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ARTICLE

Silencing p75[NTR] prevents proNGF-induced endothelial cell death and development of acellular capillaries in rat retina

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Accumulation of the nerve growth factor precursor (proNGF) and its receptor p75[NTR] have been associated with several neuro-degenerative diseases in both brain and retina. However, whether proNGF contributes to microvascular degeneration remain unexplored. This study seeks to investigate the mechanism by which proNGF/p75[NTR] induce endothelial cell (EC) death and development of acellular capillaries, a surrogate marker of retinal ischemia. Stable overexpression of the cleavage-resistant proNGF and molecular silencing of p75[NTR] were utilized in human retinal EC and rat retinas *in vivo*. Stable overexpression of proNGF decreased NGF levels and induced retinal vascular cell death evident by 1.9-fold increase in acellular capillaries and activation of JNK and cleaved-PARP that were mitigated by p75[NTR]shRNA. *In vitro*, overexpression of proNGF did not alter TNF-α level, reduced NGF, however induced EC apoptosis evident by activation of JNK and p38 MAPK, cleaved-PARP. Silencing p75[NTR] using siRNA restored expression of NGF and TrkA activation and prevented EC apoptosis. Treatment of EC with human-mutant proNGF induced apoptosis that coincided with marked protein interaction and nuclear translocation of p75[NTR] and the neurotrophin receptor interacting factor. These effects were abolished by a selective p75[NTR] antagonist. Therefore, targeting p75[NTR] represents a potential therapeutic strategy for diseases associated with aberrant expression of proNGF.

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INTRODUCTION

The retina, a typical neurovascular unit is composed of vascular cells (endothelial and pericyte) that interact with neurons and glia to maintain retinal homeostasis under variant conditions.[1] Retinal ischemia gauged as an inadequacy, but not essentially a complete block of blood flow can result in failure to meet the high energy demand of retina cells. Central and branched retinal vein occlusion and diabetic retinopathy are the most well-defined retinal micro-angiopathy diseases in adults.[2] Due to lack of effective treatments, retinal ischemia remains a common cause of visual impairment and blindness in the working age. Therefore, understanding of the molecular events that govern microvascular degeneration is critical for devising new treatments for such blinding diseases.

Neurons and glia are the main source of neurotrophins, essential factors for differentiation, development, and maturation of neuro-nal cells.[3–6] Although typically studied in relation to their actions on neurons, the role of neurotrophins in maintaining the proper function of the vasculature has gained recent attention. The first discovered neurotrophin, nerve growth factor (NGF), is secreted as a proform NGF (proNGF) that is traditionally cleaved intracellular by furin and extracellular by the matrix metalloproteineases including MMP-7 and -9 into the mature ligand.[7] NGF promotes cell survival by preferentially binding the prosurvival receptor tyrosine kinase A (TrkA), while proNGF has high affinity to bind and activate p75[NTR],

resulting in cell death.[3,8] Accumulation of proNGF has been associated with neurodegenerative diseases such as Alzheimer's,[9] Down's syndrome,[10] and retinal neurodegenerative diseases.[11,12] Our group was the first to demonstrate that diabetes-induced oxidative milieu impairs MMP-7 activity and maturation of NGF, resulting in accumulation of its precursor, proNGF, in clinical and experimental diabetes.[13–15] Next, we attempted to mimic accumulation of proNGF via an artificial model of overexpressing cleavage-resistant proNGF in rat retina to examine molecular events triggered by proNGF apart from diabetic milieu.[16–18] These studies elucidated that proNGF overexpression causes glial activation, retinal inflammation, and neurodegeneration.

Deleterious effects of the proNGF/p75[NTR] pathway on retinal neurodegeneration have been shown to occur via direct action on neurons[16] or indirectly via paracrine stimulation of proinflammatory mediators in Müller cells.[12,16,17] While the role of proNGF/p75[NTR] in mediating neuronal cell death is well-documented, the extent and mechanism by which proNGF/p75[NTR] contributes to reti-nal endothelial cell (EC) death *in vitro* and development of acellular capillaries *in vivo* remains unexplored. P75[NTR] lacks intrinsic kinase activity; instead, p75[NTR] undergoes two-step proteolytic cleavage by α- and γ-secretase to release the intracellular domain (p75[ICD]) that can recruit different protein adaptors to dictate specific signaling pathways.[19,20] In neurons, a number of interacting proteins have

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been identified that can bind the p75^{ICD} in the cytosol and activate proapoptotic signaling including neurotrophin receptor interacting factor (NRIF)[21] and tumor necrosis factor receptor associated factor-6 (TRAF6).[22] The significance of NRIF has been established based on phenotypic similarities between NRIF$^{-/-}$ and p75NTR$^{-/-}$ mice[23] and on its essential role in p75^{NTR}-mediated apoptosis in the developing mouse retina.[24] Nevertheless, the impact of modulating expression p75^{NTR} on EC death and the crosstalk between p75^{NTR} and TrkA in EC remain largely unexplored.

The aim of this study was to elucidate the molecular mechanisms by which p75^{NTR} contributes to proNGF-induced acellular capillary formation, a hallmark of retinal ischemia. A comprehensive approach using stable overexpression of cleavage-resistant proNGF, and pharmacological and molecular modulators of p75^{NTR} expression and activity were used to probe the mechanisms in vivo and in vitro. The results demonstrate protective effect of modulating p75^{NTR} expression at two levels: by inhibiting proapoptotic action of proNGF/p75^{NTR} and by restoring endogenous survival pathways of NGF/TrkA.

RESULTS

Silencing p75^{NTR} prevents proNGF accumulation and restores NGF levels in rat retina

To examine the contribution of proNGF to retinal microvascular degeneration, we used our established protocol of overexpressing cleavage-resistant proNGF in rat retina.[18] Before electroporation, rats received single intraocular injection of one of the following: pGFP plus scrambled shRNA (GFP+Scr), pGFP plus short hairpin RNA (shRNA) against p75^{NTR} (GFP+shRNA); pGFP-proNGF123, a cleavage-resistant form of proNGF plus scrambled shRNA (proNGF+Scr); pGFP-proNGF123 plus shRNA against p75^{NTR} (proNGF+shRNA). After 6-weeks, retinas were collected and
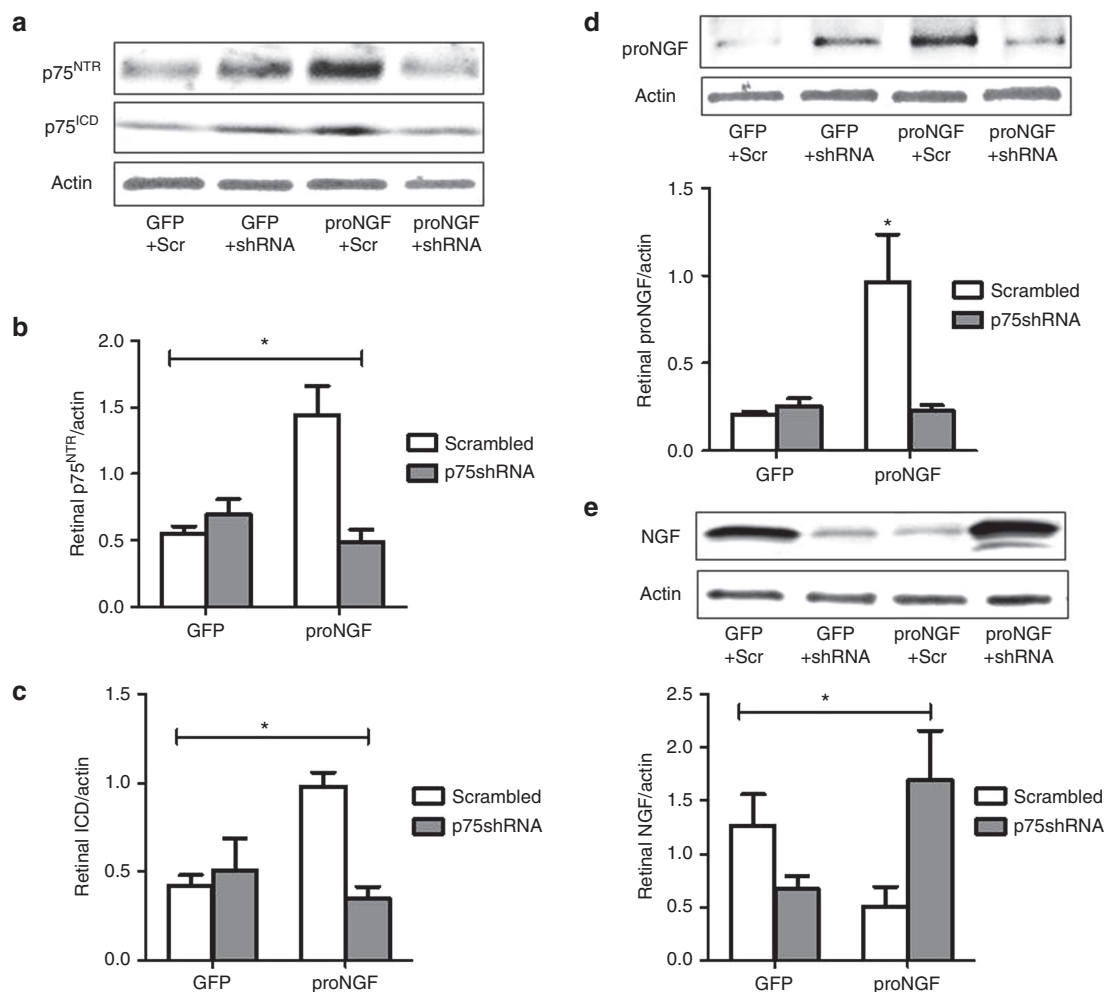

