Role of Cell and Matrix-Bound VEGF Isoforms in Lens Development

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Abstract

PURPOSE.—To determine the role of vascular endothelial growth factor (VEGF) in embryonic eye development and lens differentiation.

METHODS.—Expression of components of the VEGF signaling pathway during lens development and in adults was characterized by β-galactosidase staining of VEGF-LacZ mice, immunohistochemistry, and real-time (q) PCR. Embryonic eyes from wild-type mice and VEGF120/120 mice were analyzed by light microscopy and immunohistochemistry. VEGF function during lens development was analyzed using eye explants treated with VEGF-neutralizing antibody. Direct function of VEGF was demonstrated on the human lens epithelial cell line, HLE-B3.

RESULTS.—Embryonic lens epithelium and posterior lens fibers expressed VEGF and VEGFR2. qPCR revealed VEGF164 as the major isoform in embryonic lens. Transgenic mice expressing only VEGF120 (VEGF120/120 mice) showed major defects in eye development, including microphthalmia, failed lens differentiation, and hyperplastic hyaloid vessels. The lens displayed abnormal cell patterning and differentiation associated with altered c-Maf, Prox1, and p57 expression pattern in the anterior epithelium. The number of proliferating epithelial cells was drastically reduced in VEGF120/120 lenses. Altered MIP26 cellular localization and reduced E-cadherin expression in the lens epithelium were observed. VEGF-neutralization led to reduced fiber elongation of eye explants. Exogenous VEGF increased survival and proliferation of HLE-B3 cell in a dose-dependent manner.

CONCLUSIONS.—Abnormalities in ocular development in VEGF120/120 mice suggest a role for VEGF not only in the formation of ocular vascular beds but also in the differentiation of the lens itself.

Vertebrate eye development begins with the formation of the optic vesicle when the inner layer of the cup gives rise to the sensory retina and the outer layer forms the pigment epithelium. The lens is derived from the surface ectoderm, with the formation of the lens

placode at embryonic (E) day 9. The lens invaginates with the optic cup to form the lens vesicle, a hollow sphere of lens epithelial cells. The separation of the lens vesicle from the surface of the optic cup is accompanied by the differentiation of the anterior lens epithelium and the posterior primary fibers. Differentiation of the lens fibers from the epithelium is a complex process, involving withdrawal from cell cycle and elongation. This process obliterates the lens cavity and is marked by the expression of lens-specific proteins such as the crystallins. The final phase of lens fiber differentiation includes the loss of the nucleus and all intracellular organelles, an event essential for the lens transparency.[1]

Early eye development is associated with the formation of a capillary network, the pupillary membrane on the anterior lens and the tunica vasculosa lentis on the posterior lens, which provides nutrients to the intraocular components. This dense vasculature undergoes nearly complete regression in the latest stages of ocular development as the retinal vasculature matures.[2]

Significant progress has been made in understanding the signaling molecules that mediate early lens formation.[1,3] A number of transcription factors, including Pax6, Six3, Prox1, and Sox2 have been shown to be involved. Though roles for the bone morphogenic proteins and FGF pathways have been identified, the upstream regulators of these transcription factors have not been fully defined.

Vascular endothelial growth factor (VEGF) is critical for early vascular development, and heterozygous deletion of the VEGF gene leads to lethality at E9.5.[4,5] However, inducible and/or tissue-specific VEGF deletions have revealed central roles for VEGF in the vascularization of most tissues and organs, including the lung[6] and the kidney.[7] More recently, VEGF has been shown to be required for the maintenance of the adult vasculature, with virtually all adult tissues expressing VEGF in an organ-specific fashion[8] and systemic neutralization leading to loss of fenestrations and significant vessel regression.[9,10]

VEGF is produced as multiple isoforms by alternative splicing (reviewed in Ref. 11). There are three main isoforms in mouse that differ in their binding to heparin, which determines their extracellular localization: VEGF120 lacks heparan sulfate proteoglycan (HSPG) binding and is freely diffusible, VEGF188 binds strongly to HSPG and remains cell- and matrix-associated, and VEGF164 has intermediate properties. In addition, the isoforms differ in their binding to neuropilin (Nrp)-1 and Nrp-2, which have been shown to serve as co-receptors for VEGF164 (and possibly VEGF188) and to potentiate VEGFR2 signaling.[12] Differential expression of VEGF isoforms during development and in the adult indicates that the isoforms serve distinct roles.[13,14] Conclusive evidence was provided by mice engineered to express single VEGF isoforms. Mice expressing only VEGF120 develop to term (at a reduced ratio) and die perinatally due to defects in cardiac and pulmonary angiogenesis,[15] whereas mice expressing only VEGF188 develop to term and are viable and fertile, but display a variety of non-lethal vascular defects, including abnormalities in cartilage development[16] as well as retinal vascularization.[17]

The major VEGF signaling receptor, VEGFR2, was initially reported to be restricted to the vascular endothelium and thus VEGF was thought to be an endothelial-specific mitogen.[18]

However, there is increasing evidence for the expression of VEGFR2 by nonvascular cells, including dendritic cells,[19] ganglion cells,[20] pulmonary epithelium,[21] and podocytes.[22] Furthermore, VEGF has been shown to act on a variety of neural cells, stimulating neurogenesis as well as acting as a neuroprotective factor (reviewed in Ref. 23). In the embryonic retina, VEGFR2 has also been shown to be expressed by retinal progenitor cells, [24,25] and recent studies have suggested that VEGF may regulate both the proliferation and differentiation of the retinal progenitors.[26,27]

We and others have previously described the expression of VEGF during lens development, [24,28–30] where it was assumed to mediate the growth of the tunica vasculosa lentis and the pupillary membrane. However, motivated by our observation of VEGFR2 expression by cells of the early lens vesicle (E10.5), we examined the possibility that VEGF might be involved in lens development. Analysis of VEGF isoform distribution revealed that the developing lens produces predominantly VEGF164. We, therefore, used mice that express only VEGF120 to investigate the impact of the absence of HSPG-binding isoforms on lens development. VEGF120/120 mice displayed abnormal eye formation with significant defects in the growth and differentiation of the lens, indicating a role for VEGF in lens development.

Methods

Animals

Timed-pregnant C57B1/6 VEGF-LacZ (provided by Andras Nagy, Samuel Lunenfeld Research Institute, Ontario, Canada)[31] and C57B1/6 VEGF120/120[15] mice were used in this study. The VEGF-LacZ mice contain a LacZ reporter gene with a nuclear localization inserted in the *3′* untranslated region of the *vegf* gene.[31]

Homozygous VEGF120/120 embryos were generated by intercrossing of heterozygous VEGF120/+ mice and genotyped by PCR on tail biopsy DNA. Wild-type littermates were used as controls for the VEGF120/120 embryos. All animal experiments were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research under protocols approved by the Schepens Eye Research Institute ACUC.

Histology and Immunohistochemistry

VEGF expression was visualized in embryos from VEGF-LacZ mice by staining wholemounted E10.5 and E13.5 embryos for LacZ using the in situ β-galactosidase staining kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. As a negative control, whole embryos and cryosections from wild-type (WT) type mice were stained for LacZ and showed no positive staining in the eye (data not shown).

Embryos and lens explants were fixed overnight in 10% buffered formalin or 4% paraformaldehyde. The heads were placed in embedding molds, insuring proper orientation. For paraffin and cryosections, serial transverse sections covering the entire optic cup were cut and sections containing the largest area of the lens were selected for histology and immunochemistry. For histology, paraffin sections were stained with hematoxylin and periodic acid Schiff. For immunohistochemistry, rehydrated paraffin sections were boiled 30 minutes in citrate buffer pH 6 for antigen retrieval and pretreated with 1% H_2O_2 in methanol

for 10 minutes to block endogenous peroxidase activity. Primary antibodies included rat anti-endomucin (1:500; provided by Dietmar Vestweber, Max-Planck-Institute, Bad Nauheim, Germany),[32] rabbit anti-mouse VEGFR2 T1014 (1:500; provided by Rolf Brekken, University of Texas Southwestern Medical Center, Dallas, TX)[33,34] and rabbit anti-mouse c-Maf (1:500, Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies were visualized using avidin-biotin-peroxidase and DAB substrate (Vector ABC kit; Vector Laboratories, Burlingame, CA). For each experiment, a section was incubated with isotype-matched IgG as a negative control. After mounting, the sections were visualized and photographed using a microscope (Axioskop 2 FS mot; Carl Zeiss Microimaging GmbH, Göttingen, Germany).

For fluorescent immunodetection, antisera included polyclonal antisera against αB-crystallin (1:1000; Serotec, Raleigh, NC), β-catenin (1:1000; Upstate, Billerica, MA), E-cadherin (1:500; Abcam, Cambridge, MA), N-cadherin (1:500; R&D Systems, Minneapolis, MN), MIP26 (1:500; kindly provided by Joseph Horwitz [Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, CA]), Phospho-histone H3 (Ser10; 1:40; Upstate), Prox1 (1:1000; Covance, Trenton, NJ) and p57^{kip2} (1:100; Abcam), followed by a rabbit or donkey raised Cy3-conjugated or FITC-conjugated secondary antibody (1:300; Jackson ImmunoResearch, West Grove, PA). Some sections were co-stained for F-actin using FITC-phalloidin (1:200; Molecular Probes, Carlsbad, CA). Cell nuclei were identified by DAPI labeling.

