TWEAK/Fn14 Pathway Is a Novel Mediator of Retinal Neovascularization

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PURPOSE. Retinal neovascularization (NV) is a major cause of vision loss in ischemia-induced retinopathy. Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and its receptor, fibroblast growth factor inducible-14 (Fn14), have been implicated in angiogenesis, but their role in retinal diseases is unknown. The goal of this study was to investigate the role of TWEAK/Fn14 pathway in retinal NV.

METHODS. Studies were performed in a mouse model of oxygen-induced retinopathy (OIR) and in primary human retinal microvascular endothelial cells (HRMECs). Hyperoxia treatment was initiated on postnatal day (P)14. Immunohistochemistry and quantitative PCR (qPCR) were used to assess retinal vascular changes in relation to expression of Fn14 and TWEAK.

RESULTS. Fibroblast growth factor-inducible 14 mRNA was prominently increased from P13 to P17 in OIR retinas, whereas TWEAK level was slightly decreased. These alterations were normalized by hyperoxia treatment and were more striking in isolated retinal vessels. There was a discernible shift in the immunoreactivity of Fn14 and TWEAK from the neuronal layers in the healthy retina to the neovascular tufts in that of OIR. Blockade of TWEAK/Fn14 significantly prevented retinal NV while slightly accelerated revascularization. In contrast, activation of Fn14 positively regulated survival pathways in the B-cell lymphoma-2 (Bcl2) family and robustly enhanced HRMEC survival. Furthermore, gene analysis revealed the regulatory region of Fn14 gene contains several conserved hypoxia inducible factor (HIF)-1 α binding sites. Overexpression of HIF-1 α prominently induced Fn14 expression in HRMECs.

CONCLUSIONS. We found that the TNF-like weak inducer of apoptosis (TWEAK)/fibroblast growth factor inducible-14 (Fn14) pathway is involved in the development of pathologic retinal neovascularization. Hypoxia inducible factor-1 α is likely implicated in the upregulation of Fn14.

Keywords: ischemia-induced retinopathy, neovascularization, TWEAK, Fn14, retina

Retinal neovascularization (NV) is a major cause of irreversible vision loss in ischemia-induced retinopathy such as retinopathy of prematurity, diabetes, and retinal vein occlusion. The standard options to treat retinal NV, include retinal laser photocoagulation, cryotherapy, and vitrectomy; however, they are destructive and the functional outcomes are often suboptimal.^{1–3} In recent years, intravitreal injection of anti-VEGF agents shows promise to cause regression of the retinal NV,⁴ but there are significant concerns such as systemic off-target effects, short-lived effects, and potential neuronal and glial toxicity.^{5–7} Severe side effects have also been reported when anti-VEGF agents were used in preterm infants to treat retinopathy of prematurity.⁸ Thus, it is imperative to further understand mechanisms of NV in order to develop new therapeutic targets.

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK), a member of TNF family, is initially synthesized as a type II transmembrane protein that can be cleaved to a soluble factor with biological activity.^{9,10} Fibroblast growth factor inducible-14 (Fn14), the smallest member of the TNF receptor superfamily, has been recognized as TWEAK receptor.¹¹ The TWEAK/Fn14 pathway has been shown to play an essential role in the pathogenesis of stroke, kidney injury, muscle atrophy, and a number of inflammatory diseases.^{12–14} It also has been implicated in angiogenesis by studies that have reported its role in endothelial cell migration, proliferation, and tube formation.^{15–17} While in vivo studies have linked the TWEAK/Fn14 pathway to angiogenesis using the rodent corneal pocket assay model,^{11,18} the role of TWEAK/Fn14 pathway in retinal NV remains completely unknown.

METHODS

Treatment of Animals

All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Texas Medical Branch (UTMB) Institutional Animal Care and Use Committee. Oxygen-induced retinopathy (OIR) was induced by maintaining C57BL/6J mice in 75% oxygen from postnatal day (P)7 to P12, followed by a return to room air (RA) from P12 to P17.^{19,20} Age-matched mice maintained in RA from birth to P17 served as RA control. To investigate the effect of hyperoxia treatment (HT), OIR mice were maintained in RA from P12 to P14 and then returned to 75% oxygen from P14 to P17. At the end of treatment, mice were euthanized and their eyes or retinas were prepared for morphology or molecular biology studies.

Intravitreal Injection

Mice were anesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg). Intravitreal injections were performed as described previously.^{20,21} In brief, at P12, 0.5 μ L PBS containing 2.5 μ g soluble Fn14-Fc decoy receptor (R&D Systems, Minneapolis, MN)¹³ or vehicle only was delivered to the vitreous with a 35-gauge needle mounted to a 10- μ L NanoFil syringe (World Precision Instruments, Sarasota, FL). The tip of the needle was inserted under the guidance of a Leica Wild M650 dissecting microscope (Leica, Bannockburn, IL) through the dorsal limbus of the eye.

Immunostaining of Retinal Whole Mounts

After fixation in 4% paraformaldehyde, retinas were dissected from the choroid and sclera, blocked, and permeabilized in PBS containing 10% normal goat serum and 1% Triton X-100 for 30 minutes, and then incubated with Alexa Fluor 594-labeled isolectin B4 (*Griffonia simplicifolia*; 1:200; Invitrogen, Carlsbad, CA) overnight at 4°C. For Fn14 expression, retinas were incubated with anti-Fn14 (1:200; Biolegend, San Diego, CA) and isolectin B4 overnight at 4°C, which was followed by the incubation with Alexa Fluor 488-labeled goat anti-mouse secondary antibody (1:400; Invitrogen) for 4 hours at 4°C. Next, retinas were washed with PBS, mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and examined with an Olympus 1X71 fluorescence microscope (Olympus, Center Valley, PA) or confocal microscopy (Zeiss 510; Carl Zeiss, Thornwood, NY). Areas of vasoobliteration and retina NV were quantified using ImageJ software (National Institutes of Health, Bethesda, MD), as previously reported.²²

Immunostaining of Retinal Sections

After fixation in 4% paraformaldehyde, retinas were equilibrated in 30% sucrose and embedded in optimal cutting temperature compound (Tissue Tek; Sakura Finetek, Torrance, CA). They were then frozen in liquid nitrogen and cut into 10- μ m sections. Retinal sections were placed on a glass slide, washed with PBS, and permeabilized with PBS containing 1% Triton X-100 for 30 minutes at room temperature before blocking with PBS containing 10% normal goat serum for 1 hour. Sections were incubated overnight at 4°C with Alexa Fluor 594-labeled isolectin B4 (1:200; Invitrogen) and primary antibody against Fn14 (1:1000; Abcam, Cambridge, MA) or TWEAK (1:1000; Novus Biologicals, Littleton, CO) in PBS

containing 3% normal goat serum. Next, sections were washed with PBS, incubated with Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (1:400; Invitrogen) at room temperature for 1 hour, washed with PBS, mounted with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories), and examined with a fluorescence microscope (Olympus 1X71; Olympus).