Figure 1 Silencing p75^{NTR} prevents proNGF accumulation and restores NGF levels in rat retina. Rats received single intraocular injection of one of the following: pGFP plus scrambled shRNA (GFP+Scr), pGFP plus shRNA against p75^{NTR} (GFP+shRNA); pGFP-proNGF123, a cleavage-resistant form of proNGF plus scrambled shRNA (proNGF+Scr); pGFP-proNGF123 plus shRNA against p75^{NTR} (proNGF+shRNA). Retinas were lysed after 6 weeks. (**a**) Representative western blot image and (**b,c**) statistical analysis of total p75^{NTR} (75 kDa) and its intracellular domain (ICD, 25 kDa) normalized to actin. A 2×2 analysis showed a significant interaction between proNGF overexpression and silencing p75^{NTR}. Overexpression of proNGF induced expression of both total p75^{NTR} and p75^{ICD} compared with GFP. Silencing p75^{NTR} with shRNA prevented the increase in p75^{NTR} and p75^{ICD} expression in proNGF group but not in GFP group. $n = 4$–5 per group, *$P < 0.05$ versus other groups. (**d**) Representative western blot image and statistical analysis of proNGF expression normalized to actin showing significant increases in proNGF in proNGF overexpressing group that were mitigated by silencing p75^{NTR} compared with GFP controls. (**e**) Representative western blot images and statistical analysis of NGF expression normalized to actin. A 2×2 analysis showed a significant interaction between proNGF overexpression and silencing p75^{NTR}. ProNGF overexpression resulted in significant decreases in NGF levels compared with GFP. Silencing p75^{NTR} with shRNA significantly reduced NGF levels in GFP-controls and restored NGF levels in proNGF overexpressing retina. Results presented as mean ± SD. $n = 4$–5 per group. *$P < 0.05$ versus other groups.

examined to verify proNGF and p75[NTR] expression. As shown in Figure 1a, overexpression of proNGF induced expression of both total p75[NTR] and its intracellular domain (p75[ICD]) compared with GFP-control. A 2×2 analysis showed a significant interaction between proNGF overexpression and silencing p75[NTR]. Silencing p75[NTR] with shRNA prevented the increase in p75[NTR] and p75[ICD] expression in proNGF group but not in GFP controls (Figure 1b,c). Silencing p75[NTR] mitigated the increase in proNGF level in the proNGF-over-expressing group (proNGF+shRNA) comparable to GFP-controls (Figure 1d). We next examined impact of proNGF overexpression on NGF levels in rat retina. A 2×2 analysis showed a significant interaction between proNGF overexpression and silencing p75[NTR]. As shown in Figure 1e, proNGF overexpression resulted in significant decreases in NGF levels compared with GFP-control similar to our prior report.[18] Silencing p75[NTR] with shRNA significantly increased NGF levels in proNGF overexpressing retina (Figure 1e). However, silencing p75[NTR] with shRNA significantly reduced NGF levels in GFP+p75shRNA compared with GFP+Scrambled controls.

Silencing p75[NTR] mitigates proNGF-induced retinal acellular capillaries formation

Prior work showed that proNGF overexpression can induce formation of acellular capillaries,[18] a surrogate marker for retinal microangiopathy.[25] To dissect the contribution of p75[NTR] in proNGF-mediated acellular capillary formation, p75[NTR] expression was silenced using single intravitreal delivery of lentiviral particles containing shRNA against p75[NTR]. Representative images of

periodic acid–Schiff and hematoxylin stained, trypsin-digested, and flat-mounted retinas show acellular capillaries (arrows) identified as capillary-sized vessel tubes having no nuclei anywhere along their length (see typical example in Figure 2f). 2×2 way analysis showed interaction between proNGF overexpression and silencing p75[NTR]. Overexpression of GFP-proNGF induced significant (1.9-fold) increase in acellular capillaries compared with the control GFP-plasmid (Figure 2e). Silencing expression of p75[NTR] using shRNA mitigated proNGF-induced acellular-capillary formation but did not alter the number in GFP-plasmid control group.

Silencing p75[NTR] mitigates proNGF-induced apoptotic markers in vivo

ProNGF-induced vascular cell death was also verified by performing TUNEL-staining on digested and periodic acid–Schiff and hematoxylin stained retina vasculature (trypsinized retinas). As shown in (Figure 3a–d), overexpression of GFP-proNGF induced numerous TUNEL-positive cells in retinal vasculature compared with GFP-control. This observation prompted us to investigate the impact of proNGF/p75[NTR] expression on activation of apoptotic signals in retina lysate. 2×2 analysis showed interaction of proNGF overexpression and silencing p75[NTR] expression. Overexpression of GFP-proNGF induced twofold increase in the phosphorylation of the stress kinase C-Jun N-terminal kinase (JNK; Figure 3e) and 1.6-fold in cleaved- PolyAdp-Ribose Polymerase (PARP; Figure 3f). Silencing p75[NTR] prevented the proNGF-induced numerous TUNEL-positive nuclei (Figure 3d) and suppressed activation of apoptotic signals
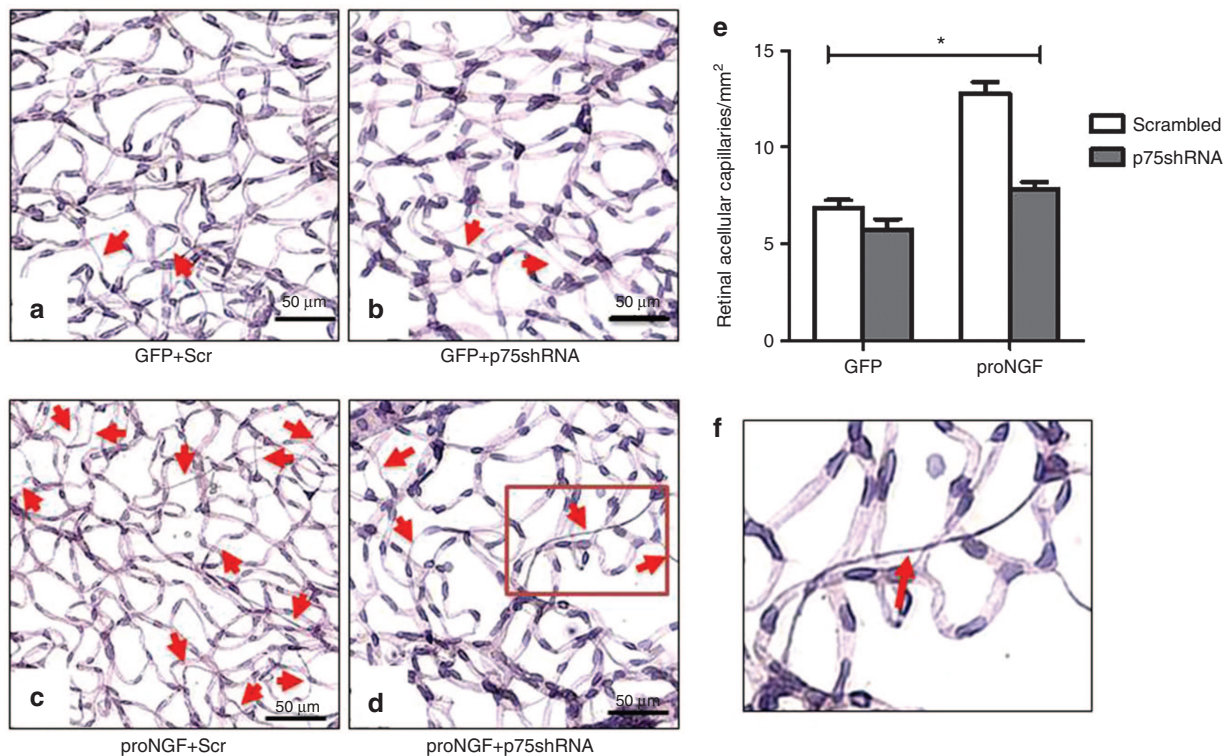

