allows endothelial cells to form tube-like structures. Geltrex matrix was added to a pre-chilled 96-well plate using pre-chilled pipette tips (50 µL/cm^2) and then kept at room temperature for 1 hour to polymerize. HUVEC were seeded (42,000 viable cells/cm^2) on the matrix gel-coated plate using Medium 200 with 1% foetal bovine serum and incubated at 37˚C and 5% CO_2 for 14 - 16 hours. The medium was supplemented with or without proBDNF (50 ng/mL). After incubation, the cell dye Calcein, AM (ThermoFisher Scientific) was added (2 µg/mL) and incubated for 30 minutes. The nonfluorescent Calcein AM is converted to a green fluorescent Calcein in live cells. The dye-containing media were gently removed and replaced with the medium 200. Phase-contrast images and fluorescent images were taken using a light microscope and fluorescence microscope, respectively. Triplicate wells were used for both control and proBDNF groups and repeated twice. Total mesh areas and total branch lengths of capillary networks were measured by means of the Angiogenesis Analyzer plugin for ImageJ [28].

6) Matrigel plug assay (*In vivo* angiogenesis assay)

The animal experiment was approved by the Animal Welfare Committee of Flinders Medical Centre and conducted in accordance with the National Health and Medical Research Council of Australia. C57Bl/6J mice were used for Matrigel plug assay. All animals were bred under a 12-hour light/dark cycle and had free access to food and water. Twelve mice were randomly divided into four groups of 3 mice in each group (PBS, proBDNF, mBDNF, and anti-proBDNF). Mice were subcutaneously injected in the flank of mice bilaterally, with 0.3 mL PBS, proBDNF, mBDNF, or anti-proBDNF (concentration, 100 µg/mL), combined with 0.3 ml Matrigel, respectively (Becton Dickinson Biosciences). After seven days, mice were humanly killed, and the Matrigel gel plug was harvested for staining.

7) Hematoxylin and eosin staining

The abdominal skin (5-mm width) around the Matrigel plug, including the skin, muscle, and peritoneum, was removed for H&E staining. The samples were fixed in 4% PFA overnight at room temperature. Graded ethanol dehydration and xylene were then applied before embedding in paraffin wax blocks. After cutting on a microtome, the sections went through a hydration process and stained in hematoxylin for 10 min, followed by eosin for 5 min. After dehydration treatment again, the slides were mounted with a coverslip and mounting medium and observed under the microscope. **Scoring of tissue:** Angiogenesis in

Matrigel plug tissue was assessed based on a three-point scale as follows: 1) No obvious or few cells were observed in the Matrigel; 2) Significant cells were invading the Matrigel; 3) Vascular structures presented in Matrigel, with substantial cell invasion [29].

8) Immunohistochemistry

After cutting, sections from the Matrigel plug assay were processed through xylene and an ethanol series. After rehydration, heat-induced epitope recovery was performed by placing slides into 0.01 M Citrate buffer (pH 6.0) at 98˚C in a water bath for 15 min. After cooling down and washing with PBS, slides were treated with 3% H_2O_2 for 5 min to block endogenous peroxidase. 10% Normal Goat Serum blocking solution was then applied to sections for 30min to block non-specific antigen binding. Without washing, Primary antibody Rat Anti-Mouse CD31 (BD Pharmingen) and mouse monoclonal α-Smooth Muscle antibody (α-SMA) (Sigma-Aldrich) were added respectively and incubated at 4˚C overnight. After washing with PBS, corresponding biotinylated secondary antibodies were added for 30 min at room temperature. ABC (Vector Lab) solution was then added after washing and incubated for 1 hour at room temperature. DAB (diaminobenzidine) colour generation system was used for chromogenic visualization. The expression of CD31 and α-SMA were observed and photographed using a microscope. Positively stained area per unit area was analysed among different groups by ImageJ software (NIH, USA).

9) Statistical analyses

The results were reported as M ± SD, and the statistical significance was analysed by GraphPad Prism 7 software (USA). Parametric tests were applied after testing for normal distributions and equal variances. Student t-test was used for comparing the difference between two groups, and one-way ANOVA followed by Tukey's multiple comparison post hoc test was utilized when comparing differences among more than two groups. $p < 0.05$ was used as the standard for statistical significance.

3. Results

1) Expression of BDNF, ProBDNF, TrkB, Sortilin and p75^NTR in HUVEC

Von Willebrand factor (*vWF*) is a glycoprotein synthesized by endothelial cells and is a commonly used marker for HUVECs. Immunofluorescence staining of *vWF* exhibited a cobblestone-like or spindle-shaped appearance in the HUVEC cytoplasm (Figure 1(a)). ProBDNF and mBDNF are mainly expressed in the cytoplasm as homogeneous red fluorescence staining. ProBDNF has stronger staining around the nuclear membrane compared with mBDNF (Figure 1(b) and Figure 1(c)). Sortilin and p75^NTR are expressed in both the cytoplasm and nucleus, shown as green fluorescence staining (Figure 1(d) and Figure 1(e)). TrkB exhibits a cytoplasmic network shaped pattern in HUVEC cytoplasm, with stronger cell membrane staining (Figure 1(f)). No fluorescence is detected in the control cells. Western blot assays further confirm that HUVEC ex-

presses mBDNF, proBDNF, TrkB, sortilin and p75^{NTR} (**Figure 1(g)**). ProBDNF staining is colocalized with p75^{NTR} or sortilin in HUVEC cytoplasm (**Figure 2**).

