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Isolation and characterization of murine retinal astrocytes

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Purpose: To isolate and characterize primary retinal astrocytes in culture (RAC) from wild-type and transgenic mice to aid the study of their properties in vitro.

Methods: Astrocytes were isolated from wild-type and transgenic Immortomice by collagenase digestion of the retina. Affinity purification using magnetic beads coated with anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) was used to remove retinal endothelial cells. The remaining cells were cultured and expanded. The majority of these cells were identified as astrocytes. These cells were characterized for expression of astrocytic markers using fluorescence-activated cell sorting (FACS) and immunostaining analysis. The expression of various integrins and other cell adhesion molecules on the surface of retinal astrocytes, their adhesion to various matrix proteins, their migration, and their ability to organize on Matrigel were determined.

Results: Here we describe a method for the isolation of RAC from wild-type and thrombospondin-1 deficient ($TSP1^{-/-}$) mice. Our results indicated that nearly 100% of cells isolated expressed the astrocytic markers GFAP, NG2, Pax2, and vimentin. These cells were successfully passaged and maintained in culture for several months without a significant loss in expression of astrocytic markers. The RAC expressed $\alpha v\beta 3$ integrin and other cell adhesion molecules on their surface. The $TSP1^{-/-}$ RAC adhered more strongly to fibronectin and vitronectin compared to the wild-type cells, while neither cell types adhered to collagen and laminin. Wild-type and $TSP1^{-/-}$ RAC exhibited similar migratory characteristics despite alterations in their adhesive properties and production of various matrix proteins. Also, these cells, like endothelial cells, similarly organized into a network in Matrigel.

Conclusions: The RAC can be readily obtained from wild-type and transgenic mice. This facilitates the comparison and identification of specific gene functions in RAC compared to astrocytes prepared from other sites of central nervous system.

Astrocytes are glial cells that provide support, nutrients, and insulation for neurons in the central nervous system and are important for maintaining normal extracellular glutamate levels [1,2]. They promote synapthogenesis in neurons [3] and may provide a source of neural stem cells in adult central nerves system [4,5]. Astrocytes play a role in endothelial cell differentiation and blood-brain and blood-retina barrier functions [6-9]. Retinal astrocytes in culture (RAC) are derived from astrocyte precursor cells in the embryonic optic nerve that migrate from the optic nerve into the retina [10-13]. RAC are critical to the formation of primary vasculature of the retina, and are believed to migrate ahead of the developing vascular network laying down the scaffolding that is followed by endothelial cells [12,14,15]. In the murine retina, the superficial vascular network forms along an existing template of astrocytes, using endothelial cell filapodial extensions and R-cadherin cell adhesion molecules for direction [16]. Once vessel maturation begins, the retinal astrocytes become associated exclusively with endothelial cells.

In the retina, vascular development is characterized by the initial formation of a superficial primordial layer of vessels [17] followed by vascularization of the peripheral and inner retina, and thought to occur through both vasculogenesis and angiogenesis in humans [18]. Studies of retinal vascularization have sought to study the relationships among vascular cells including endothelial cells, pericytes, and astrocytes, and determine the production and effect of positive and negative regulatory factors [19]. RAC play an important role in maintaining the integrity of the retinal vascular function and their alterations under pathological conditions, such as diabetes and ischemia, contributes to vascular malfunctions and neovascularization [6,20]. Unfortunately, there is little known about how RAC accomplish these functions and the identity of the key players involved.

Understanding retinal vascular development is crucial in discovering mechanisms whose alterations contribute to various eye diseases, such as retinopathy of prematurity, proliferative diabetic retinopathy, and age-related macular degeneration. The developing mouse retinal vasculature provides a unique opportunity to study all aspects of vascular development and angiogenesis, and the mouse provides the ability to assess genetically modified and engineered effects on retinal vascularization. The pattern of retina vascularization is similar between the mouse and human [21,22]. In both species vessels originate at the optic nerve head and spread over the inner surface of the retina, forming a dense vasculature network that astrocytes associate with [18,21,23]. The study of

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RAC will provide further insight into the relationships between these cells and the developing retinal vasculature, and their role in other processes such as injury response or eye diseases [24].

Astrocytes are often identified by their morphology and the presence of glial fibrillary acid protein (GFAP), NG2, Pax2, nestin, and vimentin [9,13]. GFAP, vimentin, and nestin are the main intermediate filament proteins in astrocytes and are differentially regulated [25]. Expression of vimentin and nestin is characteristic of intermediate filament of the immature astrocytes, whereas vimentin and GFAP are expressed in the mature astrocytes. GFAP expression is generally upregulated in activated astrocytes. Pax2 is a member of Pax family of transcription factors with important roles in tissue morphogenesis and pattern formation. Pax2 expression is essential for appropriate development of many organs including eye and kidney, and the central nervous system.

Here we describe a method for routine isolation and propagation of mouse RAC from wild-type and Thrombospondin-1 deficient ($TSP1^{-/-}$) mice. We demonstrate that mouse RAC can be readily isolated, expanded, and retain their astrocytic markers in culture. To our knowledge this is the first report of culturing of murine RAC.

METHODS

Experimental animals: The mice used for these studies were maintained and treated in accordance with the Association for Research in Vision and Ophthalmology resolution for the use of animals in research. Immortomice expressing a temperature sensitive SV40 large T antigen were obtained from Charles River Laboratories (Wilmington, MA). $TSP1^{-/-}$ mice in the C57BL/6 background were generated as previously described [22,26]. $TSP1^{-/-}$ mice were crossed with an Immortomouse, and the $TSP1^{+/-}$ Immortomice were crossed to obtain $TSP1^{-/-}$ Immortomice. These mice were identified by PCR analysis of DNA isolated from tail biopsies. The PCR primer sequences were as follows in Table 1.

Table 1. Screening primers

Name	Primer
immorto	F: 5'-CCTCTGAGCTATTCCAGAAGTAGTG-3'
	R: 5'-TTAGAGCTTTAAATCTCTGTAGGTAG-3'
Neo	F: 5'-TGCTCTCCATCTGCACGAGACTAG-3'
	R: 5'GAGTTGCTTGTGGTGAACGCTCAG-3'
TSP1	F: 5'-AGGGCTATCTGGAATTAATATCGG-3'
	R: 5'-GAGTTTGCTTGTGGTGAACGCTCAG-3'

These primers were used for mouse genotyping by PCR. The PCR conditions were as follows: 94 °C 3 min, and 35 cycle of 94 °C 20 s, 59 °C 10 s, and 72 °C 45 s. The PCR products were analyzed on a 2% agarose gel. The TSP1 reverse primer was used with the Neo forward primer for the mutant allele or TSP1 forward primer for the wild-type allele. The wild-type and mutant alleles yield PCR products of 700 and 400 bp, respectively. The Neo and immorto primer pairs yield PCR products of 650 and 350 bp, respectively.