Figure 2 Silencing p75[NTR] mitigates acellular capillaries in proNGF-overexpression model. After 6 weeks of intraocular injection, retina vasculature was isolated using trypsin digest and stained with periodic acid–Schiff hematoxylin showing acellular capillaries. Representative images of trypsin digested retinas are shown for (**a**) GFP+Scr, (**b**) GFP+p75shRNA, (**c**) proNGF+Scr, and (**d**) proNGF+p75shRNA. Red arrows indicate acellular capillaries. (**e**) A 2×2 analysis showed a significant interaction between proNGF overexpression and silencing p75[NTR]. Overexpression of proNGF induced a significant increase of acellular capillaries compared with GFP-controls. These effects were mitigated by silencing of p75[NTR]. Results presented as mean ± SD. $n = 6$ per group. *$P < 0.05$ versus other groups. (**f**) A typical acellular capillary identified as capillary-sized vessel tubes having no nuclei anywhere along their length.
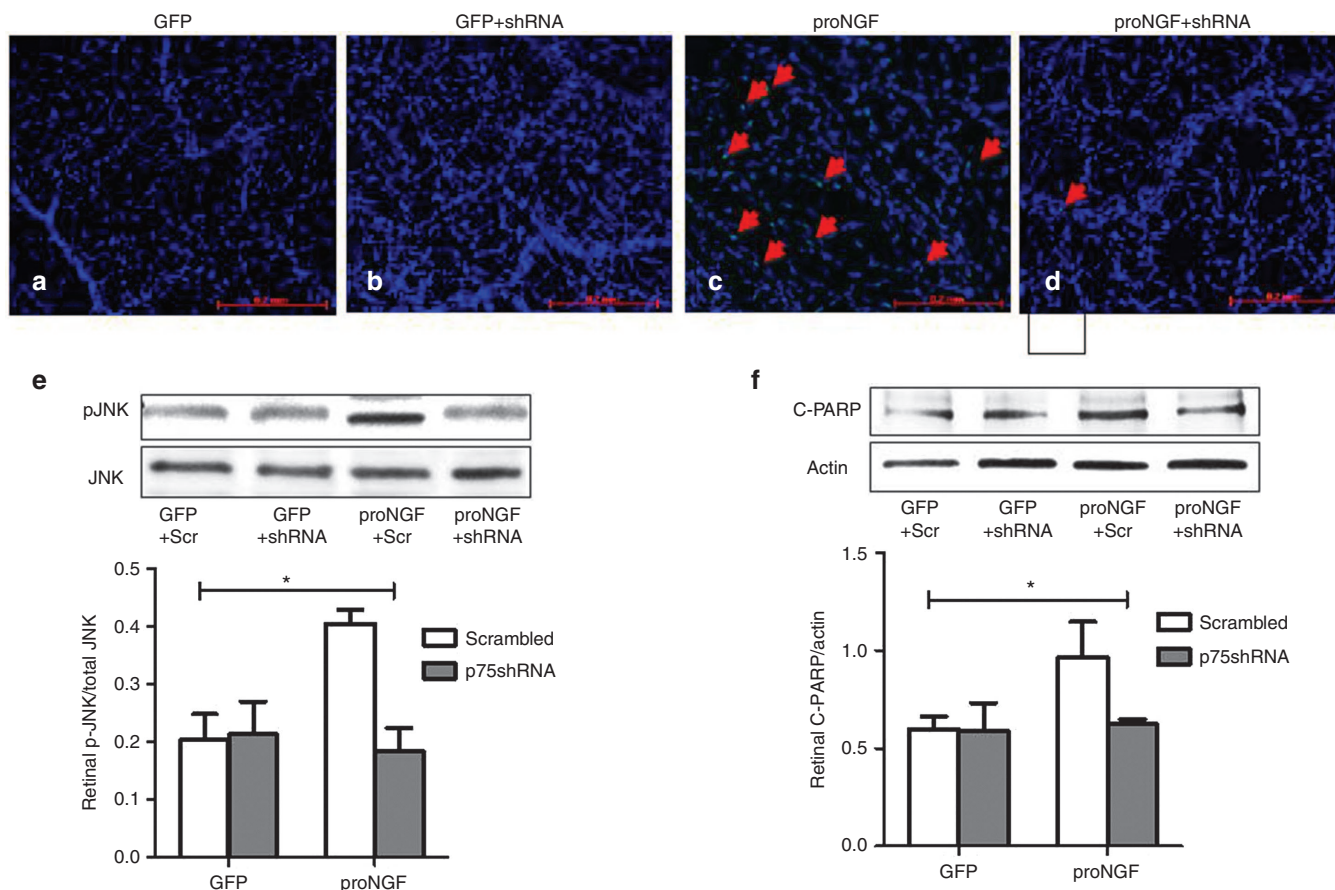
Figure 3 Silencing p75[NTR] suppresses proNGF-induced vascular cell death and apoptotic marker in rat retina. Representative images of trypsin-digested retinas stained with TUNEL (green) and counterstained with DAPI (blue) are shown for (**a**) GFP+Scr, (**b**) GFP+p75shRNA, (**c**) proNGF+Scr, and (**d**) proNGF+p75shRNA. TUNEL assay showed several positive cells in proNGF-overexpressing retinas, suggesting that capillary degradation occurs through apoptosis. (**e**) Representative western blots and statistical analysis of pJNK and JNK and (**f**) cleaved-PARP normalized to actin. 2×2 analysis showed interaction between proNGF expression and silencing p75[NTR]. Overexpression of proNGF induced significant activation of JNK and cleavage of PARP compared with GFP-controls. These effects were mitigated by p75shRNA but not scrambled. Results presented as mean ± SD. $n = 4$–6 per group. *$P < 0.05$ versus other groups.

suggesting that p75[NTR] is a critical mediator of proNGF-induced capillary degeneration.

