Knockout of αA-Crystallin Inhibits Ocular Neovascularization

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EXTRACTION

Intraocular angiogenesis is a major cause of vision loss associated with various diseases, such as retinopathy of prematurity (ROP), neovascular age-related macular degeneration (nAMD), and diabetic retinopathy (DR).1,2 Neovascularization may lead to vitreous hemorrhage, proliferative membranes, and the subsequent induction of visual loss.3,4 The occurrence of ocular neovascularization (NV) relies on the balance between proangiogenic (e.g., vascular endothelial growth factor [VEGF]) and antiangiogenic factors. Pathologic factors such as hypoxia promote the expression of proangiogenic factors, resulting in NV.5 Therefore, it is important to identify the molecules that regulate NV to facilitate the discovery of effective therapeutic targets.

To date, several target molecules have been identified and characterized. Among these, VEGF and its receptors play major roles in the generation and progression of neovascular ocular diseases.6–12 Anti-VEGF treatment can reduce ocular NV and tumor NV; however, resistance occurs during treatment with antiangiogenic therapy.13–15 Moreover, anti-VEGF agents might induce local and systemic adverse effects, including ocular inflammation,16–18 cardiovascular toxicities,19 nonocular hemorrhage, and stroke.20 Further exploration and evaluation of the precise mechanisms of NV are necessary to identify more effective therapeutic targets.

Crystallins primarily serve as the major structural proteins of the ocular lens, and they are categorized into three distinct families: α, β, and γ. α-Crystallins can exert a chaperone-like activity, remodel and protect the cytoskeleton, prevent aberrant protein interactions, inhibit apoptosis, and enhance the resistance of cells to stress.21–25 α-Crystallin consists of two similar subunits: αA-crystallin (CRYAA) and αB-crystallin.24 An increasing number of studies have demonstrated that CRYAA exists in the normal retina.25–28 and participates in a variety of retinopathies. For example, CRYAA expression has been recently shown to be upregulated in DR29 and
inflammatory eye diseases,\textsuperscript{28,30} as well as during tumor angiogenesis.\textsuperscript{31}

The role of crystallins in NV has attracted the attention of researchers. \textsuperscript{32}B-crystallins have been proposed to regulate retinal vascular remodeling during vision system development.\textsuperscript{32,33} While \textsuperscript{32}B-crystallin acts as a modulator of VEGF-A in oxygen-induced retinopathy (OIR) and laser-induced chorioidal neovascularization (CNV),\textsuperscript{34} \textsuperscript{32}B-crystallin also enhances vascular formation under conditions of tumorigenesis.\textsuperscript{35} Several studies\textsuperscript{36,37} have suggested that CRYAA might play an important role in corneal neovascularization by regulating soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) expression. The aim of this study was to investigate the effects of CRYAA on intraocular pathologic neovascularization and to explore the mechanisms underlying this regulation by using CRYAA-knockout mice and CRYAA-depleted human umbilical vein endothelial cells (HUVECs). The results of our study could provide a useful therapeutic strategy for the treatment of NV.

\section*{Methods}

\subsection*{Cells and Small Interfering RNA (siRNA) Transfection}

The HUVECs used in this study were obtained from the American Type Culture Collection (CRL-1730; Manassas, VA, USA).\textsuperscript{38} The HUVECs were cultured in Dulbecco's modified Eagle's Media (DMEM; Hyclone, Grand Island, NY, USA) containing 10\% fetal bovine serum (FBS; Hyclone; Hyclone). To knock down CRYAA expression in HUVECs, the following siRNA targeting human \textsuperscript{2}A-crystallin (sc-40430; Santa Cruz Biotechnology, Dallas, TX, USA) was applied: forward, 5'-AGCTACGATCATATGACCGT-3'; reverse, 5'-AGGGCGTTGGTC TATGCTG-3'; the following negative control (NC) siRNA, designed by the manufacturer (catalog No. 1121; SBS Genetech, Beijing, China), was also used: forward, 5'-ACGG GACACGUUCGGAGAATT-3'; reverse, 5'-GACACGUUCGGAGAATT-3'.

\subsection*{RNA Extraction and Quantitative PCR for CRYAA}

The HUVECs were lysed in Trizol reagent (catalog No. 15596; Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Each RNA extract (2 \mu g) was reverse transcribed into cDNA by using the RevertAid First-Strand cDNA Synthesis Kit and oligo-dT primers (catalog No. 1622; Fermentas, Pittsburgh, PA, USA). Fluorescent quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed with the SYBR Green PCR mix (catalog No. L0905; Thermo, Pittsburgh, PA, USA) by using an ABI 7300 real-time PCR system (Applied Biosystems, Life Technologies, Foster City, CA, USA). The following primers were used in the RT-PCR analysis: CRYAA forward, 5'-GAACGGGGACAAGTTCGTCAT-3'; reverse, 5'-GCCGTTGTGGTTCTTCC-3'; GAPDH forward, 5'-CTGGGAGCAGACATGGAGAAGA-3'; reverse, 5'-AAAGGAACCTGTTAGAAAGAC-3'.

