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Tubedown-1 in Remodeling of the Developing Vitreal Vasculature *in Vivo* and Regulation of Capillary Outgrowth *in Vitro*

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Tubedown-1 (tbdn-1) is a mammalian homologue of the N-terminal acetyltransferase subunit NAT1 of *Saccharomyces cerevisiae* and copurifies with an acetyltransferase activity. Tbdn-1 expression in endothelial cells becomes downregulated during the formation of capillary-like structures *in vitro* and is regulated *in vivo* in a manner which suggests a functional role in dampening blood vessel development. Here we show that tbdn-1 is expressed highly in the vitreal vascular network (tunica vasculosa lentis and vasa hyaloidea propria) during the pruning and remodeling phases of this transient structure. The vitreal blood vessels of mice harboring a targeted inactivation of *TGF-β2* fail to remodel and abnormally accumulate, a phenomenon reminiscent of the ocular pathology resembling persistent fetal vasculature (PFV) in humans. Since suppression of normal tbdn-1 expression has been previously observed in retinal vessel proliferation, we analyzed vitreal vascular changes and tbdn-1 expression in *TGF-β2*$^{-/-}$ eyes. The nuclei of vitreal vessel endothelial cells in *TGF-β2*$^{-/-}$ eyes express proliferating cell nuclear antigen (PCNA) and exhibit increased levels of active $^{P42/44}$mitogen-activated protein kinase (phospho-$^{P42/44}$MAPK), characteristics consistent with proliferative endothelial cells. In contrast to normal vitreal vessels, collagen IV expression exhibited a disorganized pattern in the *TGF-β2*$^{-/-}$ vitreal vessels, suggesting vessel disorganization and possibly a breakdown of vessel basal laminae. Moreover, vitreal vessels of *TGF-β2*$^{-/-}$ mice lack expression of pericyte markers (CD13, alpha smooth muscle actin) and show ultrastructural changes consistent with pericyte degeneration. The accumulating vitreal blood vessels of *TGF-β2*$^{-/-}$ mice, while maintaining expression of the endothelial marker von Willebrand Factor, show a significant decrease in the expression of tbdn-1. We addressed the functional role of tbdn-1 in the regulation of vitreal blood vessels using an *in vitro* model of choroid–retina capillary outgrowth. Clones of the RF/6A fetal choroid–retina endothelial cell line showing suppression of tbdn-1 levels after overexpression of an antisense *TBDN-1* cDNA display a significant increase in the formation of capillary-like structures *in vitro* compared with controls. These findings suggest that tbdn-1 inhibits capillary-like formation *in vitro* and may serve to dampen vitreal blood vessel formation preceding the regression of the vitreal vasculature during development. Our results also suggest that tbdn-1 may participate with *TGF-β2* in regulating normal development of the vitreal vasculature.

Key Words: **tubedown-1; vitreal vasculature; TGF-β2; endothelium; pericyte; capillary outgrowth.**

INTRODUCTION

In order to gain new insights into the processes controlling development and remodeling of the vasculature, we previously isolated and characterized a novel protein, tubedown-1 (tbdn-1), which shows homology to yeast NAT1, N-terminal acetyltransferase 1 subunit (Gendron *et al.,* 2000). Tbdn-1 copurifies with an acetyltransferase activity, suggesting that tbdn-1 may serve a regulatory function by participating in the acetylation of other proteins. Tbdn-1 is expressed in vascular structures during embryogenesis, and tbdn-1 protein expression is significantly downregulated during the formation of capillary-like struc-

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tures by endothelial cells *in vitro* on Matrigel (Gendron *et al.*, 2000, 2001a). In adulthood in mice, expression of tbdn-1 is restricted to only a few tissues, including the ocular endothelium, the bone marrow, blood vessels in regressing ovarian follicles, and atrial endocardium. In human, the suppression of normal retinal vascular tbdn-1 expression has been observed in retinal vessel proliferation in proliferative diabetic retinopathy (Gendron *et al.*, 2001a).

During eye development, the posterior chamber (vitreal) vasculature is composed of the hyaloid blood vessel network (vasa hyaloidea propria), which extends in a network of capillaries from the hyaloid artery at the posterior portion of the eye and the tunica vasculosa lentis surrounding the developing lens (Ito and Yoshioka, 1999). The developing vitreal vasculature can be viewed as a good *in vivo* model of vascular remodeling, maturation, and regression since it has a finite lifespan encompassing all of these processes. In the mouse, the vitreal vasculature starts to develop at E11.5 and matures and progressively regresses almost to completion at approximately P10 (Mitchell *et al.*,1998; Ito and Yoshioka, 1999; Zhu *et al.*, 1999). The vitreal vasculature temporarily nourishes posterior chamber tissues. Formation and maturation of the vitreal vasculature involves extensive initial vasculogenesis followed by combinations of vasculogenesis and angiogenesis events which are accompanied by pruning and remodeling of the maturing vitreal vessel network (Lang *et al.*, 1993; Yang *et al.*, 1996, Zhu *et al.*, 1999; Mitchell *et al.*,1998; Ito and Yoshioka, 1999; Ash and Overbeek, 2000). Both the maturation and the regression processes of the vitreal vasculature in the mouse are associated with apoptosis which commences at approximately E17.5 (Lang *et al.*, 1993; Mitchell *et al.*,1998; Ito and Yoshioka, 1999; Ash and Overbeek, 2000).

Persistent fetal vasculature (PFV; Goldberg, 1997) includes a number of vascular abnormalities, such as persistent hyperplastic primary vitreous (PHPV), persistent pupillary membrane, persistent iridohyaloid vessels, the Mittendorf's Dot, Bergmeister's papilla, remnants of the hyaloid artery, persistent vasa hyaloidea propria, and congenital nonattachment of the retina. PFV is characterized by the failure of the hyaloid and tunica vasculosa lentis vasculature to regress (Boeve *et al.*, 1990; Steichen-Gersdorf *et al.*, 1997; Castillo *et al.*, 1997; Goldberg, 1997; Silbert and Gurwood, 2000). In the most severe form, this condition can lead to vision loss and blindness. Several transgenic mouse models display abnormalities of vitreal vascular development and remodeling. Transgenic mice in which ocular macrophages are disrupted display persistence of the vitreal vasculature (Lang *et al.*, 1993). A PFV-like condition also develops in p53-deficient mice (Reichel *et al.*, 1998). Mice harboring specific NOTCH2 mutations display hyperplasia of cells associated with the hyaloid vasculature (McCright *et al.*, 2001). It was noticed that mice lacking *TGF-β2* show abnormal accumulation of the developing vitreal vasculature (Saika *et al.*, 2001). However, the molecular and anatomical changes associated with accumulation of vitreal vessels in *TGF-β2*$^{-/-}$ eyes have not yet been characterized. To gain further insight into the role of tbdn-1 in the present work, we have analyzed the expression of tbdn-1 in normal development of the vitreal vasculature and in accumulated vitreal vessels of *TGF-β2*$^{-/-}$ eyes. We have characterized in detail the cellular and structural changes in the *TGF-β2*$^{-/-}$ vitreal vasculature. In addition, the functional role of tbdn-1 in endothelial cells was investigated by evaluating its effects on the formation of capillary-like structures by a fetal choroid–retina endothelial cell line *in vitro*.

EXPERIMENTAL PROCEDURES

Immunohistochemistry

Immunohistochemistry was performed on paraformaldehyde-fixed, paraffin-embedded sections of developing mouse tissue and human eye specimens to detect tbdn-1 and other markers. The anti-tbdn-1 chicken IgY antibody (Ab1272) was described previously and was proven useful for immunocytochemistry, Western blot, and immunoprecipitation analysis (Gendron *et al.*, 2000). All uses of mouse tissues in this study were adherent to the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals). Human specimens were from autopsy material obtained postmortem from consenting donors under the approval of the Institutional Review Boards of the Smith Kettlewell Eye Research Institute, San Francisco and Childrens Hospital Medical Center, Cincinnati. All research on the human specimens followed the tenets of the Declaration of Helsinki at all times. Reactions for tbdn-1 and negative control chicken IgY preimmune staining were carried out by using modified salt conditions in Tris-buffered saline (TBS) with 25 mM NaCl. All other reactions were carried out in TBS with 150 mM NaCl. Following a 1-h blocking step in 6% lowfat powdered milk in TBS, sections were incubated with 1/200 dilutions of anti-tbdn-1 antibody Ab1272 (Gendron *et al.*, 2000) or the preimmune IgY in 3% lowfat powdered milk in TBS. The endothelial cell marker rabbit anti-von Willebrand Factor (vWF) antibody (used undiluted; directly conjugated to peroxidase; Dako, Denmark), the mouse monoclonal vascular smooth muscle marker anti-alpha smooth muscle actin (ASMA) antibody (dilution of 1/100; Sigma Immunochemicals, St. Louis, MI), mouse monoclonal anti-CD13 (dilution of 1/50; clone SJ1D1 from Coulter Immunology, Hialeah, FL), rabbit anti-phospho $^{P42/44}$MAPK (dilution of 1/500; New England Biolabs), rabbit anti-PCNA (dilution of 1/25; Santa Cruz Biotechnology, Inc.), and the vascular wall basement membrane marker anti-collagen IV antibody (dilution of 1/100; Saika *et al.*, 2001) were also used for labeling endothelial cells and vascular wall structures in sections from blood vessels. The sections used for staining vitreal structures were always taken from the same level, at the eye equator, in order to maintain consistency in section locations between eye specimens. After rinsing in the appropriate concentration of TBS, reactions were developed by using alkaline phosphatase-conjugated species-specific secondary antibodies (anti-chicken IgY second antibody reagents from Promega, Madison, WI, were also incubated and washed in 25 mM NaCl TBS). Red color reactions for tbdn-1 and ASMA staining were generated by using naphthol-AS-MX Phosphate in the presence of Fast Red and Levamisole (to block endogenous tissue alkaline phosphatase activity). Dark brown color reactions for vWF staining were generated by using diaminobenzidine (DAB) in the presence of hydrogen peroxide (sections were previously pretreated with hydrogen peroxide to quench endogenous peroxidase activity). Reacted sections were lightly counterstained by using a

0.5% aqueous solution of methyl green, rinsed in water, dried, and mounted in Permount (Fisher, Pittsburgh, PA). Sections were viewed and photographed by using a Nikon microscope system with a Kodak digital camera attachment.

Preparation and Analysis of TGF-β2$^{-/-}$ Mouse Embryos

Wild type mouse embryos and mouse embryos either heterozygous or homozygous null for a disrupted *TGF-β2* gene were generated via gene targeting in 129-C57BL/6 mice and genotyped as previously reported (Sanford *et al.*, 1997). Tissue collection and processing of wild type embryos and embryos from *TGFβ2*$^{-/-}$ and heterozygous littermates were also performed as previously described (Saika *et al.*, 2001). Five to ten embryos from each genotype were morphologically and immunohistochemically analyzed.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed as previously described (Saika *et al., 2001*). Briefly, both *TGFβ2*$^{+/-}$ and *TGFβ2*$^{-/-}$ eyes from E18.5 stage were dehydrated and embedded in Epon 812 mixture. Semithin sections were prepared and stained with toluidine blue for inspection at 1000× high power by light microscopy. Ultrathin sections were stained with electron dense uranyl acetate and lead citrate, and vitreal tissues were observed under TEM (Saika *et al.*, 2001).

