Thrombospondin-2 Expression During Retinal Vascular Development and Neovascularization

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Abstract

Purpose: To determine thrombospondin-2 (TSP2) expression and its impact on postnatal retinal vascular development and retinal neovascularization.

Methods: The TSP2-deficient (TSP2^{-/-}) mice and a line of TSP2 reporter mice were used to assess the expression of TSP2 during postnatal retinal vascular development and neovascularization. The postnatal retinal vascularization was evaluated using immunostaining of wholemount retinas prepared at different postnatal days by collagen IV staining and/or TSP2 promoter driven green fluorescent protein (GFP) expression. The organization of astrocytes was evaluated by glial fibrillary acidic protein (GFAP) staining. Retinal vascular densities were determined using trypsin digestion preparation of wholemount retinas at 3- and 6-weeks of age. Retinal neovascularization was assessed during the oxygen-induced ischemic retinopathy (OIR). Choroidal neovascularization (CNV) was assessed using laser-induced CNV.

Results: Using the TSP2-GFP reporter mice, we observed significant expression of TSP2 mRNA in retinas of postnatal day 5 (P5) mice, which increased by P7 and remained high up to P42. Similar results were observed in retinal wholemount preparations, and western blotting for GFP with the highest level of GFP was observed at P21. In contrast to high level of mRNA at P42, the GFP fluorescence or protein level was dramatically downregulated. The primary retinal vasculature developed at a faster rate in TSP2^{-/-} mice compared with TSP2^{+/+} mice up to P5. However, the developing retinal vasculature in TSP2^{+/+} mice caught up with that of TSP2^{-/-} mice after P7. No significant differences in retinal vascular density were observed at 3- or 6-weeks of age. TSP2^{-/-} mice also exhibited a similar sensitivity to the hyperoxia-mediated vessel obliteration and similar level of neovascularization during OIR as TSP2^{+/+} mice. Lack of TSP2 expression minimally affected laser-induced CNV compared with TSP2^{+/+} mice.

Conclusions: Lack of TSP2 expression was associated with enhanced retinal vascularization during early postnatal days but not at late postnatal times, and minimally affected retinal and CNV. However, the utility of TSP2 as a potential therapeutic target for inhibition of ocular neovascularization awaits further investigation.

Introduction

THROMBOSPONDINS (TSPs) are a family of at least 5 secreted, matricellular glycoproteins (TSP1–5), with diverse biological functions.^{1–3} The first 2 members of the family, TSP1 and TSP2, are homotrimeric molecules in which each chain has a molecular mass of ~145 kDa. TSP1 and TSP2 share a high degree of similarity, but they are encoded by different genes.⁴ They exhibit differences in the structure, distribution, and their molecular interactions.

TSP1 has been extensively studied and has antiangiogenic activity. In contrast, TSP2 has not been as extensively studied but it is involved in modulation of cell adhesion, migration, and proliferation with a strong antiangiogenic activity.² Regulation of angiogenesis by TSP2 is due, in part, to modulation of matrix metalloproteinases (MMP) levels through its binding of MMP and promotion of their endocytosis.⁵

The phenotype of TSP2-null mice has been very helpful in providing clues to TSP2 functions. In addition to

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histological and functional abnormalities in connective tissues, these mice display an increased vascularity of the dermis and subdermal tissues, increased endosteal bone growth, a bleeding defect, and a marked adhesive defect of dermal fibroblasts.⁶ The increased vascular density, however, appears to be restricted to certain tissues, as brains from TSP2-null mice display no change in vascular density.⁷ However, the physiological role of TSP2 in vascular development and neovascularization requires further investigation.

The developing mouse retinal vasculature provides a unique opportunity to study all aspects of vascular development postnatally and is readily amenable to biochemical evaluations. Mice are born without any retinal blood vessels, which develop immediately after birth. During the first week of life, a superficial layer of vessels is formed, which sprouts perpendicularly deep into the retina forming the deep and intermediate retinal vascular plexuses by the third week of postnatal life. Retinal vasculature continues to undergo pruning and remodeling during the next 3 weeks, and a complete vasculature is established by 6 weeks of age.^{8–10} Although it was previously reported that TSP2 is not expressed in mouse eyes,¹¹ we have shown that the retinal endothelial cells (EC) and pericytes (PC) express TSP2.^{12,13} Thus, TSP2 may play a significant role in the development of retinal vasculature and neovascularization. We have shown mice lacking TSP1 exhibit increased retinal vascular density due to failure in proper remodeling and pruning at late stages of retinal vascularization.¹⁰

Oxygen-induced ischemic retinopathy (OIR) in the mouse is a highly reproducible model of angiogenesis *in vivo* and recapitulates the human condition retinopathy of prematurity, which occurs in babies born prematurely.¹⁴ In this model, postnatal day 7 (P7) mice are exposed to 75% oxygen for 5 days and then brought to room air for 5 days, during which maximum retinal neovascularization occurs. We showed TSP1^{-/-} mice are protected from hyperoxia-mediated vessel obliteration, and exhibited less nonperfused area after exposure to high oxygen. However, the degree of retinal neovascularization was not significantly affected.¹⁰ In contrast, TSP1^{-/-} mice exhibited significantly higher degree of neovascularization during laser-induced choroidal neovascularization (CNV) compared with wild-type mice.¹⁵ How TSP2 expression impacts retinal vascular homeostasis remains unexplored.

To gain further insight into the physiological role TSP2 plays during vascular development and neovascularization, we compared the normal postnatal development of retinal vasculature, retinal neovascularization during OIR, and laser-induced CNV in TSP2^{+/+} and TSP2^{-/-} mice. In addition, using a line of TSP2-GFP reporter mice we determined the changes in expression of TSP2 in retinal vasculature during development and neovascularization. Here, we demonstrate that the primary retinal vasculature develops at a faster rate in TSP2^{-/-} mice compared with TSP2^{+/+} mice in early stages of retinal vascularization. However, the progression of retinal vasculature in TSP2^{+/+} catches up with that of TSP2^{-/-} mice with no significant differences in the degree of vascularization in adult mice. The regression of the hyaloid vessels also occurred similarly in TSP2^{-/-} compared with TSP2^{+/+} mice. These mice had similar sensitivity to the hyperoxia-mediated vessel obliteration and exhibited a similar level of neovascularization

during room air exposure. Although lack of TSP1 is associated with increased CNV,¹⁵ lack of TSP2 expression minimally affected laser-induced CNV in adult mice. Thus, TSP2 shows an effect in the very early stages of normal retinal vascular development, but it may play a lesser important role in the later stages of vascular development, vascular remodeling, and neovascularization.

Methods

Tissue preparation

All animal studies were carried out in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care Committee of the University of Wisconsin-Madison School of Medicine and Public Health. The generation of TSP2^{-/-} mice was previously described and back crossed to C57BL/6J strain.¹⁶ The screening primers used for genotyping were as follows: TSP2 KO forward: 5'-GTG GGGGTGGGGTGGGA TTAGATA-3'; TSP2 WT forward: 5'-ACACATGTGCACC AC ACTTC-3'; and TSP2 common reverse: 5'-AAACCCC AAGAAAGCCATTAGAA-3'. The generation of a line of TSP2 reporter transgenic mice, where endogenous TSP2 promoter drives expression of green fluorescent protein (GFP), was previously described.¹⁷ The following primers were used for screening of the transgene: GFP forward: 5'-CCTACGGC GTGCAGT GCTTCAGC-3'; and GFP reverse: 5'-CGGCG AGCTGCACGCTGCGTCCTC-3'.

Mice were bred for different experimental time points. For OIR, 7-day-old (P7) pups and their mother were placed in an airtight incubator and exposed to an atmosphere of 75% ± 0.5% oxygen for 5 days. Incubator temperature was maintained at 23°C ± 2°C, and oxygen was continuously monitored with a PROOX model 110 oxygen controller (Reming Bioinstruments Co.). Mice were brought to room air for 5 days, and then pups were sacrificed for retinal wholemount preparations and analysis of neovascularization as described below.

