Vascular Endothelial Growth Factor Expression and Signaling in the Lens

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PURPOSE. Previous studies have identified sequences encoding vascular endothelial growth factor (VEGF)-A and one of the VEGF receptors (VEGFR2, Flk-1, KDR) in lens fiber cells. The current study was undertaken to determine the distribution of VEGF-A protein in the lens, whether signaling through VEGF receptors occurs in lens cells, the pattern of VEGF-A expression during lens development, and the effect of hypoxia on VEGF-A expression.

METHODS. VEGF-A and VEGFR2 were localized using immuno-cytochemistry. VEGF-A and VEGFR2 protein were identified and quantified by Western blot analysis. Activated (tyrosine phosphorylated) VEGFR2 was detected by immunoprecipitation with an anti-phosphotyrosine antibody followed by Western blot analysis with antibody to VEGFR2. Levels of VEGF-A mRNA were measured by quantitative PCR. Suturing the lids of adult mouse or rabbit eyes for 3 days was used to induce lens hypoxia.

RESULTS. VEGFR2 sequences were present in adult human lens epithelial cells, and VEGF-A transcripts were detected in chicken embryo, adult human, and mouse lens epithelial cells. VEGF-A protein localized to the ends of mouse embryo lens fiber cells at developmental stages when the fetal vasculature was forming. At later stages, VEGF-A was distributed uniformly throughout the cytoplasm of cortical fiber cells. VEGFR2 was present in mouse lens epithelial and fiber cells and was tyrosine phosphorylated at all stages examined. VEGF-A protein was barely detectable in lens epithelial cells during the first postnatal week, but increased as the capillaries of the anterior pupillary membrane regressed. VEGF-A levels were highest in adult lenses. Suturing the eyelid caused an increase in VEGF-A mRNA and protein in lens epithelial and fiber cells.

CONCLUSIONS. VEGF-A secreted by lens cells may stimulate the formation of the fetal vasculature, but regression of these vessels is not likely to be caused by a reduction in VEGF-A production by the lens. An active VEGF-A signaling system of unknown function appears to be active in the lens. It is likely that VEGF-A expression is regulated by tissue hypoxia at all stages of lens development. (*Invest Ophthalmol Vis Sci.* 2003; 44:3911–3919) DOI:10.1167/iovs.02-1226

Vascular endothelial growth factor-A is the founding member of a family of growth factors (VEGF-A through -D and placental growth factor [PlGF]) that play essential roles in the formation and maintenance of the blood and lymph vascular systems.[1,2] VEGF-A also plays an important role in physiological vascularization during wound healing and in the female reproductive cycle and contributes to pathologic neovascularization induced by local tissue ischemia and in the vascularization of tumors.[2] VEGF-A action is mediated primarily by binding to the transmembrane tyrosine kinase receptor VEGFR2 (Flk-1, KDR). A second transmembrane tyrosine kinase, the receptor VEGFR1 (Flt-1), modulates the levels of VEGF-A during embryonic development and mediates its effects in selected cell types.[3–5] Targeted disruption in mice of VEGFR2 genes or a single VEGF-A allele leads to death early in the second week of embryonic life, due to failure of vascular development.[6,7] VEGF-A levels are increased when cells are deprived of oxygen or glucose or by the activation of one of several growth factor–mediated signaling pathways.[2] Hypoxia stimulates the transcription of the VEGF-A gene by stabilizing and activating the transcription factor hypoxia inducible factor (HIF-1α), although VEGF-A protein levels are also regulated by posttranscriptional mechanisms.[8–13]

In mammals, a capillary plexus transiently surrounds the lens beginning early in its development. The capillary network at the posterior surface of the lens, the tunica vasculosa lentis (TVL), arises from the hyaloid artery. The vessels that cover the anterior lens surface form the anterior pupillary membrane (APM) and are derived from vasculature of the iris stroma. Together with the hyaloid artery, these vessels are referred to as the fetal vasculature of the eye. The fetal vasculature normally regresses prenatally in humans and during the first 2 weeks of postnatal life in rodents.[14–16] Several human diseases are associated with failure of the fetal vessels to regress completely,[16] and gene targeting studies in mice have identified genes that play a role in this process.[17,18] However, the factors that regulate the formation of the fetal vasculature and its orderly regression are not known.

VEGF-A mRNA has been detected in the lens fiber cells of mouse embryos, and VEGF-A mRNA and protein were found in adult rat lens epithelial cells.[19,20] Based on changes in the expression of VEGF-A mRNA during mouse lens development, Mitchell et al.[20] proposed that VEGF-A secreted from the lens attracts and stabilizes the capillaries of the TVL. They further suggested that a decrease in VEGF-A expression in lens cells causes the regression of the fetal vasculature. Studies with transgenic animals showed that overexpression of VEGF-A in the lens fiber cells caused overgrowth of the capillaries of the fetal vasculature and failure of vascular regression.[21] Other investigators provided evidence that circulating levels of VEGF-A are necessary to maintain the APM. Their data suggest that decreases in plasma VEGF-A contribute to the normal

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TABLE 1. Sequence of PCR Primers and Probes

Name	Sequence
Total VEGF-A PCR primers	5′-AGGCTGCTGTAACGATGAAAG-3′
	5′-GTCCTGTCTTTCTTTGGTCTGC-3′
VEGF-A$_{120}$ isoform PCR primers	5′-AGGCTGCTGTAACGATGAAAG-3′
	5′-CTGAACAAGGCTCACAGTGA-3′
VEGF-A$_{164}$ isoform PCR primers	5′-AGGCTGCTGTAACGATGAAAG-3′
	5′-CTCGGCTTGTCACATTTTTCT-3′
VEGF-A$_{188}$ isoform PCR primers	5′-AGGCTGCTGTAACGATGAAAG-3′
	5′-CAGGATTTAAACCGGGATTTCT-3′
VEGF-A *Taq*Man probe	6-FAM-5′-CCACGTCAGAGAGCAACATCACCATGC-3′
β-actin PCR primers	5′-GCCTCACTGTCCACCTTCCA-3′
	5′-AAACGCAGCTCAGTAACAGTCC-3′
β-actin *Taq*Man probe	6-FAM-5′-CCGGACTCATCGTACTCCTGCTTGCTG-3′

*Taq*Man (Applied Biosystems, Inc.)

regression of the APM,[22] raising questions about the importance of VEGF-A in the lens in this process.

Chicken embryo lenses express VEGFR2,[23] and exogenously added VEGF-A stimulates the activity of phosphatidylinositol-3 kinase (PI-3 kinase) in cultured porcine lenses.[24] These observations raise the possibility that signaling through VEGFR2 plays some role in lens development and/or function.

The present study was performed to investigate the expression of VEGF-A and VEGFR2 protein and mRNA in the lens during embryonic and postnatal life and to determine whether VEGFR2 is normally activated in vivo. We also tested whether hypoxia can regulate VEGF-A expression in the adult lens in vivo. The results of these studies are consistent with a role for VEGF-A in the formation of the fetal vasculature, but do not support the view that the regression of these vessels is regulated by a decrease in VEGF-A from the lens. VEGF-A mRNA and protein were present in adult lenses and were increased by hypoxia, suggesting that the lens is normally in a hypoxic state and that VEGF-A expression is regulated by hypoxia in the lens, as it is in many other tissues.