Cell Culture

IEM cells are an immortalized embryonic endothelial cell line originally derived using differentiation products of mouse embryonic stem cells (Gendron *et al.*, 1996). MK/T-1 cells are an immortalized mouse fibroblast cell line derived using cultures of mouse corneal stroma cells (Gendron *et al.*, 2001b). Primary culture of human umbilical vein endothelial cells (HUVEC) was obtained from Clonetics (San Diego, CA). RF/6A cells (American Type Culture Collection, Manassas, VA) are an immortalized endothelial cell line derived from rhesus macaque fetal choroid–retinal tissue (Lou and Hu, 1987a,b). BREC cells are primary bovine retinal endothelial cells prepared as previously described (Schor and Schor, 1986). Cells were grown and maintained in culture in low glucose Dulbecco's Modified Eagle Media (DMEM) supplemented with 2 mM glutamine and either 10% (IEM, RF/6A, MK/T-1, and HUVEC) or 5% (BREC) of fetal bovine serum (FBS). For RF/6A cells, culture media was further supplemented with 50 μM of nonessential amino acids, while HUVEC medium was supplemented with 1 ng/ml of bFGF and a mixture of insulin, transferrin, and selinium (Gibco/BRL).

For treatment of RF/6A, HUVEC, BREC, and MK/T-1 cells with TGF-β *in vitro*, cells were cultured for 16–24 h in reduced FBS (1%)-containing media and then stimulated for 24 and 48 h with 3 ng/ml of TGF-β1 (R&D Systems, Minneapolis, MN).

Western Blotting

Western blotting analysis of whole cell lysates was performed by standard procedures using chemiluminescence detection (ECL Plus reagent; Amersham). Cell lysates were prepared by using Triton X-100 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100) supplemented with 1 mM DTT, protease inhibitors (1 mM PMSF, 0.3 U/ml aprotinin, and 10 μg/ml leupeptin) and phosphatase inhibitors (1 mM sodium orthovanadate, 25 mM sodium fluoride, and 10 mM β-glycerophosphate). Lysates were clarified by centrifugation, and protein was quantified and analyzed by SDS–PAGE. Gels were processed for Western blotting by using the Ab1272 chicken IgY antibody directed against tbdn-1 (Gendron *et al.*, 2000). Expression of ASMA in RF/6A and MK/T-1 cells (Gendron *et al.*, 2001b) was analyzed by Western blot using an anti-ASMA antibody (Sigma). VEGFR-2 Western blot analysis was performed by using a polyclonal anti-VEGFR-2 antibody (Santa Cruz Biotechnology).

Immunoprecipitation

Cell lysates for immunoprecipitation were prepared by using TNB lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 0.5% Brij 96) supplemented with protease inhibitors and phosphatase inhibitors as described above. Lysates were clarified by centrifugation, and protein concentrations were quantified. For analysis of VEGFR-2 expression, VEGFR-2 was immunoprecipitated from RF/6A cell lysates by incubation for 6 h to overnight at 4°C with 7.5 μg of a polyclonal anti-VEGFR-2 antibody (Santa Cruz Biotechnology). Immune complexes and whole cell lysates were analyzed by SDS–PAGE. Gels were processed for Western blotting analysis with a different anti-VEGFR-2 antibody (Santa Cruz Biotechnology) than that used for immunoprecipitation.

Recombinant cDNA Constructs and Proteins, and Transfections

For assessing the effects of downregulating the level of tbdn-1 expression on capillary formation *in vitro*, IEM and RF/6A cells were transfected by using lipofection with the vector pcDNA3.1/Zeo (Invitrogen) alone, or with a construct of the pcDNA3.1/Zeo vector harboring *TBDN-1* cDNA nucleotide sequences 1–1413 in an antisense orientation (*ASTBDN-1*). This latter *ASTBDN-1* construct was described previously and shown to block tbdn-1 protein expression in IEM cells (Gendron *et al.*, 2000). Two days after transfection, cells were selected with Zeocin (Invitrogen). After selection, 30 clones from each transfection were isolated with cloning cylinders, expanded, and characterized. Stable IEM cell transfectants and RF/6A clones were maintained in the presence of 75 and 100 μg/ml of Zeocin, respectively.

Acetyltransferase Activity

For determination of acetyltransferase activity copurifying with tbdn-1, tbdn-1 was immunoprecipitated by using the Ab1272 antibody from equal amounts of protein lysates of control IEM cells or IEM cells showing reduced levels of tbdn-1 by overexpression of *ASTBDN-1* construct as previously described (Gendron *et al.*, 2000). Immunoprecipitates prepared using anti-tbdn-1 Ab1272 were next processed for acetyltransferase reactions in the presence of [^{3}H]acetyl-Co-enzyme A as described previously (Gendron *et al.*, 2000).

Capillary Outgrowth Assays

Capillary outgrowth of parental and transfected RF/6A cell clones on Matrigel (Collaborative Biomedical Products, Bedford, MA) was induced by 10 ng/ml of basic fibroblast growth factor (bFGF) essentially as described (Gendron *et al.*, 1996). Two days after stimulation with bFGF, cells were transferred to Matrigel in

the presence of fresh bFGF. Relative capillary abundance was quantitated by using methods previously described (Gendron *et al.*, 1996; Paradis and Gendron, 2000). Colonies of cells sprouting capillary structures on Matrigel were photographed, and the number of sprouts per individual colony was counted. To account for variation in colony sizes, the capillary abundance was expressed as the number of capillaries per individual colony divided by the approximate diameter of the colony. The diameters of individual colonies were approximated by calculating the average of four measurements evenly spaced around the circumference of each colony. At least three separate capillary outgrowth assays were performed in duplicate on several of the different RF/6A transfectants and parental cells. Colonies of two control clones and two *ASTBDN-1* clones showing downregulation of tbdn-1 protein were analyzed in larger numbers for more precise quantification of relative capillary abundance. Results were expressed as mean of relative capillary abundance ± standard error of the mean (sem).

RESULTS

Tbdn-1 Is Expressed in Developing Mouse and Human Vitreal Vasculature

In order to study the temporal dynamics and spatial localization of tbdn-1 expression in the developing posterior chamber ocular vasculature, immunolocalization of tbdn-1 protein was performed on specimens of developing mouse eye. Our results revealed that tbdn-1 protein expression in vitreal blood vessels appears to increase between embryonic day (E) 13.5 of embryogenesis and postnatal day (P) 1 of postnatal development (Figs. 1A–1D). Tbdn-1 expression was also detected at P5 in the vitreous (Fig. 1E). The embryonic vitreal blood vessels were negative when stained with preimmune IgY, a negative control for the tbdn-1 antibody (see Fig. 1F). Hyalocytes scattered around the vitreal vascular networks at the E16.5 and E18.5 stages also expressed tbdn-1 at similar levels as found in endothelial cells lining the vitreal blood vessels. These results indicate that the mouse vitreal vasculature expresses tbdn-1 during late gestation to early postnatal stages as the embryonic vitreal vasculature matures during ocular development.

Tbdn-1 immunolocalization was also performed on a human embryonic eye specimen to study the spatial localization of tbdn-1 protein expression in the developing human ocular vitreal vasculature. Analysis of a 14-week human embryonic eye revealed that tbdn-1 was also expressed in human vitreal vessels (Figs. 1G and 1H). The human embryonic vitreal vascular networks also expressed the endothelial marker vWF (Fig. 1I) and were negative when stained with preimmune IgY, a negative control for the tbdn-1 antibody (Fig. 1J).

Analysis of Morphology of Accumulated Vitreal Vasculature of Mice Lacking TGF-β2

In a previous study, it was noticed that, in contrast to wild type or heterozygote *TGF-β2*$^{+/-}$ mice, the posterior chamber of *TGF-β2*$^{-/-}$ eyes show an accumulation of a vitreal mass during late gestation (Saika *et al.*, 2001). As described above, since tbdn-1 is normally expressed in developing vitreal cells, the *TGF-β2*$^{-/-}$ vitreal defect was further characterized. In order to assess in more detail the morphological changes of the posterior chamber tissues of *TGF-β2*$^{-/-}$ eyes, light microscopy analysis of toluidine blue-stained semithin sections and TEM of thin sections were performed on E18.5 specimens (Figs. 2 and 3, respectively). There was no observable difference in the morphology of the vitreous between *TGF-β2*$^{+/-}$ and wild type eyes, either at the level of the semithin toluidine blue sections or the TEMs (not shown). Toluidine blue-stained semithin sections clearly show that a large mass of vitreal and vascular tissues is present in the posterior chamber in *TGF-β2*$^{-/-}$ eyes, but not in *TGF-β2*$^{+/-}$ eyes (Fig. 2). TEM (Fig. 3) revealed that vitreal vessel pericytes of *TGF-β2*$^{+/-}$ eyes appeared to retain a normal cellular morphology and maintained tight cell–cell and cell–basal laminal adhesions (Figs. 3A and 3C). In sharp contrast, *TGF-β2*$^{-/-}$ vitreal vessels appeared to contain degenerated pericytes compared with *TGF-β2*$^{+/-}$ eyes (Figs. 3B and 3D–3F). The pericytes in *TGF-β2*$^{-/-}$ vessels show nuclear and cytoplasmic degeneration with lack of normal organelles present, rare degenerated mitochondria, loss of cell-to-cell contact. and loss of contact with the surrounding endothelial cell basal laminae.

Expression Analysis of tbdn-1 and Blood Vessel Markers in Accumulated Vitreal Vasculature of Mice Lacking TGF-β2

We next determined whether the expression of tbdn-1 in the posterior chamber vasculature was affected in TGF-*β2*$^{-/-}$ developing eyes. Using immunohistochemistry, we observed a large decrement in tbdn-1 expression in the accumulated blood vessels of the vitreal vasculature in *TGF-β2*$^{-/-}$ developing eyes (Figs. 4D and 4H) as compared with robust tbdn-1 expression in the wild type (Figs. 4A–4C) and *TGF-β2*$^{+/-}$ heterozygous littermates (see Figs. 4E–4G). This loss of tbdn-1 expression in *TGF-β2*$^{-/-}$ vitreal vessels is observable at E16.5 (not shown) and E18.5 (Fig. 4) stages. The cells surrounding the accumulated *TGF-β2*$^{-/-}$ vessels (presumably hyalocytes) also displayed a significant decrease in tbdn-1 expression compared with hyalocytes in wild type and *TGF-β2*$^{+/-}$ heterozygous eyes. Like the *TGF-β2*$^{+/-}$ heterozygous eyes, the vitreal blood vessels in *TGF-β2*$^{-/-}$ eyes express the endothelial marker vWF (Figs. 5A and 5B, respectively).

