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The function of VEGF-A in lens development: Formation of the hyaloid capillary network and protection against transient nuclear cataracts

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Abstract

A network of capillaries branches from the hyaloid vascular system and surrounds the mammalian lens throughout much of its embryonic development. These vessels are presumed to be important for the growth and maturation of the lens, although the lenses of non-mammalian vertebrates have no comparable vessels. Over expression of VEGF-A in the lens increases the extent of these capillaries, but it is not known whether VEGF-A from the lens is necessary for their formation or survival. To address this question, we deleted *Vegfa* in the lens. This prevented the formation of the capillary networks adjacent to the lens capsule, but did not alter nearby hyaloid vessels at the surface of the retina. Postnatal lenses lacking *Vegfa* were smaller than wild type and, by 1 month of age, many had mild nuclear opacities. These opacities regressed with age. The lens is hypoxic throughout most of life and VEGF-A expression is often regulated by the transcription factor, hypoxia inducible factor-1. Lenses lacking *Hif1a* were of apparently normal size, had markedly reduced levels of mRNA for VEGF-A and glyceraldehyde-3-phosphate dehydrogenase, but had normal-appearing capillaries covering their surface. We conclude that VEGF-A from the lens is necessary for the formation of the normal hyaloid vascular system and that lack of these capillaries was the most likely cause of growth retardation during fetal and early postnatal lens development. In the absence of HIF-1 function, sufficient VEGF-A is produced by the lens to promote capillary formation. Further study is needed to explain the formation of the mild opacities seen in some lenses lacking *Vegfa* and their regression later in life.

Keywords

tunica vasculosa lentis; anterior pupillary membrane; hyaloid vascular system; lens growth; VEGF-A; HIF-1; vascular regression; nuclear cataract

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1. Introduction

Early in its development, the mammalian lens becomes enveloped by a network of capillaries that arises from the hyaloid artery in the posterior of the eye. A capillary network also covers the anterior surface of the lens. The posterior capillary network is called the tunica vasculosa lentis (TVL) and the anterior is the anterior pupillary membrane (APM). These networks anastomose to form the capillaries of the hyaloid vascular system (HVS). Later in development, the capillaries of the TVL and APM undergo an orderly process of vascular regression that involves substantial programmed cell death. Regression occurs in the first 2 weeks of postnatal life in rodents (Latker et al., 1981; Ito et al., 1999) and in the second trimester of fetal life in humans (Zhu et al., 2000). Because failure of proper regression can cause visual impairment, attention has been directed to the mechanisms that promote or inhibit this process (Lang et al., 1994; Reichel et al., 1998; Meeson et al., 1999; McKeller et al., 2002; Ohlmann et al., 2004; Dean et al., 2005; Hahn et al., 2005; Rao et al., 2007). Less is known about the mechanisms responsible for the formation of the TVL and APM and their possible function in lens development.

When considering factors that might be responsible for the formation of the hyaloid vasculature, most attention has been directed to the angiogenic factor, vascular endothelial growth factor (VEGF-A). VEGF-A mRNA and protein have been detected in the lens when the TVL is beginning to form (Mitchell et al., 1998; Shui et al., 2003; Gogat et al., 2004) and over expression of VEGF-A in the lenses of transgenic mice leads to the formation of excessive intraocular vasculature adjacent to the lens (Ash et al., 2000; Mitchell et al., 2006; Rutland et al., 2007). Regression of the APM is hastened by blocking VEGF function with antibodies or soluble VEGF receptors in postnatal rodents (Meeson et al., 1999; Feeney et al., 2003). Although these studies are consistent with a role for VEGF-A in the formation and survival of the capillaries of the HVS, they do not test whether VEGF-A is required for these processes.

VEGF-A expression is regulated by several factors. Chief among these is tissue hypoxia. Hypoxic cells accumulate the transcription factor HIF-1. HIF-1 is comprised of two subunits, HIF-1 α , which is degraded when oxygen is sufficient and accumulates during hypoxia, and HIF-1 β , which is expressed constitutively. HIF-1 promotes the expression of genes that support cell survival under hypoxic conditions, including glycolytic enzymes, glucose transporters and factors that increase delivery of oxygen to tissues, like erythropoietin and VEGF-A (Semenza et al., 1994).

