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PEDF-Deficient Mice Exhibit an Enhanced Rate of Retinal Vascular Expansion and are More Sensitive to Hyperoxia-Mediated Vessel Obliteration

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Abstract

Pigment epithelium derived factor (PEDF) is an endogenous inhibitor of angiogenesis. However, its physiological role during vascular development and neovascularization remains elusive. Here we investigated the role of PEDF in normal postnatal vascularization of retina and retinal neovascularization during oxygen-induced ischemic retinopathy (OIR) using PEDF-deficient (PEDF^{minus/minus}) mice. The β -galactosidase staining of eye sections from PEDF^{minus/minus} mice confirmed the expression pattern of endogenous PEDF previously reported in mouse retina. However, strongest staining was observed in the retinal outer plexiform layer. Retinal trypsin digests indicated that the ratio of endothelial cells (EC) to pericytes (PC) was significantly higher in PEDF^{minus/minus} mice compared to wild type (PEDF^{+/+}) mice at postnatal day 21 (P21). This was mainly attributed to increased number of EC in the absence of PEDF. There was no significant difference in the number of PC. We observed increased rate of proliferation in retinal vasculature of PEDF^{minus/minus} mice, which was somewhat compensated for by an increase in the rate of apoptosis. Staining of the retinal wholemounts and eye frozen sections indicated postnatal retinal vascularization expansion occurred at a faster rate in the absence of PEDF, and was more prominent at early time points (prior to P21). The retinal vascularization in PEDF^{+/+} mice reaches that of PEDF^{minus/minus} mice such that no significant difference in vascular densities was observed by P42. Lack of PEDF had minimal effect on the regression of hyaloid vasculature and VEGF levels. PEDF^{minus/minus} mice also exhibited enhanced sensitivity to hyperoxia-mediated vessel obliteration during OIR compared to PEDF^{+/+} mice despite higher levels of VEGF. However, there was no significant difference in the degree of retinal neovascularization. Our studies indicate that PEDF is an important modulator of early postnatal retinal vascularization and in its absence retinal vascularization proceeds at a faster rate and is more susceptible to hyperoxia-mediated vessel obliteration.

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Keywords

Oxygen-induced ischemic retinopathy; Angiogenesis; Endothelial Cells; Pericytes; Apoptosis; Proliferation; VEGF

1. Introduction

Pigment epithelium-derived factor (PEDF) was first identified as a 50 kDa secreted protein in conditioned medium from cultured fetal human retinal pigment epithelial (RPE) cells (Tombran-Tink and Johnson, 1989). It shares sequence and structural homology with the serine protease inhibitor (Serpin) family but does not inhibit proteases (Becerra et al., 1995; Steele et al., 1993). PEDF is produced by a variety of cell types including RPE cells, muller cells, endothelial cells (EC), and pericytes (PC), and has multiple biological activities (Tombran-Tink and Barnstable, 2003). In addition to neurotrophic and neuprotective properties, PEDF is a potent antiangiogenic factor. PEDF inhibits angiogenesis in a number of *in vivo* assays including the rat corneal pocket assay, the oxygen-induced ischemic retinopathy, and laser-induced choroidal neovascularization (Dawson et al., 1999; Mori et al., 2002; Stellmach et al., 2001). The relevance of PEDF to human ocular neovascular disease has also been demonstrated in a number of clinical studies. The decreased levels of PEDF in the vitreous and ocular tissues were associated with proliferative diabetic retinopathy and choroidal neovascularization in age-related macular degeneration (Bhutto et al., 2006; Funatsu et al., 2006; Ogata et al., 2007; Ogata et al., 2002). Thus, PEDF may play important roles in the development and maintaining of ocular vascular homeostasis.

PEDF is expressed in the neural retina early in life in a developmentally regulated fashion in both mouse and human tissues (Behling et al., 2002; Karakousis et al., 2001). Expression of PEDF in the ganglion cell layer is present near term and increases over the first two weeks of life in the mouse, coinciding with the development of inner retinal vascular plexuses. The retinas of newborn mice are void of any vessels, and normal retinal vasculature develops after birth with a highly restricted pattern (Fruttiger, 2007). These restricted developmental patterns of retinal vascularization suggest that PEDF, as well as other endogenous inhibitors of angiogenesis, may be important as regulators of retinal vascular homeostasis in the eye (Bhutto et al., 2004; Sheibani et al., 2000; Uno et al., 2006).

Increased expression of PEDF during retinal vascular development does not exert a significant impact on the expansion of retinal vessels from optic nerve to the periphery, or the neural retina development (Wong et al., 2004). However, a decrease in the rate of blood vessel growth in the deeper layers and a decreased rate of maturation of nascent blood vessels were observed. These differences were not as prominent after P21, when normal differentiated capillaries were present. In addition, an increase in retinal vascular density of 3-month-old PEDF null mice was observed (Doll et al., 2003). However, the impact lack of PEDF has on normal postnatal vascularization of retina and its neovascularization during oxygen-induced ischemic retinopathy (OIR) requires further investigation.

To gain further insight into the physiological role PEDF plays in normal postnatal retinal vascular development and retinal neovascularization during OIR, we have used PEDF^{minus}/minus mice. Here we demonstrate that PEDF^{minus}/minus mice exhibit increased retinal vascular density during normal postnatal development of retinal vasculature. This was not associated with significant changes in the vascular endothelial growth factor (VEGF) levels. In addition, the regression of hyaloid vasculature, an apoptosis dependent process, was not affected in PEDF^{minus}/minus mice. However, PEDF^{minus}/minus mice did exhibit enhanced sensitivity to hyperoxia-mediated vessel obliteration during OIR compared to PEDF

+/- mice. There was no significant difference in the degree of retinal neovascularization in the absence of PEDF. These studies demonstrate an important role for PEDF during early development of retinal vasculature and its protection from hyperoxia.

2. Materials and methods

2.1. Tissue preparation

All animal studies were carried out in accordance to the Association for Research in Vision and Ophthalmology Statement for the Use of animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care Committee of the University of Wisconsin School of Medicine and Public Health. PEDF^{minus}/minus; mice, on a C57BL/6J, were generated using a novel knock-in methodology (Valenzuela et al., 2003) and were provided by Dr. S.J. Wiegand (Regeneron Pharmaceuticals, Tarrytown, NY) (Wiegand et al., 2004). The entire PEDF gene was replaced with a targeting vector encoding a lacZ reporter cassette inserted downstream of the PEDF ATG site allowing determination of temporal and spatial expression of PEDF by β -galactosidase staining. The litters produced by mating mutant mice were genotyped by PCR of genomic DNA extracted from tail biopsies. The PCR primers were PEDF forward- 5'-AAGACC TCAAGTCAAGGGTC-3', PEDF-reverse 1- 5'-CATAAGGACCAGTTTCTCTCC-3', PEDF reverse 2- 5'-CTGCCCTCCCTGCACTGTCTC-3', and neo forward-5'-TCATTCTCAGTATTGTTTGGCC-3'.

