*Molecular Vision* 2001; 7:277-82 <http://www.molvis.org/molvis/v7/a39>
Received 4 September 2001 | Accepted 28 November 2001 | Published 2 December 2001



# Regulation of choroid development by the retinal pigment epithelium

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**Purpose:** The choroidal vasculature is essential for normal retinal function. However, mechanisms that control choroid development are unknown. In the present study, we provide evidence that the retinal pigment epithelium (RPE) plays an essential role in regulation of the choroid development in the mouse eye.
**Methods:** Transgenic mice that transiently express FGF9 in the embryonic RPE were generated. Postnatal eyes were analyzed by histology and in situ hybridizations.
**Results:** In the transgenic mice, most of the RPE was converted to neural retina. The choroid formed only in regions where patches of RPE were present. The choroid failed to develop in the absence of the RPE.
**Conclusions:** The presence of the RPE appears to be required for choroid development, suggesting that molecular interactions between RPE and periocular mesenchyme are essential for melanocyte differentiation and vascular development in the choroid.

The choroid is a layer of highly vascularized tissue surrounding the eye. Choroidal blood nourishes the retinal pigment epithelium (RPE) and the photoreceptors at the outer layer of the retina. The choroid develops from two embryonic tissues, the mesoderm and cranial neural crest cells. The endothelial cells of choroidal blood vessels are of mesodermal origin while all other cells including stromal cells, melanocytes, and pericytes are derived from neural crest cells [1-3]. During early eye development, tubes and spaces form in the periocular region next to the optic vesicle. These tubes are lined by endothelium of mesodermal origin. As the eye grows, they expand from the central axis to the caudal end of the optic vesicle and form a plexus. When the optic vesicle invaginate to form the optic cup and pigmentation appears in the presumptive RPE, primitive capillaries develop from the plexus adjacent to the pigmented RPE. Subsequently, the ophthalmic artery and the posterior ciliary arteries enter the choriocapillary layer while large capillaries fuse to form the veins. In the human embryo, pigmentation of choroidal melanocytes occurs in late gestation and is complete at birth [2].

The periocular mesenchyme remains close contact with the RPE during eye development, suggesting that interactions between these tissues may be important for normal ocular development. We have previously reported that transient expression of transgenic FGF9 in embryonic RPE can switch its differentiation to a neuronal pathway, resulting in a duplicate neural retina in transgenic mice [4,5]. We report here that the choroid fails to develop in these mice, indicating that RPE provides inductive signals for choroid development. In the mouse, pigmentation of choroidal melanocytes begins soon after birth and is complete by two weeks of age.

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## METHODS

We previously described transgenic mice that express FGF9 in the presumptive RPE under the control of a tyrosinase-related protein 2 (TRP2) promoter [4]. In these mice, the embryonic RPE is induced to differentiate into neural retina. In the present study, we further examined whether the consequent lack of an RPE affected development of periocular tissues in these mice. Since FVB/N mice from which the transgenic mice were originally derived carry an autosomal recessive mutation causing retinal degeneration [6,7], these mice were mated with wild type pigmented inbred C57BL/6 mice or albino outbred ICR mice for further analysis. Postnatal day 1 (P1) and day 7 (P7) mouse eyes were collected and fixed in 10% formalin overnight at room temperature and then rinsed in 70% ethanol for 24 h. The eyes were next dehydrated using ethanol solutions of increasing concentration, cleared in xylene, and embedded in paraffin wax. Sections (5 μm) were cut on a microtome, dewaxed with xylene, rehydrated using decreasing concentrations of ethanol, and then used for hematoxylin/eosin staining and for in situ hybridizations. Animals were handled following the guidelines provided in US Public Health Service Policy on Humane Care and Use of Laboratory Animals.

To examine the possible factors that are likely to be involved in development of choroidal vasculature, in situ hybridizations were carried out with $^{35}$S-labeled antisense RNA probes specific to mouse vascular endothelial growth factor (VEGF) and its receptors Flt-1 and Flk-1 as previously described [4]. After washing, RNase treatment, and dehydration, the slides were coated with Kodak NTB-2 emulsion for autoradiography. The slides were developed, counter-stained with hematoxylin, and then mounted with a cover slip for examination of silver grain distribution under bright-field or dark-field illumination. Both the bright- and dark-field images were collected by computer through a CCD camera and subse-

quently superimposed onto each other using Adobe Photoshop software. Silver grains in the dark-field images were pseudocolored red to improve contrast in the superimposed images.

