Attenuation of proliferation and migration of retinal pericytes in the absence of thrombospondin-1

Elizabeth A. Scheef,1 Christine M. Sorenson,2 and Nader Sheibani1

Departments of 1Ophthalmology and Visual Sciences and 2Pediatrics, University of Wisconsin School of Medicine, Madison, Wisconsin

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Scheef EA, Sorenson CM, Sheibani N. Attenuation of proliferation and migration of retinal pericytes in the absence of thrombospondin-1. Am J Physiol Cell Physiol 296: C724–C734, 2009. First published February 4, 2009; doi:10.1152/ajpcell.00409.2008. —Perivascular supporting cells, including endothelial cells and pericytes (PCs), provide instructive signals to adjacent endothelial cells helping to maintain vascular homeostasis. These signals are provided through direct contact and by the release of soluble factors by these cells. Thrombospondin (TSP)1 is a matricellular protein and an autocrine factor for VSMCs. TSP1 activity, along with that of PDGF, regulates VSMC proliferation and migration. However, the manner in which TSP1 and PDGF impact retinal PC function requires further investigation. In the present study, we describe, for the first time, the isolation and culture of retinal PCs from wild-type (TSP1+/+) and TSP1-deficient (TSP1−/−) immortomice. We showed that these cells express early and mature markers of PCs, including NG2, PDGF receptor-β, and smooth muscle actin as well as desmin, calbindin, and mesenchymal stem cell markers. These cells were successfully passaged and maintained in culture for several months without significant loss of expression of these markers. TSP1−/− PCs proliferated at a faster rate compared with TSP1+/+ PCs. In addition, TSP1−/− PCs, like VSMCs, responded to PDGF-BB with enhanced migration and proliferation. In contrast, TSP1+/+ PCs failed to respond to the promigratory and proliferative activity of PDGF-BB. This may be attributed, at least in part, to the limited interaction of PDGF-BB with TSP1 in null cells, which is essential for PDGF proliferative and migratory action. We observed no significant differences in the rates of apoptosis in these cells. TSP1−/− PCs were also less adherent, expressed increased levels of TSP2 and fibronectin, and had decreased amounts of N-cadherin and α,β3-integrin on their surface. Thus, TSP1 plays a significant role in retinal PC proliferation and migration impacting retinal vascular development and homeostasis.

retinal vasculature; platelet-derived growth factor; platelet-derived growth factor receptor-β; mesenchymal stem cells; perivascular cells; cell adhesion

ANGIOGENESIS, the formation of new blood vessels from preexisting capillaries, is a complex and multistep process. Pathological growth of new blood vessels contributes to a large number of eye diseases, including retinopathy of prematurity, proliferative diabetic retinopathy, and age-related macular degeneration (4, 6, 13). However, the molecular and cellular events that contribute to the initiation of angiogenic events require further delineation. Specific alterations in vascular cells, including endothelial cells (ECs) and perivascular support cells [vascular smooth muscle cells (VSMCs) and pericytes (PCs)], may play an important role in these processes.

The mouse retinal vasculature develops postnatally, providing a unique opportunity to study all aspects of vascular development and remodeling. In addition, there is a similar pattern of vascular development in the mouse retina compared with the human (11). In both species, the first vessels originate at the optic nerve head and spread over the inner surface of the retina, forming a dense network. Thus, results from mouse studies should give us a better understanding of human retinopathy. This, combined with the need to better understand the mechanisms of neovascularization in diseases like diabetic retinopathy and retinopathy of prematurity, further emphasizes the importance of developing retinal vascular cells with characteristics similar to in vivo vascularization.

Retinal vascularization is very tightly regulated by coordinated interactions of vascular cells, including ECs, PCs, and astrocytes, and by a tightly balanced production of positive and negative regulatory factors by these cells (8–10, 12, 42). However, the study of these coordinated interactions and the identity of the factors involved in vivo has been very challenging. As an important component in the process of vasculogenesis and angiogenesis, the biology of mouse vascular cells has been a recent focus of many studies. Mice offer the added benefits of well-established genetic modification techniques. Many genetically modified mouse strains have been established in the past two decades. Studies on the effect of certain single or multiple genetic modifications have revealed an advanced understanding of their roles in many basic biological processes.

Thrombospondin (TSP)1 is a member of the matricellular family of TSP proteins with potent antiangiogenic activity. TSP1 inhibits angiogenesis in vivo and EC proliferation and migration in vitro (25, 41). In contrast, TSP1 is an important autocrine factor for VSMC proliferation and migration (31). We (46) have recently shown that mice deficient in TSP1 (TSP1−/−) exhibit increased retinal vascular density. This was mainly attributed to the failure of the developing retinal vasculature to undergo appropriate pruning and remodeling in the absence of TSP1. Furthermore, we (49) have shown that overexpression of TSP1 in the eye results in the attenuation of retinal vascular development and ischemia-driven neovascularization. Therefore, appropriate expression of TSP1 plays an essential role in retinal vascular homeostasis.

