Isolation and characterization of corneal endothelial cells from wild type and thrombospondin-1 deficient mice

Elizabeth A. Scheef,¹ Qiong Huang,¹ Shoujian Wang,¹ Christine M. Sorenson,² Nader Sheibani¹,³

¹Departments of Ophthalmology and Visual Sciences, ²Pediatrics, and ³Pharmacology, University of Wisconsin School of Medicine and Public Health, Madison, WI

Corneal endothelial cells (CEC) are derived from the neural crest and form a single layer of cells that line the most posterior segment of the cornea [1,2]. The corneal endothelium serves a barrier role and functions to maintain the corneal stroma in a state of relative dehydration, thereby preserving corneal transparency. This is accomplished by intercellular tight junctions and ion pumps in the CEC membrane [3,4]. Damage to CEC compromises barrier and pump functions, resulting in corneal edema, opacification, and impaired vision. Human CEC possess a limited capacity to regenerate and their loss can lead to permanent denudation of the corneal endothelium [2]. Therefore, CEC are considered an irreplaceable resource. Although in rabbit and rat CEC proliferate to heal the wound, CEC of cats, monkeys, and human rarely undergo mitosis after injury [5-8]. The human CEC heal the wound by migration and hypertrophy [8]. However, they do have the capacity to undergo mitosis in vitro under appropriate conditions [9].

The vascular EC, which line the inner layer of blood vessels, differ from CEC in their tissue of origin. Vascular EC are mesodermally derived while CEC are derived from the neural ectoderm [1]. Many cell surface markers have been described for vascular EC, which have been useful in isolation and culture of vascular EC, including PECAM-1 and VE-cadherin [10]. The availability of such markers has been very limited and, to our knowledge, no one has used such markers for identification and/or isolation of CEC. Despite a number of attempts to identify CEC specific markers, such as generation of antibodies that specifically recognize CEC, the identity of such antigens remains unknown [11].

Primary CEC have been successfully cultured from eyes of many species including human, monkey, bovine, rabbit, rat, and mouse for several decades [9,12-16]. Tissue culture models have made significant contribution to our understanding of growth factors and extracellular matrix molecules during the wound healing process [17-22]. These studies have been limited by the inability for long term cultures. Many of the current culture methods require young donors or special selection procedures to eliminate contaminating fibroblasts. A method of choice for isolation of primary CEC has been isolating descemet’s membrane and allowing the endothelial cells to proliferate from the explants. The majority of these cells exhibit limited capacity to proliferate in culture and require continuous isolation of primary cells from fresh and young tissue. To further expand the life span of these cells, viral oncogenes including SV40 virus large T antigen and human papiloma viruses E6/E7 oncogenes have been utilized for their

Purpose: To isolate and characterize primary corneal endothelial cells (CEC) from wild type and transgenic mice to facilitate the study of their properties in vitro.

Methods: CEC were isolated from wild type or transgenic-immortomice corneas. The Descemet’s membrane was gently peeled from the periphery of the cornea towards the central region and placed into wells of a 96 well tissue culture plate coated with fibronectin in growth medium. Cells that grew out were trypsinized and expanded on fibronectin-coated wells and used for further characterization. CEC were evaluated for expression and localization of specific markers and adhesion molecules by FACS analysis and indirect immunofluorescence staining. The migration properties of CEC were evaluated using a scratch wound and transwell assay, while their ability to undergo capillary morphogenesis was assessed on Matrigel.

Results: Isolation of CEC from transgenic mice has been somewhat challenging and not previously reported. Here we describe a method for isolation of CEC from wild type and thrombospondin-1 deficient (TSP1-/-) immortomice. Our results indicate that nearly 100% of selected cells express B4-lectin and VE-cadherin, but not PECAM-1. These cells were successfully passaged and maintained in culture for several months without a significant loss in expression of these markers. The wild type CEC, like vascular EC, organized and formed a capillary-like cell network on Matrigel. The ability of the CEC from TSP1-/- mice to form such a network was somewhat compromised. This may be attributed, at least in part, to altered adhesive and migratory properties of these cells.

