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Vascular Endothelial Growth Factor Expression in the Retinal Pigment Epithelium Is Essential for Choriocapillaris Development and Visual Function

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The choroid in the eye provides vascular support for the retinal pigment epithelium (RPE) and the photoreceptors. Vascular endothelial growth factor (VEGF) derived from the RPE has been implicated in the physiological regulation of the choroidal vasculature, and overexpression of VEGF in this epithelium has been considered an important factor in the pathogenesis of choroidal neovascularization in age-related macular degeneration. Here, we demonstrate that RPE-derived VEGF is essential for choriocapillaris development. Conditional inactivation of VEGF expression in the RPE (in *VEGF^{rpe-/-}* mice) results in the absence of choriocapillaris, occurrence of microphthalmia, and the loss of visual function. Severe abnormalities of RPE cells are already observed when VEGF expression in the RPE is only reduced (in *VEGF^{rpe+/-}* mice), despite the formation of choroidal vessels at these VEGF levels. Finally, using *Hif1a^{rpe-/-}* mice we demonstrate that these roles of VEGF are not dependent on hypoxia-inducible factor-1 α -mediated transcriptional regulation of VEGF expression in the RPE. Thus, hypoxia-inducible factor-1 α -independent expression of VEGF is essential for choroid development. (Am J Pathol 2005, 167:1451–1459)

The choroidal vessels between the retina and the sclera of the eye originate from mesodermal tissue surrounding the newly formed optic cup early in development. The

choroidal endothelial cells are derived from the perocular mesenchyme, whereas choroidal melanocytes are derived from cranial neural crest cells.^{1,2} It is thought that the choroidal vessels are induced by the retinal pigment epithelium (RPE) during development. In mice in which the RPE is converted to neural retina through transgenic expression of FGF-9 in the RPE, choroidal vessels and melanocytes are absent.³ RPE cells are likely to maintain choroidal vessels also in the adult. For example, in the advanced form of dry age-related macular degeneration, localized RPE degeneration precedes atrophy of the underlying choroidal vessels.^{4–7} Furthermore, localized chemical or mechanical destruction of the RPE in animal models results in atrophy of choroidal vessels, loss of endothelial fenestrations, and increased deposition of collagen in the choroid.^{8–10} These observations are consistent with ultrastructural studies that provide evidence for a continuous remodeling of the choroidal vessels in the adult,^{9,10} with large vessels showing extensive fenestrations at the side facing the RPE. Thus, RPE cells have an essential role in the development and the maintenance of a fenestrated choroidal vasculature in the eye.

We speculate that vascular endothelial growth factor (VEGF), expressed by the RPE, might be a critical mediator of these RPE functions. This hypothesis is based on several observations. First, RPE cells stimulate tube formation of endothelial cells in co-cultures with choroidal endothelial cells, a process that was reduced by adding VEGF-neutralizing antibodies to the cell cultures.¹¹ Second, VEGF expression by the RPE occurs at the time of choroidal vessel formation (shown in humans), and persists throughout adulthood (shown in humans and mice).^{3,12,13} Third, cell culture studies demonstrate the presence of VEGF at the basal side of the RPE.^{14,15} This polarized localization is consistent with secretion of VEGF from the basal side of RPE cells. Fourth, VEGF induces

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endothelial fenestrations in several tissues,¹⁶ and the preferential localization of the choroidal endothelial fenestrations at the side facing the RPE suggests a role for the VEGF-expressing RPE cells in maintaining a fenestrated phenotype of choroidal vessels. To test the hypothesis that RPE-derived VEGF is critical for choroidal vascular development or postnatal function of the choroidal vessels, we used a conditional gene-targeting approach to generate mutant mice that specifically lack VEGF expression in the RPE.

Materials and Methods

Generation of Mutant Mice

A transgenic mouse line expressing Cre recombinase under the control of the tyrosinase-related protein (*Trp*)-1 promoter was used to generate site-specific somatic mutations in the RPE.¹⁷ All mice harboring the *Trp1*-Cre transgene were genotyped for the *rd1* (retinal degeneration 1) mutation that was present in the founder mouse of FVB/N background, and mice that did not carry the *rd1* mutation were used. In this mouse line, Cre was expressed in the RPE from embryonic day (E) 10.5 (time of RPE differentiation) to postnatal stages resulting in efficient excision of floxed DNA from E10.5 to adulthood.¹⁷ The presence of Cre expression in the RPE of this mouse line has been shown by *in situ* hybridization, which correlated with Cre-mediated excision of floxed DNA in polymerase chain reaction (PCR) experiments using isolated RPE cells from *Trp1*-Cre mice.¹⁷ *Trp1*-Cre mice (*Trp1-Cre^{tg/o}*) were interbred with *floxed-Vegfa* mice to generate homozygous mutant mice that lack the expression of VEGF-A (VEGF) in the RPE (*Trp1-Cre^{tg/o}/VEGF^{flox/flox}* mice, referred to as *VEGF^{rpe-/-}* mice).