PEDF^{minus}/minus; mice and PEDF^{+/+} C57BL/6J mice (Jackson Labs) were bred for different experimental time points. For OIR, 7-day-old (P7) pups and mothers were placed in an airtight incubator and exposed to an atmosphere of $75 \pm 0.5\%$ oxygen for 5 days. Incubator temperature was maintained at $23 \pm 2^\circ\text{C}$, and oxygen was continuously monitored with a PROOX model 110 oxygen controller (Reming Bioinstruments Co., Redfield, NY). Mice were then brought to room air for 5 days (unless specified other wise), and then were sacrificed for retinal wholemount preparations as described below.

2.2. Trypsin-digested retinal vessel preparation

Eyes were enucleated from P21 or P42 PEDF^{+/+} and PEDF^{minus}/minus; mice, fixed in 4% paraformaldehyde for at least 24 h. The eyes were bisected equatorially and the entire retina was removed under the dissecting microscope. Retinas were washed overnight in distilled water and incubated in 3% trypsin (Difco^{TM} Trypsin 250, Becton Dickinson and Company, Sparks, MD) prepared in 0.1 M Tris, 0.1 M maleic acid, pH 7.8 containing 0.2 M NaF for approximately 1.5 h at 37°C . Following completion of digestion, retinal vessels were flattened by four radial cuts and mounted on glass slides for periodic acid-schiff (PAS) and hemotoxylin staining. Nuclear morphology was used to distinguish pericytes (PC) from endothelial cells (EC). The nuclei of EC are oval or elongated and lied within the vessel wall along the axis of the capillary, while PC nuclei are small, spherical, stain densely, and generally have a protuberant position on the capillary wall. The stained and intact retinal wholemounts were coded, and subsequent counting was performed masked.

The number of EC and PC was determined by counting respective nuclei under the microscope at a magnification of x400. Only retinal capillaries were included in the cell count, which was performed in the mid-zone of the retina. We counted the number of EC and PC in four field of view from the four quadrants of each retina. The ratio of EC to PC was then calculated. To evaluate the density of cells in the capillaries, the mean number of EC or PC was recorded from the four quadrants of each retina.

2.3. Visualization of retinal vasculature and quantification of avascular areas

Vessel obliteration and the retinal vascular pattern were analyzed using retinal wholemounts stained with anti-collagen IV antibody. At various times, the eyes of mice were enucleated and briefly fixed in 4% paraformaldehyde (4 min on ice). The eyes were fixed in methanol for at least 24 h at -20°C . Retinas were dissected in phosphate buffered saline (PBS; Sigma, St. Louis, MO) and then washed with PBS three times, 10 min each. Following incubation in a blocking buffer (50% fetal calf serum, 20% normal goat serum in PBS) for 1 h, the retinas were incubated with rabbit anti-mouse collagen IV antibody (Chemicon, Temecula, CA; diluted 1:500 in PBS containing 20% fetal calf serum, 20% normal goat serum) at 4°C overnight. Retinas were then washed three times with PBS, 10 min each, and incubated with secondary antibody Alexa Fluor 594 goat-anti-rabbit (Invitrogen, Carlsbad CA; diluted 1:500 in PBS containing 20% fetal calf serum, 20% normal goat serum) for 2 h at room temperature, washed three times with PBS, 10 min each, and mounted on a slide with PBS/glycerol (2 vol/1 vol). Retinas were viewed by fluorescence microscopy and images were captured in digital format using a Zeiss microscope (Zeiss, Chester, VA). The retinal wholemounts were also stained with antibodies to NG2 (Chemicon), GFAP (Dako, Denmark), and endoglin (BD Biosciences, San Jose, CA) to visualize PC, astrocytes, and EC, respectively, at dilutions recommended by the supplier. The central capillary dropout area was quantified, as a percentage of whole retina area, from the digital images in masked fashion using Axiovision software (Zeiss).

2.4. Quantification of neovascular proliferative retinopathy

Quantification of vitreous neovascularization on P17 was performed as previously described (Wang et al., 2003). Briefly, mouse eyes were enucleated, fixed in formalin for 24 h, and embedded in paraffin. Serial sections (6 μm thick), each separated by at least 40 μm , were taken from around the region of the optic nerve. The hematoxylin- and PAS- stained sections were examined in masked fashion for the presence of neovascular tufts projecting into the vitreous from the retina. The neovascular score was defined as the mean number of neovascular nuclei per section found in eight sections (four on each side of the optic nerve) per eye.

2.5. Staining of the hyaloid vasculature

Following the removal of eyes, the sclera, choroids, and retinas were dissected anteriorly from the optic nerve to limbus. The remaining wholemount specimen was stained with anti-collagen IV antibody as described above to visualize the hyaloid vasculature. Wholemount specimens were viewed by fluorescence microscopy, and images were captured in digital format using a Zeiss microscope (Zeiss). The dissection resulted, in most cases, in the loss of hyaloid artery and vasa hyaloidea propria vessels. However, the tunica vasculosa lentis vessels were clearly visible following collagen IV staining.

2.6. Immunohistochemical and β -galactosidase staining of the frozen sections

Mouse eyes were enucleated and embedded in optimal cutting temperature (OCT; VWR Scientific, West Chester, PA) compound at -80°C . Sections (9 μm) were cut on a cryostat, placed on glass slides and allowed to dry for 2 h in a desiccator at 4°C . For fluorescence microscopy, sections were fixed in cold acetone on ice for 10 min, followed by three washes with PBS, 5 min each. Sections were incubated in blocking buffer (1% bovine serum albumin (BSA), 0.2% skim milk, and 0.3% Triton X-100 in PBS) for 15 min at room temperature. Sections were then incubated with rabbit anti-mouse collagen IV antibody (Chemicon; diluted 1:500 in blocking buffer) overnight at 4°C in a humid environment. After three 5 min washes with PBS, section were incubated with secondary antibody Alexa Fluor 594 goat-anti-rabbit (Invitrogen; diluted 1:500 in blocking buffer) for 2 h at room temperature. Sections were washed three times in PBS, covered with PBS/glycerol (2 vol/1 vol) and mounted with a

coverslip. Retinal sections were viewed by fluorescence microscopy, and images were captured in digital format using a Zeiss microscope (Zeiss).

β -galactosidase staining of eye frozen sections was performed as previously described (Emert et al., 1998). Briefly, sections were fixed in 1.25% glutaraldehyde prepared in PBS for 10 min, and washed with PBS three times, 5 min each. Sections were incubated overnight at 37°C with 0.5 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-pyranoside (X-Gal; Invitrogen) prepared in 44 mM HEPES, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 15 mM NaCl, and 1.3 mM $MgCl_2$. After two washes in PBS, 5 min each, sections were incubated with nuclear fast red (Invitrogen). Sections were washed three times in PBS, covered with PBS/glycerol (2 vol/1 vol), and mounted with a coverslip. Retina sections were viewed by microscopy and images were captured in digital format.

