



Pigment epithelium-derived factor expression in the developing mouse eye

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Purpose: Pigment Epithelium-Derived Factor (PEDF) is a 50 kDa secretable protein with neuroprotective, neurotrophic, and antiangiogenic properties. Expression patterns in the human eye suggest that modulation of this protein over time and place may play a role in development of normal ocular vasculature. Because of the potential importance of normal PEDF expression patterns in controlling ocular blood vessel growth in health and disease, we characterized these patterns over the period of retinal vascular development in the mouse.

Methods: Eyes from CD1 mice (embryonic days E 14.5, 18.5, P0, P4, P7, P14, and Adult) were cryosectioned and examined. A polyclonal PEDF antibody was used to locate PEDF protein using immunohistochemical techniques while a PEDF RNA probe was used to localize PEDF mRNA by in situ hybridization.

Results: Immunohistochemical and in situ hybridization showed initial expression in the ciliary body and choroid during mid-gestation. Near term, relative protein levels increased in the ganglion cell layer and remained high through the first two weeks postnatal. Levels qualitatively decreased after this point but persisted through adulthood. Relative levels of expression in the inner retina were much higher at all timepoints than in the outer retina.

Conclusions: These expression patterns likely maintain the vitreous and aqueous humors as avascular spaces and may also control vascular development in the inner/outer retina.

The mammalian retina develops as clearly stratified cell layers, some of which house an ordered array of blood vessels and capillary networks. Rodents offer a unique opportunity for studying development of retinal vasculature as much of this occurs late in gestation or in the early postnatal period. The largest blood vessel network is present in the choroid, and this completes its development early in life. In rodents, choroidal vasculature is mature by postnatal day 1 (P1), before differentiation of the retina has taken place [1]. In contrast, retinal vascular development takes place as the retinal neurons are differentiating over the first two weeks of postnatal life [2]. The net result is a further stratification of the retina based on presence or absence of blood vessels. Blood vessels are limited to the inner retina (ganglion cell layer, inner nuclear layer) and the choroid while the photoreceptor cell layer (including subretinal space, outer nuclear layer, and outer plexiform layer) and the retinal pigment epithelium (RPE) are devoid of vessels.

The patterns of retinal vascular development have been well characterized in rodents, and several stimulators of vascular growth have been identified [1-3]. Insulin-like growth factor-1 (IGF-1) and Vascular Endothelial Growth Factor (VEGF) are two examples of stimulators of vascular development in the mouse [4,5]. The role of inhibitors of angiogenesis with respect to normal vascular development in the eye has not received much attention.

A candidate for this role is pigment epithelium derived factor (PEDF), a 50 kDa secreted protein that has been shown to have antiangiogenic properties [6]. PEDF inhibits neovascularization in the corneal pocket assay and decreases migration in an endothelial cell migration assay. PEDF is present in ocular fluids and cells, and delivery of antibodies to PEDF to vitreous and corneal extracts decreases the antiangiogenic activity of these materials in in vitro assays. PEDF protein levels in the eye are responsive to ambient oxygen tension with protein levels increasing with increasing oxygen in vitro [6]. In fetal and adult human eye tissue, PEDF is expressed by the cornea, ciliary body, and cells of the inner and outer retina [7,8]. It is theorized that PEDF secreted by these cells accumulates in avascular spaces of the eye such as the aqueous humor, vitreous humor, and the interphotoreceptor matrix where it acts as a major inhibitor of angiogenesis [7]. Several groups have suggested that shifts in the balance between pro-angiogenic factors such as VEGF and anti-angiogenic factors such as PEDF may be responsible for the pathology seen in choroidal and inner-retinal neovascularization [9-15]. In fact, delivery of PEDF protein through virus-mediated gene transfer and direct protein introduction has been used successfully to inhibit angiogenesis in a number of models of retinal and choroidal neovascularization [16-20].

In addition to its antiangiogenic properties, PEDF is a non-inhibitory member of the serine protease inhibitor family [21]. It also has neurotrophic and neuroprotective properties. Y-79 cells exposed to PEDF will extend neurites and express neuronal specific markers [22]. PEDF protects cerebellar granule cells, chick spinal motor neurons, hippocampal neurons, retinal ganglion cells, and photoreceptors from cell death due

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to various stimulators [23-32]. In cerebellar granule cells, PEDF has been shown to inhibit apoptosis through the NF κ B pathway [33].