The generation and genotyping of *floxed-Vegfa* mice have been described previously.¹⁸ *Trp1*-Cre mice were also interbred with *floxed-Hif1a* mice to generate homozygous mutant mice that lack the expression of hypoxia-inducible factor-1 α (HIF-1 α) in the RPE (*Trp1-Cre^{tg/o}/Hif1a^{flox/flox}* mice, referred to as *Hif1a^{rpe-/-}* mice). The generation and genotyping of *floxed-Hif1a* mice have been described previously.¹⁹ We confirmed that expression of Cre was maintained in the RPE of the mutant mice that we generated with immunostainings using a polyclonal anti-Cre antibody (Novagen, Madison, WI) (Figure 2C). In addition, the presence of Cre-mediated excision of floxed DNA in the eyes of mutant mice was confirmed by PCR analysis. Posterior eye-cups (after removal of retinal tissue) of mutant mice were used as a DNA source. PCR primers were designed to flank the loxP sites at which Cre-mediated excision of floxed DNA occurs (*VEGF^{flox/flox}* PCR primers: UP 5'-gtgtgggcctgtggtcctttc-3', DW 5'-ctggcctcacctgcattcacatct-3'; *Hif1a^{flox/flox}* PCR primers: UP 5'-ggaagtaagcacctggaagtagta-3', DW 5'-aaaaagtattgttggtggcagta-3'). To assess expression of VEGF at a single-cell level in embryonic and postnatal RPE cells, we used a mouse line in which *Vegf* expression is associated with *LacZ* expression, and performed sub-

sequent staining for β -galactosidase activity.²⁰ In all timed pregnancies, plug date was defined as E0.5. Genomic DNA, isolated using standard protocols from portions of the embryos, was used for genotyping.

Morphological Examination of Mouse Eyes

For immunohistochemical analysis whole eyes were fixed in 4% paraformaldehyde, followed by infiltration in 30% sucrose. Eyes were embedded in OCT (Tissue-Tek; EMS, Hatfield, PA) and 7- μ m cryostat sections were made. Sections were incubated with monoclonal rat anti-mouse CD31 (BD PharMingen, San Jose, CA) and rabbit polyclonal anti-mouse Cre antibodies (Novagen). Sections were incubated with biotinylated secondary IgG (Vector Laboratories, Burlingame, CA), and an ABC kit (Vector Laboratories) was used for detection. For histological analysis eyes from mutant and wild-type littermates were fixed in 2.5% glutaraldehyde and 1.25% paraformaldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 1 day. After postfixation in 4% osmium tetroxide, and dehydration steps, the eyes were embedded in TABB epon (Marrivac Ltd., Halifax, Canada). Semithin sections were used for methylene blue staining. Ultrathin sections were contrasted with uranyl acetate and lead citrate before evaluation in a 1200EX JEOL electron microscope. For macroscopic examination of choroid tissue, eyes were enucleated, the posterior eyeball was dissected, the retina was removed, and choroidal flat mounts were examined under a dissection microscope.

Staining for β -Galactosidase Activity

Staining for β -galactosidase activity was performed on embryos at E12.5 and postnatal day (P)1. Embryos were fixed in a fixative, containing 0.2% glutaraldehyde, 5 mmol/L EGTA, pH 7.3, 2 mmol/L MgCl₂, and 2% paraformaldehyde in 0.1 mol/L sodium phosphate. After washing in 0.1 mol/L sodium phosphate (pH 8), tissues were incubated in a solution containing 1 mg/ml X-Gal (Sigma, St. Louis, MO), 5 mmol/L K-ferrocyanide, and 5 mmol/L ferricyanide for 5 to 16 hours at 37°C. Tissues were then infiltrated with 30% sucrose and embedded in Tissue-Tek OCT. Sections were cut on a cryostat, mounted on Superfrost Plus slides, and dried at room temperature.