Detection of Apoptotic Cells

TUNEL-positive cells were stained using a cell death detection kit (In Situ Cell Death Detection Kit, TMR red; Roche Applied Science, Indianapolis, IN), according to the manufacturer's recommendation. Air-dried cryosections were permeabilized in 0.2% Tween-20 at 4°C and incubated with the TUNEL reaction mix containing TdT and tetramethylrhodamine-5-dUPT.

Real-Time PCR Analysis

Lenses were dissected under RNase-free conditions from E13.5 C57B1/6 embryos. Total RNA was extracted from 20 lenses (RNAqueousTM-4PCR kit; Ambion Inc., Austin, TX). Residual DNA was removed by treatment with one unit DNase I (Ambion) at 37°C for 20 minutes. RNA (0.5 μg) was reverse-transcribed (Superscript II reverse transcriptase; Invitrogen, Carlsbad, CA). Real-time PCR was performed in a thermocycler workstation (ABI Prism 9700 Sequence Detection System; Applied Biosystems, Foster City, CA). VEGF isoform RNA was quantified as previously described.[24] Each sample was run in triplicate and each experiment included three non-template control wells. Results were expressed as mean ± standard deviations (SD).

Eye Explants

Eye explants were prepared and cultured as previously described.[35] Briefly, E11.5 embryos from one litter were collected in PBS at 4°C, optic cups were dissected under a dissecting microscope and the primitive RPE layer was removed. Optic cup explants were embedded in 33% collagen I gels (vol/vol in Dulbecco's modified Eagle's medium [DMEM]) and

immersed in DMEM media with 10% FCS in the presence or absence of goat anti-mouse VEGF neutralizing antibody (10 μg/mL; R&D Systems) with equal goat IgG added to control explants. Explants were cultured for 48 hr at 37°C in 5% CO_2, then processed for paraffin sectioning and hematoxylin/eosin staining. All eye explants were serial sectioned and the height and width of each lens were quantified in the largest section in each sample series. Results were expressed as mean ± SD ($n = 4$ for control; $n = 7$ for anti-VEGF). Statistical analysis was performed using an unpaired Student's t-test for the height and width measurements, **$P < 0.01$. The statistical significance of the ratio height/width was analyzed using a non-parametric Mann-Whitney test comparison (**$P = 0.0061$).

Cell Culture

Mouse primary lens epithelial cell (mLEC) were isolated accordingly to previously published method.[36] Briefly, eyes were dissected from 8-week-old mice and transferred to lens epithelial cell (LEC) culture medium composed of DMEM (Invitrogen Gibco) supplemented with 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin (all from Gibco), and 20% fetal bovine serum (Sigma). Under a dissecting microscope, lenses were dissected out and all tissue attached removed. Lens capsules were removed and transferred to a six-well plate with eight capsules per well. After three days, LEC migrated from the capsules and after seven days were harvested for RNA isolation. Large antigen-T transformed human lens epithelial cell line (HLE-B3)[37] was obtained from ATCC and cultured in LEC culture medium.

Cell Survival Study

HLE-B3 cells were seeded at a density of 1×10^4 cells/cm^2 into 24-well plate in 20% serum. One day later, cell medium was changed, replaced with low serum (0.1% FBS) medium, and cultured for three days in absence or presence of various concentration of VEGF165 (R&D Systems). Cell number per well was determined by Coulter counting.

Standard RT-PCR

Total RNA was isolated from primary lens epithelial cell and hLEC-B3 (RNA-bee; IsoTex Diagnostics, Friendswoods, TX) according to the manufacturer's protocol. RNA was reverse-transcribed (Superscript III; Invitrogen). Standard PCR was performed (1 U *Taq* DNA polymerase; Roche) and 0.2 μM of appropriate primer pair (Table 1).

Results

Expression of VEGF and VEGF Receptors during Early Lens Development

To examine VEGF expression in the developing lens, we used mice in which the gene that encodes for LacZ with a nuclear localization signal had been introduced into the VEGF locus.[31] The weak nuclear β-gal staining at E10.5 suggests a low VEGF expression in the lens vesicle (Fig. 1A, 1B). VEGFR2 immunoreactivity was localized to the EC of the forming pupillary membrane and to the lens epithelium, where VEGFR2 was associated with the membranes of the equatorial lens epithelial cells (Figs. 1A, 1B). Strong VEGF expression was detected in the primary lens fibers at E13.5 and, to a lesser extent, in the lens epithelial cells. At the same stage, VEGFR2 was detected on the pupillary vessels and tunica

vasculosa lentis, as well as on the lens cells and the mesenchyme of the primitive cornea. VEGFR2 was clearly localized to the surface of the lens epithelium and the lens fibers, particularly in the bow zone of active lens fiber differentiation (Figs. 1D–1E). Real-time PCR of E13.5 lens revealed that the primitive lens expressed mainly VEGF164 (95%) with 4% VEGF120 and virtually undetectable (<1%) VEGF188 (Fig. 1G).

Developmental Defects in Eye and Lens in Mice Expressing Only VEGF120

As VEGF164 is the predominant isoform expressed in primitive lens (Fig. 1G), we examined eye development in mice expressing only VEGF120. In these mice, *VEGF* gene modification results in the removal of the exons 6 and 7, leading to the production of only VEGF120, while the total VEGF level remains unchanged.[15] Macroscopic evaluation of E13.5 VEGF120/120 mice revealed major defects in the eye (Fig. 2). Two qualitatively distinct phenotypes, which we refer to as type I (moderate) and type II (severe), representing approximately $\frac{2}{3}$ and $\frac{1}{3}$ of the VEGF120/120 mice, respectively, were observed. No changes were observed in the eyes of VEGF120/+ littermates (data not shown).

In the type I phenotype, microphthalmia was associated with a reduced optic cup opening and altered orientation (Figs. 2A–2C) as well as a smaller lens (Fig. 2F). No gross changes were detected in the neuroblastic layers of the type I VEGF120/120 retinas. The type II VEGF120/120 mice were characterized by a significant reduction in optic cup size and opening (Figs. 2A–2D). Histologic analysis of serial sections revealed thickening and folding of the retina that dislodged the lens from its central location above the optic nerve axis, so that the section with the largest lens was regularly found away from the section containing the optic nerve (Fig. 2G). Examination of all VEGF120/120 embryos by serial section thorough the entire eye showed no evidence of incomplete ocular fissure closure.

Abnormal Hyaloid Vasculature in Eyes of VEGF120/120 Mice

Organ size and differentiation is controlled in large part by its vasculature. We have previously shown that VEGF120/120 lungs have reduced vascular density and are therefore smaller in size and developmentally delayed.[14] However, sections of eyes from E13.5 WT and type I VEGF120/120 mice stained for the endothelial-specific marker endomucin[32] did not reveal any reduction in the ocular vasculature that might account for the reduced optic cup and lens size (Figs. 3A, 3B). The apparent failure of the anterior part of the optic cup to completely open led to a reduced pupillary membrane. The primitive choroid was present and appeared to be normally differentiated into main choroidal vessels and choriocapillaris (Figs. 3C, 3D). There were notable differences between the inner ocular vessels of the WT and VEGF120/120 mice. The hyaloid vessels of the VEGF120/120 mice were markedly dilated and hyperplastic, completely filling the space between the lens and the retina (Figs. 3E, 3F).

Altered Lens Differentiation in VEGF120/120 Eyes

High magnification of hematoxylin and PAS-stained sections of lenses from VEGF120/120 mice revealed various types of abnormalities. In type I VEGF120/120 mice, lens differentiation was sufficient to distinguish between anterior epithelium and posterior fibers, and a lens capsule was present (Figs. 4B, 4E). However, the primary lens fibers lacked the

characteristic equatorial organization and the posterior elongation was significantly reduced (Fig. 4B). In the anterior lens, the morphology of the epithelium was abnormal; the characteristic cuboidal epithelium was replaced by a disorganized and thickened epithelium (Fig. 4E). The lenses of type II mice also exhibited abnormal lens cells organization, defective epithelial differentiation, lack of proper fibers and absence or significant reduction of the lens capsule as shown by the undetectable PAS staining (Fig. 4C). The organization of the primitive corneal was significantly altered with an accumulation of mesenchymal cells and a lack of distinct corneal endothelium (Fig. 4F).

To better characterize the altered lens differentiation in VEGF120/120 eyes, we examined the expression of αB-crystallin, which is primarily expressed by the developing lens epithelium and is down-regulated in differentiated lens fibers.[38] Lenses from VEGF120/120 mice displayed an increased expression of αB-crystallin in both the lens epithelium and the primitive lens fibers, leading to its accumulation in the anterior portion of the fibers (Figs. 4G–4I). Crystallin expression and lens differentiation are controlled by the transcription factor c-Maf.[39] In lenses from wt mice, c-Maf was expressed by the fiber cells, with the highest expression in the equatorial margins, the zone of active differentiation, whereas it was absent from the lens epithelium (Fig. 4J). In contrast, lenses from VEGF120/120 mice revealed an abnormal expression pattern with ectopic c-Maf in the lens epithelium (Figs. 4K–4L).

Premature Lens Epithelial Differentiation and Reduced Proliferation Caused by Expression of VEGF120 Alone

The expression of proteins involved in the differentiation of lens fiber cells was next examined. Prox1 is a homeobox protein whose expression is required for fiber cell differentiation and elongation.[40] In wt eyes, Prox1 reactivity was weak in the cytoplasm of the lens epithelial cells but intense in the nuclei of the postmitotic fiber cells, as previously described.[41] In VEGF120/120 lenses, Prox1 translocation from cytoplasm to nucleus was detected more anteriorly in the lens epithelium and was prominent in the lens fiber nuclei (Figs. 5A, 5B). The expression of p57 (kip2), a cyclin-dependent kinase inhibitor expressed at the earliest stages of lens fiber differentiation,[42] where it controls cell cycle withdrawal,[43] was also shifted anteriorly in the lens epithelium of the VEGF120/120 mice (Figs. 5C, 5D).

