

An essential role for RPE-derived soluble VEGF in the maintenance of the choriocapillaris

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Clinical and experimental observations indicate a role for VEGF secreted by the retinal pigment epithelium (RPE) in the maintenance of the choriocapillaris (CC). VEGF in mice is produced as three isoforms, VEGF120, VEGF164, and VEGF188, that differ in their ability to bind heparan sulfate proteoglycan. RPE normally produces the more soluble isoforms, VEGF120 and VEGF164, but virtually no VEGF188, reflecting the fact that molecules secreted by the RPE must diffuse across Bruch's membrane (BrM) to reach the choriocapillaris. To determine the role of RPE-derived soluble VEGF on the choriocapillaris survival, we used mice that produce only VEGF188. VEGF188/188 mice exhibited normal choriocapillaris development. However, beginning at 7 months of age, we observed a progressive degeneration characterized by choriocapillaris atrophy, RPE and BrM abnormalities, culminating in areas of RPE loss and dramatic choroidal remodeling. Increased photoreceptor apoptosis in aged VEGF188/188 mice led to a decline in visual acuity as detected by electroretinogram (ERG). These changes are reminiscent of geographic atrophy (GA) and point to a role for RPE-derived VEGF in the maintenance of the choriocapillaris.

age-related macular degeneration | Bruch's membrane | geographic atrophy | retinal pigmented epithelium

The vascular endothelial growth factor (VEGF) family is comprised of a number of closely related ligands, including VEGF-A, B, C, D, E, and placental growth factor (PlGF) (1). VEGF-A (hereafter referred to as VEGF) is a critical angiogenic factor associated with several ocular pathologies, including wet age-related macular degeneration (AMD) and proliferative diabetic retinopathy. In AMD, increased VEGF expression leads to proliferation of pathological and highly permeable vessels into the subretinal space, resulting in severe vision loss. The clear involvement of VEGF in proliferative AMD has led to the development of multiple anti-VEGF drugs, with two—Macugen (OSI/Eyetech Pharmaceuticals) and Lucentis (Genentech)—approved by the Food and Drug Administration for the treatment of choroidal neovascularization.

VEGF binds with high affinity to the tyrosine kinase receptors, VEGFR1 and VEGFR2; VEGFR2 appears to be the major signal transducer of VEGF in noncirculating cells, whereas VEGFR1 is considered as a natural decoy for VEGF by regulating its ability to bind VEGFR2. In contrast to VEGFR1, VEGF binding to VEGFR2 leads to a robust autophosphorylation and the induction of various signaling cascades including, MAPK, PLC γ , Akt, and Src, critical for endothelial cell proliferation, migration, and survival (2). VEGFR2 activity is not only regulated by the availability of VEGF but also by the presence of the coreceptors neuropilins (NP-1). Binding of VEGF to NP-1 mediates formation of complexes between it and VEGFR2, potentiating the effects of VEGF (3).

Although the function of VEGF during development and pathology is well established, its role in the adult is only beginning to be appreciated. Analysis of VEGF expression in adult has yielded some insight into its postdevelopmental role. The use of *VEGF-LacZ* mice revealed that VEGF is expressed in virtually every tissue (4). The highest level of VEGF expression was localized to the

epithelia adjacent to the fenestrated vessels including the choriocapillaris (CC), kidney, and choroid plexus.

Studies in rodents have begun to elucidate the function of VEGF in quiescent tissues. Inhibition of VEGF or its receptors has been shown to lead to vessel regression in a number of tissues. In the kidney, VEGF neutralization resulted in glomerular endotheliosis and proteinuria (5–7). Vessel regression was also noted in the pancreas (8, 9), trachea, thyroid, and small intestine (8). Recently, we have reported that systemic inhibition of VEGF using adenoviral expression of sFlt-1 leads to nonperfusion of vessels in the choroid plexus (10). The CC of the eye has been shown to be similarly vulnerable to VEGF inhibition. Decreased fenestrations and vessel occlusion were observed after intravitreal injection of anti-VEGF (bevacizumab) in primate eyes (11).

In addition, there is growing acknowledgment of a role for VEGF on nonvascular cells (12), including a neurotrophic role in the brain (13). Systemic VEGF neutralization in mice led to a breakdown of the ventricular barrier function, and a direct role for VEGF as a survival factor for ependymal cells was indicated by their expression of VEGFR2 (10). We recently demonstrated a paracrine relationship between Müller cell-derived VEGF and VEGFR2 expressing photoreceptors in the murine retina (14). The physiologic importance of this relationship was evidenced by a significant loss of photoreceptors following systemic VEGF inhibition (11).