MATERIALS AND METHODS

Human lenses were obtained from eyes donated for corneal transplantation to Mid America Transplant Services. The use of human lenses was approved by the Washington University Institutional Review Board and conformed to the Declaration of Helsinki. All experiments using animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Washington University Animal Studies Committee. Adult mice and rats were killed by CO_2 inhalation and rabbits by overdose of pentobarbital. Eyes were removed immediately and either fixed or kept on ice. Lenses were removed and epithelia and fibers separated by dissection. To decrease oxygen levels in the anterior chamber, mice or rabbits were anesthetized with ketamine (30 mg/kg) and medetomine (1 mg/kg; for mice) or xylazine (5 mg/kg; for rabbits), and one eyelid from each animal was closed with 6-0 (rabbits) or 7-0 (mice) silk sutures for 3 days. Care was taken that the sutures did not penetrate the inner surface of the lid where they might irritate the corneal epithelium.

For immunocytochemistry, mouse embryos and adult eyes were fixed in 10% neutral buffered formalin and tissue sections prepared after embedding in paraffin. Sections pretreated by boiling for 15 minutes in 0.01 M citrate buffer (pH 6.0) and stained with a 1:500 dilution of polyclonal rabbit anti-VEGF-A antibody (SC-507; Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:2000 dilution of rabbit anti-VEGFR2 (SC-315; Flk-1; Santa Cruz Biotechnology). Control experiments were performed by omitting the primary antibody or by pretreating the VEGF-A antibody with a 10-fold excess (by weight) of human VEGF-A (R&D Systems, Minneapolis, MN) or a 5-fold excess of

the peptide against which the VEGFR2 antibody had been generated (SC-315p; Santa Cruz Biotechnology).

Western blot analysis was performed by standard methods, and bands were visualized using chemiluminescence. Each experiment was performed a minimum of three times. Tissues were prepared in radio-immunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail at the recommended concentration (Complete; Roche Diagnostics, Indianapolis, IN) and 0.2 mg/mL sodium orthovanadate. Protein concentration was measured using a reagent (D_C Protein Assay Reagent; Bio-Rad Laboratories, Hercules, CA) in microtiter plates with bovine serum albumin as a standard. Each blot was stained with ponceau S to confirm that equal amounts of total protein were loaded. The specificity of the blots was determined by adding a 10-fold excess (by weight) of human VEGF-A or a 5-fold excess of the immunizing peptide for VEGFR2 (SC-315p; Santa Cruz Biotechnology) to the diluted antibody and incubating at 4°C overnight before adding the mixture to the blot. In some experiments, antigens were first immunoprecipitated with one primary antibody and the immunoprecipitated proteins Western blotted with a second antibody. For VEGF-A, five whole adult mouse lenses were incubated in 1 mL of lysis buffer for 1 hour at 4°C and the lysate centrifuged for 5 minutes at approximately 2000g. Mouse anti-VEGF-A monoclonal antibody (12 μL SC-7269) was added to 100 μL of the supernatant and incubated at 4°C for 2 hours, and 30 μL of protein A/G beads (SC-2003; Santa Cruz Biotechnology) were then added and incubated overnight in a cold room with gentle rocking. The beads were pelleted by gentle centrifugation, the supernatant discarded, and the beads washed once with PBS, resuspended in 15 μL of electrophoresis loading buffer, and heated at 80°C for 3 minutes before electrophoresis. The blot was probed overnight at 4°C or for 2 hours at room temperature with anti-VEGF-A rabbit polyclonal antibody (SC-507; Santa Cruz Biotechnology) at a dilution of 1:1,000 and the bound antibody detected with anti-rabbit secondary antibody conjugated to horseradish peroxidase at a dilution of 1:10,000 (SC-2030; Santa Cruz Biotechnology). A similar procedure was used to detect VEGFR2 (Flk-1) extracted from adult mouse lenses, except that the mouse monoclonal antibody used for immunoprecipitation was SC-6251, and the rabbit polyclonal antibody used for blotting was SC-315 (both from Santa Cruz Biotechnology) at a dilution of 1:500. Tyrosine phosphorylated VEGFR2 was immunoprecipitated by using agarose beads coupled to monoclonal anti-phosphotyrosine (SC-707; Santa Cruz Biotechnology), the bound material was dissolved in electrophoresis sample buffer, and the blot probed with anti-VEGFR2 (SC-315; Santa Cruz Biotechnology).

VEGF-A mRNA levels were measured and compared with β-actin mRNA levels in mouse lenses at postnatal day 5 (P5), P10, P12, P16 and in adults using *Taq*Man probes (Applied Biosystems, Inc., Foster City, CA) to perform quantitative PCR. PCR primers were designed to amplify mouse β-actin, total mouse VEGF-A, and each of the three isoforms of mouse VEGF-A (VEGF-A$_{120}$, -A$_{164}$, and -A$_{188}$). The PCR

FIGURE 1. Distribution of VEGF-A immunoreactivity in the lens during pre- and postnatal development. (A) At E12.5 the lens fiber cell cytoplasm had granular immunostaining with increased staining at the posterior ends of the fiber cells (*arrow*). Staining was stronger in the lens than in other periocular tissues. (B) A section of an E15.5 lens stained with anti-VEGF-A antibody that had been treated with an excess of human VEGF-A. Only background staining was seen. Similar control experiments or controls in which the primary antibody was omitted were performed at each stage examined. None of the controls showed any staining. (C) At P3, staining for VEGF-A was uniform throughout the lens fiber cell cytoplasm. (D) By P13, staining persisted in the superficial fiber cells, but the cells in the center of the fiber mass no longer stained for VEGF-A. c, cornea; l, lens; le, lens epithelium; r, developing retina; v, fetal vasculature.
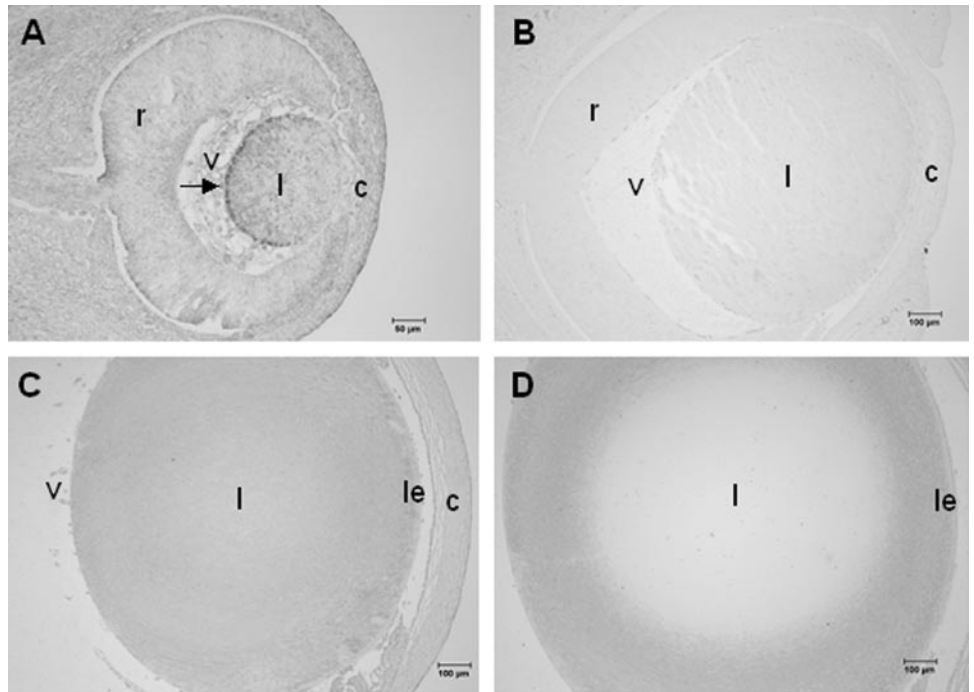
primers and probe sequences are listed in Table 1. The single *Taq*Man probe detected total VEGF-A mRNA and each of the isoforms of VEGF-A, depending on the primer pairs used. Total RNA was extracted with an RNA isolation reagent (RNAwiz; Ambion, Austin, TX) and treated with DNase (DNA-free; Ambion) to remove any contaminating genomic DNA, and 1 μg was reverse transcribed (RetroScript reverse transcriptase; Ambion) in a total volume of 20 μL according to the instructions provided by the manufacturer. For each 50-μL PCR reaction, 0.6 μL of the reverse transcription product was amplified in a thermal cycler (iCycler; Bio-Rad), using the *Taq*Man probe at a final concentration of 0.2 μM in a standard PCR mixture with *Taq* polymerase (Ampli*Taq* Gold; Applied Biosystems, Inc.). PCR efficiency was determined using serial dilution of a known concentration of a plasmid containing the β-actin sequence. Each sample was run in triplicate, and each experiment was performed at least twice.

