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Technical Brief

Isolation and characterization of murine retinal endothelial cells

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Purpose: To isolate and characterize primary retinal endothelial cells (REC) from wild type and transgenic mice to facilitate the study of their properties in vitro.
Methods: REC were isolated from wild type or transgenic-immortomouse by collagenase digestion of retina and affinity purification using magnetic beads coated with platelet/endothelial cell adhesion molecule-1 (anti-PECAM-1). The bound cells were plated on fibronectin-coated wells and expanded. The REC were characterized for expression and localization of endothelial cell markers by fluorescence-activated cell sorting (FACS) analysis and indirect immunofluorescence staining. The ability of these cells to form capillary like networks was assessed on Matrigel while the migration properties were examined in wound closure assays.
Results: Isolation of REC from mouse has been very difficult and has not been previously reported. Here, we describe a method for isolation of retinal endothelial cells from wild type and thrombospondin-1 deficient ($TSP1^{-/-}$) immortomice. Our results indicate that nearly 100% of selected cells express the endothelial cell marker PECAM-1 and vascular endothelial-cadherin (VE-cadherin). The cells were successfully passaged and maintained in culture for several months without a significant loss in expression of endothelial cell markers. The wild type REC, like most primary endothelial cells, formed capillary-like networks on Matrigel. The ability of the REC from $TSP1^{-/-}$ mice to form capillary-like networks on Matrigel was severely compromised. This may be attributed, at least in part, to the enhanced migratory and less differentiated phenotype of these cells.
Conclusions: The retinal endothelial cells can be readily obtained from wild type and transgenic mice, which facilitate the comparison and identification of the physiologic role of specific genes in endothelial cell function.

Angiogenesis, the formation of new vessels from pre-existing capillaries, is a complex and multi-step process. The ability to culture endothelial cells has been instrumental in developing assays that recapitulate different stages of angiogenesis. These assays have provided great knowledge regarding the biochemical events that modulate angiogenesis and the players involved [1,2]. Pathological growth of new vessels contributes to a large number of eye diseases, including retinopathy of prematurity, proliferative diabetic retinopathy, and macular degeneration [3-5]. However, the molecular and cellular events that contribute to initiation of the angiogenic events require further delineation. Specific alternations in endothelial cells may play an important role in these processes.

Retinal vascularization is very tightly regulated by coordinated interactions of vascular cells, including endothelial cells, pericytes, and astrocytes, and a balanced production of positive and negative regulatory factors [1,6]. However, the study of these coordinated interactions and the identity of the soluble factors involved in vivo has been very difficult. The ability to culture vascular cells has allowed the study of these interactions in in vitro co-culture experiments both in two- and three-dimensional cultures [7,8]. Although endothelial cells have been purified from retina of other species, the success with murine retinal endothelial cells (REC) has been limited. The advances in mouse genetics and availability of mutant mice that lack a specific gene allows assessment of the consequence of specific genetic changes on vascular interactions.

The retinal vasculature provides a unique opportunity to study all aspects of vascular development and remodeling. It is restricted to two dimensions, which simplifies the study of a vascular plexus in its entirety [9]. As an important component in the process of vasculogenesis and angiogenesis, the biology of murine REC is a recent focus of many studies. Mice offer the added benefits of a well-established genetic modification technique. 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. In addition, there is similar pattern of vascular development in mouse retina compared to the human [9]. In both species the first vessels originate at optic nerve head and spread over the inner surface of the retina, forming a dense network. Thus, the results from mouse studies should give us a better understanding of human

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retinopathology. This, combined with the need to better understand the mechanisms of neovascularization and retinopathy derived from diabetic retinopathy or retinopathy of prematurity, further emphasizes the importance of developing REC with characteristics similar to in vivo vascularization.

Different methods are utilized to isolate REC from various species. Culture of REC is reported for human [10], rhesus monkey [11], bovine [12,13], feline [14], and rat [15]. Initially, most of the REC cultures from human and bovine were obtained by mechanical separation of the retina microvessels and later isolation of endothelial cells. In some experiments, microvessels were partially digested with enzymes and cultured to allow cell outgrowth manually selecting endothelial-like clones [11]. Others have digested microvessels purifying REC by gradient centrifugation [16,17]. The isolation and culture of mouse REC has proven difficult, perhaps due to its relatively low tissue supplement compared to other animal sources. Recently the endothelial cell marker, PECAM-1, has proven to be a useful, efficient way to isolate endothelial cells from different tissue and animal sources [15,18,19]. Here we describe a method for the routine isolation and propagation of mouse REC from wild type and TSP1$^{-/-}$ immortomice. We demonstrate that mouse REC can be readily expanded, retaining their endothelial cell markers, and can aid in defining the functional consequences of gene targeting on endothelium properties.