2) Effect of proBDNF on HUVEC Viability

To investigate the effect of proBDNF on HUVEC viability, MTT assay was performed by dividing HUVEC into treatment groups: control, BSA, proBDNF 1 ng/mL, proBDNF 3 ng/mL, proBDNF 10 ng/mL, proBDNF 30 ng/mL, proBDNF 100 ng/mL and proBDNF 100 ng/mL with anti-proBDNF (10 μg/mL). Treatment groups with proBDNF concentrations of 30 ng/mL and 100 ng/mL decreased HUVEC viability significantly ($p < 0.05$), compared with control and BSA groups. The suppressive effect of proBDNF on HUVEC can be counteracted by introducing anti-proBDNF into the culture medium (**Figure 3**).
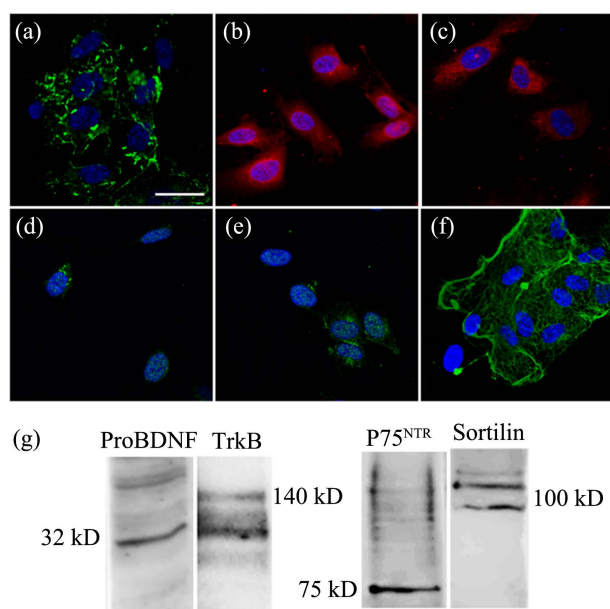

Figure 1. Immunofluorescence staining of HUVECs. (a) von Willebrand factor. (b) ProBDNF, (c) mBDNF, (d) P75^{NTR}, (e) Sortilin, (f) TrkB. (Bar = 40 μm). (g) Western blot of ProBDNF, p75NTR, Sortilin and TrkB in HUVEC.
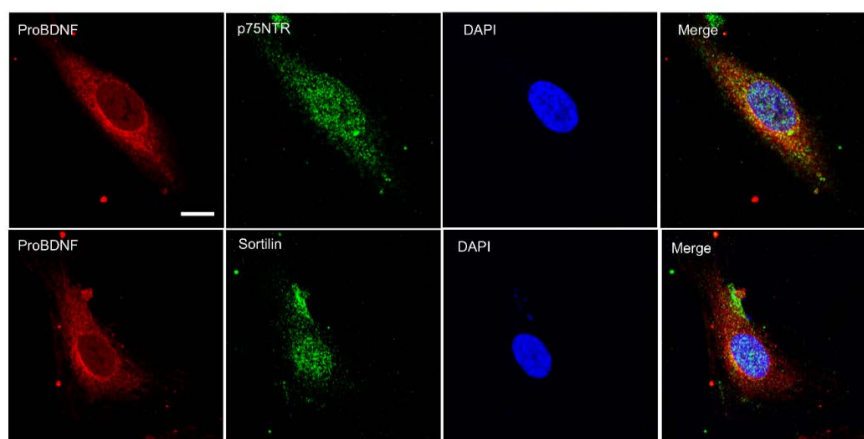

Figure 2. Double staining of ProBDNF with p75^{NTR} and Sortilin (Bar = 10 μm).

3) Effects of proBDNF on Angiogenesis *in Vitro*

HUVEC seeded onto Geltrex matrix coated plates developed well-formed tube networks after 14 - 16 hours incubation (**Figure 4(a)**). HUVEC that were incubated with proBDNF (50 ng/mL) also developed tube networks. However, the total mesh areas ($p < 0.01$) and the total branch lengths ($p < 0.05$) were significantly less than the control group (**Figure 4(b)** and **Figure 4(c)**).
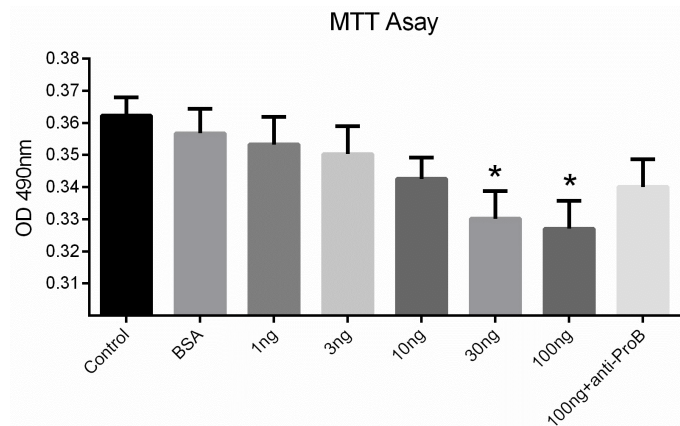