The ability to culture vascular cells has been instrumental in developing assays that recapitulate the different stages of angiogenesis as well as studying mechanisms that impact vascular cell phenotypes. These assays have provided great
knowledge regarding the biochemical events that modulate angiogenesis and the molecules involved. We (48) have recently shown that retinal ECs prepared from TSP1−/− mice exhibit a proangiogenic phenotype in culture. TSP1−/− retinal ECs are more proliferative and migratory and exhibit sustained activation of Akt1 and increased levels of its downstream effector, endothelial nitric oxide (NO) synthase (eNOS) (48). We (37, 38) have also shown that lack of TSP1 significantly impacts the adhesive and migratory properties of retinal astrocytes and corneal ECs. These results are consistent with the antiangiogenic activity demonstrated for TSP1 and its role as an important modulator of retinal vascular homeostasis. However, the impact that lack of TSP1 has on the characteristics of PCs needs further investigation. Although PCs have been cultured from retinas of other species, including the human, monkey, bovine, pig, dog, rat, and rabbit (2, 3, 15, 17, 32, 36, 40, 45), the success with mouse retinal PCs has been very limited and, to our knowledge, has not been previously reported.

Different methods have been used to isolate and enrich for retinal PCs from various species. Initially, most of the retinal PC cultures from the human and bovine were obtained by allowing the outgrowth of PCs from retinal vessels and their identification by morphology and expression of smooth muscle actin. Isolation and culture of mouse retinal PCs have proven difficult, perhaps due to its relatively low tissue supplement compared with other animal sources. PC markers PDGF receptor-β (PDGF-Rβ) and NG2 have proven to be a useful, efficient way to identify PCs from different cell types (16, 43). In the present study, we describe a method for the routine isolation and propagation of mouse retinal PCs from wild-type (TSP1+/+) and TSP1−/− immortomice. Furthermore, we demonstrate that mouse retinal PCs can be readily expanded, retaining their PC markers, and can aid in defining the functional consequences of gene targeting on PC properties. We show retinal PCs prepared from TSP1−/− mice are less proliferative and less migratory compared with TSP1+/+ mice and fail to respond to the promigratory and proliferative effects of PDGF-BB.

MATERIALS AND METHODS

Experimental animals. All experiments 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 School of Medicine and Public Health. Immortomice expressing a temperature-sensitive simian virus (SV)40 large T antigen were obtained from Charles River Laboratories (Wilmington, MA). TSP1−/− mice on a C57BL/6 background were generated as previously described (26). TSP1−/− mice were crossed with immortomice, and immorto-TSP1−/− mice were identified by PCR analysis of DNA isolated from tail biopsies. The PCR primer sequences were as follows: immorto, forward 5′-TTAGAGCTTAAATCTGTTAGTAG-3′; Neo, forward 5′-TGCTCTCATCATGCAGAAGTAGTG-3′; reverse 5′-TTAGAGCTTTAAATCTGTTAGTAG-3′; Neo, forward 5′-GTCTCTCATCATGCAGAAGTAGTG-3′; and reverse 5′-GAGTTGTGGTTGAGCTCAG-3′.

Tissue preparation and culture of retinal PCs. PCs were isolated from mouse retinas by collecting retinas from one litter (6–7 pups, 4 wk old) using a dissecting microscope. Twelve to fourteen retinas were rinsed with serum-free DMEM (Invitrogen, Carlsbad, CA), pooled in a 60-mm dish, minced, and digested for 45 min with collagenase type II (1 mg/ml, Worthington, Lakewood, NJ) with 0.1% BSA in serum-free DMEM at 37°C. Cells were rinsed in DMEM containing 10% FBS and centrifuged for 5 min at 400 g. Digested tissue was resuspended in 4 ml DMEM containing 10% FBS, 2 mM glutamine, 100 μg/ml streptomycin, 1000/mL penicillin, and murine recombinant IFN-γ (R&D Systems, Minneapolis, MN) at 44 U/ml. Cells, tissue, and medium were evenly divided into 4 wells of a 24-well tissue culture plate and maintained at 33°C with 5% CO2. Cells were progressively passed to larger plates and maintained in 60-mm dishes.

FACScan analysis. Retinal PCs from 60-mm culture plates were rinsed with PBS containing 0.04% EDTA and incubated with 1.5 ml of Cell Dissociation Solution (Sigma, St. Louis, MO). Cells were rinsed from plates with DMEM containing 10% FBS, washed once with 5 ml Tris-buffered saline (TBS; 20 mM Tris·HCl and 150 mM NaCl; pH 7.6), and then blocked in TBS with 1% goat serum for 20 min on ice. Cells were incubated with rabbit anti-NG2 (Millipore, Temecula, CA), rabbit anti-mouse α-smooth muscle actin (Sigma), rat anti-mouse PDGF-Rβ, rat anti-mouse CD11b (eBioscience, San Diego, CA), mouse anti-mouse CD36, rat-anti mouse CD45, rat-anti-mouse CscAl (BD Pharmingen, San Diego, CA), rat-anti-mouse CD47 (a gift of Dr. William A. Frazier, Washington University, St. Louis, MO), R-phycocerythrin-conjugated rat anti-mouse CD73, PerCP mouse anti-mouse CD90.1, rat anti-CD105, Armenian hamster anti-mouse ICAM-1, rat anti-mouse VCAM-1, mouse anti-N-cadherin (BD Pharmingen), rabbit anti-β3-integrin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-β1-integrin, rabbit anti-α3-integrin, rabbit anti-α3β1-integrin, rabbit anti-α3β2-integrin, and rabbit anti-α3β3-integrin (Millipore) or rat anti-PECAM-1 (Mec13.3, BD Pharmingen) for 30 min on ice. Cells were washed twice with TBS with 1% BSA and incubated with the appropriate FITC-conjugated secondary antibody for 30 min on ice. Cells were then washed twice with TBS with 1% BSA, resuspended in 0.5 ml TBS with 1% BSA, and analyzed by a FACScan caliber flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). All antibodies were used at the dilutions recommended by the supplier. Cells incubated with secondary antibodies in the absence of primary antibodies were used as controls.