Because the hyaloid vasculature is a universal feature of mammalian eye development, many authors have concluded that it is required to 'nourish' the lens during fetal development. However, many non-mammalian species never form a capillary network around the lens. Therefore, the importance of the TVL and APM in mammalian lens development has not been tested.

VEGF receptors are expressed in the lens and these receptors are activated (tyrosine phosphorylated) in vivo (Shui et al., 2003). Therefore, VEGF signaling, may independently contribute to the growth, survival, or differentiation of lens cells.

We used conditional gene targeting to delete *Vegfa* early in the development of the mouse lens. These studies demonstrate that VEGF-A is required for the formation of the TVL and APM. The lenses of mice lacking the TVL and APM formed in an apparently normal manner, but were smaller than in wild type littermates that had an intact hyaloid vasculature. The knockout lenses remained smaller through the first several months of postnatal life. VEGF-A null lenses often had small nuclear opacities that regressed with age. VEGF expression in the fetal lens was regulated by HIF-1, but the low level of VEGF mRNA produced in the absence of HIF-1 α was sufficient to support the formation of the hyaloid capillary network. Thus, VEGF-

A is required for the formation of the capillaries of the HVS and promotes lens growth during fetal life. A proportion of *Vegfa* conditional knockout (*Vegfa*^{CKO}) lenses had mild nuclear cataracts. These opacities regressed during postnatal life.

2. Methods

2.1. Animals

Mice that had loxP sites flanking exon 3 of the *Vegfa* gene (Gerber et al., 1999) were separately mated to two Cre lines, Le-Cre and MLR10-Cre. In the Le-Cre line the Pax6 P0 promoter/enhancer drives Cre recombinase in the lens, corneal epithelium, eyelids and endocrine pancreas from E9.0 onward (Ashery-Padan et al., 2000). The MLR-Cre transgene is expressed under the a combined Pax6/ α A-crystallin promoter in the lens epithelial and fiber cells from E10 (Zhao et al., 2004). Genetically modified mice with loxP sites flanking exon 2 of *Hif1a* (Ryan et al., 1998) were crossed to the Le-Cre line. In all cases, mice that were loxP/loxP, Cre-positive were mated to mice that were loxP/loxP, Cre-negative to ensure that the conditional knockouts had only one copy of the Cre transgene and to obtain littermates that were conditional knockouts (Cre-positive) or wild type control (Cre-negative). *Vegfa* floxed mice were genotyped by PCR using the primers described previously (Gerber et al., 1999). For Cre and Hif-1 α genotyping, the Universal PCR protocol (Stratman et al., 2003) was used with the following primers:

Cre forward, 5'-GCATTACCGGTCGATGCAACGAGTGATGAG-3';

Cre reverse, 5'-GAGTGAACGAACCTGGTCGAAATCAGTGCG-3';

Hif-1 α forward, 5'-ATATGCTCTTATGAAGGGGCCTATGGAGGC-3';

Hif-1 α reverse, 5'-GATCTTTCCGAGGACCTGGATTCAATTCCC-3'.

2.2. Histology

Whole embryos or postnatal eyes were dissected at different developmental stages and fixed in 10% formalin overnight at room temperature, embedded in paraffin and sectioned. Sections were counterstained with Hematoxylin and Eosin and photographed with a Spot digital camera (Diagnostic Instruments, Inc. Sterling Heights, MI) attached to an Olympus BX60 microscope (Olympus America, Inc., Melville, NY).

2.3. Lens diameter

Postnatal lenses were dissected at different ages and photographed using dark field microscopy with a Spot digital camera (Diagnostic Instruments, Inc.) attached to a Zeiss Stemi 2000c dissecting microscope (Carl Zeiss, Thornwood, NY). The lens diameter was measured using the Spot digital camera software; two measurements were taken from each lens, one vertical and one horizontal, and then averaged to calculate lens diameter.

2.4. Capillary density

Capillary lumens were counted on central sections of P3 WT and *Hif1a*^{CKO} lenses. Only lumens touching the posterior lens capsule were included. To avoid counting retinal vessels, counts were started on the posterior capsule at a position 50 μ m from the retina.