In order to further characterize the role of PEDF in controlling retinal vascular development, we examined the expression of *PEDF* in developing and adult mouse retinas using immunohistochemistry and in situ hybridization. Both techniques were used in order to more accurately localize *PEDF* expression given the secretable nature of PEDF protein.

METHODS

The experiments in this study were performed in accordance with the guidelines set by the Institutional Animal Care and Use Board at the University of Pennsylvania.

Tissue preparation: Eyes were enucleated from cohorts of embryonic day (E) 14.5, 18.5, P0, P4, P7, P14, and adult (3 month old) CD1 mice. CD1 mice were selected as they are albino (thereby simplifying detection of cells that are positive by immunohistochemistry and in situ hybridization) and because they have wildtype retinas. The eyes were immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). The eyes were then placed in 30% sucrose PBS at 4 °C overnight. The eyes were embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) and stored at -80 °C until sectioning. Eyes were cryosectioned at 14 μ m and stored at -80 °C before staining. Sections from two different eyes were examined at each time point using immunohistochemistry and in situ hybridization as described below. Data shown is representative of that seen in both eyes.

Immunohistochemistry: Sections were processed for immunohistochemistry with modifications of previously published methods [7]. A rabbit polyclonal antibody to a peptide that corresponds to amino acids 327-343 of PEDF was used in these studies (1:200; Research Genetics, Inc., Huntsville AL). A cy3 goat anti-rabbit secondary antibody was used (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Slides were mounted with Vectashield mounting medium for fluorescence containing DAPI (Vector Laboratories, Inc., Burlingame, CA). Control slides were either treated with no primary antibody or with the polyclonal anti-PEDF antibody preabsorbed to 50 μ g/ml of the immunizing peptide at 4 °C overnight. The slides were examined with a Leica DM R Microscope (Leica, Wetzlar, Germany), photographed, and digitized using a Hamamatsu CCD camera (Hamamatsu Photonics, Japan) and Openlab software (Improvision, Boston, MA).

In situ hybridization: Sense and antisense probes were generated using a pBluescript plasmid containing a 356 bp insert of PEDF cDNA as previously described [7]. This plasmid was linearized with *SpeI* (antisense) and *NcoI* (sense) and transcribed with T3 and T7 respectively using the DIG RNA labeling kit (Roche Pharmaceuticals, Indianapolis, IN). In situ hybridization was performed on E 14.5, 18.5, P4, P7, P14, and adult sections as previously described with the following modifications: Proteinase K digestion was conducted for 25 min, hybridization and prehybridization occurred at 55 °C, and embryo powder was not used as a blocking agent for the anti-DIG antibody [7]. Slides were examined using a Leica

DM R microscope, photographed using a Fuji Fine Pix S1Pro camera (Fuji, Tokyo, Japan), and digitized using the accompanying software.