Conclusions: The CEC can be readily obtained from wild type and transgenic mice, which facilitate the comparison and identification of the physiologic role of specific genes in CEC function.
immortalization in culture [16,23,24]. Furthermore, the expression of these viral oncogenes minimally affects the morphology and proliferative properties of these cells [25,26]. Therefore, these immortalized cells can be readily passaged and propagated for a variety of studies, including identification of autocrine and paracrine factors that influence their phenotype and transplantation of wounded corneas [26-33]. Despite much advancement in culturing CEC, information regarding many of the cellular characteristics and biochemical and cell biological properties of these cells require further investigation.

CEC express many of the genes known to be expressed in vascular EC including plasminogen activators, thrombin receptor, thrombospondin-1 (TSP1), vascular endothelial cell growth factor and its receptors, platelet-derived growth factor receptors, fibroblast growth factor receptors, junctional adhesion molecule-C, LDL-receptors, sphingosine-1-phosphate receptors, nitric oxide synthase, endoglin, and inflammatory markers [18,34-43]. CEC also respond to TGF-β by growth inhibition and to cellular stress by upregulation of bcl-2 expression, similar to vascular EC [29,44-47]. Additional insight into the functional importance of CEC has come from recent transgenic mouse studies. For example, mice expressing EGF/TGF-α in their lens or lacking collagen VIII genes exhibit abnormalities in the development of corneal endothelium and anterior segment [48,49].

Thrombospondin-1 (TSP1) or platelet TSP, is a member of TSP family of matricellular proteins, which currently contains five members (TSP1-5) [50]. Expression of TSP1, TSP2, and TSP3 in corneal epithelial, stromal, and endothelial cells has been previously demonstrated especially during wound repair [41,42,51-54]. Therefore, TSPs may play a pivotal role in maintaining the integrity of the cornea, as well as its avascularity, due to anti-angiogenic properties of TSP1 and TSP2. To investigate the molecular and cellular mechanisms of TSP action in the cornea, we isolated CEC from wild type and TSP1−/− immortomice. These cells express a temperature sensitive SV40 large T antigen when propagated at 33 °C, thus eliminating the need for the immortalization of established primary cultures of CEC. Furthermore, simple incubation of these cells at 37 °C for 48 h will eliminate the potential effects of large T antigen on cellular processes. Here we show these cells express B4-lectin and VE-cadherin, two markers that are generally associated with vascular EC, and previously not reported in CEC. Furthermore, CEC adhesion, migration, and capillary-like morphogenesis were compromised in the absence of TSP1.

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. Immortomouse expressing a temperature-sensitive SV40 large T antigen were obtained from Charles River Laboratories (Wilmington, MA). Thrombospondin-1 deficient (TSP1−/−) mice in the C57BL/6J background were generated as previously described [55]. TSP1−/− mice were crossed with immortomice and the immorto/TSP1−/− mice were identified by PCR analysis of DNA isolated from tail biopsies. The PCR primer sequences were as follows: immorto-forward: 5'-CCT CTG AGC TAT TCC AGA AGT AGT G-3', immorto-reverse: 5'-TTA GAG CTT TAA ATC TCT GTA GGT AG-3'; Neo-forward: 5'-TGC TCT CCA TCT GCA CGA GAC TAG-3', Neo-reverse: 5'-GAG TTC TGT GGT GAA CGC TCA G-3'; TSP1-forward: 5'-AGG GTC ATC TGG AAT TAA TAT CGG-3', and TSP1-reverse: 5'-GAG TTT GCT TGT GGT GAA CGC TCA G-3'.

Tissue preparation and culture of corneal endothelial cells: Corneal endothelial cells (CEC) were isolated from mouse cornea by collecting corneas from one litter (6 to 7 pups, 4 weeks of age) using a dissecting microscope. Descemet’s membrane was gently peeled from the periphery of the cornea towards the central region and placed into wells of a 96 well tissue culture plate coated with fibronectin (2 µg/ml) in growth medium. The Descemet’s membranes were incubated at 33 °C in 5% CO2, and CEC were allowed to migrate out until confluent. CEC were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 20% fetal bovine serum (FBS), 2 mM L-glutamine, 2 mM sodium pyrovate, 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, St. Louis, MO), and murine recombinant interferon-γ (R&D Associates, Minneapolis, MN) at 44 units/ml. Cells were progressively passed to larger plates, maintained at 33 °C with 5% CO2, and propagated in 1% gelatin-coated 60 mm dishes. In all the studies presented here TSP1−/− CEC are compared with TSP1+/+ CEC prepared and cultured under identical conditions.