RESULTS

Preliminary and published studies from our laboratory and others showed that VEGF-A and VEGFR2 mRNAs were present in the lenses of several species, including human, rat, and chicken embryo.[19,20,23] Based on these findings, we initiated studies to determine whether the proteins encoded by these mRNAs were expressed and functioning in the lens.

We first determined whether VEGF-A protein was expressed in the lens at the time that the TVL was forming. Mouse embryos at embryonic day (E)11.5 or E12.5 were fixed, and the eyes were sectioned and stained with a polyclonal antibody to VEGF-A. Granular immunostaining was present throughout the lens fiber cell cytoplasm with stronger staining concentrated at the ends of the fiber cells (Fig. 1A). In some embryos, the strongest staining was present only at the posterior pole of the fiber cells, near the forming TVL, whereas staining in others localized to both ends of the fiber cells. At these stages, staining for VEGF-A was more intense in the lens than in any of the surrounding ocular tissues. Addition of excess recombinant human VEGF-A to the antibody reduced immunostaining markedly at all stages (Fig. 1B). At later stages of embryonic and early postnatal development, VEGF-A immunoreactivity was diffusely distributed throughout the cytoplasm of the cortical fiber cells (Fig. 1C). Immunostaining decreased markedly in the nucleus of older lenses (Fig. 1D). At all stages, immunostaining was lower in the epithelial cells than in the fiber cells.

VEGF-A expression in postnatal lenses was examined by Western blot analysis. When extracts from lens fiber or epithelial cells were separated by electrophoresis and probed with a polyclonal antibody to VEGF-A, a single major band was detected at a molecular mass of ~25 kDa. We performed two experiments to verify the identity of this putative VEGF-A band. Lysates of whole adult mouse lens were immunoprecipitated with a monoclonal antibody to VEGF-A, and the immunoprecipitates were separated on polyacrylamide gels, transferred to nitrocellulose membranes, and probed with an anti-VEGF-A polyclonal antibody. A single band was detected at the appropriate relative molecular mass (Fig. 2A). We also pretreated the anti-VEGF-A polyclonal antibody with an excess of recombinant human VEGF-A and compared the ability of the untreated and the pretreated antibody to detect VEGF-A by Western blot. In this experiment, fiber masses from lenses of different postnatal ages were isolated and Western blotted with an antibody to VEGF-A. Pretreatment of the antibody with VEGF-A markedly reduced the reactivity of the antibody with the putative VEGF-A band (Fig. 2B). This experiment also showed that VEGF-A increased in fiber cells with age.

In mice, the fetal vasculature begins to regress during the first postnatal week and regression is nearly complete by the time of eye opening at postnatal day (P)14.[15] We used Western blot analysis for VEGF-A in lens epithelial cells during and after this period to determine whether a decrease in VEGF-A levels was associated with vascular regression, as predicted by Mitchell et al.[20] Lens epithelia were dissected from P5, P10, P16, and adult lenses. At P5 and P10, epithelia either included the associated TVL and APM, or these vascular tissues were carefully removed during dissection. We did not try to remove the few remnants of the fetal vasculature that were associated with lens epithelia isolated at P16. As in the lens fiber cells shown in Figure 2B, VEGF-A levels were lowest in epithelia isolated at P5 and increased at later stages, reaching a maximum in adults (Fig. 3). The fetal vasculature accounted for most of the VEGF-A at P5, because when this tissue was removed, little
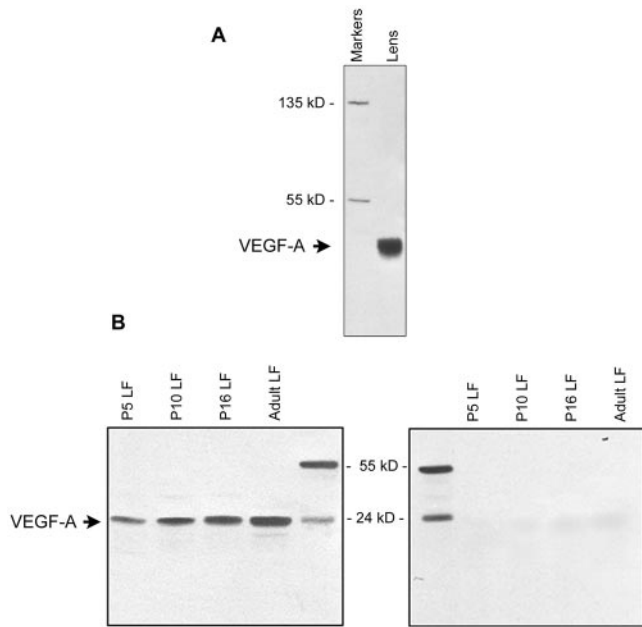
FIGURE 2. VEGF-A is present in lens cells at all postnatal ages. (**A**) VEGF-A was immunoprecipitated from an extract of whole adult lenses with a mouse monoclonal antibody to VEGF-A and Western blotted using a polyclonal antibody. The molecular weight marker at ~24 kDa does not show up in this image, because the VEGF-A band was strong and the exposure was therefore short. Longer exposures showed the marker and revealed that the stained band was of the correct size for VEGF. (**B**) *Left*: bands detected by a polyclonal anti-VEGF-A antibody in 10 micrograms of protein extracted from lens fibers (LF). *Right*: results obtained when the same primary antibody was mixed with human VEGF-A before being added to the blot. In lens fiber cells, VEGF-A levels increased with increasing age.