Figure 3. Effect of proBDNF on HUVEC viability. ProBDNF concentrations of 30 ng/mL and 100 ng/mL inhibit HUVEC viability significantly, compared with control and BSA groups. (*$p < 0.05$). This inhibitive effect is counteracted by anti-proBDNF (10 μg/mL).
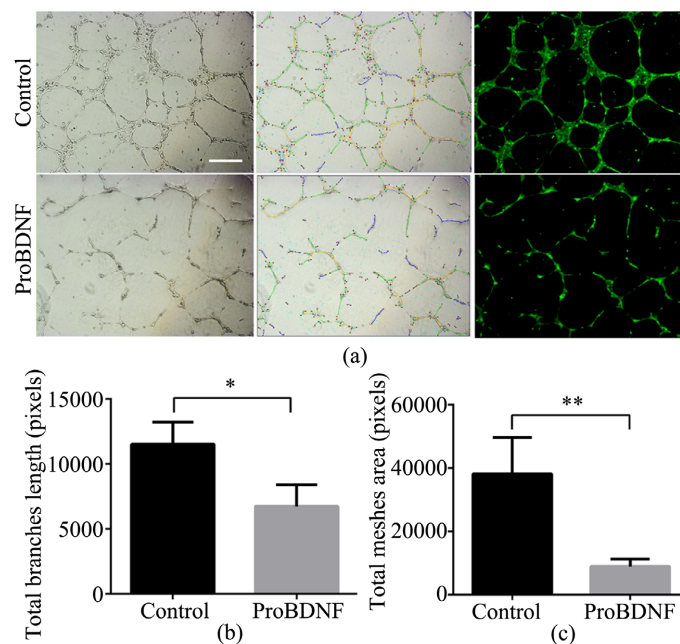

Figure 4. ProBDNF inhibitive effects on HUVEC angiogenesis *in vitro*. (a) HUVEC angiogenesis in the Control group and proBDNF (50 ng/mL) group. Left: phase-contrast images; middle: Analysing images using Angiogenesis Analyzer for ImageJ (tube structures are cyan, branches are green, segments are yellow, nodes are red, isolated structures are blue); right: fluorescent image with Calcein AM; (Bar = 100 μm). (b) Total branch lengths difference between control and proBDNF group (*$p < 0.05$). (c) Total mesh areas difference between control and proBDNF group (**$p < 0.01$).

4. Matrigel Plug Angiogenesis Assay

1) **Tissue scores were significantly decreased by pre-treatment with proBDNF**

H & E staining of Matrigel plug tissue revealed substantial vascular structures surrounding and infiltrating to the centre of the Matrigel in the mBDNF group and anti-proBDNF group (**Figure 5(a)**). There are several endothelial cells in the Matrigel of PBS group; however, there are few endothelial cells around the Matrigel and rare inside the centre of the Matrigel in the proBDNF group. Tissue scores in the proBDNF group were significantly lower than the PBS group ($p < 0.05$), anti-proBDNF group ($p < 0.001$), and mBDNF group ($p < 0.001$, not shown) (**Figure 5(b)**). Tissue score in the mBDNF group is higher than the anti-proBDNF group (*$p < 0.05$).

2) **CD31 and a-SMA expression were decreased by pre-treatment with proBDNF**

CD31 and a-SMA are the common markers for angiogenesis [30]. Immunohistochemical staining showed significantly lower expression of CD31 in the
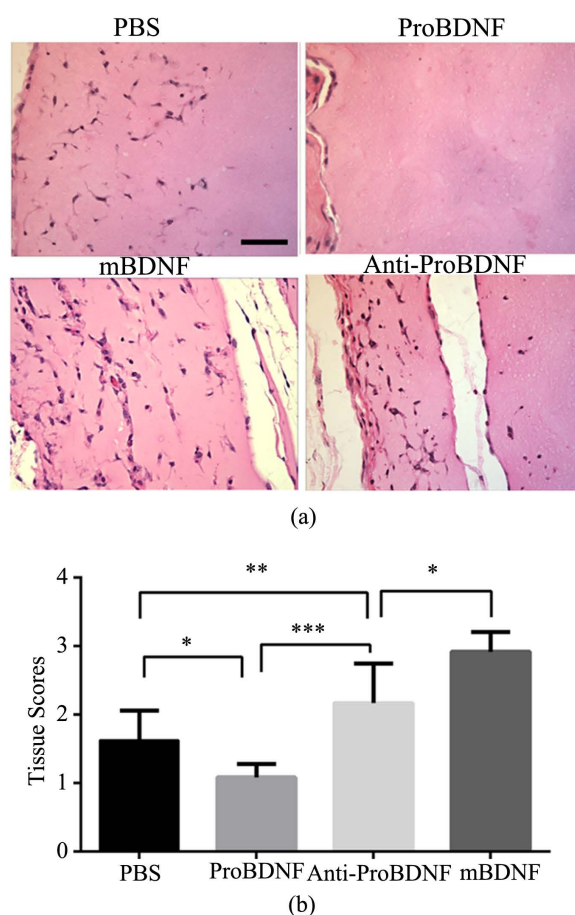

(a)

(b)

Figure 5. (a) H & E staining of Matrigel plug tissue in the different groups (Bar = 50 μm). (b) Comparison of tissue scores in different groups. Tissue scores were significantly decreased in the proBDNF group, compared with the PBS group (*$p < 0.05$), anti-proBDNF group (***$p < 0.001$) and mBDNF group (***$p < 0.001$, not shown). Tissue score in the mBDNF group is higher than the anti-proBDNF group (*$p < 0.05$) (n = 6).