2.7. BrdU labeling and collagen IV staining of wholemount retinas

The detection of cell proliferation on the retinal blood vessels was assessed by immunohistochemistry for 5-bromo-2-deoxyuridine (BrdU) incorporation followed by collagen IV staining of blood vessels. Mice were injected intraperitoneally with BrdU (Sigma) at 120 mg/Kg of body weight. One and a half hours later, mice were sacrificed, eyes were removed, and fixed immediately in 4% paraformaldehyde for 4 min on ice. The eyes were then transferred to methanol and stored at -20°C for at least 24 h. Retinas were dissected in PBS, washed in PBS, and stained with anti-collagen IV antibody as described above for staining of wholemount retinas. Retinas were then washed for 30 min in PBS containing 1% Triton X-100 to permeabilize cell membranes, placed in 2 M HCl at 37°C for 1 h, washed in 0.1 M sodium borate for 30 min to neutralize the HCl, washed in PBS containing 1% Triton X-100 for 30 min, and incubated with a monoclonal antibody to BrdU (Roche, Indianapolis, IN; diluted 1:250 in PBS containing 1% BSA) at 4°C overnight. Following incubation, the retinas were washed for 30 min in PBS containing 1% Triton X-100 and incubated with anti-mouse CY2 antibody (Jackson ImmunoResearch Laboratories, West Grove, PA; diluted 1:250 in PBS containing 1% BSA) at room temperature for 2 h. After the final wash in PBS for 30 min, the retinas were mounted on a slide with PBS/glycerol (2 vol/1 vol), viewed by fluorescence microscopy, and images were captured in digital format using a Zeiss microscope (Zeiss). For quantitative assessment of the data the mean number of BrdU-positive nuclei on the blood vessels was determined per retina.

2.8. TdT-dUTP Terminal Nick-End Labeling (TUNEL) on retinal vessels

Apoptotic cell death on the retinal vasculature was assessed by TdT-dUTP Terminal Nick-End Labeling (TUNEL) staining of trypsin-digested retinal vessels. The eyes were removed and fixed in 10% neutral formalin at 4°C for 48 h. The retinas were trypsin digested as described above and retinal vessels were mounted on glass slides. After washing in PBS for 20 min, the retinal vessels were rehydrated with PBS containing 0.5% Triton X-100 for 1 h. The retinal vessels were washed in PBS for 20 min, and TUNEL staining was performed with the Fluorescein *in situ* Cell Death Detection kit as recommended by the supplier (Roche). After washing in PBS for 30 min, the retinal vessels were counterstained with Hoechst 33258 (Invitrogen) for nuclei staining. The retinal vessels were washed in PBS for 20 min, covered with PBS/glycerol (2 vol/1 vol) and mounted with a coverslip. Retinal vascular wholemounts were viewed by fluorescence microscopy, and images were captured in digital format (Zeiss). For quantitative assessment of the data the mean number of TUNEL positive cells was determined per retina.

2.9. Western blot analysis

VEGF protein levels were determined by Western blotting of whole eye extracts prepared from P15 mice (5 days of hyperoxia and 3 days of normoxia), when VEGF is maximally expressed

(Pierce et al., 1995), during OIR. Eyes were dissected, homogenized in RIPA buffer (10 mM HEPES pH 7.6, 142.5 mM KCl, 1% NP-40, and protease inhibitor cocktail; Roche), sonicated briefly, and incubated at 4°C for 20 min. Homogenates were centrifuged at 4°C for 30 min (16,000 $\times g$) to remove insoluble material. Clear supernatants were transferred to clean tubes and protein concentrations were determined using DC protein assay (Bio-Rad, Hercules, CA). Approximately 20 μg of protein lysate was analyzed by SDS-PAGE (4–20% ; Tris-Glycin gel, Invitrogen) under reducing conditions and transferred to a nitrocellulose membrane. The blot was incubated with a rabbit polyclonal anti-mouse VEGF antibody (1:2000; PeproTech, Roch Hill, NJ), washed, and developed using a goat anti-rabbit HRP-conjugated secondary antibody (1:5000; Jackson ImmunoResearch Laboratories) and ECL system (Amersham Biosciences, Pittsburgh, PA). The same blot was also probed with a monoclonal antibody to β -actin (1:5000; Sigma) to verify equal protein loading in all lanes. The levels of VEGF were also evaluated in retina extracts prepared from PEDF^{+/+} and PEDF^{minus;/minus} mice at different postnatal days in room air and during OIR using a mouse VEGF immunoassay kit as recommended by the supplier (R&D Systems, Inc., Minneapolis, MN).

2.10 Clinical evaluation of murine eyes

Mouse eyes were examined for ocular abnormalities using a Kowq SL-2 hand-held slit lamp and an ophthalmoscope. Mice were physically restrained during the analysis and their eyes were dilated with 1% tropicamide at least 15 min before evaluation. Both PEDF^{+/+} (8 eyes) and PEDF^{minus;/minus} (12 eyes) mice were analyzed without the observers' prior knowledge of genotype. We also examined retinal functions in PEDF^{+/+} and PEDF^{minus;/minus} mice using electroretinogram (ERG) analysis (Mizota and Adachi-Usami, 2002). The ERG analysis were performed by delivering a series of flashes at different intensities under dark adapted condition (Scotopic Intensity Series) using a UTAS-4000 ERG instrument (LKC Technologies, Gaithersburg, MD). ERGs were recorded using modified DTL fiber electrodes placed on the corneas and referenced to an ipsilateral subdermal electrode.

2.10. Statistical analysis

Statistical differences between groups were evaluated with Student's unpaired *t*-test (two-tailed). Means $\pm$ SD are shown. $P \leq 0.05$ is considered significant.

3. Results

3.1. PEDF^{minus;/minus} mice and spatial and temporal expression of PEDF

The PEDF^{minus;/minus} mice were generated by insertion of a transgene cassette replacing the entire PEDF gene (Valenzuela et al., 2003; Wiegand et al., 2004). The transgene contained the β -galactosidase coding sequence inserted at the PEDF ATG codon allowing its expression from the endogenous PEDF promoter. We determined the expression pattern of PEDF by staining for β -galactosidase activity. Figure 1 shows β -galactosidase staining of eye sections prepared from PEDF^{minus;/minus} mice at different postnatal days during normal retinal vascularization (P0-P42) and during OIR (P7-P17). Very little staining was observed in neural retina prior to P7, which was increased to maximum level by P14. The β -galactosidase staining then decreased to almost undetectable levels by P42. The maximum staining was initially observed in the outer plexiform layer (P7-P14), but later sporadic staining was also observed in other retinal inner layers (P14-P21). However, strong β -galactosidase staining was observed in the choroid and RPE throughout postnatal life. During OIR strong β -galactosidase staining was observed in the retina after exposure to hyperoxia (P12). The staining remained relatively strong during active neovascularization (P17). These expression patterns are similar to those reported for endogenous PEDF in the mouse retina (Behling et al., 2002; Dawson et al., 1999). However, the strongest staining was localized to the outer plexiform layer.