This alteration in the expression pattern of Prox1 and p57 is consistent with the observation of c-Maf in the lens epithelium and suggests that reduced lens epithelium proliferation due to premature cell cycle arrest might be a primary defect in the VEGF120/120 lens. We investigated this hypothesis by staining E13.5 lenses for phosphohistone H3 (phh3; Figs. 5E–5H), a marker of cell division. Whereas numerous lens epithelial cells in the WT mice were labeled for phh3, there was a marked reduction in the number of phh3-positive lens epithelial cells in VEGF120/120 lenses, though labeling in other part of the eye, including as the retina, were unchanged. The reduced epithelial cell proliferation in the VEGF120/120 lenses was not associated with increased apoptosis, as examined by TUNEL staining (Figs. 5I–5J), nor was increased apoptosis detected in the neuroblastic layers of the VEGF120/120 eyes (data not shown).

Abnormal MIP26 Localization and E-Cadherin Expression in VEGF120/120 Mice

Histologic observation suggested that the reduced size of VEGF120/120 lenses could be due, at least in part, to aberrant lens fiber elongation and/or morphology. We therefore examined the expression of proteins involved in cell-cell interactions and cytoskeleton organization. MIP26, also called aquaporin-0, is a lens fiber-specific protein that functions as a cell-cell adhesion molecule and water channel.[44] In lens of E13.5 WT mice, MIP26 expression was restricted to the plasma membrane of the fiber cells (Figs. 6A, 6C). As predicted by the normal expression of Prox1 in the posterior fiber nuclei, the lens fibers of type I VEGF120/120 lenses displayed a similar level of MIP26 immunoreactivity (Fig. 6B). However, the localization of MIP26 in the anterior part of the lens fibers was more diffuse with perinuclear distribution and a lack of distinct membrane labeling; membrane-associated MIP26 was seen in the posterior lens (Figs. 6C, 6D).

To determine whether the aberrant MIP26 localization was associated with abnormal cytoskeletal architecture, lenses were co-stained for membrane associated proteins including E- and N-cadherin, filamentous (F) actin, and β-catenin. In WT lenses, E-cadherin expression was restricted to the proliferating lens epithelium,[45] where it was preferentially localized to the apical and lateral cell junctions (Figs. 6E, 6G). E-cadherin expression was significantly reduced in the VEGF120/120 lenses whereas N-cadherin was normally distributed at the cell membrane (Figs. 6E–6H). These findings suggest a defect in lens epithelial differentiation in VEGF120/120 mice and are consistent with the observations of an abnormal epithelial architecture and premature cell cycle exit.

In WT lenses, β-catenin, which is involved in cell-cell adhesion, was also highly expressed at the junctions of the epithelial cells and lens fiber and was colocalized with F-actin. β-catenin staining was more intense in the marginal zone of WT lenses (Figs. 7A–7C). In contrast, β-catenin staining of VEGF120/120 lens was weaker and lacked the intense staining observed in the equatorial region of the WT (Fig. 7D). Whereas β-catenin and F-actin were localized to intercellular junctions in WT lenses, they appeared more diffuse in the central fiber cells VEGF120/120 lenses (Figs. 7D–7F). Together, these results suggest that defective lens fiber differentiation in the VEGF120/120 mice is associated with alteration of the cell junction organization.

Reduction of Lens Fiber Elongation by VEGF Inhibition during Lens Development Ex Vivo

We used an ex vivo model to assess the effect of VEGF neutralization under conditions independent of endogenous blood supply. Eye rudiments were isolated from WT E11.5 embryos, a developmental stage where lens induction and early formation has already occurred. The ocular explants were cultured in collagen gels for 48 hr in the presence of VEGF neutralizing antibodies and primary fiber lens elongation was examined. More than half of the explants cultured in the presence of VEGF neutralizing antibody failed to obliterate the lens vesicle (4 out of 7 total) whereas the control explants showed complete vesicle obliteration (Figs. 8A, 8B). This defect was the result of incomplete lens fiber elongation, evidenced by a significant reduction in lens height and height to width ratio ($P <$ 0.01; Figs. 8C, D). These support the hypothesis that VEGF signaling is involved in primary lens fiber differentiation.

Expression of VEGF and Its Receptors by Adult Lens

We characterized the expression pattern of VEGF and its receptor in adult mice and epithelial lens cells. Similar to the developing lens, β-galactosidase staining of adult VEGF-lacZ eyes revealed an intense staining of the differentiating lens fibers; no LacZ staining was observed in the adult lens epithelium (Fig. 9A). Detection of VEGFR2 by immunohistochemistry indicated strong expression in both the lens epithelium and differentiating lens fibers (Fig. 9B). Mouse primary lens epithelial cell (mLEC) also expressed VEGFR1 and VEGFR2 by RT-PCR (Fig. 9B). Though not detected in adult lens epithelium in vivo, VEGF was expressed by primary lens epithelial cells, suggesting that cell culture conditions may induce VEGF expression. Because of the limited proliferative capacity of primary mLEC in vitro, we used the well-defined human lens epithelial cell line HLE-B3.[46] RT-PCR analysis confirmed that HLE-B3 cells express the same components of the VEGF pathway as primary mLEC (Fig. 9B). Addition of VEGF led to a significant dose dependent increase in HLE-B3 cell number (Fig. 9C).

DISCUSSION

VEGF function was initially thought to be restricted to endothelial cells, but mounting evidence has revealed that VEGF also acts on a wide variety of nonvascular VEGFR2-expressing cells, including neuronal and cardiac progenitors.[25,47] Our findings add lens cells to this growing list. We observed the expression of VEGFR2 by the lens epithelium and the differentiating lens fibers of the margin zone. Co-localization of VEGF and VEGFR2 in the embryonic lens, together with the defects observed in VEGF120/120 eyes, implicates an autocrine or paracrine VEGF pathway in the expansion and differentiation of the lens epithelium and fibers, and the continued expression of VEGF and VEGFR2 by postnatal lens suggests a role in the adult lens.[30]

We have also demonstrated that VEGFR2 is expressed by the mesenchymal cells of the cornea. Corneal morphogenesis is characterized by the colonization of the space between the corneal epithelium and the lens with cranial neural crest cells (NCC), which give rise to the corneal endothelium and stromal keratinocytes.[48] Findings from Wnt1-LacZ mice confirmed that NCC arise in the corneal space around E12.5, as the lens detaches from the epithelium, [49] which corresponds to the time of VEGF induction in the lens. VEGFR2-mediated migration has been documented during other developmental processes. During early embryogenesis, VEGF mediates the migration of VEGFR2-positive cells toward the anterior part of the head[50] and has been shown to be a chemoattractant for neural progenitors in the brain.[51] These observations support the notion that lens-derived VEGF could participate in the homing of VEGFR2-expressing NCC to the corneal mesenchyme.

The variability of ocular defects in the VEGF120/120 mice parallels the variable penetrance in the overall development of VEGF120/120 mice, ranging from very early embryonic to perinatal death.[15] However, all the eyes of VEGF120/120 mice were significantly smaller than those in wt or 120/+ littermates, and all displayed major developmental defects. Though the outer ocular vascular bed of VEGF120/120 mice was normal, the hyaloid vessels appeared hyperproliferative and dilated. Transgenic mice overexpressing VEGF120, 164 or 188 in the lens have been shown to display specific vascular phenotypes.[52,53] Interestingly,

the vascular changes observed in the VEGF120/120 eyes resemble the abnormalities noted with lens-specific overexpression of VEGF120 and 164.[52] Major nonvascular changes, some in the lens itself, were also observed with overexpression of VEGF188 leading to reduced lens size and cataract formation[54] whereas VEGF164 and VEGF120 overexpression was associated with delayed lens epithelium differentiation and vacuolization.[52] While the authors attributed most of the lens defects to changes in the hyaloid vasculature, our findings suggest that at least some of the observed lens phenotypes may be due to abnormal VEGF signaling in the lens cells.

Reciprocal interactions between the developing lens and other ocular tissue such as the retina and the anterior segment have been well characterized.[55] Thus, it is possible that some of the developmental changes observed in the VEGF120/120 lenses might be secondary to other ocular anomalies. Yet, we did not detect any gross anomalies, changes in cell proliferation or apoptosis in the retinas of VEGF120/120 mice with a moderate phenotype (Type I). Strong support for a role for VEGF in lens fiber elongation was provided using an ex vivo lens development system where VEGF neutralization led to reduced fiber elongation and incomplete lens vesicle obliteration. Because retina is present in the eye explants and retinal progenitors express VEGFR2,[24,25] we cannot exclude a role for retinal anomalies in the observed lens phenotype. In spite of this, using HLE-B3 lens cells, we demonstrated a direct effect of VEGF on lens epithelial cell survival and proliferation in vitro.

Formation of a mature lens is associated with the deposition of a thick basement membrane, the lens capsule, which surrounds both lens epithelium and fiber cells. Like most basement membranes, the lens capsule is comprised of collagen IV, laminins, nidogen/entactin, and proteoglycans including HSPGs (for review see Ref. 56), which can bind growth factors such as FGF-2 and VEGF and thus may act as a reservoir for growth and survival factors. Consistent with this concept, metalloprotease treatment of the lens capsule releases FGF-2 that acts to mediate lens epithelial cell survival.[57] VEGF164, which we demonstrate is the major VEGF isoform produced by the embryonic lens, contains one heparin-binding site, which allows it to both associate with the HSPGs of the lens capsule[58] and diffuse into the vitreous. VEGF120's lack of heparin binding domains would lead to reduced or lack of local VEGF in the lens, which we speculate result in defective lens epithelial differentiation. At the same time, excess diffusion of VEGF120 into the vitreous could account for the observed dilation of hyaloid vessels and the accumulation of mesenchymal cells in the prospective cornea.