Electroretinogram (ERG) Analysis

Groups ($n = 5$) of *VEGF^{rpe-/-}*, *VEGF^{rpe+/-}*, and control mice were used for ERG measurements. Scotopic ERG analysis was performed as described.²¹ Mice were dark-adapted and anesthetized by using xylazine (20 mg/kg) and ketamine (80 mg/kg). Full-field ERGs were recorded from both eyes by using the electrophysiological system 2000 (UTAS E-2000; LKC Technologies, Gaithersburg, MD). ERGs were recorded in response to 10- μ s families of single flashes with a series of light intensities (the unattenuated flash is 2.48 photopic m⁻²cd·s) under scotopic conditions.

Rhodopsin Measurements

The method for measuring rhodopsin in mouse retinas has been described previously.^{21,22} Briefly, retinas were homogenized in 10 mmol/L Tris buffer (pH 7.5) containing 1 mmol/L ethylenediamine tetraacetic acid, 1 mmol/L 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, protease inhibitor cocktail, and 10 μ g DNase I. After centrifugation at $14,200 \times g$ for 15 minutes, pellets were solubilized in 1% dodecylmalto-side (sodium phosphate buffer, pH 7.4) at 4°C for 2 hours, centrifuged ($88,000 \times g$ for 10 minutes), and the supernatant analyzed in a spectrophotometer. Difference spectra were determined from measurements before and after bleaching with white light in the presence of freshly prepared 20 mmol/L hydroxylamine, pH 6.0 to 7.0. The concentrations of rhodopsin were calculated using the following extinction coefficient: $\epsilon(\text{rhodopsin}) = 40,000 \text{ mol/L}^{-1}\text{cm}^{-1}$.

Analysis of Ocular Vasculature with Fluorescein Isothiocyanate (FITC)-Coupled Dextran and Concanavalin A Lectin

The ocular vasculature in eyes of 3- to 12-week-old control, *VEGF^{rp+/-}*, and *VEGF^{rp-/-}* albino mice was analyzed using FITC-coupled dextran and concanavalin A lectin perfusion. After the induction of deep anesthesia, the chest cavity was opened, a needle was inserted into the right ventricle, and a perfusion cannula was inserted into the left ventricle. The mice were first perfused with phosphate-buffered saline (PBS) to wash out erythrocytes. Then fixation was achieved with 1% paraformaldehyde and 0.5% glutaraldehyde. The perfusion was continued with FITC-coupled concanavalin A lectin (40 μ g/ml in PBS) (Vector Laboratories). Lectin staining was followed by PBS perfusion to remove excess concanavalin A. The eyes of the mice were enucleated and lens and retina were removed. The remaining posterior eyeballs were flat-mounted in a water-based fluorescence anti-fading medium (Vectashield; Vector Laboratories). In a second set of experiments, mice were perfused with FITC-coupled high-molecular weight dextran (Sigma), before perfusion with 1% paraformaldehyde and 0.5% glutaraldehyde.

Fluorescein Angiography

Fluorescein angiography was performed on the Topcon Imagenet digital angiography system (TRC 50 VT camera and IMAGENet 1.53 system; Topcon, Paramus, NJ). The photographs were captured with a 20-D lens in contact with the fundus camera lens, after intraperitoneal injection of 0.1 ml of 1% fluorescein sodium (Akorn, Decatur, IL).

Figure 1. VEGF expression during ocular development. Sections of *VEGF-lacZ* mice were stained for β -galactosidase activity: **A**, E12.5; **B**, E12.5; **D**, P1; **E**, P1. CD31 immunostaining of endothelial cells in choroidal vessels: **C**, E12.5; **F**, P1. **Arrowheads** indicate RPE cells. **Arrows** indicate choroidal vessels. L, Lens. Original magnifications: $\times 10$ (**A**, **D**); $\times 40$ (**B**, **E**); $\times 60$ (**C**, **F**).

Results

VEGF Expression during Eye Development

Primitive periorbital vessels, first seen at E11.5, form the choroidal vascular network at E13.5 in mouse eyes. Choroidal melanocytes migrate between these vessels in the first 3 weeks postnatally.²³ We analyzed the expression of *Vegf* in embryonic and postnatal RPE cells using *Vegf-lacZ* mice. At the time of choroidal vessel formation, at E12.5, *Vegf* expression was prominent in the RPE (Figure 1, A and B). Expression was also detected in the lens and in the retinal ganglion cell layer (Figure 1, A and B). Immunostaining for blood vessels with anti-CD31 antibodies showed the presence of choroidal vessels at E12.5 (Figure 1C). After development of the choroid, *Vegf* continued to be expressed in the RPE and was also detected postnatally (Figure 1, D and E). At P1, *Vegf* expression continued to be expressed in the lens and the retinal ganglion cell layer as well (Figure 1, D and E).