Isolation of Retinal Vessels

Retinal vessels were isolated from other retinal cellular components as previously described.²⁰ Briefly, retinas were dissected and incubated in sterile water at 4°C for 1 hour, and then incubated in 4 mL distilled water containing 500 U DNase I (Worthington Biochemical Corp., Lakewood, NJ) in a 60-mm dish for 10 minutes. During the latter incubation, the solution was gently and repeatedly pipetted onto the retinal tissue until the preparation became transparent. The retina was then transferred back to water and retinal vessels were separated from the remaining debris. The purity of the isolated vessels was assessed by microscopic examination after staining with periodic acid Schiff and hematoxylin.

RT-PCR Assay

Total RNA was isolated with an RNAqueous-4 PCR Kit (Invitrogen). Complementary DNA was produced by reverse transcription with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed with StepOne PCR system (Invitrogen) using Power SYBR Green (Invitrogen).²¹ The fold difference in various transcripts was calculated by the $\Delta\Delta$ CT method using hypoxanthine guanine phosphoribosyltransferase (Hprt) or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal controls according to the Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR (Invitrogen). Subsequently, a melting curve, constructed in the range of 60°C to 95°C, was used to evaluate the specificity of the amplification products. Primer sequences for mouse transcripts were as follows: Hprt For-5'-GAA AGA CTT GCT CGA GAT GTC ATG-3'; Hprt Rev-5'-CAC ACA GAG GGC CAC AAT GT-3'; Fn14 For-5'-CTG GTT TTG GCG CTG GTT-3'; Fn14 Rev-5'-TCT CTC CGG CGG CAT CT-3'; TWEAK For-5'-TGG GAA GAG ACC AAA ATC AAC A-3'; and TWEAK Rev-5'-CCC AAT CTG GCG GTC GTA-3'. Primer sequences for human transcript were as follows: GAPDH For-5'-ATG GAA ATC CCA TCA CCA TCT T-3'; GAPDH Rev-5'-CGC CCC ACT TGA TTT TGG-3'; Fn14 For-5'-GCT CTG AGC CTG ACC TTC GT-3'; Fn14 Rev-5'-TCC GCC GGT CTC CTC TAT G-3'; TWEAK For-5'-GGA AAA CAC GGG CTC GAA-3'; TWEAK Rev-5'-CCA GGT CGT GGA TGA ACT TCA-3'; Bcl2 For-5'-GCT GGG AGA ACA GGG TAC GA-3'; Bcl2 Rev-5'-TCT GCG ACA GCT TAT AAT GGA TGT-3'; Bim For-5'-TGG CAA AGC AAC CTT CTG ATG-3'; Bim Rev-5'-GCA GGC TGC AAT TGT CTA CCT-3'; Bcl-xL For-5'-CCA TGG CAG CAG TAA AGC AA-3'; Bcl-xL Rev-5'-CCG GTA CCG CAG TTC AAA CT-3'; Bfl-1 For-5'-CCT GGA TCA GGT CCA AGC AA-3'; Bfl-1 Rev-5'-TTG GAC TGA GAA CGC AAC ATT T-3'; Bax For-5'-CCA AGG TGC CGG AAC TGA-3'; Bax Rev-5'-TCC CGG AGG AAG TCC AAT G-3'; Bcl-w For-5'-CCA GGC TCA GCC CAA CAA-3'; Bcl-w Rev-5'-GCC CCC TTG AAA AAG TTC ATC-3'; Mcl-1 For-5'-TCA AAA ACG AAG ACG ATG TGA AA-3'; and Mcl-1 Rev-5'-CCG TCG CTG AAA ACA TGG A-3'.

Western Blotting

Cells were lysed in a radioimmunoprecipitation assay lysis buffer (Millipore, Billerica, MA) supplemented with protease inhibitors (Roche Applied Science, Indianapolis, IN). The

FIGURE 1. The expression of Fn14 and TWEAK is altered in retinas of OIR. Mice were subjected to OIR or maintained in RA as control. Retinas were collected at indicated time points (P12–P17), and Fn14 (A) or TWEAK mRNA (B) in the retinas was examined by qPCR and normalized to the age-matched RA control ($n = 6$). * $P < 0.05$ compared with relevant control.

lysates were cleared of debris by centrifugation. Protein concentration in the lysates was determined by a bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL). The lysates were mixed with 4× SDS sample buffer and subjected to 10% SDS-PAGE, transferred onto a nitrocellulose membrane that was incubated with primary antibodies against Tubulin (1:10,000; Sigma-Aldrich, St. Louis, MO), HIF-1 α (1:200; Santa Cruz Biotechnology, Dallas, TX), Fn14 (1:500) or Phospho-Akt (Ser473; #4058, 1:1000; Cell Signaling Technology, Danvers, MA) followed by horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were detected using the enhanced chemiluminescence (ECL) system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Analysis of Transcription Factor Binding Sites

A 6-kb genomic sequence of human Fn14 gene (−4176 to 1802 bp) was aligned with the representative sequence from mouse Fn14 gene using rVista2.0 ([in the public domain] <http://rvista.dcode.org>).²³ Alignments were accomplished using the Mulan program, and conserved transcription factor binding sites were identified using the multiTF ([in the public domain] <http://mulan.dcode.org>). In addition to the HIF-1 α binding sequence

included in the multiTF program, the consensus core of hypoxia-responsive elements (RCGTG)²⁴ was used in the search of additional potential HIF-1 α binding sites.

Construction of HIF-1 α Adenovirus

Hypoxia inducible factor-1 α adenovirus was made as described by Corley et al.²⁵ with modifications.²⁶ Human HIF-1 α was cloned into the pAdTrack shuttle vector cytomegalovirus (CMV) promoter using KpnI and EcoRI restriction sites, and adenovirus plasmids containing green fluorescent protein (GFP) alone (AdGFP) and GFP together with HIF-1 α (AdHIF-1 α) were produced using the AdEasy XL System (Agilent Technologies, Santa Clara, CA). The AdGFP and AdHIF-1 α were transfected into AD-293 cells and the viral particles were purified using the Adeno-X Virus Purification Kit according to manufacturer's instructions (Clontech Laboratories, Mountain View, CA).