## RESULTS

In the wild type eyes, pigmented cells appeared in the developing choroid at P7 (Figure 1A,C). Small blood vessels and blood cells were present in the developing choroid adjacent to the RPE (Figure 1C). In P7 eyes from the TRP2-FGF9 transgenic mice, the RPE was converted to a second layer of neural retina (Figure 1B). Both the original and the RPE-derived neural retinae differentiated, laminated, and expressed retina-specific markers such as rhodopsin [4,5]. The majority of the presumptive RPE was converted to neural retina except for a patch in the posterior region of the eye (Figure 1B). The failure of this RPE patch to convert to neural retina was presumably due to low levels of transgene expression in this region. Pigmented choroid tissue was found only in the region where the RPE was still present (Figure 1B,D), indicating that RPE is required for choroid development. Small blood vessels and blood cells were observed in this region (Figure 1D). In regions where the RPE had been converted to neural retina, no such vascular structures were present (Figure 1E). Occasionally, thin monolayers of melanocytes were found in regions where the RPE had been converted to the neural retina (red arrow in Figure 1B). However, these thin cell layers do not resemble the developing choroid. They possibly migrated there from adjacent regions.

To examine what factors might be involved in development of the choroidal vasculature, the expression pattern of VEGF in wild type eyes was analyzed by in situ hybridization. At P1, VEGF mRNA was expressed predominantly in