Preparation of antibody coated magnetic beads: Sheep anti-Rat Dynabeads (20 μl; Dynal Biotech, Lake Success, NY) were rinsed in serum-free Dulbecco's Modified Eagle's Medium (DMEM) and incubated at 4 °C overnight with the rat anti-mouse PECAM-1 (3 μg; Mec13.3; BD Biosciences). Antibody coated magnetic beads were then rinsed three times in DMEM containing 10% fetal bovine serum (FBS).

Tissue preparation, isolation, and culture of retinal astrocytes: RAC were isolated from mouse retina by collecting retinas from one litter of 4 week old (6 to 7) mice using a dissecting microscope. Retinas (12 to 14) were rinsed with serum-free DMEM, pooled in a 60 mm dish, minced and digested for 45 min with collagenase Type I (1 mg/ml; Worthington, Lakewood, NJ) in serum-free DMEM at 37 °C. Cells were rinsed in DMEM containing 10% FBS and centrifuged for 5 min at 400x g. Digested cells were rinsed again in DMEM containing 10% FBS, and filtered through a double layer of sterile 40 μm nylon mesh (Sefar America Inc., Fisher Scientific, Hanover Park, IL). Cells were centrifuged for 5 min at 400x g and medium was aspirated. Cells were washed twice with DMEM containing 10% FBS, resuspended in 1 ml of DMEM containing 10% FBS in a 1.5 ml microfuge tube with Mec13.3 coated sheep anti-rat magnetic beads, and were gently rocked for 1 h at 4 °C. Using a Dynal magnetic tube holder, cells not bound to magnetic beads were collected and washed in DMEM containing 10% FBS. Cells were plated in growth medium in a single well of a 24 well plate coated with human fibronectin (2 μg/ml in serum-free DMEM; BD Biosciences, Bedford, MA), and incubated at 33 °C with 5% CO_2. The cells bound to magnetic beads are generally used for culturing retinal endothelial cells as we described recently [27]. RAC were grown in DMEM containing 10% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% non-essential amino acids, 100 μg/ml streptomycin, 100 U/ml penicillin, freshly added heparin at 55 U/ml (Sigma, St. Louis, MO), endothelial growth supplement 100 μg/ml (Sigma), and the murine recombinant interferon-γ (R&D, Minneapolis, MN) at 44 U/ml. Cells were maintained at 33 °C with 5% CO_2. Cells were progressively passed to larger plates, maintained, and propagated in 1% gelatin-coated 60 mm dishes.

FACScan analysis: RAC from 60 mm culture plates were rinsed with phosphate buffered saline (PBS) containing 0.04% EDTA and incubated with 2 ml of cell dissociation solution (Catalog number C5914, Sigma). Cells were removed from the plates with 5 ml of DMEM containing 10% FBS, washed once with 5 ml of TBS (20 mM Tris, pH 7.6, 150 mM NaCl), resuspended in 0.5 ml of blocking solution (TBS with 1% goat serum), and kept on ice for 20 min. Cells were then incubated with 0.5 ml of rabbit anti-GFAP (Dako, Carpinteria, CA), rabbit anti-NG2, mouse anti-αvβ3 integrin, rabbit anti-α2 or α3 integrin, rat anti-α6 integrin (Chemicon, Temecula, CA), rat anti-mouse VCAM-1 or ICAM-1 (BD Pharmingen), rat anti-mouse CD47 (a gift from Dr. W. A. Frazier, Washington University, St. Louis, MO), mouse anti-SHPS-1 (BD Transduction, San Jose, CA) antibodies prepared in TBS with 1% BSA at 2 μg/ml, and kept on ice for 30 min. Cells were then washed twice with TBS containing 1% BSA, incubated with 0.5 ml of

an appropriate secondary antibody conjugated with FITC prepared in TBS with 1% BSA, and kept on ice for 30 min. Following incubation, cells were washed twice with TBS containing 1% BSA, resuspended in 0.5 ml TBS with 1% BSA, and analyzed with a FACScan caliber flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Immunoblot analysis: For TSP1 analysis, cells were plated at $1x10^6$ cells per 60 mm dishes and allowed to reach approximately 95% confluence. Cells were then rinsed once with serum-free medium and incubated with serum-free DMEM for two days. Conditioned medium was collected and clarified by centrifugation. Samples (100 µl each) were mixed with 6X SDS sample buffer and analyzed by 4-20% SDS-PAGE (Invitrogen, Carlsbad, CA). Proteins were transferred to nitrocellulose membrane, and the blot was incubated with anti-TSP1 (A6.1, Neo Markers, Fremont, CA) antibody. The blot was washed, incubated with an appropriate secondary antibody, and developed using enhanced chemiluminescence detection system (ECL; Amersham, Piscataway, NJ). The blot was stripped and probed with rat anti-tenascin-C (Chemicon), rabbit anti-rat fibronectin (Invitrogen, Carlsbad, CA) and anti-mouse TSP2 (BD Pharmingen, San Diego, CA). The cells were also lysed in 20 mM Tris pH 7.4, 2 mM EDTA solution, sonicated briefly, and similarly analyzed along with the conditioned medium.

Indirect immunofluorescence assays: RAC were plated on fibronectin coated glass coverslips (2 µg/ml in serum-free medium) and allowed to reach 70% confluence. Cells were then rinsed twice with PBS, fixed with 4% paraformaldehyde (PFA) for 10 min on ice, and washed three times with PBS. Cells were then incubated with a rabbit anti-vimentin (Sigma) or a rabbit anti-Pax2 (Santa Cruz) antibody for 30 min at 37 °C, rinsed twice with TBS, and incubated with an appropriate CY3-conjugated secondary antibody for 30 min at 37 °C. After washing three times with TBS, the coverslips containing cells were mounted onto glass slides and photographed using a Zeiss fluorescence microscope (Axiophot, Zeiss, Germany) in a digital format. Negative controls in which the primary antibodies were omitted were analyzed under similar conditions.

Cell adhesion assays: The RAC adhesion to various extracellular matrix proteins was determined as recently described [28]. 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+} (Ca/Mg) overnight at 4 °C. The next day plates were rinsed four times with TBS containing Ca/Mg, blocked for 1 h with 200 µl 1% BSA prepared in TBS with Ca/Mg for at least 1 h at room temperature. Cells were removed using 3 ml of dissociation solution (Sigma), washed once with TBS, and resuspended in HEPES buffered saline (25 mM HEPES pH 7.6; 150 mM NaCl) containing 4 mg/ml of BSA at $6x10^5$ cells/ml. Next, blocking solution was removed, 50 µl of TBS with Ca/Mg was added to each well, and 50 µl of cell suspension was added to each of triplicate wells. Cells were then allowed to adhere for 90 min and non-adherent cells were removed by gently washing the wells with 200 µl of TBS with Ca/Mg until no cells were left in BSA-coated wells. The number of adherent cells was quantified by measuring the intracellular phosphatase activity [28].