The altered pattern of c-Maf and E-cadherin localization and early expression of Prox1 and p57 suggests premature cell cycle exit and differentiation of the lens epithelium, which is consistent with the observed decrease in proliferation. The Notch signaling has been shown to regulate lens differentiation via suppression of p57 expression.[59] VEGF has been reported to increase Notch signaling in vascular endothelial cells[60] and a reduction of cleaved Notch1 has been observed in the endocardium of VEGF120/120 mice.[61] Our observation of premature p57 expression in VEGF120/120 mice may be associated with an alteration of Notch signaling in the lens epithelium, which has been shown to express Notch2 and Notch3.[62]

VEGF120/120 lenses also displayed reduced elongation of the posterior fiber cells. Lens cell migration and elongation have been shown to be regulated, in part, by the activation of Rho GTPase-dependent pathways[63] and inactivation of lens-specific Rho GTPases has been demonstrated to lead to major lens defects, including abnormal fiber cells shape, migration, and organization.[64] Similarly, recent studies of the vascular endothelium have demonstrated that the Rho proteins are a direct downstream target of VEGF signaling.[65,66] Mice engineered to express C3-exoenzyme, a Rho inhibitor, in the lens display a phenotype similar to that observed in the VEGF120/120 mice, including shortening of the lens fibers, abnormal MIP26 cellular localization, and multilayered epithelium.[67] These similarities support the notion that impaired lens development in the VEGF120/120 mice may be due, in part, to alterations in the Rho GTPase pathway.

Fiber cell elongation and differentiation require the dynamic reorganization of the cytoskeleton and extracellular matrix interactions. The differentiation of the lens epithelial cells into mature fiber cells is a highly complex process involving the coordinated expression of multiple growth factors and transcription factors. In support a role for VEGF in lens differentiation, analysis of the mouse VEGF promoter reveals consensus binding sites for many transcription factors and growth factors known to be involved in lens induction and formation, including Pax6, TGFβ family members (TGFβ2, BMP4 and Smad4), MITF, Maf proteins, Meis1 and Sox proteins. VEGF is not expressed in early lens development, rather its induction correlates temporally with lens fibers differentiation (in this report and Ref. 24). Our findings of significant defects in lens development in mice lacking the normally expressed VEGF164 (expressing only the soluble form of VEGF) together with the results of VEGF inhibition in eye explants and cell culture provide strong support a role for VEGF in lens differentiation and provide additional evidence that VEGF plays important roles in the development of nonvascular tissues/organs.

Acknowledgments

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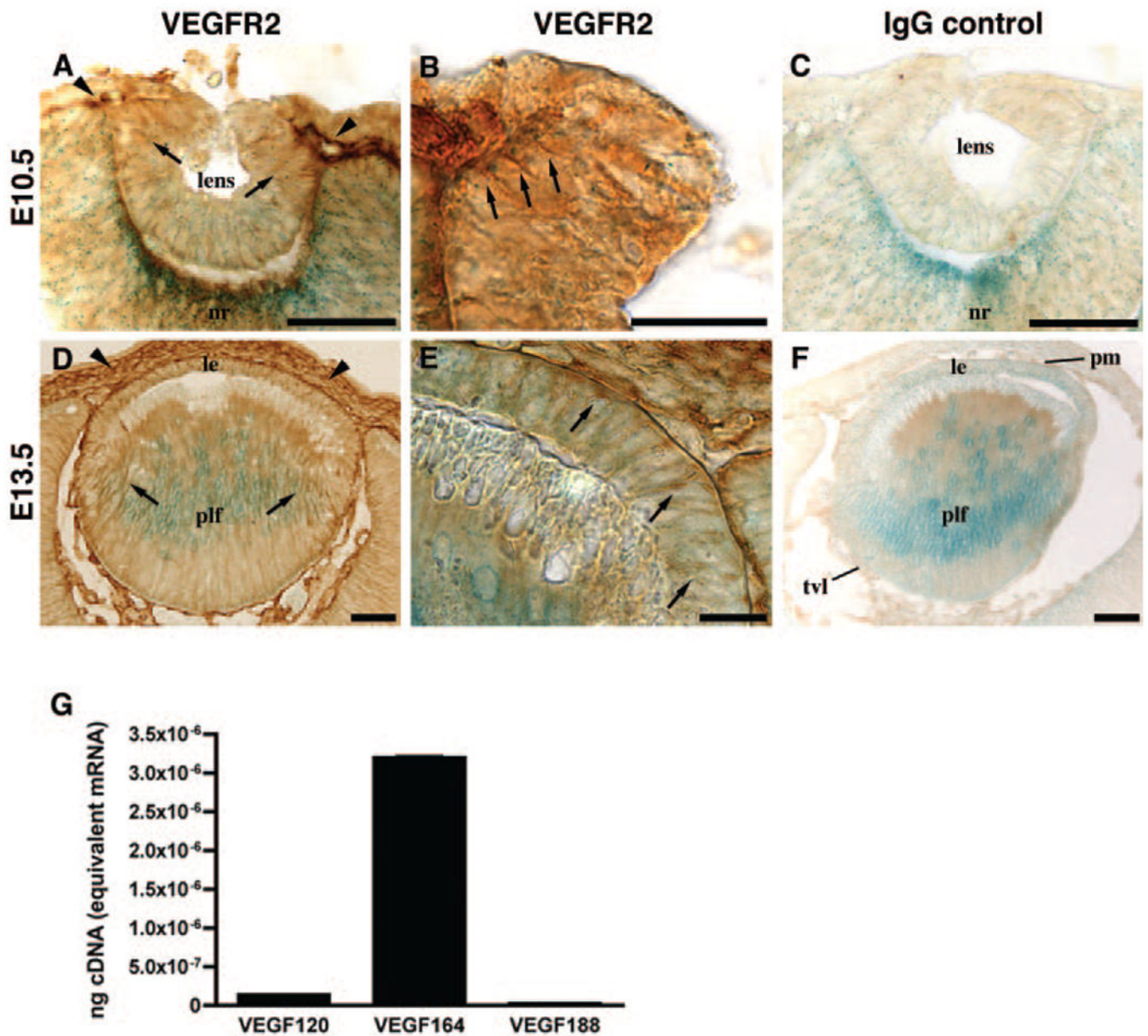
Figure 1.
Expression of VEGF and VEGF receptors during early lens development. (**A–F**)
Cryosections of eyes from E10.5 and E13.5 VEGF-LacZ/+ mice were co-stained for β-
galactosidase and VEGFR2, or with IgG as a control. (**A–C**) At E10.5, VEGF was strongly
expressed in the innermost layer of the neural retina, but weakly by the invaginating lens.
VEGFR2 was highly expressed by the endothelial cells of the forming pupillary membrane
(*arrowheads*) and was associated with the membranes of the lens cells in the equatorial
region (*arrows*). (**B**) Higher magnification of the equatorial region of the lens vesicle
showing VEGFR2 localization on the membrane of the lens epithelium. (**D–F**) At E13.5,
VEGF expression was detected in the primary lens fibers, and to a lesser extent, in the lens
epithelium. VEGFR2 was detected in the endothelium of the hyaloid vasculature (pupillary
membrane and tunica vasculosa lentis) as well as in the lens epithelium and the posterior

lens fibers, specifically at the equatorial margin (*arrows*). In the anterior part of the optic cup, VEGFR2 was also observed in the mesenchymal cells of the primitive cornea (*arrowheads*). (**E**) Higher magnification showing VEGFR2 localization on the lens cell membranes (*arrows*). nr, neuronal retina; mL, choriocapillaris; le, lens epithelium; plf, primary lens fiber; pm, pupillary membrane; tvl, tunica vasculosa lentis. Scale bar, (**A–F**) 50 μm. (**G**) qPCR quantification of VEGF isotorms mRNA expression in embryonic lens. E13.5 lens expressed mainly VEGF164.