In mice, VEGF is produced as three isoforms (VEGF120, VEGF164, and VEGF188) by alternative splicing of a single gene (15). The isoforms differ in their inclusion of exons that encode for charged domains that mediate binding to heparan sulfate or to NP-1 which, in turn, determines the extracellular localization of VEGF. Upon secretion, VEGF120, which lacks heparan sulfate binding, is freely diffusible, whereas VEGF188, which contains two heparan sulfate binding sites, is sequestered on the cell surface or in the extracellular matrix, and VEGF164, with one heparan sulfate binding site, has intermediate distribution. Evidence that individual VEGF isoforms serve specific functions was provided by mice engineered to express single VEGF isoforms (16). Consistent with the high levels of VEGF188 in the lung (17), mice expressing only VEGF120 displayed defective pulmonary development (16, 18).

AMD is a pathology of the retinal pigment epithelium (RPE)-Bruch's membrane (BrM)-CC complex. In the dry AMD, accumulation of extracellular material or drusen under the RPE and within the BrM leads to RPE dysfunction, and in some cases, the loss of RPE, regression of the underlying CC, and death of the overlying photoreceptors, a pathology known as geographic atrophy (GA). In about 10% of cases, breaks in BrM lead to invasion of the CC into the retinal space. The association between RPE and CC loss in GA points to a role for the RPE

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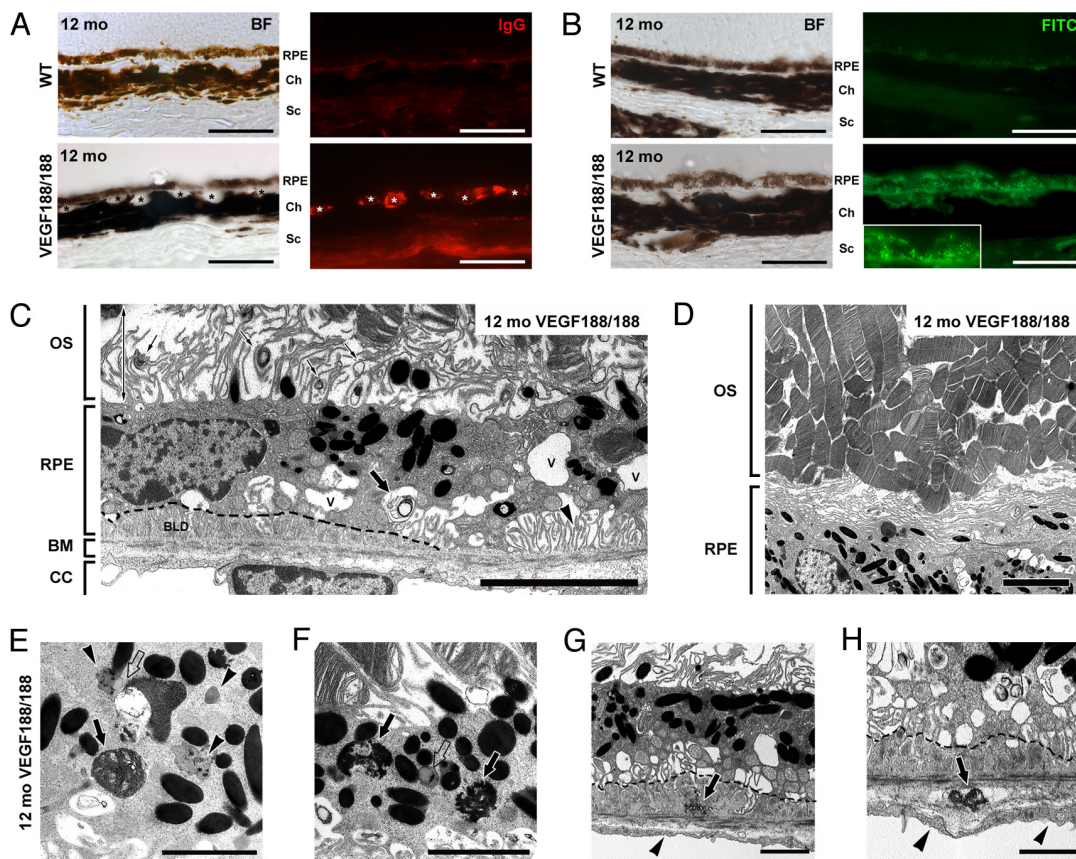