Western blot analysis. For TSP1 analysis, cells were plated at 8 × 104 cells/60-mm dish and allowed to reach ~95% confluence (2 days). Cells were rinsed once with serum-free DMEM and incubated with serum-free medium for 2 days. Conditioned medium was collected and clarified by centrifugation. Samples (100 μl each) were analyzed by Western blot analysis as recently described (38). The antibodies used were mouse anti-human TSP1 (A6.1, Neo Markers, Fremont, CA), rabbit anti-TSP2 (BD Pharmingen), rabbit anti-rat fibronectin (Invitrogen), rat anti-chicken tenasin-C (Millipore), goat anti-mouse osteopontin (R&D Systems), mouse anti-human β-catenin and N-cadherin (BD Transduction), rabbit anti-desmin (Sigma), and goat anti-mouse calbindin (Santa Cruz Biotechnology) or rabbit anti-mouse β-actin (Sigma) to control for loading. Cells were also lysed in 0.2 ml of 20 mM Tris (pH 7.4) and 2 mM EDTA solution, sonicated briefly, and similarly analyzed along with the conditioned medium.

To assess Akt1 and eNOS activation, cells were plated at 8 × 104 cells/60-mm dish and allowed to reach ~95% confluence. Plates were then rinsed twice with cold serum-free medium containing 1 mM Na3VO4, lysed in 0.5 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 1 mM CaCl2, 1 mM MgCl2, 1% Nonidet P-40, 0.5% deoxycholate, 1% Triton X-100, 50 mM NaF, 3 mM Na3VO4, and a cocktail of protease inhibitors, Roche Applied Sciences, Indianapolis, IN), and transferred to a microtube on ice. Samples were sonicated briefly, rocked for 30 min at 4°C, and centrifuged, and cleared lysates were transferred to clean tubes. Protein concentrations were determined by the DC protein assay kit (Bio-Rad, Hercules, CA), and aliquots corresponding to equal amounts of protein (20 μg) were analyzed by Western blot analysis as described above. The
antibodies to phospho-Akt1, total Akt1, total eNOS, inducible NO synthase (iNOS), and neuronal NO synthase (nNOS) were from Cell Signaling (Cambridge, MA) and used at the dilutions recommended by the supplier.

Indirect immunofluorescence. PCs were cultured on glass chamber slides (Nalge Nunc, Naperville, IL) coated with 2 μg/ml fibronectin and allowed to reach 50% confluence (next day). Cells were rinsed twice with cold PBS, fixed with 4% paraformaldehyde for 10 min on ice, and washed three times with PBS. Cells were then incubated with mouse anti-vinculin (Sigma) for 30 min at 37°C, rinsed twice with TBS, and incubated with anti-mouse CY2-conjugated secondary antibody (1:500, Jackson Immunoresearch Laboratories, West Grove, PA) for 30 min at 37°C. Cells were rinsed twice with TBS and incubated with TRITC-conjugated phalloidin (Sigma) for 10 min at 37°C. Cells were rinsed three times with TBS, and the chambers were removed from the slide, mounted with a coverslip, and photographed using a Zeiss fluorescence microscope (Axiophot, Zeiss) in digital format. Pericytes were also stained with rabbit anti-desmin (Dako, Carpentaria, CA) and goat anti-calbindin (Santa Cruz Biotechnology), smooth muscle actin. These experiments were repeated with three isolations of PCs with similar results.

Cell proliferation assays. Retinal PCs were plated in multiple sets in triplicate at 5 × 10^4 cells/well, 4 wells, Nalge Nunc) coated with 2 μg/ml fibronectin. The next day, cells were processed for the detection of apoptotic cells as recommended by the supplier. Similar experiments were also conducted using PCs challenged with 1 mM 5-fluorouracil (Sigma) for 24 h to induce apoptosis. Data are reported as mean percentages of cells undergoing apoptosis relative to the total number of cells counted.

Transwell migration assays. The migration ability of PCs was determined as recently described (38). Briefly, the bottom side of an 8-μm-pore size Costar transwell (Corning, Acton, MA) was coated with 2 μg/ml fibronectin, and cells were incubated overnight at 4°C. The bottom of the transwell was rinsed with PBS and blocked with 0.5 ml of PBS with 2% BSA for 1 h at room temperature. The transwell was rinsed with PBS, and 0.5 ml serum-free DMEM or serum-free DMEM containing 20 ng/ml PDGF-BB (PeproTech) was added to the 24-well dish containing the transwell. Cells were then trypsinized, resuspended in serum-free DMEM or DMEM containing 20 ng/ml PDGF-BB, plated at 1 × 10^5 cells in 0.1 ml on the top of the transwell.

Fig. 1. Isolation and characterization of retinal pericytes (PCs). Top left: morphology of wild-type (thrombospondin (TSP1^+/+)) and TSP1-deficient (TSP1^-/-) mouse retinal PCs cultured on uncoated plates. Magnification: ×40. Bottom: expression of PC markers in TSP1^+/+ (left) and TSP1^-/- (right) PCs determined by FACS analysis in early passage (passage 5 (P5)) or late passage (passage 22 (P22)). The shaded traces show staining in the absence of primary antibody. Please note the decreased levels of N-cadherin (N-Cad) in TSP1^-/- PCs. Expression of NG2 (top right) varied between isolations and was affected by the confluence of cultures. PDGFRb, PDGF receptor-β; SMA, smooth muscle actin. These experiments were repeated with three isolations of PCs with similar results.