WT **Type I 120/120** **Type II 120/120**
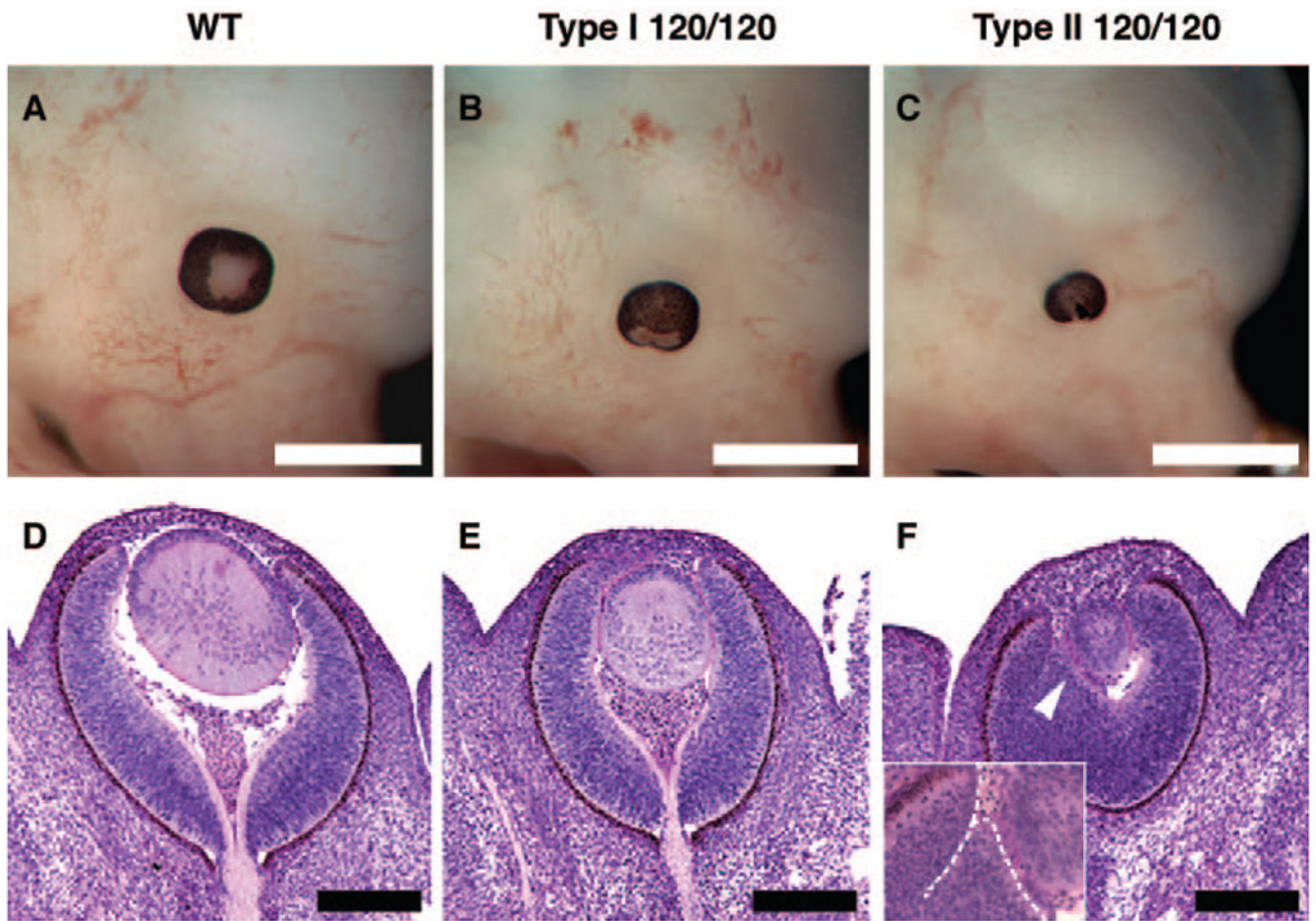
Figure 2.
Ocular phenotype of VEGF120/120 mice. (**A–C**) E13.5 WT and VEGF120/120 heads. (**D–F**) Paraffin sections of E13.5 WT and VEGF120/120 mice were stained with hematoxylin-periodic acid-Schiff. (B, E) In type I VEGF120/120 mice, all the eye structures were evident but smaller. Note the reduced optic cup opening compared to WT eye. (**C**, **F**) Type II VEGF120/120 mice displayed a more severe phenotype with a closed optic cup. Retinas were present but lenses were extremely reduced with no apparent differentiation of lens epithelium or posterior lens fibers and displaying prominent retinal folding (white arrowhead and inset). Scale bar: (**A–C**) 1 mm; (**D–F**) 200 μm.
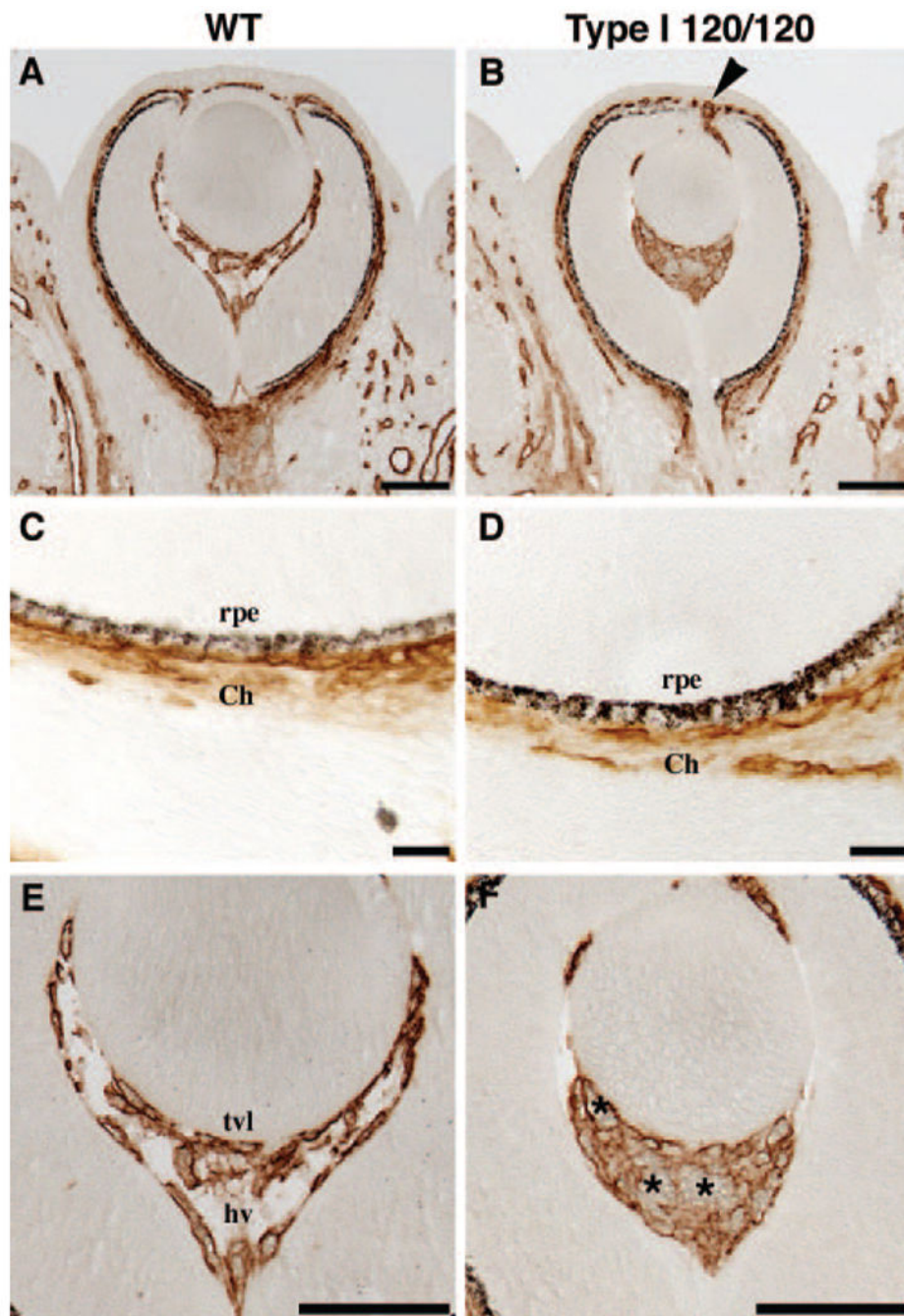
Figure 3.
Abnormal hyaloid vasculature in eyes of VEGF120/120 mice. Paraffin sections of E13.5 WT and type I VEGF120/120 eyes were stained with the endothelial-specific marker, endomucin. (**A–B**) Low magnification showing the presence of the external and internal ocular vasculatures in type I VEGF120/120 mice, but an abridged opening of the optic cup (*arrow*) was associated with an abnormal pupillary membrane that failed to cover the lens epithelium. (**C–D**) Higher magnification of the choroidal vessels, which appear normal. (**E–F**) Higher magnification of the hyaloid vessels revealed a significant expansion, compared to