Fig. 2. Early AMD features in aged VEGF188/188 mice. (A) IgG staining (red) of the choroids of 12-month-old mice reveal the deposition of IgG in the walls of the choroidal vessel of the transgenic mice (asterisks) but not in aged-matched controls. (B) Increased lipofuscin autofluorescence was detected with a FITC filter. Multiple bright dots and clusters were evident in the RPE layer of the VEGF188/188 mice, especially in the clumped cells (see insert). (C–H) Representative electron micrographs showing RPE and BrM anomalies in 12-month-old VEGF188/188 mice. (C) Unhealthy RPE cells with reduced and disorganized basal infoldings (arrowhead) displaying large vacuoles (V) and membranous debris (large arrow). Formation of continuous subRPE deposits is outlined by a dotted line. Note the loss of intimate association between the RPE and the OS. The OS were separated from the RPE by a large space (double-headed arrow) containing disorganized RPE apical digitations and lamellar bodies (small arrows). (D) Lower magnification showing highly disorganized OS. (E and F) Higher magnification showing lipofuscin granules (arrowheads), abnormal phagosomes containing melanin (arrows in panel E), and undigested photoreceptor outer-segments in VEGF188/188 RPE (arrow in panel F). Lipid droplets were also observed in the lipofuscin bodies (open arrows). (G and H) Basal laminal deposits were associated with dense and membranous debris in the subRPE space or in the outer layers of the BrM (arrows in panels G and H, respectively). Note the loss of fenestrations and thickening of the endothelium membrane in the CC underlying the subretinal lesions. OS, outer segment; BrM, Bruch's membrane; CC, choriocapillaris; BLD, basal laminal deposits. [Scale bar, 50 μ m (A and B); scale bar, 5 μ m (C and D); scale bar, 2 μ m (E–H).]

Aged VEGF188/188 Mice Display Increased Photoreceptor Apoptosis and Altered Visual Function. To determine if the focal disruptions in RPE-retina interactions were associated with cell death, retinal sections were stained for apoptotic cells (Fig. 3A). Clusters of TUNEL-positive cells were detected in the ONL of retinas of 14-month-old VEGF188/188 mice but not in the aged-matched wt mice. Apoptotic cells in the GCL were frequently observed in the same location as the dying photoreceptors (Fig. 3A). Clustering of apoptotic photoreceptors has been reported in advanced GA where expanding areas of RPE loss leads to photoreceptor death at the edge of the atrophic zone (24). Visual function in VEGF188/188 mice was assessed using electroretinogram (ERG) recordings, which revealed a mild but significant reduction of both a- and b-wave amplitude (Fig. 3B). Reduction of the a-wave amplitude is indicative of a loss of photoreceptor function and is consistent with our previous observations. The reduction of the b-wave is a sign of an altered photoreceptor postsynaptic cascade that would accompany the increased photoreceptor cell death. A similar decrease in the amplitude of the foveal ERG response has been reported in patients with AMD (25).

Altered RPE Barrier Function in VEGF188/188 Mice. RPE barrier function is determined primarily by tight junctions, which limit

diffusion from the extracellular space through the RPE monolayer and regulate transepithelial flow (26). RPE-blood barrier is also controlled by adherens junctions, which mediate intercellular adhesion and preservation of the epithelial morphology (26). RPE junctional integrity in aged VEGF188/188 mice was evaluated by co-immunostaining RPE flat-mounts for the adherens junction protein β -catenin and F-actin. β -catenin, which is normally membrane-associated and colocalizes with F-actin, showed an aberrant distribution at the site of RPE lesions in 14-month-old VEGF188/188 eyes (Fig. 4A). Western blot analysis demonstrated a modest but significant reduction in the level of β -catenin expression in RPE lysates from VEGF188/188 mice (Fig. 4B). Since β -catenin localization was altered only at the sites of RPE lesions, the moderate change at the protein level suggests a more drastic loss at the site of RPE damage.