Cell Culture

Primary human retinal microvascular endothelial cells (HRMECs) were purchased from Cell Systems (Kirkland, WA)

FIGURE 2. Hyperoxia treatment reverses Fn14 and TWEAK expression in OIR mice. Oxygen-induced retinopathy mice were treated with hyperoxia (75% oxygen; HT) or maintained in RA (OIR) from P14 to P17. Mice maintained in RA from P1 to P17 are control. Fibroblast growth factor inducible-14 mRNA (A) and TWEAK mRNA (B) were determined by qPCR and normalized to RA control ($n = 6$). * $P < 0.05$ compared with RA. # $P < 0.05$ compared with OIR.

FIGURE 3. The expression of Fn14 and TWEAK is redistributed in the retinas of OIR. Retinal frozen sections from P17 OIR mice or RA mice were stained with isolectin B4 (red) to highlight the retinal vessels, DAPI (blue) for nuclei, and Fn14 antibody (green; [A]), or TWEAK antibody (green; [B]). The immunoreactivity of Fn14 and TWEAK shifted from the neuronal layers in healthy retinas to the neovascular tufts in OIR. Representative fluorescence microscopy images are shown (200 $\times$, $n = 3$). All images in OIR and RA were taken at the same setting except for isolectin B4 images that were adjusted individually in each photograph for better visibility of vessels. IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

and cultured in medium constituted of 50% CSC Serum-Containing Medium (Cell Systems) and 50% EGM Endothelial Cell Medium (Lonza Walkersville, Inc., Walkersville, MD). Confluent HRMECs were infected with adenovirus carrying HIF-1 α or GFP produced as described previously.^{25,26} The following day, cells were incubated overnight with serum free EGM. At 48 hours after infection, cells were stimulated with vehicle (PBS containing 0.1% BSA), VEGF (50 ng/mL; R&D systems), bFGF (20 ng/mL; PeproTech, Rocky Hill, NJ), or IL-1 β (10 ng/mL; PeproTech) for 2 hours. Cells were then collected for analysis of the expression of HIF-1 α and Fn14.

Analysis of Cell Survival

Human retinal microvascular endothelial cells were plated at a density of 4×10^4 cells/chamber in fibronectin-coated chambers of the eight-well electrode arrays (8W10E; Applied Biophysics, Inc., Troy, NY). The following day, cells were incubated overnight with serum free EGM and then treated with vehicle (serum free EGM) or TWEAK (200 ng/mL) for 4

days. The electrical cell-substrate impedance was measured at days 0, 1, 2, 3, and 4 after drug treatment using electrical cell-substrate impedance sensing (ECIS; Applied Biophysics, Inc.). The basal level of impedance was measured in the absence of cells. Cells treated with vehicle at day 0 were used as reference. The percentage of normalized impedance was calculated as $[\text{Impedance}_{\text{treated}} - \text{Impedance}_{\text{basal}}] / [\text{Impedance}_{\text{vehicle at day 0}} - \text{Impedance}_{\text{basal}}] \times 100\%$. To examine cells at day 4 after treatment, cells were washed twice with PBS, fixed with 4% paraformaldehyde, and imaged by fluorescence microscope (Olympus 1X71) after mounted with mounting medium containing DAPI.

MTT

Human retinal microvascular endothelial cells were seeded at a density of 6×10^3 cells/well in collagen/fibronectin-coated 96-well plates. The following day, cells were incubated with serum-free EGM overnight and then treated with vehicle (serum-free EGM) or TWEAK (200 ng/mL) for 4 days. After

FIGURE 4. Fibroblast growth factor inducible-14 is highly expressed in the neovascular tufts in the retinas of OIR. Retinas isolated from P17 OIR mice or RA mice were immunostained with isolectin B4 (red) to highlight the retinal vessels and Fn14 antibody (green). The Fn14 protein was consistently highly expressed in neovascular tufts of central, intermediate, and peripheral retinal areas. Representative confocal images were taken at 200 $\times$ magnification ($n = 3$).

treatment, 20 μ L of 5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) was added to each well and plates were incubated at 37°C for 3 hours. After removing the media, 100 μ L of DMSO (Sigma-Aldrich) was added to each well and shaken at room temperature for 10 minutes to dissolve intracellular MTT formazan crystals, followed by absorbance measurement at 540 nm. Wells containing medium only were used as blank and the value was subtracted from each value.

Statistical Analysis

Data are presented as mean $\pm$ SEM. Statistical analysis was performed using SigmaStat 2.03 (SPSS, Inc., Chicago, IL). Group differences were evaluated with one-way ANOVA followed by post hoc Student's *t*-test using the Student-Newman-Keuls method. Results were considered significant at *P* less than 0.05.

RESULTS

Fn14 Is Upregulated in Ischemia-Induced Retinopathy

Studies were performed in a mouse model of OIR in which obliteration of the immature retinal vessels was induced by maintaining mice in 75% oxygen from P7 through P12.¹⁹ Upon returning to RA, the avascular central retina became hypoxic, leading to upregulation of many angiogenic and inflammatory genes and, subsequently, retinal NV.²⁷ This model has been used extensively to study mechanisms and design strategies for blockade of pathologic NV in ischemia-induced retinopathy.^{28–31} To investigate the potential involvement of TWEAK/Fn14 in NV, mRNA levels of Fn14 and TWEAK were measured from P12 to P17. Compared with the age-matched control mice kept in RA, Fn14 mRNA increased significantly in OIR retinas soon after relative hypoxia (P13) was initiated, and remained high (2.3- to 4.5-fold versus control) through P17 (Fig. 1A). During the same period, TWEAK mRNA was marginally decreased (less than 15%) in OIR (Fig. 1B). These findings indicate that upregulation of Fn14 was associated with relative

hypoxia and development of NV during ischemia-induced retinopathy.

Hyperoxia Treatment Normalizes Fn14 and TWEAK

To further explore the association of the Fn14/TWEAK pathway with OIR pathology, we determined mRNA levels of Fn14 and TWEAK at P17 in retinas from OIR mice treated with hyperoxia (75% oxygen, from P14–P17). This approach has been shown to eliminate retinal hypoxia, accelerate the process of revascularization and prevent development of pathologic NV in ischemia-induced retinopathy.²¹ Our data showed that compared with OIR mice without treatment, hyperoxia treatment reduced Fn14 mRNA and increased TWEAK mRNA to RA levels (Fig. 2).