3.2. PEDF^{minus}/^{minus}; mice exhibited increased retinal vascular density

To compare retinal vascular density in PEDF^{+/+} and PEDF^{minus}/^{minus}; mice, we prepared retinal trypsin digests at different postnatal days and determined EC and PC ratios and their densities. In wholemount retinal trypsin digests, the nuclei of EC are oval or elongated and lied within the vessel wall along the axis of the capillary, while PC nuclei are small, spherical, stain densely, and generally have a protuberant position on the capillary wall. Figure 2 shows retinal vessels prepared from P21 PEDF^{+/+} and PEDF^{minus}/^{minus}; mice. The PEDF^{minus}/^{minus}; retinal vasculature exhibited greater cellularity and more capillaries, suggesting an increase in retinal vascular density (see below). Minimal differences were observed in retinal trypsin digests from P42 mice (data not shown). Thus, the impact lack of PEDF has on retinal vascularization is more prominent during early vascularization of retina.

Table 1 shows the mean EC and PC ratios (EC/PC) of retinal microvessels from PEDF^{+/+} and PEDF^{minus}/^{minus}; mice. At P21, the mean EC/PC ratio for PEDF^{minus}/^{minus}; mice was 2.73:1, while that of the PEDF^{+/+} mice was 2.35:1. However, EC/PC ratio at P42, when the vascular remodeling and maturation is complete, was decreased to 2.1:1 regardless of PEDF expression. Thus, the potential contribution of PEDF to vascular remodeling and pruning, which occurs at later stages of retinal vascularization, is minimal.

To determine whether the observed changes in the EC/PC ratios were due to changes in the number of EC and/or PC, we determined the EC and PC densities. Tables 2 and 3 show the mean density of retinal EC and PC from PEDF^{+/+} and PEDF^{minus}/^{minus}; mice. The mean EC density of P21 PEDF^{minus}/^{minus}; was significantly higher than PEDF^{+/+} mice, while the mean PC density was not significantly different. The mean densities of EC and PC in P42 PEDF^{+/+} and PEDF^{minus}/^{minus}; mice were similar. Thus, the retinas from P21 PEDF^{minus}/^{minus}; mice had a higher number of EC resulting in increased EC/PC ratio.

3.3. The primary retinal vasculature developed at a faster rate in PEDF^{minus}/^{minus}; mice

The murine retinal vasculature develops postnatally. A superficial layer of vessels is formed during the first week of postnatal life (P7). After that, the deep vascular plexuses begin to form at the outer edge of the inner nuclear layer. This is followed by the formation of an intermediate vascular plexuses between the primary and the deep vascular layer during the third postnatal week. The course of retinal vessel sprouting and assembly can be readily visualized by collagen IV, a major component of the basement membrane of retinal vessels, immunostaining of wholemount retinal preparations and/or eye frozen sections.

We compared retinal vascular development by collagen IV staining of wholemount retinas prepared from mice during the first week of postnatal life (P3, P5 and P7). Figure 3 shows the first-week of the developing retinal vessels in PEDF^{+/+} and PEDF^{minus}/^{minus}; mice. Blood vessels originate from the center of retina near the optic nerve, and spread toward the retinal periphery. At each time point, P3, P5 and P7, retinal blood vessels expanded at a significantly faster rate toward the retinal periphery in PEDF^{minus}/^{minus}; mice compared to PEDF^{+/+} mice ($P < 0.05$). We observed no significant differences in spreading pattern of astrocytes ahead of the developing retinal vasculature (not shown) or along the developing retinal vasculature (Figure 4) in PEDF^{minus}/^{minus}; mice compared to PEDF^{+/+} mice. However, we consistently observed lower levels of glial fibrillary acidic protein (GFAP), a marker of mature astrocytes (Scheef et al., 2005) in retinal extracts prepared from PEDF^{minus}/^{minus}; mice compared to PEDF^{+/+} mice, both during room air and OIR (data not shown).

The developing retinal vasculature was also appropriately covered by PC, immediately following the expanding vasculature (Figure 5) in both PEDF^{+/+} and PEDF^{minus}/^{minus}; mice. We observed no significant differences in the organization or number of tip cells ahead

of the expanding retinal vasculature (not shown). We also compared vessel sprouting and penetration into the deep retina after P7. Figure 6 shows retinal vessels in the frozen eye section from P10 PEDF^{+/+} and PEDF^{minus/minus} mice. There were significantly more intermediate retinal vessels developed in P10 PEDF^{minus/minus} mice compared to P10 PEDF^{+/+} mice. However, retinal vessel development in PEDF^{+/+} mice reached that of PEDF^{minus/minus} mice at later postnatal days (Tables 1–3).

3.4. Increased retinal vascular cells proliferation and apoptosis in PEDF^{minus/minus} mice

Retinal vessel development depends on the balance between retinal vascular cells proliferation and apoptosis. We used BrdU incorporation of retinal vascular cells to determine the rate of proliferation of cells in the developing retinal vasculature of PEDF^{+/+} and PEDF^{minus/minus} mice. PEDF^{+/+} and PEDF^{minus/minus} mice of different ages were injected with BrdU, and labeled cells in retinal vascular wholemount were detected by staining with anti-BrdU and collagen IV (stains blood vessels). Figures 7A–D show double labeling of P7 PEDF^{+/+} (A and C) and PEDF^{minus/minus} (B and D) mice retinal vasculature with anti-BrdU and collagen IV. Figure 7E shows the mean number of proliferating cells in the retinal vessels of PEDF^{+/+} and PEDF^{minus/minus} mice prior to P21, when active vascular cell proliferation is normally occurring. We observed a peak of retinal vascular cell proliferation at P10 in both PEDF^{+/+} and PEDF^{minus/minus} mice, which occurred at similar levels. However, at P7 there were more proliferating vascular cells in PEDF^{minus/minus} mice than in PEDF^{+/+} mice, while at P14 there were less proliferating vascular cells in PEDF^{minus/minus} mice than in PEDF^{+/+} mice. At P21, only few cells were proliferating in the retinal vasculature of both PEDF^{+/+} and PEDF^{minus/minus} mice.

We next used the TUNEL method to determine the rate of apoptosis in the developing retinal vasculature of PEDF^{+/+} and PEDF^{minus/minus} mice. Figures 8A, B show the TUNEL labeling of retinal vasculature in the trypsin digested retinal vessels from P10 PEDF^{+/+} and PEDF^{minus/minus} mice, respectively. Figures 8C, D show merged images following Hoechst nuclear labeling of the vascular cells in A and B. The mean number of apoptotic cells in the trypsin digested retinal vessels from PEDF^{+/+} and PEDF^{minus/minus} mice prior to P21, which shows a similar trend to that of proliferation in these mice, are shown in figure 8E. We observed the highest rate of apoptosis in retinal vessels from P10 mice. At this time point there were more apoptotic vascular cells in PEDF^{minus/minus} mice compared to PEDF^{+/+} mice. However, by P14 there were less apoptotic vascular cells in PEDF^{minus/minus} mice compared to PEDF^{+/+} mice. At P21, only few cells undergo apoptosis in retinal vasculature, which is significantly less in PEDF^{minus/minus} mice. Thus, the retinal vasculature undergoes active proliferation and apoptosis prior to P10, which is enhanced in the absence of PEDF. Proliferation and apoptosis of vascular cells slows down significantly after P10, and is rarely observed by P21. This decline is more dramatic in PEDF^{minus/minus} mice compared to PEDF^{+/+} mice.