Fig. 2. Retinal PCs expressed mature PC markers. TSP1^+/+ and TSP1^-/-retinal PCs were cultured on glass slides and stained with specific antibodies to desmin and calbindin as described in MATERIALS AND METHODS. Please note that the expression of these markers was minimally affected by the lack of TSP1. These experiments were repeated with three isolations of PCs with similar results.
Fig. 3. Retinal PCs expressed other vascular cell markers. Expression levels of various markers were determined by FACS analysis using specific antibodies as described in MATERIALS AND METHODS. The shaded traces show staining in the absence of primary antibody. Please note the lack of PECAM-1 expression, an endothelial cell marker, in retinal PCs, as expected. TSP1+/+ PCs expressed lower levels of endoglin. Expression levels of CD36, VCAM-1, and ICAM-1 were similar in TSP1+/+ and TSP1−/− retinal PCs. These experiments were repeated with three isolations of PCs with similar results.

Fig. 4. Retinal PCs expressed mesenchymal stem cell markers. Expression levels of various markers were determined by FACS analysis using specific antibodies as described in MATERIALS AND METHODS. The shaded traces show staining in the absence of primary antibody. Please note the expression of Sca1, CD11b, CD45, and CD90 in retinal PCs. Expression levels of CD11b, CD45, and CD90 were lower in TSP1−/− compared with TSP1+/+ PCs. These cells did not express CD73. These experiments were repeated with three isolations of PCs with similar results.
membrane, and incubated for 3 h at 37°C. The membrane was fixed, stained with hematoxylin-eosin, and mounted on a slide. The mean number of cells that migrated through the membrane was determined by counting the number of cells in 10 high-power fields (×400).

**Cell adhesion assays.** Adhesion of PCs to different extracellular matrix (ECM) proteins was determined as recently described (34). Briefly, 96-well plates (Maxisorb, Nunc) were coated with various concentrations of fibronectin, human type I collagen, vitronectin, and laminin (BD Biosciences) prepared in TBS with 2 mM Ca$^{2+}$ and 2 mM Mg$^{2+}$ (TBS with Ca$^{2+}$-Mg$^{2+}$) overnight at 4°C. Plates were then rinsed four times with TBS containing Ca$^{2+}$-Mg$^{2+}$ and blocked for

![Graph](image)

**A**

- **TSP+/+ PC**
- **TSP1−/− PC**

**B**

- **TSP1+/+ PC**
- **TSP1+/+ PC + PDGF-BB**
- **TSP1−/− PC**
- **TSP1−/− PC + PDGF-BB**

**C**

- **TSP1+/+**
- **TSP1−/−**

Fig. 5. TSP1−/− PCs proliferated at a slower rate. The rate of cell proliferation was determined by counting the number of cells for 10 days under regular growth conditions (A) or in the presence of PDGF-BB (B) as described in MATERIALS AND METHODS. Please note the significant decrease in the rate of proliferation of TSP1−/− PCs ($n = 3$, *$P < 0.05$). TSP1−/− PCs also failed to respond to the proliferative effect of PDGF-BB ($n = 3$, *$P > 0.05$), whereas TSP1+/+ PC exhibited a significant increase in the rate of proliferation in response to PDGF-BB ($n = 3$, *$P < 0.05$). C: rates of cell proliferation in TSP1+/+ and TSP1−/− PCs were also evaluated by 5-ethyl-2′-deoxyuridine (EdU) incorporation as described in MATERIALS AND METHODS. A significant decrease in the percentage of PCs that incorporated EdU was observed in the absence of TSP1 (see text for quantifications). TSP1−/− PCs also failed to respond to the stimulatory effects of PDGF-BB in EdU labeling compared with TSP1+/+ PC (not shown).

![Graph](image)

**A**

- **TSP1+/+ PC**
- **TSP1+/+ PC 20 ng PDGF-BB**
- **TSP1−/− PC**
- **TSP1−/− PC 20 ng PDGF-BB**

**B**

- **TSP1+/+ PC**
- **TSP1−/− PC**

Control

PDGF-BB

![Images](image)

Fig. 6. TSP1−/− retinal PCs did not respond to the promigratory effect of PDGF-BB. Migration of TSP1+/+ and TSP1−/− retinal PCs was determined in transwell assays with and without PDGF-BB as described in MATERIALS AND METHODS. No significant differences in the basal migration of TSP1+/+ PCs was observed compared with TSP1+/+ PCs. A significant increase in the migration of TSP1+/+ PCs in response to PDGF-BB was observed ($n = 3$, *$P < 0.05$). TSP1−/− retinal PCs did not show a significant response to the promigratory effects of PDGF-BB ($n = 3$, *$P > 0.05$). The migratory characteristic of TSP1+/+ PCs in response to PDGF-BB was also demonstrated by staining with phalloidin (actin) and vinculin (focal adhesions). Please note the morphology changes in TSP1+/+ PCs incubated with PDGF-BB compared with untreated or TSP1−/− PCs with and without PDGF-BB. TSP1+/+ PCs incubated with PDGF-BB showed elongated morphology with numerous peripheral focal adhesions localizing to lamellipodia extensions along with increased actin stress fibers.
1 h with 200 μl of 1% BSA prepared in TBS with Ca2+-Mg2+ at room temperature. Cells were removed using 1.5 ml dissociation solution (Sigma), washed once with TBS, and resuspended in HEPES-buffered saline [25 mM HEPES (pH 7.60) and 150 mM NaCl] containing 4 mg/ml BSA at 5 × 10^5 cells/ml. Blocked wells then received 50 μl of cell suspension and 50 ml of TBS with Ca2+-Mg2+, and cells were allowed to adhere for 90 min at 37°C. Nonadherent cells were removed by gently washing the wells with 200 μl of TBS with Ca2+-Mg2+ until no cells were left in BSA-coated wells. Numbers of adherent cells were quantified by measuring the intracellular phosphatase activity as previously described (34).