Fn14 Is Localized in NV Tufts in Ischemia-Induced Retinopathy

We next determined the retinal localization of Fn14 and TWEAK. Isolectin B4 was used as a marker for the retinal vasculature and DAPI was used to identify nuclei. In RA-control mice, the immunoreactivity of Fn14 was localized to inner nuclear layer (INL) and ganglion cell layer (GCL) with negligible expression in normal vessels (Fig. 3A). In contrast, the OIR retina showed predominant localization of Fn14 in neovascular tufts, whereas the expression of Fn14 was less obvious in neuronal layers (Fig. 3A). The immunoreactivity of TWEAK was diffused throughout the healthy retina, albeit more intense in the GCL, but it was increased predominantly in the neovascular tufts in OIR (Fig. 3B). Fibroblast growth factor inducible-14 expression was also assessed by immunostaining whole-mount retinas, which showed the Fn14 protein was highly expressed in neovascular tufts in OIR rather than normal vessels in RA (Fig. 4).

Fn14 Is Upregulated in Retinal Vasculature in Ischemia-Induced Retinopathy

To evaluate whether Fn14 and TWEAK mRNA expressions were correlated with their increased immunoreactivity in the vasculature during OIR, retinal vessels were isolated and mRNA levels were determined by qPCR. Consistent with increased Fn14 immunoreactivity in neovascular tufts, Fn14 mRNA showed a 6.6-fold increase in OIR versus RA control (Fig. 5A). In contrast, TWEAK mRNA was decreased by 46% in retinal vessels in OIR (Fig. 5B).

The TWEAK/Fn14 Pathway Contributes to Retinal Neovascularization in Ischemia-Induced Retinopathy

The selective upregulation of Fn14 in retinal vasculature during OIR suggests a potential involvement of the TWEAK/Fn14 pathway in retinal NV. To test this possibility, an Fn14-Fc decoy receptor was injected into the vitreous cavity of the OIR mice at P12 to block the interaction between Fn14 and TWEAK.¹³ Compared with the OIR control group that received intravitreal injection of vehicle, blockade of the TWEAK/Fn14 pathway significantly reduced retinal NV by 45% (Figs. 6A, 6B). While the retinal avascular area was reduced by 24%, it did not reach statistical significance (Figs. 6A, 6C). These findings indicate that the TWEAK/Fn14 pathway plays a key role in the pathogenesis of ischemia-induced retinopathy.

FIGURE 5. The expression of Fn14 and TWEAK is altered in retinal vessels of OIR. Retinal vessels were isolated from P17 OIR or RA control mice, and RNA was extracted from pooled retinal vessels. Fibroblast growth factor inducible-14 mRNA (A) or TWEAK mRNA (B) in retinal vessels was quantified by qPCR and normalized to the RA control. $n = 3$ (each n represents a pool of six retinas). * $P < 0.05$ compared with RA.

Blockade of the TWEAK/Fn14 Pathway Does not Alter VEGF Expression in Ischemia-Induced Retinopathy

Vascular endothelial growth factor is a potent angiogenic factor that plays a critical role in retinal NV in ischemia-induced retinopathy.^{21,32–34} The TWEAK/Fn14 pathway has been

shown to promote ovarian cancer cell metastasis by upregulating VEGF expression.³⁵ To investigate whether the TWEAK/Fn14 pathway contributes to retinal neovascularization by similar mechanism, the level of VEGF mRNA was analyzed after intravitreal injection of Fn14-Fc decoy receptor (Fig. 7). Vascular endothelial growth factor expression was unaffected by blocking the interaction between Fn14 and TWEAK,

FIGURE 6. The blockade of TWEAK/Fn14 results in decreased retinal NV in OIR. Oxygen-induced retinopathy mice were intravitreally treated with a soluble Fn14-Fc decoy receptor (2.5 $\mu\text{g}/\text{eye}$) or vehicle (PBS) at P12. Retinas were collected at P17 and stained with isolectin B4. Representative images of the retinal flat mounts are shown (40 $\times$; [A]). Neovascularization (B) and avascular areas (C) were quantified using ImageJ software ($n = 8$). * $P < 0.05$ compared with vehicle-treated mice.

FIGURE 7. The blockade of TWEAK/Fn14 does not influence VEGF expression in OIR. Oxygen-induced retinopathy mice were intravitreally treated with a soluble Fn14-Fc decoy receptor (2.5 μ g/eye) or vehicle (PBS) at P12. Retinas were collected at P17 and VEGF mRNA level was measured by qPCR ($n = 5$). * $P < 0.05$ compared with vehicle-treated mice.

suggesting that the TWEAK/Fn14 pathway is involved in retinal NV via a mechanism independent of VEGF expression in ischemia-induced retinopathy.

Activation of the TWEAK/Fn14 Pathway Enhances Survival of Retinal Microvascular Endothelial Cells

Previous studies have consistently shown that the TWEAK/Fn14 pathway induces angiogenic responses such as endothelial cell migration, proliferation, and tube formation.^{15–17} However, it remains controversial whether this pathway is involved in endothelial cell survival.^{15,16} As enhanced endothelial survival is critical for the development of retinal NV given that endothelial cells in the NV tufts are sensitive to apoptotic stimuli compared with normal retinal vessels,²⁰ we determined the survival of HRMECs in serum-deprived medium when cells were treated with TWEAK. Cell density was determined by measurement of electrical cell-substrate impedance with an ECIS. The degree of impedance change can be determined by multiple factors, including cell density, barrier function, cell-cell interaction, and quality of interaction of cells with the electrodes.³⁶ However, there is a strong correlation between the fall of impedance and extent of cell death when cell loss is significant.³⁷ As shown in Figure 8A, there was no impedance change in vehicle-treated cells 24 hours after being placed in culture media containing no serum. From days 2 to 4, the impedance fell to 49%, 26% and 9% of the initial level, respectively, indicating significant and continuous loss of cells. In contrast, TWEAK treatment significantly reduced HRMEC death. The impedance was 2.3- and 4.2-fold of vehicle-treated cells at days 3 and 4, respectively. Correlated with the reduced fall of impedance, there were more cells preserved in the electrodes 4 days after TWEAK treatment compared with vehicle treatment (Fig. 8B). The effect of TWEAK in HRMEC survival was further confirmed by the MTT assay that showed a 2-fold increase in the number of viable cells after TWEAK treatment (Fig. 8C). These results suggest that TWEAK/Fn14 may modulate retinal NV by promoting survival of retinal microvascular endothelial cells.