FACScan analysis: CEC from 60 mm culture plates were rinsed with phosphate buffered saline (PBS) containing 0.04% EDTA and incubated with 1.5 ml of Cell Dissociation Solution (Sigma, St. Louis, MO). Cells were then rinsed from plates with DMEM containing 10% FBS, washed once with Tris-buffered saline (TBS; 20 mM Tris-HCl, 150 mM NaCl, pH 7.6), blocked in TBS with 1% goat serum for 20 min on ice, then incubated with rat-anti-mouse PECAM-1 (MEC13.3; BD Pharmingen, San Diego, CA), rabbit-anti-mouse VE-cadherin (Alexis, San Diego, CA), mouse anti-CD36 (BD Pharmingen), FITC-conjugated B4-Lectin (Sigma), mouse anti-SHPS-1 (BD Pharmingen), rat anti-IAP (CD47; a gift of Dr. William A. Frasier, Washington University, St. Louis, MO), mouse anti-β3 integrin, rabbit anti-α3 integrin, rabbit anti-α5 integrin, or anti-αv integrin (Chemicon, Temecula, CA) for 30 min on ice. Cells were then washed twice with TBS + 1% BSA and incubated with the appropriate FITC-conjugated secondary antibody for 30 min on ice. Following incubation, cells were washed twice with TBS + 1% BSA and resuspended in 0.5 ml TBS + 1% BSA, and analyzed by FACScan caliber flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). All antibodies were used at the dilutions recommended by the supplier.

Corneal wholemount staining: Wild type and TSP1−/− mouse (4 weeks of age) eyes were enucleated and briefly fixed in 4% paraformaldehyde (4 min on ice), and then were fixed...
in methanol for at least 24 h at -20 °C. Corneas were dissected from the limbus in PBS and washed with PBS three times. Following incubation in blocking buffer (20% FBS, 20% normal goat serum, and 0.1% Triton X-100 in PBS) for 1 h, the corneas were incubated with FITC-conjugated BS-I isolectin B4 from Bandeiraea simplicifolia (Sigma) diluted 1:100 in blocking buffer (20% fetal calf serum, 20% normal goat serum, and 0.1% Triton X-100 in PBS) at 4 °C overnight. Corneas were then washed with PBS three times, mounted on slides, and photographed in digital format using a Zeiss fluorescence microscope (Carl Zeiss, Chester, VA).

Indirect immunofluorescence: CECs were cultured on glass coverslips coated with 2 µg/ml of fibronectin and allowed to reach 70% confluence (next day). Cells were then rinsed twice with PBS, fixed with 4% PFA containing 0.1% Triton X-100 for 10 min on ice, washed three times with PBS, and incubated with either rabbit anti-ZO-1 (Invitrogen, Carlsbad, CA), rabbit anti-VE-cadherin (Dako, Carpinteria, CA), rabbit anti-β-catenin (BD Transduction), goat anti-aquaporin-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-N-cadherin (Cell Signaling Technologies, Danvers, MA) and FITC conjugated B4-Lectin (Sigma) for 30 min at 37 °C. Cells were then rinsed twice with TBS and incubated with appropriate CY3 conjugated secondary antibody for 30 min at 37 °C. This step was skipped for cells stained with FITC-conjugated B4-lectin. After washing three times with TBS, coverslips containing cells were mounted onto glass slides and photographed using a Zeiss fluorescence microscope (Axiophot; Zeiss, Oberkochen, Germany) in digital format. Similar experiments were also performed with confluent monolayers of CEC to better evaluate cell junctional staining.

Western blot analysis: For TSP1 analysis, cells were plated at 8x10^5 cells per 60 mm dishes and allowed to reach approximately 95% confluence (2 days). 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). 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 (ECL; Amersham, Piscataway, NJ). The blot was stripped and probed with a rabbit anti-TSP2 (BD Pharmingen), rabbit anti-human eNOS (Santa Cruz Biotechnology, Inc.), rabbit anti-VE-cadherin (Invitrogen), rabbit anti-human nNOS (BD Biosciences), and mouse anti-human β-catenin (BD Transduction) to control for loading. The cells were also lysed in 0.2 ml of 20 mM Tris pH 7.4, 2 mM EDTA solution, sonicated briefly, and similarly analyzed along with the conditioned medium.