Capillary morphogenesis assay. We (37, 44) have previously shown that retinal ECs and astrocytes rapidly organize into a capillary-like network when plated on Matrigel. The ability of TSP1+/+ and TSP1−/− PCs to organize on Matrigel was determined as previously described (44). Briefly, 2 × 10^6 cells in 2 ml growth medium were gently layered on 35-mm tissue culture dishes coated with 0.5 ml Matrigel (10 mg/ml, BD Biosciences), which was allowed to harden at room temperature. Cells were removed using 1.5 ml dissociation buffer containing 4 mg/ml BSA at 5

RESULTS

Isolation of mouse retinal PCs. Isolation of mouse retinal PCs have proven very difficult and, as far as we are aware of, have not been previously reported. Using TSP1+/+ and TSP1−/− immortomice, we isolated and characterized retinal PCs. Figure 1 shows the morphology of retinal PCs prepared from TSP1+/+ and TSP1−/− mice. These cells exhibited a similar irregular and stellate morphology when plated on uncoated plates (Fig. 1, top left). To confirm that these cells were PCs, we examined the expression of the PC markers PDGF-Rβ, NG2, and α-smooth muscle actin by FACS analysis. Figure 1 shows that retinal PCs prepared from TSP1+/+ and TSP1−/− mice expressed significant amounts of these markers on their surface. The long-term culture of these cells for several months did not significantly affect the expression pattern of these markers (Fig. 1, bottom left, passage 5 vs. passage 22). PCs also expressed N-cadherin, which plays an important role in perivascular cell migration and vascular stabilization (33, 35). We observed significant expression of N-cadherin in TSP1+/+ retinal PCs, which was decreased in TSP1−/− cells (2.3-fold as determined by densitometry, Fig. 1; see also Fig. 10A). We also determined the expression of additional markers associated with mature PCs, including desmin and calbindin, in retinal PCs. Figure 2 shows indirect immunofluorescence staining of TSP1+/+ and TSP1−/− retinal PCs with antibodies to desmin and calbindin. No staining was observed in the absence of primary antibody (not shown). This observation was further confirmed for desmin by Western blot analysis of cell lysates from TSP1+/+ and TSP1−/− retinal PCs (see Fig. 10A) showing similar levels. We were unable detect calbindin by Western blot analysis, perhaps a limitation of the antibody, regardless of TSP1 status (not shown).

We also determined the expression of other vascular cell markers in TSP1+/+ and TSP1−/− PCs by FACS analysis. Although retinal PCs did not express the EC marker PECAM-1, as expected, they expressed significant amounts of endoglin, CD36, VCAM-1, and ICAM-1 (Fig. 3). These cells also expressed CD47, the TSP1 carboxyl terminal receptor, with an important role in the modulation of VSMC migration (47). We also examined the expression of markers reported to be expressed by mesenchymal stem cells, which are
known for their potential to become vascular supporting cells, including Sca1, CD11b, and CD45 (hematopoietic stem cell markers) and CD73 and CD90.1 (mesenchymal precursor cell markers) (Fig. 4). TSP1+/+ PCs expressed significant amounts of CD11b, CD45, and CD90.1. The expression of these markers was reduced in TSP1−/− PCs. These cells lacked the expression of CD73 but expressed similar levels of Sca 1 regardless of TSP1 status.

TSP1−/− retinal PCs proliferate at a slower rate. TSP1 is an autocrine factor for VSMCs and is essential for their proliferation and migration in response to PDGF (31). However, the specific effects of TSP1 on PC proliferation and apoptosis require further study. We next examined the rates of proliferation in TSP1+/+ and TSP1−/− retinal PCs by counting the numbers of cells for several days. We observed that TSP1−/− retinal PCs proliferated at a significantly slower rate compared with TSP1+/+ cells (Fig. 5A). A similar result was observed when retinal PCs were incubated with PDGF-BB (Fig. 5B). We observed no significant response to proliferative effects of PDGF-BB in TSP1−/− retinal PCs, whereas TSP1+/+ cells responded as expected.

The decreased rate of proliferation observed in TSP1−/− retinal PCs suggests a decrease in the percentage of cells actively dividing or an increased rate of apoptosis. To distinguish between these possibilities, we next evaluated the percentage of TSP1+/+ and TSP1−/− retinal PCs undergoing active DNA synthesis using EdU incorporation (Fig. 5C). The percentage of TSP1+/+ cells that were EdU positive was significantly higher than that in TSP1−/− PCs (TSP1+/+ cells: 54 ± 4.3% vs. TSP1−/− cells: 40 ± 2.8%, P < 0.05, n = 5). The percentage of EdU-positive TSP1+/+ PCs was also significantly enhanced in the presence of PDGF-BB compared with TSP1−/− PCs (not shown). Thus, TSP1 expression positively influenced the proliferation of PCs.