Ablation of VEGF Expression in the RPE

To ablate VEGF expression in the RPE, *VEGF^{flox/flox}* mice were bred with *Trp1-Cre^{tg/o}* transgenic mice to generate *Trp1-Cre^{tg/o}/VEGF^{flox/flox}* mice (referred to as *VEGF^{rp-/-}*). *Trp1-Cre^{tg/o}/VEGF^{+/+}*, *Trp1-Cre^{tg/o}/VEGF^{flox/flox}*, and *Trp1-Cre^{tg/o}/VEGF^{flox/+}* littermates served as controls. None of the control mice displayed morphological eye abnormalities. *VEGF^{rp-/-}* mice showed expression of Cre in the RPE (Figure 2C). Excision of floxed DNA was confirmed by PCR analysis of posterior eyecups derived from *VEGF^{rp-/-}* mice (Figure 2C). Thus, VEGF is efficiently ablated in the RPE of *VEGF^{rp-/-}* mice. The ocular phenotype described here was observed in black, brown, and white mice.

Figure 2. $VEGF^{rpe-/-}$ mice display microphthalmia. **A:** Eye regions of 3-week-old $VEGF^{rpe-/-}$ mouse and control littermate. **B:** The enucleated eye of an 8-week-old $VEGF^{rpe-/-}$ mouse is much smaller in size than the eye of the control littermate. The posterior eyeball is lighter in appearance in the $VEGF^{rpe-/-}$ eye. **C:** Cre-mediated excision of floxed DNA occurs in $VEGF^{rpe-/-}$ eyes. PCR with DNA from posterior eyecup homogenates from $VEGF^{flox/flox}$ mice (WT) reveals a single band (~ 2000 bp), whereas Cre activity in the RPE of $VEGF^{rpe-/-}$ mice (KO) leads to an additional smaller PCR product (~ 550 bp). Immunostaining for Cre reveals positive staining (gray color of DAB/Ni) within the RPE cells (arrowhead; brown pigment is visible within the RPE cells). **D:** The optic nerve of an eye of an adult $VEGF^{rpe-/-}$ mouse is indicated with an arrowhead (left). No vitreous or anterior chamber are visible; the lens is in direct contact with the retina and iris. Arrowhead shows clustered melanocytes surrounding the optic nerve (right). Original magnifications: $\times 4$ (D, left); $\times 20$ (D, right).

Severe Microphthalmia in $VEGF^{rpe-/-}$ Mice

$VEGF^{rpe-/-}$ mice displayed severe microphthalmia with complete penetrance. All $VEGF^{rpe-/-}$ mice investigated had a significant reduction in the size of the eyeball ($n > 100$ $VEGF^{rpe-/-}$ mice), whereas all control littermates had normally sized eyes. The extent of microphthalmia showed some variability between the $VEGF^{rpe-/-}$ mice, but in all of these mice the microphthalmia phenotype was very striking and could easily be detected macroscopically (Figure 2A). After enucleation, eyes of $VEGF^{rpe-/-}$ mice appeared to be significantly smaller than control eyes (Figure 2B). In addition, an almost translucent appearance of the posterior eyeball was evident in $VEGF^{rpe-/-}$ mice (Figure 2B). Histological analysis of eyes in these mice revealed some melanocytes at the area of the optic nerve region (Figure 2D). The lens occupies the complete interior of the eyeball, and no vitreal space can be seen in $VEGF^{rpe-/-}$ mice (Figure 2D).

Absence of Choroid in $VEGF^{rpe-/-}$ Mice

Eyes of $VEGF^{rpe-/-}$ mice at E13.5, P1, P14, P21, P28, and P56 were examined by light microscopy and electron microscopy. Absence of choroid was observed at all stages examined, whereas normal choroid was evident in control littermates (Figure 3A). Almost no choroidal vasculature was observed in the eyes of $VEGF^{rpe-/-}$ mice (Figure 3B). Instead, underneath the RPE dense collagenous tissue resembling sclera could be seen. Absence of choroidal vessels was further investigated with immunostainings for endothelial cell markers (eg, CD31). Whereas strong immunostaining for CD31 was detected underneath the RPE in control littermates (Figure 3C),

almost no immunostaining underneath the RPE was observed in $VEGF^{rpe-/-}$ mice, confirming the absence of a significant choroidal vasculature in these mice (Figure 3D). The retinal vasculature did not appear to be affected in $VEGF^{rpe-/-}$ mice (Figure 3D). Perfusion of $VEGF^{rpe-/-}$ mice with FITC-coupled high-molecular weight dextran or concanavalin A lectin confirmed the absence of choroidal vasculature in most areas of the posterior eyeball (Figure 4G), and only a fine vascular network with thin vessels was seen in some areas of the posterior eyeball (Figure 4, H and I), lacking the typical choriocapillaris as seen in control mice (Figure 4; A to C). Furthermore, no choroidal melanocytes were present, giving the posterior eyeball a translucent appearance when compared with control eyes. Notably, clusters of melanocytes were seen in the area near the optic nerve (Figure 2D).