Expression of proNGF is dependent on p75[NTR] in retinal endothelial cells

Overexpression of proNGF has been shown to induce triad of events in the retina including glial inflammation and neurodegeneration that can contribute to microvascular degeneration. To dissect the detrimental effects of proNGF in vasculature, we used proNGF overexpression model in human retinal EC. ECs were transduced with one of the followings: pGFP plus scrambled siRNA (GFP+Scr), pGFP plus siRNA against p75[NTR] (GFP+siRNA); pGFP-proNGF123, plus scrambled siRNA (proNGF+Scr); pGFP-proNGF123 plus siRNA against p75[NTR] (proNGF+siRNA) and cell lysate and supernatant were collected after 48 hours. 2×2 analysis showed interaction between proNGF overexpression and silencing p75[NTR]. Silencing p75[NTR] expression was first verified by in both GFP-controls and proNGF groups (Figure 4a,b). Silencing p75[NTR] downregulated proNGF protein expression back to GFP controls in EC (Figure 4a,c). We have verified that the proNGF detected in EC is expressed mainly by the cleavage-resistant plasmid. As shown in Supplementary Figure S1a, PCR analysis using mouse proNGF primers showed 4000-fold increase in the proNGF-GFP group that was significantly reduced

by silencing p75[NTR] using siRNA. These results demonstrated that proNGF expression is p75[NTR] dependent in retinal EC. Moreover, EC cultures were treated with an inhibitor for Furin, the main endogenous protease responsible for intracellular cleavage of proNGF to NGF. As shown in Supplementary Figure S1b, inhibiting Furin did not result in further increase of proNGF levels in EC cultures transduced with proNGF alone or proNGF with p75siRNA. Interestingly, furin inhibitor significantly increased accumulation of proNGF only in GFP-controls, suggesting that the proNGF protein is mainly due to translation of the mouse proNGF plasmid and not from endogenous human proNGF.

Overexpression of proNGF significantly reduces NGF level and activation of TrkA in EC

NGF is the mature ligand and well-known survival factor for both neurons and vasculature. In EC, overexpression of proNGF resulted in 75% reduction in NGF levels compared with GFP-controls (Figure 4d). The decision of survival or death may be dictated by the ratio of p75[NTR] and TrkA receptor.[26] Overexpression of proNGF in EC resulted in 60% decrease in TrkA phosphorylation, compared with GFP-controls (Figure 4e). Silencing p75[NTR] increased NGF levels and TrkA activation back to normal levels in proNGF overexpressing cells but not in GFP-controls.
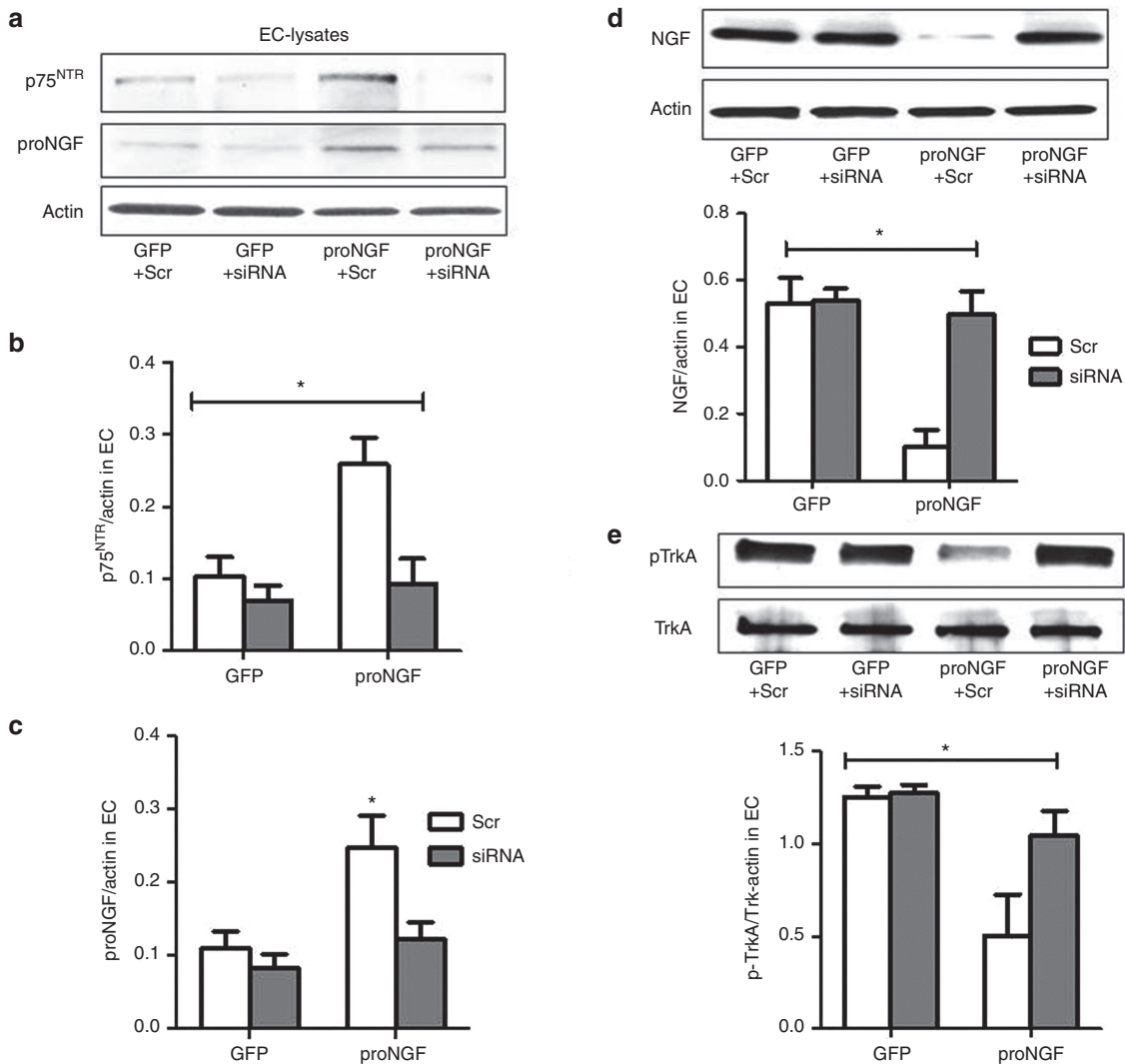
Figure 4 Expression of proNGF is dependent on p75^NTR in EC. Human retinal ECs were transduced with one of the followings: pGFP plus scrambled siRNA (GFP+Scr), pGFP plus siRNA against p75^NTR (GFP+siRNA); pGFP-proNGF123, a mutant cleavage resistant form of proNGF, plus scrambled siRNA (proNGF+Scr); pGFP-proNGF123 plus siRNA against p75^NTR (proNGF+siRNA), and cell lysate and supernatant were collected after 48 hours. (**a,b**) Representative western blots statistical analysis of total p75^NTR expression normalized to actin. (**a,c**) Representative western blots and statistical analysis of proNGF expression normalized to actin. 2×2 analysis showed interaction between proNGF overexpression and silencing p75^NTR. Silencing p75^NTR significantly reduced its expression in both GFP-controls and proNGF-groups. Results showed that overexpression of proNGF induced p75^NTR expression and that silencing p75^NTR downregulated proNGF expression in EC. Results presented as mean ± SD. $n = 5–6$ per group. *$P < 0.05$ versus other groups. (**d**) Representative western blots and statistical analysis of NGF expression normalized to actin in EC. (**e**) Representative western blots and statistical analysis of TrkA activation normalized to total TrkA. 2×2 analysis demonstrated interaction between proNGF overexpression and silencing p75^NTR. Overexpression of proNGF resulted in significant decreases in NGF levels and activation of its TrkA receptor compared with GFP-controls. Silencing p75^NTR expression increased NGF levels and enhanced TrkA activation in proNGF overexpressing cells. Results are expressed as mean ± SD. $n = 5$ per group. *$P < 0.05$ versus other groups.

Overexpression of proNGF activates p75^NTR-mediated apoptotic signal in EC

We next investigated the impact of proNGF/p75^NTR expression on activation of apoptotic signals in EC. In comparison to GFP-controls, proNGF overexpression induced twofold phosphorylation of the stress kinase JNK (Figure 5a), 1.7-fold in activation of p38MAPK (Figure 5b) and 2.2-fold in cleaved-PARP (Figure 5c). 2×2 analysis showed interaction between proNGF overexpression and silencing p75^NTR. Silencing p75^NTR completely mitigated proNGF-mediated apoptotic signal in EC and have modest decreases in GFP-controls.