Scratch wound assay: Cells (4x10^5) were plated on gelatin-coated 60 mm tissue culture dishes and allowed to reach confluence (3 days). After aspirating the medium, cell layers were wounded using a 1 ml micropipette tip. Plates were then rinsed with PBS, fed with growth medium, and wound closure was monitored and photographed at 0, 24, 48, and 72 h. Similar assays were performed in the presence of 5-fluorouracil (10 µg/ml; Sigma) to rule out the potential contribution of differences in cell proliferation. These experiments were repeated at least twice with similar results using two different isolations of CEC.

Transwell migration assay: The migration ability of CEC was also determined using a transwell migration assay. The bottom side of an 8 µm pore size Costar transwell (Corning, Acton, MA) was coated by adding 0.5 ml of PBS containing 2 µg/ml fibronectin to the wells of a 24 well dish, placing the transwell in the well, and incubating overnight at 4 °C. The bottom of the transwell was rinsed once 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 of serum-free DMEM was added to the 24 well dish containing the transwell. Cells were trypsinized, resuspended in serum free DMEM, plated at 1x10^5 cells/0.1 ml on top of the transwell membrane, and incubated for 3 h at 37 °C. Medium was then aspirated and cells were fixed by adding 0.5 ml of 4% paraformaldehyde (PFA) in PBS to the well below the transwell and 0.1 ml of 4% PFA to the transwell for 15 min at room temperature. The transwell was then removed. The top of the membrane was wiped clean with both ends of a cotton swab, and placed in a well with 0.5 ml of hematoxylin below and 0.1 ml hematoxylin on top of the transwell membrane. Cells were stained for 20 min at room temperature. The transwell was wiped with cotton swab and placed in a well with 0.5 ml water to wash the membrane twice. The membrane was wiped again and stained in 1% eosin for 5 min. The membrane was then serially washed in 70%, 95%, and 100% ethanol for approximately 30 s, and wiped between each wash. The membrane was then placed in another 100% ethanol until mounted. For mounting, the membrane was removed, wiped, cut out from the transwell, placed bottom side up on a glass slide, and secured with permament and a glass coverslip.

Cell adhesion assay: CEC adhesion to various extracellular matrix proteins was determined as recently described [56]. 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/Mg) overnight at 4 °C. The next day plates were rinsed four times with TBS containing Ca/Mg and 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 5x10^5 cells/ml. Blocking solution was then removed, rinsed once with 200 µl of TBS with Ca/Mg, and 50 µl of cell suspension was added to each of triplicate wells containing 50 µl of TBS with Ca/Mg. Cells were then allowed to adhere for 90 min at 37 °C and nonadherent 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 as previously described [56].
Three dimensional culture of CEC: Vascular EC generally organize into a capillary-like network when plated on Matrigel. To determine whether CEC have the ability to undergo capillary-like morphogenesis in Matrigel, 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. Cells were incubated at 37 °C and photographed after 18-20 h in digital format.

Statistical analysis: Statistical differences between groups were evaluated with Student’s t-test (two-tailed). The mean±S.D. is shown. A p of less than or equal to 0.05 was considered significant.

RESULTS
Isolation of murine corneal endothelial cells: Successful isolation and culture of mouse CEC from transgenic mice has
not been previously reported. Using wild type and TSP1−/− immortomice, we have successfully isolated and characterized CEC. The CEC were first released from corneal tissues by dissecting the Descemet’s membrane and placing it in wells of a 96 well plate coated with fibronectin and allowed to reach confluence. The cells were then passed to wells of 24 well plate, 35 mm tissue culture dishes, and then to 60 mm tissue culture dishes. This resulted in isolation of a homogeneous population of CEC. Figure 1 shows the morphology of CEC prepared from wild type and TSP1−/− mice plated on gelatin-coated plates. The CEC appeared elongated at low confluency and upon confluency they looked more cobblestone-like (Figure 1E,F), as previously shown with primary culture of mouse CEC immortalized using SV40-large T [16]. These cells also expressed ZO-1, which localized to sites of cell-cell contact in confluent monolayers (Figure 1G,H).