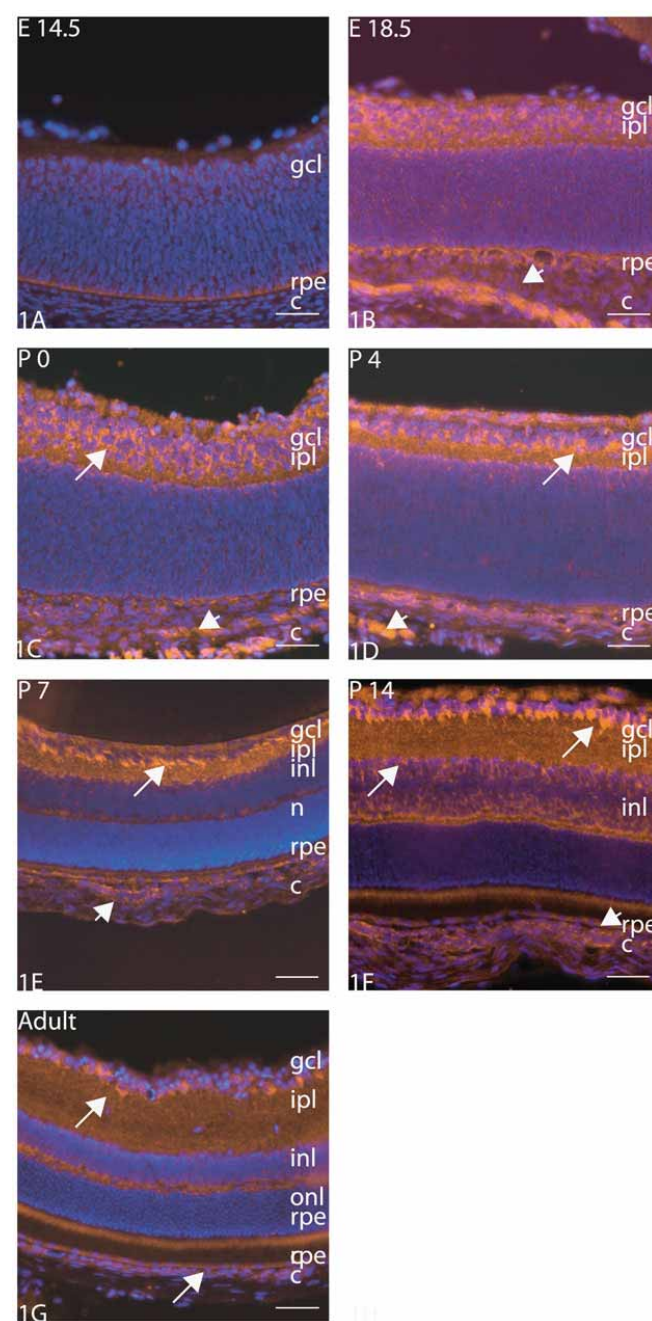


Figure 1. Immunohistochemical examination of PEDF in the neural retina and choroid. Immunohistochemical examination of PEDF in the neural retina and choroid (E14.5-Adult). PEDF was seen in the choroid at all time points shown except E 14.5 (arrowheads). PEDF levels in the ganglion cell layer qualitatively increased with increasing age (arrows; B-F) with highest levels seen at P14 (F). Relative levels decreased slightly at adulthood (G). Nuclei are counterstained with DAPI. Bar represents 50 μ m. Abbreviations: gcl = ganglion cell layer; rpe = retinal pigment; c = choroid; inl = inner nuclear layer; ipl = inner plexiform layer; n = neuroblasts; onl = outer nuclear layer.

RESULTS

Immunohistochemistry: In the retina, PEDF protein localizes to ganglion cells and cells of the inner nuclear layer at all time points except E 14.5 with the polyclonal PEDF antibody (Figure 1A-G). Relative intensity of PEDF immunofluorescence in the ganglion cell layer increases with increasing age of the mouse. At E 18.5 and P0, PEDF immunofluorescence in the ganglion cell layer is relatively weak, but by P14, the PEDF immunofluorescent signal is stronger (Figure 1B-F). PEDF staining qualitatively decreases slightly by adulthood (Figure 1G). PEDF staining can be seen in the inner plexiform layer at all timepoints after E 14.5 (Figure 1B-G). PEDF staining is also seen in retinal pigment epithelial cells (RPE) but not photoreceptors or Muller cells at all timepoints (Figure 1A-G). Immunofluorescence patterns are uniform across the retina.

PEDF immunofluorescence was also detected in the cili-

ary body and choroid (Figure 1A-G and Figure 2A-F). Signal strength in the ciliary body does not appear to change with age. PEDF protein in the choroid is not detected at E 14.5 but is present from E 18.5 through adulthood (Figure 1A-G). There is no immunofluorescence detectable in the inner retina, choroid, and ciliary body after using preadsorbed or no primary antibody controls (not shown).

In situ hybridization: PEDF mRNA is detected throughout the ganglion cell layer and inner nuclear layer at all time points examined after E 18.5 (Figure 3A,C,E,G). PEDF expression is not seen in the photoreceptors, RPE, or Muller cells at any timepoint (Figure 3A,C,E,G). Staining in the ganglion cell layer is qualitatively the greatest at P7 and P14 (Figure 3C,E). PEDF expression is observed in the ciliary body and choroid at all time points after E 18.5 including adulthood (Figure 3A,C,E,G and Figure 4A,C,E,G,I) and does not vary in intensity with age. Staining is not observed in any of the sense controls (Figure 3B,D,F,H and Figure 4B,D,F,H,J).