3.5. Regression of hyaloid vasculature was not affected in the PEDF^{minus/minus} mice

The pupillary membrane and hyaloid vessels (hyaloids arteries, tunica vasculosa lentis, and vasa hyaloidea propria) provide nourishment in the immature lens, retina and vitreous (Ito and Yoshioka, 1999). However, they regress during the later states of ocular development. Figure 9 shows the staining of the vasculature in wholemount ocular specimens prepared from PEDF^{+/+} and PEDF^{minus/minus} mice at P10, P21 and P42. We observed a significant amount of hyaloid vessels in P10 PEDF^{+/+} and PEDF^{minus/minus} mice, however, these vessels regressed similarly in PEDF^{+/+} and PEDF^{minus/minus} mice by 6-weeks of age. Thus, absence of PEDF minimally affects the regression of hyaloid vasculature.

3.6. The developing retinal vasculature of PEDF^{minus}/^{minus}; mice is more sensitive to hyperoxia-mediated vessel obliteration during OIR

We next compared the response of PEDF^{+/+} and PEDF^{minus}/^{minus}; mice during OIR. The mouse OIR is a highly reproducible model for studying all aspects of angiogenesis. In this assay, P7 mice are exposed to 75% oxygen for 5 days, and then returned to room air for 5 days. The exposure of developing retinal vasculature to high oxygen prevents further development of retinal vessels as well as promoting obliteration of vessels in the center of retina, resulting in loss of perfusion to this area. When animals are returned to room air, the retina becomes ischemic and promotes neovascularization, which results in reestablishment of vasculature in the nonperfused area and growth of new vessels into the vitreous. We examined retinal nonperfused area in P12 retinas, when maximum vessel obliteration is observed (5 days of hyperoxia). Figure 10 shows that retinal vasculature from PEDF^{minus}/^{minus}; mice are significantly more sensitive to hyperoxia-mediated vessel obliteration exhibiting a significantly larger nonperfused area relative to the whole retina compared to PEDF^{+/+} mice ($P < 0.05$).

3.7. Quantification of preretinal neovascularization

Quantification of preretinal neovascularization on P17 mice (when maximum preretinal neovascularization occurs) was performed as described previously (Wang et al., 2003). Briefly, 6- μ m-thick serial sections, each separated by at least 40 μ m, were obtained from the region around the optic nerve. The sections were stained with hematoxylin and PAS and examined in masked fashion for the presence of neovascular cell nuclei projecting into the vitreous from the retina. The neovascular cell nuclei score was defined as the mean number of neovascular nuclei per section found in eight sections (four on each side of the optic nerve) per eye. Figure 11 shows the mean number of vascular cell nuclei projecting into the vitreous of eyes from P17 PEDF^{+/+} and PEDF^{minus}/^{minus}; mice. We consistently observed similar numbers of vascular cell nuclei in the PEDF^{+/+} and PEDF^{minus}/^{minus}; mice, which matches well with the collagen IV staining of wholemount P17 retinas.

3.8. Modulation of retinal VEGF levels during room air or oxygen-induce ischemic retinopathy

Expression of VEGF during hypoxia is believed to be the major contribution factor to the development of new blood vessels (Pierce et al., 1995). The ability of the PEDF^{+/+} and PEDF^{minus}/^{minus}; mice to elicit a similar neovascular response during OIR suggests that similar expression of VEGF should occur in these mice. We examined expression of VEGF in eyes from P15 PEDF^{+/+} and PEDF^{minus}/^{minus}; mice during OIR (5 days of hyperoxia and 3 days of normoxia) when VEGF expression is maximally induced. Figure 12A shows a Western blot of protein lysates prepared from whole eye extracts of P15 PEDF^{+/+} and PEDF^{minus}/^{minus}; mice during OIR. We observed similar levels of VEGF expression in eyes from PEDF^{+/+} and PEDF^{minus}/^{minus}; mice during OIR. We also observed similar levels of VEGF in retinas from PEDF^{+/+} and PEDF^{minus}/^{minus}; mice during room air postnatal retinal vascularization using an ELISA assay as described in Methods (Figure 12B). However, we observed higher level of VEGF in retinas of PEDF^{minus}/^{minus}; at P12 (5 days of hyperoxia) and P21 (5 days of hyperoxia and 9 days of normoxia) during OIR (Figure 12C). Similar levels of VEGF were observed in P15 (5 days of hyperoxia and 3 days of normoxia) retinas of PEDF^{+/+} and PEDF^{minus}/^{minus}; mice during OIR using the ELISA assay, as was shown by Western (Figure 12 A). Thus, the absence of PEDF does not affect VEGF expression during room air retinal vascularization or during OIR at P15. However, significantly higher levels of VEGF were detected at P12 (right after 5 days of hyperoxia) and at P21 (when the majority of the newly formed vessels are regressing) during OIR. Therefore, expression of PEDF may be essential for dampening of the VEGF levels under hyperoxic conditions.

3.9. Histological and functional analysis of retinas from PEDF^{+/+} and PEDF^{minus/minus} mice

To determine whether retinal development proceeded normally in PEDF^{minus/minus} mice, histological examination of eyes from PEDF^{+/+} and PEDF^{minus/minus} mice was performed by hematoxylin and PAS staining of eye sections. Figures 13 A, B show the retina structure and organization prepared from P21 PEDF^{+/+} and PEDF^{minus/minus} mice, respectively. The ganglion cells, inner nuclear cells and photoreceptors of the PEDF^{+/+} retinas were histologically similar to that of PEDF^{minus/minus} mice. We did not observe significant changes in the neural retinal organization from older PEDF^{minus/minus} mice (not shown). This is consistent with slit-lamp analysis which revealed no significant phenotype in the PEDF^{minus/minus} mice compared to PEDF^{+/+} mice.

We next examined retinal function by ERG analysis of PEDF^{+/+} and PEDF^{minus/minus} mice. Figure 13 C shows intensity vs. response in the growth of ERG a- and b-wave with increased flash intensity in P42 PEDF^{+/+} and PEDF^{minus/minus} mice. The prominent oscillatory potentials riding on the b-wave indicate normal retinal function in these mice. Thus, lack of PEDF has minimal effect on ocular development, and the formation and function of the retina.

4. Discussion

Normal retinal vascular development and homeostasis is achieved by a fine temporal and spatial balanced production of proangiogenic and antiangiogenic factors. A number of proangiogenic and antiangiogenic factors have been identified in the eye and several appear to play major roles in normal development and in the disease states. Insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF) and VEGF are examples of stimulators of angiogenesis in the eye. PEDF, a protein originally identified in conditioned medium from RPE cells, has received a great deal of attention as a potent inhibitor of angiogenesis (Bouck, 2002). However, its physiological role during retinal vascular development and neovascularization requires further investigation.