the control with enlarged vessels (*asterisks* show enlarged lumen). Note the absence of vitreal space. Scale bar: (**A**, **B**, **E**, **F**) 100 μm; (**C**, **D**) 20 μm. rpe, retina pigmented epithelium; Ch, choroid; tvl, tunica vasculosa lentis; hv, hyaloid vessels.
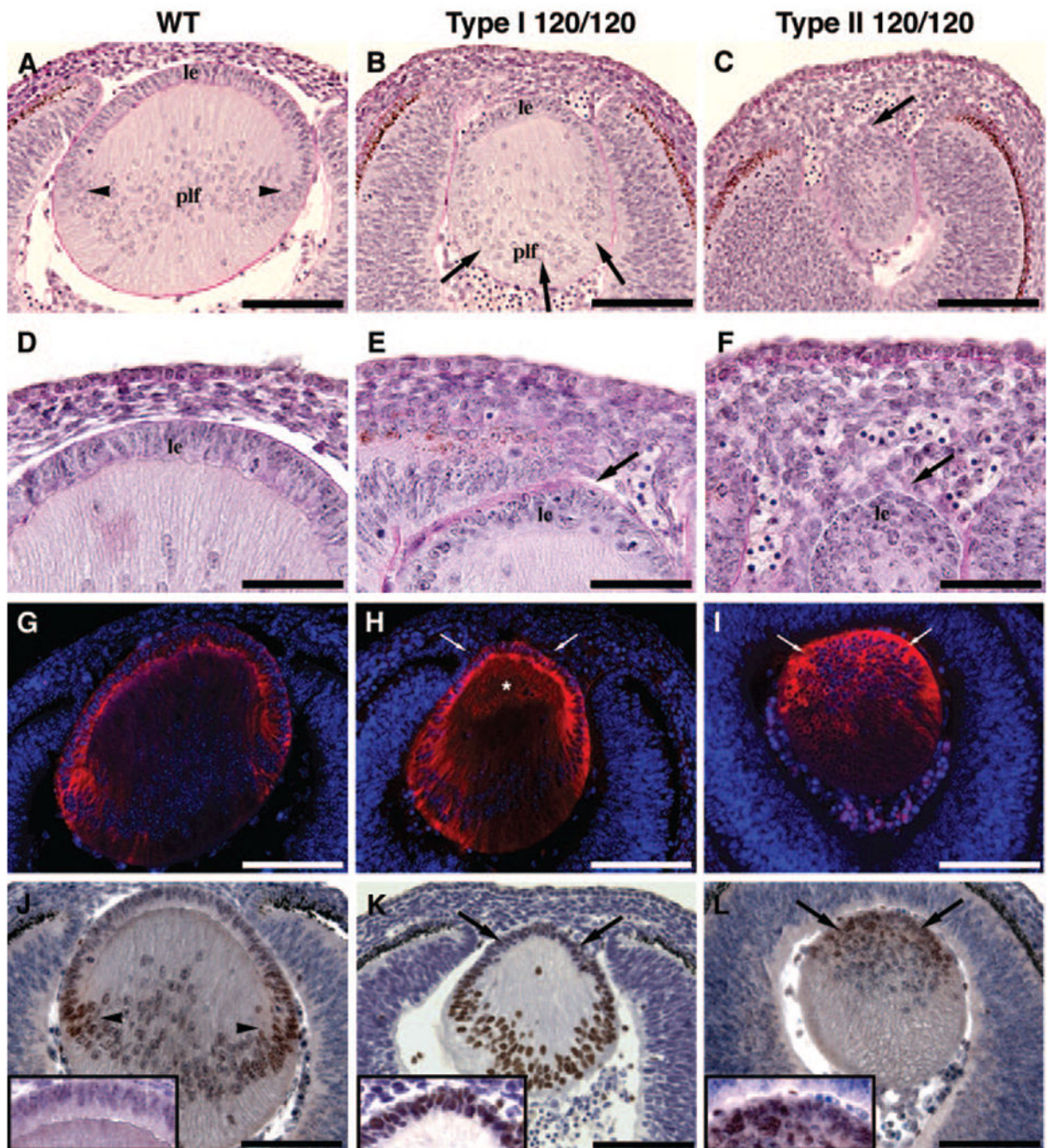
Figure 4.
Defective lens differentiation and altered expression of αB-crystallin and c-Maf in VEGF120/120 mice. (**A–F**) Sections of E13.5 wt, type I and type II VEGF120/120 lenses stained with hematoxylin and PAS. (**D–F**) Higher magnification of the anterior part of the lens. (**A–C**) The normal organization of the primitive lens fibers along the equatorial zone (*arrowhead*) was absent in VEGF120/120 lenses (**B**, **C**). (**B**) Nuclei of the type I 120/120 lens fibers appeared to be pushed toward the posterior pole as the elongation of the fibers is strongly reduced (*arrows*). (**C**, **F**) The lens epithelium and fibers of type II VEGF120/120

lenses failed to differentiate and the lens epithelium formed a multilayered structure, lacking proper lens capsule, as shown by the absence of PAS staining (*arrow*). (**D**) WT lens showing normal cuboidal epithelium. (**E**) Type I phenotype was associated with an irregular epithelium but the lens capsule was present (*arrow*). (**F**) Type II VEGF120/120 displayed aberrant epithelial differentiation (*arrow*). (**G–I**) Sections of E13.5 eyes from WT and VEGF120/120 embryos were stained for αB-crystallin. (**G**) At this stage in WT lenses, αB-crystallin is normally strongly detected in the lens epithelium and the fibers of the equatorial region. (**H–I**) In VEGF120/120 lenses, the αB-crystallin expression pattern was altered with overexpression in the lens epithelium (*white arrows*) and fibers, leading to an ectopic accumulation in lens fibers (*asterisk*). (**J–L**) Sections of E13.5 eyes stained for c-Maf. (**J**) In WT lenses, c-Maf was expressed preferentially at the equatorial margin where cells begin to differentiate (*black arrowheads*). (**K**, **L**) In VEGF120/120 lenses, c-Maf expression was abnormally detected in the lens epithelium (*black arrows* and *inserts*). Scale bar: (**A–C**, **G–L**) 100 μm; (**D–F**) 50 μm.
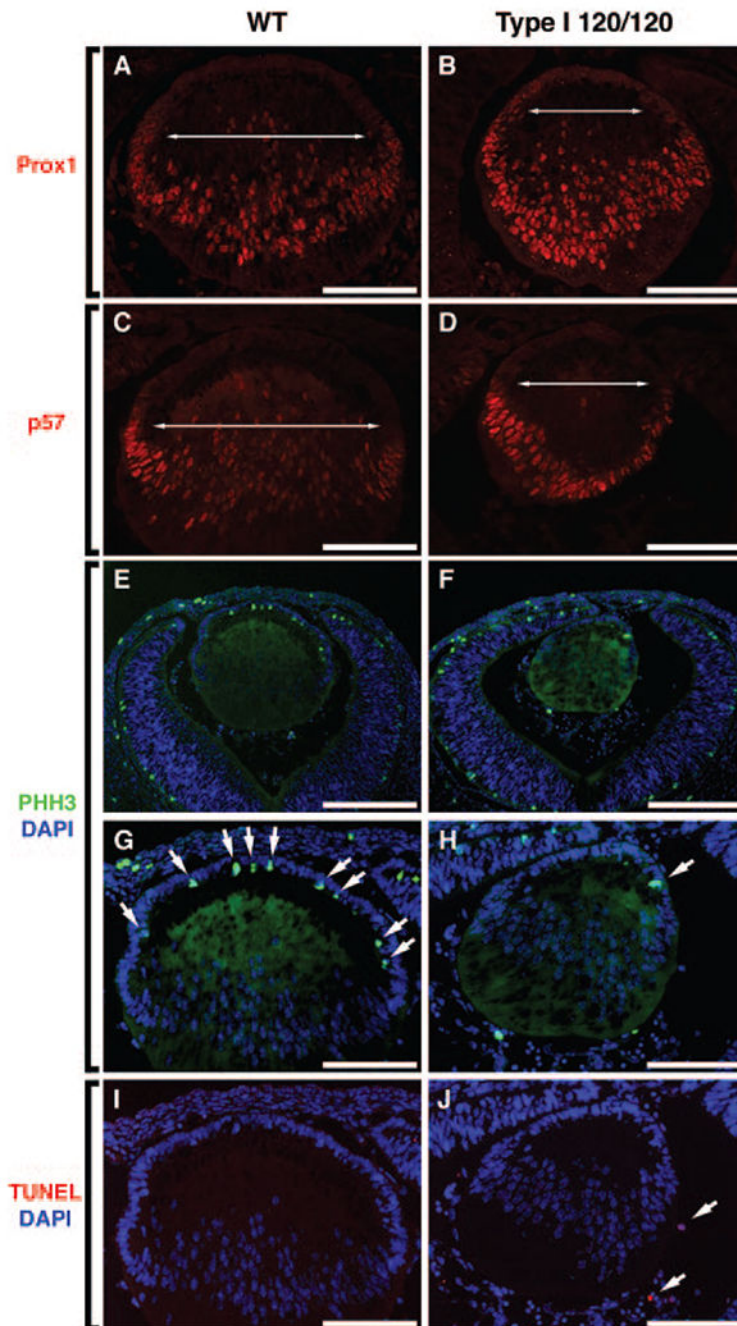
Figure 5.
Premature differentiation and decreased proliferation of the VEGF120/120 lens epithelium.
Cryosections of E13.5 eyes from WT and type I VEGF120/120 embryos were stained for
Prox1 and p57, markers of lens epithelium differentiation, for proliferation using phh3 and
for apoptosis by TUNEL staining. (**A**) In WT lenses, Prox1 labeling was weak in the
cytoplasm of the epithelial cells but was strong in the nuclei of cells undergoing
differentiation at the equatorial zone. (**B**) In type I VEGF120/120 lenses, nuclear Prox1 was
more anteriorly localized (shown by the *horizontal lines* marking the onset of Prox1