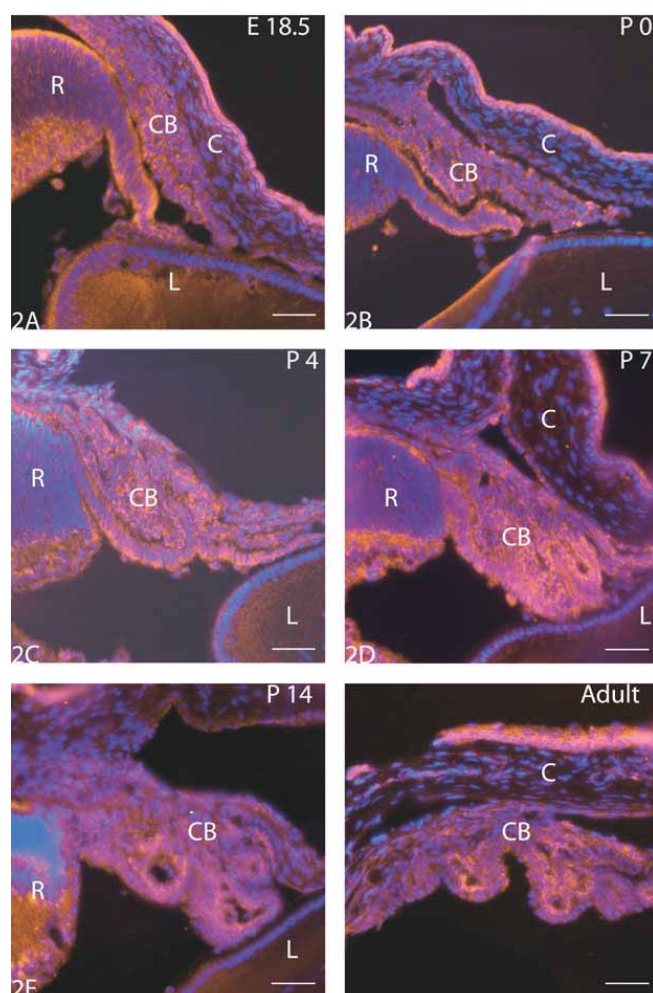


Figure 2. Immunohistochemical localization of PEDF in the ciliary body. Immunohistochemical localization of PEDF in the ciliary body (E18.5-Adult). PEDF was expressed by cells of the ciliary body throughout development (A-F). There were no changes in intensity of PEDF immunofluorescence with developmental age. Nuclei are counterstained with DAPI. Bar represents 50 μ m. Abbreviations: CB = ciliary body; C = cornea; R = retina; L = lens.

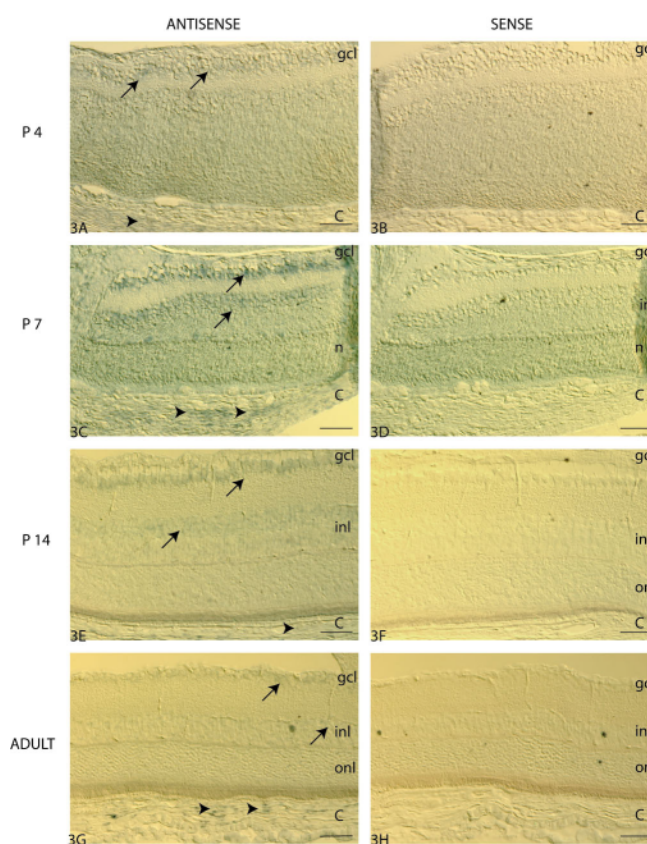


Figure 3. PEDF expression in the neural retina and choroid. In situ hybridization demonstrating PEDF expression in the neural retina and choroid in the developing mouse retina (E 14.5-Adult). PEDF mRNA was found in cells of the ganglion cell layer at P4-adult (arrows; A, C, E, G). Expression can also be seen in the inner part of the inner nuclear layer at P7-adult (arrows; C, E, G). Expression is also seen in the choroid at P4-adult (3). No staining was seen in the sense controls (B, D, F, H). Bar represents 50 μ m. Abbreviations: gcl = ganglion cell layer; rpe = retinal pigment; c = choroid; inl = inner nuclear layer; n = neuroblasts; onl = outer nuclear layer.