There are no good cell surface markers reported for identification of mouse CEC. Vascular EC commonly express PECAM-1 and VE-cadherin [10]. In addition, B-4 lectin is commonly used to stain mouse retinal vasculature [57]. We next determined whether CEC express any of these markers by FACS analysis. Figure 2A shows that CEC from wild type and TSP1−/− do not express PECAM-1 but have significant amount of VE-cadherin. CEC, like vascular EC, also stained with B4-lectin. We also observed B4-lectin staining in corneas from wild type and TSP1−/− in vivo using corneal wholemount staining (Figure 2B,C). B4-lectin staining showed a junctional staining at cell borders. The CEC also expressed significant amounts of CD36, the angioinhibitory receptor for TSP1 (not shown). The lack of TSP1 did not significantly affect the expression of these markers. Therefore, CEC, like murine microvascular EC, express VE-cadherin, CD36, and are positive for B4-lectin.

Figure 2. Expression of vascular endothelial cell markers in CEC. Mouse CEC were examined for expression of PECAM-1, VE-cadherin, and B4-lectin by FACS analysis (A). Please note that CEC express significant amounts of VE-cadherin and B4-lectin, but lack PECAM-1 expression. The shaded graphs show staining in the absence of primary antibody. B and C show corneal whole mounts prepared from wild type (B) and TSP1−/− (C) and stained with B4-lectin. Please note junctional localization of B4-lectin at sites of cell-cell contact.
Figure 3. Localization of VE-cadherin, B4-lectin, β-catenin, aquaporin-1, and N-cadherin in CEC. Confluent monolayer of wild type (A, C, E, G, I) or TSP1−/− (B, D, F, H, J) CEC on glass coverslips were stained with anti-VE-cadherin (A, B), FITC-B4-lectin (C, D), anti-β-catenin (E, F), anti-aquaporin-1 (G, H), and anti-N-cadherin (I, J). Please note the junctional localization of B4-lectin and β-catenin (arrow heads), while other proteins showed diffuse staining throughout the cells. These experiments were repeated at least twice with two different isolations of CEC. The scale bar is equal to 20 µm.
VE-cadherin normally organizes to the sites of cell-cell contact as a major component of vascular EC adherens junction complexes. We next determined the organization of VE-cadherin in CEC (Figure 3A,B). VE-cadherin did not localize to sites of cell-cell contact. N-cadherin is another major cadherin expressed in vascular EC which lacks junctional localization. CEC are also shown to express N-cadherin upon their terminal differentiation from the neural crest mesenchymal cell and lack P- and E-cadherin [48,58]. We observed N-cadherin expression in wild type and TSP1\(^{-/-}\) CEC, which did not localize to sites of cell-cell contact (Figure 3I,J). However, B4-lectin demonstrated a junctional localization in these cells (Figure 2B,C, and Figure 3C,D; arrow heads). Another component of adherens junction is \(\beta\)-catenin. Wild type and TSP1\(^{-/-}\) CEC expressed \(\beta\)-catenin with a junctional localization (Figure 3E,F; arrow heads). Both wild type and TSP1\(^{-/-}\) CEC also expressed aquaporin-1 (Figure 3G,H), as reported by others [59]. No staining was observed when primary antibodies were omitted (not shown).

Expression of ECM proteins by CEC: TSP1 is an endogenous inhibitor of angiogenesis whose endothelium expression favors a differentiated, quiescent phenotype. Although the expression of TSP1 in the cornea, and more specifically in CEC, has been previously reported, the role TSP1 plays in modulation of CEC phenotype requires further study. The abil-

![Figure 4. Western blot analysis of conditioned medium and cell lysates prepared from CEC. Levels of TSP1, TSP2, eNOS, nNOS, tenascin-C, and fibronectin were analyzed by western blot analysis of serum free-conditioned medium and cell lysates prepared from CEC as described in Methods. Please note the absence of TSP1 in TSP1\(^{-/-}\) CEC. The TSP1\(^{-/-}\) CEC express less fibronectin, both secreted and cell-associated, compared to wild type CEC. Tenascin-C was only detectable in TSP1\(^{+}\) CEC. Both wild type and TSP1\(^{-/-}\) CEC did not express detectable levels of TSP2 or eNOS, but expressed nNOS. The blot was also incubated with anti-\(\beta\)-catenin as a loading control for intercellular protein levels.](http://www.molvis.org/molvis/v13/a165/)