expression). (**C–D**) The premature nuclear expression of Prox1 in the type I 120/120 lens was associated with a more anterior expression of p57, indicative of early cell cycle exit (the *horizontal lines* show the onset of p57 expression). (**E**, **G**) In WT lenses, phh3 staining revealed significant epithelial cell proliferation (*arrows* in **G**). (**F**, **H**) In VEGF120/120 lenses, there were few replicating epithelial cells (*arrow* in **H**). No changes were observed in other parts of the eye including the retina. (**I**, **J**) No increase in apoptosis was evident in VEGF120/120 lenses and as for WT eyes, only a few cells positive for TUNEL staining were detected in the cornea stroma and the hyaloid vessels (*arrows* in **J**) similar to that seen in the WT. Scale bar: (**A**, **B**, **E–H**) 100 μm; (**C**, **D**) 200 μm.
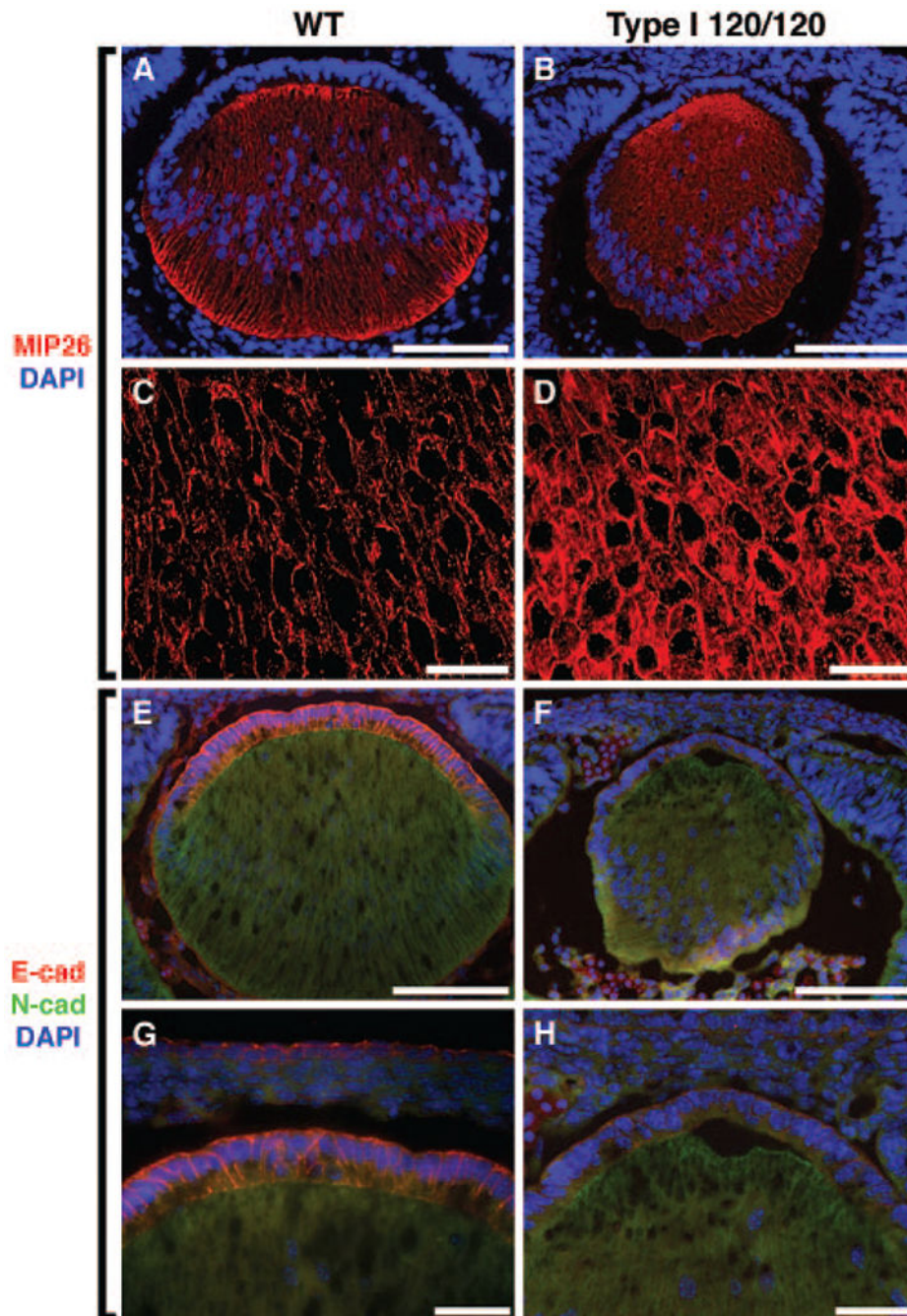
Figure 6.

Abnormal expression of MIP26 and E-cadherin in VEGF120/120 lenses. Cryosections of E13.5 eyes from WT and type I VEGF120/120 embryos were stained for MIP26 or co-stained for E-cadherin and N-cadherin. (**A**, **C**) In WT lens, MIP26 expression was restricted to the lens fiber membranes. (**B**, **D**) In type I lens, MIP26 was detected in the lens fibers with no apparent change in the level of expression. (**C–D**) High power confocal images of the central lens fibers revealed a diffuse cytosolic distribution of MIP26 in the lens of VEGF120/120 mice contrary to the WT lenses where MIP26 appears strongly associated to

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the fiber cell membrane. (**E**, **G**) In WT lens, E-cadherin expression was restricted to the lens epithelium and was prominent at the apical and lateral cell membrane. (**F**, **H**) In VEGF120/120 lenses, E-cadherin expression in the epithelium was strongly reduced whereas N-cadherin expression appeared unaffected. Scale bar: (**A**, **B**, **E**, **F**) 100 μm; (**C**, **D**, **G**, **H**) 20 μm.