We next determined the rate of apoptosis in TSP1+/+ and TSP1−/− PCs in the presence or absence of 5-fluorouracil (1 mM), as previously described (23). We observed no significant differences in the rates of apoptosis in these cells (TSP1+/+ PCs: 1.2 ± 0.09% vs. TSP1−/− PCs: 1.4 ± 0.12%, n = 5, P > 0.05). Although incubation of PCs with 5-fluorouracil resulted in increased apoptosis, there were no significant differences between the rates of apoptosis in TSP1−/− PCs compared with TSP1+/+ PCs (TSP1−/− PCs: 5.7 ± 0.46% vs. TSP1−/− PCs: 6.2 ± 0.5%, n = 5, P > 0.05). Thus, lack of TSP1 has minimal impact on the rate of apoptosis in PCs.

TSP1−/− retinal PCs are less migratory. Migration of PCs is fundamental to their ability to cover and stabilize newly formed blood vessels (1). PDGF-BB secreted by ECs is believed to play a vital role in the recruitment of PCs and coverage of blood vessels. We next evaluated the migration of TSP1+/+ and TSP1−/− PCs in the presence or absence of PDGF-BB. TSP1+/+ PCs responded to PDGF with a significant increase in migration (P < 0.05, n = 3; Fig. 6A). In contrast, TSP1−/− PCs did not respond to the promigratory effects of PDGF-BB. Thus, TSP1 expression may be essential for the appropriate migration of PCs in response to PDGF-BB. The migratory phenotype of TSP1+/+ retinal PCs incubated with PDGF-BB was further confirmed by increased actin stress fibers, focal adhesions, and lamellipodia after staining with phalloidin for actin filaments and vinculin immunostaining for focal adhesions (18) (Fig. 6B).

Fig. 8. TSP1−/− retinal PCs expressed reduced levels of αvβ3-integrin. Expression levels of various integrins were determined by FACS analysis using specific antibodies as described in MATERIALS AND METHODS. The shaded traces show staining in the absence of primary antibody. Please note the similar expression levels of β1-integrin, α6-integrin, and CD47 (integrin-associated protein, a TSP1 receptor) in TSP1+/+ and TSP1−/− PCs. Expression levels of αvβ3-integrin were significantly lower in TSP1−/− PCs (see text for quantifications). These experiments were repeated with three isolations of PCs with similar results.
attributed to changes in the levels and/or activities of integrins expressed on the cell surface. We examined the expression of a number of integrins on the surface of TSP1<sup>+/+</sup> and TSP1<sup>−/−</sup> PCs by FACS (Fig. 8). We observed similar expression of β1-, β2-, and α<sub>6</sub>-integrin in both cell types. Similar levels of α<sub>2</sub>, α<sub>3</sub>, and α<sub>5</sub>-integrin were also expressed in these cells (not shown). In contrast, decreases in the levels of α<sub>3</sub>-integrin (not shown) and α<sub>6</sub>β<sub>3</sub>-integrin expression were observed in TSP1<sup>−/−</sup> PCs. The mean fluorescent intensities for α<sub>6</sub>β<sub>3</sub>-integrin were as follows: 58 ± 2.3 in TSP1<sup>+/+</sup> PCs vs. 26 ± 1.0 in TSP1<sup>−/−</sup> PCs (P < 0.05, n = 3).

Retinal PCs fail to undergo morphogenesis in Matrigel. We (37, 44) have previously shown that retinal ECs and astrocytes rapidly organize into a network of interconnecting cells, which resemble a capillary-like network when plated in Matrigel. However, the ability of PCs to organize into a network of interconnecting cells has not been previously evaluated. TSP1<sup>+/+</sup> and TSP1<sup>−/−</sup> PCs, unlike retinal ECs and astrocytes, minimally organized into a network when plated in Matrigel (Fig. 9). They formed small cell aggregates with a limited ability to migrate and connect with other groups of cells as normally seen with retinal ECs and astrocytes (37, 44). Thus, PCs, in contrast to astrocytes and ECs, may not actively participate in patterning of the developing retinal vasculature.

**TSP1<sup>−/−</sup> PCs express increased levels of TSP2 and fibronectin.** ECM proteins have a significant impact on PC proliferation and differentiation (39). We next examined the production of various ECM proteins in conditioned medium and cell lysates prepared from TSP1<sup>+/+</sup> and TSP1<sup>−/−</sup> PCs by Western blot analysis. We observed an increase in the level of TSP2, a closely related family member of TSP1, in TSP1<sup>−/−</sup> PCs (Fig. 10A). These cells also expressed increased levels of fibronectin compared with TSP1<sup>+/+</sup> PCs. Retinal PCs also expressed low amounts of osteopontin (not shown) and tenascin C, which was not affected by the status of TSP1. TSP1<sup>+/+</sup> PCs expressed a significant amount of TSP1, which was mainly cell associated, whereas TSP1<sup>−/−</sup> PCs lacked TSP1 expression, as expected. β-Actin was used as a loading control for cell lysates.