Scratch wound assays: The migratory characteristics of RAC were determined by scratch wound assays. Confluent

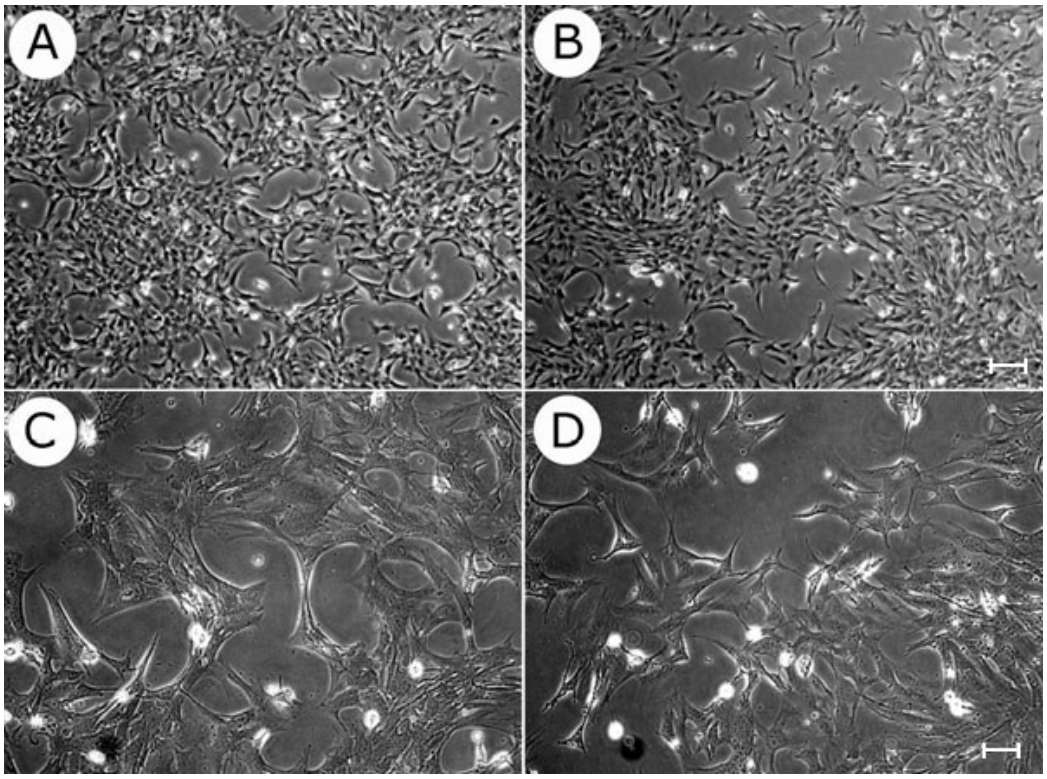

Figure 1. Morphology of mouse retinal astrocytes in culture. Retinal astrocytes in culture (RAC) from wild-type (**A**,**C**) and $TSP1^{-/-}$ (**B**,**D**) mice were cultured on gelatin coated plates. Cells were photographed using a Nikon phase microscope in a digital format (top 2 panels, 40x; bottom 2 panels, 100x). RAC from wild-type and $TSP1^{-/-}$ mice share a similar morphology. The scale bar represents 40 µm in **A**,**B** and 100 µm in **C**,**D**.

monolayers of cells plated on 60 mm dishes were wounded using a micropipette tip, rinsed with PBS to remove detached cells, and wound closure was monitored using still photography at indicated time points [28].

Three dimensional culture of retinal astrocytes: To determine the ability of cells to form three dimensional structures, approximately $2x10^5$ cells were plated in 2 ml of serum-free growth medium on a 35 mm dish coated with 0.5 ml of 10 mg/ml Matrigel (BD Biosciences). Cells were incubated at 37 °C for 24 h and photographed in a digital format [27,28].

RESULTS

Isolation of murine RAC: The majority of primary astrocyte cultures are prepared from brain and/or optic nerve head [13,29,30]. To our knowledge, the isolation and culture of RAC from wild-type and transgenic mice has not been previously reported. Using wild-type and TSP1$^{-/-}$ Immortomice, we have successfully isolated and characterized RAC. The immortomouse ubiquitously expresses a temperature sensitive large T antigen. Therefore, crossing of wild-type or transgenic mice with immorto mice allows isolation of desired cells from these mice which express the large T antigen at the permissive temperature (33 °C). This allows the cells to readily proliferate. However, the growth of cells at the nonpermissive temperature (37 °C) results in loss of large T antigen and its potential unwanted effects on cellular behavior [27]. These cells were prepared by digestion of retinal tissue with collagenase type I and removal of retinal endothelial cells using PECAM-1 bound magnetic beads. The remaining cells were then plated in a single well of a 24-multiwell plate coated with fibronectin and allowed to reach confluence for 2-3 weeks. Cells were then passed to a single 60 mm tissue culture dish. This resulted in the isolation of a pure population of retinal astrocytes whose identity was confirmed as outlined below. Figure 1 shows the morphology of retinal astrocytes prepared from wild-type and TSP1$^{-/-}$ mice. These cells exhibit a similar morphology when plated on gelatin-coated (Figure 1) or uncoated plates (data not shown). Primary astrocytes from brain and/or optic nerve typically exhibit a flattened, polygonal morphology with numerous contractile actinomyosin stress fibers [13,29,30].

To confirm that the isolated retinal cells are astrocytes, we examined the expression of astrocytic markers including GFAP, NG2, vimentin, and Pax2. GFAP is member of the intermediate filament family that provides support and strength to cells and is expressed in astrocytes and neural stem cells. NG2 is a cell membrane-associated chondroitin sulfate proteoglycan expressed by several types of immature progeni-