Overexpression of proNGF does not exert proinflammatory effect in EC

We and others have shown that proNGF overexpression caused production and release of TNF-α in glial Müller cells.[12,17] To explore whether proNGF can be proinflammatory in EC, we examined TNF-α expression as well as release of TNF-α and IL-1β in EC supernatant. Overexpression of proNGF in EC did not result in significant changes in release of proNGF (Figure 6a,b) TNF-α (Figure 6a,c) IL1β (data not shown) or TNF-α mRNA expression (Figure 6d). These results suggest that unlike the proinflammatory effect in glial Müller cells, proNGF does not induce a proinflammatory action in EC.
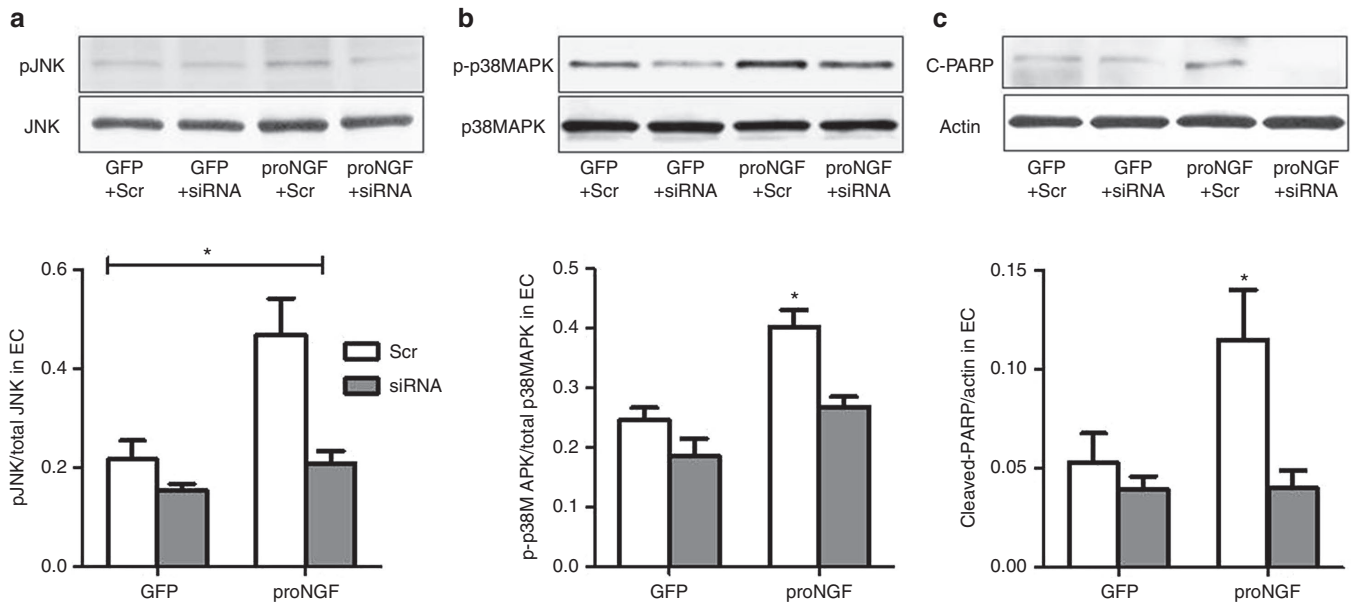
Figure 5 Silencing p75[NTR] suppresses proNGF-induced apoptotic markers in EC. Representative western blots and statistical analysis of (**a**) pJNK, total JNK, (**b**) pP38MAPK, total p38MAPK, and (**c**) cleaved-PARP (C-PARP), actin in EC lysates. 2×2 way analysis showed interaction between proNGF overexpression and silencing p75[NTR]. Overexpression of proNGF induced EC apoptosis evident by activation of JNK, p38MAPK, and cleavage of PARP. These effects were blunted by silencing p75[NTR]. Results presented as mean ± SD. $n = 4$–6 per group. *$P < 0.05$ versus other groups.
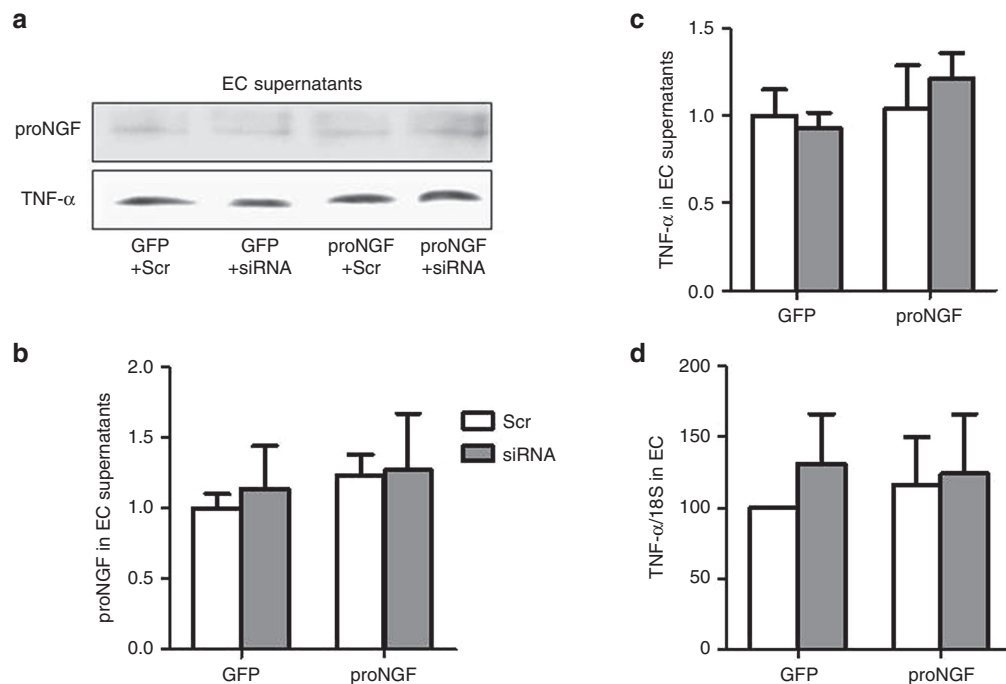

Figure 6 Overexpression of proNGF does not exert proinflammatory effect in EC. (**a–c**) Representative western blots statistical analysis of proNGF and TNF-α of concentrated EC-supernatants normalized to BSA-IgG. (**d**) Statistical analysis of mRNA expression of TNF-α normlaized to 18S. 2×2 analysis showed no effect of proNGF overexpression or silencing p75[NTR] on altering levels of TNF-α expression in EC lysates or released proNGF or TNF-α into supernatant of EC cultures. Results presented as mean ± SD. $n = 4$ per group.

ProNGF induces p75[NTR]-dependent nuclear translocation of NRIF-p75ICD in EC

To elucidate how proNGF/p75[NTR] activates JNK apoptotic signal in EC, P75[NTR], we switched to using human mutant (hm-proNGF)[12] instead of proNGF-plasmid electroporation. We first verified the apoptotic effects of hm-proNGF on human EC. As shown in Supplementary Figure S2, hm-proNGF induced cell death assessed by a death and