DISCUSSION

Interestingly, PEDF was not detected in any cells of the retina until late in gestation (E 18.5). At this timepoint, *PEDF* expression is detectable in a variety of ocular cell types. The expression patterns evolve over the next two weeks, coinciding with the period during which retinal vasculature develops. These patterns suggest that PEDF may be acting as an important regulator of vascular development in the eye.

Relatively high levels of expression of *PEDF* are observed in cells of the ciliary body, and choroid from E 18.5 on. Expression of *PEDF* in cells of the ciliary body likely result in secretion of this antiangiogenic factor into the vitreous cavity and anterior chamber. Its presence in these regions may inhibit the growth of blood vessels into the cornea and lens. Opacities caused by blood vessels in these regions would block the pathway of light going to the retina. The production of

PEDF in the ciliary body over the life of the animal therefore likely contributes to the clarity of these cavities/structures.

Qualitatively high levels of PEDF are also produced in the choroid at all developmental timepoints after E 18.5. The choroid is rich in vessels that develop early in eye morphogenesis and persist throughout adulthood. Stone et al have shown that in the rat, the choroid is well developed by P1 [1]. PEDF is not apparent in the choroid until its development is close to completion. *PEDF* expression remains high in the choroid throughout the life of the animal. *PEDF* expression in the choroid may play a role in preventing new choroidal vessels from forming, breaking through Bruch's membrane, and invading the subretinal space. Its presence does not seem to disrupt the normal, mature choroidal vasculature.

PEDF protein was absent from neural progenitor cells at gestational/early postnatal timepoints and in photoreceptors and interphotoreceptor matrix (IPM) at late postnatal timepoints/adulthood. Nevertheless, PEDF protein was present in the RPE at all timepoints. This is puzzling as *PEDF* expression was not found in the RPE via in situ hybridization. This could be due to differences in the sensitivity of immunohistochemistry vs. in situ hybridization for examining PEDF protein and mRNA levels respectively. PEDF was first discovered as a neurotrophic protein found in the conditioned media of human fetal RPE cells under cell culture conditions [22]; hence its name. In vivo, *PEDF* protein was also detected in the washes of the interphotoreceptor matrix of human and bovine eyes [34,35]. In vivo, PEDF expression was also identified in the RPE of bovine and human fetal RPE cells [34,36]. In the human fetal retina, PEDF protein was found in the RPE and developing cones while PEDF immunolocalization was seen in the rods and cones of adult retinas. *PEDF* expression was also seen in the RPE and photoreceptors in the adult retina. PEDF protein was found in the IPM in human fetal tissue, but only with the monoclonal PEDF antibody [7]. The lack of *PEDF* expression in photoreceptors in this study may be related to species differences in levels and localization of *PEDF* expression.

Interestingly, *PEDF* expression in the neural retina appears to be temporally regulated. *PEDF* expression is seen in the ganglion cell layer from E 18.5 through adulthood, and in the inner-most cells of the inner nuclear layer from P7 through adulthood. Qualitatively, there is an increase in *PEDF* expression in the ganglion cell layer at P 7 which anticipates the qualitative increase in PEDF protein levels seen in the ganglion cell layer at P14. The results are similar to those we reported for human retina [7], where fetal and adult ganglion cells also express high levels of PEDF. It has been previously shown that PEDF mRNA is not present in the mouse retina by Northern blot analysis [37]. In situ hybridization may be a more sensitive technique for detecting PEDF mRNA in the retina thereby allowing us to see its expression. In any case, a recent report from Ogata et al [15] provides evidence that *PEDF* expression patterns similar to what we have detected in the adult mouse are also present in the adult rat retina (i.e., high levels in ciliary body, ganglion cells and RPE but no indication of *PEDF* expression in photoreceptors).