The TWEAK/Fn14 Pathway Regulates the Expression of Anti- and Proapoptotic Molecules

The B-cell lymphoma-2 (Bcl2) family of proteins regulate both intrinsic and extrinsic mechanisms of apoptosis and are essential for cell survival.³⁸ This family of proteins can be divided into two groups that have opposite functions on cell

survival. B-cell lymphoma-2, Bcl-xL, Bcl-w, Mcl-1, and Bfl-1 are antiapoptotic proteins, whereas Bax, Bak, and Bim are proapoptotic proteins. To examine molecular mechanisms underlying TWEAK/Fn14-promoted retinal endothelial cell (EC) survival, the expression of several anti- and proapoptotic molecules in the Bcl2 family was determined by qPCR. These studies showed that serum deprivation significantly induced the expression of proapoptotic molecule Bim, and TWEAK treatment blocked this effect (Fig. 9A). In addition, TWEAK induced the expression of antiapoptotic molecule Bfl-1 and slightly increased the levels of other two antiapoptotic molecules, Mcl-1 and Bcl-w (Fig. 9B). Neither serum deprivation nor TWEAK treatment significantly changed the levels of Bcl2, Bcl-xL, Bax, and Bak (Fig. 9). Akt phosphorylation, another pathway involved in cell survival, was assayed and no difference between vehicle and TWEAK treatment was observed (data not shown). These findings suggest that TWEAK/Fn14 promote retinal endothelial cell survival by simultaneously activating survival pathways and inhibiting apoptotic pathways in the Bcl2 family.

HIF-1 α Is Implicated in Upregulation of Fn14

Given that upregulation of Fn14 was involved in retinal NV in ischemia-induced retinopathy, it would be important to understand potential mechanisms underlying Fn14 expression during OIR. The upregulation of Fn14 during OIR and its normalization by oxygen supplementation suggested that hypoxia-induced factors are involved in Fn14 expression. To investigate how hypoxia-induced factors regulate Fn14 expression in ischemia-induced retinopathy, rVISTA2.0 software was used to perform a comparative sequence analysis of mouse and human genome and predict transcription factor binding sites in a 6-kb region of Fn14 gene. This analysis was based on the hypothesis that regulatory modules are under positive selection during evolution and are often conserved between species.²³ This approach has dramatically enhanced prediction accuracy compared with the analysis based only on the short sequence of transcription factor binding sites.²³ Our analysis revealed several conserved clusters in the noncoding DNA regions between human and mouse Fn14 genes (upper panel of Fig. 10). Within these clusters, there were 395 multi-conserved transcription factor binding sites, including binding sites for nuclear factor-kappa B (NF- κ B) that is known to regulate Fn14 transcription.³⁹ Among these transcription factor binding sites, there were 11 binding sites for HIF-1 α (lower panel of Fig. 10). Since HIF-1 α is known to play a pivotal role in hypoxia-induced responses, our analysis suggested that HIF-1 α is a potential transcription factor involved in Fn14 expression during hypoxia.

To test this possibility, HIF-1 α was introduced into HRMECs by adenovirus-mediated gene delivery and the level of Fn14 mRNA was analyzed. Compared with cells infected with adenovirus carrying GFP (AdGFP, control), the level of HIF-1 α was high in cells infected with adenovirus carrying HIF-1 α (AdHIF-1 α ; Fig. 11A). Associated with increase in HIF-1 α , the Fn14 mRNA level was significantly increased by 123% (Fig. 11B). Consistently Fn14 protein was also significantly induced by HIF-1 α (Fig. 11C).

Given that levels of proangiogenic and proinflammatory cytokines, including VEGF, bFGF, or IL-1 β are increased in ischemia-induced retinopathy and these molecules have been shown to induce Fn14 expression in other cells,^{15,21,40,41} it is possible that these cytokines also contribute to Fn14 upregulation in hypoxic retinas. To test this possibility, HRMECs were stimulated with VEGF, bFGF, or IL-1 β in the presence or absence of HIF-1 α . Although VEGF treatment induced 52% upregulation of Fn14 mRNA and had an additive effect with

FIGURE 8. Tumor necrosis factor-like weak inducer of apoptosis treatment improves the survival of HRMECs. **(A)** Human retinal microvascular endothelial cells were treated with vehicle (EGM without serum) or TWEAK (200 ng/mL) for the time as indicated. Cell viability was determined by measuring the electrical cell-substrate impedance and normalized to that of cells treated with vehicle for 0 hour ($n = 4$). $*P < 0.05$ compared with cells treated with vehicle at the corresponding time. **(B)** After 4 days of treatment, cells in the electrode were stained with DAPI and representative images are shown (40 $\times$). *Arrows* show examples of cell nuclei. **(C)** Human retinal microvascular endothelial cells were treated with vehicle (EGM without serum) or TWEAK (200 ng/mL) for 4 days. Cell viability was determined by MTT assay ($n = 3$). $*P < 0.05$ compared with control.

HIF-1 α , bFGF and IL-1 β did not induce upregulation of Fn14 in HRMECs and did not affect HIF-1 α -induced Fn14 expression (Fig. 11D). Taken together, these results indicate that HIF-1 α is likely involved in Fn14 expression and may work together with other cytokines to induce Fn14 expression in retinal endothelial cells during ischemia-induced retinopathy.

DISCUSSION

Retinal NV occurs after a period of ischemia due to the cessation of retinal vascular development, vessel regression, or occlusion. It can lead to vitreous hemorrhage, epiretinal or subretinal fibrosis, and tractional retinal detachment, resulting in profound irreversible vision loss. Laser photocoagulation is the common therapy for retinal NV; however, this method

destroys retinal neurons and has significant side effects, including loss of visual field, reduced visual acuity, and impaired night vision.^{4,42} Anti-VEGF agents are now in widespread use for treatment of the subretinal NV associated with AMD. In ischemia-induced retinopathy, anti-VEGF drugs show promise, but issues such as efficacy, impairment of revascularization, and the long-term safety of VEGF antagonism have emerged as significant concerns for anti-VEGF therapy.^{4-7,21} In this study, we provide the first evidence that the TWEAK/Fn14 pathway is prominently upregulated in retina during ischemia-induced retinopathy and blockade of the TWEAK/Fn14 pathway significantly reduces the extent of retinal NV. Unlike VEGF trap (VEGFR1-Fc decoy receptor), which reduces retinal NV but blocks retinal revascularization and increases avascular area,²¹ the Fn14-Fc decoy receptor did not prevent retinal revascularization and the avascular area was

FIGURE 9. Tumor necrosis factor–like weak inducer of apoptosis treatment regulates expression of Bcl2 family genes. Human retinal microvascular endothelial cells were treated with vehicle (EGM without serum) or TWEAK (200 ng/mL) for the times indicated. Ribonucleic acid was extracted and the expression of proapoptotic genes (Bim, Bax, and Bak; [A]) and antiapoptotic genes (Bcl2, Bcl-xL, Bfl-1, Mcl-1, and Bcl-w; [B]) were determined by qPCR analysis ($n = 3$). * $P < 0.05$ compared with 6-hours vehicle control. # $P < 0.05$ compared with relevant vehicle-treated values.

actually slightly reduced. Considering the limitations of photocoagulation and anti-VEGF therapy, blocking TWEAK/Fn14 pathway may provide an alternative therapeutic approach for preventing vision loss due to retinal NV in ischemia-induced retinopathy.