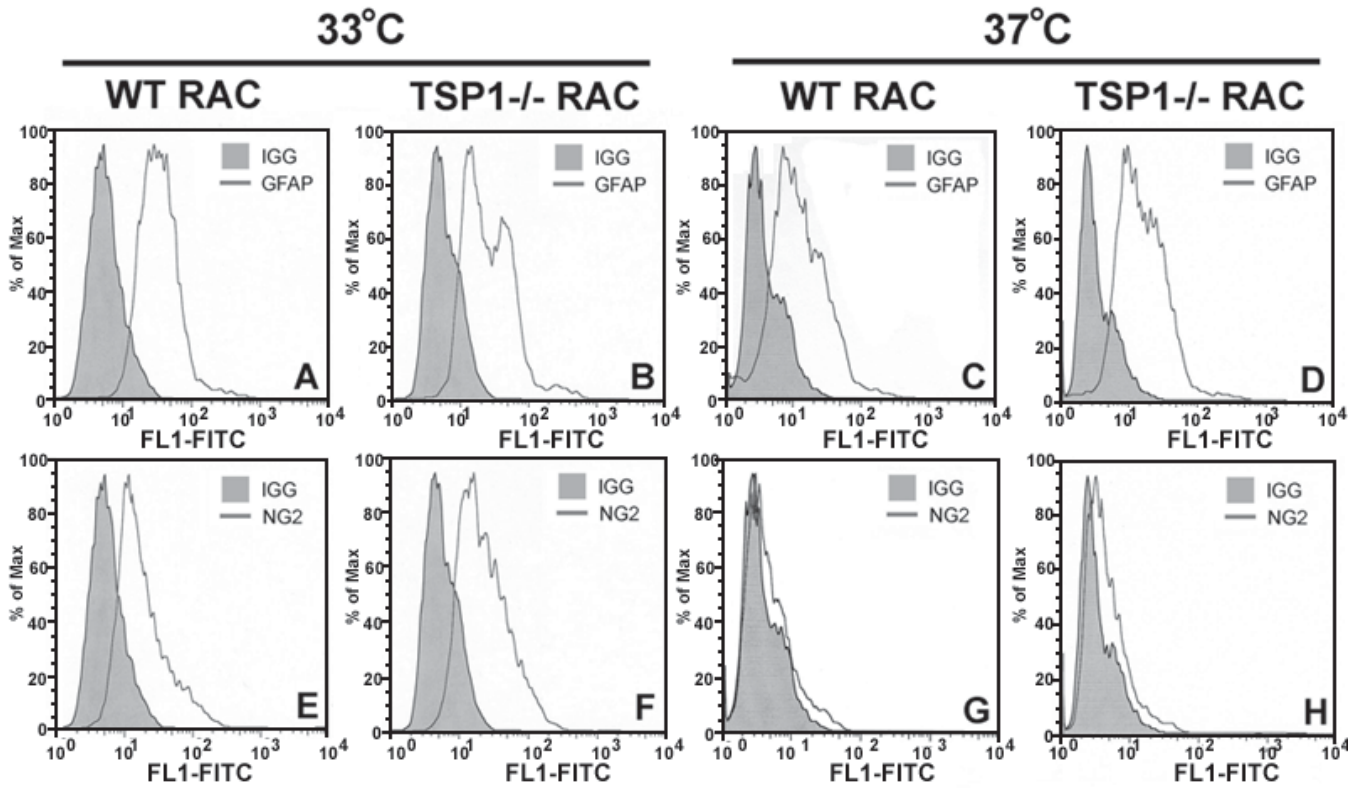

Figure 2. Expression of astrocytic markers in retinal astrocytes in culture. Mouse RAC prepared from wild-type (**A**,**C**,**E**,**G**) and TSP1$^{-/-}$ (**B**,**D**,**F**,**H**) knockout mice were examined for expression of GFAP and NG2 at permissive (33 °C; **A**,**B**,**E**,**F**) or nonpermissive (37 °C without interferon-γ; **C**,**D**,**G**,**H**) by FACS analysis. The shaded areas show staining in the absence of primary antibody. Note similar expression of NG2 and GFAP in these cells under permissive growth conditions. However, only NG2 expression was lost when cells were grown under nonpermissive conditions. These experiments were performed at least three times with similar results.

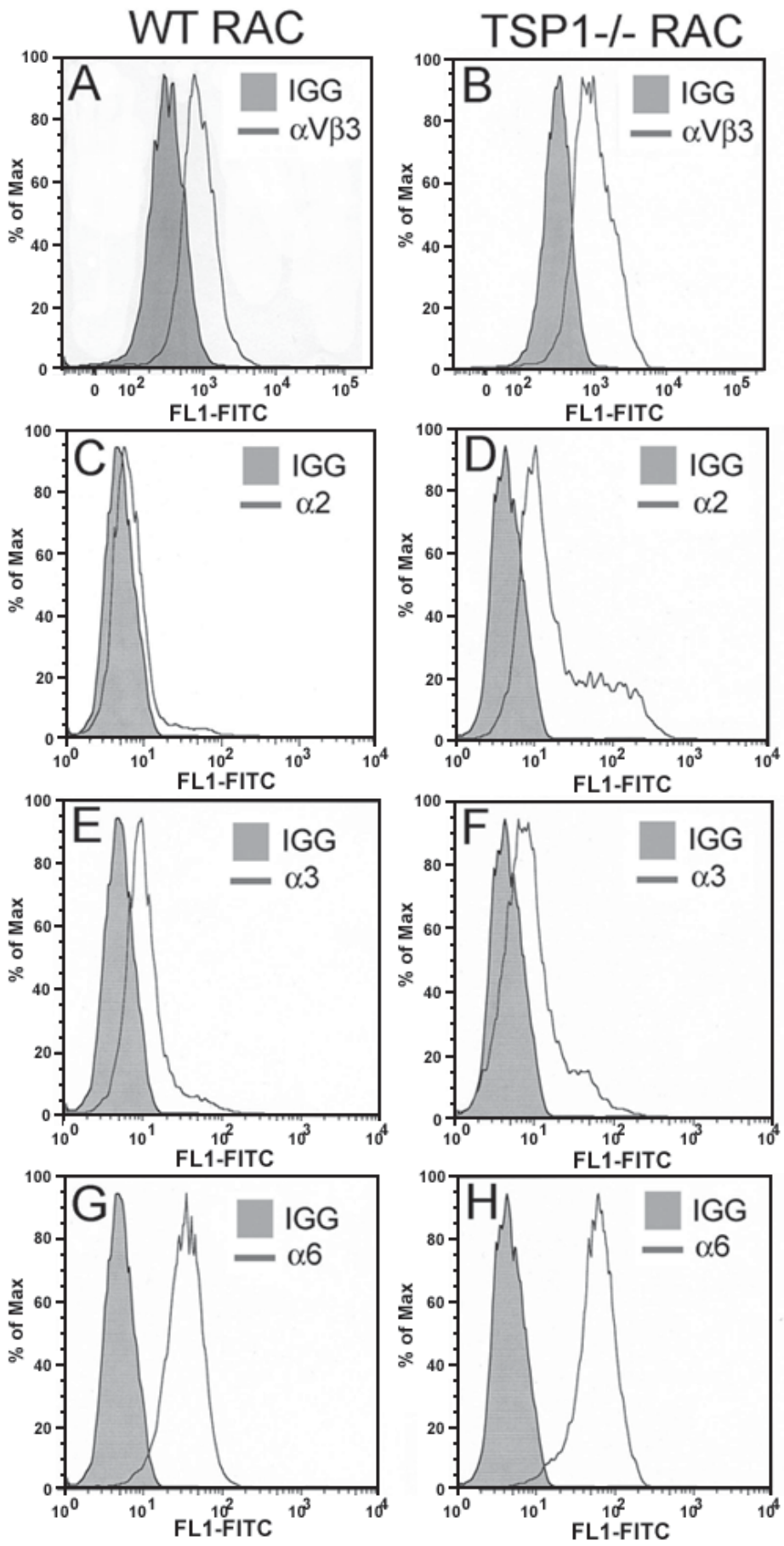

Figure 3. Expression of integrins in retinal astrocytes in cultures. Expression of αvβ3 (**A**,**B**), α2 (**C**,**D**), α3 (**E**,**F**), and α6 (**G**,**H**) integrins was determined using FACS analysis as described in Methods. The shaded graphs show staining in the absence of primary antibody (control IgG). Note similar expression of these integrins in wild-type and TSP1$^{-/-}$ RAC with the exception of α2 integrin. Each of these experiments was performed at least three times with similar results.