\subsection*{HUVEC Proliferation Assays}

The Cell Counting Kit-8 (CCK-8; Dojindo, Shanghiai, China) assay was used to evaluate the effects of CRYAA knockdout on proliferation, as shown in previous studies.\textsuperscript{39} Briefly, HUVECs (normal cells, normal cells with HiPerFect reagent, cells transfected with NC siRNA, or cells transfected with CRYAA siRNA) were synchronized in DMEM at a density of 1 \times 10^4 cells per well in 96-well plates in the absence of FBS for 24, 48, or 72 hours. After the addition of 10 \mu L CCK-8 to each well, the cells were incubated at 37\(^\circ\)C for 30 to 60 minutes. The absorbance was measured by using an enzyme-linked immunosorbent assay (ELISA) microplate reader at 450 nm (Finsturments Multiskan Models 347; MTX Lab Systems, Inc., Vienna, VA, USA). Each sample was repeated in five wells, and each experiment was performed at least three times.

\subsection*{HUVEC Migration Assay}

To evaluate the effects of CRYAA knockout on cell migration capacity, we used a Transwell system (catalog No. 3422; Corning Life Sciences, Lowell, MA, USA) with a pore size of 8.0 \mu m, as described previously.\textsuperscript{39} Briefly, 2 \times 10^4 HUVECs (normal cells or cells transfected with NC or CRYAA siRNA for 24, 48, or 72 hours) were placed in the top chamber of a Transwell system. Afterward, DMEM containing 10\% FBS was added to the bottom chamber, resulting in a final volume of 600 \mu L. All of the migration assays were conducted at 37\(^\circ\)C for 4 hours. At the end of the assay, the cells were fixed in 4\% paraformaldehyde and stained with 4,6-diamidino-2-phenylindole (DAPI; Roche Diagnostics, Indianapolis, IN, USA) for 15 minutes. The cells that had not migrated were removed with a cotton swab, and the membrane was imaged. We selected five random areas from the upper, right, lower, left, and middle regions of the field of view. The cells within those regions were counted and photographed. The total number of cells was also recorded. Each experiment was repeated three times.

\subsection*{Tube Formation Study}

Tube formation studies are used to test the ability of endothelial cells to form capillary-like structures. Following the manufacturer's instructions and the protocol described in our previous report,\textsuperscript{39} 200 \mu L Matrigel (catalog No. 354234; BD Biosciences, San Jose, CA, USA) solution was placed into the wells of 24-well plates, and the plates were incubated at 37\(^\circ\)C for 30 minutes in a 5\% CO\textsubscript{2} incubator. A total of 5 \times 10^4 HUVECs per well (normal cells or cells transfected with NC and CRYAA siRNA for 24, 48, or 72 hours) were seeded onto the Matrigel and were cultured in DMEM for 6 to 8 hours. The networks in the Matrigel were counted and photographed in five randomly chosen fields. The number of closed tubes was counted by three different individuals in blinded fashion, and the average number was recorded. The experiments were performed in triplicate.

\subsection*{Expression of VEGF in HUVECs by ELISA}

To determine whether VEGF secretion was affected by CRYAA depletion, ELISAs were performed. We seeded HUVECs (normal cells with HiPerFect reagent, cells transfected with NC siRNA, or cells transfected with CRYAA siRNA) in 96-well plates (1 \times 10^4 cells per well) and incubated them at 37\(^\circ\)C without FBS. After 24, 48, or 72 hours of incubation, the cell culture supernatant was harvested and centrifuged. The levels of VEGF protein secreted by the HUVECs into the culture medium were measured by using an ELISA kit (catalog No.
Detection of HUVEC Apoptosis by Flow Cytometry Analysis

To determine the apoptosis rate of HUVECs after CRYAA knockout, an FITC Annexin V experiment was performed by using the Annexin V Detection Kit (catalog No. 556547; BD Science, San Diego, CA, USA). Briefly, 1 × 10⁶ HUVECs (normal cells, cells transfected with NC siRNA, or cells transfected with CRYAA siRNA) were seeded in 6-well plates and were incubated for 24, 48, or 72 hours. Then, we stained the cells with Annexin-V-FITC and propidium iodide (PI) according to the manufacturer’s instructions. The cells were immediately analyzed by using a flow cytometer (FACS Calibur, excitation wavelength/emission wavelength = 488/530 nm; BD Biosciences). Approximately 10⁴ cells were collected and divided into four groups: dead cells (Annexin V+PI−, upper left [UL]); late apoptotic cells (Annexin V+/PI+, upper right [UR]); viable cells (Annexin V−/PI−, lower left [LL]); and early apoptotic cells (Annexin V−/PI+, lower right [LR]). The rate of apoptosis was calculated as the percentage of early apoptotic cells (LR) plus the percentage of late apoptotic cells (UR).