2.5. Reverse transcription and quantitative RT-PCR (qPCR)

Lenses were dissected at P3 and the posterior lens capsule was removed. Epithelia and peripheral fibers were collected and RNA was extracted with the RNeasy Micro Kit (Qiagen, Inc., Valencia CA). 200 ng of total RNA was reversed transcribed at 42 °C for 1 h in a total

volume of 20 μ l (SuperScript II Reverse Transcriptase Kit, Invitrogen Corp., Carlsbad, CA), with oligo dT primers. At the end of the reaction the reverse transcriptase was heat inactivated at 70 °C for 15 min. For qPCR, cDNA was diluted 1:10 and 1 μ l was amplified for 40 cycles using the SYBR Green JumpStart Taq Ready Mix for Quantitative PCR (Sigma-Aldrich Chemical Co., St. Louis, MO) in a thermocycler (iCycler iQ Real-Time Detection System, Bio-Rad Laboratories, Hercules, CA). β -Actin primers were used to generate a standard curve and the level of fluorescence generated by each PCR product was compared with this standard. In each qPCR run, samples were analyzed in triplicate. The expression levels of each PCR product were compared to β -Actin and the ratios were plotted. VEGF-A primers were designed to amplify all splice forms of the mRNA. qPCR primers were designed with PrimerQuest (Integrated DNA Technologies, Inc. Coralville, IA). These were:

β -Actin forward, 5'-GCTGTATTCCCCTCCATCGTG-3';

β -Actin reverse, 5'-CACGGTTGGCCTTAGGGTTCA-3';

VEGF-A forward, 5'-GGAGAGCAGAAGTCCCATGA-3';

VEGF-A reverse, 5'-ACTCCAGGGCTTCATCGTTA-3';

Hif-1 α forward, 5'-TCTCGGCGAAGCAAAGAGTCTGAA-3';

Hif-1 α reverse 5'-TAGACCACCGGCATCCAGAAGTTT-3';

GAPDH forward 5'-CCCAATGTGTCCGTCGTGGAT-3';

GAPDH reverse 5'-TGTAGCCCAAGATGCCCTTCAG-3'.

3. Results

3.1. The development of *Vegfa* knockout lenses

Vegfa was deleted in the lens by expressing Cre recombinase at the lens placode or lens vesicle stage in transgenic mice that expressed Cre recombinase and were homozygous for a floxed allele of *Vegfa* (Gerber et al., 1999). Most studies were performed using the Le-Cre strain, which expresses Cre at the lens placode stage (E9) (Ashery-Padan et al., 2000). Le-Cre deletes floxed alleles in the lens, corneal epithelium, conjunctival epithelium and epithelium of the eyelids. Knockouts made using MLR10-Cre, which expresses Cre in the lens beginning at the lens vesicle stage (E10.5), were used to study lenses at postnatal stages (Zhao et al., 2004). Matings were set up so that half of the littermates expressed the Cre transgene (*Vegfa* conditional knockout; *Vegfa*^{CKO}) and half did not ('wild type' controls). Previous studies from our laboratory showed that Le-Cre efficiently deleted a variety of targeted alleles by E10.0 (Rajagopal et al., submitted).

Lens formation and eye development in *Vegfa*^{CKO} embryos was indistinguishable from wild type through embryonic day 12.5 (E12.5). At E11.5, a sinusoidal vessel, the initial ingrowth of the future hyaloid artery, was present between the lens vesicle and the developing retina in control and *Vegfa*^{CKO} embryos (Fig. 1A,B). By E12.5, primary lens fiber cell formation was complete and a capillary network was developing between the posterior of the lens and the retina, in the prospective vitreous cavity (Fig. 1C,D). Measurement of lens diameter in tissue sections at E12.5 showed that *Vegfa*^{CKO} lenses were similar in size to wild type (*t*-test, $n \geq 6$; $P = 0.82$). No difference in vascular development was evident between wild type and *Vegfa*^{CKO} embryos at these stages.