Morphological Abnormalities of RPE Cells and Bruch's Membrane in $VEGF^{rpe-/-}$ Mice

RPE cells in adult $VEGF^{rpe-/-}$ mice showed dystrophic changes with a reduction in the content of melanin granules. The granules that were present appeared irregularly shaped and melanin granules with the typical fusiform shape were almost lacking (Figure 5; B to E). The basal infoldings of the RPE were disorganized and reduced. Furthermore, the apical villi of the RPE did not interdigitate with photoreceptors regularly (Figure 5B). The thickness of the RPE was reduced as well (Figure 5; B to E). Ectopic RPE cells were occasionally observed in the photoreceptor outer segment layer. Notably, a single basal lamina layer was seen underlying the RPE in $VEGF^{rpe-/-}$ mice, adjacent to the dense collagenous scleral tissue (Figure 5F). Thus, only the innermost layer of the five-layered Bruch's membrane was present in these mice, suggesting that the formation of a proper Bruch's membrane depends on the presence of choroidal vessels (Figure 5F). Notably, RPE differentiation seemed to occur normally in $VEGF^{rpe-/-}$ embryos (Figure 3F).

Retinal Abnormalities in $VEGF^{rpe-/-}$ Mice

Photoreceptor outer segments did not display the regular alignment seen in control eyes, and were significantly shorter and disorganized. Focal areas of neural retina had only very few photoreceptor outer segments and some areas displayed even complete absence of outer segments (Figure 5B). Outer segments contained also irregular disk membranes (Figure 5E). Photoreceptor inner segments appeared disorganized as well (Figure 5B). There was a significant reduction in outer segment lengths in retinas of $VEGF^{rpe-/-}$ mice at all ages examined. Furthermore, photoreceptor nuclei were occasionally dislocated within the retina, some nuclei being localized right next to RPE cells (Figure 5, C and D). The morphological abnormalities of the RPE cells and the photoreceptors suggest an impaired visual function in $VEGF^{rpe-/-}$ mice. To assess photoreceptor function, scotopic ERG responses were recorded and rhodopsin content in eye homogenates was measured. Both a- and

Figure 3. Loss of choroid in $VEGF^{rpe-/-}$ mice. **A** and **B:** Methylene blue-stained section of an eye of a 4-week-old $VEGF^{rpe-/-}$ mouse (**B**) and the control (**A**). **B:** Loss of choroid can be observed in $VEGF^{rpe-/-}$ mice with absence of vessels and melanocytes underlying the RPE (arrowhead). **A:** Control littermate eye shows normal choroid (arrow) with choroidal vessels and melanocytes, and a continuous RPE cell layer (arrowhead). Photoreceptor outer segments (*) and inner segments are significantly shortened in $VEGF^{rpe-/-}$ mice (**B**), when compared to those in control mice (**A**). **C:** CD31 immunostaining in a 4-week-old control eye shows staining for retinal vessels (arrowhead) and choroidal vessels underlying the RPE (arrow). **D:** Lack of choroidal vessels (arrow) in a $VEGF^{rpe-/-}$ eye, with presence of retinal vessels (arrowhead). **E:** Vessels were already observed at E13.5 in control littermates, with an endothelial lining (arrowhead) and intraluminal erythrocytes (black arrow), which were lacking in $VEGF^{rpe-/-}$ mice at E13.5 (**F**). RPE cell morphology shows no significant difference at E13.5 (white arrow). R, Retina. Original magnifications: $\times 20$ (**A**, **B**); $\times 40$ (**E**, **F**).

b-wave amplitudes were significantly reduced in $VEGF^{rpe-/-}$ mice when compared to ERGs of control mice (Figure 6). Consistent with the abnormal ERGs, retinal rhodopsin content in $VEGF^{rpe-/-}$ eyes was also significantly reduced (9.45 ± 13.04 pmol/retina; mean $\pm$ SD) when compared to control littermates (486.6 ± 43.27 pmol/retina; mean $\pm$ SD) ($P < 0.005$) (Figure 6). These results indicate an abnormal retinal function in $VEGF^{rpe-/-}$ mice.