ZO1 and occludin are membrane-associated proteins whose association account for a majority of the blood retinal barrier (27). Abnormal ZO1 localization was also observed at the site of RPE lesions in aged VEGF188/188 mice (Fig. 5C). However, no changes in total ZO1 and occludin protein levels were detected (Fig. S6). Immunoprecipitation of occludin followed by Western blotting for ZO1 revealed a 45% reduction in association in VEGF188/188

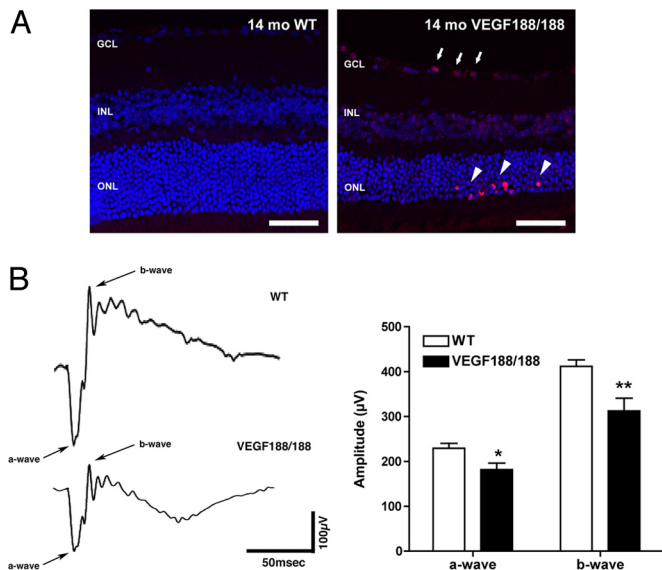


Fig. 3. Increased photoreceptor apoptosis and abnormal ERGs in VEGF188/188 mice. (A) TUNEL staining revealed a markedly increased apoptosis in the ONL of 14-month-old VEGF188/188 mice. Apoptotic photoreceptors appeared as clusters in the outermost part of the ONL layer (arrowhead) and were often associated with increased cell death in the GCL (arrows). (B) Scotopic ERG recordings of 14-month-old mice using a flash intensity of +10 dB revealed a marked reduction of both a- and b-wave amplitudes in VEGF188/188 mice ($n = 14$ for wt and $n = 12$ for VEGF188/188). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. (Scale bars, 50 μm .)

RPE (Fig. 4D). Local RPE atrophy observed in 14-month-old VEGF188/188 mice (Fig. 5A and Fig. S7) expanded over time to form large, discrete areas of RPE loss that were associated with the involution of the underlying CC (Fig. 4E).

Choroidal Atrophy in Aged VEGF188/188 Animals. Choroidal atrophy secondary to RPE degeneration is a key feature of GA (28). FITC-lectin cardiac perfusion followed by flat-mount preparation of the posterior eyecup was used to visualize the choroidal vasculature. Compared to the dense vascular plexus of the wt choroid, the CC of 18-month-old VEGF188/188 mice was severely atrophied, with widened intercapillary spaces and reduced branching both in the central and peripheral retina (Fig. 5A). Choroidal atrophy was reflected by a significant increase in the avascular area of VEGF188/188 choroids (Fig. 5B). A reduction in the ratio of phosphorylated VEGFR2 to total VEGFR2 in the RPE-choroid complex of VEGF188/188 mice demonstrated that the absence of soluble VEGF resulted in a reduced availability of VEGF for the CC and led to age-dependent choroidal involution (Fig. 5C).

Discussion

VEGF isoform mRNA levels vary among tissues (17), with the pattern of isoform expression reflecting the association between the source of the VEGF and its target cells. For instance, in the lung where VEGF188 is the predominant isoform (17), VEGF is produced by type 2 pneumocytes, which are separated from the alveolar capillary endothelial cell by a very thin basement membrane, an arrangement that maximizes oxygen diffusion. On the other hand, in the choroid plexus of the brain, the epithelium produces primarily VEGF120, which supports not only the underlying fenestrated microvasculature, but diffuses into the cerebral spinal fluid to act on the VEGFR2-expressing ependymal cell ventricular lining (10). The fact that RPE express only the soluble VEGF isoforms, VEGF120 and VEGF164, is consistent with the physical barrier posed by the presence of BrM

elastic lamina between the VEGF-producing RPE cells and the target CC.

Here, we demonstrate that the absence of soluble VEGF isoforms in mice leads to age-dependent, degenerative changes in the RPE-choroid complex that recapitulate the classical features of dry AMD such as signs of RPE dysfunction, including increased autofluorescence, accumulation of drusen-like basal deposits, and loss of barrier properties. These changes preceded the development of both focal choroidal atrophy and RPE attenuation that evolved into larger regions of RPE loss, a progression similar to the natural course of GA (28). The loss of RPE and CC led to photoreceptor death and reduced visual function, again recapitulating the course of human GA.