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/6 background were generated as previously described [20]. TSP1$^{-/-}$ mice were crossed with immortomouse 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 TGT CCA TCT GCA CGA GAC TAG-3', Neo-reverse: 5'-GAG TTT GCT TGT GGT GAA CGC TCA G-3'; TSP1-forward: 5'-AGG GCT ATG TGG AAT TAA TAT CGG-3', and TSP1-reverse: 5'-GAG TTT GCT TGT GGT GAA CGC TCA G-3'.

Preparation of antibody coated magnetic beads for endothelial cell isolation: Sheep anti-rat Dynabeads (Dynal Biotech, Lake Success, NY) were washed three times with serum-free DMEM (Dulbecco's Modified Eagle's Medium; Invitrogen, Carlsbad, CA) and then incubated with rat anti-mouse PECAM-1 monoclonal antibody MEC13.3 (BD Pharmingen, San Diego, CA) overnight at 4 °C (10 µl/50µl beads in DMEM). Following incubation, beads were washed three times with DMEM containing 10% fetal bovine serum (FBS) and resuspended in the same medium.

Tissue preparation, isolation, and culture of mouse retinal endothelial cells: Eyes from one litter (6 to 7 pups) of 4 week-old wild type and TSP1$^{-/-}$ immortomice were enucleated and hemisected. The retinas were dissected out aseptically under a dissecting microscope and kept in HBSS buffer containing penicillin/streptomycin (Sigma, St. Louis, MO). Retinas (12 to 14 from one litter) were pooled together, rinsed with HBSS buffer, minced into small pieces in a 60 mm tissue

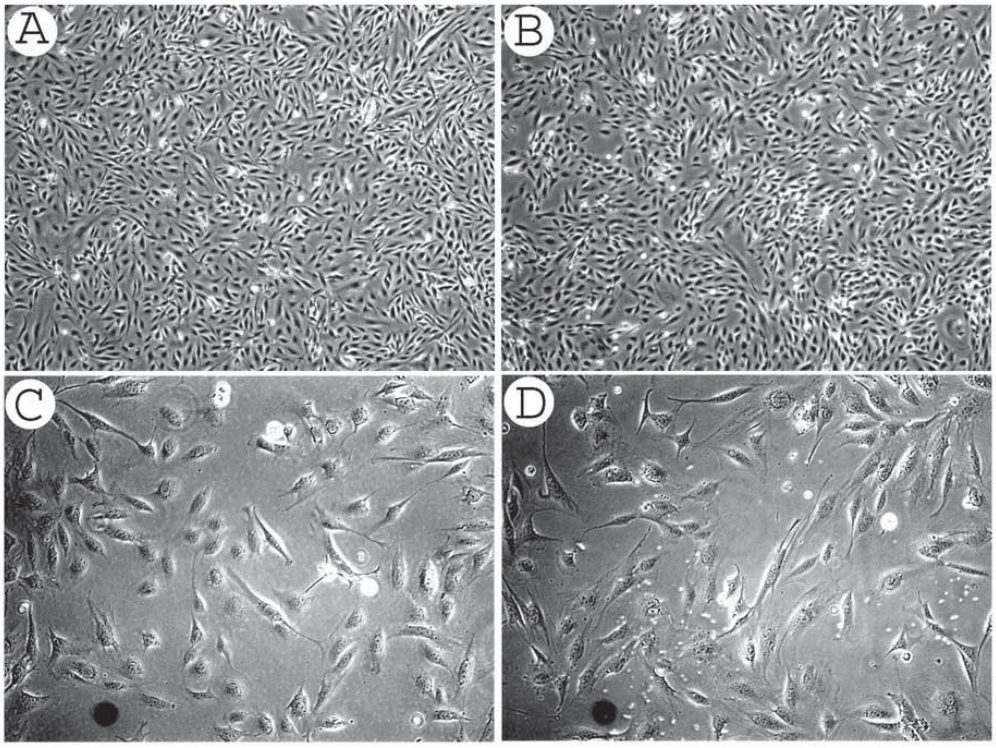


Figure 1. Morphology of mouse retinal endothelial cells (REC) cultured on gelatin. Wild type REC (**A**) and TSP1$^{-/-}$ REC (**B**) were cultured on gelatin-coated plates (40x). The TSP1$^{-/-}$ REC cultured on fibronectin (**C**) or gelatin (**D**) coated plates (100x magnification) exhibit a similar polygonal morphology with close cell-cell apposition.