VEGF-A was detected in the remaining lens epithelial cells. The fetal vasculature accounted for about half of the VEGF-A detected at P10. The level of VEGF-A in fiber cells was generally higher than in the epithelial cells at a given stage (data not shown). If the VEGF-A levels detected in these studies reflect the amount of VEGF-A secreted by the lens, decreases in
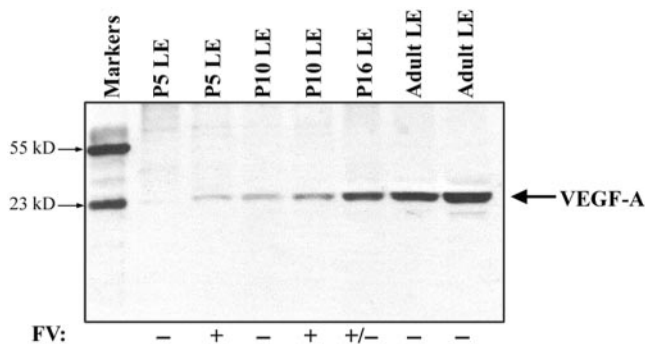

FIGURE 3. VEGF-A protein levels increase in postnatal mouse lens epithelial cells (LE) from ages P5 to adult. In some samples at P5 and P10 the capillaries around the lens (FV; fetal vasculature) were removed during dissection, whereas they were not removed in others. At P5, most of the VEGF-A immunoreactivity was associated with the fetal vasculature, whereas at P10 roughly equal levels were present in the lens epithelial cells and the vessels. Higher levels of VEGF-A accumulated in the epithelial cells as the fetal vasculature regressed, reaching a maximum in the adult. Duplicate samples from adult epithelial cells are shown. Fifteen micrograms of total protein was loaded in each lane.
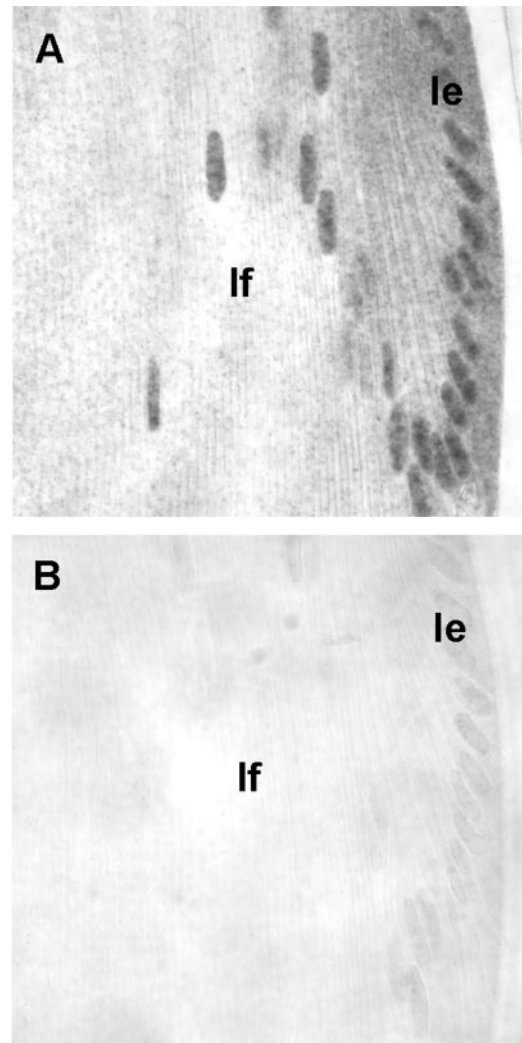

FIGURE 4. The distribution of VEGFR2 in lens cells. (**A**) Antibody to VEGFR2 (Flk-1) stained the membranes and nuclei of epithelial cells at the lens equator and elongating fiber cells. le, lens epithelial cells; lf, lens fiber cells. (**B**) Addition of the immunizing peptide to the antibody substantially reduced staining over the entire section, indicating that all staining in (**A**) was specific.

VEGF-A from the lens are not likely to be responsible for the regression of the fetal vasculature.

We also examined the expression of VEGFR2 in the lens. With a polyclonal antibody, immunostaining was present throughout the lens epithelium and nucleated fiber cells of the P10 (not shown) and adult lens (Fig. 4A). Staining was particularly prominent along the fiber cell membranes and in the nuclei of both epithelial and fiber cells. Translocation of VEGFR2 to the nucleus after stimulation with VEGF-A has been reported.[25] Immunostaining was reduced substantially by adding an excess of the peptide against which the antibody had been raised (Fig. 4B).

We confirmed the presence of VEGFR2 in the mouse lens by immunoprecipitation followed by Western blot analysis (Fig. 5A) and by blocking the antibody with the immunizing peptide (Fig. 5B). Several lower molecular mass bands were detected by both tests, suggesting that VEGFR2 is degraded by sequential processing. A modest increase in the amount of receptor protein was noted when fiber cell extracts from ages P5 through adult were compared (Fig. 5C).
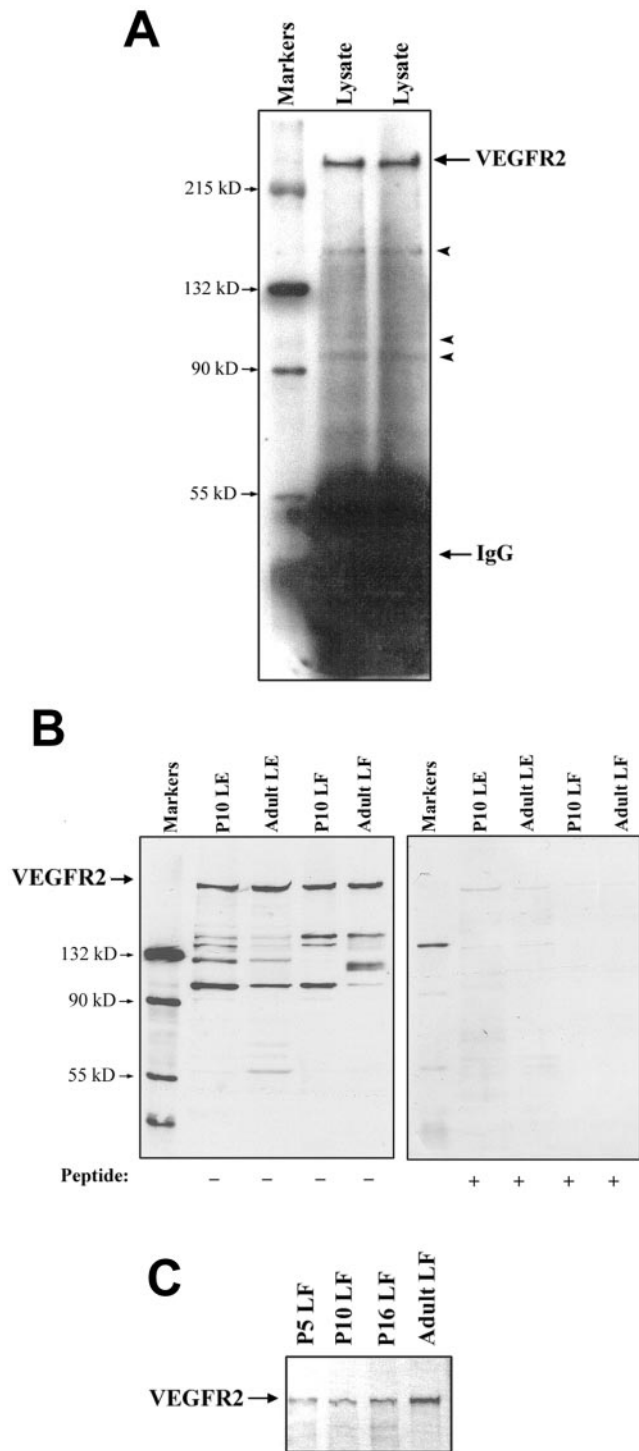
FIGURE 6. Extracts of 38 P10 and P20 adult mouse lens epithelia and fiber masses were immunoprecipitated with antibody to phosphotyrosine and Western blotted with a polyclonal antibody to VEGFR2. Bands corresponding to tyrosine phosphorylated FGFR2 (pY-FGFR2) were detected in P10 and adult lens fiber cells in this experiment. The bands due to cross reactivity between the primary antibody used for immunoprecipitation and the secondary antibody used for detecting polyclonal anti-VEGFR2 are labeled IgG.