In embryos and young adult VEGF188/188 mice up to 4 months of age, the RPE-choroid complex appeared normal, an observation that was initially surprising since it had been previously shown that RPE-specific deletion of VEGF leads to defective choroidal development (29). However, understanding the time frame of BrM development provides an explanation. In mice, BrM is not fully formed until around 6 weeks of age, so that the development of the choroidal vasculature, which is initiated as early as E10.5 (19), would be unaffected by the reduced VEGF bioavailability in the VEGF188/188 mice. However, once BrM is in place, the diffusion of VEGF188 to the CC would be significantly decreased, explaining the RPE-choroid complex in aged VEGF188/188 mice.

A thickening of BrM and an alteration in its permeability are part of the normal human aging (30, 31). The accumulation of lipids in BrM observed in aging eyes, and particularly in patients with AMD, is suspected to limit the diffusion of water-soluble proteins like VEGF (32) and has led to the concept that a reduction in the diffusion of RPE-derived factors such as VEGF could be a causal in the development of GA (20, 33). Consistent with this notion, COX2-deficient mice, which exhibit progressive choroidal degeneration, have been reported to display reduced VEGF expression (34). Inhibition of VEGF signaling has been shown to be associated with rapid loss of fenestration and EC apoptosis in a variety of tissues (6, 8, 10, 35). Our observation of CC atrophy in mice expressing only VEGF188 is consistent with our hypothesis that the reduced ability of VEGF188 to diffuse through the BrM would impair CC survival. The lower level of VEGFR2 phosphorylation in the choroid of VEGF188/188 mice is strong evidence of the limited availability of VEGF188 to the choroidal capillary EC.

Our characterization of the ocular phenotype of VEGF188/188 mice, has not definitively identified the site of primary degeneration. Histological analyses revealed anomalies in both the RPE and the CC at the earliest time studied. We have recently shown that whereas embryonic RPE do not express VEGFR2, the receptor is expressed by adult, differentiated RPE (19). The expression of both VEGF and VEGFR2 by adult RPE suggests the existence of an autocrine function for VEGF in vivo, an observation previously shown for RPE in vitro (36). Thus, a direct effect of the absence of soluble VEGF isoforms on the RPE themselves cannot be excluded, although this seems unlikely, since there is no reason to suspect that VEGF188 produced by RPE would not have access to VEGFR2 on RPE. Due to technical difficulties encountered in the production of recombinant VEGF188, few studies looked at VEGF188 specific signaling and functions. Human VEGF189 stimulates endothelial proliferation, migration, and VEGFR2 up-regulation in vitro to levels comparable to VEGF165 (37). In addition, our preliminary data suggest that VEGF188 is binds NRP-1, as would be expected based on the presence of NRP binding domains (Fig. S8).

Our data strongly support the hypothesis that RPE-derived VEGF is necessary for the maintenance of RPE-complex integrity and suggest that reduced VEGF signaling, secondary to RPE dysfunction and/or BrM thickening, may contribute to the patho-