The TWEAK/Fn14 pathway is involved in various processes such as proinflammatory response, cell growth, cell survival, and cell death dependent on the cellular context.⁴³ It has been reported to be a key mediator in kidney injury, neuronal damage, and muscle atrophy.^{12–14} Although the angiogenic function of this pathway was recognized soon after TWEAK was identified,¹⁸ its role in physiological or pathologic

angiogenesis in diseases has not been explored. Here, we found that Fn14 expression was upregulated at the beginning of the relative hypoxia period, prior to the appearance of NV tufts, and remained high throughout the period of NV formation during ischemia-induced retinopathy. Concurrently, the immunoreactivity of Fn14 and TWEAK shifted from neuronal layers in the healthy retina to neovascular tufts in OIR. This shift suggests the involvement of the TWEAK/Fn14 pathway in the formation and maintenance of ischemia-induced pathologic retinal angiogenesis. This possibility was supported by our finding that retinal NV was significantly reduced by blocking the TWEAK/Fn14 pathway.

FIGURE 10. Identification of transcription factor binding sites in Fn14 gene region. *Upper panel*, rVISTA plot shows regions of homology between mouse and human in a 6-kb region of the Fn14 gene. The *shaded area* represents greater than 70% identity between two species. *Lower panel*, schematic diagram represents potential HIF-1 α binding sites in the 6-kb region of Fn14 gene predicted by rVISTA2.0.

At present, it is unknown how TWEAK/Fn14 is involved in retinal NV. The selective upregulation of Fn14 in the NV tufts may highly sensitize endothelial cells to TWEAK, and therefore initiate angiogenesis processes such as cell proliferation, migration, and tube formation during retinal ischemia.^{15–17} Moreover, our data indicate that activation of TWEAK/Fn14 pathway promoted HRMEC survival in a process that may involve TWEAK/Fn14-induced expression of survival molecules and inhibition of apoptotic molecules in the Bcl2 family. This property is important for the process of retinal NV, as neovascular tufts, unlike mature vessels, lack proper pericyte coating and interaction with glia, and are therefore sensitive to apoptotic signals.²⁰ Unlike previous studies that induced endothelial cell death by removing growth factors,^{15,16} we included bovine brain extract in the medium to mimic the *in vivo* condition in which endothelial cells are exposed to multiple survival cytokines secreted from neuronal tissue. The strikingly enhanced HRMEC survival by TWEAK treatment in our study highlights the role of TWEAK/Fn14 as an independent survival factor during NV. The precise mechanisms by which TWEAK/Fn14 regulates expression of Bcl2 family genes remain to be elucidated. Presumably, Fn14, after engaged by TWEAK, forms a trimmer that recruits TNFR-associated factor (TRAF) and induces rapid activation of canonical NF- κ B followed by prolonged activation of noncanonical NF- κ B.⁴⁴ Nuclear factor- κ B then binds to the transcription regulator regions of Bcl2 family genes and regulates their transcription.^{45,46} In addition to the direct angiogenic impact on endothelial cells, the development of retinal NV depends on multiple other factors including upregulation of angiogenic factors, inflammation, and breakdown of internal limiting membrane. Our data indicate that VEGF is not a downstream mediator of TWEAK/Fn14-modulated retinal NV in the OIR model. However, TWEAK/Fn14 can induce expression of other angiogenic and inflammatory factors such as IL-6 and IL-8.⁴⁷ Tumor necrosis factor-like weak inducer of apoptosis/Fn14 pathway can also promote production of matrix metalloproteinase proteins, which may facilitate the breakdown of the internal limiting membrane.^{48,49} Further studies are required to explore the precise mechanisms of TWEAK/Fn14-induced retinal NV.

Fibroblast growth factor inducible-14 is normally expressed at low level under physiological conditions. Its expression is upregulated during tissue injury or diseases probably due to increases in growth factors and/or inflammatory factors under these conditions.⁴³ The highly inducible pattern of Fn14 expression is well conserved between mouse and human, suggesting this pathway is evolutionarily conserved.⁴³ Nevertheless little is known about mechanisms underlying regulation of Fn14 expression. To our knowledge, NF- κ B is the only transcription factor that has been identified to induce Fn14 upregulation during inflammation.³⁹ In the current study, we provide new evidence that Fn14 expression was upregulated during the relative hypoxia period and was normalized with hyperoxia, suggesting hypoxia responsive factors are involved in Fn14 expression. With comparison analysis, we identified 11 predicted HIF-1 α binding sites, which are conserved between the mouse and human gene. Our data that HIF-1 α induces Fn14 expression in HRMECs support the hypothesis that Fn14 expression is hypoxia driven and HIF-1 α is likely implicated in this process. This observation is different from the report by Vendrell et al,⁵⁰ in which upregulation of Fn14 expression in adipocytes and macrophages is induced by inflammatory cytokines, but not by hypoxia. Therefore HIF-1 α -induced Fn14 expression is dependent on cellular contexts such as co-activators, cell-specific DNA methylation patterns, and post translational histone modifications in specific cell types.⁵¹ Future studies that would include reporter gene assay, gel electrophoresis mobility shift assay, chromatin immunoprecipitation, histone acetylation/methylation analysis, and DNA methylation analysis are needed to identify HIF-1 α binding sites in the Fn14 transcriptional regulation region, elucidate co-activators and determine the chromatin-based mechanisms in order to better understand the mechanisms underlying hypoxia-induced Fn14 expression in specific cell types. Although we show that HIF-1 α is likely involved in Fn14 expression, other molecules produced in hypoxic retina, such as VEGF and IL-6, may further boost HIF-1 α -induced Fn14 expression by activating other transcription regulatory elements.