VEGF Expression in the RPE Is Not Dependent on HIF-1 α Expression during Ocular Development

A key transcriptional regulator of VEGF expression in many cells is hypoxia-inducible factor-1 α (HIF-1 α),²⁴ which is also expressed in RPE cells.^{25–27} VEGF expression can be induced by exposure to low oxygen tension,

Figure 4. Analysis of vasculature in flat mounts of control eyes (A–C), *VEGF^{rpe+/-}* eyes (D–F), and *VEGF^{rpe-/-}* eyes (G–I). **A** and **B**: A dense vascular network consisting mostly of capillaries (arrows) characterizes the choroid in control mice, supplied by choroidal arteries (arrowhead) (FITC-dextran). **C**: Concanavalin A lectin staining of the choroidal capillaries (arrow) and arteries (arrowhead) seen in flat mounts of control eyes. *VEGF^{rpe+/-}* eyes show areas with presence of choriocapillaries (**D**), while at some areas choriocapillaris was lacking and only thin vessels are seen at these areas (**E**, **F**) (FITC-dextran). **G**: Flat mount of a FITC-dextran-perfused *VEGF^{rpe-/-}* eye shows iris vessels (arrowhead) at the left. The area of the ora serrata is indicated (*), with the posterior eyeball being located at the right. Absence of choroidal vasculature is seen in most areas of the posterior eyeball in *VEGF^{rpe-/-}* mice, with the exception of only some small vessels (arrow). **H**: These vessels form a fine vascular network (arrow) that lacks the typical fenestrated vessels of the choriocapillaris (FITC-dextran). **I**: Concanavalin A lectin staining of the remaining vessels seen in flat mounts of *VEGF^{rpe-/-}* eyes. Original magnifications: $\times 20$ (**A**, **C**, **D**–**F**, **I**); $\times 10$ (**B**, **H**); $\times 2$ (**G**).

and HIF-1 α is a key mediator of hypoxia-induced VEGF expression.²⁴ To assess if VEGF expression in the RPE is dependent on HIF-1 α , we generated mutant mice that specifically lack HIF-1 α expression in the RPE (*Hif1 α ^{rpe-/-}* mice) and compared the eyes of these mice with *VEGF^{rpe-/-}* eyes. Eyes of *Hif1 α ^{rpe-/-}* mice appeared normal in size and shape, and histological examination showed normal choroid tissue with choroidal vasculature and melanocytes (Figure 7B). Electron microscopic examination of the choroidal vasculature in *Hif1 α ^{rpe-/-}* mice revealed the presence of endothelial fenestrations (Figure 7C), as seen in control mice. Thus, *Hif1 α ^{rpe-/-}* mice do not have the abnormal choroidal phenotype seen in *VEGF^{rpe-/-}* mice. This demonstrates that HIF-1 α is not a critical transcriptional regulator of VEGF expression in the RPE during ocular development.

VEGF^{rpe+/-} Mice Show RPE Defects Despite the Presence of Choroidal Vessels

VEGF^{rpe+/-} mice show no significant microphthalmia. However, a focal loss of choroidal pigmentation could still be observed in the eyes of these mice (Figure 8, A and B). In areas at which choroidal vessels and melanocytes were present, RPE cells showed abnormalities similarly

as seen in *VEGF^{rpe-/-}* eyes (Figure 8C). In such areas, RPE cells next to choroidal vessels were flattened and contained abnormal pigment granules that were reduced in number (Figure 8D). Apical interdigitations of RPE cells with photoreceptors were also reduced. Fluorescein angiography demonstrates the focal loss of RPE cell pigmentation in *VEGF^{rpe+/-}* eyes as distinct areas of hyperfluorescence, due to the presence of fluorescein in the underlying choroidal vasculature (Figure 8, E and F). The presence of choroidal vessels in albino *VEGF^{rpe+/-}* mice was further shown in choroidal flat mounts of FITC-dextran perfused eyes. Notably, focal loss of choriocapillaris was observed in eyes of *VEGF^{rpe+/-}* mice, which showed only some thin vessels at these areas (Figure 4; D to F).

Discussion

Our data show that deficiency of VEGF expression in the RPE results in the absence of choroid. This lack of choroid tissue in *VEGF^{rpe-/-}* mice was also reflected in the absence of a proper Bruch's membrane, and only a single basal lamina under the RPE was seen adjacent to scleral collagen fibers. Thus, VEGF is essential for choroid development.