culture dish using sterilized razor blades, and digested in 5 ml of collagenase type I (1 mg/ml in serum free DMEM, Worthington, Lakewood, NJ) for 30-45 min at 37 °C. Following digestion, DMEM with 10% FBS was added and cells were pelleted. The cellular digests then were filtered through a double layer of sterile 40 μm nylon mesh (Sefar America Inc., Fisher Scientific, Hanover Park, IL), centrifuged at 400x g for 10 min to pellet cells, and cells were washed twice with DMEM containing 10% FBS. The cells were resuspended in 1.5 ml medium (DMEM with 10% FBS), and incubated with sheep anti-rat magnetic beads pre-coated with anti-PECAM-1 as described above. After affinity binding, magnetic beads were washed six times with DMEM with 10% FBS and bound cells in endothelial cell growth medium were plated into a single well of a 24 well plate pre-coated with 2 μg/ml of human fibronectin (BD Biosciences, Bedford, MA). Endothelial cells were grown in DMEM containing 20% 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, Minneapolis, MN) at 44 units/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: Monolayers of retinal endothelial cells (1-2x10^6 in 60 mm dishes) from wild type and TSP1$^{-/-}$ immortomouse were washed once with PBS containing 0.04% EDTA, and incubated with 1 ml of cell dissociation solution (Sigma, St. Louis, MO) to release the cells. Cells were washed once with DMEM with 10% FBS, blocked in TBS with 1% goat serum for 20 min on ice, and incubated with rat anti-mouse PECAM-1 or rabbit anti-mouse VE-cadherin antibody (prepared in TBS with 1% BSA at 2 μg/ml) for 30 min on ice. Following incubation, cells were washed twice with TBS/1% BSA, then incubated with appropriate FITC-conjugated secondary antibody for 30 min on ice. The stained cells were washed twice with TBS/1% BSA and resuspended in 0.5 ml of TBS/1% BSA, and analyzed by FACScan caliber flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Three-dimensional culture of endothelial cells: Matrigel 10 mg/ml (BD Biosciences, Bedford, MA) was applied at 0.5 ml/35 mm tissue culture dish and incubated at 37 °C for at

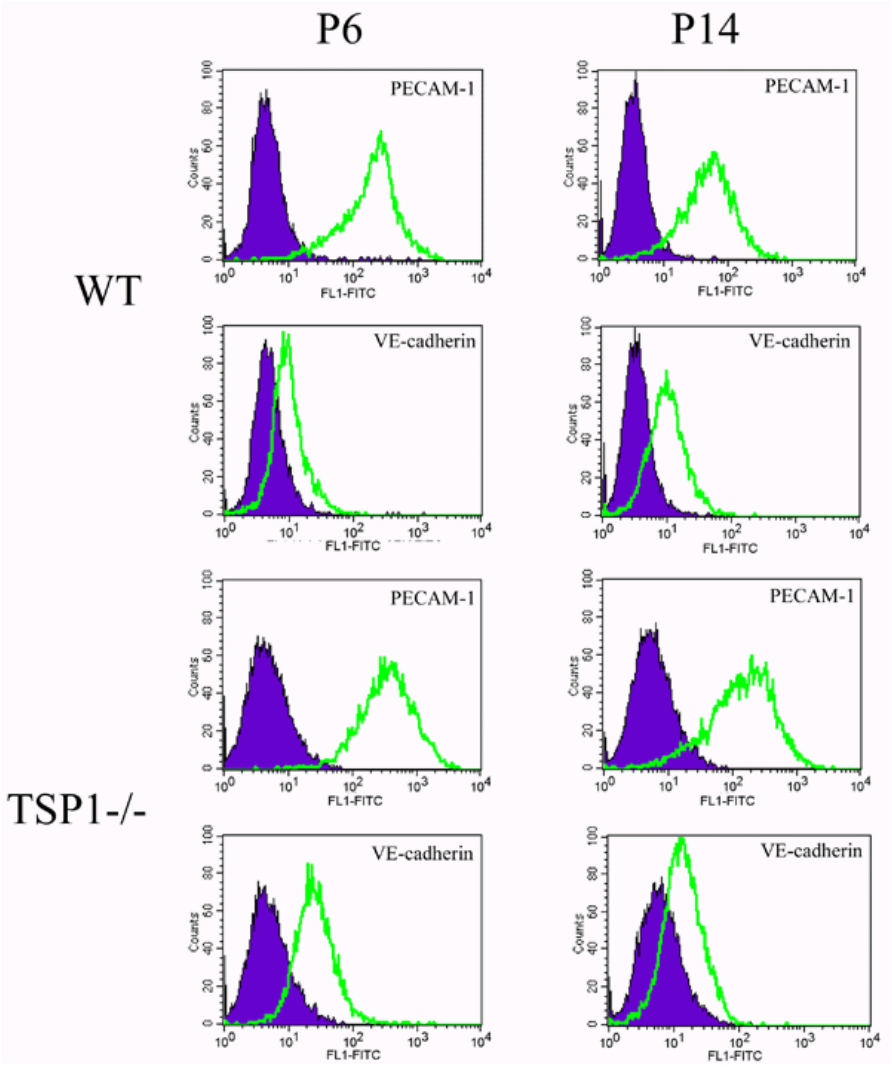


Figure 2. Expression of endothelial cell markers in REC. Mouse REC were examined for expression of PECAM-1 and VE-cadherin on their surface at different passages by FACS analysis. Note that the expression of these markers is not significantly affected with time in culture. The shaded graphs show staining in the absence of primary antibody.