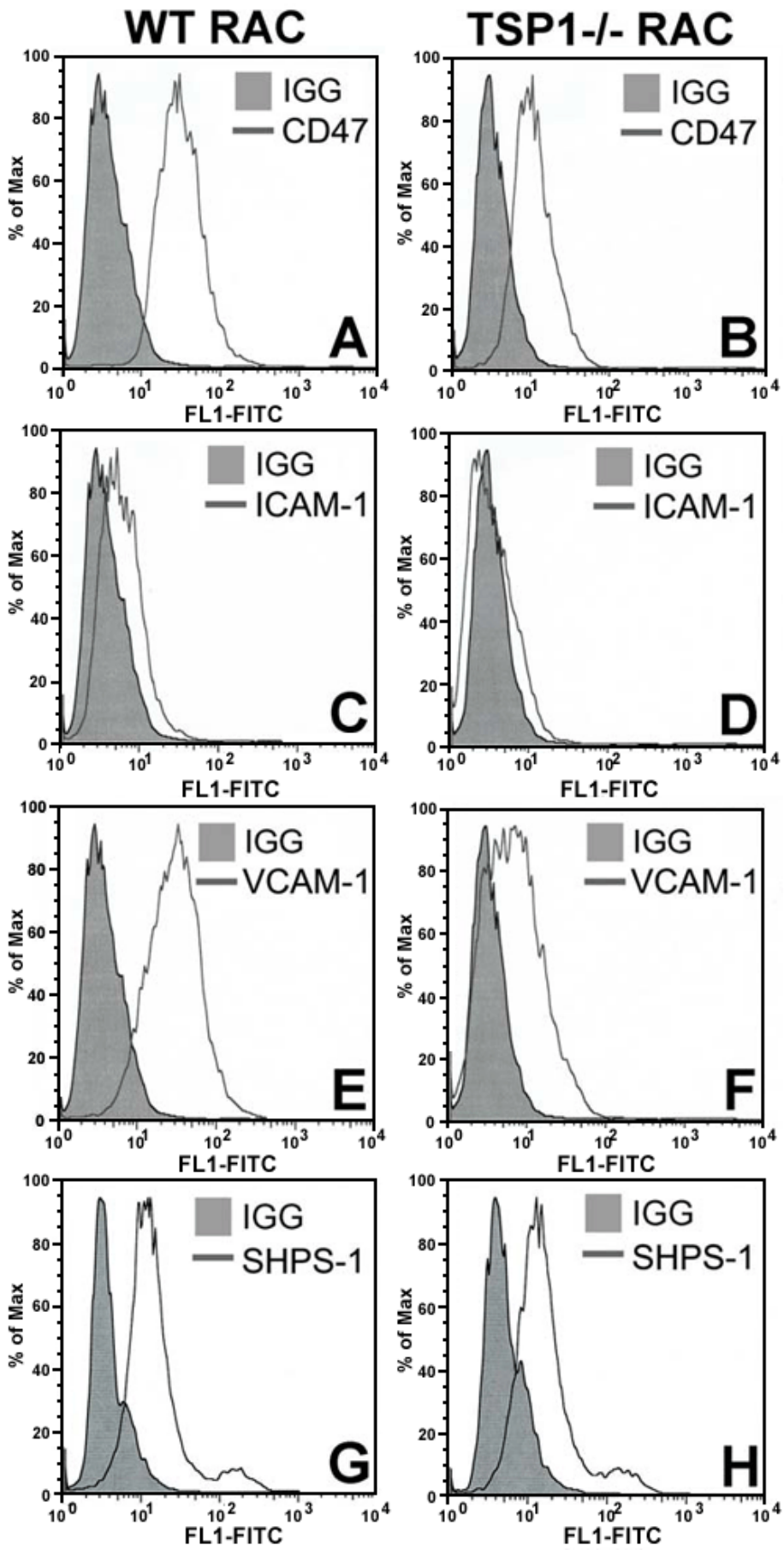

Figure 4. Expression of other cell adhesion molecules in retinal astrocyte cultures. Expression of CD47 (**A**,**B**), ICAM-1 (**C**,**D**), VCAM-1 (**E**,**F**), and SHPS-1 (**G**,**H**) was determined using FACS analysis as described in Methods. The shaded graphs show staining in the absence of primary antibody (control IgG). Note decreased expression of CD47, ICAM-1, and VCAM-1 in TSP1$^{-/-}$ RAC, while expression of SHPS-1 was similar in these cells. Each of these experiments was performed at least three times with similar results.

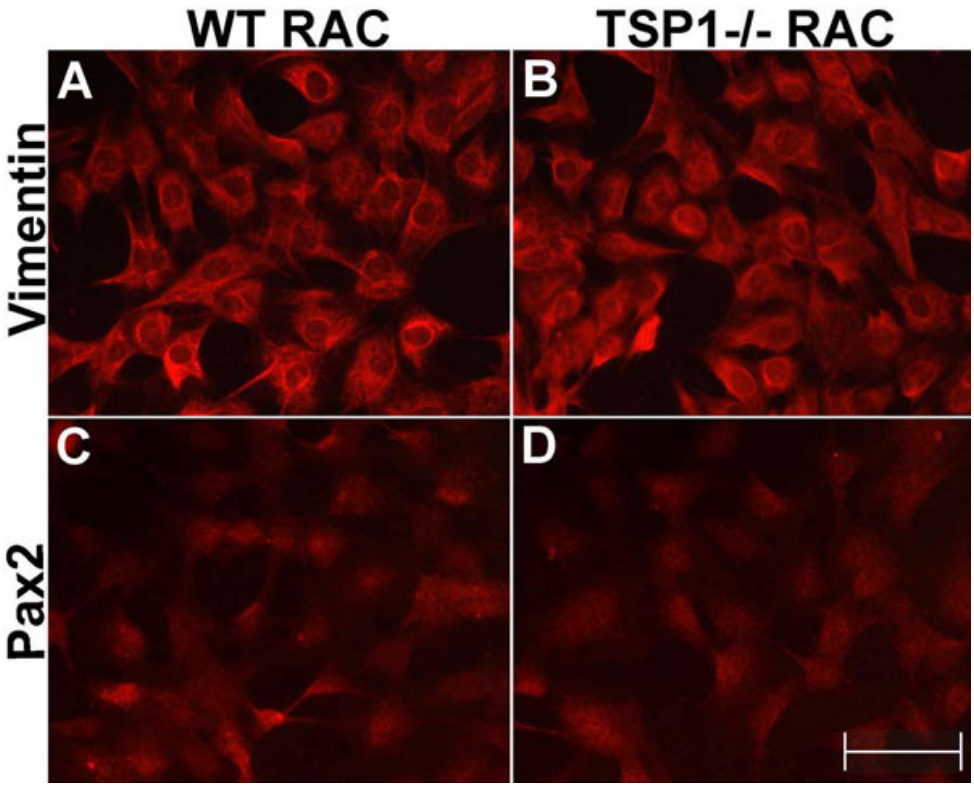

Figure 5. Expression of vimentin and Pax2 in retinal astrocyte cultures. Expression of vimentin and Pax2 in wild-type (**A**,**C**) and TSP1$^{-/-}$ (**B**,**D**) RAC were determined by indirect immunofluorescence analysis. RAC were plated on glass cover slips coated with fibronectin and stained with antibodies to vimentin (**A**,**B**) or Pax2 (**C**,**D**) as described in Methods. Note similar expression of vimentin and Pax2 in these cells. Vimentin was expressed throughout the cells, while Pax2 staining was nuclear. These experiments were repeated three times with similar results. The scale bar represents 40 μm.