Experimental OIR Model

CRYAA(−/−) and wild-type (WT) mice were used to create an OIR model. The CRYAA(−/−) mice were originally generated at the Qingdao Eye Institute through targeted gene disruption and were maintained in the 129 S6/SvEvTac background. This study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was performed in accordance with the guidelines provided by the Animal Care and Use Committee of Peking University.

Retinal neovascularization was induced by using a well-established murine model of OIR, as previously reported. Neonatal mouse pups at postnatal day seven (P7) and their nursing mothers were maintained for five days in 75% ± 2% oxygen and were subsequently returned to room air (relative hypoxia) at P12. The mouse eyes were enucleated at P18 (CRYAA(−/−), n = 12; WT, n = 12) to obtain protein extracts.

At P18, murine retinal NV was evaluated by using angiography, as described previously. Briefly, the mice were perfused with fluorescein-dextran-FITC (catalog No. FDX-100; Sigma, St. Louis, MO, USA). The eyes were removed and fixed in 4% paraformaldehyde (PFA) for 30 minutes, and the retinas were flat-mounted by using four peripheral retinal cuts. The retinas were then imaged with fluorescence microscopy. The nonperfused areas were analyzed with ImageJ software (http://imagej.nih.gov/ij/: provided in the public domain by the National Institutes of Health, Bethesda, MD, USA), and a ratio was calculated by comparing the nonperfused area to the entire retina. All of the data were analyzed by three individuals, two of whom were completely blinded to the study groups. The average ratio was used for the statistical analyses.

For the retinal section study, retinal NV was evaluated by using paraffin-embedded sections of OIR mouse eyes. P18 mice were killed by intraperitoneal injection of an overdose of sodium pentobarbital. The eyes were enucleated, fixed with 4% PFA in PBS, and embedded in paraffin. The sections were stained with hematoxylin-eosin (HE) to visualize the cell nuclei. Cross-sections that included the optic nerve were not sampled. Three individuals counted the cell nuclei above the internal limiting membrane in blinded fashion.

Western Blot Analysis

HUVECs and mouse retinas were used for Western Blot analysis. For the OIR model mice (CRYAA(−/−) and WT), retinal proteins were collected on P18; for the CNV model, the pooled RPE/choroid layers were collected 14 days after laser photocoagulation.

We measured the protein content by using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated by 10% SDS-PAGE and were electrophoretically transferred to nitrocellulose membranes (catalog No. IPVH00010; Amersham, Little Chalfont, UK). After blocking, the membranes were incubated with rabbit monoclonal antibodies against phospho-VEGFR2 (1:1000), phospho-Akt (1:1000), phospho-PLCγ1 (1:500), phospho-p38 MAPK (1:1000), phospho-FAK (1:1000), phospho-p44/p42 MAPK (1:1000), phospho-Src (1:1000) (these seven antibodies were purchased from Cell Signaling, Danvers, MA, USA), and antibodies against phospho-VEGFR2 (1:1000), phospho-Akt (1:1000), phospho-PLCγ1 (1:500), phospho-p38 MAPK (1:1000), phospho-FAK (1:1000), phospho-p44/p42 MAPK (1:1000), phospho-Src (1:1000), FAK (1:500), phospho-p38 MAPK (1:500), phospho-p44/p42 MAPK (1:500), phospho-Src (1:1000).
USA). A mouse monoclonal antibody against β-actin (1:600, catalog No. ab8226; Abcam) was used as a loading control.

The effects of siRNA transfection were verified by examining HUVEC proteins as previously described. These samples were incubated with a mouse monoclonal antibody against αA-crystallin (1:1000, catalog No. ab5595; Abcam). The signals were visualized (ECL Kit; GE Healthcare Life Sciences, Buckinghamshire, UK) according to the manufacturer’s protocol. The Western Blot analyses were repeated three times, and qualitatively similar results were obtained during each replicate experiment.

**Statistical Analysis**

The data analysis was performed by using Prism 6 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). All of the data are presented as the mean ± SEM and were evaluated for normality of distribution. Differences were evaluated by using an analysis of variance followed by the Student-Newman-Keuls test for multiple comparisons and Student’s t-test for pairwise comparisons. P < 0.05 was considered a statistically significant difference.