The first difference between wild type eyes and those with *Vegfa* null lenses was noted at E15.5. By this age, the vitreous cavity had formed and the two divisions of the hyaloid vasculature were apparent in wild type embryos (Fig. 2A). A vascular plexus covered the inner surface of the retina, while a branch of the hyaloid artery had given rise to the capillaries of the TVL, which were closely apposed to the posterior lens capsule. In *Vegfa*^{CKO} embryos, the superficial retinal plexus was present, but the hyaloid artery did not branch toward the lens and no capillaries were seen at the posterior surface of the lens (Fig. 2B). In the anterior chamber at E15.5, capillaries adhered to the anterior capsule of wild type lenses and were present in the space between the ciliary epithelium and the lateral surface of the lens (Fig. 2C). Although capillaries were present in the anterior chamber of the *Vegfa*^{CKO} lenses, none was closely apposed to the anterior surface of the lens. Capillaries with erythrocytes were seen between the ciliary epithelium and the lens capsule in the knockout lenses. A similar vascular pattern was present at E17.5 (Fig. 2E,F) and was observed in the first few days after birth in sections and by examination of whole, dissected lenses (not shown). At all stages from E15.5 onward, *Vegfa*^{CKO} lenses appeared to be smaller than in their wild type littermates. In spite of their apparently smaller size, no other morphological abnormalities were evident in tissue sections of *Vegfa*^{CKO} lenses.

3.2. Postnatal *Vegfa* knockout lenses are smaller than wild type and often have mild nuclear cataracts

Vegfa^{CKO} lenses were typically transparent and appeared to have normal morphology, except for their smaller size (Fig. 3A–C). Lens diameter was measured at 2 week intervals between 4 and 10 weeks after birth. At each age, *Vegfa*^{CKO} lenses were significantly smaller than wild type (Fig. 3C). Although lens size varied considerably, even between littermates of the same genotype, the difference in diameter between wild type and *Vegfa*^{CKO} lenses was consistently 150–250 μ m at the stages examined. From the data in Figs. 2 and 3, it seemed most likely that *Vegfa*^{CKO} lenses were smaller at birth and that this initial difference in size persisted, at least during the initial 2.5 months of life.

Nuclear opacities were seen in a decreasing fraction of *Vegfa*^{CKO} lenses after 4 weeks of age, whether deletion was mediated by Le-Cre or MLR10-Cre, a Cre transgene that is expressed only in the lens (Zhao et al., 2004) (Fig. 3B; Table 1). Like Le-Cre; *Vegfa*^{CKO} lenses, MLR10-Cre; *Vegfa*^{CKO} lenses did not form capillaries around the lens and were smaller than their wild type littermates (not shown). As 4 weeks was the first time that lenses were examined for cataracts, we do not know when the opacities first appeared. By 10 weeks, no wild type or *Vegfa*^{CKO} lenses had nuclear opacities. Nuclear opacities were evident by slit lamp evaluation in living mice and did not increase in severity when the lens was removed and cooled to room temperature (not shown). A small proportion of knockout and a few wild type lenses in the Le-Cre group were much smaller in size and had severe defects in lens morphogenesis. Since these defects were occasionally seen when the Le-Cre transgene was used to delete other conditional alleles and were not seen in the MLR-Cre knockouts, this severe phenotype was attributed to the action of a modifier gene in the Le-Cre background and not to deletion of *Vegfa*.

3.3. HIF-1 is not required for the formation of the TVL

In tissue sections, *Hif1a*^{CKO} lenses appeared to be of normal size at birth, although their fiber cells were often swollen and appeared to be degenerating (Fig. 4A,B). Other studies from our laboratory found that fiber cells from *Hif1a*^{CKO} lenses were undergoing apoptosis by P30 and completely degenerated thereafter (Shui et al., IOVS in press). Although HIF-1 is thought to be a major regulator of VEGF expression, *Hif1a*^{CKO} lenses formed a TVL. Counts showed that the number of capillaries at the posterior of *Hif1a*^{CKO} lenses tended to be lower than in wild type eyes, but this difference did not reach statistical significance (lumens per lens: WT, 8.83 ± 2.69 ; $n = 8$; *Hif1a*^{CKO}, 7.23 ± 1.88 ; $n = 9$; $P = 0.10$; t -test).