Expression of PEDF in the neural retina is spatially and temporally regulated and may play important roles in retinal vascularization and homeostasis. In mouse eyes, PEDF is not detected in the neural retina until late in gestation (E18.5). PEDF expression detected in retinal ganglion cell layer increases after birth and remains high through P14 and decreases thereafter, but persists through adulthood (Behling et al., 2002). Here we did not observe significant β -galactosidase staining in retinal ganglion cell layer during postnatal vascularization of retina until later postnatal days (P14-P17; Fig. 1). The strongest staining was observed in the outer plexiform layer followed by some sporadic staining in the inner retina. The staining pattern decreased after P14 and was almost undetectable in the neural retina by P42. Thus, normal postnatal retinal vascularization corresponds to the time points of higher PEDF expression in the neural retina. Given the antiangiogenic nature of PEDF, it is reasonable to speculate that expression of PEDF may act as a feed back mechanism to dampen proangiogenic signaling and promoting a more quiescent, differentiated state of the endothelium. These expression patterns are also consistent with the reported VEGF expression pattern which also decreases as retinal vascularization reaches completion (Ohno-Matsui et al., 2001; Suzuma et al., 1999). In addition, PEDF inhibits VEGF expression in retinal EC and muller cells (Zhang et al., 2006), and its expression is up-regulated by exposure to hyperoxia when VEGF is minimally expressed during OIR (Dawson et al., 1999), and Fig. 1). Furthermore in the absence of PEDF, VEGF levels remained higher during exposure to hyperoxia or normoxia in OIR (Fig. 12C), thus, further supporting a role for PEDF in down regulation of VEGF expression under hyperoxic conditions.

PEDF inhibits EC proliferation, migration, and capillary morphogenesis resulting in death of EC by apoptosis (Chen et al., 2006; Ho et al., 2007; Kanda et al., 2005). In contrast, PEDF promotes PC survival and proliferation, and protects PC from oxidative damage in culture (Amano et al., 2005; Yamagishi et al., 2005). We compared the EC and PC density of retinal vessel trypsin digests from PEDF^{+/+} and PEDF^{minus/minus} mice at P21 and P42. At P21, when formation of the retinal primary vascular plexuses is complete, the mean EC density was significantly higher in PEDF^{minus/minus} retinal microvessels compared to PEDF^{+/+} mice. However, the mean density of PC was not significantly affected in the absence of PEDF. Thus, increased number of EC results in a higher EC/PC ratio in PEDF^{minus/minus} mice. The retinal vascular remodeling and pruning occurs after P21 and is complete by P42 (Wang et al., 2003). We observed a similar EC and PC ratios and densities in P42 PEDF^{+/+} and PEDF^{minus/minus} (Tables 1–3). Thus, our data suggest that PEDF deficiency promotes the early vessel growth in the inner neural retina by permitting more EC growth during the formation of retinal vascular plexuses without impacting its pruning and remodeling at later postnatal age when PEDF level is decreased.

We used retinal wholemount and frozen eye sections stained for collagen IV to further evaluate retinal vascular development during early postnatal days. During the first week of life (P7), when a superficial layer of vasculature spreads from the optic disc towards the peripheral retina, vessel spreading occurred at a significantly faster rate in PEDF^{minus/minus} mice compared to PEDF^{+/+} mice (Fig. 3). After P7, when sprouting of the retinal superficial layer of blood vessels into the deep retina occurs, we observed significantly more intermediate retinal vessels (between the superficial and the deep layer) developing in P10 PEDF^{minus/minus} mice compared to PEDF^{+/+} mice (Fig. 6). This is consistent with the increased rate of proliferation and apoptosis observed in retinal vasculature of PEDF^{minus/minus} mice (Figs. 7 and 8). Our results are consistent with those reported in mice where increased expression of PEDF resulted in a delay of deep retinal vascularization and vessel maturation (Wong et al., 2004). However, our results are in contrast to those reported in 3-month-old mice where lack of PEDF was associated with an increase in retinal vascular density (Doll et al., 2003). Our analysis failed to detect significant differences in vascular density at P42, and later time points (Tables 1–3 and not shown). The analysis of retinal vasculature in the study by Doll and colleagues was limited to a single time point (3 months, when PEDF is normally undetectable). In addition, no images of stained retinal vasculature, demonstrating these differences, were provided. Perhaps strain differences and the exact methods used to generate the null mice may contribute to these differences.

Here we show that early postnatal retinal vascularization occurs at a faster rate in PEDF^{minus/minus} mice. However, the development of retinal vasculature in PEDF^{+/+} mice catches up with that of PEDF^{minus/minus} mice at later postnatal days, exhibiting spatial and density patterns similar to those of PEDF^{minus/minus} mice at P42. Thus, PEDF deficiency may disturb the normal balance between proliferation and apoptosis impacting early retinal vascularization. The most significant effect on the developing retinal vasculature occurred during the first two weeks of life, when PEDF expression is normally at its highest level ((Behling et al., 2002) and Fig. 1), perhaps acting as a feed back mechanism to dampen the VEGF proangiogenic signaling in an ischemic retina. This is consistent with increased rate of proliferation, and its compensation by increased rate of apoptosis, in PEDF^{minus/minus} mice when active angiogenesis is occurring (P7-P10). Later, retinal angiogenesis reaches completion, and as a result, the degree of proliferation and apoptosis is significantly reduced, especially in the absence of PEDF (P14-P21). This is perhaps due to exaggeration of these activities in the absence of PEDF during earlier postnatal days. PEDF expression normally decreases in the retina after P21, and the deficiency of PEDF appear to play a less significant role in retinal vascularization. This is consistent with significant decrease in rates of proliferation and apoptosis observed in retinal vasculature of P21 PEDF^{+/+} and PEDF^{minus/minus}.

minus;mice. However, it is possible that other antiangiogenic factors, such as thrombospondin-1, may play a significant role during later stages of retinal vascular development and homeostasis (Bhutto et al., 2004; Uno et al., 2006; Wang et al., 2003). We did not observe a significant impact on expansion and organization of astrocytes, PC, the number of endothelial tip cells, or VEGF levels during early retinal vascularization (Figs. 4, 5, 12 and not shown). Thus, a tightly balanced production of pro- and anti-angiogenesis factors is essential for retinal vascular homeostasis.

The pupillary membrane and hyaloid vasculature (hyaloids arteries, tunica vasculosa lentis, and vasa hyaloidea propria) provide nourishment in the immature lens, retina and vitreous (Ito and Yoshioka, 1999). However, they regress during the later stages of ocular development by apoptosis. The contribution of PEDF to these processes has not been previously addressed. To determine the potential role of PEDF in the regression of hyaloid vasculature, an apoptosis-dependent process, we examined the hyaloid vasculature at different postnatal days. Our results indicated that the regression of hyaloid vessels, mainly the tunica vasculosa lentis, was not significantly affected in the PEDF^{minus;/minus;} mice compared to PEDF^{+/+} mice. Thus, the regression of ocular embryonic vessels is not compromised in the absence of PEDF.