Our results showed that VEGF-A and VEGFR2 are expressed in the same regions of the lens. We, therefore, tested whether autocrine or paracrine activation of VEGF-A receptors occurs in vivo. Lenses were removed immediately after death, and epithelial and fiber cells were dissected in ice-cold medium. Proteins containing phosphotyrosine were immunoprecipitated, blotted, and probed with polyclonal antibody to VEGFR2. Figure 6 shows that bands corresponding to tyrosine-phosphorylated VEGFR2 were detectable in the fiber cells of P10 and adult lenses. Although tyrosine-phosphorylated VEGFR2 was not detected in extracts of epithelial cells in this experiment, the activated receptor was detected in P10 and adult lens epithelial cells in other tests (data not shown).

The increase in VEGF-A protein in lens epithelial and fiber cells that occurred as the fetal vasculature regressed suggests that VEGF-A levels in the lens may be regulated by hypoxia. To test this directly, we closed one eyelid of adult mice for 3 days and analyzed VEGF-A levels in lenses removed from eyes with closed or open lids. Eyelid closure was associated with a substantial increase in VEGF-A accumulation in the lens epithelium (Fig. 7A). Similar experiments performed on rabbits also demonstrated a large increase in VEGF-A levels after lid closure (Fig. 7B).

To examine further the regulation of VEGF-A expression, VEGF-A transcript levels were compared to the levels of β-actin mRNA using quantitative PCR. In general agreement with measures of VEGF-A protein, VEGF-A sequences increased during postnatal development (Fig. 8A). However, the maximum expression of VEGF-A, relative to β-actin, occurred at P10 and

FIGURE 5. (A) Proteins extracted from five whole adult mouse lenses were immunoprecipitated with a monoclonal antibody to VEGFR2 and Western blot analysis conducted with an anti-VEGFR2 polyclonal antibody. The blot shows the results obtained with duplicate samples. In addition to the native receptor, bands of lower molecular weight were detected (*arrowheads*). This suggests that lens cells sequentially process the receptor to lower molecular mass forms. The large smear at ~50 kDa is due to cross reactivity between the monoclonal antibody used for immunoprecipitation (IgG) and the peroxidase-labeled secondary antibody used for detection. (B) VEGFR2 was detected in P10 and adult lens epithelial and fiber cells by Western blot analysis. Multiple lower molecular mass bands were detected. The most abundant of these correspond to the lower molecular mass bands in (A) after immu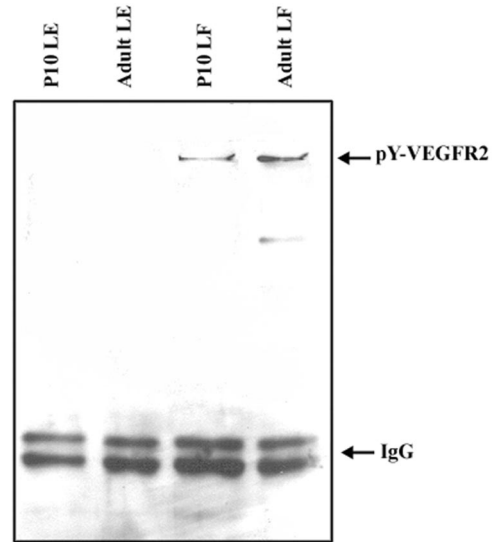noprecipitation. Pretreatment of the antibody with the immunizing peptide greatly reduced staining in all bands, supporting the view that these bands were due to proteolytic processing of the receptor. Fifteen micrograms of total protein was loaded in each lane. (C) VEGFR2 levels increased in lens fiber cells during postnatal development. Only bands corresponding to the full-length protein are shown in this view. Fifteen micrograms of total protein was loaded in each lane.
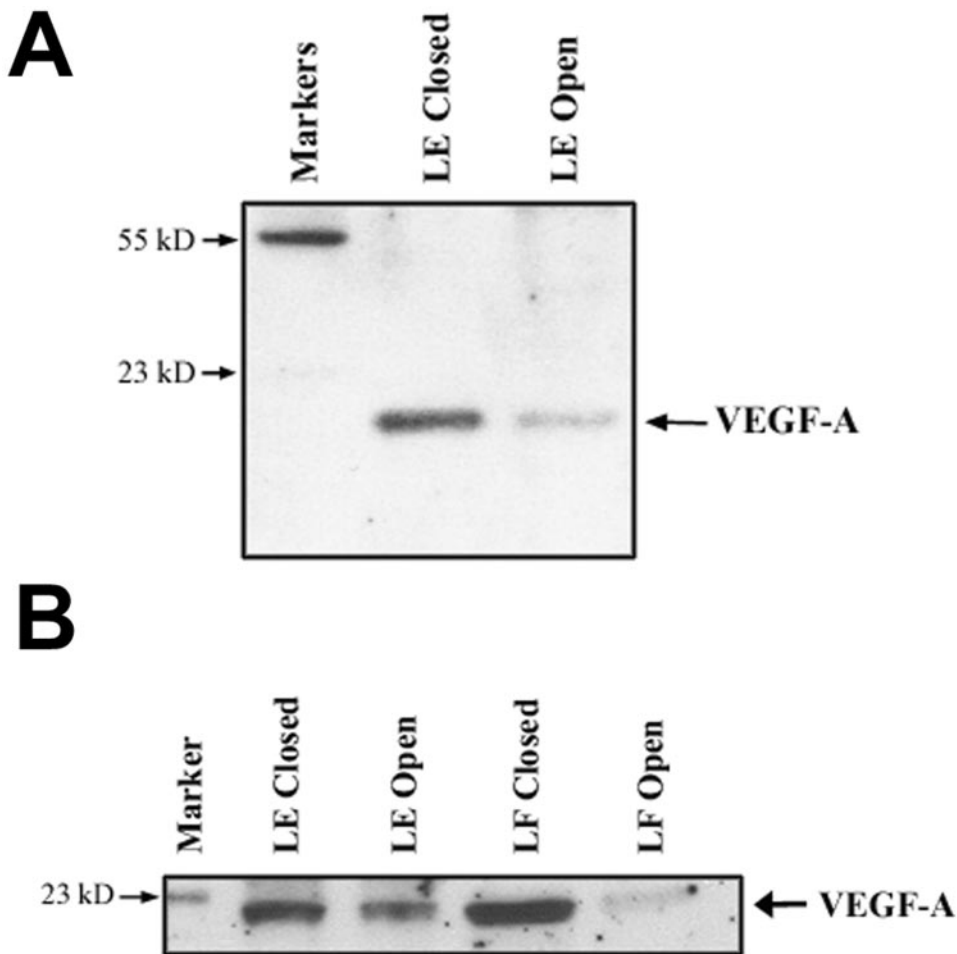
FIGURE 7. (A) Mice were anesthetized, and one eyelid was sutured. After 3 days, the animals were killed, and the lens epithelia were removed and the epithelial proteins analyzed by Western blot analysis. VEGF-A levels increased after eyelid closure. VEGF-A levels were not measured in fiber cells in this experiment. Ten micrograms of total protein was loaded in each lane. (B) In a similar experiment performed on rabbits, VEGF-A levels increased in lens epithelial and fiber cells after eyelid closure. Ten micrograms of total protein was loaded in each lane.