3.4. mRNA levels in *Vegfa*^{CKO} and *Hif1a*^{CKO} lens epithelial and fiber cells

VEGF-A mRNA was greatly reduced ($>4 \times 10^3$ times lower than wild type) in the epithelial cells of *Vegfa*^{CKO} lenses at P3 (Fig. 5A). Given that *Vegfa*^{CKO} lenses did not have capillaries at their surface, we expected that they might be more hypoxic than normal. Although HIF-1 level is largely regulated by protein stability, increased hypoxia might also result in higher HIF-1 α mRNA levels. However, HIF-1 α mRNA levels were nearly 60% lower in *Vegfa*^{CKO} lens epithelia than in wild type.

To determine the extent to which HIF-1 promotes VEGF-A mRNA expression in the lens, HIF-1 α and VEGF-A mRNA levels were quantified by qPCR in lens epithelial and fiber cells from wild type mice and their *Hif1a*^{CKO} littermates at P3 (Fig. 5B). As a separate measure of HIF-1 transcriptional activity, *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) mRNA, a gene known to be regulated by HIF-1 (Ryan et al., 1998), was also measured. *Hif1a*^{CKO} lens epithelial and fiber cells had HIF-1 α mRNA levels that were $>5 \times 10^3$ times lower than wild type, confirming the efficiency of deletion. In these lenses, VEGF-A mRNA levels were 2% and 17% of wild type in epithelial and fiber cells, respectively, demonstrating that HIF-1 is one of the factors that regulates VEGF-A mRNA accumulation in the lens. However, even these lower levels of VEGF-A mRNA produced sufficient VEGF-A to attract the capillaries of the TVL and APM. As expected, GAPDH mRNA levels were reduced to levels that were 32% and 1% of wild type in epithelial and fiber cells, respectively, in *Hif1a*^{CKO} lenses.

4. Discussion

The results of these studies show that VEGF-A is required to attract the capillaries of the HVS to the lens capsule to form the TVL and APM. HIF-1 promotes the accumulation of VEGF-A transcripts in lens epithelial and fiber cells. However, other factors contribute to VEGF-A mRNA accumulation, as the capillaries of the HVS form in the absence of HIF-1 α . Lenses lacking VEGF-A appeared smaller than wild type in fetal life and maintained their smaller size for more than 2 months after birth. At 4 weeks of age, *Vegfa*^{CKO} lenses often had mild nuclear opacities. These regressed with age, resulting in no lenses with cataracts by 10 weeks of age.

4.1. The function of VEGF-A in the lens

Previous studies showed that VEGF-A is required for the vascularization of many tissues, including vascular beds important to eye growth and function (Ferrara, 2001). In mouse embryos, loss of a single allele of *Vegfa* caused lethality between E10 and 11 (Ferrara et al., 1996), a phenotype that was associated with defects in angiogenesis and the formation of blood islands. In the eye, conditional deletion of *Vegfa* in the retinal pigmented epithelium (RPE) prevented the formation of the choriocapillaris in the adjacent choroid layer, resulting in microphthalmia (Marneros et al., 2005). The choriocapillaris is a layer of capillaries that is closely apposed to Bruch's membrane, the thickened basement membrane of the RPE. It is, therefore, structurally analogous to the TVL and APM, which are composed of capillaries closely applied to the lens capsule, the thickened basement membrane of lens epithelial and fiber cells. As with the formation of the TVL and APM at the surface of the lens, deletion of *Hif1a* in the RPE did not prevent the formation of the choriocapillaris (Marneros et al., 2005). As levels of VEGF-A mRNA were not measured in the RPE, it is not clear to what extent HIF-1 regulates the accumulation of *Vegfa* transcripts in this tissue.

The decrease of HIF-1 α mRNA levels in the *Vegfa*^{CKO} lenses was unexpected. These lenses should be more hypoxic than normal, due to absence of the TVL and APM. Increased hypoxia would be expected to increase, not decrease HIF-1 α mRNA levels. However, a recent report demonstrated that HIF-1 α mRNA levels can be increased by treating capillary endothelial cells with VEGF-A, even in the absence of hypoxia (Deudero et al., 2008). Therefore, reduced

exposure of lens cells to VEGF-A may be the cause of the decreased levels of HIF-1 α mRNA in the *Vegfa*^{CKO} lenses.