**Increased expression and activation of Akt1 in TSP1<sup>−/−</sup> PCs.** We (48) have previously shown that TSP1<sup>−/−</sup> retinal ECs express increased levels of Akt1 and its phosphorylated (activated) form. This was also associated with the activation of its downstream effector, eNOS. We first examined the expression and phosphorylation of Akt1 in TSP1<sup>+/+</sup> and TSP1<sup>−/−</sup> retinal PCs. We observed an approximately twofold increase in the levels of Akt1 as well as its phosphorylated form (Fig. 10B, as determined by densitometry). We next examined the expression of NO synthases, including eNOS, iNOS, and nNOS. The expression of iNOS, but not eNOS, has been previously reported in bovine retinal PCs (22). The production of NO by eNOS in ECs impacts VSMC/PC contractility and vasodilatation. This effect may be hampered by TSP1 through cGMP-dependent and -independent mechanisms (20). TSP1<sup>+/+</sup> and TSP1<sup>−/−</sup> retinal PCs expressed undetectable levels of eNOS (Fig. 10B). We also did not detect iNOS or nNOS expression in retinal PCs regardless of TSP1 status (not shown).

**DISCUSSION**

Here, we report the successful isolation and culture of retinal PCs from TSP1<sup>+/+</sup> and TSP1<sup>−/−</sup> mice. These cells expressed NG2, PDGF-Rβ, and α-smooth muscle actin and were readily propagated in culture for several passages without a significant loss in the expression of these markers. Retinal PCs also expressed other mesenchymal and hematopoietic stem cell markers, supporting their ability to further differentiate into other mesenchymal cells, including fat and bone cells (5, 7). TSP1<sup>−/−</sup> PCs proliferated at a slower rate and failed to respond to the proliferative and migratory effects of PDGF-BB. This may be attributed, at least in part, to the reduced expression of N-cadherin in TSP1<sup>−/−</sup> PCs (35). In addition, lack of TSP1 did not affect the rate of apoptosis in these cells. TSP1<sup>−/−</sup> PCs expressed increased levels of TSP2 and fibronectin and decreased levels of α<sub>6</sub>β<sub>3</sub>-integrin and were less adhesive compared with TSP1<sup>+/+</sup> PC. TSP1<sup>−/−</sup> PCs did not express detectable amounts of eNOS or iNOS but expressed higher levels of Akt1. To our knowledge, this is the first report on the isolation and culture of retinal PCs from wild-type and transgenic mice demonstrating an important role for TSP1 in the proliferation and migration of PCs.

The ability to culture PCs has resulted in a dramatic increase in our understanding of their function in vascular homeostasis. The culture of PCs from genetically modified mice will allow us to gain a more detailed understanding of the functional consequences that specific genes have on vascular function. Previously, preparation of mouse retinal PCs has been difficult and tedious. Here, we report a method for the routine isolation and propagation of retinal PCs from immunomice. The immunomouse expresses a thermolabile strain (tsA58) of the SV40 large T antigen (taA58 Tag) driven by an inducible major histocompatibility complex H-2K promoter, thus eliminating many intrinsic problems with immortalized lines (28). T antigen expression is functionally evident at the reduced temper...
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Fig. 10. TSP1−/− retinal PCs exhibited altered production of ECM proteins and Akt1 activation. A: levels of various ECM proteins, including TSP1, TSP2, fibronectin (FN), and tenascin C, were determined by Western blot analysis of cell lysates and conditioned medium as described in MATERIALS AND METHODS. TSP1+/+ PCs expressed significant amounts of TSP1, which was mainly cell associated, whereas TSP1−/− cells lacked TSP1. TSP1−/− PCs produced increased levels of TSP2 and FN but showed similar levels of tenascin C. β-Actin was used as a loading control for cell lysates. B: levels of Akt1 and its phosphorylated (active) form (pAkt1), as well as its downstream target endothelial nitric oxide synthase (eNOS), were evaluated by Western blot analysis of cell lysates as described in MATERIALS AND METHODS. Please note the increased levels of Akt1 and its phosphorylated (active) form in TSP1+/+ compared with TSP1−/− PCs. We were unable to detect eNOS expression in retinal PCs. These experiments were repeated with three isolations of PCs with similar results.

The staining of the tumor vasculature with PDGF-Rβ and mature PC markers, including NG2, desmin, and α-smooth muscle actin, showed only a partial overlap, suggesting that only a subset of PDGF-Rβ-positive perivascular cells express mature PC markers (43). It was, therefore, hypothesized that the subset of perivascular cells that express PDGF-Rβ and mature PC markers represents an intermediate, more differentiated pool of cells derived from a progenitor pool that further matures into PDGF-Rβ-negative cells. Our study (7) demonstrated that the cells isolated here express significant amounts of perivascular and mature PC markers as well as a number of mesenchymal stem cell markers. However, the expression of these perivascular markers in vivo may be influenced by the interaction of perivascular cells with vascular ECs as well as in response to factors produced by ECs (43). These intercellular interactions are essential for the survival of EC and the integrity of the blood vessels and require further study.

The interactions of perivascular cells with each other and with ECs through N-cadherin are important in the recruitment of VSMCs/PCs and stabilization of blood vessels (33, 35). We showed that retinal PCs expressed significant amounts of N-cadherin, which was decreased in TSP1−/− PCs (Fig. 1). This is consistent with reduced migration of these cells in response to PDGF in vitro (Fig. 4) and lack of maturation and stabilization of the developing retinal vasculature in vivo, without a significant impact on the number of PCs/VSMCs (35, 46). Our hypothesis is that the predominant expression of TSP1 at later stages of vascular development is essential for the appropriate development and maturation of retinal blood vessels and promotion of the quiescent, differentiated state of the endothelium. This could be mediated, at least in part, through the homotypic interaction of N-cadherin on ECs and PCs and/or PCs (35). Lack of N-cadherin in ECs has been recently shown to severely compromise vascular development and angiogenesis (29). Thus, appropriate expression of N-cadherin, both on ECs and PCs, is essential for the proper migration of PCs, maturation, and stabilization of developing blood vessels, which may be impacted by TSP1 expression.