RNA purification and quantitative polymerase chain reaction

The total retina RNA from C57BL/6J TSP2^{+/+} mice was extracted by mirVana PARIS kit (Ambion) according to the manufacturer's instructions. The cDNA synthesis was performed from 1 µg of total RNA using Sprint RT Complete-Double PrePrimed kit from (Clontech). Each cDNA (1 µL; diluted 1:10) was used as template in quantitative polymerase chain reaction (qPCR) assays, performed in triplicate of 3 biological replicates on Mastercycler Realplex (Eppendorf) using the SYBR qPCR Premix (Clontech). Amplification parameters were as follows: 95°C for 2 min; 40 cycles of amplification (95°C for 15 s, 60°C for 40 s); dissociation curve step (95°C for 15 s, 60°C for 15 s, 95°C for 15 s).

Standard curves were generated from known quantities for each target gene of linearized plasmid DNA. Ten times dilution series were used for each known target, which were amplified using SYBR-Green qPCR. The linear regression line for ng of DNA was determined from relative fluorescent units (RFU) at a threshold fluorescence value (Ct) to quantify gene targets from cell extracts by comparing the RFU at the Ct to the standard curve, normalized by the simultaneous

amplification of Calnexin, which was used as a housekeeping gene to normalize all samples. The primer sequences were Calnexin NM_007597.3: Calnexin forward 5'-GCTGCTGG TCCTTGGAAGT-3', Calnexin reverse 5'-TCAATATCAA TCGCGTCATCATC-3', TSP2 NM_011581.3: TSP2 forward 5'-CCCCAAAGTCCAAATTCC-3', and TSP2 reverse 5'-TCGTCACAAGCATCT CCGATT-3', and TSP1 NM_011580: TSP1 forward 5'-TGGCCAGCGTTGCCA-3' and TSP1 reverse 5'-TCTGCAGCACCCCTGAA-3'.

Visualization of GFP expression in the retina of TSP2-GFP transgenic mice during normal development and OIR

At various times during normal development and OIR TSP2-GFP transgenic mouse eyes were enucleated and briefly fixed in 4% paraformaldehyde (10 min on ice). Retinas were dissected in phosphate-buffered saline (PBS; Sigma) and mounted on a slide with PBS/glycerol (2 vol/1 vol). Retinas were viewed by fluorescence microscopy and images were captured in digital format using a Zeiss microscope (Carl Zeiss).

Western blot analysis

GFP protein levels were determined by western blotting of whole retina extracts prepared from P7, P14, P21, P42, and OIR P12, P15, P17 TSP2-GFP transgenic mice [OIR P15, when vascular endothelial growth factor (VEGF) is maximally expressed].¹⁸ Eyes were dissected, homogenized in RIPA buffer (10 mM HEPES pH 7.6, 142.5 mM KCl, 1% NP-40, and protease inhibitor cocktail; Roche), sonicated briefly, and incubated at 4°C for 20 min. Homogenates were centrifuged at 4°C for 30 min (15,000 g) to remove insoluble material. Clear supernatants were transferred to clean tubes and protein concentrations were determined using DC protein assay (Bio-Rad). Approximately 50 µg of protein lysate was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 4%–20%; Tris–Glycine gel; Invitrogen) under reducing conditions and transferred to a nitrocellulose membrane. The blot was incubated with an anti-GFP antibody (1:2,000; Millipore), washed, and developed using an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000; Jackson Immunoresearch Laboratories) and ECL system (Amersham Biosciences). The same blot was also probed with a monoclonal antibody to β-actin (1:5,000; Sigma) to verify equal protein loading in all lanes. Data were analyzed by ImageJ for quantification.

Trypsin-digested retinal vessel preparations

Eyes were enucleated from P21 or P42 mice and fixed in 4% paraformaldehyde for at least 24 h. The eyes were bisected equatorially and the entire retina was removed under the dissecting microscope. Retinas were washed overnight in distilled water and incubated in 3% trypsin (trypsin 1:250; Difco) prepared in 0.1 M Tris, 0.1 M maleic acid, pH 7.8 containing 0.2 M NaF for ~1–1.5 h at 37°C. Following completion of digestion, retinal vessels were flattened by 4 radial cuts and mounted on glass slides for periodic acid-Schiff (PAS) and hematoxylin staining. Nuclear morphology was used to distinguish PC from EC. The nuclei of EC are oval or elongated and lie within the vessel wall along the axis of the capillary, while pericyte nuclei are small,

spherical, stain densely, and generally have a protuberant position on the capillary wall. The stained and intact retinal wholemounts were coded, and subsequent counting was performed masked. The numbers of EC and pericytes were determined by counting respective nuclei under the microscope at a magnification of 400×. A mounting reticle (10×10 µm) was placed in one of the viewing oculars to facilitate counting. Only retinal capillaries were included in the cell count, which was performed in the mid-zone of the retina. We counted the number of EC and pericytes in 4 reticles from the 4 quadrants of each retina. The total number of EC and pericytes for each retina was determined by adding the numbers from the 4 reticles. The ratio of EC to pericytes was then calculated. To evaluate the density of cells in the capillaries, the mean number of EC or pericytes was recorded in 4 reticles from each of the 4 quadrants of each retina.

Visualization of retinal vasculature and quantification of the rate of vascular expansion and avascular areas during OIR

The retinal vascular pattern and vessel obliteration were analyzed using retinal wholemounts stained with anti-collagen IV antibody. At various times mouse eyes were enucleated and briefly fixed in 4% paraformaldehyde (4 min on ice). The eyes were fixed in methanol for at least 24 h at –20°C. Retinas were dissected in PBS (Sigma) and then washed with PBS 3 times, 10 min each. Following incubation in blocking buffer (50% fetal calf serum and 20% normal goat serum in PBS) for 1 h, the retinas were incubated with rabbit anti-mouse collagen IV antibody (diluted 1:500 in PBS containing 20% fetal calf serum and 20% normal goat serum; Millipore) at 4°C overnight. Retinas were then washed 3 times with PBS, 10 min each, and incubated with secondary antibody Alexa Fluor 594 goat-anti-rabbit (diluted 1:500 in PBS containing 20% fetal calf serum and 20% normal goat serum; Invitrogen) for 2 h at room temperature, washed 3 times with PBS, 10 min each, and mounted on a slide with PBS/glycerol (2 vol/1 vol). Retinas were viewed by fluorescence microscopy and images were captured in digital format using a Zeiss microscope (Carl Zeiss). The retinal wholemounts were also stained with antibodies to GFAP (1:500 dilution; Dako), and FITC-conjugated B4-lectin (1:100 dilution; Sigma) to visualize astrocytes, and retinal vasculature, respectively. The relative rate of retinal vascular expansion was determined by measuring the radius of expanding retinal blood vessels relative to the radius of the retina from the optic nerve head in P5 and P7 TSP2^{+/+} and TSP2^{-/-} mice. The central capillary dropout area during OIR was quantified as percentage of whole retina area. The quantitative assessments were made from the digital images in masked fashion using Axiovision software (Carl Zeiss).

Quantification of neovascular proliferative retinopathy

Quantification of vitreous neovascularization on P17 was performed as previously described.¹⁰ Briefly, mouse eyes were enucleated, fixed in formalin for 24 h, and embedded in paraffin. Serial sections (6 µm thick), each separated by at least 40 µm, were taken from around the region of the optic nerve. The hematoxylin- and PAS-stained

sections were examined in masked fashion for the presence of neovascular tufts projecting into the vitreous from the retina. The neovascular score was defined as the mean number of neovascular nuclei per section found in 8 sections (4 on each side of the optic nerve) per eye.