least 30 min to allow harden. REC from wild type and TSP1$^{-/-}$ were prepared by trypsinization, washed with growth medium once, and resuspended at 1.5x10^5 cells per ml in endothelial cell growth medium. Cells (2 ml) were gently added to the Matrigel coated plates, incubated at 33 °C, monitored for 6-24 h, and photographed using a digital camera.

Western blot analysis: For TSP1 analysis, cells were plated at 2x10^4 per 60 mm dishes and allowed to reach approximately 90% confluence. The cells were then rinsed once with serum free medium and incubated with serum free endothelial cell (EC) growth medium (complete medium without serum) for two days. Then conditioned medium was collected, clarified by centrifugation, and protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL). Samples were adjusted for protein content and mixed with appropriate volume of 6X SDS 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 appropriate secondary antibody, and developed using ECL (Amersham, Piscataway, NJ). The blot was stripped and probed with anti-fibronectin (Invitrogen, Carlsbad, CA) and β-catenin (BD Transduction, San Diego, CA; for loading control). Cells were also lysed in 20 mM Tris pH 7.6, 2 mM EDTA and protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN) and similarly analyzed along with the conditioned medium.

Indirect immunofluorescence staining: REC were plated on chamber slides (Falcon) and allowed to reach confluence. Cells were then rinsed with PBS, fixed with 3% of paraformaldhyde (PFA) for 15 min on ice, washed three times with PBS, and incubated with anti-PECAM-1 (MEC13.3, 1 μg/ml), VE-cadherin (2 μg/ml, Alexis Biochemical, San Diego, CA), or ZO-1 (2 μg/ml, Zymed Laboratory, South San Francisco, CA) for 30 min at 37 °C. After washing three times with TBS, cells were incubated with appropriate CY-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA) at 37 °C for 30 min. Cells were washed three times with TBS, mounted and photographed using a Zeiss fluorescence microscope (Axiophot, Zeiss, Germany) equipped with a digital camera.

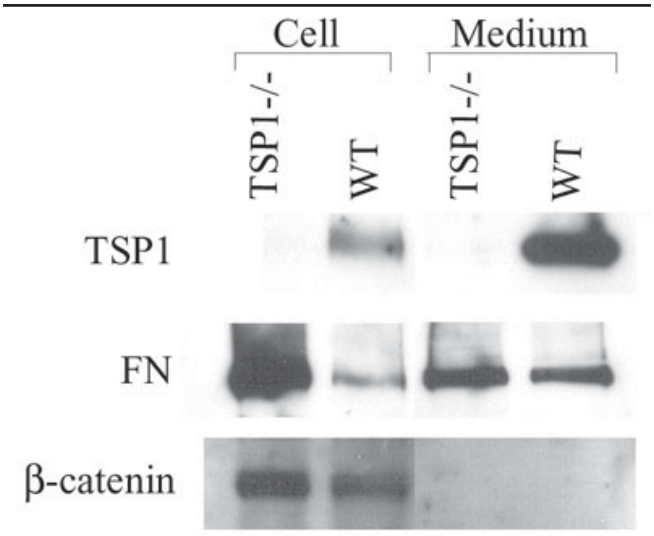


Figure 3. Western blot analysis of conditioned medium and cell lysates prepared from REC. The levels of TSP1 and fibronectin were analyzed by western blot analysis of serum free-conditioned medium prepared from REC as described in Methods. Note the absence of TSP1 in TSP1$^{-/-}$ REC. The TSP1$^{-/-}$ REC express more fibronectin, especially cell-associated fibronectin, compared to wild type REC. The blot was also incubated with anti-β-catenin as a loading control for intercellular protein levels.

Scratch wound assays: The migration of cells was assessed in scratched wound assays. Confluent monolayers of REC were wounded using a micropipette tip (1 ml blue tip), rinsed with growth medium and wound closure was monitored for 24 h and photographed using a digital camera. Similar assays were performed in the presence of 5-flurouracil (1 mM, Sigma, St. Louis, MO) to rule out the potential contribution of differences in cell proliferation. These experiments were repeated at least twice with two-different strains of REC with similar results. For quantitative presentation of the data, the percent of the total distance migrated was determined by dividing the distance migrated by the total distance using Axiovision software (Zeiss, Germany).