Another important intercellular interaction between ECs and perivascular cells that is important in blood vessel maturation is mediated through αvβ3-integrin (on proliferating ECs) and VCAM-1 (on proliferating perivascular cells). The antagonism of these interactions prevents the adhesion of perivascular cells to proliferating ECs, resulting in the death of ECs and perivascular cells and inhibition of angiogenesis (14). We observed significant expression of VCAM-1 on proliferating PC (Fig. 3), which was minimally affected by the absence of TSP1. These cells also expressed ICAM-1, as previously reported in rat PCs (24). Thus, expression of VCAM-1 in PCs is minimally influenced by TSP1 levels. This is also consistent with the recruitment and coverage of retinal blood vessels in TSP1−/− mice (46). Thus, lack of TSP1 minimally affects EC/PC interactions through αvβ3-integrin/VCAM-1, which may be important in the recruitment and coverage of blood vessels, but decreased N-cadherin in null cells may impact the maturation and functionality of blood vessels (35). The potential role of ICAM-1 in these intercellular interactions remains elusive. PCs also expressed CD36 (the TSP1 angioinhibitory receptor) and endoglin (a transforming growth factor-β accessory receptor) on their surface. Endoglin haploinsufficiency is associated with abnormal vascular development and functions and failure in the appropriate recruitment of perivascular supporting cells (27).
However, little is known about the biological roles of these receptors in PC function and retinal vascularization.

The ability to culture retinal PCs from TSP1−/− mice allowed us to delineate the impact that lack of TSP1 has on retinal PC properties. Our results showed that TSP1−/− retinal PCs proliferated at a significantly slower rate compared with TSP1+/+ retinal PCs (Fig. 5). Expression of PDGF-BB by ECs is important in the recruitment of perivascular supporting cells into newly forming blood vessels. These interactions also require the expression of PDGF-Rβ on the surface of perivascular cells allowing for paracrine signaling (1). Although we observed no significant differences in the basal migration of retinal PCs, lack of TSP1 was associated with an attenuation of PC migration in response to PDGF-BB (Fig. 6). We also observed a proliferative response to PDGF-BB in TSP1+/+ PCs, which was attenuated in TSP1−/− PCs (Fig. 5). Thus, TSP1 expression is crucial for the promigratory and proproliferative responses of PCs to PDGF, as previously demonstrated for aortic VSMCs (19, 31).

The observed decreased rate of proliferation in TSP1−/− retinal PCs is in contrast to the similar rate of proliferation reported for TSP1−/− and TSP1+/+ VSMCs from the mouse aorta (19). This may be attributed, at least in part, to the tissue of origin (the retina vs. aorta) and perhaps other experimental conditions. However, the increased production of TSP2 observed in TSP1−/− PCs (Fig. 10A) suggests that the role of TSP1 in the proliferation and migration of PCs, and their appropriate response to PDGF-BB, is unique to TSP1 and cannot be compensated for by an increase in TSP2 expression. The increased Akt1 expression and activity may contribute to the survival of retinal PCs in the absence of TSP1, an autocrine factor important in PC function. This is consistent with the similar rates of apoptosis observed in TSP1−/− compared with TSP1+/+ cells. The specific impact of increased TSP2 and fibronectin expression, and perhaps their downstream effectors, including Akt1, on PC function remains the subject of future investigation.

Alterations in the production of ECM proteins and cell adhesion molecules have a significant impact on cell proliferation and migration. TSP1−/− PCs were less adherent on fibronectin and vitronectin. This may be attributed, at least in part, to changes in integrin activity and/or expression. Although we observed similar levels of α5β1-integrin expression (the major fibronectin receptor), αvβ3-integrin expression (a receptor for fibronectin and vitronectin) was significantly reduced in TSP1−/− PCs (Fig. 8). TSP1 also interacts with αvβ3-integrin through CD47 (integrin-associated protein) impacting VSMC adhesion and migration (21). Furthermore, expression of CD47 and its interaction with TSP1 have been shown to be important in the proliferative and migratory responses of VSMCs to IGF-I (30). However, the role of these interactions in the modulation of retinal PC adhesion and migration requires further investigation.

In summary, we described a simple method for the isolation and culture of retinal PCs from TSP1+/+ and TSP1−/− immunomice. These cells can be readily propagated at permissive temperature and retained their PC characteristics in long-term culture. The ability to rapidly propagate the cells at permissive temperature and then switch them to nonpermissive temperature eliminates the effects of large T antigen on cellular properties. The comparison of retinal PCs from TSP1+/+ and TSP1−/− mice demonstrated an important role for TSP1 in PDGF-mediated promigratory and proproliferative functions. The comparison of these cells under normal or pathological conditions, such as hypoxia or hyperglycemia, will provide additional insight into the role that TSP1 may play in retinal vascular homeostasis and neovascularization. Furthermore, these cells may provide an unlimited supply of mesenchymal stem cells for the study of their contribution to physiological and pathological retinal vascularization.

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