In contrast to Fn14 expression, the TWEAK mRNA was downregulated in the retina and retinal vasculature during ischemia-induced retinopathy. However, the magnitude of

FIGURE 11. Hypoxia inducible factor-1 α induces Fn14 expression in HRMECs. Human retinal microvascular endothelial cells were infected with adenovirus expressing HIF-1 α (AdHIF-1 α) or GFP (AdGFP) as control for 48 hours. Hypoxia inducible factor-1 α protein was determined by Western blot and Tubulin was used as loading control (A). Fibroblast growth factor inducible-14 mRNA (B) and protein (C) in HRMECs were determined by qPCR ($n = 5$) and Western blot ($n = 3$). * $P < 0.05$ compared with control. (D) At 48 hours after infection, HRMECs were stimulated with VEGF (50 ng/mL), bFGF (20 ng/mL), or IL-1 β (10 ng/mL) for 2 hours. Fibroblast growth factor inducible-14 mRNA was determined by qPCR and normalized to control cells that were infected with AdGFP and treated with vehicle ($n = 3$). * $P < 0.05$ compared with control.

Fn14 upregulation was higher than the magnitude of TWEAK downregulation; in other words, the ratio of Fn14 to TWEAK increased in hypoxic retina. We postulate that a high Fn14/TWEAK ratio may offer selective activation of endothelial cells that show higher Fn14 upregulation. This notion is supported by our observation that the immunoreactivity of TWEAK was redistributed from neuronal layers in normal retina to the NV tufts during ischemia-induced retinopathy. Similar phenomenon of high receptor/ligand ratio as an activation mechanism has been previously described; for example, it has been shown that high ratio of Eph receptor to its ligand, ephrin, facilitates tumorigenesis.^{52,53}

In summary, our data provide the first evidence that TWEAK/Fn14 pathway is activated and involved in pathologic retinal NV during ischemia-induced retinopathy. Given that angiogenesis is involved in other diseases such as tumors, limb ischemia, and neovascular AMD, this novel finding would encourage further exploration of the role of TWEAK/Fn14 in the pathogenesis of these diseases.

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References

1. Multicenter trial of cryotherapy for retinopathy of prematurity. 3 1/2-year outcome-structure and function. Cryotherapy for Retinopathy of Prematurity Cooperative Group. *Arch Ophthalmol*. 1993;111:339-344.

2. Phelps DL. Retinopathy of prematurity. *Pediatr Rev.* 1995;16:50-56.
3. Tsilimbaris MK, Kontadakis GA, Tsika C, Papageorgiou D, Charoniti M. Effect of panretinal photocoagulation treatment on vision-related quality of life of patients with proliferative diabetic retinopathy. *Retina.* 2013;33:756-761.
4. Mintz-Hittner HA, Kennedy KA, Chuang AZ. Efficacy of intravitreal bevacizumab for stage 3+ retinopathy of prematurity. *N Engl J Med.* 2011;364:603-615.
5. Kurihara T, Westenskow PD, Bravo S, Aguilar E, Friedlander M. Targeted deletion of VEGF-A in adult mice induces vision loss. *J Clin Invest.* 2012;122:4213-4217.
6. Nishijima K, Ng YS, Zhong L, et al. Vascular endothelial growth factor-A is a survival factor for retinal neurons and a critical neuroprotectant during the adaptive response to ischemic injury. *Am J Pathol.* 2007;171:53-67.
7. Fleck BW. Management of retinopathy of prematurity. *Arch Dis Child Fetal Neonatal Ed.* 2013;98:F454-F456.
8. Jalali S, Balakrishnan D, Zeynalova Z, Padhi TR, Rani PK. Serious adverse events and visual outcomes of rescue therapy using adjunct bevacizumab to laser and surgery for retinopathy of prematurity. The Indian Twin Cities Retinopathy of Prematurity Screening database Report number 5. *Arch Dis Child Fetal Neonatal Ed.* 2013;98:F327-333.
9. Winkles JA. The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. *Nat Rev Drug Discov.* 2008;7:411-425.
10. Chicheportiche Y, Bourdon PR, Xu H, et al. TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. *J Biol Chem.* 1997;272:32401-32410.
11. Wiley SR, Cassiano L, Lofton T, et al. A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis. *Immunity.* 2001;15:837-846.
12. Sanz AB, Sanchez-Nino MD, Ortiz A. TWEAK, a multifunctional cytokine in kidney injury. *Kidney Int.* 80:708-718.
13. Yepes M, Brown SA, Moore EG, Smith EP, Lawrence DA, Winkles JA. A soluble Fn14-Fc decoy receptor reduces infarct volume in a murine model of cerebral ischemia. *Am J Pathol.* 2005;166:511-520.
14. Kumar A, Bhatnagar S, Paul PK. TWEAK and TRAF6 regulate skeletal muscle atrophy. *Curr Opin Clin Nutr Metab Care.* 2012;15:233-239.
15. Donohue PJ, Richards CM, Brown SA, et al. TWEAK is an endothelial cell growth and chemotactic factor that also potentiates FGF-2 and VEGF-A mitogenic activity. *Arterioscler Thromb Vasc Biol.* 2003;23:594-600.
16. Jakubowski A, Browning B, Lukashev M, et al. Dual role for TWEAK in angiogenic regulation. *J Cell Sci.* 2002;115:267-274.
17. Kawakita T, Shiraki K, Yamanaka Y, et al. Functional expression of TWEAK in human hepatocellular carcinoma: possible implication in cell proliferation and tumor angiogenesis. *Biochem Biophys Res Commun.* 2004;318:726-733.
18. Lynch CN, Wang YC, Lund JK, Chen YW, Leal JA, Wiley SR. TWEAK induces angiogenesis and proliferation of endothelial cells. *J Biol Chem.* 1999;274:8455-8459.
19. Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci.* 1994;35:101-111.
20. Liu H, Zhang W, Xu Z, Caldwell RW, Caldwell RB, Brooks SE. Hyperoxia causes regression of vitreous neovascularization by downregulating VEGF/VEGFR2 pathway. *Invest Ophthalmol Vis Sci.* 2013;54:918-931.
21. Zhang W, Yokota H, Xu Z, et al. Hyperoxia therapy of preproliferative ischemic retinopathy in a mouse model. *Invest Ophthalmol Vis Sci.* 2011;52:6384-6395.
22. Stahl A, Connor KM, Sapieha P, et al. Computer-aided quantification of retinal neovascularization. *Angiogenesis.* 2009;12:297-301.
23. Loots GG, Ovcharenko I. rVISTA 2.0: evolutionary analysis of transcription factor binding sites. *Nucleic Acids Res.* 2004;32:W217-221.
24. Wenger RH, Stiehl DP, Camenisch G. Integration of oxygen signaling at the consensus HRE. *Sci STKE.* 2005;306:re12.
25. Corley KM, Taylor CJ, Lilly B. Hypoxia-inducible factor 1alpha modulates adhesion, migration, and FAK phosphorylation in vascular smooth muscle cells. *J Cell Biochem.* 2005;96:971-985.
26. Liu H, Zhang W, Kennard S, Caldwell RB, Lilly B. Notch3 is critical for proper angiogenesis and mural cell investment. *Circ Res.* 2010;107:860-870.
27. Ishikawa K, Yoshida S, Kadota K, et al. Gene expression profile of hyperoxic and hypoxic retinas in a mouse model of oxygen-induced retinopathy. *Invest Ophthalmol Vis Sci.* 2010;51:4307-4319.
28. Al-Shabrawey M, Mussell R, Kahook K, et al. Increased expression and activity of 12-lipoxygenase in oxygen-induced ischemic retinopathy and proliferative diabetic retinopathy: implications in retinal neovascularization. *Diabetes.* 2011;60:614-624.
29. Chen J, Connor KM, Aderman CM, Smith LE. Erythropoietin deficiency decreases vascular stability in mice. *J Clin Invest.* 2008;118:526-533.
30. Gardiner TA, Gibson DS, de Gooyer TE, de la Cruz VE, McDonald DM, Stitt AW. Inhibition of tumor necrosis factor-alpha improves physiological angiogenesis and reduces pathological neovascularization in ischemic retinopathy. *Am J Pathol.* 2005;166:637-644.
31. Park K, Chen Y, Hu Y, et al. Nanoparticle-mediated expression of an angiogenic inhibitor ameliorates ischemia-induced retinal neovascularization and diabetes-induced retinal vascular leakage. *Diabetes.* 2009;58:1902-1913.
32. Avery RL, Pearlman J, Pieramici DJ, et al. Intravitreal bevacizumab (Avastin) in the treatment of proliferative diabetic retinopathy. *Ophthalmology.* 2006;113:e1691-e1615.
33. Spaide RF, Fisher YL. Intravitreal bevacizumab (Avastin) treatment of proliferative diabetic retinopathy complicated by vitreous hemorrhage. *Retina.* 2006;26:275-278.
34. Aiello LP, Pierce EA, Foley ED, et al. Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. *Proc Natl Acad Sci U S A.* 1995;92:10457-10461.
35. Dai L, Gu L, Ding C, Qiu L, Di W. TWEAK promotes ovarian cancer cell metastasis via NF-kappaB pathway activation and VEGF expression. *Cancer Lett.* 2009;283:159-167.
36. Xi B, Yu N, Wang X, Xu X, Abassi YA. The application of cell-based label-free technology in drug discovery. *Biotechnol J.* 2008;3:484-495.
37. Glamann J, Hansen AJ. Dynamic detection of natural killer cell-mediated cytotoxicity and cell adhesion by electrical impedance measurements. *Assay Drug Dev Technol.* 2006;4:555-563.
38. Liu Q, Wang HG. Anti-cancer drug discovery and development: Bcl-2 family small molecule inhibitors. *Commun Integr Biol.* 2012;5:557-565.
39. Tran NL, McDonough WS, Savitch BA, et al. Increased fibroblast growth factor-inducible 14 expression levels promote glioma cell invasion via Rac1 and nuclear factor-kappaB and correlate with poor patient outcome. *Cancer Res.* 2006;66:9535-9542.
40. Demircan N, Safran BG, Soylu M, Ozcan AA, Sizmaz S. Determination of vitreous interleukin-1 (IL-1) and tumour necrosis factor (TNF) levels in proliferative diabetic retinopathy. *Eye (Lond).* 2006;20:1366-1369.