![Figure 5. Scratch wound and transwell migration assays of CEC. Confluent monolayers of wild type or TSP1\(^{-/-}\) CEC (A) were wounded and wound closure was monitored after 72 h and photographed in digital format. Please note that the wild type CEC migrated and covered the wound, while significant area of the wound remains uncovered in TSP1\(^{-/-}\) CEC. B shows the migration of wild type and TSP1\(^{-/-}\) CEC through a transwell as described in Methods. A significant decrease in the mean number of TSP1\(^{-/-}\) CEC migrating through the filter compared to the wild type cells was observed (p<0.05). These experiments were performed at least twice with two different isolations of CEC.](http://www.molvis.org/molvis/v13/a165/)
ity to culture CEC has allowed us to directly study the role of TSP1 in modulation of CEC properties. Figure 4 shows that CEC from wild type mice produce a significant amount of TSP1 in their conditioned medium. Some cellular associated TSP1 was also observed in these cells. However, as expected, the CEC prepared from TSP1−/− mice did not produce TSP1. The wild type and TSP1−/− CEC did not express detectable levels of TSP2, a closely related member of TSP family. These cells also expressed very low levels of neuronal nitric oxide synthase (nNOS), but had undetectable levels of endothelial or inducible NOS (eNOS and iNOS; Figure 4, and not shown).

Fibronectin and tenascin C are major component of the extracellular matrix elaborated by many cell types including CEC with important roles in cell migration and wound repair [60-63]. Retinal vascular EC that lack TSP1 express increased amounts of fibronectin and are more migratory [10,64]. We next examined the level of fibronectin in wild type and TSP1−/− CEC by western blotting of the conditioned medium and cell lysates. Figure 4 shows wild type and TSP1−/− CEC produced significant amounts of fibronectin with lower levels in TSP1−/− CEC. However, a significant amount of tenascin-C (TN-C) was only detected in TSP1−/− CEC. The same blot was also probed for cellular levels of β-catenin as a loading control, which shows similar levels of protein.

TSP1−/− CEC are less migratory: TSP1 greatly impacts vascular EC migration. Retinal vascular EC which lack TSP1 are more migratory compared to wild type cells [10]. Furthermore, TSP1 expression is upregulated during CEC wound repair [41]. We next evaluated the migratory ability of CEC using a scratch wound assay. Confluent monolayers of wild type and TSP1−/− CEC were wounded and wound closure was monitored up to 72 h. Figure 5A shows that wild type CEC migrate and cover the wound by 72 h, while a significant area of the wound remains uncovered in TSP1−/− CEC. A similar result was observed during transwell migration of these cells. Wild type CEC migrated through the transwell. In contrast, we observed a significant decrease in the migration of TSP1−/− CEC (Figure 5B, p<0.01). Thus, TSP1 may play a major role in migration of CEC.

The observed migratory defects in TSP1−/− CEC suggested that alterations in cell adhesive mechanisms may exist. We next evaluated the adhesion of CEC to various matrix proteins. Figure 6A shows that TSP1−/− CEC were less adhesive on fibronectin, especially at higher concentrations, but were more adhesive on vitronectin at all the matrix concentrations (p<0.05). Neither cell strains adhered to laminin or collagen (not shown). Thus, lack of TSP1 may affect the adhesive properties of CEC impacting their migration.

In an attempt to determine whether the altered adhesive properties are due to changes in expression and/or activity of integrins on CEC, we examined the expression of various integrins by FACS analysis. Figure 7 shows wild type and TSP1−/− CEC express similar levels of integrin associated protein (IAP/CD47) and its ligand SHPS-1. Although these cells expressed limited amounts of αvβ3 integrin, they expressed significant amounts of αv integrin. The αv integrin level was reduced in TSP1−/− CEC compared to wild type cells. The CEC also expressed significant amounts of α3 and α5 integrins. TSP1−/− CEC expressed higher levels of α3 but lower levels of α5 integrin compared to wild type cells (Figure 7).

In vitro capillary-like morphogenesis of CEC: Most vascular EC rapidly organize and form capillary-like networks when plated in Matrigel. Though not absolutely specific for vascular EC, it still can distinguish vascular EC from some common contamination cell types [65]. This recapitulates the later stages of angiogenesis with minimal amounts of cell proliferation. Figure 8A,C show that CEC from wild type mice organize in Matrigel forming an extensive cell network very similar to vascular EC. However, the ability of CEC from TSP1−/− mice to form these structures was somewhat compromised (Figure 8B,D). TSP1−/− CEC formed larger islands of cells
which failed to migrate out and form an extensive cell network. These observations are consistent with the reduced migratory phenotype of TSP1−/− CEC.