The present and previous studies provide at least two explanations for the smaller size of *Vegfa*^{CKO} lenses. It is possible that the TVL and APM do 'nourish' the lens, promoting lens growth during fetal and early postnatal life, as assumed by previous investigators. This would be consistent with the observation that *Vegfa*^{CKO} lenses grew at the normal rate from 4 to 10 weeks of postnatal life, after these capillaries had regressed. A similar situation exists in mice lacking the gene for connexin50 (*Gja8*) (White et al., 1998; Sellitto et al., 2004). These lenses grow more slowly than wild type for about 1 week during the early postnatal period. Thereafter, they grow at the normal rate, never making up the size differential. Alternatively, VEGF-A may directly contribute to cell proliferation or growth early in lens development, but not during the postnatal period. This would be consistent with the presence of activated (tyrosine phosphorylated) VEGF receptor-2 in rodent lenses (Shui et al., 2003). However, no other evidence suggests that VEGF-A is a mitogen for lens cells or promotes their growth or differentiation. Since *Hif1a*^{CKO} lenses did not appear to be smaller than wild type, yet had substantially lower levels of mRNA for VEGF-A, it seems likely that the absence of the capillaries of the HVS, rather than the absence of VEGF-A signaling in the lens, accounted for the smaller size of the *Vegfa*^{CKO} lenses. This conclusion could be tested by examining the phenotype of lenses lacking VEGF receptor-2 (*Kdr*), which should be able to secrete VEGF-A, but not respond to it. However, to our knowledge, floxed alleles of *Kdr* are not yet available.

Previous studies showed that levels of VEGF-A increased in lens cells during and after the regression of the TVL and APM (Shui et al., 2003). This suggests that VEGF-A from the lens is not sufficient to sustain the viability of the vascular endothelial cells of these capillaries (Meeseon et al., 1999) and that decline in VEGF from the lens does not occur and, therefore, cannot account for the regression of the HVS (Mitchell et al., 1998). Together with the results of the present study, these observations suggest that, in spite of its abundance in the adult lens, VEGF-A from the lens may play no significant role in the eye after the HVS is formed.

4.2. Nuclear cataracts in *Vegfa*^{CKO} lenses and their regression with age

A proportion of *Vegfa*^{CKO} lenses had mild nuclear opacities at 4 and 6 weeks of age. No abnormalities were observed in histological sections, making it unlikely that the opacities involved swelling or disruption of lens fiber cells. Opacities were seen in *Vegfa*^{CKO} lenses generated from matings of *Vegfa*^{fllox} mice with two Cre transgenic lines, Le-Cre and MLR10-Cre. This makes it unlikely that the cataracts were due to mutations caused by insertion of the transgene or the presence of a modifier gene closely linked to the transgene. Like lens growth, our studies do not reveal whether these cataracts resulted from absence of the capillaries of the HVS or decreased VEGF receptor activation during lens development.

One of the most surprising observations made in this study was that the nuclear opacities observed at 4 weeks of age were seen less frequently in mice 6 weeks of age or older and, by 10 weeks, no cataracts were observed. We are not aware of other instances in which cataracts, present at 4 weeks of age, regress in older lenses. However, this situation is reminiscent of the 'cold cataracts' that can be elicited by cooling rodent lenses from birth into the 2nd week of life and in the lenses of other species throughout life (Tanaka et al., 1975, 1977). These opacities, which are due to reversible phase separation in the cytoplasm of the nuclear fiber cells, can be made to increase or disappear by cooling or warming the lens, respectively. Because the critical phase separation temperature, T_c, declines steadily during rodent development, greater cooling is required to cause phase separation and opacification as the lens matures (Clark et al., 1987). Most of the lenses in this study were examined at room temperature (~23 °C) at ages when T_c is normally below this temperature. It is possible that loss of the HVS or VEGF signaling caused changes in the cytoplasm of a small group of lens

fiber cells that resulted in a local increase in Tc. As lens Tc declined postnatally, the critical temperature in these cells was eventually exceeded, phase separation was prevented and the opacities disappeared. Arguing against this interpretation is the observation that opacities were present by slit lamp examination in vivo and did not increase in severity when lenses from 4- to 8-week-old animals were cooled to room temperature. In initial tests, warming lenses with cataracts from room temperature to ~38 °C also did not alter the density or extent of the cataract. Additional biochemical, biophysical and morphological analyses are needed to define the nature of these opacities and to provide an explanation for their disappearance.