BrdU and collagen IV staining of wholemount retinas

The detection of cell proliferation on the retinal blood vessels was assessed by immunohistochemistry for 5-bromo-2-deoxyuridine (BrdU) incorporation and type IV collagen (staining blood vessels). Mice were intraperitoneally injected with BrdU (Sigma) 0.12 g/kg of body mass dissolved in water. Mice were sacrificed 1.5 h later; eyes were removed and fixed immediately in 4% paraformaldehyde for 4 min on ice. The eyes were then transferred to methanol and stored at -20°C for at least 24 h. Retinas were dissected in PBS, washed in PBS, and stained with anti-collagen IV antibody as described above for staining of wholemount retinas. Retinas were then washed for 30 min in PBS containing 1% Triton X-100 to permeabilize cell membranes, placed in 2 M HCl at 37°C for 1 h, washed in 0.1 M sodium borate for 30 min to neutralize the HCl, washed in PBS containing 1% Triton X-100 for 30 min, and incubated with a monoclonal antibody to BrdU [diluted 1:250 in PBS containing 1% bovine serum albumin (BSA); Roche] at 4°C overnight. Following incubation, the retinas were washed for 30 min in PBS containing 1% Triton X-100 and incubated with anti-mouse CY2 antibody (diluted 1:250 in PBS containing 1% BSA; Jackson ImmunoResearch Laboratories) at room temperature for 2 h. After the final wash in PBS for 30 min, the retinas were mounted on a slide with PBS/glycerol (2 vol/1 vol), viewed by fluorescence microscopy, and images were captured in digital format using a Zeiss microscope (Carl Zeiss). For quantitative assessment of the data the mean number of BrdU⁺ nuclei on the blood vessels was determined per retina.

TdT-dUTP terminal nick-end labeling of retinal vasculature

Apoptotic cell death on the retinal vasculature was assessed by TdT-dUTP terminal nick-end labeling (TUNEL) staining on trypsin-digested retinal vessels. The eyes were removed, fixed in 10% neutral formalin at 4°C for 48 h, bisected equatorially, and the entire retina was removed under the dissecting microscope. Retinas were washed in distilled water for 1 h and incubated in 3% trypsin (trypsin 1:250; Difco) prepared in 0.1 M Tris, 0.1 M maleic acid, pH 7.8 containing 0.2 M NaF for ~ 40 min at 37°C . Following completion of digestion, retinal vessels were flattened by 4 radial cuts, placed on a poly-L-lysine-coated glass slide, and allowed to dry. The retinal vessels were then rehydrated in PBS for 10 min, twice, permeabilized with PBS containing 0.5% Triton X-100 for 1 h at room temperature, and washed in PBS twice for 10 min each. The TUNEL staining was performed using the Click-iT[®] TUNEL Alexa Fluor[®] Imaging Assay kit as recommended by the supplier (Invitrogen). The retinal vessels were counterstained with Hoechst 33342 (included in the Click-iT TUNEL Alexa Fluor Imaging Assay kit) for nuclei staining. Retinal vessels were viewed by fluorescence microscopy and images were captured in digital format using a Zeiss microscope (Carl Zeiss). For

quantitative assessment of the data the mean number of TUNEL-positive cells was determined per retina.

Immunohistochemical staining of the frozen sections

Mouse eyes were enucleated and embedded in optimal cutting temperature compound at -80°C . Sections ($9\ \mu\text{m}$) were cut on a cryostat, placed on glass slides, and allowed to dry for 2 h. For fluorescence microscopy, sections were fixed in cold acetone (4°C) on ice for 10 min, followed by 3 washes with PBS, 5 min each. Sections were incubated in blocker (1% BSA, 0.2% skim milk, and 0.3% Triton X-100 in PBS) for 15 min at room temperature. Sections were then incubated with rabbit anti-mouse type IV collagen antibody (Millipore) overnight at 4°C in a humid environment. After 3 washes in PBS, 5 min each, sections were incubated with secondary antibody Alexa 594 goat-anti rabbit (1:500 dilution prepared in blocking solution; Invitrogen). Sections were washed 3 times in PBS, covered with PBS:glycerol (2 vol/1 vol), and mounted with a coverslip. Retina sections were viewed by fluorescence microscopy, and images were captured in digital format using a Zeiss microscope (Carl Zeiss).

Staining of the hyaloid vasculature

Following the removal of eyes, the sclera, choroids, and retina were dissected anteriorly from the optic nerve to limbus. The remaining wholemount specimen was stained with FITC-conjugated B4-lectin (1:100 dilution; Sigma) to visualize the hyaloid vasculature. Wholemount specimens were viewed by fluorescence microscopy and images were captured in digital format using a Zeiss microscope (Carl Zeiss). The dissection resulted, in most cases, in the loss of hyaloid artery and vasa hyaloidea propria vessels. However, the tunica vasculosa lentis vessels were clearly visible following lectin staining.

Laser-induced CNV

CNV was induced in TSP2^{+/+} and TSP2^{-/-} mice (6 weeks old, female) by laser photocoagulation-induced rupture of Bruch's membrane on day 0. Mice were anesthetized with ketamine hydrochloride (100 mg/kg) and pupils were dilated. Laser photocoagulation ($75\ \mu\text{m}$ spot size, 0.1 s duration, 120 mW) was performed in the 9, 12, and 3 o'clock positions of the posterior pole of each eye with the slit lamp delivery system of an Oculight GL diode laser (Iridex) and a handheld cover slip as a contact lens to view the retina. After 14 days, the eyes were removed and fixed in 4% paraformaldehyde at 4°C for 2 h. Following 3 washes in PBS, the eyes were sectioned at the equator, and the anterior half, vitreous, and retinas were removed. The remaining eye tissue was incubated in blocking buffer (5% fetal calf serum and 20% normal goat serum in PBS) for 1 h at room temperature, followed by incubation with anti-ICAM-2 (1:500 in PBS containing 20% fetal calf serum, 20% normal goat serum; BD Biosciences) overnight at 4°C . The remaining eye tissue was then washed 3 times with PBS and incubated with the appropriate secondary antibody. The retinal pigment epithelium-choroid-sclera complex was dissected through 5 to 6 relaxing radial incisions and flat mounted on a slide with VectaMount[™] AQ (Vector Laboratories, Inc.). The remaining eye tissue was viewed by fluorescence

microscopy and images were captured in digital format using a Zeiss microscope (Zeiss). ImageJ software (National Institute of Mental Health, Bethesda, MD; <http://rsb.info.nih.gov/ij>) was used to measure the total area (in μm^2) of CNV associated with each burn.

Statistical analysis

Statistical differences between groups were evaluated with Student's unpaired *t*-test (2-tailed). Mean $\pm$ standard error is shown. *P*-values of <0.05 are considered significant.

Results

Expression of TSP2 during postnatal development of retinal vasculature

Although a previous study suggested lack of TSP2 expression in mouse retina,¹¹ our studies have shown that TSP2 is expressed in the mouse retina, especially in the vascular cells. Retinal pericytes are the major source of TSP2 in the retinal vasculature or in culture.^{12,17} We performed qPCR to detect TSP2 RNA expression in the retina at critical time points of postnatal retinal vascularization. We detected considerable amounts of TSP2 RNA in retina samples from the wild-type mice. The TSP2 RNA level was

low at P5, and increased in subsequent postnatal days reaching a constant level (Fig. 1A).

To know whether the TSP2 protein is produced in the retina during normal postnatal vascularization, we prepared retinal wholemounts from TSP2-GFP reporter mice to look at the GFP expression and distribution at different time points (P7, P14, P21, and P42). The green fluorescence showed significant vascular staining, which increased during the postnatal vascular development and was almost undetectable at the late time point (P42) (Fig. 1B). We also examined GFP levels by western blot analysis of the retinal lysates prepared from the TSP2-GFP transgenic mice at different postnatal days. A similar result to that detected in the retinal wholemount preparations was observed. The GFP protein level increased in P7, P14, and P21 compared with P5, but was almost undetectable at P42 (Fig. 1C, D). Thus, TSP2 expression is detectable in developing retinal vasculature.