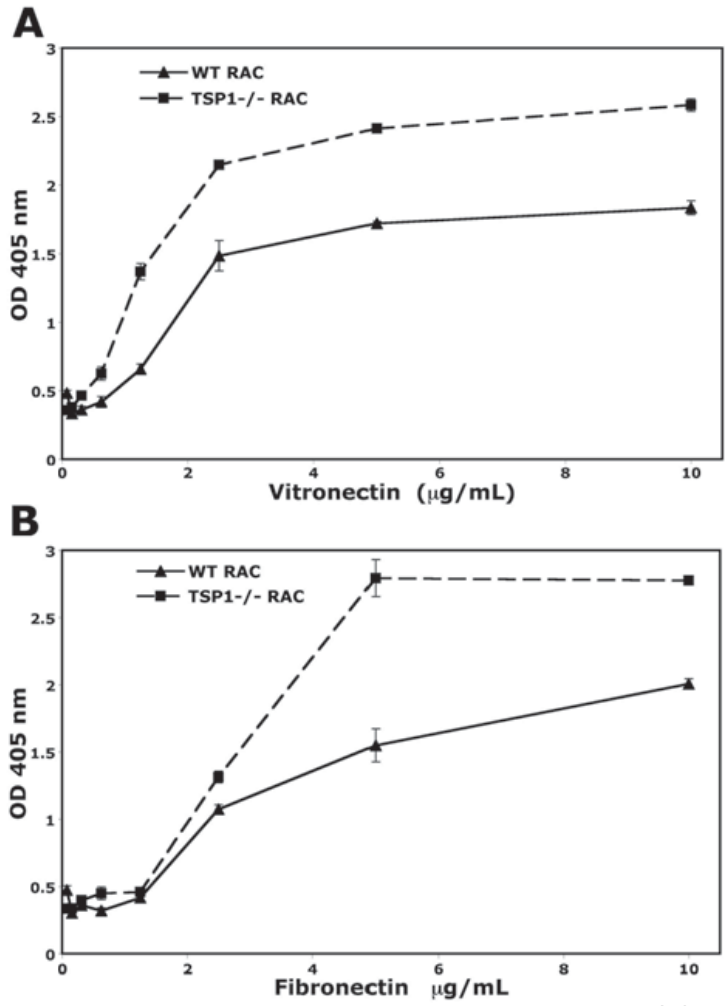

Figure 6. Adhesion of retinal astrocytes to extracellular matrix proteins. Adhesion of wild-type and TSP1$^{-/-}$ RAC to vitronectin (**A**) or fibronectin (**B**) was determined as described in Methods. Briefly, cells were plated on wells coated with different concentration of vitronectin or fibronectin and allowed to adhere for 90 min. Non-adherent cells were removed by rinsing the wells. The number of adherent cells was determined by measuring intracellular phosphatase activity utilizing absorbencies at 405 nm. Note TSP1$^{-/-}$ RAC adhered more strongly to fibronectin and vitronectin compared to wild-type RAC. These experiments were repeated four times with similar results.

tor cells including neuronal cells that have not yet specialized into oligodendrocytes [31]. These cells are generally mitotic and in some cases highly motile. Furthermore, the role of NG2 in proliferation and migration is initiated by its PKC-α mediated threonine phosphorylation [32]. Therefore, NG2 is involved in the signaling mechanisms that control cell proliferation and motility [33].

Figure 2A,B,E,F show that the wild-type and TSP1$^{-/-}$ retinal astrocytes expressed GFAP and NG2 by FACS analysis. To determine whether this expression of NG2 is associated with the proliferative characteristic of cells in the permissive temperature (33 °C) and presence of interferon-γ we examined the expression of GFAP and NG2 in cells grown at the non-permissive temperature (37 °C) in the absence of interferon-γ for 4 days (Figure 2C,D,G,H). Growing the cells that expressed the temperature sensitive large T antigen at 37 °C in the absence of interferon-γ for 48 h resulted in complete loss of large T antigen expression, thus, eliminating its effects on cell proliferation [34]. Interferon-γ is also shown to enhance proliferation of astrocytes [35]. Figure 2G,H show that when cells were grown at the nonpermissive temperature without interferon-γ they lost NG2 expression without a significant effect on GFAP expression (Figure 2C,D). Therefore, expression of NG2 in these cells was associated with their proliferative phenotype in the presence of large T antigen. Astrocytes also express αvβ3 integrin [36,37], its associated protein (CD47), and CD47 receptor SHP substrate 1 (SHPS-1) on their surface. Figure 3 and Figure 4 show that wild-type and TSP1$^{-/-}$ retinal astrocytes expressed similar levels of αvβ3 (Figure 3A,B), α3 (Figure 3E,F), α6 (Figure 3G,H) integrins, and SHPS-1 (Figure 4G,H). However, TSP1$^{-/-}$ cells expressed higher levels of α2 integrin (Figure 3C,D) but reduced levels of CD47 (Figure 4A,B), ICAM-1 (Figure 4C,D), and VCAM-1 (Figure 4E,F) compared to wild-type cells. These cells also expressed similar levels of β1 and β4 integrin on their surface (not shown).

Expression of vimentin and Pax2 in RAC: Our FACS analysis demonstrated that wild-type and TSP1$^{-/-}$ retinal astrocytes expressed similar levels of GFAP and NG2 (Figure 2). To determine whether these cells also express other astrocytic markers, namely vimentin and Pax2, we examined their expression by indirect immunofluorescence. Figure 5A,B showed strong expression of vimentin in retinal astrocytes from wild-type and TSP1$^{-/-}$ mice, respectively. Vimentin staining showed