proBDNF group, compared with the PBS group (p < 0.05), anti-proBDNF group (p < 0.001), and mBDNF group (p < 0.001, not shown) (**Figure 6(a)** and **Figure 6(c)**). The anti-proBDNF group also showed considerably higher CD31 expression than the PBS group (p < 0.01). The CD31 expression in mBDNF group was more elevated than anti-proBDNF group (p < 0.01) (**Figure 6(a)** and **Figure 6(c)**). The expression of α-SMA was also significantly lower in the proBDNF group, compared with the PBS group (p < 0.05), anti-proBDNF group (p < 0.001), and mBDNF group (p < 0.001, not shown) (**Figure 6(b)** and **Figure 6(d)**). The expression of α-SMA in the anti-proBDNF group was also substantially higher than in the PBS group (p < 0.001). The mBDNF group demonstrated higher expression than the anti-proBDNF group (p < 0.01) (**Figure 6(b)** and **Figure 6(d)**).
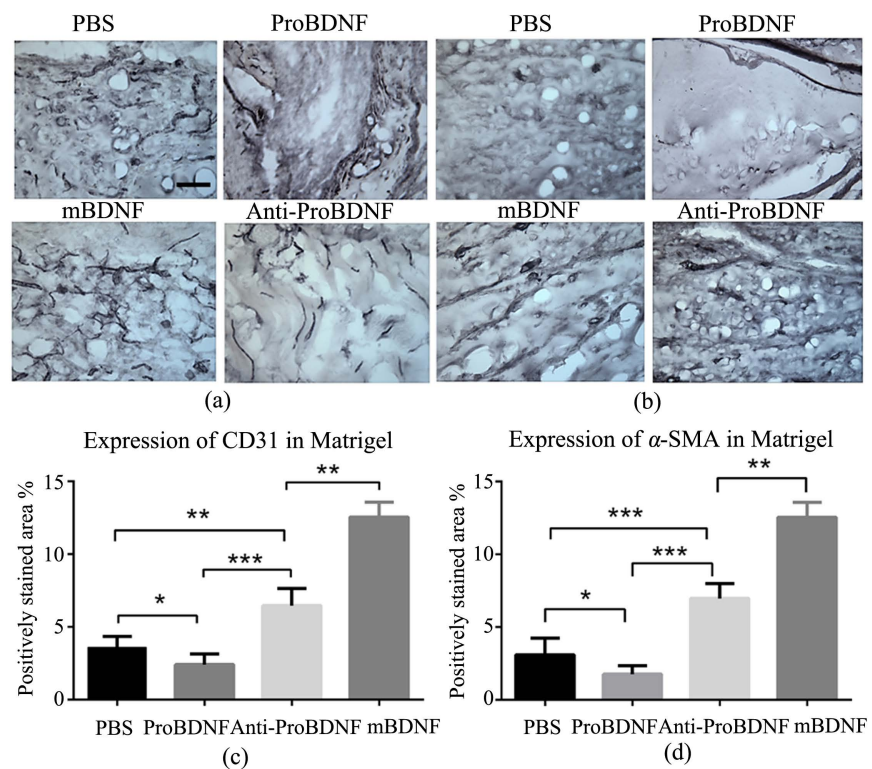

Figure 6. (a) The expression of CD31 in different treatment groups (Bar = 50 μm). (b) The expression of α-SMA in different treatment groups. (c) Comparison of CD31 expression in Matrigel plugs. CD31 was significantly decreased in the proBDNF group, compared with the PBS group (*p < 0.05), anti-proBDNF group (***p < 0.001), and mBDNF group (***p < 0.001, not shown). Anti-proBDNF showed increased CD31 expression significantly, compared with the PBS group (**p < 0.01), and the mBDNF group has more CD31 expression than the anti-proBDNF group (**p < 0.01). (d) Comparison of α-SMA expression in Matrigel plugs. α-SMA was significantly decreased in the proBDNF group, compared with the PBS group (p < 0.05), anti-proBDNF group (p < 0.001), and mBDNF group (p < 0.001, not shown) (***p < 0.001). The expression of α-SMA in the anti-proBDNF group was also substantially greater than the PBS group (p < 0.001). The mBDNF group exhibited higher expression than the anti-proBDNF group (p < 0.01) (n = 6).

5. Discussion

BDNF is a widely known neurotrophic factor, which regulates neuronal survival and differentiation, and is involved in long-term potentiation and learning memory. ProBDNF, as the precursor of BDNF, is cleaved to yield mBDNF by either intracellular or extracellular enzymes. ProBDNF and mature BDNF often manifest opposing biological effects. Recent studies show that BDNF is not only expressed in the nervous system but also in a wide range of non-neural tissues and cells, for example, heart, ovaries, bone marrow, vascular smooth cells and endothelial cells [31] [32]. In the present study, we also found that proBDNF and its receptors Sortilin and p75^{NTR} were expressed in HUVEC.

In addition to its neuropoietic effects, BDNF is unveiled to promote endothelial cell survival and induce angiogenesis. It is shown that BDNF plays a vital role in modulating vascular development. Compared to VEGF, which is crucial for the early stages of vascular development, BDNF has indispensable effects in perinatal and adult vasculature. BDNF is detectable in endothelial cells of the heart, and skeletal muscle at late gestation and the expression persists at elevated levels into adulthood [33]. Hempstead speculated that VEGF initiates the angiogenic process, but BDNF stabilizes and maintains the vasculature [34]. In the BDNF knockout mouse (BDNF−/−) model, it was shown that embryonic vasculature could be formed in the heart and be able to transform into arteries, capillaries and veins, but the endothelial cells cannot function through the late gestational to early postnatal stages, and vascular haemorrhages occur [33]. BDNF knockout impairs endothelial cell-cell contacts and causes endothelial cell apoptosis, which results in hypo-contractile heart and perinatal mortality. These results indicate BDNF plays a crucial role in regulating cardiac vascular endothelial cells and maintaining cardiac vessel stability in the gestational and postnatal periods [33]. BDNF has recently been described as a new mediator of angiogenesis [34]. BDNF significantly stimulates the migration of HUVEC *in vitro* and induces angiogenesis in tube formation assay and Matrigel plug in a mouse model [35]. BDNF induces angiogenin secretion and is involved in the nuclear translocation of angiogenin in HUVEC [36]. In a conditional genetic switching of the VEGF model, VEGF induction can lead to new vessel formation, but the vessels can be leaky and disorganized, causing severe tissue oedema [37]. In comparison, BDNF promotes an angiogenic response after cerebral ischemia without inducing fragile and malformed vessels, in contrast to VEGF [38] [39]. The angiogenic effects of BDNF on ischemia are not only by stimulating existing blood vessels to sprout but also by activating and organizing stem cells to the vasculature site [10].