**DISCUSSION**

The ability to culture CEC has resulted in a dramatic increase in our understanding of CEC function and biology. Culture of CEC from genetically modified mice will allow us to gain a more detailed understanding of the functional consequences that specific genes and their product may have on corneal endothelium homeostasis. Previous preparations of mouse CEC have been difficult and tedious involving multiple steps. Here we report a method for routine isolation and propagation of CEC from wild type and transgenic immortomice. Descemet’s membranes from wild type and TSP1−/− immortomice were removed and CEC were allowed to grow out. The immortomice express a thermolabile strain (tsA58) of the simian virus (SV)40 large T antigen (taA58 Tag) driven by an inducible major histocompatibility complex H-2K promoter, thus, eliminating many intrinsic problems with immortalized lines [66]. The T-antigen expression is functionally evident at the reduced temperature of 33 °C and enhanced in the presence of interferon-γ. Generally, incubation at 37 °C for 48 h in the absence of interferon-γ results in loss of large T antigen [66]. We successfully isolated and cultured CEC from wild type and TSP1−/− mice. FACScan and immunostaining analysis showed nearly 100% of the isolated cells express B4-lectin and VE-cadherin. These cells were readily passaged and propagated in culture for up to six months without significant loss in expression of these markers. To our knowledge, this is the first report of isolation and culture of CEC from transgenic mice in a single step.

The wild type and TSP1−/− CEC exhibited similar morphology and growth characteristics comparable to those previously reported for SV40-large T-antigen immortalized mouse CEC [16]. These cells expressed N-cadherin and aquaporin-1 as previously reported for CEC [48,59]. We showed that CEC, like vascular EC, express VE-cadherin but lack PECAM-1 expression. CEC were positive for B4-lectin, which is generally associated with mouse vascular EC. CEC also expressed CD36, IAP/CD47, and its ligand SHPS-1. To our knowledge the expression of these vascular EC markers have not been previously reported in CEC.

The expression of VE-cadherin is thought to be specific to vascular EC and generally used as a marker of mesenchymal precursor cells that may develop into vascular EC and/or hematopoietic cells. The cells that line the Schlemm’s canal

![Figure 7. Expression of integrins in CEC. Mouse CEC were examined for expression of SHPS-1, IAP/CD47, αvβ3, α5, α3, and αv integrins by FACS analysis. Please note that CEC express significant amounts of IAP/CD47 and its ligand SHPS-1. These cells lacked expression of αvβ3 but expressed significant amounts of α3, α5, and αv integrins. The level of α3 was increased in TSP1−/− CEC while that of α5 and αv was decreased compared to wild type cells. These experiments were performed at least twice with two different isolations of CEC.](http://www.molvis.org/molvis/v13/a165/)
of the outflow pathway are also derived from the ectoderm and express VE-cadherin [67]. The expression of VE-cadherin in CEC suggests that the CEC may share a similar mesenchymal stem cell of origin as vascular and hematopoietic cells. Therefore, it is tempting to speculate that such mesenchymal precursor cells may contribute to repopulation of corneal endothelium during wound repair as they do during formation of new blood vessels in response to ischemia and/or injury.

The VE-cadherin expressed in CEC, however, did not localize to sites of cell-cell contact, as it does in vascular EC. Vascular EC, like CEC, also express N-cadherin, which is absent from interendothelial adherens junctions [68]. The N-cadherin expressed in the CEC prepared here also lacked junctional localization (Figure 3). Although the spatial organization of corneal endothelial cytoskeletal proteins and their relationship to the apical junctional complexes have been examined [69], the identity of the cadherin involved in the formation of adherens junctions remains elusive and requires further study.

TSP1 is expressed by a variety of cell types, including CEC (Figure 4). The increased expression of TSP1 in wounded cornea and its localization as migration tracks oriented in the direction of cellular migration into the wound site, suggest an important role for TSP1 in corneal injury induced cell migration [41,42]. The ability to culture CEC from TSP1−/− mice will allow us to delineate the role of TSP1 in corneal endothelium homeostasis and wound healing. Our results showed, despite similar morphology and growth characteristics, the wild type and TSP1−/− CEC exhibit different adhesive and migratory characteristics. The TSP1−/− CEC were significantly less migratory in scratch wound and transwell migration assays compared to wild type cells (Figure 5). This is consistent with increased TSP1 expression observed in migrating CEC during wound healing [41].