RESULTS

Isolation of murine retinal endothelial cells: The isolation and culture of mouse REC has proven very difficult and, as far as we are aware of, has not been previously reported. Using wild type and TSP1$^{-/-}$ immortomice we have successfully isolated and characterized retinal REC. The REC were first released from retina tissues by incubating with collagenase type I, and selectively separated from contaminating cells using magnetic beads pre-coated with anti-PECAM-1. The magnetic bead coated cells were then plated in a single well of a 24 multi-well plate coated with fibronectin and allowed to reach confluence. The cells were passed to 2 wells of a 24 multi-well plate and then to a 60 mm tissue culture dish. This resulted in isolation of a homogeneous population REC. Figure 1 shows the morphology of REC prepared from wild type and TSP1$^{-/-}$ mice. These cells exhibit a similar morphology when plated on gelatin-coated plates (Figure 1A,B). The REC isolated from TSP1$^{-/-}$ (or wild type) mice exhibited a similar morphology when plated on gelatin or fibronectin coated plates (Figure 1C,D).

To confirm that these cells are EC, we examined the expression of two endothelial cell specific markers [21], PECAM-1 and VE-cadherin, by FACS analysis. Figure 2 shows that nearly 100% of wild type and TSP1$^{-/-}$ REC express high levels of these markers on their surface. To ensure that no other contaminating cells are present, a second enrichment for PECAM-1 positive cells may be required. The long-term culture of these cells for several months did not significantly effect the expression of these markers. The data for passage 6 and 14 are shown in Figure 2. The expression of these markers did not significantly change up to passage 24 and can be readily detected on the cell surface (not shown).

Characterization of REC from wild type and TSP1$^{-/-}$ mouse: Thrombospondin-1 (TSP1) is a natural inhibitor of

angiogenesis whose expression favors a differentiated quiescent phenotype of endothelium [1]. We have recently shown that TSP1 plays an important role in retinal vascular homeostasis, particularly during remodeling and maturation of vasculature (unpublished observation). However, the further evaluation of the specific effect lack of TSP1 had on REC properties in vivo is difficult to assess. The ability to culture REC has allowed us to directly study the role of TSP1 in modulation of endothelial cell properties. We next examined the expression levels of TSP1 in REC from wild type and TSP1$^{-/-}$ mice by western blot analysis. Figure 3 shows that REC 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 REC prepared from TSP1$^{-/-}$ mice do not produce TSP1. Both cell types produce fibronectin, another secreted matrix protein, although at different levels. The REC from TSP1$^{-/-}$ produce more fibronectin compared to wild type cells and exhibit higher levels of cell associated fibronectin. The same blot was also probed for cellular levels of β-catenin as a loading control, which shows similar levels of protein.

In vitro angiogenesis assays: Most EC rapidly organize and form capillary-like structures when plated on Matrigel. Though not absolutely specific for EC, the ability to form capillary-like structures on Matrigel still can distinguish EC from some common contamination cell types [19]. This recapitulates the later stages of angiogenesis with minimal amounts of cell proliferation. Figure 4A shows that REC from wild type mice organize nicely on Matrigel forming extensive capillary networks. In sharp contrast, the ability of REC from TSP1$^{-/-}$ mice to form capillary-like structures was severely compromised (Figure 4B). There was no significant enhancement of tube formation when cells were incubation with PMA during the assay (Figure 4C,D).

Localization of PECAM-1, VE-cadherin, and ZO-1 in REC: PECAM-1 is a member of immunoglobulin gene superfamily, which is highly expressed on the surface of EC and at moderate levels on platelets and hematopoietic cell [1]. In endothelial cells, generally PECAM-1 localizes to the sites of cell-cell contact. VE-cadherin, an endothelial specific cadherin also known as cadherin-5, mediates cell-cell interactions through formation of adherens junctions, which are important in maintaining vascular integrity [22]. Our FACS analysis data showed that both wild type and TSP1$^{-/-}$ REC express similar levels of PECAM-1 and VE-cadherin on their surface (Figure 2). To determine if the organization of these proteins is affected in the absence of TSP1, we examined their localization by indirect immunofluorescence staining. Figure 5A,D show localization of PECAM-1 in REC from wild type and TSP1$^{-/-}$ mice, respectively. PECAM-1 staining showed a typical junctional localization as seen in other endothelial cells. Figure 5B,E show VE-cadherin while Figure 5C,F show ZO-1 localization in wild type and TSP1$^{-/-}$ REC, respectively. These proteins exhibited a junctional localization as has been previously demonstrated in other endothelial cells.