Based on the known effect of BDNF as an angiogenesis mediator and the importance of balancing mBDNF and proBDNF, it is intriguing to know how proBDNF affects the process of angiogenesis. Our study shows that proBDNF inhibits HUVEC viability significantly, as revealed by the MTT assay. This inhibition can be counteracted by introducing anti-proBDNF. ProBDNF can also

significantly suppress angiogenesis *in vivo* and *in vitro*. Endothelial cell tube formation assay exhibits total mesh areas and total branch lengths of tube networks that are substantially less in the proBDNF group than the control group. In Matrigel plug assay, the mean score for cell infiltration is significantly less in the proBDNF group, while anti-proBDNF pre-treatment increase cell infiltration scores. The immunostaining of CD31 and α-SMA (markers for angiogenesis) in Matrigel also confirms the inhibitive effect of proBDNF on angiogenesis. ProBDNF is likely via activating p75^{NTR}/sortilin signals by an autocrine/paracrine mechanism as we showed that HUVEC expressed proBDNF colocalized with p75^{NTR}/sortilin in these cells. These results indicate that proBDNF has an opposite effect to BDNF, acting as an angiogenesis inhibitor, providing more evidence for the balanced function of pro-neurotrophin and mature neurotrophin. Our results are consistent with other studies that show the inhibitive effect of pro-neurotrophins on vascular structure and function. Research has shown that proNGF induced endothelial cell death via the p75^{NTR} in an oxygen-induced retinopathy mouse model, introducing potential therapeutic targets for the treatment of proliferative retinopathies [40]. In a hypoxia/reoxygenation model, proBDNF mediated myocardial microvascular endothelial cell (MMEC) injury by reducing MMEC migration and decreasing the formation of capillary-like structures [41]. ProBDNF and proNGF are highly expressed in the infarcted rat heart, triggering damage to the pericytes of blood vessels, and p75ECD-Fc, the scavenger of proneurotrophin can protect the structure and function of the ischemic heart [42]. P75^{NTR}, as the preferred receptor of proneurotrophin, can promote endothelial cell apoptosis and inhibit angiogenesis. P75^{NTR} impairs postischemic neovascularization and blood flow recovery response to limb ischemia in diabetes. It suggests that inhibiting p75^{NTR} could be a potential therapy for treating diabetes-induced microvascular diseases. [43]. Silencing p75^{NTR} can also prevent proNGF-induced endothelial cell death and the development of acellular capillaries in rat retina, and deletion of p75^{NTR} can protect against retinal ischemia and prevent retinal neovascularization in the ischemic retinopathy model [44] [45]. These studies support that proneurotrophins have opposite effects on endothelial cell survival and angiogenesis compared with mature neurotrophins.

Understanding the opposite functions of BDNF and proBDNF on angiogenesis is essential to broaden the insight for treating diseases like cerebral ischemia and cancer. Current research shows that neuro-restoration, including angiogenesis, neurogenesis and synaptic plasticity, are likely to have a far greater window of time for intervention than acute neuroprotective treatments [46]. The advantage of BDNF as a selective angiogenic factor is minimal secondary side effects such as the induction of vascular permeability, which could lead to the development of novel and specific angiogenic therapies for cerebral ischemia. On the other hand, angiogenesis inhibitors pose a promising therapeutic strategy for tumours [47]. Research shows the efficacy of anti-angiogenic monotherapies is compromised due to the redundancy of angiogenic signals. When treating

with a single pro-angiogenic molecule, the tumours tend to evade the pathway and become refractory by upregulating alternative angiogenic factors as compensation. Therefore, more and more research focuses on combining different anti-angiogenic elements and finding accurate time windows for anti-angiogenic action on tumours [48]. Our study provides a new anti-angiogenic factor, proBDNF, which could contribute to rational anti-angiogenic drug combination therapy and personalized molecular therapy for tumour or other proliferative vascular diseases.

6. Conclusion

In contrast to mature BDNF, proBDNF acts as an inhibitor of angiogenesis as revealed by the reduction of survival in endothelial cells, and a decrease in blood vessel formation *in vitro* and *in vivo*. Therefore, suppression of endogenous proBDNF in endothelial cells can promote the survival of endothelial cells and angiogenesis. This finding could provide more therapeutic strategies for diseases, such as cerebral ischemia, tumours, and other vascular diseases.

Acknowledgements

Authors' Contributions

XFZ supervised and coordinated all aspects of the work. HL performed the experiments, analysed data, prepared figures, and wrote the paper. FFB assisted with animal experiments. AB helped with immunostaining and provided technical support. LB provided material support and revised the manuscript. All authors have read and approved the final manuscript for submission. The authors declare no competing financial interests.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Lee, R., Kermani, P., Teng, K.K. and Hempstead, B.L. (2001) Regulation of Cell Survival by Secreted Proneurotrophins. *Science*, **294**, 1945-1948.
https://doi.org/10.1126/science.1065057

[2] Pang, P.T., Teng, H.K., Zaitsev, E., Woo, N.T., Sakata, K., Zhen, S., Teng, K.K., Yung, W.H., Hempstead, B.L. and Lu, B. (2004) Cleavage of proBDNF by tPA/Plasmin Is Essential for Long-Term Hippocampal Plasticity. *Science*, **306**, 487-491.
https://doi.org/10.1126/science.1100135