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Fig. 1.

Eyes with lenses lacking *Vegfa* form and develop normally at early stages. At embryonic E11.5 wild type (A) and *Vegfa*^{CKO} (B) eyes formed a lens vesicle. A sinusoidal vessel, the initial ingrowth of the hyaloid artery (arrows) is present between the lens and inner surface of the retina. By E12.5, in wild type (C) and the *Vegfa*^{CKO} (D) lenses the lens primary fibers have formed and there is a developing capillary network between the posterior of the lens and the optic cup (arrows). LV, lens vesicle; L, lens; OC, optic cup. Scale bar = 50 μ m.

Fig. 2.

VEGF-A is necessary for the formation of the TVL and APM. By E15.5, the hyaloid artery has given rise to the capillaries of the TVL (arrows) that cover the posterior of wild type lenses (A). However, in the *Vegfa*^{CKO} lenses (B) no TVL was present, as there were no capillaries adhering to the posterior lens capsule. Wild type and *Vegfa*^{CKO} eyes had a well formed vascular plexus on the inner surface of the retina (arrowheads). A few tissue macrophages (hyalocytes) were present in the vitreous cavity. In the anterior chamber, although capillaries are present in wild type (arrows) (C) and *Vegfa*^{CKO} (D) eyes, no capillaries were closely apposed to the anterior surface of lenses lacking VEGF-A (D). Capillaries were present between the ciliary epithelium and the lateral surface of the lens in wild type and *Vegfa*^{CKO} eyes (arrows with asterisk). *Vegfa*^{CKO} lenses appeared smaller than the wild type lenses from E15.5 onward. At E17.5 the TVL was still only present in wild type embryos (E, arrows). However, both wild type and *Vegfa*^{CKO} (F) embryos had a similar vascular plexus on the inner surface of the retina (arrowheads). Scale bars in A–B and E–F, 100 μ m and in C–D, 50 μ m.

Fig. 3. *Vegfa*^{CKO} lenses are smaller than wild type; some have nuclear cataracts. (A) Most *Vegfa*^{CKO} lenses examined with a dark field dissecting microscope (right) were transparent and appeared to have normal morphology, although they consistently appeared smaller than the lenses of their wild type littermates (left). (B). Nuclear cataracts were seen in a substantial proportion of the *Vegfa*^{CKO} lenses prior to 6 weeks postnatal (right). A wild type lens is shown on the left. (C) *Vegfa*^{CKO} lenses were significantly smaller by ~150–250 μm than their wildtype littermates at 4, 6, 8 and 10 weeks of age. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bar in A and B = 500 μm.

Fig. 4. HIF-1 is not required for the formation of the TVL. *Hif1a*^{CKO} lenses examined 2 days after birth had a TVL; capillaries (arrows) were present on the posterior surface of the lens (B), comparable to those in wildtype littermate lenses (A). The *Hif1a*^{CKO} lenses appeared to be the same size as the wildtype lenses, however there was some swelling of the fiber cells and they appear to be degenerating.

Fig. 5. Quantitative PCR confirmed the deletion of *Vegfa* and *Hif1a* and showed that HIF-1 regulates VEGF-A and GAPD mRNA levels in the lens. (A) VEGF-A and HIF-1α transcript levels in wild type (solid bars) and *Vegfa*^{CKO} lens epithelial cells (open bars). (B) HIF-1α, VEGF-A and GAPD transcripts in wild type (solid bars) and *Hif1a*^{CKO} lens epithelial (open bars) or fiber cells (gray bars).

Table 1

The number (#) and percentage of lenses with nuclear opacities

Age (weeks)	Wild type # opaque (%)	Le-Cre # opaque (%)	MLR10-Cre # opaque (%)
4	2/24 (8.3)	7/18 (39)	4/6 (67)
6	0/12 (0)	2/10 (20)	2/2 (100)
8	0/16 (0)	0/10 (0)	8/10 (80) (4+; 4+/-)
10	0/4 (0)	0/6 (0)	0/10 (0)

In MLR10-Cre; *Vegfa*^{CKO} lenses at 8 weeks, four of the opacities were of similar severity to those at earlier stages (+), while four others were barely detectable (+/-).