P16, with a decrease in transcript levels between P16 and adulthood. Similar relative changes in abundance occurred for two of the VEGF-A isoforms that are generated by alternative splicing of the VEGF-A transcript, $VEGF-A_{120}$ and $VEGF-A_{164}$ (Fig. 8B). A third isoform, $VEGF-A_{188}$, was present but accounted for less than 10% of the total VEGF-A transcripts and was not examined further in this study. Eyelid closure for 3 days resulted in an increase in VEGF-A transcripts, compared with the contralateral, control eye (Figs. 8A, 8B). The change in total VEGF-A after eyelid closure did not reach statistical significance. When the data for the measurements of total VEGF-A and the two VEGF-A isoforms were combined, the increase after eyelid closure was statistically significant ($P = 0.015$). These data suggest that increases in VEGF-A protein levels during postnatal development or after eyelid closure are partly due to increases in the levels of VEGF-A mRNA. The increase in VEGF-A levels in the face of declining VEGF-A mRNA levels after P16 suggests that VEGF-A levels are also controlled by posttranscriptional mechanisms, as described previously in other cell types.[13]

DISCUSSION

Mammalian lenses are avascular throughout most of life, and the lenses of nonmammalian vertebrates are never associated with a vascular plexus. For this reason, it was initially surprising to find VEGFR2 and VEGF-A mRNA expressed in chicken embryo and adult human lenses, because these proteins are best known as key components of a signaling system that regulates the differentiation, growth, and survival of vascular endothelial cells. However, it is possible that the expression of VEGF-A in the lens is related to the unusual structure of this tissue. As it forms, the lens becomes a solid mass of epithelial cells. Compared with most epithelia that are closely associated with well-vascularized connective tissues, lens cells are separated from a vascular supply for most of their lives and are likely to be in a state of relative hypoxia. In most tissues, VEGF-A mRNA and protein levels increase greatly during hypoxia as a consequence of the action of the oxygen-regulated transcription factor, HIF.[2,26] Stabilization of VEGF-A mRNA[12] and an increase in the relative efficiency of its translation[13] also contribute to the increases in VEGF-A that are induced by hypoxia.

There are four aspects of the present work that are consistent with the hypothesis that VEGF-A expression in the lens is regulated by hypoxia. In early lens development, just as the lens becomes a solid mass of epithelial cells, VEGF-A is expressed at higher levels in the lens than in the surrounding ocular tissues. It is at this stage that the capillaries of the fetal vasculature begin to surround the lens. Later, as these capillaries regress, our data show that VEGF-A levels increase in lens epithelial and fiber cells. This suggests that vascular regression causes the lens to become more hypoxic and, consequently, to express more VEGF-A. It is noteworthy that the fiber cells consistently produced more VEGF-A than the epithelial cells. The epithelial cells are at the surface of the lens and would be maximally exposed to oxygen diffusing from the fetal capillaries or other nearby tissues. Many of the fiber cells are buried deep within the lens. Oxygen consumption by overlying fiber cells should make these cells more hypoxic and thus produce
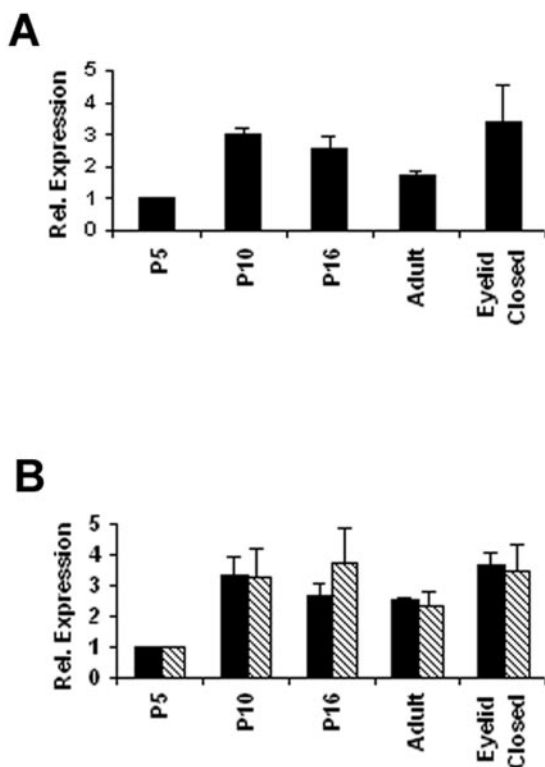
FIGURE 8. (A) Changes in the relative expression of total VEGF-A during postnatal development and after 3 days of lid suture in adult mice. VEGF-A transcript levels were measured by quantitative PCR and expressed relative to the levels of β-actin mRNA. Values are normalized to the levels of VEGF-A transcripts at P5 and represent triplicate determinations from two experiments. Error bars, SD. (B) Relative expression of VEGF-A$_{120}$ (■) and VEGF-A$_{164}$ (▨) transcripts during postnatal development and after 3 days of lid suture in adult mice. Transcript levels are normalized as in (A). Data are triplicate determinations from two experiments. Error bars, 1 SD.

more VEGF-A. Third, adult mouse and rabbit lens epithelial cells express VEGF-A. This is likely to be associated with the low levels of oxygen that have been measured in the anterior chamber. Below 5% O_2, most tissues show signs of hypoxia.[27] The partial pressure of oxygen in the anterior chamber measured in humans, rats, rabbits, and dogs ranges between 8 and 64 mm Hg ($\sim$1%–8% O_2), with an average of 38 mm Hg ($\sim$5% O_2; reviewed in Ref. 28). When measurements are made close to the anterior surface of the lens, oxygen levels are at the lower end of this range.[29–31] Closing the lids lowers the oxygen in the middle of the anterior chamber by approximately 50%.[29] We found that VEGF-A protein is expressed constitutively by adult lens epithelial cells and increases substantially after lid closure. Taken together, these studies suggest that, throughout development, VEGF-A expression in the lens is at least partially regulated by hypoxia.