life assay. Interestingly, 10, 20, and 50 ng/ml but not 100 ng/ml of hm-proNGF induced significant EC death compared with control. Therefore, we selected 50 ng/ml to probe the apoptotic signal in EC. As shown in Figure 7a, hm-proNGF (50 ng/ml) induced 2.5-fold in caspase-activity compared with controls that was blunted by a small peptide and a selective antagonist (A) against p75[NTR] receptor.[17,27] To explore the mechanism by which proNGF/p75[NTR]
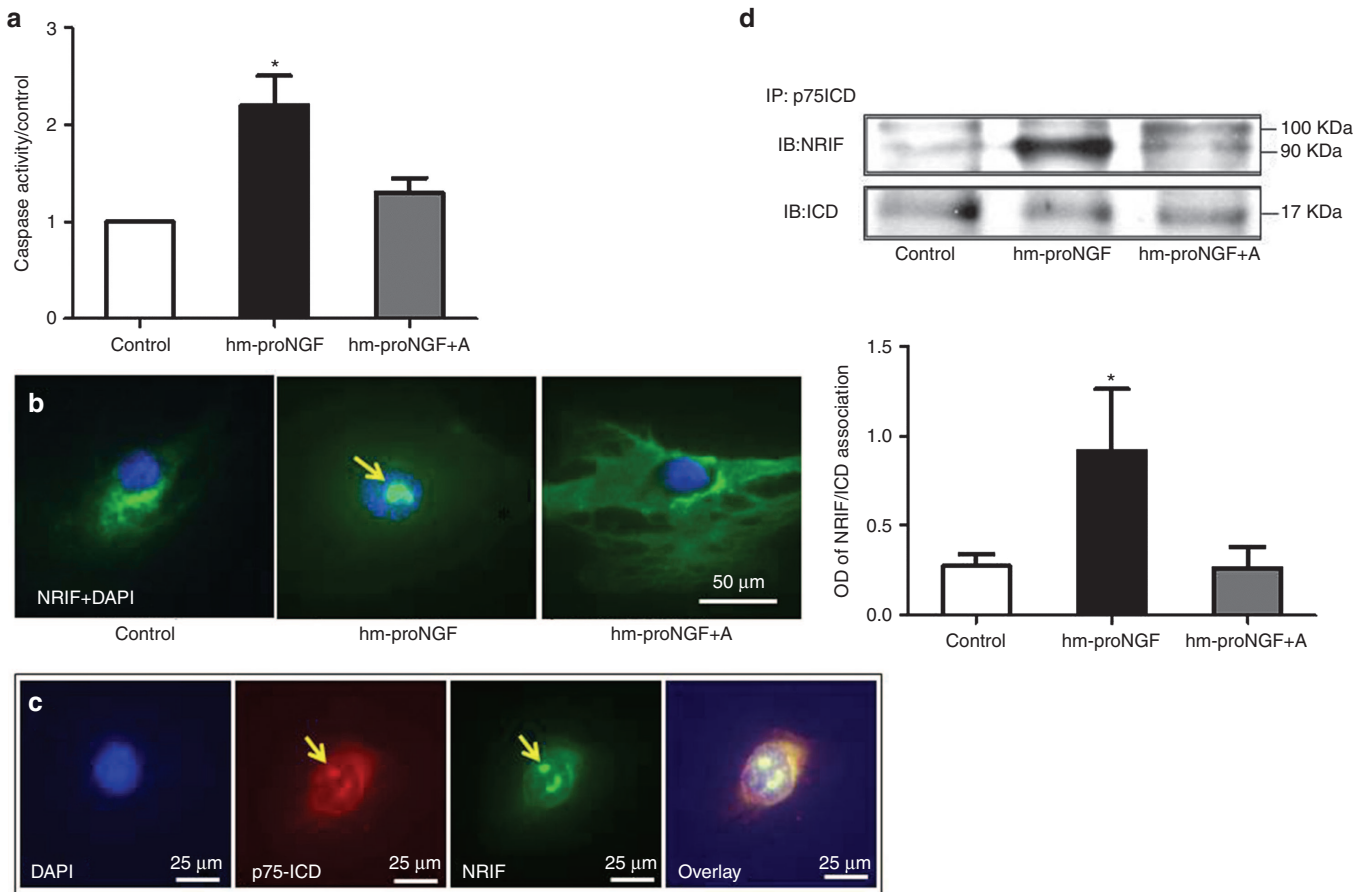
Figure 7 ProNGF causes protein interaction and nuclear translocation of p75^{NTR} and neurotrophin interacting factor (NRIF). Human retinal endothelial cells were treated with human mutant (hm-proNGF, 50 ng/ml) in the presence or absence of a selective p75^{NTR} antagonist (A, 20 μmol/l) for 48 hours. (**a**) Cell death was assessed by measuring activity of caspase enzyme and the results were presented as mean ± SD. *n* = 3–5 per group. *$P < 0.05$ versus other groups. (**b**) Representative images of immunolocalization showing that hm-proNGF induced nuclear translocation of NRIF (green) within the nucleus (blue) that was blocked by p75^{NTR} antagonist (A) (proNGF+A). (**c**) Enlarged micrographs of EC treated with hm-proNGF showing colocalization of p75^{NTR} (red), NRIF (green) within the nuclei (blue). Similar results were observed in two independent experiments. (**d**) Representative blots and statistical analysis showing that hmproNGF induced protein association between NRIF (90 kDa) and p75^{ICD} (17 kDa) in EC lysate immunoprecipitated with anti-p75^{NTR} and immunoblotted by anti-NRIF. Additional band for NRIF was detected at 100 kDa. Results presented as mean ± SD. *n* = 4 per group. *$P < 0.05$ versus all groups.

mediates JNK-apoptotic signals in EC, we investigated whether increases in p75^{ICD} in the cytosol can recruit the DNA binding protein, neurotrophin interacting factor (NRIF), resulting in NRIF-p75^{ICD} nuclear translocation. Immunocytochemistry clearly showed that hm-proNGF (50 ng/ml) induced redistribution of NRIF (green) from the cytoplasm and perinuclear to be in the nuclei (blue) of EC after 48 hours (Figure 7b). Treatment with hm-proNGF also induced translocation of p75^{NTR} (red), NRIF (green) to the nuclie (blue) (Figure 7c). Nuclear translocation of NRIF was blocked by the specific p75^{NTR} antagonist (A, 20 μmol/l). Of note, the antibody used for immunocytochemistry can recognize both total p75^{NTR} and p75^{ICD}. To further characterize whether the total p75^{NTR} receptor or the p75^{ICD} is translocated to the nuclei, we attempted to inhibit γ-secretase-mediated cleavage of p75^{NTR} using compound E or DAPT. However, results were not conclusive due to the harsh effects of vehicle used (dimethylsulfoxide and ethanol) for extended time (48 hours of exposure). Next, we examined NRIF and p75^{ICD} protein interaction using immunoprecipitation. As shown in Figure 7d, proNGF markedly increased protein interactions between p75^{ICD} (detected ~17kDa) and NRIF (detected ~90kDa) compared with controls. Interestingly, the NRIF band had additional band at 100kDa that was detected in all groups. The interaction between NRIF and p75^{ICD} was abolished by a pharmacological antagonist of p75^{NTR}. These results demonstrate that proNGF-induced NRIF nuclear translocation plays an essential role in executing p75^{NTR}-mediated EC death.

DISCUSSION

The main findings of this study are: (i) Similar to neurodegenerative disease, overexpression of proNGF shift the balance to less neurotrophic support evident by less NGF and impaired TrkA activation. Silencing p75^{NTR} expression restored balance by decreasing proNGF/p75^{NTR} levels and increasing NGF/TrkA in proNGF-overexpression models *in vitro* and *in vivo* (Figures 1 and 4). (ii) ProNGF induced NRIF nuclear translocation and EC apoptosis in a p75^{NTR}-dependant manner (Figures 5 and 7). (iii) Modulating p75^{NTR} expression prevented proNGF-induced development of acellular capillaries and hence retinal ischemia *in vivo* (Figures 2 and 3). We believe that this is the first report dissecting the contribution of upregulated proNGF/p75^{NTR} and impaired NGF/TrkA in mediating EC death. A scheme of the proposed molecular mechanism and findings is depicted in (Figure 8).