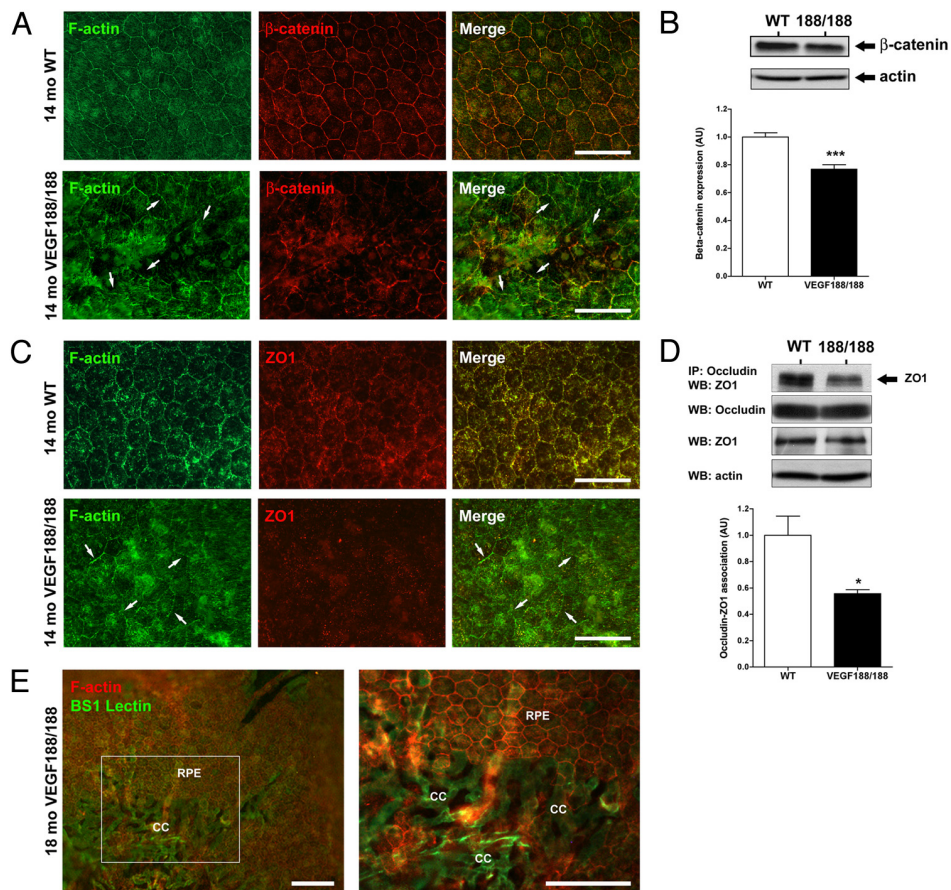


Fig. 4. RPE dystrophy is associated with barrier function loss in VEGF188/188 mice. (A) Flat-mount preparation of 14-month-old wt mice and VEGF188/188 showed abnormal distribution of the junctional proteins in RPE layer of transgenic mice. In wt, RPE formed a regular honeycomb pattern. In VEGF188/188, the RPE organization is disturbed and atrophic revealing the choroidal vessel underneath (CC). In the RPE lesions, the gap-junction protein β -catenin has lost its characteristic membranous association (arrows). (B) Abnormal β -catenin distribution was accompanied by a reduction of protein expression showed by Western blot on 16-month-old choroid-RPE samples ($n = 6$). (C) Aberrant ZO1 localization was also observed in the VEGF188/188 mice. (D) No changes in ZO1 protein level were detected. Immunoprecipitation for occludin followed by ZO1 Western blotting revealed a 45% reduction in association between ZO1 and occludin ($n = 3$). (E) Over time, the limited RPE lesions observed at 14 months (see panels A and C) expanded significantly leading to a major loss in the integrity of the RPE layer of 18-month-old VEGF188/188 mice. [Scale bar, 50 μm (A and C); scale bar, 100 μm (E).]

genesis of GA. Anti-VEGF is currently the first line therapy for wet macular degeneration, and it is being increasingly used in the treatment of a number of other ocular pathologies characterized by edema and/or neovascularization, including diabetic macular edema, proliferative diabetic retinopathy, retinal vein occlusion, and the retinopathy of prematurity. In light of the postulated essential role for RPE-derived VEGF in the maintenance of CC, the chronic use of anti-VEGF therapies in the eye should be closely monitored. However, the relatively short intraocular half-life of

both Avastin and Lucentis indicate that VEGF neutralization is neither total nor chronic and thus may achieve neutralization of the VEGF involved in pathologic vessel growth without influencing endogenous levels.

Methods

Materials. C57BL/6J (Jackson Laboratories) and C57BL/6 VEGF188/188 (38) were used in this study. All animal protocols were approved by the Schepens Eye Research IACUC. Embryos were generated by breeding VEGF188/+ mice. Littermates VEGF188/188 and wt were used for our study.