In vitro cell migration: The migratory properties of REC were assessed using a scratch wound assay. Confluent monolayers of REC were wounded using a micropipette and wound closure monitored by still photography. Figure 6 shows that REC from TSP1$^{-/-}$ mice exhibit an enhanced ability to migrate and wound closure is complete by 24 h while a significant area of wound remains uncovered in wild type REC. Figure 6E shows the quantitative assessment of the data. Therefore, the absence of TSP1$^{-/-}$ in REC affects their migration com-

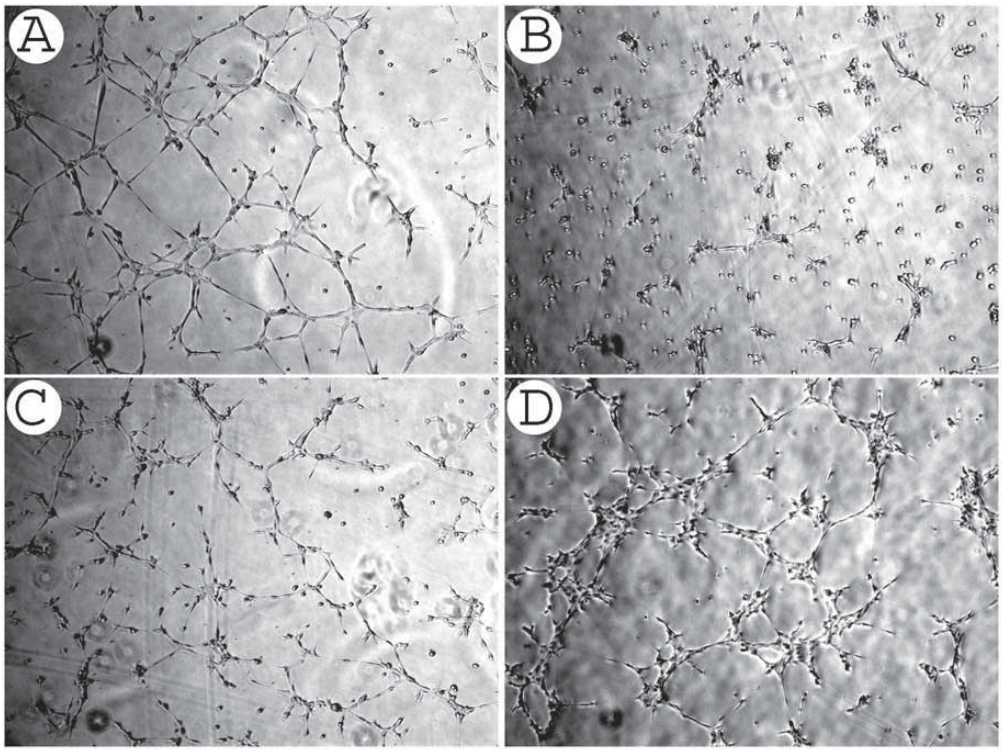


Figure 4. Capillary formation of REC on Matrigel. The REC from wild type (**A** and **C**) and TSP1$^{-/-}$ (**B** and **D**) were plated on Matrigel as described in Methods. After 7 h of incubation, wild type REC formed well-organized capillary-like structures (**A**), while TSP1$^{-/-}$ REC ability to organize was severely compromised (**B**). The effects of PMA on capillary morphogenesis were minimal (**C** and **D**). These experiments were repeated twice with two-different strains of REC with similar results.

pared to wild type cells. Observation of similar results in the presence of 5-flurouracil indicates that differences observed are not due to differences in the rates of proliferation (not shown).

DISCUSSION

The ability to culture EC has resulted in a dramatic increase in our understanding of EC function. Culture of EC from genetically modified mice will allow us to gain a more detailed understanding of the functional consequences that specific genes have on endothelium homeostasis. Previous preparation of mouse REC has been difficult and tedious. Here we report a method for routine isolation and propagation of REC from immortomice. The magnetic beads coated with antibodies against the endothelial cell specific marker PECAM-1 were used to enrich REC from wild type and TSP1$^{-/-}$ immortomice. The immortomouse expresses a thermolabile strain (tsA58) of the simian virus (SV)40 large T antigen (taA58 Tag) driven