To further examine vessel organization and vascular basal lamina integrity, the vitreal vasculature was stained for collagen IV expression, which is a marker for the vessel wall and labels the normal basal laminal components of developing vitreal blood vessels (Sarthy, 1993). Vitreal blood vessels in *TGF-β2*$^{+/-}$ eyes at the E18.5 (Fig. 5) stages showed a well-defined collagen IV expression pattern appearing as rings of staining of the basal laminae in the cross-sectioned vitreal vessels (Fig. 5C). Vitreal blood vessels of *TGF-β2*$^{-/-}$ mice also expressed collagen IV, but the staining for this marker in the *TGF-β2* null vitreal vessels

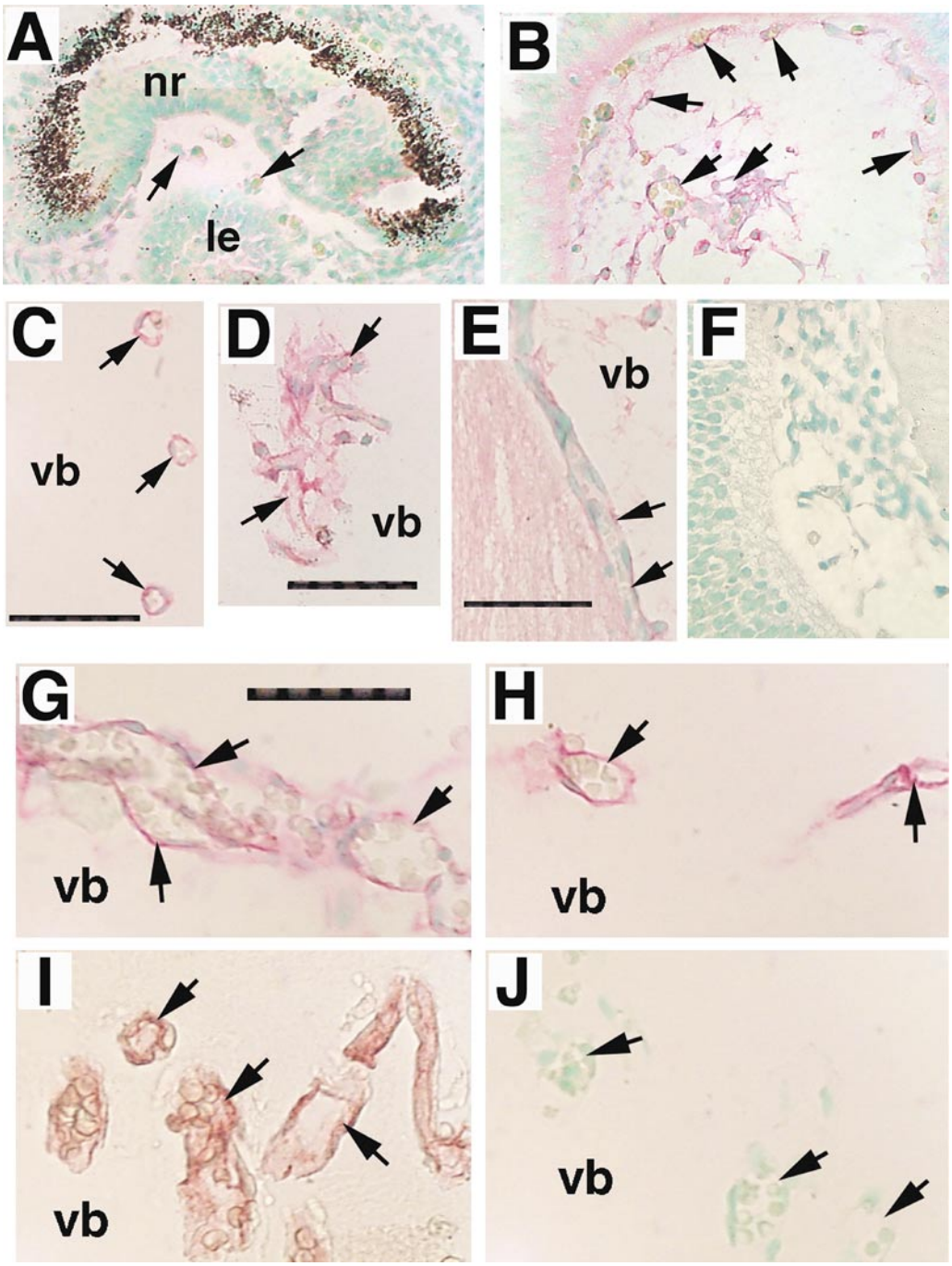

FIG. 1. Tbdn-1 expression in the developing mouse and human ocular vitreal vasculature. (A) Tbdn-1 protein staining as revealed by red stain in the developing mouse E13.5 eye (arrows indicate the early developing hyaloid network with in the vitreous body). (B) Tbdn-1 protein staining (red) in the hyaloid vascular network of developing mouse E16.5 eye (arrows indicate the hyaloid vascular network within the vitreous body, which is visibly larger at this stage than at the E13.5 stage). (C) Tbdn-1 protein staining (red) in cross-sections of vitreal blood vessels in the E18.5 embryonic mouse eye (arrows indicate tbdn-1 positivity in the vitreal vascular endothelial cells). (D) Tbdn-1 protein staining (red) in endothelial cells (arrowed) of a vessel tuft of the vitreal vasculature in the postnatal P1 mouse eye. (E) Tbdn-1 protein staining in sections of vitreal vasculature in the postnatal P5 mouse eye (arrows indicate tbdn-1-positive endothelial cells lining a longitudinally sectioned blood vessel on the posterior surface of the lens). (F) A section adjacent to that shown in (B) stained with preimmune IgY, which is a negative control for the anti-tbdn-1 antibody staining, showing no staining. nr, neural retina; le, lens; vb, vitreous body. (A), (B), (C), and (F) are shown at the same magnification (×400), and scale bar in (C) equals 50 μm. (D, E) Digitally magnified slightly from ×400 to ×570 to better show sparsely distributed vessels at the E18.5–P5 stages, and scale bars equal 35 μm. (G) Tbdn-1

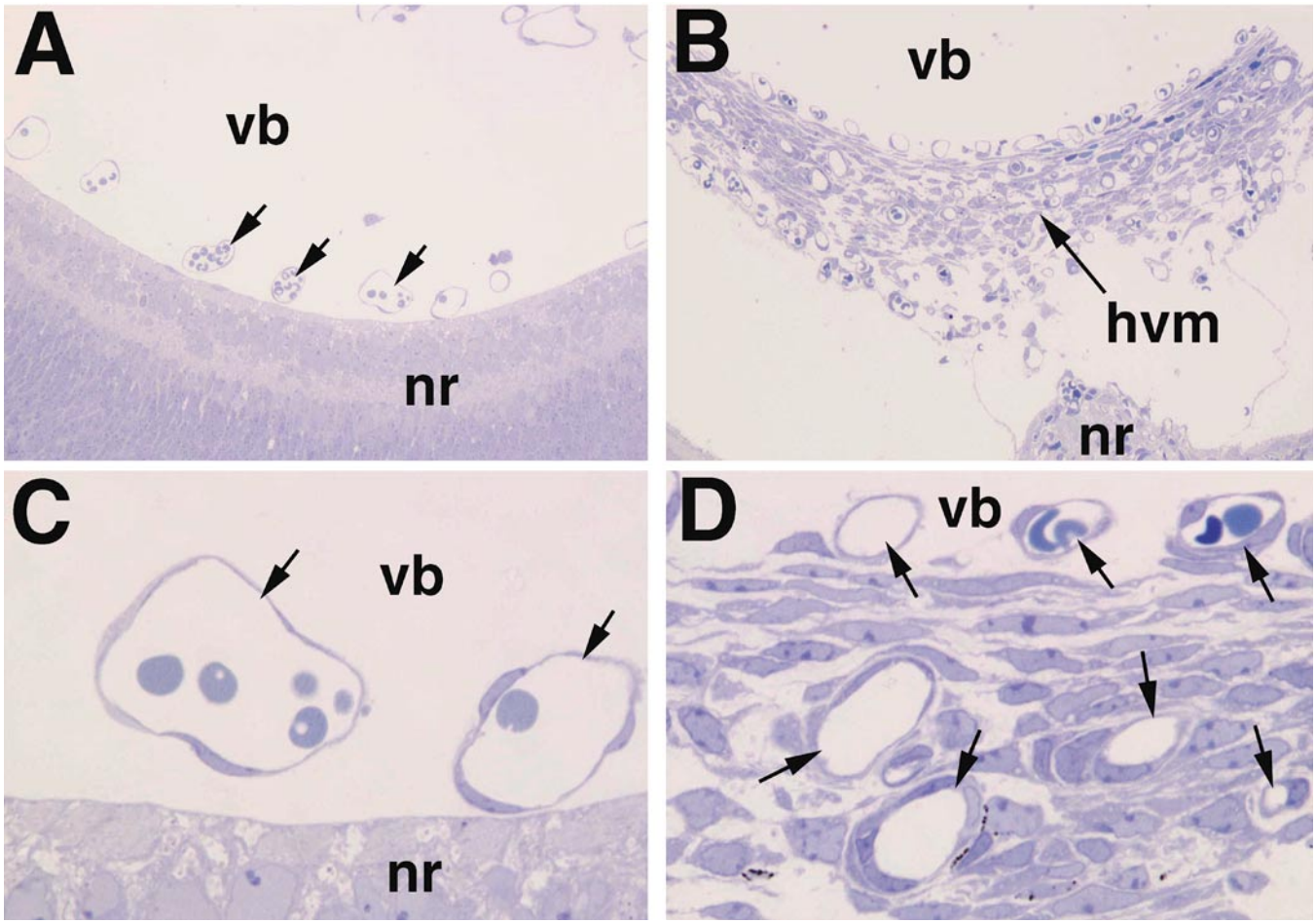

FIG. 2. Morphological analysis of the vitreous in *TGF-β2*$^{-/-}$ mice. Toluidine blue-stained semithin sections of the vitreous of *TGF-β2*$^{+/-}$ (A, C) and *TGF-β2*$^{-/-}$ (B, D) eyes at E18.5 as indicated. The bottom panels show magnified areas of top panels. Orientation is the same for all panels with the neural retina positioned at the bottom. hvm, hyaloid vascular mass; nr, neural retina; vb, vitreous body. Arrows indicate the positions of vitreal blood vessels in each case.

did not appear in well-defined rings in cross-sectioned vessels and was disorganized and diffusely distributed throughout the accumulated *TGF-β2*$^{-/-}$ vitreal tissue, suggesting that the *TGF-β2*$^{-/-}$ vitreal vessels were disorganized and that the basal laminae may be breaking down (Fig. 5D).

In order to assess the proliferative state of the vitreal endothelial cells, we also compared the expression of proliferating cell nuclear antigen (PCNA) and activated $^{P42/44}$MAPK expression in cross-sections of vitreal vessels of *TGF-β2*$^{+/-}$ and *TGF-β2*$^{-/-}$ mice. Endothelial cells lining the accumulating vitreal vessels of E18.5 *TGF-β2*$^{-/-}$ mice showed a striking upregulation of both PCNA expression and increased number of nuclei staining for the activated $^{P42/44}$MAPK compared with the vitreal vessels of *TGF-β2*$^{+/-}$ eyes (Figs. 5E–5H) at the same stage. These results indicate that the endothelial cells lining the accumulating vitreal

protein staining of the hyaloid vessels within Cloquet's canal in the developing human eye at approximately 14 weeks of gestation. (H) Tbdn-1 protein staining in the tunica vasculosa lentis vessels of the same section as (G). (J) A section adjacent to (H) stained with preimmune IgY, which is a negative control for the anti-tbdn-1 antibody staining. (I) a section adjacent to (H) stained with anti-vWF antibody, which stains and labels endothelial cells (arrows indicate vWF-positive endothelial cells). Arrows in all panels indicate the hyaloid and/or tunica vasculosa lentis blood vessels; vb, vitreous body; magnification of (G–J) is ×1000, and scale bar in (G) equals 25 μm and is representative for all panels in (G–J).

vessels of E18.5 *TGF-β2*$^{-/-}$ mice have a more proliferative status than normal.