We next examined the response of developing retinal vasculature to oxygen-induced ischemic retinopathy. P7 PEDF^{+/+} and PEDF^{minus;/minus;} mice were exposed to 75% oxygen for 5 days. Hyperoxia negates the increase in VEGF expression during vascular development resulting in underdeveloped retinal vasculature and obliteration by apoptosis of existing vessels. This is mainly attributed to decreased VEGF level (Pierce et al., 1995). An increase in PEDF level, at P12 (5 days of hyperoxia) when the most vascular obliteration occurs, has been reported ((Dawson et al., 1999) and Fig. 1). We observed that the developing retinal vasculature of PEDF^{minus;/minus;} mice is more sensitive to oxygen-induced vessel obliteration resulting in increased nonperfused areas compared with the PEDF^{+/+} mice despite higher levels of VEGF. This may be attributed, at least in part, to the underdeveloped glial cells in the retina of PEDF^{minus;/minus;} mice (Eichler et al., 2004; Yafai et al., 2007). We consistently observed lower levels of GFAP in retinal extracts prepared from PEDF^{minus;/minus;} mice during room air or OIR (not shown). However, whether the presence of astrocytes with reduced levels of GFAP in PEDF^{minus;/minus;} mice contributes to enhanced sensitivity of the retinal vasculature to hyperoxia remains to be determined.

PEDF is also a neuroprotective factor, which in its absence dying signals, especially under hyperoxia, may be exaggerated (Becerra, 1997; Cao et al., 1999; Tombran-Tink and Barnstable, 2003). PEDF protects the retina from the stress of hyperoxia through its anti-oxidant and/or PC protective activity (Amano et al., 2005; Yamagishi et al., 2006). Increased oxidative stress, in the absence of PEDF, may also account for the increased VEGF levels observed in PEDF^{minus;/minus;} mice during hyperoxia or normoxia in OIR. However, the molecular and cellular mechanisms which mediate PEDF antioxidant and/or protective effects require further delineation.

Exposure of mice after 5 days of hyperoxia to room air for 5 days (normoxia) stimulates growth of new blood vessels into the vitreous (Smith et al., 1994). Our results indicated that preretinal neovascularization occurred similarly in the PEDF^{+/+} and PEDF^{minus;/minus;} mice at P17. This was consistent with similar levels of VEGF expression observed in P15 PEDF^{+/+} and PEDF^{minus;/minus;} (5 days of hyperoxia and 3 days of normoxia), when VEGF is maximally induced ((Pierce et al., 1995) and Figs. 12A,C). Thus, lack of PEDF does not affect VEGF expression during normal postnatal retinal vascularization or active neovascularization in OIR. However, increased levels of VEGF were observed after exposure to hyperoxia or after retinal neovascularization in PEDF^{minus;/minus;} mice during OIR. Thus, although the impact lack of PEDF has on retinal neovascularization during OIR is minimal, its expression may be

essential for appropriate down-regulation of VEGF in response to hyperoxia and excessive neovascularization.

In summary, our results indicate that PEDF is an important modulator of retinal vascular homeostasis. It provides a protective role during formation of primary retinal vascular plexuses by maintaining the pro- and anti-angiogenic balance. Thus, alterations in PEDF levels during development and/or under pathological conditions may contribute to retinal vasculopathies. Modulation of PEDF levels has already proven as a suitable target for manipulation of retinal vascularization under some pathological conditions. However, additional studies are needed to elucidate the molecular and cellular mechanisms underlying the antiangiogenic and neuroprotective activities of PEDF.

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Fig. 1.

The spatial and temporal pattern of PEDF expression in the eye. Frozen eye sections prepared from PEDF^{minus}/_{minus} mice, at indicated time points, were stained for β -galactosidase activity. Eyes from PEDF^{+/+} mice were used as negative control. Please note strong staining in the outer plexiform layer of eyes from P14 mice and, increased staining upon exposure to hyperoxia in P12 mice. These experiments were repeated with eyes from 3 different mice with similar results. A representative image is shown. V: vitreous; Bar = 100 μ m. GCL: ganglion cell layer; IPL: inner plexiform layer; OPL: outer plexiform layer; and RPE: retinal pigment epithelium.

Fig. 2. PEDFminus;/minus; mice exhibit increased retinal vascular density during normal postnatal retinal vascularization. Mice retinal vasculature was prepared by trypsin digestion of retinal wholemounts. Retinas were obtained from P21 PEDF+/+ (A, C) and PEDFminus;/minus; (B, D) mice. Lower panels (C and D; x400) are higher magnification of upper panels (A and B; x100). The arrows indicate endothelial cells, while arrowheads show pericytes. Please note the increased densities of capillaries (see Tables 1–3) in retinas from PEDFminus;/minus; mice. Bars, A and B=100 μ m; C and D=20 μ m.

Fig. 3. The primary retinal vasculature expands at a faster-rate in PEDF^{minus}/^{minus} mice. Collagen IV staining of the retinal wholemounts prepared from PEDF^{+/+} (A, C, E) or PEDF^{minus}/^{minus} (B, D, F) mice at postnatal day 3 (P3) (A, B), P5 (C, D) and P7 (E, F). The quantitative assessment of the data is shown in G. Please note that the spreading of retinal vessels is significantly faster in PEDF^{minus}/^{minus} mice compared to PEDF^{+/+} mice (** $P < 0.01$; $n=10$, * $P < 0.05$; $n=10$). Bar = 200 μ m.

Fig. 4. Similar astrocytic organization during retinal vascularization in PEDF^{+/+} and PEDF^{minus;/minus;} mice. GFAP (astrocytes, red; A and B) and endoglin (EC, green; C and D) staining of the retinal wholemounts prepared from P7 PEDF^{+/+} (A, C, E) or PEDF^{minus;/minus;} (B, D, F) mice. The merged images are shown in panels E and F. Please note similar astrocytic and EC organization in these mice. Similar astrocytic organizations were observed in areas not covered by EC (not shown). These experiments were repeated with eyes from 5 different mice with similar results. A representative image is shown. Bar=20 μ m.

Fig. 5. Similar organization of pericytes during retinal vascularization of PEDF^{+/+} and PEDF^{minus;/minus;} mice. NG2 (PC, red; A and B) and endoglin (EC, green; C and D) staining of the retinal wholemounts prepared from P7 PEDF^{+/+} (A, C, E) or PEDF^{minus;/minus;} (B, D, F) mice. The merged images are shown in panels E and F. Please note similar PC and EC organization in these mice. These experiments were repeated with eyes from 5 different mice with similar results. A representative image is shown. Bar = 20 μ m.

Fig. 6.