TSP1−/− CEC were less adhesive on fibronectin compared to wild type CEC (Figure 6). This may be explained, at least in part, by reduced expression of α5 and αv integrins and increased expression of tenascin-C in TSP1−/− CEC compared to

Figure 8. Capillary-like morphogenesis of CEC in Matrigel. The CEC from wild type (A, C) and TSP1−/− (B, D) were plated on Matrigel as described in Methods. After 16 h of incubation, wild type CEC formed well-organized capillary-like network (A), while TSP1−/− CEC ability to organize was somewhat compromised (B). C and D (x100) are higher magnificationS of A and B (x40), respectively. These experiments were repeated twice with two different isolations of CEC with similar results.
wild type cells (Figure 7). Tenascin-C is a multi-domain glycoprotein that is present transiently in the extracellular matrix with important roles in morphogenetic migration of neural crest cells, patterning, and formation of cornea [62]. It has both adhesive and anti-adhesive properties through its interactions with cells and other extracellular matrix proteins. Interaction of tenascin-C with fibronectin regulates expression of genes involved in migration of fibroblasts [63]. Thus, increased expression of tenascin-C in TSP1−/− CEC may have a significant impact on their adhesion and migration properties. The ability of TSP1−/− CEC to undergo capillary-like morphogenesis in Matrigel was also compromised. These cells formed large islands of cells which failed to migrate to form a more extensive network as seen with wild type cells. These observations together support the important role proposed for TSP1 in CEC migration.

We have shown that TSP1 expression in vascular EC promotes a differentiated, quiescent phenotype [70]. Our recent in vivo studies with TSP1−/− mice indicated that expression of TSP1 is essential for proper remodeling and maturation of retinal vasculature [71]. In addition, TSP1−/− retinal EC exhibit proangiogenic characteristics in culture and are more migratory [64]. In contrast, CEC migration is severely compromised in the absence of TSP1. Therefore, the intracellular signaling pathways activated in CEC may be very different from those activated in vascular EC. However, CEC, like vascular EC, express CD47/IAP and CD36, the two known TSP1 receptors that are essential for antiangiogenic activity of TSP1 in vascular EC [72]. The identities of the signaling pathways that are activated in CEC upon interaction of TSP1 with its receptor(s) remain elusive and need investigation.

Integrin α5β1 and its ligand fibronectin are expressed at significantly increased levels in new blood vessels induced by growth factors or solid tumors [73]. Furthermore, interaction of fibronectin and its receptor integrin α5β1 is essential for angiogenesis in vitro and in vivo [74]. We observed increased expression of fibronectin in TSP1−/− retinal vascular EC consistent with their promigratory phenotype [64]. This is consistent with our observation in brain endothelial (bEND) cells which are highly angiogenic and express little or no TSP1 [75]. However, lack of TSP1 resulted in decreased expression of fibronectin (Figure 4) and α5 integrin (Figure 7) in CEC. Therefore, the absence of TSP1 oppositely effects the migration of vascular EC compared to CEC. TSP1 expression appears to be essential for migration of CEC, while its expression in vascular EC is antimigratory. The molecular and cell type specific mechanisms responsible for these opposing effects of TSP1 on cell migration are intriguing and are currently under investigation.

In summary, we described a simple method for the isolation of CEC from wild type and TSP1−/− immortomice. These cells can be readily propagated at the permissive temperature and retain their characteristics in long-term cultures. The ability to rapidly propagate the cells in permissive temperature and then switching them to the non-permissive temperature eliminates the effects of large T antigen on cellular properties. The comparison of CEC from wild type and TSP1−/− mice under normal or pathological conditions such as wound healing will enhance our understanding of the role TSP1 plays in corneal endothelium homeostasis and wound repair.

ACKNOWLEDGEMENTS
This work was supported by grants EY13700 and EY16695 (N.S.) and DK67120 (C.M.S.). N.S. is a recipient of a research award from American Diabetes Association and Retina Research Foundation.

REFERENCES


54. Sekiyama E, Nakamura T, Cooper LJ, Kawasaki S, Hamuro J, Fullwood NJ, Kinoshita S. Unique distribution of