It is likely that hypoxia controls the expression of other genes in the lens, in addition to VEGF-A. In most cell types, HIF increases the expression of mRNAs that encode genes that are of benefit to cells in a hypoxic environment, including glucose transporters and enzymes of the glycolytic pathway.[27] It has long been known that the lens can survive in the absence of oxygen if sufficient glucose is provided.[32] It has abundant glycolytic enzymes[33] and normally derives much of its ATP from glycolysis.[34] Lens epithelial and fiber cells also have abundant glucose transporters.[35] Therefore, some of the metabolic characteristics of lens cells may be specified by HIF in response

to the hypoxic environment around the lens, rather than being "hard wired" by the lens differentiation program.

Our observation that VEGF-A protein is expressed in the lens near the forming TVL is consistent with the suggestion of Mitchell et al.[20] that VEGF-A from the lens may be necessary for the formation of the fetal vasculature.[20] However, neither study provides evidence that VEGF-A is required for this process. Experiments under way in our laboratory may resolve this question by using the Cre-loxP system to delete the VEGF-A gene selectively from the lens early in its development.

The VEGF-A that is produced in the lens may affect other intraocular tissues. Low levels of VEGF-A have been detected in human vitreous humor in patients with no ischemia or active vasoproliferation,[36–38] although other studies have not found detectable levels of the growth factor in the vitreous unless retinal detachment or some sign of ischemia was also present.[39,40] Differences in the detection of VEGF-A in the vitreous humor could be due to differences in the sensitivity of the assays used. It has been suggested that the balance in the vitreous humor between the levels of VEGF-A and the putative antiangiogenic factor, pigment epithelium-derived factor (PEDF) is an important determinant of retinal neovascularization.[36,39,40] In one study, PEDF levels were lower in patients with choroidal neovascular membranes than in age-matched patients with no neovascularization. Both groups had similar low levels of VEGF-A in the vitreous humor. These investigators suggested that when PEDF levels decrease, even the low level of VEGF-A found in the vitreous humor can stimulate neovascularization.[36] The lens is a possible source for the VEGF-A that has been detected in normal vitreous humor. Comparing the levels of VEGF-A in the vitreous humor of age-matched individuals with phakic or aphakic eyes could test this possibility.

Little is known about the regulation of VEGFR2 expression in nonvascular tissues. It is not clear whether VEGFR2 expression in the lens is dynamically regulated by the levels of VEGF-A, as is seen in endothelial cells,[41,42] or whether the expression of this receptor is a normal aspect of the lens differentiation program.

Our studies suggest that the VEGF-A signaling system functions in the lens, because VEGFR2 is tyrosine phosphorylated in vivo. In addition, antibody to VEGFR2 stained the nuclei of lens epithelial and fiber cells. Previous studies have shown that nuclear localization of VEGFR2 protein is triggered by exposure to VEGF-A.[25] In vascular endothelial cells, VEGF-A stimulates cell differentiation, proliferation, survival, migration, and an increase in epithelial permeability.[2] However, it is not yet known whether endogenous VEGF-A transduces a signal that alters the behavior of lens cells. It is possible that the major function of VEGFR2 is to prevent most of the VEGF-A that is produced by the lens from diffusing into the ocular media by binding to and internalizing the growth factor, thereby targeting it for intracellular degradation. This mechanism could protect other parts of the eye from the harmful effects of high levels of VEGF-A.

An active VEGF-A signaling system is present in other avascular tissues during their differentiation. Hypertrophic chondrocytes secrete VEGF-A and contain tyrosine phosphorylated VEGFR2 in vivo.[43] Inhibition of VEGF-A signaling by administration of a soluble chimeric receptor protein to postnatal mice inhibited the development of long bones by reducing blood vessel invasion, reducing trabecular bone formation, and expanding the zone of hypertrophic cartilage.[44] Removal of the inhibitor restored vascularization and normal cartilage remodeling. However, this study could not show whether the chimeric receptor blocks cartilage remodeling indirectly, by reducing vascularization; directly, by preventing autocrine or paracrine VEGF-A signaling in chondrocytes; or both. Signaling by VEGF-A is also essential in bone repair, where it functions by

increasing vascularization and by autocrine stimulation of osteoblast activity.[45] In this case, the function of VEGF-A on nonvascular cells can be separated from its effect on the vascular system. VEGFR2 is expressed in retinal progenitor cells adjacent to cells expressing VEGF-A.[46,47] Administration of an inhibitor that blocks signaling through VEGFR1 and VEGFR2 to postnatal mice at a stage when the peripheral retina is still avascular causes thinning of the inner layers of the retina.[46] Unfortunately, the cellular changes brought on by this treatment were not investigated. These studies show that the molecular events triggered by activation of VEGFR2 in nonvascular tissues are largely unknown. Selective deletion of the VEGF-A gene in the lens may provide insight about the function of signaling mediated by VEGFR2 in this tissue.

Acknowledgments

References

1. Dumont DJ, Fong GH, Puri MC, Gradwohl G, Alitalo K, Breitman ML. Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Dev Dyn*. 1995;203:80–92.

2. Ferrara N. Molecular and biological properties of vascular endothelial growth factor. *J Mol Med*. 1999;77:527–543.

3. Fong GH, Zhang L, Bryce DM, Peng J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. *Development*. 1999;126:3015–3025.

4. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*. 1995;376:66–70.

5. Zachary I, Gliki G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. *Cardiovasc Res*. 2001;49:568–581.

6. Ferrara N, Carver-Moore K, Chen H, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*. 1996;380:439–442.

7. Carmeliet P, Ferreira V, Breier G, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*. 1996;380:435–439.

8. Kevil CG, De Benedetti A, Payne DK, Coe LL, Laroux FS, Alexander JS. Translational regulation of vascular permeability factor by eukaryotic initiation factor 4E: implications for tumor angiogenesis. *Int J Cancer*. 1996;65:785–790.

9. Prats AC, Prats H. Translational control of gene expression: role of IRESs and consequences for cell transformation and angiogenesis. *Prog Nucleic Acids Res Mol Biol*. 2002;72:367–413.

10. Ikeda E, Achen MG, Breier G, Risau W. Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. *J Biol Chem*. 1995;270:19761–19766.

11. Levy AP, Levy NS, Goldberg MA. Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. *J Biol Chem*. 1996;271:2746–2753.

12. Stein I, Neeman M, Shweiki D, Itin A, Keshet E. Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregulation with other ischemia-induced genes. *Mol Cell Biol*. 1995;15:5363–5368.