41. Munoz-Garcia B, Martin-Ventura JL, Martinez E, et al. Fn14 is upregulated in cytokine-stimulated vascular smooth muscle cells and is expressed in human carotid atherosclerotic plaques: modulation by atorvastatin. *Stroke*. 2006;37:2044-2053.
42. Fong DS, Girach A, Boney A. Visual side effects of successful scatter laser photocoagulation surgery for proliferative diabetic retinopathy: a literature review. *Retina*. 2007;27:816-824.
43. Burkly LC, Michaelson JS, Hahm K, Jakubowski A, Zheng TS. TWEAKing tissue remodeling by a multifunctional cytokine: role of TWEAK/Fn14 pathway in health and disease. *Cytokine*. 2007;40:1-16.
44. Saitoh T, Nakayama M, Nakano H, Yagita H, Yamamoto N, Yamaoka S. TWEAK induces NF-kappaB2 p100 processing and long lasting NF-kappaB activation. *J Biol Chem*. 2003;278:36005-36012.
45. Luqman S, Pezzuto JM. NFkappaB: a promising target for natural products in cancer chemoprevention. *Phytother Res*. 2010;24:949-963.
46. Vogler M. BCL2A1: the underdog in the BCL2 family. *Cell Death Differ*. 2012;19:67-74.
47. Saas P, Boucraut J, Walker PR, et al. TWEAK stimulation of astrocytes and the proinflammatory consequences. *Glia*. 2000;32:102-107.
48. Li H, Mittal A, Paul PK, et al. Tumor necrosis factor-related weak inducer of apoptosis augments matrix metalloproteinase 9 (MMP-9) production in skeletal muscle through the activation of nuclear factor-kappaB-inducing kinase and p38 mitogen-activated protein kinase: a potential role of MMP-9 in myopathy. *J Biol Chem*. 2009;284:4439-4450.
49. Wako M, Ohba T, Ando T, et al. Mechanism of signal transduction in tumor necrosis factor-like weak inducer of apoptosis-induced matrix degradation by MMP-3 upregulation in disc tissues. *Spine (Phila Pa 1976)*. 2008;33:2489-2494.
50. Vendrell J, Maymo-Masip E, Tinahones F, et al. Tumor necrosis-like weak inducer of apoptosis as a proinflammatory cytokine in human adipocyte cells: up-regulation in severe obesity is mediated by inflammation but not hypoxia. *J Clin Endocrinol Metab*. 2010;95:2983-2992.
51. Fish JE, Marsden PA. Endothelial nitric oxide synthase: insight into cell-specific gene regulation in the vascular endothelium. *Cell Mol Life Sci*. 2006;63:144-162.
52. Dodelet VC, Pasquale EB. Eph receptors and ephrin ligands: embryogenesis to tumorigenesis. *Oncogene*. 2000;19:5614-5619.
53. Mosch B, Reissenweber B, Neuber C, Pietzsch J. Eph receptors and ephrin ligands: important players in angiogenesis and tumor angiogenesis. *J Oncol*. 2010;2010:135285.