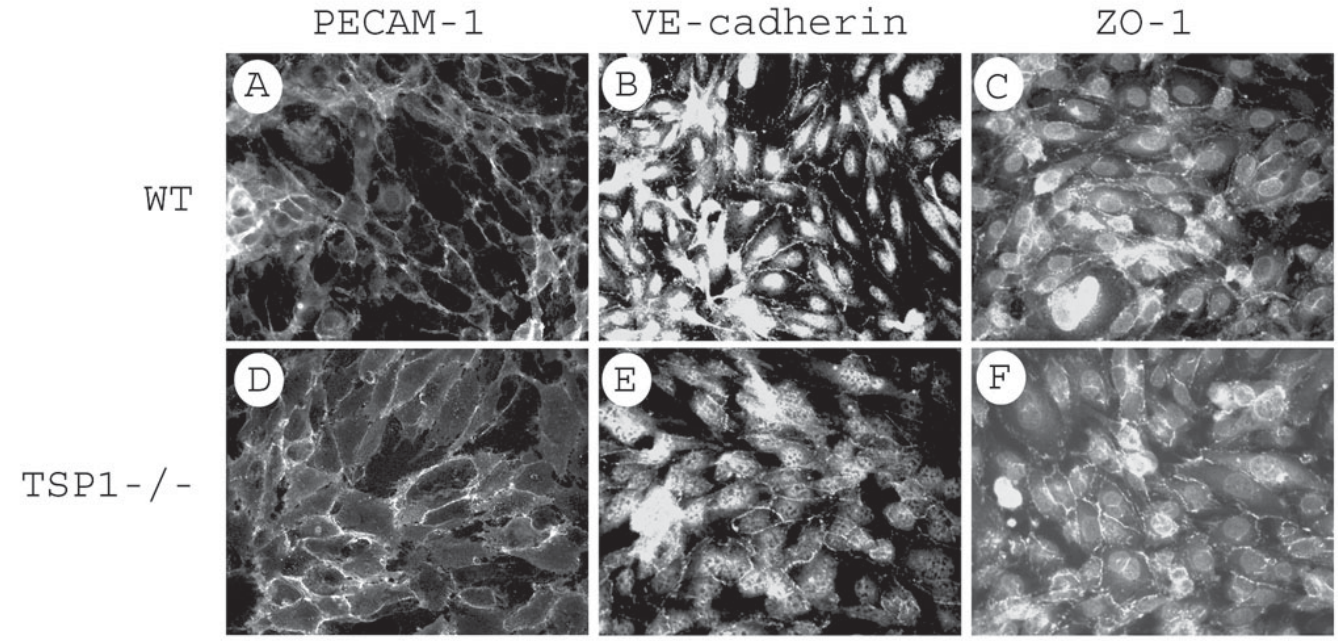


Figure 5. Localization of PECAM-1, VE-cadherin, and ZO-1 in REC. Confluent monolayer of wild type (**A**,**B**,**C**) or TSP1$^{-/-}$ (**D**,**E**,**F**) REC in multi-chamber slides were stained with anti-PECAM-1 (**A** and **D**), anti-VE-cadherin (**B** and **E**), and anti-ZO-1 (**C** and **F**). Note the localization of these proteins at sites of cell-cell contact. These experiments were repeated at least twice with two different strains of REC.

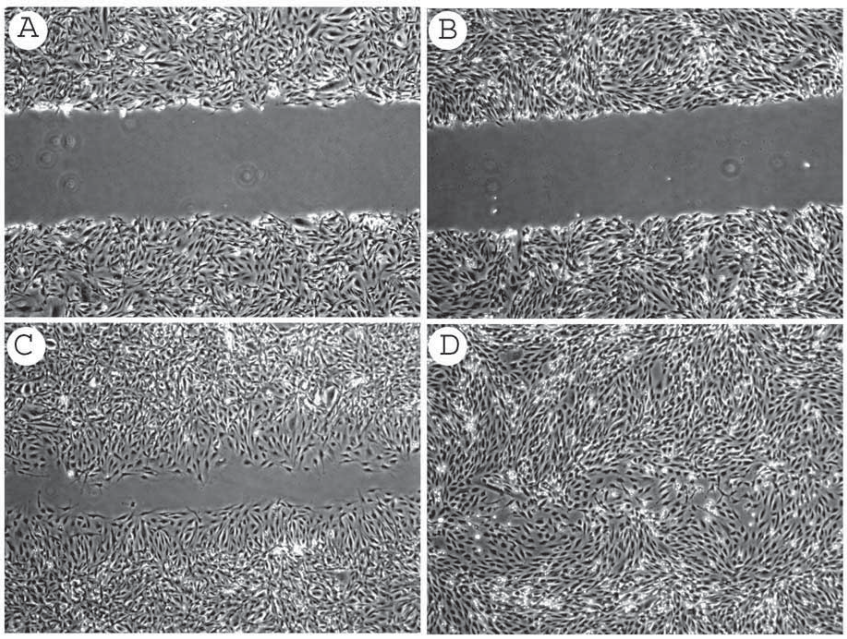
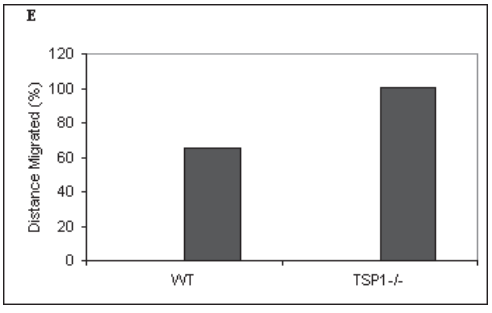