Lack of TSP2 minimally impacted retinal vascular density

To compare retinal vascular density in TSP2^{+/+} and TSP2^{-/-} mice, we prepared retinal trypsin digests at different postnatal days and determined endothelial cell and pericyte ratios and their densities. In wholemount retinal trypsin digests, the nuclei of EC are oval or elongated and

FIG. 1. TSP2 RNA expression in retinas from TSP2^{+/+} mice and GFP protein expression in the TSP2-GFP transgenic mice retina during normal postnatal retinal vascular development. (A) Shows qPCR analysis of TSP2 expression in retinas prepared from TSP2^{+/+} mice at different postnatal days. TSP2 RNA level was low at P5, but significantly increased and remained high at other postnatal time points (**P* < 0.01; *n* = 3). (B) Shows retinal wholemounts prepared from TSP2-GFP reporter mice at different postnatal days. All images were captured under identical conditions. The intensity of the green fluorescence on the vasculature increased during postnatal development of retinal vasculature, but it was undetectable at P42. Experiments were repeated with eyes from 3 mice with similar results. (C, D) Shows the western blot analysis of GFP protein in retina lysates prepared from the TSP2-GFP reporter mice at indicated postnatal days. β -Actin expression was assessed as a loading control. Please note GFP level increased at P7, P14, and P21, but expression of green fluorescent protein (GFP) was undetectable at P42. These experiments were repeated with eyes from at least 3 mice with similar results. qPCR, quantitative polymerase chain reaction; TSP, thrombospondin. Color images available online at www.liebertpub.com/jop

FIG. 2. Lack of TSP2 minimally affected the retinal endothelial cell and pericyte density. Retinas were obtained from P21 and P42 TSP2^{+/+} (A, C) or TSP2^{-/-} mice (B, D) and subjected to whole-mount trypsin digest. The *arrows* indicate endothelial cells, while *arrowheads* show the pericytes lining the retinal capillaries. Please note the similar number of capillary loops in both P21 and P42 TSP2^{+/+} and TSP2^{-/-} retina. The quantitative assessment of the data is shown in Tables 1–3.

lie within the vessel wall along the axis of the capillary, while PC nuclei are small, spherical, stain densely, and generally have a protuberant position on the capillary wall. Figure 2 shows retinal vessels prepared from P21 and P42 TSP2^{+/+} and TSP2^{-/-} mice. The retinal vasculature from TSP2^{+/+} and TSP2^{-/-} mice exhibited similar cellularity and capillary organization, suggesting there were no significant difference in retinal vascular density between P21 and P42.

Table 1 shows the mean EC and PC ratios (EC/PC) of retinal microvessels from TSP2^{+/+} and TSP2^{-/-} mice. At P21, there were no significant differences between the mean EC/PC ratio for TSP2^{+/+} and TSP2^{-/-} mice. The EC/PC ratio at P42, when the vascular remodeling and maturation is complete, was decreased to 2.3:1 in both TSP2^{+/+} and TSP2^{-/-}. Tables 2 and 3 show the mean density of retinal EC and PC from TSP2^{+/+} and TSP2^{-/-} mice. There were no significant differences between the mean EC and PC density at P21 and P42. Thus, the lack of TSP2 did not significantly affect retinal vascular density.

The primary retinal vasculature developed at a faster rate in TSP2^{-/-} mice

The murine retinal vasculature develops postnatally. A superficial layer of vessels is formed during the first week of postnatal life (P7). After that, the deep vascular plexuses begin to form at the outer edge of the inner nuclear layer.

This is followed by the formation of an intermediate vascular plexus between the primary and the deep vascular layer during the third postnatal week. The course of retinal vessel sprouting and assembly can be readily visualized by collagen IV staining, a major component of the basement membrane of retinal vessels, immunostaining of whole-mount retinal preparations and/or eye frozen sections. We compared retinal vascular development by collagen IV staining of wholemount retinas prepared from mice during the first week of postnatal life (P5 and P7).

Figure 3A shows the first week of the developing retinal vessels in TSP2^{+/+} and TSP2^{-/-} mice. Blood vessels originate from the center of the retina near the optic nerve, and spread toward the retinal periphery. We measured the distance that the retinal vessels had spread from the optic nerve head toward the retinal periphery relative to the whole retina radius (Fig. 3C). At P5, retinal blood vessels expanded at a significantly faster rate toward the retinal periphery in TSP2^{-/-} mice compared with TSP2^{+/+} mice ($P < 0.05$). However, at P7, the retinal blood vessel expansion in TSP2^{+/+} mice caught up with the TSP2^{-/-} mice. The retinal blood vessel expansion in TSP2^{+/+} mice at P7 was even faster than TSP2^{-/-} mice (Fig. 3C, D, F; $P < 0.05$). The secondary branching of the retinal vessels and their diameters were similar in TSP2^{-/-} and TSP2^{+/+} mice.

We also compared vessel sprouting and penetration into the deep retina after P7. Figure 3E and G shows retinal vessels in the frozen eye section from P10 and P42 TSP2^{+/+}

TABLE 1. ENDOTHELIAL CELLS/PERICYTES RATIOS IN TSP2^{+/+} AND TSP2^{-/-} MICE DIVIDED ACCORDING TO DIFFERENT AGE (MEAN $\pm$ SD)

Age, days	TSP2 ^{+/+}	TSP2 ^{-/-}
21	2.68 $\pm$ 0.20 (8)	2.48 $\pm$ 0.18 (8)
42	2.34 $\pm$ 0.20 (8)	2.34 $\pm$ 0.27 (8)

The number of retinas (mice) counted is given in parentheses. SD, standard deviation; TSP, thrombospondin.

TABLE 2. NUMBER OF ENDOTHELIAL CELLS PER RETICLE SQUARE (100 μ m²) IN TSP2^{+/+} AND TSP2^{-/-} MICE DIVIDED ACCORDING TO AGE (DENSITY; MEAN $\pm$ SD)

Age, days	TSP2 ^{+/+}	TSP2 ^{-/-}
21	154.8 $\pm$ 6.51 (8)	153.59 $\pm$ 2.78 (8)
42	142.38 $\pm$ 6.12 (8)	141.56 $\pm$ 4.23 (8)

The number of retinas (mice) counted is given in parentheses.

TABLE 3. NUMBER OF PERICYTES PER RETICLE SQUARE ($100\mu\text{m}^2$) IN TSP2^{+/+} AND TSP2^{-/-} MICE DIVIDED ACCORDING TO AGE (DENSITY; MEAN $\pm$ SD)

Age, days	TSP2 ^{+/+}	TSP2 ^{-/-}
21	58.13 $\pm$ 5.53 (8)	62.16 $\pm$ 3.60 (8)
42	61.06 $\pm$ 4.78 (8)	61.25 $\pm$ 7.01 (8)

The number of retinas (mice) counted is given in parentheses.

and TSP2^{-/-} mice. There were no significant differences in retinal vessel development in P10 and P42 TSP2^{-/-} mice compared with TSP2^{+/+} mice.

At P5, the superficial layer of retinal vessels has not reached the periphery, thus, allowing the evaluation of retinal vessels progression and interactions with astrocytes. Normally, astrocytes have already reached the periphery ahead of the developing vasculature at this stage. FITC-conjugated B4-lectin and antibody to GFAP were used to visualize the developing retinal vasculature and its association with the scaffolding laid down by the astrocytes. We observed no significant difference in spreading pattern of astrocytes ahead of the developing retinal vasculature in P5 TSP2^{-/-} mice compared with TSP2^{+/+} mice (Fig. 4A, B). FITC-conjugated B4-lectin staining showed similar organization of tip cells and stalk behavior in expanding retinal vasculature with and without TSP2 (Fig. 4C, D). Figure 4E and F show TSP2^{+/+} and TSP2^{-/-} EC similarly following the scaffolding laid down by astrocytes. Furthermore, the mean number of tip cell filopodias per 1 mm vessel length was similar as that of TSP2^{+/+} mice (Fig. 4G; $P > 0.05$). Thus, lack of TSP2 minimally affected the spreading of astrocytes to the retina periphery, and it had no effect on the spreading of the endothelial tip cells.