Figure 5. RPE and retinal defects in $VEGF^{rpe-/-}$ mice. Electron microscopic images of an eye of a 4-week-old $VEGF^{rpe-/-}$ mouse and the control mouse. **A:** Control eye with normal RPE, photoreceptor outer segments (arrow), and choroid. **Arrowhead** points to Bruch's membrane. **B:** RPE in the $VEGF^{rpe-/-}$ eye is flattened, with loss of interdigitations between apical villi and photoreceptor outer segments (arrow), irregular basal infoldings, and reduced pigmentation with abnormal pigment granules. **Arrowhead** points to Bruch's membrane. **C and D:** Ectopic localization of photoreceptor nuclei (arrows) next to RPE cells (arrowheads) in $VEGF^{rpe-/-}$ mice. **E:** Photoreceptor outer segments in a 4-week-old $VEGF^{rpe-/-}$ mouse eye with cystic defects and abnormal disks. **F:** Absence of Bruch's membrane in the $VEGF^{rpe-/-}$ eye. Collagen fibrils (arrow) are directly adjacent to the basal lamina (arrowhead) of the RPE (inset). Scale bars: 2.5 μ m (A, B); 3.5 μ m (D); 1.5 μ m (E); 0.5 μ m (F). Original magnification, $\times 40$ (C).

Although vascular development is a very complex process that is believed to involve a large number of growth factors (such as fibroblast growth factors, platelet-derived growth factors, VEGFs, angiopoietins, and so forth), we show here that VEGF is necessary for choroid devel-

Figure 6. Analysis of visual function in $VEGF^{rpe-/-}$, $VEGF^{rpe+/-}$, and control mice. Reduced ERG amplitudes are observed in $VEGF^{rpe+/-}$ mice when compared to control mice. ERGs demonstrate an almost absent retinal function in $VEGF^{rpe-/-}$ mice. Consistent with the ERG data, retinal rhodopsin levels are reduced in eyes of $VEGF^{rpe+/-}$ mice, whereas rhodopsin is almost lacking in eyes of $VEGF^{rpe-/-}$ mice. Mean $\pm$ SD, $n = 5$.

Figure 7. Morphological analysis of $Hif1a^{rpe-/-}$ eyes. **A:** Control eye. **B:** Choroidal vessels and melanocytes are present in $Hif1a^{rpe-/-}$ eyes. Methylene blue staining of a thin section. **C:** Electron microscopic image of a $Hif1a^{rpe-/-}$ eye shows normal choroidal endothelial fenestrations (arrowhead). **D:** Cre-mediated excision of floxed DNA occurs in $Hif1a^{rpe-/-}$ eyes. PCR with DNA from posterior eyecup homogenates from $Hif1a^{lox/flox}$ mice (WT) reveals a single band (~ 1200 bp), while Cre activity in the RPE of $Hif1a^{rpe-/-}$ mice (KO) leads to an additional smaller PCR product (~ 700 bp). Scale bar, 0.25 μ m (C). Original magnifications, $\times 40$ (A, B).

opment, and that its loss cannot be compensated for by the expression of other angiogenic growth factors. Such a critical role of a single growth factor for choroid development has not been demonstrated for any other growth factor involved in angiogenic processes. For example, lack of the angiogenic FGF-2 in $Fgf2^{-/-}$ mice had no apparent effect on choroid development.^{28,29} However, it is possible that in addition to VEGF other growth factors

Figure 8. $VEGF^{rpe+/-}$ mice show RPE cell defects. **A:** No microphthalmia is seen in $VEGF^{rpe+/-}$ mice, but focal loss of pigmentation (arrow) of the posterior eyeball can be observed. **Arrow** indicates the optic nerve. **B:** This loss of pigmentation reflects localized absence of choroid tissue (arrowhead), while most areas of eyes of $VEGF^{rpe+/-}$ mice have choroidal melanocytes and vessels (arrow). **C:** Flattening of RPE cells with loss of pigment granules (arrows) are seen next to choroid tissue (arrowhead) in $VEGF^{rpe+/-}$ mice. **D:** Arrowhead indicates a choroidal vessel, underlying flattened RPE cells with loss of pigment granules (arrow). **E:** Fluorescein angiography in $VEGF^{rpe+/-}$ mice demonstrates focal loss of RPE cell pigmentation as distinct areas of hyperfluorescence (arrows), due to the presence of fluorescein in the underlying choroidal vasculature. R, Retina; S, sclera. Original magnifications: $\times 20$ (B); $\times 40$ (C); $\times 100$ (D).