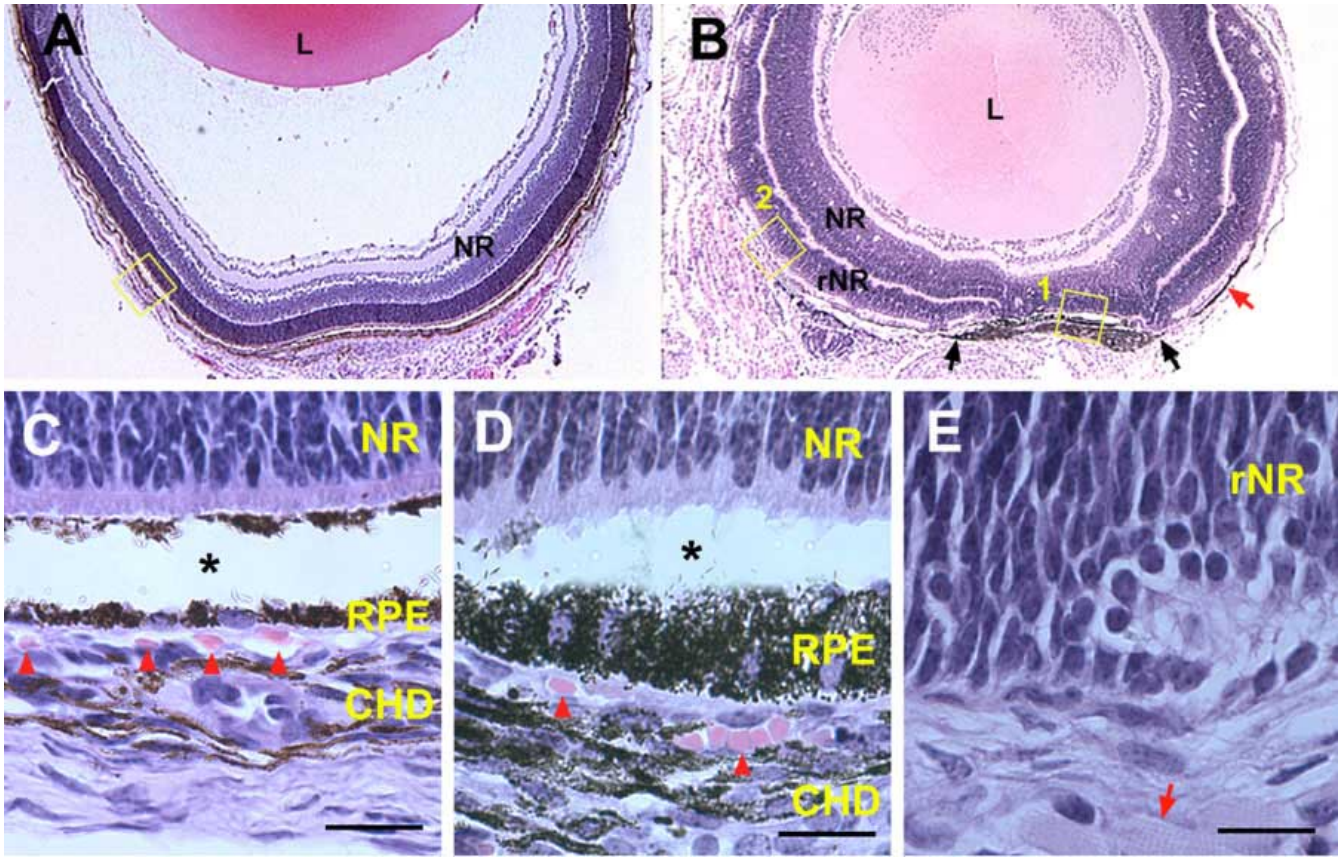


Figure 1. Failure in choroid formation in the absence of the RPE. Hematoxylin-eosin staining of P7 eye sections from wild type (**A**, **C**) and TRP2-FGF9 transgenic (**B**, **D**, **E**) mice. In wild type mice, the neural retina (NR) had differentiated and formed three distinct layers (**A**). In the TRP2-FGF9 transgenic eye (**B**), the vitreous did not form and the majority of the presumptive RPE was converted to a second layer of neural retina except for a patch of RPE in the posterior region (between the two black arrows in **B**). The developing choroid adjacent to this region had become pigmented. Thin monolayers of melanocytes were occasionally found in regions where the RPE was absent (red arrow in **B**). However, no tissues resembling developing choroid were observed in these regions. The boxed region in **A** is shown in **C** while boxes 1 and 2 in **B** are shown in **D** and **E**, respectively. In wild type P7 eyes (**C**), melanocytes already appeared in the developing choroid and small blood vessels and blood cells were present (arrowheads in **C**). In the transgenic eyes, melanocytes were present in the developing choroid next to the unconverted RPE patch (**B**, **D**). Blood vessels were seen in this choroidal tissue (arrowheads in **D**). In the region where the RPE was absent (box 2 in **B**), no blood vessels were observed in the presumptive choroid tissue adjacent to the RPE-derived neural retina (rNR) (**E**). The arrow in **E** indicates a striated muscle cell. Detachment of neural retina from RPE (asterisks in **C**, **D**) was due to artifacts in tissue processing. Abbreviations: CHD, developing choroid; L, lens. Scale bars: 20 μm.