Organization of the deep retinal vascular plexuses. Collagen IV staining of the frozen eye sections from P10 PEDF^{+/+} (A) and PEDF^{minus;/minus;} (B) mice are shown. The quantitative assessment of the data is shown in C. Please note significantly more intermediate retinal vessels are formed in PEDF^{minus;/minus;} mice (arrow) compared to PEDF^{+/+} mice (* $P < 0.01$; $n = 10$). Bar = 50 μ m.

Fig. 7. Enhanced retinal vascular cell proliferation in PEDF^{minus/minus} mice. BrdU incorporation of retinal vascular cells was used to determine the rates of proliferating cells in the postnatal developing retinal vasculature from PEDF^{+/+} and PEDF^{minus/minus} mice at indicated postnatal days. A and B show retinas from P7 PEDF^{+/+} and PEDF^{minus/minus} mice, respectively. C and D are higher magnification of above images. The quantitative assessment of the data is shown in E (*P < 0.05; *n*=10). Bar = 100 μ m (A and B) and 20 μ m (C and D).

Fig. 8.

Enhanced retinal vascular cell apoptosis in PEDF^{minus/minus} mice. TUNEL staining was used to assess the rate of apoptotic vascular cells in the trypsin digested retinal vasculature from PEDF^{+/+} and PEDF^{minus/minus} mice at indicated postnatal days. A and B show retinas from PEDF^{+/+} and PEDF^{minus/minus} mice at postnatal day 10 (P10), respectively. C and D are merged images following Hoechst 33258 staining. The quantitative assessment of the data is shown in E (** $P < 0.01$, $n=8$; * $P < 0.05$, $n=8$). Bar = 20 μm .

Fig. 9. Assessment of hyaloid vasculature in PEDF^{+/+} and PEDF^{minus/minus} mice. Collagen IV wholemount staining of hyaloid vasculature from PEDF^{+/+} (A, C, E) and PEDF^{minus/minus} mice (B, D, F) at P10 (A, B), P21 (C, D) and P42 (E, F). Please note similar degrees of vessel regression in PEDF^{+/+} and PEDF^{minus/minus} mice at all time points. These experiments were repeated with eyes from 5 different mice with similar results. A representative image is shown. Bar = 500 μm.

Fig. 10.

PEDF^{minus}/^{minus} mice are more sensitive to hyperoxia-mediated vessel obliteration during OIR. P7 PEDF^{+/+} and PEDF^{minus}/^{minus} mice were exposed to hyperoxia (75% oxygen) for 5 days. A and B show the wholemount collagen IV staining of retinal vasculature from P12 PEDF^{+/+} and PEDF^{minus}/^{minus} mice during OIR, respectively. The quantitative assessment of the non-perfused area is shown in C (*P < 0.05, *n*=12). Bar = 500 μ m.

Fig. 11. Preretinal neovascularization is not impacted by the lack of PEDF. A and B show the wholemount collagen IV staining of retinal vasculature from P17 PEDF^{+/+} and PEDF^{minus/-} mice during OIR, respectively. The number of neovascular cell nuclei projecting into the vitreous from the retina was determined from ocular serial sections as described in Methods and shown in C. The differences were not significant ($P > 0.05$, $n=16$). Bar = 500 μm .

Fig. 12.

Assessment of VEGF levels in PEDF^{+/+} and PEDF^{minus;/minus} mice. Eye extracts prepared from P15 PEDF^{+/+} and PEDF^{minus;/minus} mice (5 days of hyperoxia and 3 days of normoxia) were analyzed by SDS-PAGE and Western blotting as described in Methods. A representative blot is shown (A). Please note similar levels of VEGF in eye extracts from PEDF^{+/+} and PEDF^{minus;/minus} mice. β-actin was used for loading control. B shows VEGF levels in retina extracts prepared from PEDF^{+/+} and PEDF^{minus;/minus} mice at indicated postnatal days. The level of VEGF was determined using a mouse VEGF immunoassay as described in Methods. C shows VEGF levels in retinal extracts prepared from PEDF^{+/+} and PEDF^{minus;/minus} mice at indicated postnatal days during OIR; (P12, 5 days of hyperoxia; P15, 5 days of

hyperoxia and 3 days of normoxia; P21, 5 days of hyperoxia and 9 days of normoxia). The differences were not significant in room air and P15 OIR ($P > 0.05$; $n=5$), but significant differences were observed at P12 and P21 during OIR ($*P < 0.05$; $n=5$).

Fig. 13. Histological and functional analysis of retinas from PEDF^{+/+} and PEDF^{minus/minus} mice. A and B show the PAS/HE staining of retinas from P21 PEDF^{+/+} and PEDF^{minus/minus} mice, respectively. The ganglion cells, inner nuclear cell layer and photoreceptors of the PEDF^{+/+} eyes are histologically similar to that of PEDF^{minus/minus} retinas. C shows dark-adapted ERGs from P42 PEDF^{+/+} and PEDF^{minus/minus} mice. Traces show representative ERGs to a series of flash intensities from 0.5 cd-s m⁻² to 5.0 cd-s m⁻². Both PEDF^{+/+} and PEDF^{minus/minus} mice exhibited normal growth of the a-wave amplitude and decrease in implicit time with flash intensity. Normal amplitude oscillatory potentials on the ascending limb of the b-wave are evident in PEDF^{+/+} and PEDF^{minus/minus} mice. These experiments were repeated with eyes from 5 different mice with similar results. A representative image is shown. Bar = 50 μ m.

TABLE 1EC/PC ratios in PEDF^{+/+} and PEDF^{minus;/minus;} mice divided according to different age (Mean $\pm$ SD)^a

Age, days	PEDF ^{+/+}	PEDF ^{minus;/minus;}
21	2.35 $\pm$ 0.27 (6)	2.73 $\pm$ 0.42 (6) *
42	2.08 $\pm$ 0.27 (7)	2.10 $\pm$ 0.35 (7)

^aThe number of retinas (mice) counted is given in parentheses.

* P < 0.01

TABLE 2

Number of endothelial cells per reticle square ($100 \mu\text{m}^2$) in PEDF^{+/+} and PEDF^{minus;/minus;} mice divided according to age (Density; Mean $\pm$ SD)^a

Age, days	PEDF ^{+/+}	PEDF ^{minus;/minus;}
21	93.1 $\pm$ 7.57 (6)	112.1 $\pm$ 7.76 (6) *
42	89.7 $\pm$ 7.99 (7)	86.9 $\pm$ 8.52 (7)

^aThe number of retinas (mice) counted is given in parentheses.

* P < 0.01

TABLE 3

Number of pericytes cells per reticle square ($100 \mu\text{m}^2$) in PEDF^{+/+} and PEDF^{minus;/minus;} mice divided according to age (Density; Mean $\pm$ SD)^a

Age, days	PEDF ^{+/+}	PEDF ^{minus;/minus;}
21	39.5 $\pm$ 5.39 (6)	41.1 $\pm$ 10.2 (6)
42	43.5 $\pm$ 4.89 (7)	41.9 $\pm$ 5.61 (7)

^aThe number of retinas (mice) counted is given in parentheses.