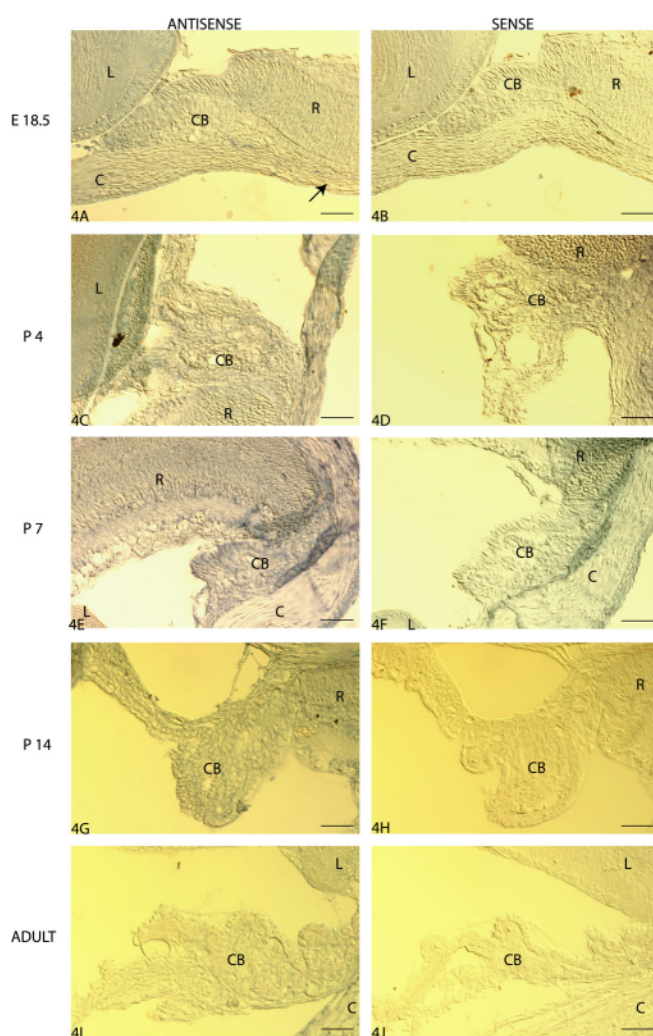


Figure 4. *PEDF* expression in the ciliary body. In situ hybridization demonstrating *PEDF* expression in the ciliary body of mouse eyes (E 18.5-Adult). PEDF mRNA was found in cells of the ciliary body at all time points shown (A, C, E, G, I). PEDF mRNA was seen in the choroid at E 18.5 (arrow). No staining was seen in the ciliary body in the sense controls (B, D, F, H, J). Bar represents 50 μ m. Abbreviations: CB = ciliary body; C = cornea; R = retina; L = lens.

The onset of *PEDF* expression in late gestation in the mouse is interesting with respect to the timing of normal retinal vascular development in the inner retina. Connolly et al have described a three-stage process of inner retinal vascular development in the mouse [2]. During the first stage (P0-P10), vessels grow from the optic disc toward the retinal periphery. Interestingly, there were no spatial changes in *PEDF* expression detected corresponding to this growth pattern. In the second stage of development starting at P4, vessels grow into the deeper layers of the retina. This is followed by development of a capillary network during the third stage at P7. By P14, inner retinal vascular development is complete [2]. The completion of retinal vascular development corresponds to the time point of highest *PEDF* expression in the ganglion cell layer. Given that *PEDF* is an antiangiogenic factor, it may be signaling the completion of vascular growth in the mid-retinal capillary bed or acting in the final stages of vascular remodeling. It is also possible that since vascular development is complete, *PEDF* is expressed in order to prevent the growth of new vessels. However, if this were true, then one would not expect *PEDF* expression to decrease in adulthood.

PEDF has already been shown to be an antiangiogenic factor in the vitreous and aqueous humor in adult human eyes [10,11,13]. Decreases in *PEDF* protein levels are seen in patients with active proliferative diabetic retinopathy and non-diabetic neovascularization as compared to those with quiescent disease as well as age-matched controls [10,11,13]. The current study suggests that *PEDF* expression is present even when inner retinal vessels are undergoing their most active periods of growth and remodeling. It may be that the accumulation of this protein in specific ocular locations (the vitreous, subretinal space, for example) plays a role in preventing the spread of blood vessels into these areas. Alternatively, the balance between pro-angiogenic and antiangiogenic factors in specific ocular locations may determine the final outcome of retinal angiogenesis.

With respect to this question, is it possible that experimentally induced high levels of *PEDF* will disrupt the normal patterns of retinal vascular development? Although this has not been studied directly, there are no reports of gross disruption of normal or developing retinal vasculature in experiments where *PEDF* [18,19] or *PEDF*-encoding DNA have been introduced exogenously at early postnatal timepoints [16,17,20]. Nevertheless, in the protein delivery studies and in some of the gene transfer studies [16,17] *PEDF*-encoding DNA was not delivered to the cells which normally express this protein. Given the promising data regarding the applications of *PEDF* in inhibition of abnormal retinal neovascularization [16-20,38], it will be important to verify that high levels of exogenously produced *PEDF* protein will not disrupt the normal retinal vasculature.

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