TSP2 deficiency minimally affected the rate of proliferation and apoptosis in retinal vasculature

Retinal vessel development depends on the balance between retinal vascular cell proliferation and apoptosis. We used BrdU incorporation in retinal vascular cells to determine the rate of proliferation of cells in the developing retinal vasculature in TSP2^{+/+} and TSP2^{-/-} mice. TSP2^{+/+} and TSP2^{-/-} mice at P14 were injected with BrdU, and labeled cells in retinal vascular wholemount were detected by staining with anti-BrdU and collagen IV antibody.

Figure 5A–D shows double staining of P14 TSP2^{+/+} and TSP2^{-/-} mice retinal vasculature with anti-BrdU and collagen IV antibody. Figure 5E shows the mean number of proliferating cells in the retinal vessels of TSP2^{+/+} and TSP2^{-/-} mice at P14, when active vascular cell proliferation is normally occurring.¹⁹ There were no significant difference between TSP2^{+/+} and TSP2^{-/-} mice.

We also determined the rate of apoptosis in the retinal vasculature of TSP2^{+/+} and TSP2^{-/-} mice by TUNEL staining of wholemount retinal trypsin digests. This allowed identification of the apoptotic cells as vascular cells that are associated with the retinal blood vessels. Figure 6 shows there were no significant difference in the rates of apoptosis observed in the retinal vasculature of P14 TSP2^{-/-} mice (Fig. 6B, D) compared to TSP2^{+/+} mice (Fig. 6A, C). The quantitative comparisons are shown in Fig. 6E. Thus, TSP2

FIG. 3. The superficial retinal vasculature expands at a faster rate in TSP2^{-/-} mice. Collagen IV wholemount staining of retinas from P5 and P7 TSP2^{+/+} (A, C) and TSP2^{-/-} (B, D) are shown. The quantitative assessment of the data is shown in (I). Please note that the spreading of retinal vessels was significantly faster at P5 in TSP2^{-/-} mice compared with TSP2^{+/+} mice, but it was caught up by the TSP2^{+/+} mice at P7 (* $P < 0.05$, ** $P < 0.01$; $n = 11$). Collagen IV staining of the frozen eye sections shows similar organization of the deep retinal vascular plexuses at P10 and P42 TSP2^{+/+} (E, G) and TSP2^{-/-} mice (F, H). These experiments were repeated with eyes from 5 different mice with similar results. Color images available online at www.liebertpub.com/jop

FIG. 4. Lack of TSP2 minimally affected astrocytic organization, formation of tip cells, and retinal vasculature expansion. Whole-mount retinas from TSP2^{+/+} (A, C, E) and TSP2^{-/-} (B, D, F) P5 mice were stained with antibodies to GFAP (A, B) or B4-lectin (C, D). The merged images are shown in (E, F). The quantitative assessment of tip cell's filopodia is shown in (G). Data in each bar are the mean number of filopodia per 1 mm vessel length counted in 4 retinas from 4 different mice. Please note similar organization and expansion of astrocytic template in TSP2^{+/+} and TSP2^{-/-}. The endothelial tip cells similarly followed the astrocytic template without a significant difference in the number of tip cell filopodia ($P > 0.05$; $n = 4$). GFAP, glial fibrillary acidic protein. Color images available online at www.liebertpub.com/jop

expression minimally affected the rate of proliferation and apoptosis of retinal vascular cells during postnatal retinal vascular development.

The development and regression of hyaloid vasculature was not affected in the TSP2^{-/-} mice

The pupillary membrane and hyaloid vessels (hyaloids arteries, tunica vasculosa lentis, and vasa hyaloidea propria)

provide nourishment in the immature lens, retina, and vitreous.²⁰ However, they regress during the later stages of ocular development. Figure 7 shows the FITC-conjugated B4-lectin staining of the vasculature in whole-mount ocular specimens prepared from TSP2^{+/+} and TSP2^{-/-} mice at P7 (Fig. 7A, B), P21 (Fig. 7C, D), and P42 (Fig. 7E, F). We observed a significant amount of hyaloid vessels in both P7 TSP2^{+/+} and TSP2^{-/-} mice, however, these vessels regressed similarly in TSP2^{+/+} and TSP2^{-/-} mice by 6

FIG. 5. Lack of TSP2 minimally affects the rate of retinal vascular cell proliferation. Proliferating cells in P14 TSP2^{+/+} (A, C) and TSP2^{-/-} (B, D) mice were labeled with BrdU and detected using an antibody to BrdU as described in the Methods section (green). Collagen IV staining was used to visualize the vasculature (red). Quantitative assessment of cell proliferation on the retinal vessels is shown in (E). Data in each bar are the mean number of BrdU⁺ cells per retina. Please note that the number of proliferating cells was similar in TSP2^{-/-} retina compared to TSP2^{+/+} retina ($P > 0.05$, $n = 6$). BrdU, 5-bromo-2-deoxyuridine. Color images available online at www.liebertpub.com/jop

weeks of age. Thus, TSP2 expression minimally affected the development and/or regression of hyaloid vasculature.

TSP2 expression during OIR

The mouse OIR is a highly reproducible model for studying all aspects of retinal angiogenesis. In this assay, P7 mice are exposed to 75% oxygen for 5 days, and then returned to room air for 5 days. The exposure of developing retinal vasculature to high oxygen prevents further development of retinal vessels and promoting obliteration of vessels in the center of the retina, resulting in loss of perfusion to this area. When animals are returned to room air, the retina becomes ischemic and promotes neovascularization, which results in reestablishment of a vasculature that is abnormal.

To determine whether TSP2 expression changes during OIR, we performed qPCR analysis of RNA prepared from

retinas at postnatal days 12–28. Compared with P7, the TSP2 RNA levels were increased 2-fold in P12, 1.6-fold in P15, and 2.65-fold in P17 during OIR. However, TSP2 RNA levels decreased to the steady state level at P28, when most of the newly formed vessels are regressing (Fig. 8A). Similar results were observed using retinal wholemount preparations from TSP2-GFP transgenic mice (Fig. 8B). The staining was diminished at P12 due to loss of blood vessels in response to high oxygen. However, staining became intensified at P15 and P17, when the neovascular sprouts were forming, consistent with increased TSP2 expression (Fig. 8B). Western blot analysis of retinal lysates showed the GFP expression in TSP2-GFP transgenic mice was similar at P12 and P17 during OIR, but less at P15, and decreased at P28 (Fig. 8C, D). Thus, TSP2 expression increased during active neovascularization and decreased with regression of newly formed vessels.

FIG. 6. Lack of TSP2 minimally affects the rate of retinal vascular cell apoptosis. TUNEL staining was used to assess the rate of apoptotic vascular cells in the trypsin-digested retinal vasculature from TSP2^{+/+} (A, C) and TSP2^{-/-} (B, D) at P14. (A, B) TUNEL-positive cells. (C, D) Merged images following Hoechst 33342 staining. The quantitative assessment of the data is shown in (E) ($P > 0.05$, $n = 7$). TUNEL, TdT-dUTP terminal nick-end labeling. Color images available online at www.liebertpub.com/jop

TSP2 deficiency minimally impacted vessel obliteration in response to hyperoxia during OIR

We next compared the response of TSP2^{+/+} and TSP2^{-/-} mice during OIR. As shown in Fig. 9A and B. The quantitative assessment of nonperfused areas in retinas from TSP2^{+/+} and TSP2^{-/-} mice is shown in Fig. 9C. We observed a similar degree of vessel obliteration in TSP2^{+/+} and TSP2^{-/-} mice after exposure to hyperoxia. Thus, lack of TSP2 did not significantly impact hyperoxia-mediated vessel obliteration during OIR.