influence choroidal vessel formation and maturation. A possible role for fibroblast growth factors in choroidal vascular maturation has been suggested based on the observation of immature choroidal vessels in mutant mice overexpressing a dominant-negative FGFR-1 in the RPE.^{23,30}

In addition to the absence of choroid, a dramatic microphthalmia was observed in *VEGF^{rpe-/-}* mice. This finding defines a potential molecular mechanism for microphthalmia seen after selective RPE cell ablation during ocular development, such as in transgenic mice expressing the diphtheria toxin-A gene under the *Trp1*-gene promoter.³¹ The observed microphthalmia in *VEGF^{rpe-/-}* mice is most likely a consequence of the absence of choroidal vessels and indicates an important role of the choroidal vascular network for the growth of the eye.

The Cre strain used, *Trp1*-Cre, expresses Cre recombinase in the RPE starting at E10.5, the stage of RPE differentiation in the mouse. Thus, excision of the *Vegfa* gene in *VEGF^{rpe-/-}* mice results in RPE-specific deficiency of VEGF expression from the onset of RPE differentiation. Notably, *Trp1*-Cre mice do not express Cre recombinase in the choroidal melanocytes and vessels, or periocular mesenchyme. Therefore, the observed absence of choroid can be attributed to the lack of VEGF expression in the RPE. It needs to be pointed out that in *Trp1*-Cre mice, Cre recombinase expression was detected also in cells of the ciliary pigment epithelium and of the ciliary margin of the retina at E10.5, in addition to its expression in the RPE.¹⁷ The ciliary margin of the retina has been reported to contain retinal progenitor cells (in fish and amphibians).^{32,33} Thus, it cannot be entirely excluded that the retinal abnormalities observed in *VEGF^{rpe-/-}* mice may, at least in part, be a consequence of the lack of VEGF expression in some cells in the neural retina.

In development, VEGF expression can be induced through various stimuli. For example, in many cells hypoxia leads to stabilization of the transcriptional regulator HIF-1 α and induced expression of VEGF. In turn, this stimulates angiogenesis. However, VEGF expression can also be induced independently of hypoxia-mediated HIF-1 α stabilization, for example by glucose deprivation. In fact, *Hif1a^{-/-}* embryos have increased VEGF expression compared to wild-type embryos.³⁴ To elucidate if hypoxia is the main stimulus for VEGF expression in the RPE preceding the development of the choroidal vascular network, we ablated HIF-1 α expression in the RPE. Our data show that VEGF expression in the RPE during development can occur independently of HIF-1 α , because *Hif1a^{rpe-/-}* eyes are not microphthalmic and develop a choroidal vasculature in contrast to *VEGF^{rpe-/-}* mice. In conclusion, RPE-derived VEGF is a key factor in choroid development, and its expression is not dependent on HIF-1 α .

VEGF may induce the formation of the choroidal vasculature through a direct mitogenic effect on endothelial precursor cells in the periocular mesenchyme. In addition to its mitogenic effects on endothelial cells,³⁵ VEGF is also a potent inducer of vascular tube formation.¹¹ The lack of these vessel-inducing properties of VEGF is con-

sistent with a diminished development of the choroidal vasculature. The absence of choroidal melanocytes, which are derived from cranial neural crest cells and which migrate into the developed choroidal vascular network postnatally, could be the result of the lack of a direct promigratory effect of VEGF on these melanocytes. Alternatively, the absence of choroidal melanocytes could be a result of the deficiency of a promigratory factor that is secreted by choroidal endothelial cells and thus be a secondary consequence of the lack of the development of the choroidal vasculature.

It is possible that the functional and morphological defects observed in the RPE and the photoreceptors of *VEGF^{rpe-/-}* mice may in part be secondary consequences of the lack of choroidal vasculature and therefore impaired nutritional support. However, there is also the possibility that VEGF itself has a role in maintaining RPE function. VEGF receptors on RPE cells have been hypothesized to mediate such an autocrine effect of VEGF on RPE cells.³⁶ The observation of abnormal RPE cells in *VEGF^{rpe+/-}* mice even adjacent to choroidal vessels, suggests an autocrine role for VEGF in maintaining RPE function. However, it cannot be excluded that the morphological abnormalities of the choroidal vessels that were observed in *VEGF^{rpe+/-}* mice may contribute to the defects in the RPE and photoreceptor cells in these mice. Although our findings demonstrate a critical role for VEGF in eye development, the role of VEGF in the aging eye remains to be determined.

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