Strikingly, the vitreal vasculature of *TGF-β2*$^{-/-}$ mice lacked staining for the pericyte marker alpha smooth muscle actin (ASMA) compared with *TGF-β2*$^{+/-}$ heterozygous vitreal vessels, which clearly showed cells staining positive for ASMA at the E18.5 stages (Figs. 5I and 5J, see arrows showing vessels lacking ASMA positivity in *TGF-β2*$^{-/-}$ eye as compared with *TGF-β2*$^{+/-}$ heterozygotes). Staining for another pericytes marker, CD13 (aminopeptidase N), was absent in the vitreal vasculature of *TGF-β2*$^{-/-}$ eyes compared with *TGF-β2*$^{+/-}$ heterozygous eyes, which showed cells staining positive for CD13 at E18.5 stage (not shown). These results indicate that the vitreal vessels of *TGF-β2*$^{-/-}$ mice do not express markers that are normally expressed by pericytes.

Reduction of tbdn-1 Expression Levels Augments Capillary Formation of the Fetal Choroid–Retina Endothelial Cell Line RF/6A

To examine the role of tbdn-1 expression in endothelial cells *in vitro*, the spontaneously immortalized endothelial cell line RF/6A derived from the choroid-retina of a rhesus macaque fetus was used (Lou and Hu, 1987a,b). The expression of the definitive endothelial marker VEGFR-2 (Millauer *et al.*, 1993; Yamaguchi *et al.*, 1993; Shalaby *et al.*, 1995) by the RF/6A cells was first confirmed to establish that these cells maintain an endothelial phenotype. As shown in Fig. 6 A, RF/6A VEGFR-2 expression is detectable both by Western blotting and by immunoprecipitation followed by Western blotting. The level of VEGFR-2 expressed by the RF/6A cells was low compared with HUVEC, which served as a positive control. The different bands recognized by the anti-VEGFR-2 antibody in RF/6A cell extracts are likely due to differential glycosylation patterns, as previously reported (Takahashi and Shibuya, 1997). Moreover, the RF/6A cells did not express any detectable levels of ASMA by Western blotting (Fig. 6B) using a monoclonal antibody raised against an N-terminal decapeptide epitope (MCEEEDSTAL) of mouse ASMA that is 100% identical with human ASMA and which has been used previously to label ASMA in rhesus tissues (Schlatt *et al.*, 1993). Furthermore, stimulation of the RF/6A cells with TGF-β1 did not induce the expression of ASMA (Fig. 6B). However, ASMA was readily detected in TGF-β1-stimulated MK/T-1 control cells (Fig. 6B). MK/T-1 cells are a fibroblast line derived from corneal stromal cell cultures which has been reported previously to upregulate ASMA upon TGF-β stimulation (Gendron *et al.*, 2001b).

To examine the effect of downregulation of tbdn-1 levels on endothelial cells *in vitro*, an antisense *TBDN-1* cDNA construct (*ASTBDN-1*) was used to decrease tbdn-1 expression in the RF/6A choroid–retina endothelial cell line. *In vitro* translation methodology confirmed that the *ASTBDN-1* construct does not encode an irrelevant protein product which could be toxic and cause nonspecific effects on cell function (data not shown). In order to verify that the antisense *TBDN-1* cDNA blocked tbdn-1 protein expression, Northern blotting, Western blotting, and acetyltransferase assays were performed on lysates of RF/6A or IEM endothelial cell clones stably overexpressing *ASTBDN-1* cDNA (Gendron *et al.*, 2000). Several RF/6A choroid–retina endothelial cell clones stably overexpressing *ASTBDN-1* showed marked inhibition of tbdn-1 protein expression levels as compared with parental clones and two control clones which expressed *ASTBDN-1* vector but showed no blockage of tbdn-1 protein production (Fig. 7A). RF/6A choroid–retina endothelial cell clones stably overexpressing *ASTBDN-1* also showed marked inhibition of tbdn-1 RNA expression levels as compared with control clones (data not shown). We have also previously shown that stable overexpression of *ASTBDN-1* can effectively block tbdn-1 protein expression in IEM embryonic endothelial cells (Gendron *et al.*, 2000). Furthermore, compared with parental cells, IEM clones exhibiting reduced levels of tbdn-1 show a significant decrease in acetyltransferase activity associated with immunoprecipitates of tbdn-1 prepared from equal quantities of protein lysates from these cells (see Fig. 7B for representative clone).

Since the growth properties of the RF/6A cell clones expressing reduced levels of tbdn-1 did not appear different from the parental cells, we used an *in vitro* capillary formation assay on Matrigel (Gendron *et al.*, 1996; Paradis and Gendron, 2000) to test whether tbdn-1 could play a role in the sprouting of capillary-like structures by these cells *in vitro*. Similar to other endothelial cell lines, the RF/6A cells can be induced by bFGF to form colonies that sprout capillary-like structures on Matrigel (Gendron *et al.*, 1996, 2001a; Paradis and Gendron, 2000). Several RF/6A cell clones exhibiting reduced levels of tbdn-1 by overexpressing *ASTBDN-1* show a significant increase in their capillary formation response compared to controls (see Figs. 8A and 8B for representative capillary colonies). The capillary-forming response of the parental RF/6A cells, two representative RF/6A *ASTBDN-1* clones showing downregulation of tbdn-1 and one representative RF/6A clone transfected with *ASTBDN-1* but not showing downregulation of tbdn-1, was quantified more precisely (Fig. 8C). Capillary colonies formed by RF/6A *ASTBDN-1* transfectant clones exhibiting reduced levels of tbdn-1 show longer, more complex and more abundant capillary sprouts (Fig. 8) than parental and control clones as assessed by precise calculation of relative capillary abundance (Paradis and Gendron, 2000). Moreover, reduction of tbdn-1 expression in the IEM embryonic endothelial cell line using the same *ASTBDN-1* cDNA construct also was associated with more abundant capillary sprouts than parental and control IEM clones (not shown). These results indicate that tbdn-1 expression in endothelial cells inhibits capillary formation.

DISCUSSION

Our results show that tbdn-1 protein is expressed by developing vitreal blood vessels during late stages of embryogenesis in the mouse. In contrast to wild type and *TGF-β2*$^{+/-}$ mice, *TGF-β2*$^{-/-}$ mice show accumulation of a

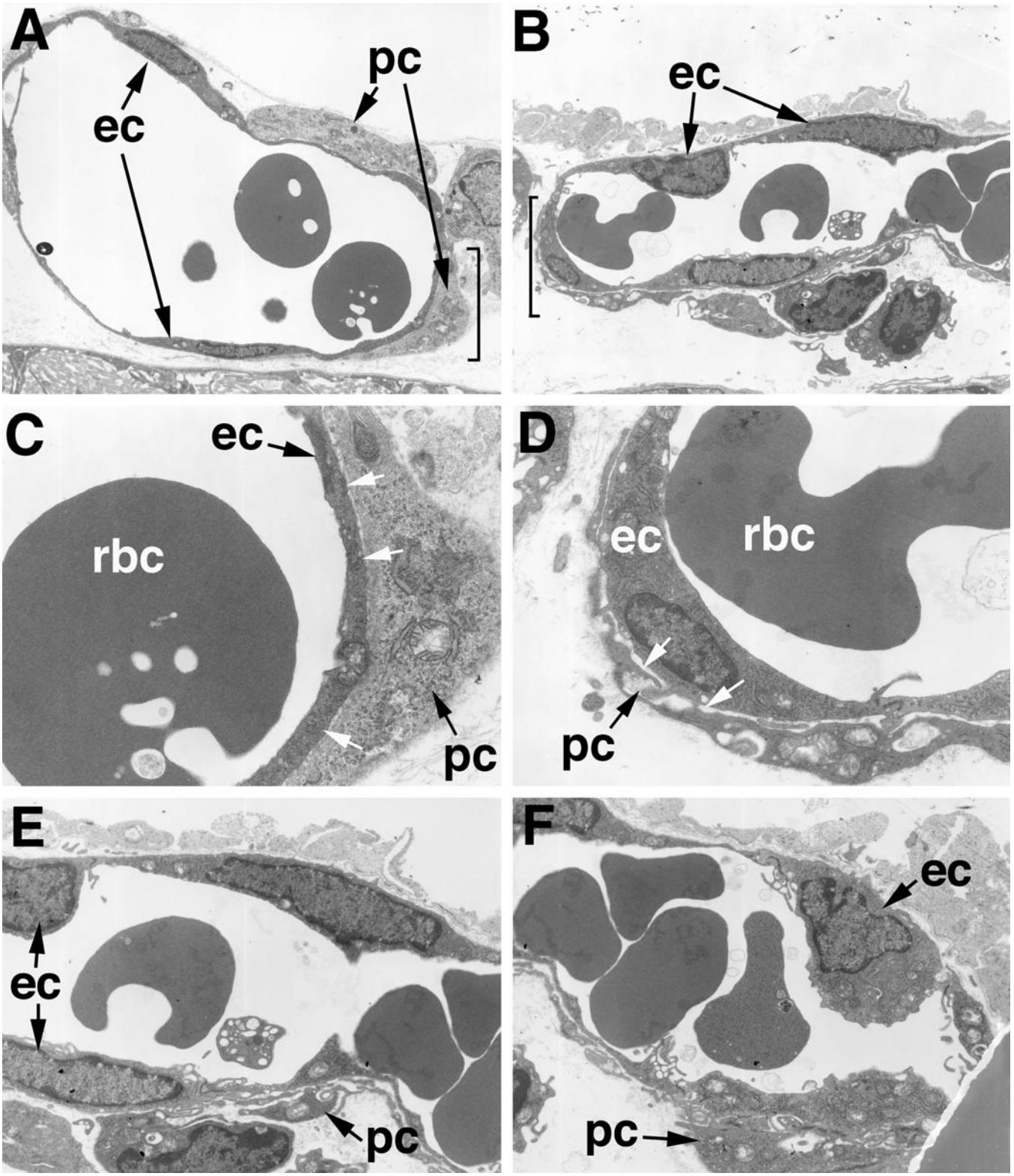

FIG. 3. Ultrastructural analysis of the vitreal vasculature in *TGF-β2*$^{-/-}$ mice. TEM of *TGF-β2*$^{+/-}$ vs *TGF-β2*$^{-/-}$ eyes at E18.5. Brackets in (A) and (B) show partial areas of the vessels magnified in (C) and (D), respectively. (A, C) *TGF-β2*$^{+/-}$ vitreal vessels. Endothelial cells and pericytes are flattened and extended in close apposition to adjacent cells and to the basal laminae (see white arrows in C indicating the tight adhesion at the endothelial–pericyte basal lamina between endothelial cell (ec) and pericyte (pc). (B, D–F) Vitreal vessels of *TGF-β2*$^{-/-}$ eyes. (B) A low-power view. (D–F) Higher power views to indicate the differences in morphology of *TGF-β2*$^{-/-}$ pericytes. Pericytes of *TGF-β2*$^{-/-}$ eyes show degeneration, cytoplasmic inclusion bodies, and loosening of contacts with adjacent endothelial cells (see white arrows in D indicating the loose endothelial–pericyte apposition), and with basal laminae compared with *TGF-β2*$^{+/-}$ eyes. ec, endothelial cells; pc, pericytes; rbc, red blood cell.