Quantification of preretinal neovascularization

Quantification of preretinal neovascularization on P17 mice (when maximum neovascularization occurs) was performed as previously described by us.^{10,21} Briefly, 6- μ m-thick serial sections, each separated by at least 40 μ m, were obtained from the region around the optic nerve. The sec-

tions were stained with hematoxylin and PAS and examined in masked fashion for the presence of neovascular cell nuclei projecting into the vitreous from the retina (Fig. 10C, D). The neovascular cell nuclei score was defined as the mean number of neovascular nuclei per section found in 8 sections (4 on each side of the optic nerve) per eye. Figure 10E shows the mean number of vascular cell nuclei projecting into the vitreous of eyes from wild-type and TSP2^{-/-} mice. There were no significant differences in the mean number of neovascular cell nuclei detected in TSP2^{+/+} and TSP2^{-/-} OIR mice. Thus, lack of TSP2 expression minimally affected retinal neovascularization during OIR.

Lack of TSP2 minimally impacted CNV

The retina can undergo 2 types of neovascularization, retinal neovascularization that occurs during ROP and diabetic retinopathy and CNV that occurs in exudative AMD. Laser-induced CNV was performed to determine whether

FIG. 7. The assessment of hyaloid vasculature regression in TSP2^{+/+} and TSP2^{-/-} mice. FITC-conjugated B4-lectin whole-mount staining of the hyaloid vasculature from TSP2^{+/+} (A, C, E) and TSP2^{-/-} mice (B, D, F) at P7 (A, B), P21 (C, D), and P42 (E, F). Please note similar degree of vessel regression in TSP2^{+/+} and TSP2^{-/-} mice at all time points. These experiments were repeated with eyes from 4 mice with similar results. Color images available online at www.liebertpub.com/jop

FIG. 8. Expression of TSP2 in retinas during OIR. (A) Shows the qPCR analysis of TSP2 RNA level in TSP2^{+/+} mice during OIR. Please note that compared with P7, TSP2 RNA level was increased by 2-fold at P12, 1.6-fold at P15, and 2.65-fold at P17 time points during OIR (** $P < 0.05$; $n = 3$). The TSP2 RNA level decreased to near base line at P28. (B) Retinal wholemounts prepared from TSP2-GFP reporter mice at different time points (P12, P15, P17) during OIR. Please note that there was low level of green fluorescence in the retina when the vessels regressed at P12 (5 days of hyperoxia). However, the green fluorescence increased at P15 and P17 (during active neovascularization) and decreased as the new vessels began to regress. (C) Western blot analysis of GFP protein in retinas prepared from TSP2-GFP transgenic mice during OIR. β -Actin staining was assessed as loading control. (D) The relative intensity of the bands in western blots. Please note the GFP expression was similar at P12 and P17, but less at P15, and decreased at P28. These experiments were repeated with eyes from at least 3 mice with similar results. OIR, oxygen-induced ischemic retinopathy. Color images available online at www.liebertpub.com/jop

FIG. 9. Lack of TSP2 minimally affected retinal vascular sensitivity to hyperoxia-mediated vessel obliteration during OIR. P7 TSP2^{+/+} and TSP2^{-/-} mice were exposed to hyperoxia (75% oxygen) for 5 days (**A**, **B**). Wholemount collagen IV staining of retinal vasculature from P12 TSP2^{+/+} and TSP2^{-/-} mice during OIR was prepared as detailed in the Methods section. The quantitative assessment of the non-perfused area is shown in (**C**). These experiments were repeated with at least 5 mice of each genotype in 3 separate experiments ($P > 0.05$; $n = 15$). Color images available online at www.liebertpub.com/jop

lack of TSP2 impacts CNV. CNV was induced in P42 TSP2^{+/+} and TSP2^{-/-} female mice by laser photocoagulation induced rupture of Bruch's membrane on day 0. The eyes were harvested 2 weeks later and the area of CNV was determined by intercellular adhesion molecule-2 (ICAM-2) staining. Figure 11 demonstrates that CNV occurred in both TSP2^{+/+} and TSP2^{-/-} mice. The degree of CNV, as determined by the area of ICAM-2 staining, was not significantly different in the absence of TSP2. Thus, lack of TSP2 expression minimally affected CNV.

Lack of TSP2 did not affect retinal TSP1 expression

We have previously shown that lack of TSP1 in endothelial cells results in increased expression of TSP2, potentially compensating for reduced expression of TSP1.^{22,23} However, whether lack of TSP2 results in increased expression of TSP1 remains unknown. Here, we examined the expression of TSP1 in retinas prepared from TSP2^{+/+} and TSP2^{-/-} mice at different postnatal days using qPCR (Fig. 12A). We observed that retinas from TSP2^{+/+} expressed higher levels of TSP1 at all time points evaluated compared to retinas from TSP2^{-/-} mice. Similar results were observed in retinas from mice during OIR. Thus, there is no increase in retinal TSP1 expression in the absence of TSP2. Furthermore, we determined the expression of TSP2 in retinas from TSP1^{-/-} mice (Fig. 12B). Although a trend in increased levels of TSP2 was observed in the absence of TSP1, they were not significantly different. Thus, the effects of TSP2 deficiency observed here are independent of any compensatory increases in TSP1 expression.

Discussion

TSP1 and TSP2 are extracellular modular glycoproteins that are best known for their anti-angiogenic properties and their ability to modulate cell-matrix interactions. We previously showed that TSP1 is a modulator of ocular vascular homeostasis and its expression is essential for appropriate remodeling and maturation of retinal vasculature.^{10,15,24–26} The retinal vascular density was increased in TSP1^{-/-} mice compared with wild-type mice. This finding was mainly attributed to increased number of retinal endothelial cells in TSP1^{-/-} mice. During OIR, the developing retinal vasculature of TSP1^{-/-} mice was less sensitive to vessel obliteration induced by hyperoxia but exhibited a similar level of neovascularization induced by normoxia compared with wild-type mice.¹⁰ Furthermore, lack of TSP1 exacerbated the pathogenesis of diabetic retinopathy and CNV.^{15,25} However, whether TSP2 expression similarly impacts these processes needs investigation.

TSP2 is a close relative of TSP1 and shares similar structure and some functions. Both TSP1 and TSP2 are potent inhibitors of angiogenesis *in vitro* and *in vivo*.^{2,27,28} TSP2 shares the same amino acid sequences, which are found to be important for the anti-angiogenic function of TSP1. They also have important roles in cell adhesion, migration, and proliferation.² Despite a number of analogous activities to TSP1, TSP2 differs in its temporal and spatial distribution in the mouse. Differences in structure and in tissue distribution between TSP1 and TSP2 indicate that the physiological roles of the 2 proteins are likely to differ, and some dissimilarity in their molecular interactions are beginning to surface.^{29–31}

FIG. 10. Lack of TSP2 minimally affected retinal neovascularization during OIR. P7 $TSP2^{+/+}$ and $TSP2^{-/-}$ mice were exposed to a cycle of hyperoxia (75% oxygen) for 5 days and then brought to room air for 5 days. (A, B) Wholemount collagen IV staining of retinal vasculature from P17 $TSP2^{+/+}$ (A) and $TSP2^{-/-}$ (B) mice prepared as detailed in the Methods section. (C, D) Representative cross sections of retinas showing neovascular tufts growing into the vitreous (arrows). The quantitative assessment of the neovascularization is shown in (E). These experiments were repeated with at least 5 mice of each genotype in 3 separate experiments ($P > 0.05$; $n = 15$). Color images available online at www.liebertpub.com/jop

TSP2 expression was previously reported to be absent in mouse retina.¹¹ However, our observations pointed out that TSP2 RNA and protein are expressed in the retina. The TSP2 RNA level was low at P5, but remained at higher level through later postnatal times. Current anti-TSP2 antibodies do not work very well, especially in mice, to show the expression of TSP2 in the immunohistochemical staining of tissue sections. To further investigate the TSP2 distribution in the retina, we utilized wholemount retinas to examine GFP expression and distribution in a TSP2-GFP transgenic line of mice. In TSP2-GFP transgenic mice, where GFP expression is driven by TSP2 promoter, the GFP fluorescence showed directly where TSP2 is distributed. We have observed TSP2-GFP expression in retinal vasculature, and predominantly in perivascular supporting cells.¹⁷

The intensity of the green fluorescence increased during the postnatal development of the retinal vasculature in older animals, but it was almost undetectable at later post-

natal time (P42). These results were further confirmed by the western blot analysis of GFP protein. Our results also showed that there was no significant differences in retinal vascular development and retinal vascular density between the $TSP2^{-/-}$ and $TSP2^{+/+}$ in adult mice. Thus, TSP2 expression minimally influenced retinal vascularization during the late stages of vascular development.