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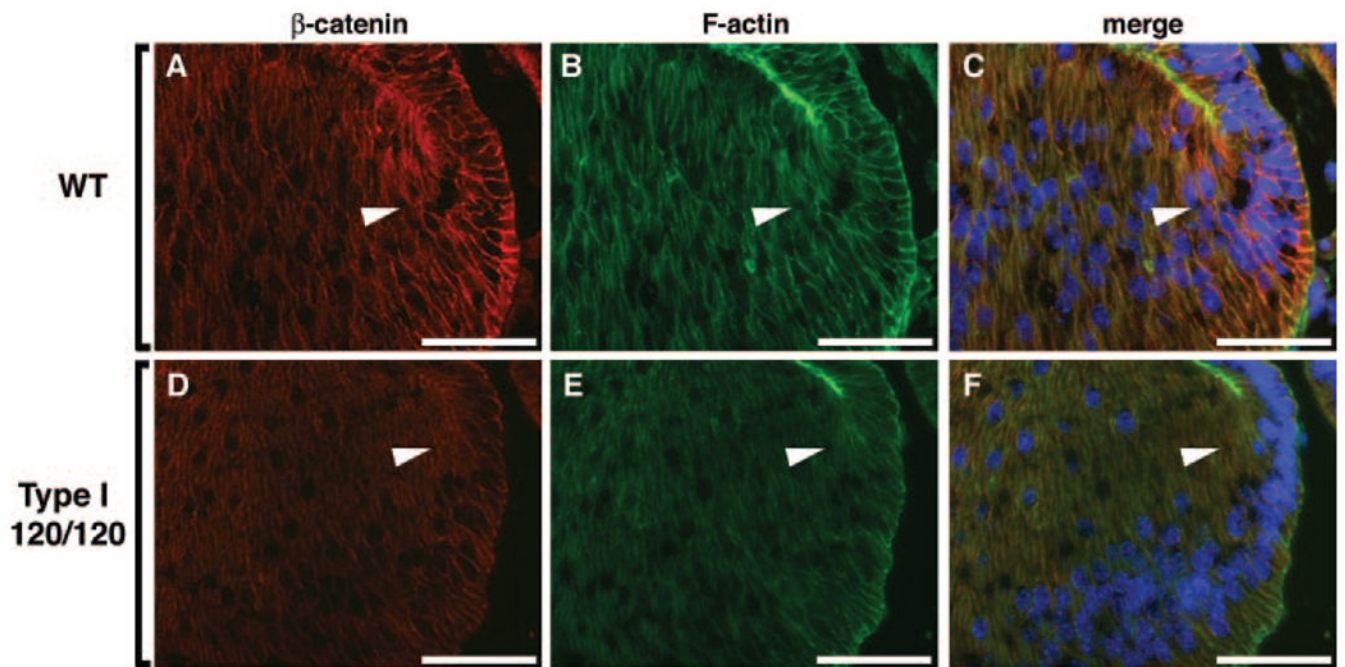
Figure 7.
Reduced expression of β-catenin in the equatorial region of type I VEGF120/120 lenses. Cryosections of E13.5 eyes from WT and type I VEGF120/120 embryos were co-stained for β-catenin and F-actin. (**A**) β-catenin was normally expressed at the membrane of the WT lens cells and was intensely stained at the margin zone (*white arrowhead*). (**B**) In WT mice, F-actin was detected at the membrane of the lens cells. (**D–F**) β-catenin and F-actin expression appeared more diffuse in the lens fiber of VEGF120/120 lenses and lacked defined membrane localization. β;-catenin expression in the marginal zone of the VEGF120/120 lens appeared weaker compared to the WT. Scale bar: 50 μm.
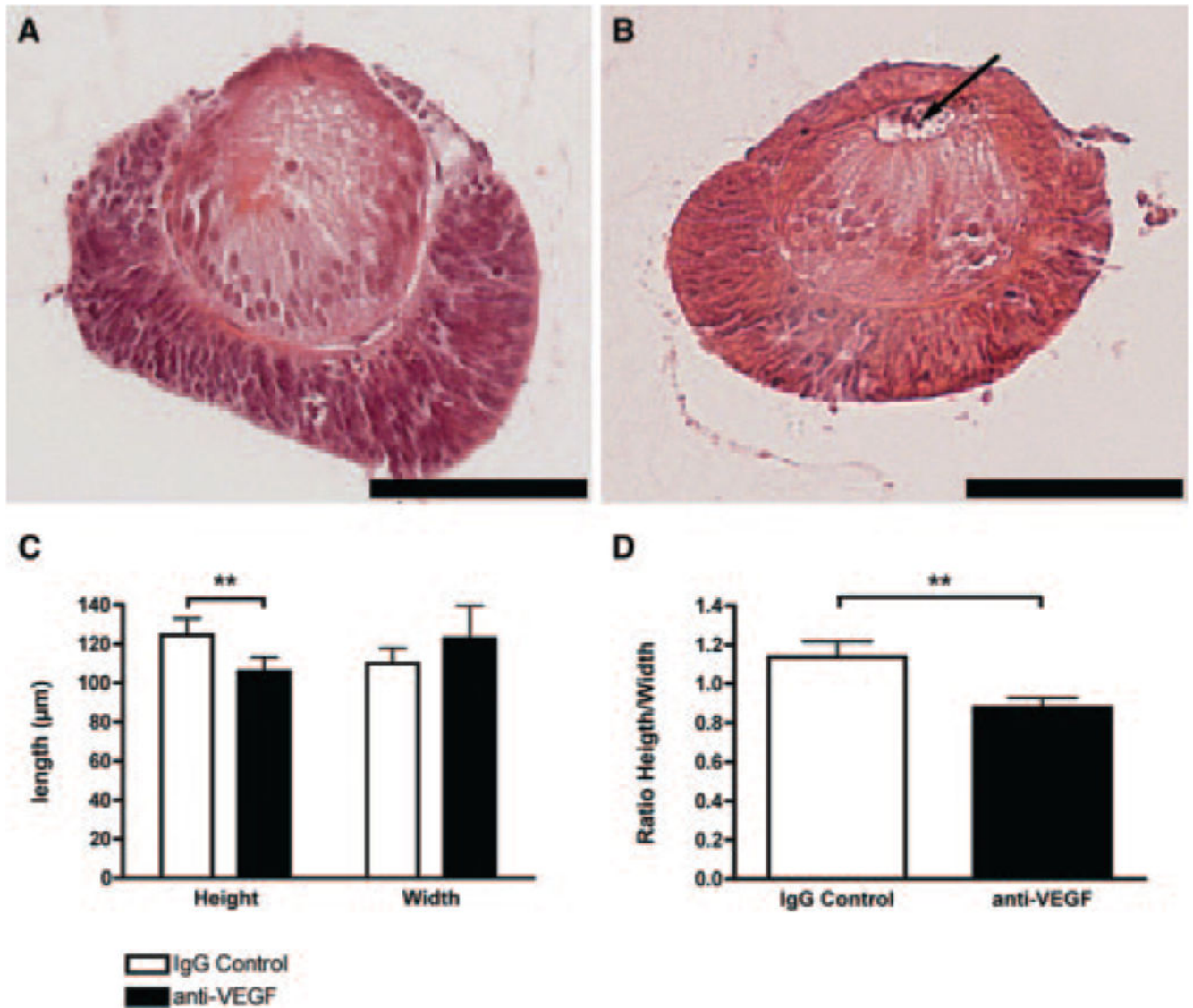
Figure 8.
VEGF neutralization in embryonic eye explants leads to reduced lens fiber extension. Eye rudiments of WT E11.5 embryos were cultured in collagen gels for 48 hr in the presence of VEGF neutralizing antibody or IgG as negative control. (**A**) In the control explants, lens differentiation and extension in vitro led to a complete closure of the lens vesicle. (**B**) Half of the eye rudiment explants treated with VEGF neutralizing antibody failed to close the lens vesicle (*arrow*). (**C**) Comparison of the height and width of lenses (in the largest lens section) revealed that the inhibition of VEGF led to a reduction in lens height while the width is unaffected. (**D**) Reduction of lens fiber extension led to a smaller height/width ratio. Statistical analysis was performed using an unpaired Studen's t-test (**$P < 0.01$) for the height and width measurements and a non-parametric Man-Whitney test (**$P = 0.0061$) for the ratio height/width. Scale bar: 100 μm.
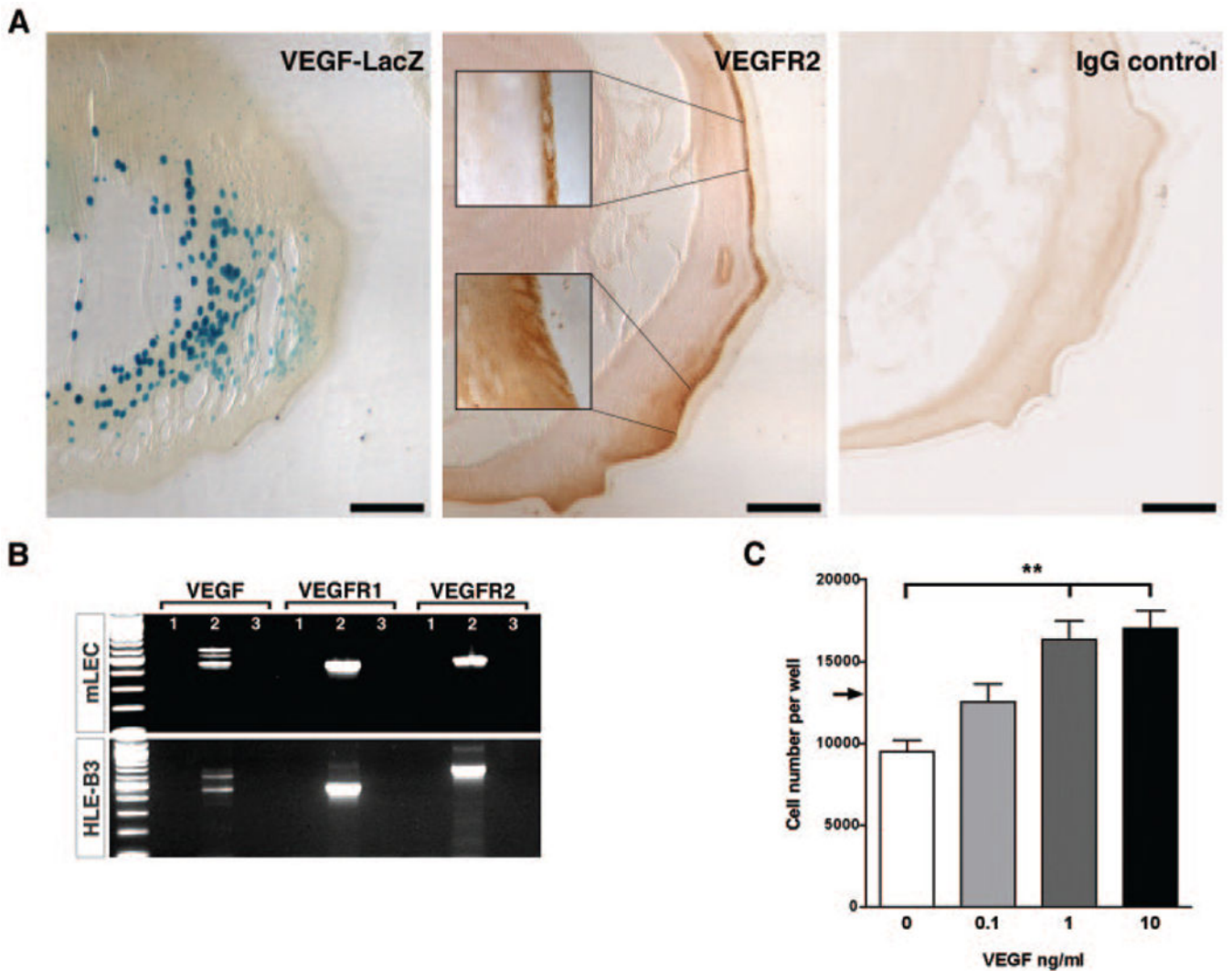
Figure 9.
VEGF and its receptors are expressed in adult lens epithelial cells and exogenous VEGF increases HLE-B3 cells proliferation. (**A**) Cryosections of eyes from 8-week-old VEGF-LacZ/+ mice were stained for β-galactosidase, VEGFR2, or with rabbit IgG as control. In adult lens, strong VEGF expression was observed in nucleated lens secondary fibers. At this stage, no LacZ staining was detected in the lens epithelium. VEGFR2 immunostaining revealed a robust expression in both the lens epithelium and the elongating lens fibers (see *insets*) while immunoreactivity was less in the differentiated lens fibers. (**B**) Expression of VEGF and its receptors VEGFR1 and VEGFR2 was detected by RT-PCR in mouse primary lens epithelial cells and the human cell line HLE-B3. *Lane 2* represents PCR reactions using cDNA template. PCR reactions without cDNA (*lane 1*) or using non-reverse-transcribed mRNA (*lane 3*) are presented as negative controls. (**C**) Addition of VEGF to HLE-B3 cultured in reduced serum increased cell proliferation in a dose dependent manner. *Arrow* indicates the cell number at the beginning of the experiment ($13,008 \pm 333$). Results are presented as mean $\pm$ SE of 3 independent experiments with 4 wells per condition for each

experiment. Statistical analysis was performed using an unpaired Studen's t-test, $**P < 0.01$. Scale bar: 100 μm.

Table 1.

RT-PCR Primers

Gene	Primer Forward (5′-3′)	Primer Reverse (5′-3′)	Product Size
Mouse			
VEGFR1	gagagcatctataaggcagcggatt	cacgtttacaatgagagtggcagtg	456 bp
VEGFR2	tacacaattcagagcgatgtgtggt	ctggttcctccaatgggatatcttc	499 bp
VEGF	cctccgaaaccatgaactttctgctc	cagcctggctcaccgcctggctt	665bp (VEGF188)
			593bp (VEGF164)
			461bp (VEGF120)
Human			
VEGFR1	caagtggccagaggcatggagtt	gatgtagtcttaccatcctgttg	498 bp
VEGFR2	gagggcctccatggtgattgt	tgccagcagtccagcatggtctg	709 bp
VEGF	cctccgaaaccatgaactttctgctg	ctgcccggctcaccgcctcggctt	665bp (VEGF189)
			593bp (VEGF165)
			461bp (VEGF121)