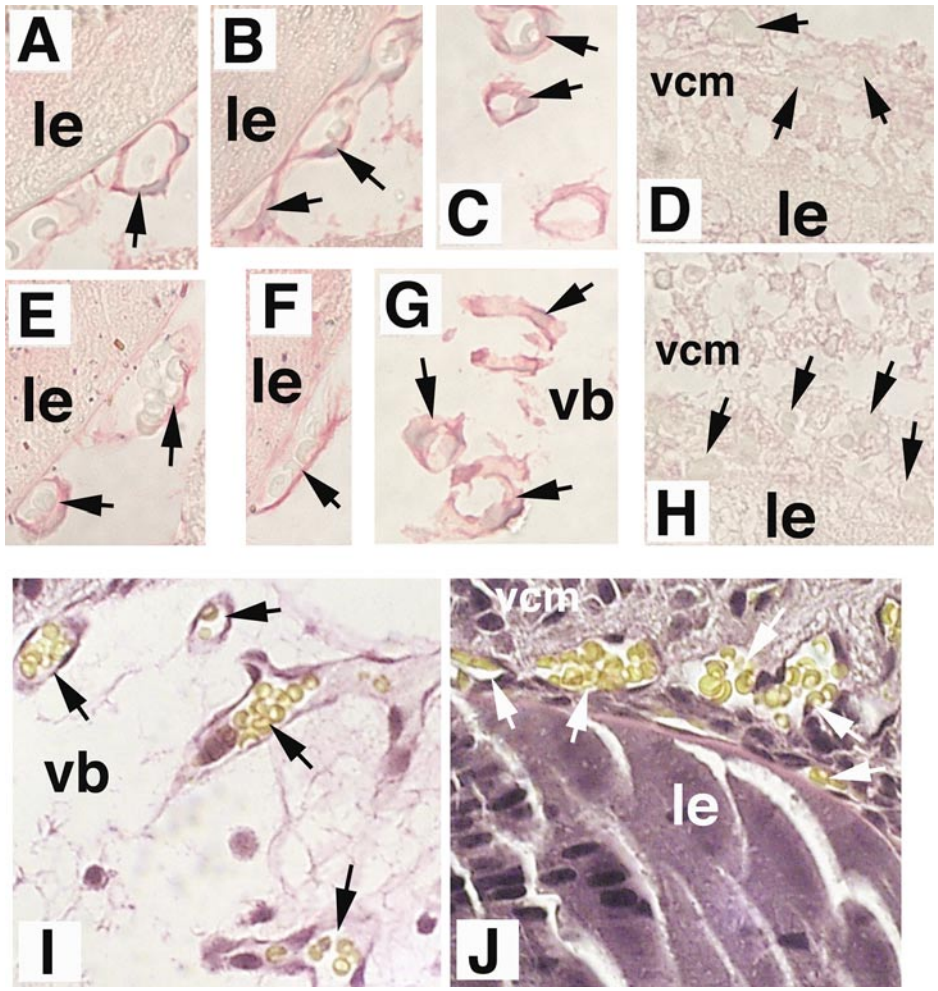

FIG. 4. Tbdn-1 expression analysis in the vitreal vasculature in wild type, *TGF-β2*$^{+/-}$, and *TGF-β2*$^{-/-}$ embryonic mouse eyes. Tbdn-1 protein expression in vitreal vessels at E18.5. Tbdn-1 protein expression, as revealed by red stain, in three separate examples of vitreal vasculature of wild type eyes (A–C), of *TGF-β2*$^{+/-}$ eyes (E–G), and in two representative examples of *TGF-β2*$^{-/-}$ eye (D, H; arrows indicate blood vessels in all panels). The expression level of tbdn-1 in vitreal vessel endothelial cells in wild type eyes is the same as that observed in *TGF-β2*$^{+/-}$ eyes. In sharp contrast to wild type and *TGF-β2*$^{+/-}$ eyes, the vitreal blood vessels in *TGF-β2*$^{-/-}$ eyes do not show expression of tbdn-1 protein (D, H). (A–H) The same magnification. (I, J) Also at the same magnification as (A–H), are adjacent sections stained with Verhoeff's elastic stain (red blood cells stain gold–yellowish and highlight the blood vessels) from *TGF-β2*$^{+/-}$ (I) and *TGF-β2*$^{-/-}$ (J) eyes. The Verhoeff's stained panels are provided here in order to more clearly indicate the positions and relative morphology of blood vessels in the sections of *TGF-β2*$^{-/-}$ eyes stained with anti-tbdn-1 antibody shown in (D) and (H). Le, lens; vcm, vitreal cell mass; vb, vitreous body; arrows in all panels indicate the positions of vitreal blood vessels.

large vitreal mass containing an increased number of vessels and hyalocyte-like cells. This accumulation of the vitreals vessels is accompanied by a loss in the expression of tbdn-1 by these vitreal vessels. In contrast to the loss of tbdn-1, our results show that accumulated vitreal vessels in *TGF-β2*$^{-/-}$ eyes still express the endothelial marker vWF. Thus, the loss of tbdn-1 in the accumulated vitreal blood vessels of *TGF-β2*$^{-/-}$ eyes is not simply due to a loss of endothelial cells from these vessels. Our TEM ultrastructural analysis also confirms that endothelial cells are present in the vitreal blood vessels of *TGF-β2*$^{-/-}$ eyes. These results suggest that removal of the *TGF-β2*-mediated influence is responsible for the loss of tbdn-1 expression in the accumulating TGF-$\beta 2^{-/-}$ vitreal vessels. We do not know at this time whether the effect of *TGF-β2* on tbdn-1 levels in vitreal endothelial cells is direct or indirect. Recent evidence (our unpublished results) indicated that mixtures of extracellular matrix (ECM) components could directly regulate tbdn-1 levels, and we are in the process of determining whether specific ECM components may be responsible for tbdn-1 regulation. In the case of the *TGF-β2*$^{-/-}$ vitreal vasculature, one might speculate that the absence of

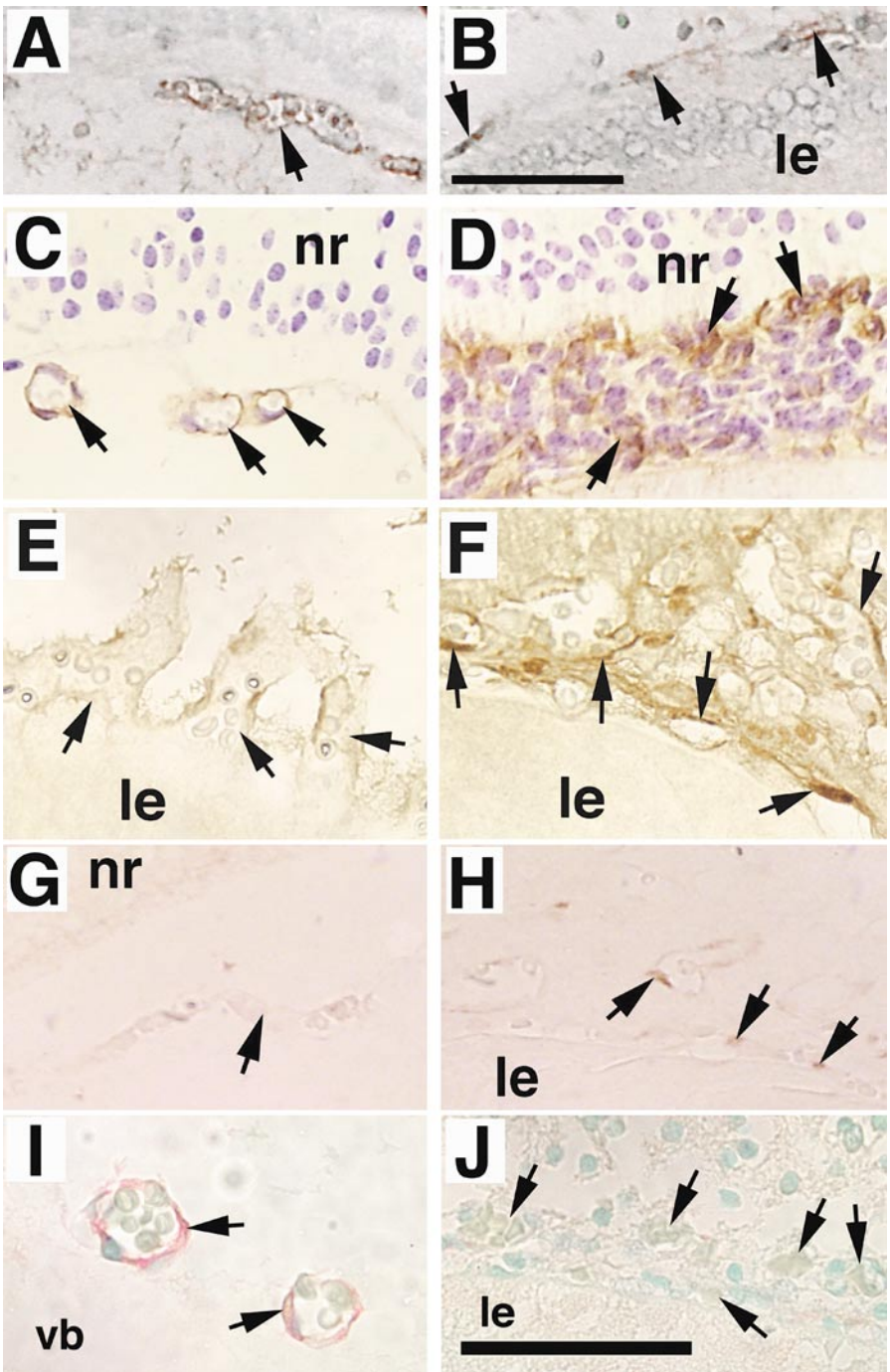

FIG. 5. Endothelial vascular wall marker analysis in the vitreal vasculature in *TGF-β2*$^{-/-}$ mice. Endothelial marker vWF expression (black stain, arrowed) is present in vitreal vasculature of both *TGF-β2*$^{+/-}$ (A) and *TGF-β2*$^{-/-}$ (B) mice. Collagen IV expression in vitreal tissue of *TGF-β2*$^{+/-}$ (C) and *TGF-β2*$^{-/-}$ (D) mice. Collagen IV stains the vascular wall basement membranes in the *TGF-β2*$^{+/-}$ and *TGF-β2*$^{-/-}$ vitreal vasculature (brown stain; arrowed). However, in TGF-*β2*$^{-/-}$ vitreous, collagen IV stains both disorganized vascular wall basement membrane material and is also expressed diffusely throughout the accumulated vitreal tissue (brown stain; arrows indicate blood vessels). Nuclear expression of PCNA (dense nuclear brown stain, arrowed) was upregulated in endothelial cells in cross-sections of vitreal vessels of *TGF-β2*$^{-/-}$ (F) as compared with the low level of PCNA in vitreal vessels of *TGF-β2*$^{+/-}$ mice (E). Nuclear expression of phosphorylated $^{p42/44}$MAPK (dense nuclear brown stain, arrowed) was upregulated in endothelial cells in cross sections of vitreal vessels of *TGF-β2*$^{-/-}$ (H) as compared with the low level of phosphorylated $^{p42/44}$MAPK in vitreal vessels of *TGF-β2*$^{+/-}$ mice (G). ASMA expression (red stain) in cross-sections of vitreal vessels of *TGF-β2*$^{+/-}$ (I) and TGF-*β2*$^{-/-}$ (J) mice. The abnormal vitreal blood vessels in TGF-*β2*$^{-/-}$ eyes do not express ASMA (J). (A–H) Shown at the same magnification. (I) is shown at the same magnification as (J). The bar in (B) of 25 mm equals 50 μm for (A–H). The bar in (J) of 35 mm equals 35 μm for (I) and (J) ($\times$1000). nr, neural retina; le, lens; arrows in all panels indicate the positions of vitreal blood vessels.