the RPE and also to some extent in the differentiated cell layer of the neural retina (the ganglion cell layer; Figure 2A,B). At P7, VEGF mRNA remained expressed in the RPE and was also present at high levels in the inner nuclear layer and in astrocytes along the vitreous surface of the neural retina (Figure 2C,D). M¸ller glial cells in the inner nuclear layer and retinal astrocytes at the vitreous surface are known to express high levels of VEGF in postnatal rodent eyes [8].

In situ hybridizations were also carried out to examine expression patterns of the VEGF receptors Flt-1 (VEGFR1) and Flk-1 (VEGFR2) in wild type mouse eyes. At P1, Flt-1 mRNA was expressed in the endothelial cells of hyaloid blood vessels (Figure 3A) and in periocular mesenchyme including a layer of cells adjacent to the RPE (Figure 3A,E). Flk-1 mRNA exhibited a similar expression pattern except that it was also expressed weakly in the proliferating retinoblast layer (RBL; Figure 3B,F). At P7, Flt-1 mRNA was expressed in the vascular endothelial cells at the vitreous surface of the neural retina and in the developing choroidal tissue near the RPE (Figure 3C). It was also detected at low levels in the inner nuclear layer (Figure 3C). In P7 eyes, Flk-1 mRNA was expressed in the ganglion cell layer, the inner nuclear layer, and in the periocular mesenchymal cells adjacent to the RPE (Figure 3D). Expression of Flk-1 mRNA in the developing neural retina has been previously reported [9]. The cells adjacent to the RPE that expressed Flt-1 and Flk-1 (Figure 3E,F) were likely to be endothelial cells of the developing choroidal vasculature.