In normal ocular tissues the distribution of the proteins as detected by immunohistochemistry may differ from that of their mRNA. For instance, TSP1 or TSP2 protein was not observed in human corneal stroma but TSP1 mRNA (not TSP2 mRNA) was detected in this structure.³² In our observation, the inconsistency in TSP2 RNA level and TSP2-GFP protein expression during the physiological retinal vasculature may be due to the post-translational differences independent of changes in RNA.

Several tissues from embryonic, neonatal, and adult TSP2-null mice, including dermis, subcutaneous adipose

FIG. 11. Lack of TSP2 minimally affects choroidal neovascularization. Choroidal neovascularization was induced in 6-week-old female TSP2^{+/+} and TSP2^{-/-} mice by laser photocoagulation-induced rupture of Bruch's membrane. After 14 days, the anti-ICAM-2 staining of the choroidal wholemount was performed to visualize the area of neovascularization (A, B). (C) The quantitative assessment of the area of neovascularization. Please note similar choroidal neovascularization occurred in the presence and absence of TSP2. These experiments were repeated with at least 10 mice of each genotype and repeated twice ($P > 0.05$; $n = 20$). ICAM-2, intercellular adhesion molecule-2. Color images available online at www.liebertpub.com/jop

tissue, and thymus, display an increase in capillary density of up to 2-fold, as judged by staining with antibodies against von Willebrand factor.¹⁶ However, this increased vascular density was restricted to certain tissues, as brains from TSP2-null mice displayed no change in vascular density.⁷ We observed no significant differences in retinal vascular density in 3- or 6-week-old TSP2^{-/-} mice. Our results are

consistent with reported lack of differences in vascular density of brain, heart, and kidney of TSP2 null mice.¹⁶

Regulation of angiogenesis by TSP2 is due, in part, to modulation of MMP. However, the role TSP2 plays during physiological vascular development and pathological neovascularization requires further delineation. The most compelling evidence for the inhibition of angiogenesis by

FIG. 12. Lack of TSP2 expression was associated with reduced expression of TSP1 in the retina. Retinal total RNA was prepared from wild-type, TSP2^{-/-}, and TSP1^{-/-} mice at different postnatal days as indicated in the figure. The expression of TSP1 (A) and TSP2 (B) was determined by real-time qPCR as described in the Methods section using specific primers for TSP1 and TSP2. Please note reduced expression of TSP1 in retinas from TSP2^{-/-} mice (* $P < 0.05$; $n = 3$), while TSP2 expression was modestly increased in retinas from TSP1^{-/-} mice ($P > 0.05$; $n = 3$). These experiments were repeated twice with similar results.

TSP2 under normal physiological circumstances comes from histological examination of TSP2-null mice. Here, we investigated the role of TSP2 during postnatal development of retinal vasculature and during OIR and laser-induced CNV.

Our results indicated that the primary retinal vasculature developed at a faster rate in TSP2^{-/-} mice compared with TSP2^{+/+} mice at P5. The reason for the enhanced spreading of the developing vasculature is not clear and could be attributed to the absence of the inhibitor TSP2, reduced expression of TSP1, and/or increased levels of VEGF in null mice. However, the developing retinal vasculature in TSP2^{+/+} mice caught up with that of TSP2^{-/-} mice after P7. No significant difference in retinal vascular density was observed at 3- or 6-weeks of age. TSP2^{-/-} mice also exhibited a similar sensitivity to the hyperoxia-mediated vessel obliteration as TSP2^{+/+} mice with similar level of neovascularization during OIR. Lack of TSP2 expression also minimally affected laser-induced CNV compared to TSP2^{+/+} mice. Thus, the potential impact of TSP2 deficiency on VEGF expression differences may be minimal since both retinal and CNV are dependent on VEGF expression. In addition, no significant difference in the rates of proliferation and apoptosis was observed in retinal vasculature of TSP2^{-/-} mice compared to TSP2^{+/+} mice.

We have shown that reduced levels of TSP1 expression in EC in culture are generally associated with increased level of TSP2.^{22,23} It is not known whether lack of TSP2 results increased level of TSP1. Here, we observed decreased levels of TSP1 expression in TSP2 null retina at various postnatal days when compared with TSP2^{+/+} mouse retina. However, consistent with our vascular cell culture studies, we observed an increasing trend in the level of TSP2 in retinas from TSP1 null mice. Thus, none of the phenotypes observed here can be attributed to a compensatory increased expression of TSP1 in TSP2^{-/-} mice.

TSP2 plays an important role in pathological angiogenesis, including corneal angiogenesis.²⁹ Additional support for TSP2 role in repressing pathological ocular angiogenesis comes from studies of ocular herpes virus infections.³³ TSP2 also promoted vascular regression in the foreign body reaction and the full-thickness excisional wounds.³⁴⁻³⁶ However, in our OIR model, TSP2^{-/-} mice exhibited a similar sensitivity to the hyperoxia-mediated vessel obliteration as TSP2^{+/+} mice with similar level of neovascularization. Furthermore, lack of TSP2 expression minimally affected laser-induced CNV compared with TSP2^{+/+} mice. Collectively, our results show that TSP2 expression does not have an effect on pathological retinal angiogenesis perhaps due to its inherently low level of expression in the mature retinal vasculature. However, the suitability of TSP2 as an inhibitor of ocular neovascularization remains to be determined.

In summary, lack of TSP2 was associated with enhanced postnatal retinal vascularization during early postnatal days but minimally affected the physiological vascularization in the late stages, and during pathological retinal and CNV, when TSP2 level is normally low. This is in contrast to the level of TSP1, which remains constant postnatally after P15.¹⁰ Although we have demonstrated that increased expression of TSP2 occurs in EC from TSP1 null mice,^{22,23} here lack of TSP2 was associated with lower expression of TSP1 in the retinas from TSP2^{-/-} mice. However, the cell autonomous impact of TSP2 expression on vascular cell

function, and whether lack of TSP2 in retinal vascular cells is compensated by increased TSP1 expression remain to be determined.

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Author Disclosure Statement

No competing financial interests exist.