[3] Nagappan, G., Zaitsev, E., Senatorov, V.V., Yang, J., Hempstead, B.L. and Lu, B. (2009) Control of Extracellular Cleavage of ProBDNF by High Frequency Neuronal

Activity. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 1267-1272. https://doi.org/10.1073/pnas.0807322106

[4] Lu, B., Pang, P.T. and Woo, N.H. (2005) The Yin and Yang of Neurotrophin Action. *Nature Reviews Neuroscience*, **6**, 603-614. https://doi.org/10.1038/nrn1726

[5] Li, C.L. (1974) A Brief Outline of Chinese Medical History with Particular Reference to Acupuncture. *Perspectives in Biology and Medicine*, **18**, 132-143. https://doi.org/10.1353/pbm.1974.0013

[6] Cantarella, G., Lempereur, L., Presta, M., Ribatti, D., Lombardo, G., Lazarovici, P., Zappalà, G., Pafumi, C. and Bernardini, R. (2002) Nerve Growth Factor-Endothelial Cell Interaction Leads to Angiogenesis *in Vitro* and *in Vivo*. *The FASEB Journal*, **16**, 1307-1309. https://doi.org/10.1096/fj.01-1000fje

[7] Lazarovici, P., Gazit, A., Staniszewska, I., Marcinkiewicz, C. and Lelkes, P.I. (2006) Nerve Growth Factor (NGF) Promotes Angiogenesis in the Quail Chorioallantoic Membrane. *Endothelium*, **13**, 51-59. https://doi.org/10.1080/10623320600669053

[8] Romon, R., Adriaenssens, E., Lagadec, C., Germain, E., Hondermarck, H. and Le Bourhis, X. (2010) Nerve Growth Factor Promotes Breast Cancer Angiogenesis by Activating Multiple Pathways. *Molecular Cancer*, **9**, Article No. 157. https://doi.org/10.1186/1476-4598-9-157

[9] Garrido, M.P., Vera, C., Vega, M., Quest, A.F.G. and Romero, C. (2018) Metformin Prevents Nerve Growth Factor-Dependent Proliferative and Proangiogenic Effects in Epithelial Ovarian Cancer Cells and Endothelial Cells. *Therapeutic Advances in Medical Oncology*, **10**, 1-16. https://doi.org/10.1177/1758835918770984

[10] Kermani, P., Rafii, D., Jin, D.K., Whitlock, P., Schaffer, W., Chiang, A., Vincent, L., Friedrich, M., Shido, K., Hackett, N.R., Crystal, R.G., Rafii, S. and Hempstead, B.L. (2005) Neurotrophins Promote Revascularization by Local Recruitment of TrkB+ Endothelial Cells and Systemic Mobilization of Hematopoietic Progenitors. *The Journal of Clinical Investigation*, **115**, 653-663. https://doi.org/10.1172/JCI200522655

[11] Usui, T., Naruo, A., Okada, M., Hayabe, Y. and Yamawaki, H. (2014) Brain-Derived Neurotrophic Factor Promotes Angiogenic Tube Formation through Generation of Oxidative Stress in Human Vascular Endothelial Cells. *Acta Physiologica*, **211**, 385-394. https://doi.org/10.1111/apha.12249

[12] Lam, C.T., Yang, Z.F., Lau, C.K., Tam, K.H., Fan, S.T. and Poon, R.T. (2011) Brain-Derived Neurotrophic Factor Promotes Tumorigenesis via Induction of Neovascularization: Implication in Hepatocellular Carcinoma. *Clinical Cancer Research*, **17**, 3123-3133. https://doi.org/10.1158/1078-0432.CCR-10-2802

[13] Zhang, Z., Zhang, Y., Zhou, Z., Shi, H., Qiu, X., Xiong, J. and Chen, Y. (2017) BDNF Regulates the Expression and Secretion of VEGF from Osteoblasts via the TrkB/ERK1/2 Signaling Pathway during Fracture Healing. *Molecular Medicine Reports*, **15**, 1362-1367. https://doi.org/10.3892/mmr.2017.6110

[14] Lin, C.Y., Hung, S.Y., Chen, H.T., Tsou, H.K., Fong, Y.C., Wang, S.W. and Tang, C.H. (2014) Brain-Derived Neurotrophic Factor Increases Vascular Endothelial Growth Factor Expression and Enhances Angiogenesis in Human Chondrosarcoma Cells. *Biochemical Pharmacology*, **91**, 522-533. https://doi.org/10.1016/j.bcp.2014.08.008

[15] Julio-Pieper, M., Lozada, P., Tapia, V., Vega, M., Miranda, C., Vantman, D., Ojeda, S.R. and Romero, C. (2009) Nerve Growth Factor Induces Vascular Endothelial Growth Factor Expression in Granulosa Cells via a trkA Receptor/Mitogen-Activated Protein Kinase-Extracellularly Regulated Kinase 2-Dependent Pathway. *The Journal of Clinical Endocrinology & Metabolism*, **94**, 3065-3071.

https://doi.org/10.1210/jc.2009-0542

[16] Felmeden, D.C., Blann, A.D. and Lip, G.Y.H. (2003) Angiogenesis: Basic Pathophysiology and Implications for Disease. *European Heart Journal*, **24**, 586-603. https://doi.org/10.1016/S0195-668X(02)00635-8

[17] Carmeliet, P. (2000) Mechanisms of Angiogenesis and Arteriogenesis. *Nature Medicine*, **6**, 389-395. https://doi.org/10.1038/74651

[18] Distler, J.H., Hirth, A., Kurowska-Stolarska, M., Gay, R.E., Gay, S. and Distler, O. (2003) Angiogenic and Angiostatic Factors in the Molecular Control of Angiogenesis. *The Quarterly Journal of Nuclear Medicine*, **47**, 149-161.