Neurotrophins are traditionally perceived as essential growth factors for differentiation, development, maturation, and regulation of
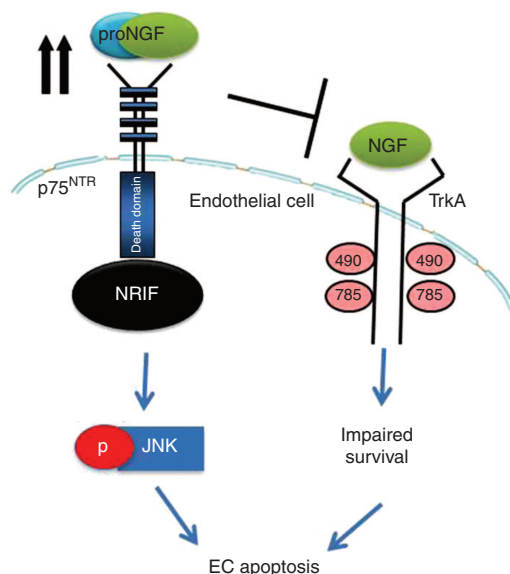
Figure 8 Schematic diagram depicting the proposed role of proNGF/p75NTR in mediating EC death and development of acellular capillaries. Overexpression of proNGF impairs endogenous NGF/TrkA survival signaling pathway and activates p75NTR-mediated apoptotic signal. ProNGF triggers interaction of intracellular domain of p75NTR (p75[ICD]) and the transcriptional factor neurotrophin interacting factor (NRIF) to activate JNK apoptotic signal. Targeting p75[NTR] prevents the apoptotic signal and stimulates endogenous cell survival.

cell death of retinal neurons.[3–6] However, over the past two decades, a growing body of evidence identified the actions of neurotrophins on other cell types in the retina.[1] Our group has been leading studies that demonstrated the multiple and complex ways by which diabetes alters homeostasis of NGF maturation resulting in upregulation of proNGF/p75[NTR] receptor and diminishing NGF/TrkA receptor activation.[13,15,17] Here, we examined the impact of accumulated proNGF/p75[NTR] on EC apoptosis and acellular capillary formation using cleavage-resistant proNGF-overexpression model. Our data showed that silencing p75[NTR] expression prevented proNGF-induced acellular capillary formation *in vivo* (Figure 2) as well as EC death *in vitro* (Figures 5 and 7). These effects were associated with restoring the balance of proNGF and NGF ratio back to the control levels, which are essential for proper retinal function (Figures 1 and 4). We next examined whether proNGF activates apoptosis via direct activation of the apoptotic pathway in EC and/or via release of proinflammatory mediators that can contribute to EC death. We and others have demonstrated proinflammatory action of proNGF in Müller cells via a p75[NTR]-dependent pathway.[12,16–18] Interestingly, overexpression of proNGF in EC did not induce significant increases in expression of TNF-α or release of proNGF, TNF-α, and IL-1β compared with controls (Figure 6), suggesting that EC apoptosis was mediated through direct activation of proNGF/p75[NTR] signaling at least within *in vitro* setting. In support of this concept, prior studies have demonstrated a direct apoptotic effect of proNGF and p75[NTR] in brain EC[28,29] and in vascular cells of the ischemic heart.[30] However, the contribution of paracrine action of retinal neuro- and glial inflammation cannot be ruled out in the *in vivo* setting.

The apoptotic p75[NTR] signal requires phosphorylation of JNK and stress kinase p38MAPK.[8,31–33] Here, we demonstrated that proNGF overexpression induced activation of JNK and p38MAPK as well as its downstream apoptotic markers caspase activation and cleaved-PARP in whole retina and EC (Figures 3 and 5). These

effects were mitigated by silencing p75[NTR] expression using shRNA *in vivo* and siRNA *in vitro*. While prior studies documented the clear apoptotic effect of proNGF or p75[NTR] in neurons,[23,31,33–36] this is the first report that demonstrated activation of p75[NTR]-dependent JNK activation in EC in response to proNGF. The use of human mutant (hm-proNGF) resulted also in EC death assessed by death/life assay and caspase activity. We used 50 ng/ml as this level induced maximum cell death (Supplementary Figure S2). A high dose of proNGF (100 ng/ml) had less cytotoxic effect possibly due to activation of other receptors including TrkA resulting in activation of survival signal.

P75[NTR], like Notch receptors, undergoes γ-secretase-intramembranal proteolysis to liberate the intracellular domain (ICD), which can recruit several apoptotic intracellular binding proteins such as NRIF (reviewed in refs. 20,37). NRIF has been described as an essential mediator of apoptotic signals by p75[NTR] in neurons.[21,38] To explore whether proNGF-mediated JNK activation and EC apoptosis involve interaction of p75[NTR] and NRIF, we switched to using human mutant (hm-proNGF) instead of proNGF-plasmid electroporation. Treatment of EC with hm-proNGF caused nuclear translocation of DNA binding NRIF as evident by localization of NRIF (green) and p75[NTR] (red) in nuclei of proNGF-treated EC, but not in controls or proNGF+A (Figure 7). Whether cleavage of p75[NTR] and release of p75[ICD] is a necessary step before nuclear translocation remains to be explored. One of the barriers to answer this question is the reliability of selective inhibitors of γ-secretase enzyme and their limited solubility in aqueous vehicles that affected cell viability. Several proteins have been shown to accumulate into the nucleolus during cell growth or death. Of note, the recruitment of NRIF and p75[NTR] in the nucleolus may represent an advanced DNA fragmentation and a stress response triggered by proNGF-apoptotic signal in endothelial cells. Furthermore, immunoprecipitation showed strong interaction between NRIF and p75[ICD] in response to proNGF, an effect that was abolished by selective antagonist of p75[NTR], inhibitor A (Figure 7). Interestingly, the NRIF band was detected higher at 100 kDa instead of 90 kDa, which suggests that NRIF may undergo protein modification such as ubiquitination, as proposed by prior studies.[38,39] Our findings suggest that NRIF/p75[ICD] translocate as a complex to mediate the cell death process in EC. Similarly, previous reports showed nuclear translocation of NRIF in neuronal cells.[23,26,40] The significance of nuclear translocation p75[NTR] to directly activate JNK or other pathways warrant investigation.

While proNGF can promote neuronal cell death by binding p75[NTR], NGF mediates survival by binding TrkA.[15] Similar to prior findings,[18] overexpression of cleavage-resistant proNGF significantly reduced mature NGF levels *in vivo* and *in vitro* in EC (Figures 1 and 4). Interestingly, these effects were associated with significant impairment of TrkA receptor phosphorylation (Figure 4). Supplementation of exogenous NGF has been shown to be neuro- and vascular protective in experimental models.[41–43] However, the distribution and actual level of NGF in the retina after topical administration remains controversial. In this study, we took a different approach by suppressing the proNGF/p75[NTR] signal which stimulated endogenous NGF/TrkA expression that together improved cell survival and preserved the retinal vasculature. Silencing p75[NTR] expression restored NGF levels and TrkA receptor phosphorylation in proNGF-overexpressing retinas and in EC (Figure 4). We also examined whether observed increases in NGF were due to endogenous production by EC as a protective mechanism or due to improved processing and cleavage of proNGF-plasmid expression. Hence, we treated EC with an inhibitor for the intracellular protease "furin"

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and results showed that inhibiting furin resulted in accumulation of proNGF levels only in GFP-controls but not in GFP-proNGF expressing cells (Supplementary Figure S1b). These results confirmed that the detected proNGF protein is primarily coming from the exogenous non-cleavable proNGF-plasmid. Overexpression of proNGF reduced NGF levels *in vivo* and in EC probably due to feedback inhibition at the transcription level (see Supplementary Figure S1a). These results came similar to our prior findings using proNGF overexpression model.[18] In support, our previous studies using diabetic model showed that deletion of p75^{NTR} resulted in restoring the ratio between proNGF and NGF that was associated with preserving retinal barrier function.[17] Studies are warranted to further elucidate the molecular events involved in how silencing p75^{NTR} restored NGF levels.

Accumulation of proNGF has been documented in neurodegenerative diseases in the brain and retina including Alzheimer's,[9] Down's syndrome,[10] as well as models of optic neuropathy, light damage and diabetic retinopathy.[11,12,15,16] In this study, we demonstrated unexplored role of p75^{NTR} in proNGF-mediated EC cell apoptosis and subsequent acellular capillary formation, beyond its known role in neurodegeneration. These findings support the notion that targeting p75^{NTR} expression represents a viable therapeutic option in diseases of the neurovascular unit. Modulating expression of p75^{NTR} can protect against proNGF mediated-EC death at two levels by inhibiting apoptotic effect of proNGF and by stimulating NGF expression and activating the TrkA survival pathway to mitigate retinal cell death and acellular capillary formation.

MATERIALS AND METHODS

Animals
All animal procedures were performed in accordance with Association for Research in Vision and Ophthalmology statement for use of animals in ophthalmic and vision research, and Charlie Norwood VA Medical Center Animal Care and Use Committee (ACORP#13–01–055).