References

- Adams, J.C. Thrombospondins: multifunctional regulators of cell interactions. *Annu. Rev. Cell Dev. Biol.* 17:25–51, 2001.
- Bornstein, P. Thrombospondins as matricellular modulators of cell function. *J. Clin. Invest.* 107:929–934, 2001.
- Sheibani, N., and Frazier, W.A. Thrombospondin-1, PECAM-1, and regulation of angiogenesis. *Histol. Histochem.* 14:285–294, 1999.
- Bornstein, P., O'Rourke, K., Wikstrom, K., Wolf, F.W., Katz, R., Li, P., and Dixit, V.M. A second, expressed thrombospondin gene (Thbs2) exists in the mouse genome. *J. Biol. Chem.* 266:12821–12824, 1991.
- Yang, Z., Strickland, D.K., and Bornstein, P. Extracellular matrix metalloproteinase 2 levels are regulated by the low density lipoprotein-related scavenger receptor and thrombospondin 2. *J. Biol. Chem.* 276:8403–8408, 2001.
- Kyriakides, T.R., Zhu, Y.H., Yang, Z., and Bornstein, P. The distribution of the matricellular protein thrombospondin 2 in tissues of embryonic and adult mice. *J. Histochem. Cytochem.* 46:1007–1015, 1998.
- Bornstein, P., Armstrong, L.C., Hankenson, K.D., Kyriakides, T.R., and Yang, Z. Thrombospondin 2, a matricellular protein with diverse functions. *Matrix Biol.* 19:557–568, 2000.
- Dorrell, M.I., Aguilar, E., and Friedlander, M. Retinal vascular development is mediated by endothelial filopodia, a preexisting astrocytic template and specific R-cadherin adhesion. *Invest. Ophthalmol. Vis. Sci.* 43:3500–3510, 2002.
- Fruttiger, M. Development of the mouse retinal vasculature: angiogenesis versus vasculogenesis. *Invest. Ophthalmol. Vis. Sci.* 43:522–527, 2002.
- Wang, S., Wu, Z., Sorenson, C.M., Lawler, J., and Sheibani, N. Thrombospondin-1-deficient mice exhibit increased vascular density during retinal vascular development and are less sensitive to hyperoxia-mediated vessel obliteration. *Dev. Dyn.* 228:630–642, 2003.
- Suzuma, K., Takagi, H., Otani, A., Oh, H., and Honda, Y. Expression of thrombospondin-1 in ischemia-induced retinal neovascularization. *Am. J. Pathol.* 154:343–354, 1999.
- Tang, Y., Scheef, E.A., Wang, S., Sorenson, C.M., Marcus, C.B., Jefcoate, C.R., and Sheibani, N. CYP1B1 expression promotes the proangiogenic phenotype of endothelium through decreased intracellular oxidative stress and thrombospondin-2 expression. *Blood.* 113:744–754, 2009.

13. Palenski, T.L., Sorenson, C.M., Jefcoate, C.R., and Sheibani, N. Lack of Cyp1b1 promotes the proliferative and migratory phenotype of perivascular supporting cells. *Lab. Invest.* 93:646–662, 2013.
14. Smith, L.E., Wesolowski, E., McLellan, A., Kostyk, S.K., D'Amato, R., Sullivan, R., and D'Amore, P.A. Oxygen-induced retinopathy in the mouse. *Invest. Ophthalmol. Vis. Sci.* 35:101–111, 1994.
15. Wang, S., Sorenson, C.M., and Sheibani, N. Lack of thrombospondin 1 and exacerbation of choroidal neovascularization. *Arch. Ophthalmol.* 130:615–620, 2012.
16. Kyriakides, T.R., Zhu, Y.H., Smith, L.T., Bain, S.D., Yang, Z., Lin, M.T., Danielson, K.G., Iozzo, R.V., LaMarca, M., McKinney, C.E., Ginns, E.I., and Bornstein, P. Mice that lack thrombospondin 2 display connective tissue abnormalities that are associated with disordered collagen fibrillogenesis, an increased vascular density, and a bleeding diathesis. *J. Cell Biol.* 140:419–430, 1998.
17. Palenski, T.L., Gurel, Z., Sorenson, C.M., Hankenson, K.D., and Sheibani, N. Cyp1B1 expression promotes angiogenesis by suppressing NF-kappaB activity. *Am. J. Physiol. Cell Physiol.* 305:C1170–C1184, 2013.
18. Pierce, E.A., Avery, R.L., Foley, E.D., Aiello, L.P., and Smith, L.E.H. Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. *Proc. Natl. Acad. Sci. U. S. A.* 92:905–909, 1995.
19. Huang, Q., Wang, S., Sorenson, C.M., and Sheibani, N. PEDF-deficient mice exhibit an enhanced rate of retinal vascular expansion and are more sensitive to hyperoxia-mediated vessel obliteration. *Exp. Eye Res.* 87:226–241, 2008.
20. Ito, M., and Yoshioka, M. Regression of the hyaloid vessels and pupillary membrane of the mouse. *Anat. Embryol. (Berl.)* 200:403–411, 1999.
21. Wang, S., Sorenson, C.M., and Sheibani, N. Attenuation of retinal vascular development and neovascularization during oxygen-induced ischemic retinopathy in Bcl-2-/- mice. *Dev. Biol.* 279:205–219, 2005.
22. Sheibani, N., and Frazier, W.A. Thrombospondin 1 expression in transformed endothelial cells restores a normal phenotype and suppresses their tumorigenesis. *Proc. Natl. Acad. Sci. U. S. A.* 92:6788–6792, 1995.
23. Fei, P., Zaitoun, I., Farnoodian, M., Fisk, D.L., Wang, S., Sorenson, C.M., and Sheibani, N. Expression of thrombospondin-1 modulates the angioinflammatory phenotype of choroidal endothelial cells. *PLoS One.* 9:e116423, 2014.
24. Wu, Z., Wang, S., Sorenson, C.M., and Sheibani, N. Attenuation of retinal vascular development and neovascularization in transgenic mice over-expressing thrombospondin-1 in the lens. *Dev. Dyn.* 235:1908–1920, 2006.
25. Sorenson, C.M., Wang, S., Genderon, R., Paradis, H., and Sheibani, N. Thrombospondin-1 deficiency exacerbates the pathogenesis of diabetic retinopathy. *J. Diabetes Metab. Suppl.* 12, 2013. DOI: 10.4172/2155–6156.S12-005.
26. Wang, S., Gottlieb, J.L., Sorenson, C.M., and Sheibani, N. Modulation of thrombospondin 1 and pigment epithelium-derived factor levels in vitreous fluid of patients with diabetes. *Arch. Ophthalmol.* 127:507–513, 2009.
27. Agah, A., Kyriakides, T.R., Lawler, J., and Bornstein, P. The lack of thrombospondin-1 (TSP1) dictates the course of wound healing in double-TSP1/TSP2-null mice. *Am. J. Pathol.* 161:831–839, 2002.
28. Lawler, J. Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth. *J. Cell. Mol. Med.* 6:1–12, 2002.
29. Good, D.J., Poverini, P.J., Rastinejad, F., Le Beau, M.M., Lemons, R.S., Frazier, W.A., and Bouck, N.P. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc. Natl. Acad. Sci. U. S. A.* 87:6624–6628, 1990.
30. Lawler, J., and Detmar, M. Tumor progression: the effects of thrombospondin-1 and -2. *Int. J. Biochem. Cell Biol.* 36:1038–1045, 2004.
31. Nicosia, R.F., and Tuszynski, G.P. Matrix-bound thrombospondin promotes angiogenesis in vitro. *J. Cell Biol.* 124:183–193, 1994.
32. Armstrong, D.J., Hiscott, P., Batterbury, M., and Kaye, S. Corneal stromal cells (keratocytes) express thrombospondins 2 and 3 in wound repair phenotype. *Int. J. Biochem. Cell Biol.* 34:588–593, 2002.
33. Hiscott, P., Paraoan, L., Choudhary, A., Ordonez, J.L., Al-Khaier, A., and Armstrong, D.J. Thrombospondin 1, thrombospondin 2 and the eye. *Prog. Retin. Eye Res.* 25:1–18, 2006.
34. Kyriakides, T.R., Leach, K.J., Hoffman, A.S., Ratner, B.D., and Bornstein, P. Mice that lack the angiogenesis inhibitor, thrombospondin 2, mount an altered foreign body reaction characterized by increased vascularity. *Proc. Natl. Acad. Sci. U. S. A.* 96:4449–4454, 1999.
35. Kyriakides, T.R., and MacLachlan, S. The role of thrombospondins in wound healing, ischemia, and the foreign body reaction. *J. Cell Commun. Signal.* 3:215–225, 2009.
36. Kyriakides, T.R., Wulsin, D., Skokos, E.A., Fleckman, P., Pirrone, A., Shipley, J.M., Senior, R.M., and Bornstein, P. Mice that lack matrix metalloproteinase-9 display delayed wound healing associated with delayed reepithelization and disordered collagen fibrillogenesis. *Matrix Biol.* 28:65–73, 2009.

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