## DISCUSSION

Our previous study showed that the TRP2 promoter activated transgene expression in the developing RPE by embryonic day 9.5 (E9.5) [4]. Our more recent study demonstrates that transgene expression directed by the TRP2 promoter was turned off by E10 as soon as RPE differentiation had been switched to the neuronal pathway [5]. That study also showed that endogenous FGF9 is normally expressed in the developing neural retina and has no apparent effect on RPE differentiation. This might be due to the high affinity of FGFs for extracellular matrix, particularly heparan sulfate proteoglycans, which can severely limit their diffusion in interstitial spaces. As a result, FGFs often have a very limited range of action [5,10]. Therefore, the transient expression of FGF9 in the presumptive RPE between E9-E10 was not very likely to have a significant impact on choroid development in postnatal mice.

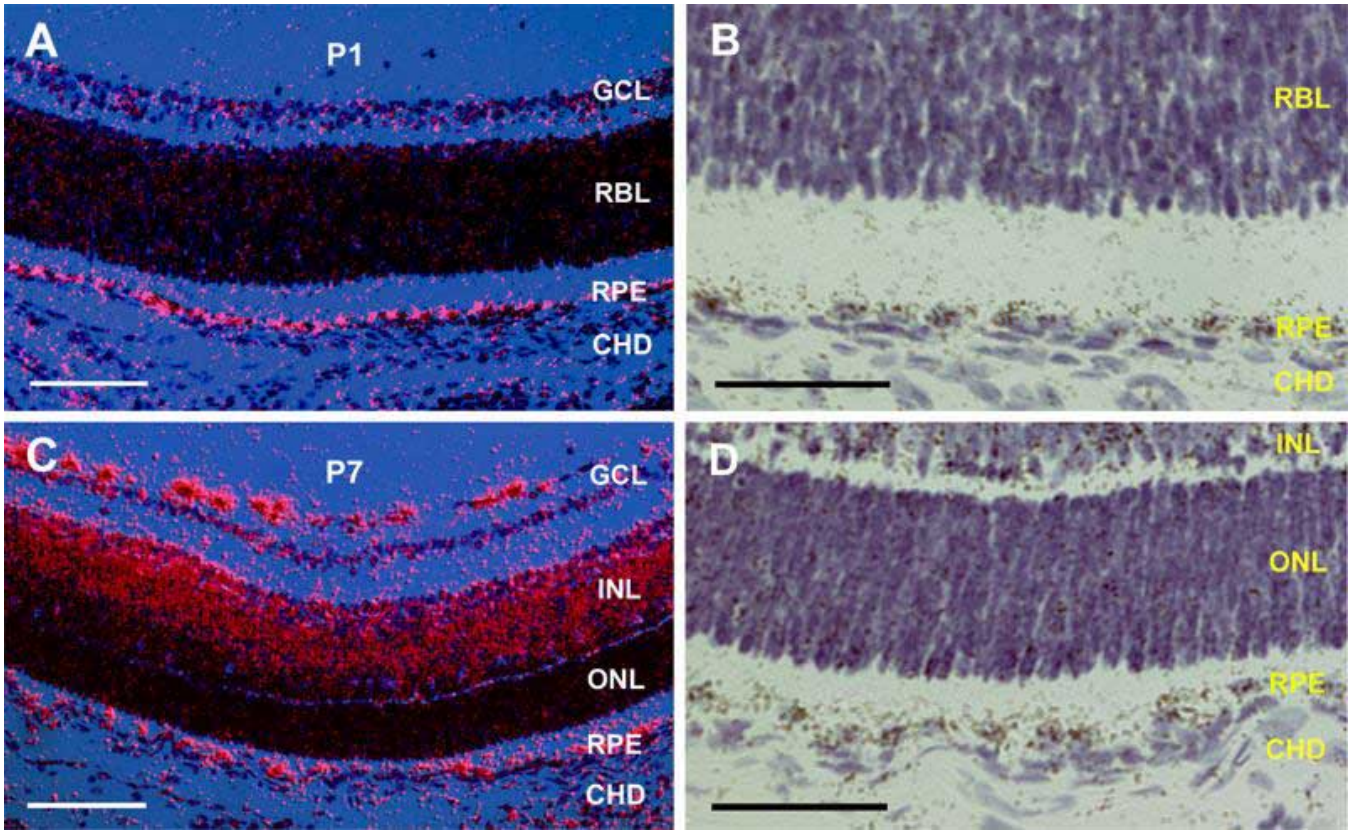


Figure 2. VEGF expression in the developing retina. Expression of VEGF mRNA was assayed by in situ hybridization using a $^{35}$S-labeled probe. At P1, levels of VEGF transcripts were highest in the RPE. Low levels of VEGF transcripts were seen in the ganglion cell layer and in the periocular mesenchyme (**A**). A bright-field image of the P1 eye section at higher magnification shows that RPE cells were densely labeled with silver grains (**B**). At P7, VEGF mRNA remained expressed in the RPE (**C**). In addition, high levels of VEGF transcripts were detected in astrocytes at the vitreous surface and in the inner nuclear layer of the neural retina (**C**). The bright-field image of the P7 eye shows heavy labeling of silver grains in RPE cells and cells in the inner nuclear layer (**D**). Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RBL, retinoblast layer. Scale bars: 100 µm (**A**, **C**) and 50 µm (**B**, **D**).

It has been observed that in human patients with colobomas, failure in RPE differentiation leads to defective choroid and sclera [2], supporting the notion that the defective RPE but not a factor such as FGF9 causes the absence of the choroid. Early in vitro studies using chick embryos also suggest a role of the RPE in regulating differentiation of periocular mesenchyme [11,12]. These studies demonstrate that RPE induced formation of cartilage, a component of the sclera. Mutations in the microphthalmia transcription factor (Mitf) cause the colobomas phenotype in mice. Recent studies show that dorsal RPE in these mice differentiate as neural retina [13,14]. Whether there were defects in periocular mesenchy-