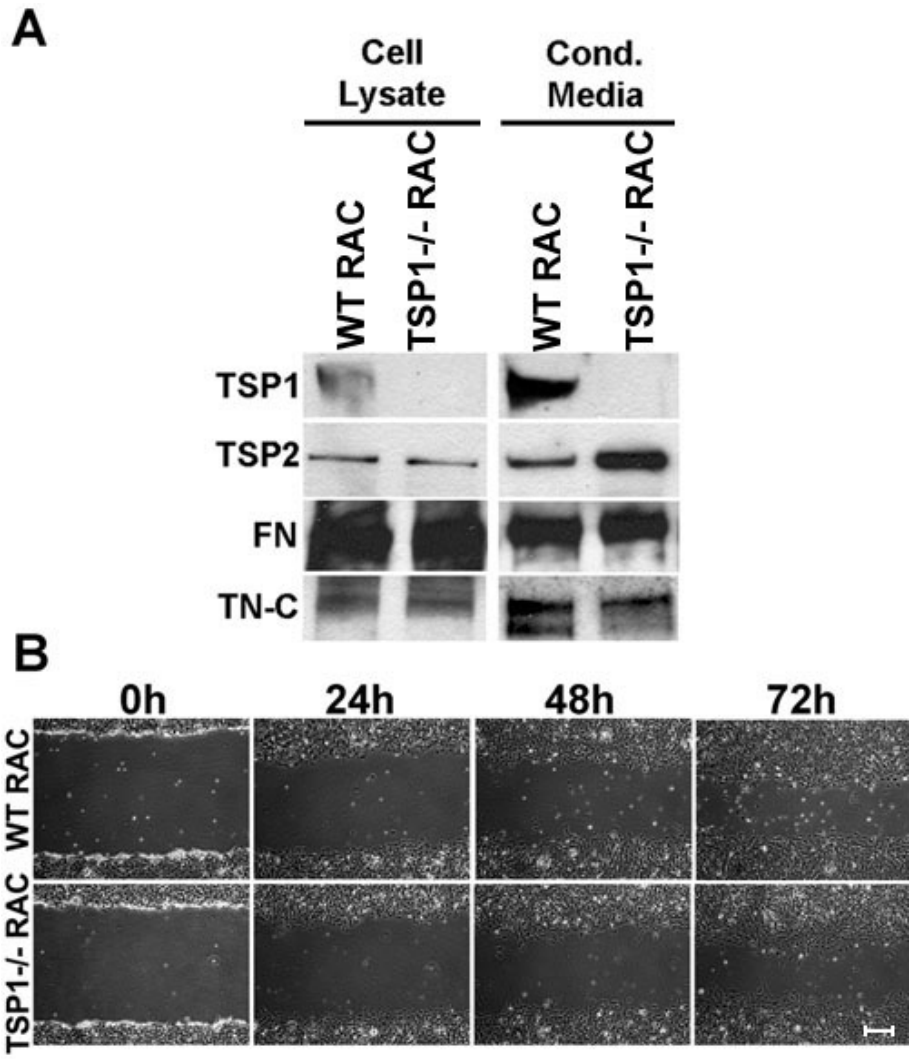

Figure 7. Expression of extracellular matrix proteins and migration of retinal astrocytes. **A**: Fibronectin, TSP1, TSP2, and tenascin-C expression of wild-type and TSP1$^{-/-}$ RAC were examined by western blot analysis of conditioned medium and cell lysates. Note expression of TSP1 in wild-type cells. The same blot was stripped and reprobed with anti-fibronectin, TSP2, and tenascin-C. Note similar expression of fibronectin in these cells. The level of secreted TSP2 was increased while that of tenascin-C was decreased in TSP1$^{-/-}$ RAC. The molecular weights of TSP1, TSP2, FN, and TN-C in monomeric form are about 180 kDa, about 180 kDa, 220 kDa, and 240 kDa, respectively. **B**: Cell migration was determined by scratch wounding of the RAC monolayers and wound closure was monitored by still photography at indicated times. These cells exhibited a similar rate of migration and wound closure. These experiments were repeated three times with similar results. The scale bar represents 40 μm.

uniform staining throughout the cells. Figure 5C,D showed strong Pax2 staining in the nuclei of wild-type and TSP1$^{-/-}$ retinal astrocytes, as expected. The corresponding negative controls in which the primary antibodies were omitted lacked any staining (not shown).

Adhesion and migration of wild-type and TSP1$^{-/-}$ RAC: Astrocytes express TSP1 and TSP2 [3]. TSP1 is an extracellular matrix protein produced by a variety of cell types including endothelial cells, astrocytes, and pericytes. It interacts with the component of the extracellular matrix through multiple receptors modulating various cellular functions and the matrix composition [38,39]. However, the potential role of TSP1 in regulation of retinal astrocytes adhesive and migratory properties remains largely unknown.

We first compared the adhesive properties of wild-type and TSP1$^{-/-}$ RAC on different matrix proteins. Both cell types adhered well to fibronectin and vitronectin (Figure 6A,B) but poorly to laminin and collagen (data not shown). TSP1$^{-/-}$ retinal astrocytes adhered more strongly to fibronectin and vitronectin compared to wild-type retinal astrocytes. We next examined expression of fibronectin, TSP1, TSP2, and tenascin-C in these cells. TSP1$^{-/-}$ RAC expressed similar levels of fibronectin but reduced levels of tenascin-C compared to wild-type cells (Figure 7A). These cells also expressed increased levels of TSP2 but exhibited similar migratory properties as wild-type cells (Figure 7B). Thus, the absence of TSP1 affected adhesive properties of retinal astrocytes and their ability to express different matrix proteins without a significant effect on their ability to migrate.

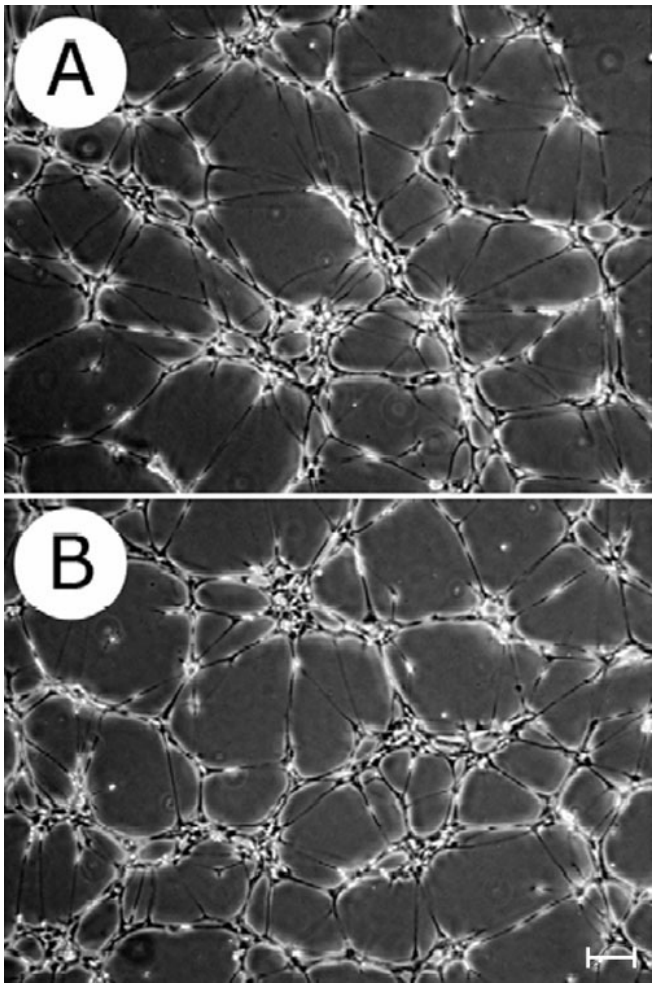

Figure 8. Organization of retinal astrocytes in Matrigel. RAC from wild-type (**A**) and TSP1$^{-/-}$ (**B**) cells were plated in Matrigel as described in Methods. The organization of cells into a network was digitally photographed using a phase-contrast microscope after incubation at 33 °C for 10 h. Both cell types exhibited a similar ability to organize into a network in Matrigel. These experiments were repeated three times with similar results. The scale bar represents 40 μm.