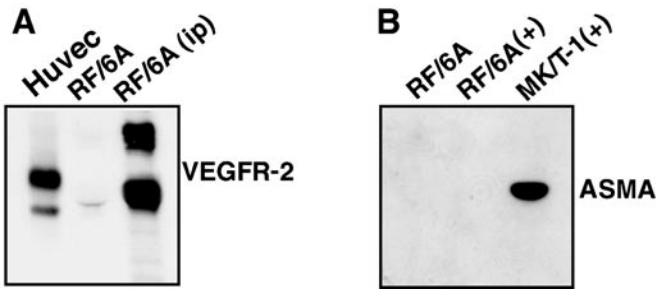

FIG. 6. RF/6A choroid retina cells retain expression of an endothelial specific marker and do not display vascular smooth muscle cell characteristics. (A) Western blot analysis of VEGFR-2 expression in whole cell lysates of RF/6A cells (RF/6A lane, 50 μg of protein loaded) and of HUVEC (10 μg of protein loaded). VEGFR-2 immunoprecipitation/Western blot analysis of RF/6A cell lysate (RF/6A [ip], lane; 1000 μg of protein was immunoprecipitated). (B) Western blot analysis of alpha smooth muscle actin (ASMA) expression in extracts of RF/6A cells (100 μg of protein loaded) nontreated (RF/6A) or stimulated with TGF-β for 72 h [RF/6A(+)] compared with an extract (20 μg of protein loaded) of a mouse corneal stromal cell line stimulated with TGF-β for 48 h [MK/T-1(+)].

TGF-β2 regulates tbdn-1 levels indirectly through disturbances in expression patterns of ECM components. Collagen IV, which is a normal component of the vascular basal lamina (Sarthy, 1993), was used here as a vessel wall marker. Collagen IV showed a diffuse pattern of expression throughout the accumulated vitreal tissue in the *TGF-β2*$^{-/-}$ eyes compared with controls, in which collagen IV expression was concentrated only in the normal vascular basal lamina. The diffuse expression pattern of collagen IV in the vitreal tissue of *TGF-β2*$^{-/-}$ eyes is consistent with a disorganization of the vessels and breakdown of vessel basal laminae. Alternatively, collagen IV expression may be more disorganized because the TGF-β2$^{-/-}$ vessels are developing too rapidly for the formation of a defined basal membrane. Whether or not collagen IV is a candidate ECM molecule that might regulate tbdn-1 expression remains to be investigated.

Hyalocytes scattered around the vitreal vascular networks at E16.5–E18.5 stages also expressed tbdn-1. Hyalocytes are thought to share lineage characteristics with monocyte/macrophages but are also known to express markers present on endothelial cells, such as binding sites for the endothelial-reactive lectin Griffonia simplicifolia (Lazarus and Hageman, 1994). Tbdn-1 expression by hyalocytes is consistent with the endothelial and monocytic characteristics of hyalocytes since tbdn-1 is also expressed in cells of myeloid origin in the bone marrow and in yolk sac endothelial and hematopoietic cells during early embryogenesis (Gendron *et al.*, 2000). The vitreal cells in accumulating *TGF-β2* null eyes show suppression of tbdn-1 expression. Besides endothelial cells expressing vWF, cells accumulating in the vitreous of TGFβ2 null mice could be either endothelial cells expressing undetectable levels of vWF, hyalocyte-like cells, or macrophages. However, the accumulating cells are unlikely to be macrophages since no staining for the macrophage marker F4/80 was observed in *TGF-β2* null vitreous either at E13.5 (Saika *et al.*, 2001) or at E18.5 (data not shown). TGFβ2 could influence the differentiation and maturation of these cells and this may impact on their expression of markers. The phenotype of the hyaloid-like cells in *TGF-β2* null eyes will require further investigation.

Our results indicate that vitreal blood vessels of *TGF-β2*$^{-/-}$ eyes contain degenerating pericytes. Pericytes in vitreal vessels of *TGF-β2*$^{-/-}$ eyes showed degeneration, cytoplasmic inclusions, and lack of contacts with adjacent cells and with surrounding basal laminae compared with *TGF-β2*$^{+/-}$ eyes. *TGF-β2*$^{-/-}$ eyes show decreases in ASMA and CD13-aminopeptidase N-positive staining (markers for pericytes) in the persisting vitreal vasculature, while *TGF-β2*$^{+/-}$ eyes clearly contained ASMA and CD13-positive cells. These results further support a conclusion that pericytes are degenerating in *TGF-β2*$^{-/-}$ vitreal vessels. Since TGF-β is known to induce expression of ASMA in pericyte cell lines *in vitro* (Verbeek *et al.*, 1994), it could be argued that loss of ASMA does not really reflect loss of pericytes in the *TGF-β2*-deficient environment but rather only loss of ASMA expression by existing pericytes. Our ultrastructural evidence showing degeneration of the pericytes in the vitreal vasculature of *TGF-β2*$^{-/-}$ eyes suggests that the existing pericytes are unhealthy and may not retain normal

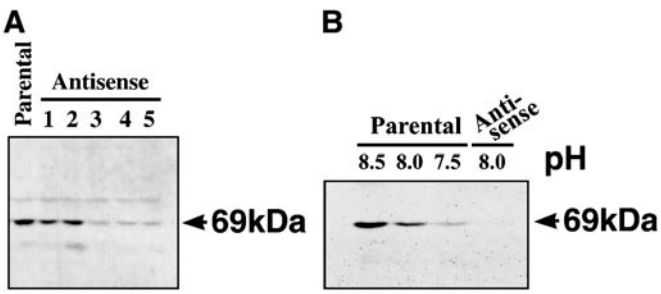

FIG. 7. Overexpression of antisense TBDN-1 cDNA blocks tbdn-1 protein expression in endothelial cell lines *in vitro*. (A) Expression analysis of tbdn-1 protein in 50 μg of whole cell lysate of the untransfected rhesus RF/6A choroid–retina endothelial cell line (Parental), in two control clones of RF/6A cells stably expressing a *TBDN-1* antisense cDNA fragment but not showing downregulation of 69 kDa tbdn-1 (Antisense 1 and 2) and in three clones of RF/6A cells stably expressing *TBDN-1* antisense cDNA fragment showing downregulation of 69 kDa tbdn-1 (Antisense 3, 4, and 5), as indicated. The 69-kDa tbdn-1 protein band is indicated by the arrow. (B) Acetyltransferase activity analysis of tbdn-1 immunoprecipitates prepared from equal protein amounts of whole cell lysates of the IEM cell line (Parental), and in IEM cells stably overexpressing a *TBDN-1* antisense cDNA fragment (anti-sense), assayed at the indicated pH. The acetylated 69-kDa band (indicated by the arrow), possibly corresponding to tbdn-1, is present at lower levels in antisense cells compared with the parental cells.

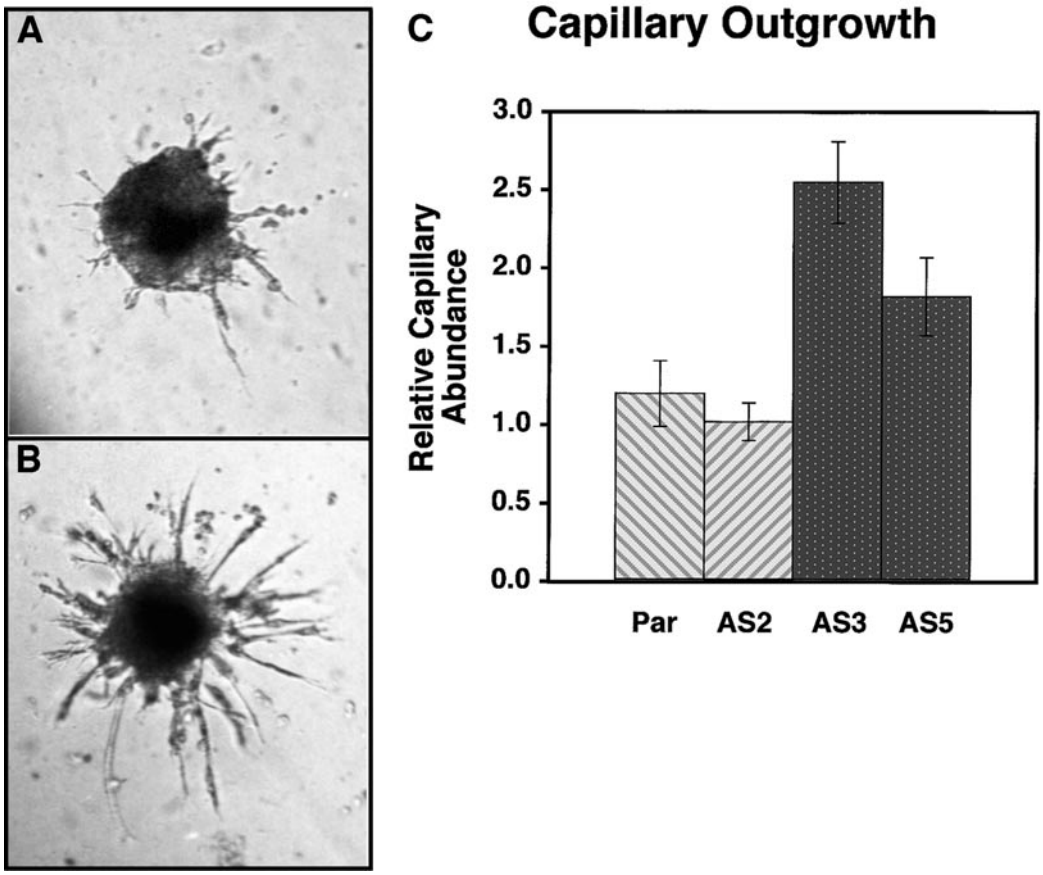

FIG. 8. Inhibition of tbdn-1 expression augments capillary formation of RF/6A choroid–retina endothelial cells in a Matrigel capillary formation assay. Capillary colonies formed by RF/6A clones overexpressing antisense *TBDN-1* cDNA and displaying suppression of tbdn-1 protein expression show longer, more complex, and more abundant capillary sprouts than parental and control cells. Representative phase-contrast images of capillary colonies are shown in (A) (parental colony) and (B) (AS3 colony [tbdn-1 suppressed]). Results are displayed in the histogram as relative capillary abundance (mean ± sem) in the histogram (C). Levels of tbdn-1 expression for each of these clones as assessed by Western blot analysis are displayed in Fig. 7.