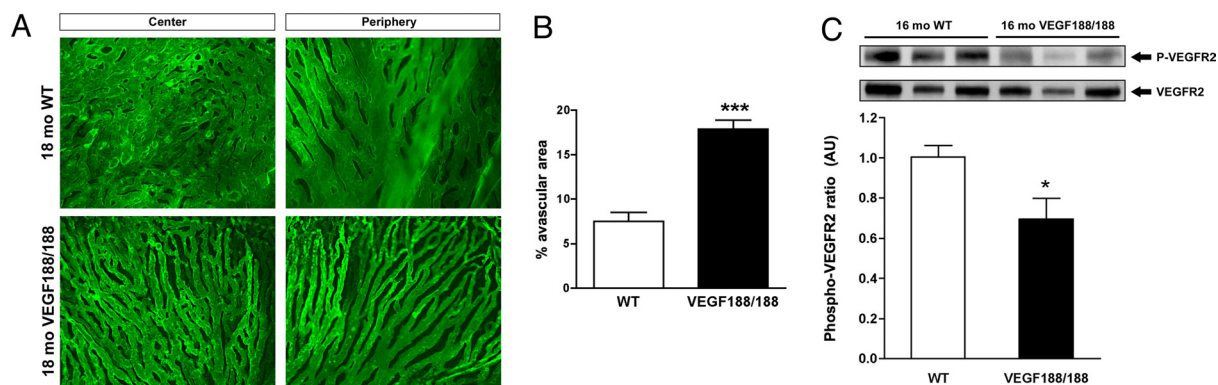


Fig. 5. CC atrophy is associated with decreased VEGFR2 phosphorylation in aged VEGF188/188 mice. (A) Choroidal vasculature of 18-month-old wt and VEGF188/188 mice was immunostained by cardiac perfusion of FITC-labeled *Bandeiraea simplicifolia* (BS-1) isolectin. In wt animals, flat-mounted choroids revealed a typical dense honeycomb vascular network. However, the choroidal vessels of VEGF188/188 animals appeared poorly branched and atrophied. The reduced vascular density was observed in both the central and the peripheral choroidal vasculature. (Scale bar, 200 μm .) (B) Quantification of the avascular area in the CC 15- to 16-month-old VEGF188/188 mice ($n = 5$) compared to aged-matched wt ($n = 5$) ($P < 0.0001$). (C) Level of VEGFR2 phosphorylation in choroid-RPE protein lysates of 16-month-old mice was quantified by Western blotting using [pY1214]-VEGFR2 specific antibody. The level of phosphorylated VEGFR2 was normalized for total VEGFR2. VEGF188/188 RPE-choroid complex displayed a significant reduction of the phosphorylation level of VEGFR2 compared to wt. (Scale bar, 100 μm .)

Immunohistochemistry, Histology, and Electron Microscopy. Whole embryos or enucleated eyes were processed and stained using previously published protocols (14) as described in the *SI Materials and Methods*.

Characterization of RPE and Choroid. Mice were perfusion via the aorta with 10 mL 4% paraformaldehyde in PBS followed by 5 mL FITC-conjugated *Griffonia simplicifolia* isolectin B4 (Vector Laboratories) and washed by 10 mL PBS to remove nonspecific binding. Flat-mounted posterior segment of the eye were immunostained using a rabbit anti mouse-ZO1 (Zymed), rabbit anti-mouse β -catenin (Upstate) antibodies, or FITC-labeled phalloidin (Molecular Probe). For analysis of the choroidal vessels, the RPE layer was removed by gentle brushing. To quantify the avascular area, six photographs were taken in the mid-periphery region of each flat-mounted choroid (representing a total area of analysis of 2.03 mm²) and analyzed using ImageJ software. Each photograph was converted to grayscale, then based on pixel brightness values ranging from 0 to 255, blood vessels stained with the isolectin were distinguished from background by using empirically determined threshold values that included only blood vessels. The avascular area was calculated as the area represented by pixels having a brightness value lower than the corresponding threshold and expressed as a percentage of the total area. The mean value was calculated for each mouse.

Western Blot Analysis and Immunoprecipitation. The choroid-RPE tissue from wt and VEGF188/188 mice were separated from the retina and homogenized in lysis

buffer (Cell Signaling). Each sample represented one mouse. Protein concentration was quantified using a BCA assay (Bio-Rad Laboratories). For Western blot analysis, identical amount of protein was separated by SDS-PAGE under reducing condition and transferred to Immobilon-P membrane (Millipore). Membranes were incubated overnight at 4 °C with β -catenin (1: 500; Upstate) or actin (1:1,000; Santa Cruz Biotechnology) antibodies. Quantification of the level of VEGFR2 phosphorylation in the RPE-choroid lysates was conducted as previously published (19). Details can be found in the *SI Materials and Methods*.

ERG Recordings. The ERGs of VEGF188/188 and wt mice ERG were assessed using a UTAS-E3000 recording system (LKC Technologies) ($n = 4$) using previously published protocols (14) as described in the *SI Materials and Methods*.

Additional methods are described in the *SI Materials and Methods*.

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