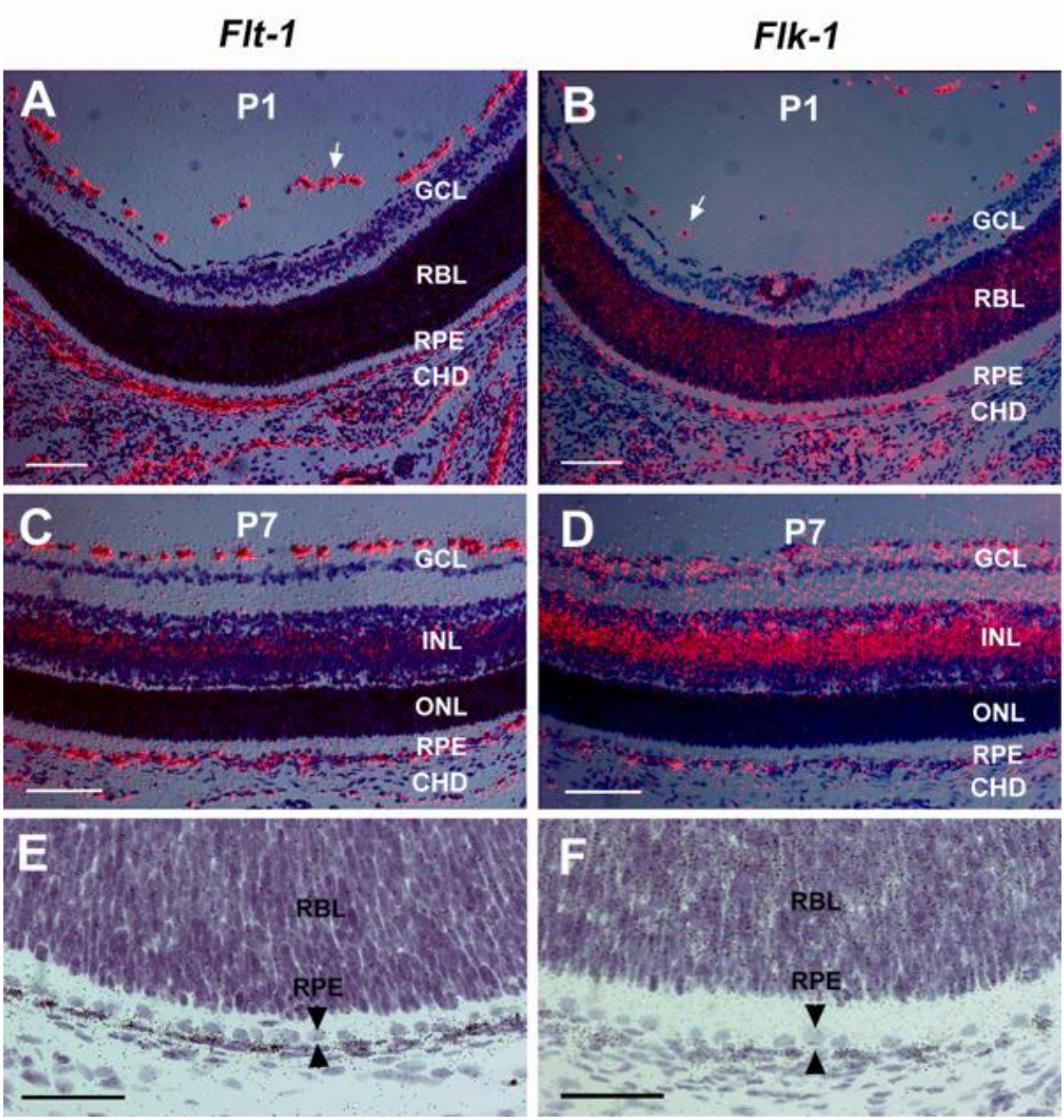


Figure 3. Expression of VEGF receptors in the developing eye. Messenger RNA transcripts of Flt-1 (**A**, **C**, **E**) and Flk-1 (**B**, **D**, **F**) were assayed by in situ hybridizations using $^{35}$S-labeled probes. At P1 (**A**, **B**), Flt-1 and Flk-1 mRNAs were expressed in the endothelial cells of hyaloid blood vessels (arrows) and in the periocular mesenchyme. Flk-1 mRNA was also expressed in the retinoblast layer (**B**). At P7 (**C**, **D**), Flt-1 and Flk-1 mRNAs were expressed in the vascular endothelial cells at the vitreous surface and in the periocular mesenchyme. The inner nuclear layer of the neural retina expressed low levels of Flt-1 mRNA (**C**) but high levels of Flk-1 mRNA (**D**). Bright-field images of P1 eye sections at higher magnification show heavy labeling of silver grains in a subset of the mesenchymal cells adjacent to the RPE expressing Flt-1 (**E**) and Flk-1 transcripts (**F**). The RPE layer is marked by a pair of arrowheads (**E**, **F**). Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RBL, retinoblast layer. Scale bars: 100 μm (**A**, **B**, **C**, **D**) and 50 μm (**E**, **F**).

mal tissues was not reported in these studies. Given the close association between the choroid and the sclera, it would not be surprising that the sclera was also defective in TRP2-FGF9 transgenic and in Mitf mutant mice although this has yet to confirmed by further studies.

We have shown that one of the signals released by RPE is VEGF. In developing rodents, VEGF expression in the RPE increases from embryonic to postnatal stages, peaks approximately a week after birth (data not shown), and then decreases with age but persists throughout adulthood [8,15]. VEGF is also present in adult human RPE [16]. In postnatal mouse eyes, VEGF receptors Flt-1 and Flk-1 were found expressed in a subset of periocular mesenchymal cells adjacent to the RPE (Figure 3). These gene expression patterns suggest that VEGF made by RPE could play a critical role in vascular development in the choroid. Elevated levels of VEGF expression by RPE have been implicated as a cause of choroidal neovascularization in human [17-21] and in animal models [22-30].

Vascular development is a complex process and requires functional interactions of many different factors (such as VEGF, PDGF, TGF-beta, angiopoietins, etc). It is unlikely that VEGF is the only factor produced by the RPE or other tissues in the vicinity that controls choroid development. For example, since there is no evidence that melanocyte differentiation requires VEGF stimulation, migration and organization of pigmented cells in the developing choroid are likely to be regulated by other as yet unidentified factors.

## ACKNOWLEDGEMENTS

This work was supported by NIH grant EY10448 (PAO), the Knights Templar Foundation and Fight For Sight, Inc. (SZ).

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The print version of this article was created on 11 December 2001. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.