function. The decreased expression of the pericyte marker CD13 (Alliot *et al.*, 1999) in *TGF-β2*$^{-/-}$ vitreal vasculature also supports this hypothesis. Interestingly, ASMA was detected at normal levels in pericytes of blood vessels in various head regions of the *TGF-β2*$^{-/-}$ embryos. This observation indicates that the decrease in ASMA-positive cells in the accumulated vitreal vasculature in *TGF-β2*$^{-/-}$ eyes is unlikely due to a global downregulation of ASMA in pericytes resulting from the lack of *TGF-β2*. TGFβ1 or TGFβ3 may have a similar role as *TGF-β2* in other tissues. However, in the case of the vitreal vasculature, loss of *TGF-β2* alone is sufficient to lead to accumulation of the abnormal vitreal vasculature. Physical properties of blood vessels may also impact upon the functional contacts of the cells composing them. Formation of blood vessels may be regulated at a paracrine level through local signaling contacts at pericyte–endothelial junctions (CIDEP; Wakui, 1992). Previous studies have shown that physical contacts between endothelial cells and pericytes are required for production of active TGF-β by these cells (Antonelli-Orlidge *et al.*, 1989; Sato and Rifkin, 1989). The importance of contacts between endothelial cells and pericytes for proper blood vessel formation and endothelial integrity has been demonstrated by gene targeting studies in mice. Mice lacking either PDGF-B or its receptor PDGFR-β display defective blood vessel development characterized by lack of pericytes, endothelial hyperplasia, and abnormal shape and ultrastructure of endothelium (Hellstrom *et al.*, 2001). TGF-β1, TGF-β2, and TGF-β3 exhibit similar activities on cells *in vitro*, but *in vivo* their pattern of expression is distinct and mice deficient for the different TGF-β isoforms exhibit distinct phenotypes ranging from multiple developmental malformations to less severe lung and palate defects (Piek *et al.*, 1999; Dunker and Krieglstein, 2000). TGF-βs exert an inhibitory influence on the *in vitro* proliferation and migration of endothelial cells (Antonelli-Orlidge *et al.*, 1989; Sato and Rifkin, 1989). Gene targeting studies have indicated that *TGF-β1* plays a role in regulating endothelial

cell differentiation during development (Dickson *et al.*, 1995). The overexpression of active TGF-β1 in the posterior chamber of the eye under the control of a lens-specific promoter blocks vitreal vessel growth (Srinivasan *et al.*, 1998). Moreover, there is evidence that TGF-β2 present in a heparin-bound fraction in aqueous humor in the adult eye can inhibit endothelial cell growth and *in vitro* angiogenesis (Hayasaka *et al.*, 1998). Furthermore, TGF-β isoforms are disregulated in proliferative diabetic retinopathy, a disease which often progresses to stages of extensive retinal and vitreal neovascularization (Spranger *et al.*, 1999). Previous studies indicated that eye developmental defects in *TGF-β2*$^{-/-}$ mice include ocular hypercellularity and malformation of the cornea and anterior segment (Sanford *et al.*, 1997; Saika *et al.*, 2001). The phenotype of the *TGF-β2*$^{-/-}$ vitreous is consistent with evidence that TGF-β plays an important role in regulating endothelial cell growth and differentiation (reviewed in RayChaudhury and D'Amore, 1991). Our results revealing that *TGF-β2*$^{-/-}$ vitreal vessels are more numerous and contain degenerated pericytes is consistent with an inhibitory role for TGF-β2 in vitreal blood vessel endothelial growth. Our results demonstrating the upregulation of PCNA and increased levels of activated $^{P42/44}$MAPK in the nuclei of *TGF-β2*$^{-/-}$ vitreal endothelial cells indicate a more proliferative state of these endothelial cells (Hall *et al.*, 1990; Paradis and Gendron, 2000; Dhanasekaran and Premkumar Reddy, 1998). Our finding that the accumulated blood vessels of the vitreal vasculature in *TGF-β2*$^{-/-}$ mice show a significant decrease in the expression of tbdn-1 is consistent with a hypothesis that tbdn-1 may play a role in a TGF-β2-related pathway in regulating vasculogenesis and/or angiogenesis events in the maturing vitreal vasculature. Our data would suggest an inhibitory role for tbdn-1 in TGF-β2 control of vitreal vascular growth, since tbdn-1 is normally expressed as the vitreal vasculature matures by remodeling and pruning while its expression is decreased in the accumulating *TGF-β2*$^{-/-}$ vitreal vasculature. Similarly, we have observed the suppression of normal retinal vascular tbdn-1 expression in retinal vessel proliferation in proliferative diabetic retinopathy (Gendron *et al.*, 2001a).

In order to address the hypothesis that tbdn-1 plays an inhibitory role in vitreal blood vessel growth, we used an *in vitro* model system to recapitulate vitreal blood vessel formation. We utilized the RF/6A cell line, originally isolated from choroid–retina tissue of a rhesus macaque fetus (Lou and Hu, 1987a,b) for modeling capillary formation of fetal posterior chamber endothelial cells. The tissue of origin and cellular phenotype of the RF/6A cell line was taken into consideration for these studies. The tunica vasculosa lentis aspect of the vitreal vasculature is thought to be composed of a mixture of blood vessels originating both from the hyaloid artery and from the choriocapillaris (reviewed in Silbert and Gerwood, 2000). Since the RF/6A cells originate from fetal choroid–retinal tissue and have been previously reported to retain endothelial characteristics, we surmised that the RF/6A cells might most closely approximate embryonic vitreal vascular endothelial cells. Furthermore, no other such cell lines are currently available. We first verified that the RF/6A cell line retained an endothelial phenotype, as reported originally (Lou and Hu, 1987a,b). Our results demonstrate that the RF/6A cell line indeed does retain the expression of endothelial markers such as VEGFR-2 tyrosine kinase, a definitive endothelial specific marker required for endothelial cell growth and development and which has been demonstrated to be upregulated during pathological retinal neovascularization (Millauer *et al.*, 1993; Yamaguchi *et al.*, 1993; Shalaby *et al.*, 1995; Robbins *et al.*, 1998; Hammes *et al.*, 1998). Furthermore, neither unstimulated nor TGF-β-stimulated RF/6A cells express ASMA, a marker for pericytes and vascular smooth muscle cells (VSMC) upregulated upon TGF-β stimulation of MK/T-1 corneal fibroblast cells as well as perivascular cells (Sawtell and Lessard, 1989; Gendron *et al.*, 2001b; Verbeek *et al.*, 1994). These characteristics indicate that RF/6A cells display an endothelial cell phenotype rather than a perivascular cell phenotype.

We have previously described that tbdn-1 expression is downregulated during capillary formation of IEM endothelial cells and RF/6A choroid–retina endothelial cells, suggesting that expression of tbdn-1 may act to dampen capillary formation (Gendron *et al.*, 2000, 2001a). An antisense TBDN-1 construct was used to assess the effects of reducing tbdn-1 expression on RF/6A capillary formation *in vitro*. As expected, the expression of ASTBDN-1 cDNA resulted in reduction of tbdn-1 protein expression as well as reduction of acetyltransferase activity present in tbdn-1 immunoprecipitates. These results confirm that the acetyltransferase activity associated with the anti-tbdn-1 antibody Ab1272 is specific for tbdn-1. Inhibition of tbdn-1 protein levels by expression of ASTBDN-1 significantly augmented capillary formation of both the RF/6A and IEM endothelial cell lines. These results indicate a role for tbdn-1 in dampening capillary formation. Based on these data, one might speculate about the functional role of tbdn-1 expression in the developing vitreal vasculature. The remodeling and pruning events which shape the vitreal vasculature late in embryogenesis precede a complete regression of the vitreal vasculature during early postnatal stages (Lang *et al.*, 1993; Yang *et al.*, 1996, Zhu *et al.*, 1999; Mitchell *et al.*, 1998; Ito and Yoshioka, 1999; Ash and Overbeek, 2000). These events likely require and include a dampening influence on vitreal capillary formation during late embryogenesis. TGF-β2 and tbdn-1 may provide this dampening influence on vitreal capillary formation in late embryogenesis. The mechanisms by which TGF-β2 and tbdn-1 might interact in this process remain to be determined.

Changes in the state of ocular pericytes are known to be associated with vasculopathy in eye diseases in humans. Pericyte death, thickening of the basement membrane, and changes in ECM expression pattern in proliferative diabetic retinopathy have been described in previous studies (Yanoff, 1966; Bloodworth and Epstein, 1967; Cogan and

Kuwabara, 1967; Addison *et al.*, 1970; Ashton, 1974; Speiser *et al.*, 1968; Podesta *et al.*, 2000). Our results raise the possibility that degenerative changes in pericytes and changes in ECM protein and tbdn-1 expression patterns may also be important for diseases affecting the developing vitreal vasculature. The vitreal vasculature of *TGF-β2*$^{-/-}$ mice mimics these defects in addition to displaying a reduction in tbdn-1 levels. Similarly, recent results show that tbdn-1 levels are markedly suppressed during neovascularization in proliferative diabetic retinopathy (Gendron *et al.*, 2001a). Our immunohistochemical analysis of a human 14-week embryonic eye revealed that tbdn-1 is expressed in human hyaloid and tunica vasculosa lentis vessels. In human, the hyaloid and tunica vasculosa lentis vasculature regresses prenatally at approximately 24 weeks (Zhu *et al.*, 1999). Therefore, the expression of tbdn-1 at 14 weeks of gestation in these vessels raises the possibility that tbdn-1 expression may also play a role in regulating maturation by dampening formation of the hyaloid and tunica vasculosa lentis vasculature in human. Failure of regression of the vitreal vasculature in PFV in humans can lead to vision loss and blindness (Boeve *et al.*, 1990; Steichen-Gersdorf *et al.*, 1997; Castillo *et al.*, 1997; Goldberg, 1997; Silbert and Gurwood, 2000). Interestingly, there is a high frequency of PFV and cataracts in p53-deficient mice (Reichel *et al.*, 1998). The persistent hyaloid and tunica vasculosa lentis vasculature of the *TGF-β2*$^{-/-}$ phenotype and the vitreal phenotype of PFV is similar. Furthermore, both the *TGF-β2* null eye phenotype and human PFV involve anterior ocular segment defects, such as corneal abnormalities (Saika *et al.*, 2001, Castillo *et al.*, 1997). It is thus conceivable that disturbances and/or disregulation in the TGF-*β*2 and/or tbdn-1 pathway are also involved in the pathophysiology of PFV. These possibilities are currently being explored in our laboratories.

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REFERENCES

Addison, D. J., Garner, A., and Ashton, N. (1970). Degeneration of intramural pericytes in diabetic retinopathy. *Br. Med. J.* **691,** 264–266.

Alliot, F., Rutin, J., Leenen, P. J., and Pessac, B. (1999). Pericytes and periendothelial cells of brain parenchyma vessels co-express aminopeptidase N, aminopeptidase A, and nestin. *J. Neurosci. Res.* **58,** 367–378.

Antonelli-Orlidge A., Saunders, K. B., Smith, S. R., and D'Amore, P. A. (1989). An activated form of transforming growth factor beta is produced by cocultures of endothelial cells and pericytes. *Proc. Natl. Acad. Sci. USA* **86,** 4544–4548.

Ash, J. D., and Overbeek, P. A. (2000). Lens-specific VEGF-A expression induces angioblast migration and proliferation and stimulates angiogenic remodeling. *Dev. Biol.* **223,** 383–398.

Ashton, N. (1974). Vascular basement membrane changes in diabetic retinopathy. Montgomery lecture, 1973. *Br. J. Ophthalmol.* **58,** 344–366.

Bloodworth, J. M., Jr., and Epstein, M. (1967). Diabetic amyotrophy. Light and electron microscopic investigation. *Diabetes* **16,** 181–190.

Boeve, M. H., van der Linde-Sipman, J. S., Stades, F. C., and Vrensen, G. F. (1990). Early morphogenesis of persistent hyperplastic tunica vasculosa lentis and primary vitreous. A transmission electron microscopic study. *Invest. Ophthalmol. Vis. Sci.* **31,** 1886–1894.

Castillo, M., Wallace, D. K., and Mukherji, S. K. (1997). Persistent hyperplastic primary vitreous involving the anterior eye. *AJNR Am. J. Neuroradiol.* **18,** 1526–1528.

Cogan, D. G., and Kuwabara, T. (1967). The mural cell in perspective. *Arch. Ophthalmol.* **78,** 133–139.