[19] Hoeben, A., Landuyt, B., Highley, M.S., Wildiers, H., Van Oosterom, A.T. and De Bruijn, E.A. (2004) Vascular Endothelial Growth Factor and Angiogenesis. *Pharmacological Reviews*, **56**, 549-580. https://doi.org/10.1124/pr.56.4.3

[20] Carmeliet, P. and Jain, R.K. (2011) Molecular Mechanisms and Clinical Applications of Angiogenesis. *Nature*, **473**, 298-307. https://doi.org/10.1038/nature10144

[21] Chen, J. and Chopp, M. (2006) Neurorestorative Treatment of Stroke: Cell and Pharmacological Approaches. *NeuroRx*: *The Journal of the American Society for Experimental NeuroTherapeutics*, **3**, 466-473. https://doi.org/10.1016/j.nurx.2006.07.007

[22] Dhanabal, M., Jeffers, M. and Larochelle, W.J. (2005) Anti-Angiogenic Therapy as a Cancer Treatment Paradigm. *Current Medicinal Chemistry Anti-Cancer Agents*, **5**, 115-130. https://doi.org/10.2174/1568011053174882

[23] Nussenbaum, F. and Herman, I.M. (2010) Tumor Angiogenesis: Insights and Innovations. *Journal of Oncology*, **2010**, Article ID: 132641. https://doi.org/10.1155/2010/132641

[24] Mancuso, M.R., Davis, R., Norberg, S.M., O'Brien, S., Sennino, B., Nakahara, T., Yao, V.J., Inai, T., Brooks, P., Freimark, B., Shalinsky, D.R., Hu-Lowe, D.D. and McDonald, D.M. (2006) Rapid Vascular Regrowth in Tumors after Reversal of VEGF Inhibition. *The Journal of Clinical Investigation*, **116**, 2610-2621. https://doi.org/10.1172/JCI24612

[25] Allegra, C.J., Yothers, G., O'Connell, M.J., Sharif, S., Petrelli, N.J., Colangelo, L.H., Atkins, J.N., Seay, T.E., Fehrenbacher, L., Goldberg, R.M., O'Reilly, S., Chu, L., Azar, C.A., Lopa, S. and Wolmark, N. (2011) Phase III Trial Assessing Bevacizumab in Stages II and III Carcinoma of the Colon: Results of NSABP Protocol C-08. *Journal of Clinical Oncology*: *Official Journal of the American Society of Clinical Oncology*, **29**, 11-16. https://doi.org/10.1200/JCO.2010.30.0855

[26] Vistica, D.T., Skehan, P., Scudiero, D., Monks, A., Pittman, A. and Boyd, M.R. (1991) Tetrazolium-Based Assays for Cellular Viability: A Critical Examination of Selected Parameters Affecting Formazan Production. *Cancer Research*, **51**, 2515-2520.

[27] Ko, J.M.Y. and Lung, M.L. (2012) *In Vitro* Human Umbilical Vein Endothelial Cells (HUVEC) Tube-Formation Assay. *Bio-protocol*, **2**, e260. https://doi.org/10.21769/BioProtoc.260

[28] Carpentier, G. (2012) Angiogenesis Analyzer for ImageJ. *ImageJ User and Developer Conference*, Luxembourg, 24-26 October 2012.

[29] Zhao, H., Zhang, Y., Zhang, Y., Shen, Y., Zhang, Y., Bi, F., Xiao, B., Zhang, H., Ye, W., Zhang, H. and Liao, Y. (2018) NGF/FAK Signal Pathway Is Implicated in Angiogenesis after Acute Cerebral Ischemia in Rats. *Neuroscience Letters*, **672**, 96-102. https://doi.org/10.1016/j.neulet.2018.02.023

[30] Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R.K. and McDonald, D.M.

(2002) Abnormalities in Pericytes on Blood Vessels and Endothelial Sprouts in Tumors. *The American Journal of Pathology*, **160**, 985-1000. https://doi.org/10.1016/S0002-9440(10)64920-6

[31] Nakahashi, T., Fujimura, H., Altar, C.A., Li, J., Kambayashi, J., Tandon, N.N. and Sun, B. (2000) Vascular Endothelial Cells Synthesize and Secrete Brain-Derived Neurotrophic Factor. *FEBS Letters*, **470**, 113-117. https://doi.org/10.1016/S0014-5793(00)01302-8

[32] Kim, H., Li, Q., Hempstead, B.L. and Madri, J.A. (2004) Paracrine and Autocrine Functions of Brain-Derived Neurotrophic Factor (BDNF) and Nerve Growth Factor (NGF) in Brain-Derived Endothelial Cells. *The Journal of Biological Chemistry*, **279**, 33538-33546. https://doi.org/10.1074/jbc.M404115200

[33] Donovan, M.J., Lin, M.I., Wiegn, P., Ringstedt, T., Kraemer, R., Hahn, R., Wang, S., Ibañez, C.F., Rafii, S. and Hempstead, B.L. (2000) Brain Derived Neurotrophic Factor Is an Endothelial Cell Survival Factor Required for Intramyocardial Vessel Stabilization. *Development* (*Cambridge, England*), **127**, 4531-4540. https://doi.org/10.1242/dev.127.21.4531

[34] Kermani, P. and Hempstead, B. (2007) Brain-Derived Neurotrophic Factor: A Newly Described Mediator of Angiogenesis. *Trends in Cardiovascular Medicine*, **17**, 140-143. https://doi.org/10.1016/j.tcm.2007.03.002