Culture of retinal astrocytes in Matrigel: Prior to retinal vascularization, retinal astrocytes migrate from the optic nerve to create a scaffold-like network for endothelial cells to follow. Endothelial cells are known to organize into a capillary-like network when plated in Matrigel [27,28]. To determine if RAC could organize into a network, and whether this ability is affected in the absence of TSP1, RAC were plated in Matrigel. Figure 8 shows that wild-type and TSP1$^{-/-}$ RAC, very much like wild-type retinal endothelial cells [27], readily organized and formed a network in Matrigel.

DISCUSSION

Astrocytes play an important role in the development of central nervous system and its vasculature. There is great interest in the study of blood-brain and blood-retina barriers and how their alterations under pathological conditions, such as ischemia and diabetes, contribute to vascular malfunctions with serious side effects. Several methods have been used to investigate developing and mature astrocytes, including immunohistochemistry staining and in situ hybridization [18,40,41]. Although the origin of astrocytes in the brain and retina may be the same their differentiated characteristics and functions may well be different. Therefore, the ability to culture astrocytes from the retina may provide additional insight into the unique characteristics of these cells compared to brain and/or optic nerve astrocytes. Furthermore, the ability to culture astrocytes from genetically modified mice will allow us to gain a more detailed understanding of how specific genes affect retinal astrocyte functions in vascular development and neuronal functions.

Here we present a method for routine isolation and propagation of astrocytes from mouse retina. This was accomplished by digesting retinal tissues with collagenase, removing endothelial cells by magnetic beads coated with anti-PECAM-1 antibody, and plating the remaining cells on fibronectin-coated dishes under similar conditions as those described for retinal endothelial cells [27]. We found that nearly all the cells grown under these conditions were astrocytes. These cells expressed GFAP and NG2 at the permissive temperature. However, when cells were grown at a nonpermissive temperature in the absence of interferon-γ they lost NG2 expression but continued expressing GFAP. Therefore, NG2 expression by these cells was consistent with their proliferative and immature characteristics at the permissive temperature in the presence of in-

terferon-γ. Degradation of NG2 by metalloproteinases is essential for the maturation and differentiation of oligodendrocytes, which are required for myelination [37]. In addition, RAC were positive for vimentin and Pax2 expression. Therefore, these cells exhibited markers that are similar to "immature perinatal astrocytes" present in human retina as described by Chang-Ling and coworkers [18] and have the potential to develop into mature astrocytes by losing Pax2 and vimentin expression.

In most mammalian retinas, the primary vascular bed forms by vasculogenesis [7,40,42-44] and is directly influenced by retinal astrocytes [7,42,43]. The production of VEGF by astrocytes at the vascular front is associated with the spread of the retinal vasculature [40,44]. However, it is thought that the contact of endothelial precursor cells with astrocytes inhibits endothelial cell growth and stimulates their elongation, alignment, and morphogenic differentiation [45]. The identity of the factor(s) produced by astrocytes that promote endothelial cell morphogenesis is unknown. However, these factors share many characteristics of TSP1 action on endothelial cells. Indeed, astrocytes in culture expressed TSP1 and, its closely related family member, TSP2 (Figure 7). We observed increased production of TSP2 in RAC from $TSP1^{-/-}$ mice, perhaps compensating for the lack of TSP1 in these cells. TSP1 and TSP2, produced by astrocytes, were recently shown to promote central nervous system synaptogenesis [3]. Interactions of retinal astrocytes with endothelial cells may also promote the differentiation of endothelial cells and the induction and maintenance of blood-retinal/brain barrier characteristics [45,46]. We recently showed $TSP1^{-/-}$ mice exhibit an increased retinal vascular density [22]. This was mainly attributed to an increased number of endothelial cells. The retinal endothelial cells prepared from $TSP1^{-/-}$ mice also maintain a proangiogenic phenotype in culture [27]. Therefore, TSP1 expression by astrocytes and endothelial cells may play an important role in promoting the differentiated, quiescent state of the endothelium and in maintaining the blood-retinal barrier. TSP1 expression by astrocytes is known to be important for migration of oligodendrocytes and appropriate myelination and synaptogenesis of central nervous system [3,37]. Understanding how these astrocytic activities contribute to the development of retinal vasculature and central nervous system will be beneficial. Furthermore, whether productions of specific factors and/or cell-cell interactions contribute to these astrocytic activities require further studies.

Cell adhesion and migration play a central role in a wide variety of biological functions, including wound healing, inflammation, and tumor metastasis. In response to pathogenic events in the brain, astrocytes change their cellular phenotype, can migrate toward the site of the lesion, and are a major cellular component of the glial scar. Such reactive astrocytes have a strong impact on the capacity of axons to regrow and reestablish synaptic connections. Directional cell migration requires an integrated response to multiple external cues, and therefore, is likely to require the participation of different families of molecules including adhesion receptors, actin cytoskeleton, and the Rho family proteins. The studies presented here show that the lack of TSP1 in retinal astrocytes impacted their adhesion to various matrix proteins without significantly affecting their migration. We recently demonstrated that retinal endothelial cells prepared from $TSP1^{-/-}$ mice express increased levels of fibronectin compared to wild-type retinal endothelial cells [27]. These cells were more motile and organized poorly when plated in Matrigel. In contrast, $TSP1^{-/-}$ RAC expressed similar levels of fibronectin compared to wild-type RAC and similarly organized into a network in Matrigel. Therefore, lack of TSP1 differentially affected expression of fibronectin, and perhaps tenascin-C, in the retinal endothelial cell compared to RAC.

Brain astrocytes express αvβ3 integrin on their surface [36,37]. Our results showed that RAC expressed αvβ3 integrin on the cell surface and its expression level was not affected by the absence of TSP1. However, $TSP1^{-/-}$ retinal astrocytes adhered more strongly to vitronectin (the main ligand of αvβ3). Therefore, lack of TSP1 may enhance the affinity and/or avidity of this integrin for its ligand. Astrocytoma cells use αvβ3 and α3β1 integrins for adhesion to TSP1 [47]. We observed similar levels of α3 (Figure 3E,F) and β1 (data not shown) integrin expression in wild-type and $TSP1^{-/-}$ RAC. However, the expression of CD47 was decreased in $TSP1^{-/-}$ RAC compared to wild-type cells (Figure 4A,B), but the levels of SHPS-1 was not affected (Figure 4G,H). Interactions of CD47 with TSP1 and with SHPS-1 affect cell adhesive and migratory properties in the central nervous system [48]. The significance of these interactions and their impact on αvβ3 integrin adhesive properties in retinal astrocytes remains to be determined.

The ability to culture retinal astrocytes from wild-type and $TSP1^{-/-}$ mice will allow us to investigate the role of TSP1 in retinal vascular development and study its contribution to interactions with endothelial cells and pericytes during vascular development in both in vivo and in vitro co-culture experiments. These studies will advance our understanding of the role retinal astrocytes play in retinal vascular development and neurogenesis. Furthermore, it will provide further insight into the role of cell-cell interactions and/or production of specific factor(s) by retinal endothelial cells and astrocytes that are essential for appropriate vascular development and function.

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