Dhanasekaran, N., and Premkumar Reddy, E. (1998). Signaling by dual specificity kinases. *Oncogene* **17,** 1447–1455.

Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S., and Akhurst, R. J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* **121,** 1845–1854.

Dunker, N., and Krieglstein, K. (2000). Targeted mutations of transforming growth factor-beta genes reveal important roles in mouse development and adult homeostasis. *Eur. J. Biochem.* **267,** 6982–6988.

Feinberg, A. P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132,** 6–13.

Gendron, R. L., Adams, L. C., and Paradis, H. (2000). Tubedown-1, a novel acetyltransferase associated with blood vessel development. *Dev. Dyn.* **218,** 300–315.

Gendron, R. L., Good, W. V., Adams, L. C., and Paradis, H. (2001a). Expression of tubedown-1 is suppressed in retinal neovascularization of proliferative diabetic retinopathy. *Invest. Opthalmol. Vis. Sci.* **42,** 3000–3007.

Gendron, R. L., Liu, C.-Y., Paradis, H., Adams, L. C., and Kao. W.-W.-Y. (2001b). MK/T-1, an immortalized fibroblast cell line derived using cultures of mouse corneal stroma. *Mol. Vis.* **7,** 107–113.

Gendron, R. L., Tsai, F. Y., Paradis, H., and Arceci, R. J. (1996). Induction of embryonic vasculogenesis by bFGF and LIF *in vitro* and *in vivo*. *Dev. Biol.* **177,** 332–346.

Goldberg, M. F. (1997). Persistent fetal vasculature (PFV): An integrated interpretation of signs and symptoms associated with persistent hyperplastic primary vitreous (PFV). LIV Edward Jackson Memorial Lecture. *Am. J. Ophthalmol.* **124,** 587–626.

Hall, P. A., Levison, D. A., Woods, A. L., Yu, C. C., Kellock, D. B., Watkins, J. A., Barnes, D. M., Gillett, C. E., Camplejohn, R., Dover, R., *et al.* (1990). Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: An index of cell proliferation with evidence of deregulated expression in some neoplasms. *J. Pathol.* **162,** 285–294.

Hammes, H. P., Lin, J., Bretzel, R. G., Brownlee, M., and Breier, G. (1998). Upregulation of the vascular endothelial growth factor/vascular endothelial growth factor receptor system in experimen-

tal background diabetic retinopathy of the rat. *Diabetes* **47,** 401–406.

Hayasaka, K., Oikawa, S., Hashizume, E., Kotake, H., Midorikawa, H., Sekikawa, A., Hoshi, K., Hara, S., Ishigaki, Y., and Toyota, T. (1998). Anti-angiogenic effect of TGFbeta in aqueous humor. *Life Sci.* **63,** 1089–1096.

Hellstrom, M., Gerhardt, H., Kalen, M., Li, X., Eriksson, U., Wolburg, H., and Betsholtz, C. (2001). Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. *J. Cell Biol.* **153,** 543–553.

Hirschi, K. K., and D'Amore, P. A. (1997). Control of angiogenesis by the pericyte: molecular mechanisms and significance. *EXS* **79,** 419–428.

Hirschi, K. K., Rohovsky, S. A., Beck, L. H., Smith, S. R., and D'Amore, P. A. (1999). Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. *Circ. Res.* **84,** 298–305.

Hirschi, K. K., Rohovsky, S. A., and D'Amore, P. A. (1998). PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J. Cell Biol.* **141,** 805–814.

Ito, M., and Yoshioka, M. (1999). Regression of the hyaloid vessels and pupillary membrane of the mouse. *Anat. Embryol. (Berl.)* **200,** 403–411.

Lang, R. A., and Bishop, J. M. (1993). Macrophages are required for cell death and tissue remodeling in the developing mouse eye. *Cell* **74,** 453–462.

Lazarus, H. S., and Hageman, G. S. (1994). In situ characterization of the human hyalocyte. *Arch. Ophthalmol.* **112,** 1356–1362.

Lou, D. A., and Hu, F. N. (1987a). Co-distribution of von Willebrand factor and fibronectin in cultured rhesus endothelial cells. *Histochem. J.* **19,** 431–438.

Lou, D. A., and Hu, F. N. (1987b). Specific antigen and organelle expression of a long-term rhesus endothelial cell line. *In Vitro Cell Dev. Biol.* **23,** 75–85.

McCright, B., Gao, X., Shen, L., Lozier, J., Lan, Y., Maguire, M., Herzlinger, D., Weinmaster, G., Jiang, R., and Gridley, T. (2001). Defects in development of the kidney, heart and eye vasculature in mice homozygous for a hypomorphic Notch2 mutation. *Development* **128,** 491–502.

Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N. P. H., Risau, W., and Ullrich, A. (1993). High affinity VEGF binding and developmental expression suggest flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* **72,** 835–846.

Mitchell, C. A., Risau, W., and Drexler, H. C. (1998). Regression of vessels in the tunica vasculosa lentis is initiated by coordinated endothelial apoptosis: A role for vascular endothelial growth factor as a survival factor for endothelium. *Dev. Dyn.* **213,** 322–333.

Mullen, J. R., Kayne, P. S., Moerschell, R. P., Tsunasawa, S., Gribskov, M., Colavito-Shepanski, M., Grunstein, M., Sherman, F., and Sternglanz, R. (1989). Identification and characterization of genes and mutants for an N-terminal acetyltransferase from yeast. *EMBO J.* **8,** 2067–2075.

Paradis, H., and Gendron, R. L. (2000). LIF transduces contradictory signals on capillary outgrowth through induction of Stat3 and $p^{41/43}$ MAP kinase. *J. Cell Sci.* **113,** 4331–4339.

Park, E. C., and Szostak, J. W. (1992). ARD1 and NAT1 proteins form a complex that has N-terminal acetyltransferase activity. *EMBO J.* **11,** 2087–2093.

Piek, E., Heldin, C. H., and Ten Dijke, P. (1999). Specificity, diversity, and regulation in TGF-beta superfamily signaling. *FASEB J.* **13,** 2105–2124.

Podesta, F., Romeo, G., Liu, W. H., Krajewski, S., Reed, J. C., Gerhardinger, C., and Lorenzi, M. (2000). Bax is increased in the retina of diabetic subjects and is associated with pericyte apoptosis in vivo and in vitro. *Am. J. Pathol.* **156,** 1025–1032.

RayChaudhury, A., and D'Amore, P. A. (1991). Endothelial cell regulation by transforming growth factor-beta. *J. Cell Biochem.* **47,** 224–229.

Reichel, M. B., Ali, R. R., D'Esposito, F., Clarke, A. R., Luthert, P. J., Bhattacharya, S. S., and Hunt, D. M. (1998). High frequency of persistent hyperplastic primary vitreous and cataracts in p53-deficient mice. *Cell Death Differ.* **5,** 156–162.

Robbins, S. G., Rajaratnam, V. S., and Penn, J. S. (1998). Evidence for upregulation and redistribution of vascular endothelial growth factor (VEGF) receptors flt-1 and flk-1 in the oxygen-injured rat retina. *Growth Factors* **16,** 1–9.

Saika, S., Saika, S., Liu, C.-Y., Sanford, L. P., Azhar, M., Doetschman, T., Gendron, R. L., Kao, C. W.-C., and Kao, W. W.-Y (2001). TGFb2 is involved in corneal morphogenesis during mouse embryonic development. *Dev. Biol.* **240,** 419–432.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanford, L. P., Ormsby, I., Gittenberger-de Groot, A. C., Sariola, H., Friedman, R., Boivin, G. P., Cardell, E. L., and Doetschman, T. (1997). TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* **124,** 2659–2670.

Sarthy, V. (1993). Collagen IV mRNA expression during development of the mouse retina: An in situ hybridization study. *Invest. Ophthalmol. Vis. Sci.* **34,** 145–152.

Sato, Y., and Rifkin, D. B. (1989). Inhibition of endothelial cell movement by pericytes and smooth muscle cells: Activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture. *J. Cell Biol.* **109,** 309–315.

Sawtell, N. M., and Lessard, J. L. (1989). Cellular distribution of smooth muscle actins during mammalian embryogenesis: Expression of the alpha-vascular but not the gamma-enteric isoform in differentiating striated myocytes. *J. Cell. Biol.* **109,** 2929–2937.

Schlatt, S., Weinbauer, G. F., Arslan, M., and Nieschlag, E. (1993). Appearance of alpha-smooth muscle actin in peritubular cells of monkey testes is induced by androgens, modulated by follicle-stimulating hormone, and maintained after hormonal withdrawal. *J. Androl.* **14,** 340–350.

Schor, A. M., and Schor, S. L. (1986). The isolation and culture of endothelial cells and pericytes from the bovine retinal microvasculature: A comparative study with large vessel vascular cells. *Microvasc. Res.* **32,** 21–38.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., and Schuh, A. C. (1995). Failure of blood island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376,** 62–66.

Silbert, M., and Gurwood, A. S. (2000). Persistent hyperplastic primary vitreous. *Clin. Eye Vis. Care.* **12,** 131–137.

Speiser, P., Gittelsohn, A. M., and Patz, A. (1968). Studies on diabetic retinopathy. 3. Influence of diabetes on intramural pericytes. *Arch. Ophthalmol.* **80,** 332–337.

Spranger, J., Meyer-Schwickerath, R., Klein, M., Schatz, H., and Pfeiffer, A. (1999). Deficient activation and different expression of transforming growth factor-beta isoforms in active proliferative diabetic retinopathy and neovascular eye disease. *Exp. Clin. Endocrinol. Diabetes* **107,** 21–28.

Srinivasan, Y., Lovicu, F. J., and Overbeek, P. A. (1998). Lens-specific expression of transforming growth factor beta1 in transgenic mice causes anterior subcapsular cataracts. *J. Clin. Invest.* **101,** 625–634.

Steichen-Gersdorf, E., Gassner, I., Unsinn, K., and Sperl, W. (1997). Persistent hyperplastic primary vitreous in a family with osteoporosis-pseudoglioma syndrome. *Clin. Dysmorphol.* **6,** 171–176.

Takahashi, T., and Shibuya, M. (1997). The 230 kDa mature form of KDR/Flk-1 (VEGF receptor-2) activates the PLC-gamma pathway and partially induces mitotic signals in NIH3T3 fibroblasts. *Oncogene* **14,** 2079–2089.

Verbeek, M. M., Otte-Holler, I., Wesseling, P., Ruiter, D. J., and de Waal, R. M. (1994). Induction of alpha-smooth muscle actin expression in cultured human brain pericytes by transforming growth factor-beta *Am. J. Pathol.* **144,** 372–382.

Wakui, S. (1992). Epidermal growth factor receptor at endothelial cell and pericyte interdigitation in human granulation tissue. *Microvasc. Res.* **44,** 255–262.

Yang, K., and Cepko, C. L. (1996). Flk-1, a receptor for vascular endothelial growth factor (VEGF), is expressed by retinal progenitor cells. *J. Neurosci.* **16,** 6089–6099.

Yamaguchi, T. P., Dumont, D. J., Conlon, R. A., Breitman, M. L., and Rossant, J. (1993). flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development* **118,** 489–498.

Yanoff, M. (1966). Diabetic retinopathy. *N. Engl. J. Med.* **274,** 1344–1349.

Zhu, M., Provis, J. M., and Penfold, P. L. (1999). The human hyaloid system: cellular phenotypes and inter-relationships. *Exp. Eye Res.* **68,** 553–563.

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