13. Stein I, Itin A, Einat P, Skaliter R, Grossman Z, Keshet E. Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implications for translation under hypoxia. *Mol Cell Biol*. 1998;18:3112–3119.

14. Latker CH, Kuwabara T. Regression of the tunica vasculosa lentis in the postnatal rat. *Invest Ophthalmol Vis Sci*. 1981;21:689–699.

15. Ito M, Yoshioka M. Regression of the hyaloid vessels and pupillary membrane of the mouse. *Anat Embryol (Berl)*. 1999;200:403–411.

16. Goldberg MF. Persistent fetal vasculature (PFV): an integrated interpretation of signs and symptoms associated with persistent hyperplastic primary vitreous (PHPV). LIV Edward Jackson Memorial Lecture. *Am J Ophthalmol*. 1997;124:587–626.

17. Kato M, Patel MS, Levasseur R, et al. Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J Cell Biol*. 2002;157:303–314.

18. Lahvis GP, Lindell SL, Thomas RS, et al. Portosystemic shunting and persistent fetal vascular structures in aryl hydrocarbon receptor-deficient mice. *Proc Natl Acad Sci USA*. 2000;97:10442–10447.

19. Gilbert RE, Vranes D, Berka JL, et al. Vascular endothelial growth factor and its receptors in control and diabetic rat eyes. *Lab Invest*. 1998;78:1017–1027.

20. Mitchell CA, Risau W, Drexler HC. Regression of vessels in the tunica vasculosa lentis is initiated by coordinated endothelial apoptosis: a role for vascular endothelial growth factor as a survival factor for endothelium. *Dev Dyn*. 1998;213:322–333.

21. Ash JD, Overbeek PA. Lens-specific VEGF-A expression induces angioblast migration and proliferation and stimulates angiogenic remodeling. *Dev Biol*. 2000;223:383–398.

22. Meeson AP, Argilla M, Ko K, Witte L, Lang RA. VEGF deprivation-induced apoptosis is a component of programmed capillary regression. *Development*. 1999;126:1407–1415.

23. Potts JD, Harocopos GJ, Beebe DC. Identification of receptor tyrosine kinases in the embryonic chicken lens. *Curr Eye Res*. 1993;12:759–763.

24. Zatechka SD Jr, Lou MF. Studies of the mitogen-activated protein kinases and phosphatidylinositol-3 kinase in the lens. 1. The mitogenic and stress responses (double dagger). *Exp Eye Res*. 2002;74:703–717.

25. Feng Y, Venema VJ, Venema RC, Tsai N, Caldwell RB. VEGF induces nuclear translocation of Flk-1/KDR, endothelial nitric oxide synthase, and caveolin-1 in vascular endothelial cells. *Biochem Biophys Res Commun*. 1999;256:192–197.

26. Forsythe JA, Jiang BH, Iyer NV, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*. 1996;16:4604–4613.

27. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol*. 1999;15:551–578.

28. Fitch CL, Swedberg SH, Livesey JC. Measurement and manipulation of the partial pressure of oxygen in the rat anterior chamber. *Curr Eye Res*. 2000;20:121–126.

29. McLaren JW, Dinslage S, Dillon JP, Roberts JE, Brubaker RF. Measuring oxygen tension in the anterior chamber of rabbits. *Invest Ophthalmol Vis Sci*. 1998;39:1899–1909.

30. Hoper J, Funk R, Zagorski Z, Rohen JW. Oxygen delivery to the anterior chamber of the eye: a novel function of the anterior iris surface. *Curr Eye Res*. 1989;8:649–659.

31. Helbig H, Hinz JP, Kellner U, Foerster MH. Oxygen in the anterior chamber of the human eye. *Ger J Ophthalmol*. 1993;2:161–164.

32. Kinoshita JH, Kern HL, Merola OH. Factors affecting the cation transport of calf lens. *Biochim Biophys Acta*. 1961;47:458–466.

33. Chylack LT Jr, Friend J. Intermediary metabolism of the lens: a historical perspective 1928–1989. *Exp Eye Res*. 1990;50:575–582.

34. Winkler BS, Riley MV. Relative contributions of epithelial cells and fibers to rabbit lens ATP content and glycolysis. *Invest Ophthalmol Vis Sci*. 1991;32:2593–2598.

35. Merriman-Smith R, Donaldson P, Kistler J. Differential expression of facilitative glucose transporters GLUT1 and GLUT3 in the lens. *Invest Ophthalmol Vis Sci*. 1999;40:3224–3230.

36. Holekamp NM, Bouck N, Volpert O. Pigment epithelium-derived factor is deficient in the vitreous of patients with choroidal neovascularization due to age-related macular degeneration. *Am J Ophthalmol*. 2002;134:220–227.

37. Simo R, Lecube A, Segura RM, Garcia Arumi J, Hernandez C. Free insulin growth factor-I and vascular endothelial growth factor in the vitreous fluid of patients with proliferative diabetic retinopathy. *Am J Ophthalmol*. 2002;134:376–382.

38. Funatsu H, Yamashita H, Ikeda T, Nakanishi Y, Kitano S, Hori S. Angiotensin II and vascular endothelial growth factor in the vitreous fluid of patients with diabetic macular edema and other retinal disorders. *Am J Ophthalmol.* 2002;133:537–543.

39. Gao G, Li Y, Fant J, Crosson CE, Becerra SP, Ma JX. Difference in ischemic regulation of vascular endothelial growth factor and pigment epithelium–derived factor in Brown Norway and Sprague Dawley rats contributing to different susceptibilities to retinal neovascularization. *Diabetes.* 2002;51:1218–1225.

40. Ogata N, Nishikawa M, Nishimura T, Mitsuma Y, Matsumura M. Unbalanced vitreous levels of pigment epithelium-derived factor and vascular endothelial growth factor in diabetic retinopathy. *Am J Ophthalmol.* 2002;134:348–353.

41. Kremer C, Breier G, Risau W, Plate KH. Up-regulation of flk-1/vascular endothelial growth factor receptor 2 by its ligand in a cerebral slice culture system. *Cancer Res.* 1997;57:3852–3859.

42. Shen BQ, Lee DY, Gerber HP, Keyt BA, Ferrara N, Zioncheck TF. Homologous up-regulation of KDR/Flk-1 receptor expression by vascular endothelial growth factor in vitro. *J Biol Chem.* 1998;273: 29979–29985.

43. Carlevaro MF, Cermelli S, Cancedda R, Descalzi Cancedda F. Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. *J Cell Sci.* 2000;113:59–69.

44. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med.* 1999;5:623–628.

45. Street J, Bao M, deGuzman L, et al. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc Natl Acad Sci USA.* 2002;99:9656–9661.

46. Robinson GS, Ju M, Shih SC, et al. Nonvascular role for VEGF: VEGFR-1,2 activity is critical for neural retinal development. *FASEB J.* 2001;15:1215–1217.

47. Yang K, Cepko CL. Flk-1, a receptor for vascular endothelial growth factor (VEGF), is expressed by retinal progenitor cells. *J Neurosci.* 1996;16:6089–6099.