Figure 6. Scratch wound assays of REC. Confluent monolayers of wild type (**A** and **C**) or TSP1$^{-/-}$ (**B** and **D**) REC were wounded and wound closure was monitored after 24 h and photographed using a digital camera. Note the rapid migration of TSP1 and closure of the wound by 24 h, while significant area of the wound remains uncovered in wild type REC. Panel **E** shows the quantitative assessment of migration assay as described in Methods. These experiments were performed at least twice with two different strains of REC; a representative experiment is shown.



by an inducible major histocompatibility complex H-2K promoter, thus eliminating many intrinsic problems with immortalized lines [23]. 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 38 °C in the absence of interferon-γresults in loss of large T antigen [23]. We successfully isolated and cultured REC from wild type and TSP1$^{-/-}$ mice. FACScan analysis showed nearly 100% of the isolated cells express PECAM-1 and VE-cadherin. These cells were readily passaged and propagated in culture for up to six months without significant loss in expression of EC markers. To our knowledge, this is the first report of isolation and culture of REC from wild type and transgenic mice.

The ability to culture REC from TSP1$^{-/-}$ mice will allow us to delineate the role of TSP1 in retinal vascular development and study its contribution to appropriate interactions with retinal pericytes and astrocytes in two- and three-dimensional cultures. Our results show, despite the similar morphology and growth characteristics, the wild type and TSP1$^{-/-}$ REC exhibit different characteristics in a number of assays. The TSP1$^{-/-}$ REC ability to form capillary-like structures on Matrigel is severely compromised, while wild type REC form extensive network of capillaries on Matrigel (Figure 3) similar to endothelial cells from vascular beds of other tissues. The TSP1$^{-/-}$ REC also exhibited an enhanced migratory phenotype compared to the wild type REC. This is consistent with a less differentiated phenotype of TSP1$^{-/-}$ REC. We have shown that TSP1 expression in endothelial cells favors a differentiated, quiescent phenotype [1]. Our recent studies of the developing retinal vasculature indicated that expression of TSP1 is essential for proper remodeling and maturation (unpublished data). The TSP1$^{-/-}$ mice exhibit increased retinal vascular density due to protection of endothelial cells from apoptosis during remodeling and maturation of retinal vasculature. TSP1 appears to play an important role in promoting and/or maintaining the differentiated state of endothelium. Therefore, the inability of TSP1$^{-/-}$ REC to organize and form capillary like structure is consistent with the role of TSP1 in promoting the differentiated phenotype.

The increased expression of fibronectin in TSP1$^{-/-}$ REC supports the notion that these cells are dedifferentiated and more migratory, as has been demonstrated in dedifferentiated epithelial cells [24]. We have recently shown that expression of a PECAM-1 isoform in Madin Darby Canine Kidney (MDCK) cells results in sustained activation of mitogen activated protein kinase/extracellular regulated kinase (MAPK/ERKs) pathway. These cells exhibit a dedifferentiated, highly migratory phenotype [24], and express high levels of fibronectin and α5β1 integrin (unpublished data). In addition, expression of dominant active MEK-1 in endothelial cells results in activation of MAPK/ERKs, increased migration, and tube formation (unpublished data). In fact, integrin α5β1 and its ligand fibronectin are expressed at significantly increased levels, in new vessels induced by growth factors or solid tumors [25]. Furthermore, interaction of fibronectin and its receptor integrin α5β1 is essential for angiogenesis in vitro and in vivo [26]. Therefore, the absence of TSP1 promotes a more dedifferentiated, angiogenic, and migratory phenotype. This is consistent with our observation in brain endothelial (bEND) cells which are highly angiogenic and express little or no TSP1 [27]. Expression of TSP1 in these cells promotes a differentiated, quiescence phenotype by down regulating expression of a number of genes with potential role in angiogenesis [1,27]. Therefore, the REC from wild type and TSP1$^{-/-}$ mice exhibit many characteristics of the vascular endothelium in vivo.

In summary, we have described a simple method for the isolation of REC from wild type and TSP1$^{-/-}$ immortomice. These cells can be readily propagated at the permissive temperature and retain their EC 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 REC from wild type and TSP1$^{-/-}$ mice under normal or pathological conditions such as hypoxia or hyperglycemia will enhance our understanding of the role TSP1 plays in retinal vascular homeostasis and neovascularization.

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