Overexpression of the cleavage-resistant proNGF in rat retinas
Four sets of, a total of 48 animals, Male Sprague-Dawley rats (~250 g body weight) were purchased from Harlan Laboratories (Indianapolis, IN) and randomly assigned to four groups. Stable overexpression of proNGF was achieved via intravitreal injection of proNGF123 plasmid and electroporation as described previously by our group.[18] Animals received either lentiviral particles containing scrambled or shRNA against p75^{NTR} (5,000 infectious units/eye) were obtained from Santa Cruz BioTechnology (Dallas, TX). Animal groups included intravitreal injection of pGFP plasmid (5 µg/eye) plus lentiviral particles containing scrambled shRNA (5,000 infectious units/eye); pGFP plasmid plus shRNA against p75^{NTR}; pGFP-proNGF123, a cleavage resistant proNGF construct, plus scrambled shRNA; or pGFP-proNGF123 plus shRNA against p75^{NTR}. Rats were sacrificed after 6 weeks, and eyes were enucleated for further investigations.

Isolation of retinal vasculature and determination of acellular capillaries
The retinal vasculature was isolated as described previously.[44] Freshly enucleated eyes were fixed with 2% paraformaldehyde overnight. Retinal cups were dissected, washed in phosphate-buffered saline, then incubated with 3% Difco-trypsin 250 (BD Biosciences, San Jose, CA) in 25 mmol/l Tris buffer, pH 8, at 37 °C for 2 hours. Vitreous and nonvascular cells were gently removed from the vasculature, which was soaked in several washes of 0.5% Triton X-100 to remove the neuronal retina. Trypsin-digested retinas were stained with periodic acid–Schiff and hematoxylin. Numbers of acellular capillaries were quantified in six different areas of the mid-retina under the microscope (×20) in a masked manner by two different researchers. Acellular capillaries were identified as capillary-sized blood vessel tubes with no nuclei along their length. TUNEL assay was performed using ApopTaG in whole flat-mounted retinas that went through tryptic digest as described previously by our group.[44]

Cell culture
All human retinal endothelial cell (EC) studies were in accordance with Association for Research in Vision and Ophthalmology and Charlie Norwood Veterans Affairs Medical Center, research and ethics committee. EC and supplies were purchased from Cell Systems Corporations (Kirkland, WA) and VEC Technology (Rensselaer, NY) as described previously.[45]

Overexpression of proNGF and manipulation of p75^{NTR} in EC culture
Plasmids used to overexpress cleavage-resistant proNGF (0.5 µg/ml) and silencing of p75^{NTR} expression using small interfering RNA siRNA (0.6 µmol/l) were performed as described by our group.[31,45] Both the SMARTpool of siRNA specific for p75^{NTR} gene (also known as Ngfr) and its scrambled form were obtained commercially from Dharmacon (Lafayette, CO). The groups included: Control, pGFP alone plus scrambled siRNA (GFP); control-treated, pGFP plus siRNA against p75^{NTR} (GFP-siRNA); proNGF, pGFP-proNGF123 plus scrambled siRNA (proNGF); and proNGF-treated, pGFP-proNGF123 plus siRNA against p75^{NTR} (pro-siRNA). EC transduction was performed using Amaxa nucleofector primary endothelial cells kit (Lonza, Germany). EC cultures were transduced for 6 hours, then left to recover in complete medium for 24 hours before performing experiments. Transfection efficiency was 60–70% for both methods as indicated by number of cells expressing green fluorescent protein (GFP) or FITC-labeled siRNA. Silencing of p75^{NTR} expression was verified by western blot analysis.

Pharmacological treatments
For cell viability experiments, human mutant proNGF (Alomone Labs, Jerusalem, Israel) was used to induce apoptosis in EC. A specific pharmacological antagonist of p75^{NTR} (A) was provided by Dr Uri Saragovi (McGill University, Montreal, QC, Canada) and was used at 20 µmol/l as previously described[17] for 48 hours. To examine whether inhibition of proteolytic activity would modify proNGF levels, a furin enzyme inhibitor Decanoyl-Arginyl-Valyl-Lysyl-Arginyl-Chloromethyl Ketone (Millipore, MA) was used at concentration 25 µmol/l, which did not affect cell viability up to 12 hours.[46]

Western blot assay and immunoprecipitation
Retinas and EC samples (30 µg protein) were homogenized in RIPA buffer and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[13] Antibodies were purchased as follows: anti-proNGF and anti-NGF (Alomone Labs, Jerusalem, Israel), JNK, p-JNK, p-p38, p38MAPK (Santa Cruz Biotechnology), and cleaved-PARP (Cell Signaling, Danvers, MA). Antibodies for p75^{NTR}, p75^{ICD}, and NRIF were kindly provided by Dr Bruce Carter (Department of Biochemistry, Vanderbilt University). Membranes were reprobed with β-actin (Sigma-Aldrich, St Louis, MO) to confirm equal loading. The primary antibody was detected using horseradish peroxidase-conjugated sheep antirabbit antibody (GE Healthcare, Piscataway, NJ) and enhanced chemiluminescence (Pierce, Thermo Fischer Scientific, Waltham, MA). For immunoprecipitation, EC lysates (150 g) were immunoprecipitated with anti-p75 antibody kindly provided by Dr Moses Chao (Skirball Institute of Biomolecular Medicine, New York University). The proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibody directed against NRIF. Band intensity was quantified using densitometry software (alphEaseFC) and normalized to p75^{ICD}.

Caspase activity assay
This assay was performed as described previously.[47] Briefly, 40 µl of 2× reaction buffer followed by 4 µl of 1 mol/l zDEVD-AFC (Enzo Life, Farmingdale, NY) were added to an equal volume of each sample. Cell lysates (10 µg) were incubated at 37 °C for 30 minutes, and caspase activity determined by measuring relative fluorescence units (RFU) at 505 nm following excitation at 400 nm using a Microplate reader BioTek (BioTek Instrument, Winooski, VT).

Death and life cell viability assay
This assay was performed following manufacturer's protocol (Thermo Fischer Scientific) as described previously by our group.[45] ECs were viewed under fluorescent microscope (Axiovert-200; Zeiss, Jena, Germany) with filter 488 nm, green for viable cells, and 568 nm, red nonviable. Death/ life ratio was counted in a masked manner.

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Quantitative real-time PCR

The One-Step qRT-PCR kit (Thermo Fischer Scientific) was used to amplify 10 ng retinal mRNA and quantification was performed as described previously.[31,45] PCR primers designed to amplify human TNF-α were purchased from Integrated DNA Technologies (Coralville, IA). Quantitative PCR was performed using a Realplex Master cycler (Eppendorf, Germany) using primers (F: 5′CCAGGGACCTCTCTCTAATCA3′; R 5′TCAGCTTGAGGGGTTTGCTAC3′). Primers for mouse proNGF are F: 5′TTTGATCGGCGTACAGGCAG3′ and R: 5′AGTGGAGTCTCGCTGCCTTAAACA3′. Expression of human TNF-α or mouse proNGF was normalized to the 18S level and expressed as relative expression to control.

Immunofluorescence

Immunofluorescence was performed as described previously.[48] EC were fixed with 2% PFA and incubated with blocking buffer (phosphate-buffered saline containing 5% goat serum, 0.02% Tween 20) followed by primary antibody (1:200) against antirabbit NRIF (provided by Dr Bruce Carter, Vanderbilt University) and antimouse p75^{NTR} (Abcam, Cambridge, MA) followed by Alexa 488-conjugated goat antirabbit or goat antimouse IgG (1:200, Thermo Fischer Scientific) in blocking buffer. Specimens were covered with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Micrographs of EC were taken using a fluorescent microscope (Axiovert-200) at ×20 and ×40.

Data analysis

Results are expressed as mean ± SD, and the data were evaluated for normality and appropriate transformations were used when necessary. Data was processed for statistical analysis with one-way ANOVA to examine the effect of human-proNGF and p75^{NTR} antagonist in endothelial cells. Two-way ANOVA was used to examine the effect of proNGF gene overexpression (GFP versus proNGF) and silencing p75^{NTR} (scrambled versus shRNA) and their interaction on cell death, mRNA, and protein expression. Two-way ANOVA followed by Bonferroni posttests were used to adjust for the multiple comparisons used to assess significant effects using Prism (Graphpad version 6). Significance, GraphPad software, La Jolla, CA was defined as $P < 0.05$.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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