[35] Sun, C., Hu, Y., Chu, Z., Huang, J. and Zhang, L. (2009) The Effect of Brain-Derived Neurotrophic Factor on Angiogenesis. *Journal of Huazhong University of Science and Technology Medical Sciences*, **29**, 139-143. https://doi.org/10.1007/s11596-009-0201-6

[36] Mori, A., Nishioka, Y., Yamada, M., Nishibata, Y., Masuda, S., Tomaru, U., Honma, N., Moriyama, T. and Ishizu, A. (2018) Brain-Derived Neurotrophic Factor Induces Angiogenin Secretion and Nuclear Translocation in Human Umbilical Vein Endothelial Cells. *Pathology, Research and Practice*, **214**, 521-526. https://doi.org/10.1016/j.prp.2018.02.013

[37] Dor, Y., Djonov, V., Abramovitch, R., Itin, A., Fishman, G.I., Carmeliet, P., Goelman, G. and Keshet, E. (2002) Conditional Switching of VEGF Provides New Insights into Adult Neovascularization and Pro-Angiogenic Therapy. *The EMBO Journal*, **21**, 1939-1947. https://doi.org/10.1093/emboj/21.8.1939

[38] Zhang, Z.G., Zhang, L., Tsang, W., Soltanian-Zadeh, H., Morris, D., Zhang, R., Goussev, A., Powers, C., Yeich, T. and Chopp, M. (2002) Correlation of VEGF and Angiopoietin Expression with Disruption of Blood-Brain Barrier and Angiogenesis after Focal Cerebral Ischemia. *Journal of Cerebral Blood Flow and Metabolism*: *Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, **22**, 379-392. https://doi.org/10.1097/00004647-200204000-00002

[39] Wang, Y., Kilic, E., Kilic, U., Weber, B., Bassetti, C.L., Marti, H.H. and Hermann, D.M. (2005) VEGF Overexpression Induces Post-Ischaemic Neuroprotection, But Facilitates Haemodynamic Steal Phenomena. *Brain*: *A Journal of Neurology*, **128**, 52-63. https://doi.org/10.1093/brain/awh325

[40] Sitaras, N., Saragovi, H.U., Chemtob, S. and Sapieha, P. (2013) Pro-NGF Induces Endothelial Cell Death via the P75 Neurotrophin Receptor (P75NTR) in a Mouse Model of Oxygen-Induced Retinopathy (OIR). *Investigative Ophthalmology and Visual Science*, **54**, 6346-6346.

[41] Yu, F., Liu, Y. and Xu, J. (2018) Pro-BDNF Contributes to Hypoxia/Reoxygenation Injury in Myocardial Microvascular Endothelial Cells: Roles of Receptors $p75^{NTR}$ and Sortilin and Activation of JNK and Caspase 3. *Oxidative Medicine and Cellular*

Longevity, **2018**, Article ID: 3091424. https://doi.org/10.1155/2018/3091424

[42] Fang, J., Wei, Z., Zheng, D., Ying, T., Hong, H., Hu, D., Lin, Y., Jiang, X., Wu, L., Lan, T., Yang, Z., Zhou, X. and Chen, L. (2020) Recombinant Extracellular Domain (p75ECD) of the Neurotrophin Receptor p75 Attenuates Myocardial Ischemia-Reperfusion Injury by Inhibiting the p-JNK/Caspase-3 Signaling Pathway in Rat Microvascular Pericytes. *Journal of the American Heart Association*, **9**, e016047. https://doi.org/10.1161/JAHA.119.016047

[43] Caporali, A., Pani, E., Horrevoets, A.J.G., Kraenkel, N., Oikawa, A., Sala-Newby, G.B., Meloni, M., Cristofaro, B., Graiani, G., Leroyer, A.S., Boulanger, C.M., Spinetti, G., Yoon, S.O., Madeddu, P. and Emanueli, C. (2008) Neurotrophin p75 Receptor (p75NTR) Promotes Endothelial Cell Apoptosis and Inhibits Angiogenesis: Implications for Diabetes-Induced Impaired Neovascularization in Ischemic Limb Muscles. *Circulation Research*, **103**, e15-e26. https://doi.org/10.1161/CIRCRESAHA.108.177386

[44] Shanab, A.Y., Mysona, B.A., Matragoon, S. and El-Remessy, A.B. (2015) Silencing p75(NTR) Prevents proNGF-Induced Endothelial Cell Death and Development of Acellular Capillaries in Rat Retina. *Molecular Therapy—Methods & Clinical Development*, **2**, 15013-15013. https://doi.org/10.1038/mtm.2015.13

[45] Elshaer, S.L. and El-Remessy, A.B. (2018) Deletion of p75NTR Prevents Vaso-Obliteration and Retinal Neovascularization via Activation of Trk-A Receptor in Ischemic Retinopathy Model. *Scientific Reports*, **8**, Article No. 12490. https://doi.org/10.1038/s41598-018-30029-0

[46] Font, M.A., Arboix, A. and Krupinski, J. (2010) Angiogenesis, Neurogenesis and Neuroplasticity in Ischemic Stroke. *Current Cardiology Reviews*, **6**, 238-244. https://doi.org/10.2174/157340310791658802

[47] Zuazo-Gaztelu, I. and Casanovas, O. (2018) Unraveling the Role of Angiogenesis in Cancer Ecosystems. *Frontiers in Oncology*, **8**, Article No. 248. https://doi.org/10.3389/fonc.2018.00248

[48] Abdollahi, A. and Folkman, J. (2010) Evading Tumor Evasion: Current Concepts and Perspectives of Anti-Angiogenic Cancer Therapy. *Drug Resistance Updates: Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy*, **13**, 16-28. https://doi.org/10.1016/j.drup.2009.12.001