**RESULTS**

**Small Interfering RNA Transfection Verification**

The RT-PCR analysis showed that CRYAA siRNA successfully inhibited the expression of CRYAA mRNA (Fig. 1A); a significant difference in expression was observed, as compared with the normal group, at 24 hours (P < 0.05), 48 hours (P < 0.001), and 72 hours (P < 0.001). Western blot analysis revealed that αA-crystallin protein expression levels were similarly inhibited at 24, 48, and 72 hours (P < 0.01) (Figs. 1B, 1C show the results at 48 hours). Thus, CRYAA siRNA could inhibit gene transcription and protein expression.

**CRYAA siRNA Inhibited HUVEC Proliferation, Migration, and Tube Formation**

A CCK-8 Assay Kit was used to evaluate the effects of CRYAA knockdown on cell proliferation in vitro, as described in the methods section. The results showed that CRYAA siRNA inhibited HUVEC proliferation at 48 and 72 hours as compared with the control (P < 0.001 and P < 0.01, respectively) and NC groups (P < 0.01 and P < 0.05, respectively; Fig. 2). No significant difference was observed at 24 hours (P > 0.05).

We also investigated whether CRYAA knockout inhibited HUVEC migration, which is an important step in vessel formation. As shown in Figure 3, the numbers of cells that passed through the membrane in the CRYAA siRNA-treated HUVEC groups were significantly lower than those in the control and NC groups at 48 hours (P < 0.001) and 72 hours (P < 0.001) (Fig. 3A).

Additionally, a Matrigel assay was performed to examine tube formation and thereby evaluate the angiogenic ability of endothelial cells in vitro. In our study, the capacity of CRYAA siRNA-treated HUVECs to form a regular network was impaired (Figs. 4B–D). Statistical analyses were performed on the number of closed tubes, which represented angiogenic capacity, and the results showed that CRYAA siRNA inhibited HUVEC tube formation, as compared with the control group at 48 hours (P < 0.001) and 72 hours (P < 0.001) (Fig. 4A).
FIGURE 3. Effects of CRYAA knockdown on the migration of HUVECs. The cell nuclei were stained with DAPI and can be observed as blue dots. Cells from five fields (upper, right, lower, left, and middle regions) were counted and photographed, and the average was used for statistical analysis. (A) Statistical analysis revealed that CRYAA siRNA inhibited HUVEC migration at 48 and 72 hours. (B) Normal group treated with 10% FBS at 48 hours. (C) Negative control siRNA-treated group at 48 hours. (D) The CRYAA siRNA-treated group at 48 hours. Each experiment was performed at least three times. The data are presented as the mean ± SEM. *P < 0.05 and ***P < 0.001.

FIGURE 4. Effects of CRYAA siRNA on HUVEC tube formation. The number of closed tubes per field of view was counted by three individuals in blinded fashion. (A) Statistical analysis. (B) Normal group treated with 10% FBS at 48 hours. (C) Negative control siRNA-treated group at 48 hours. (D) CRYAA siRNA-treated group at 48 hours. The data are presented as the mean ± SEM. Each experiment was performed at least three times. ***P < 0.001.
CRYAA Knockdown on VEGF Secretion

CRYAA siRNA-treated HUVECs showed a decrease in VEGF secretion at 48 and 72 hours. As shown in Figure 5, after treatment with CRYAA siRNA for 48 and 72 hours, VEGF was downregulated as compared with its level in the normal controls ($\text{P}<0.001$). However, after treatment for 24 hours, the CRYAA siRNA-treated group did not show a significantly different level of VEGF secretion, compared with the normal control group (Fig. 5).

CRYAA siRNA Induced HUVEC Apoptosis

Early and late apoptosis in HUVECs peaked at 48 and 72 hours after CRYAA knockdown. As shown in Figure 6, treatment with CRYAA siRNA for 48 and 72 hours led to a significantly higher percentage of apoptotic cells (UR+LR) as compared with the normal control or NC groups (48 hours, $\text{P}<0.01$; 72 hours, $\text{P}<0.05$; Fig. 6A).

The levels of cleaved caspase-9 and caspase-3 were found to increase in our study. In vitro, HUVECs were incubated with siRNA for 24, 48, and 72 hours. The levels of cleaved caspase-9 (Fig. 7A) and caspase-3 (Fig. 7B) increased at 48 hours ($\text{P}<0.001$) and 72 hours ($\text{P}<0.001$). No significant differences in $\beta$-actin concentrations were observed. In vivo, cleaved caspase-9 and caspase-3 were increased in CRYAA(-/-) mice and in both the OIR ($\text{P}<0.001$) and CNV models ($\text{P}<0.001$) (Figs. 7A, 7B), without significant changes in $\beta$-actin expression.

Attenuation of Retinal Neovascularization in the CRYAA(-/-) Murine OIR Model

CRYAA knockout led to an increase in the nonperfusion area in the CRYAA(-/-) murine OIR model (Fig. 8). The results indicated that CRYAA knockout markedly increased the avascular area in the retina at P18 in the murine OIR model ($\text{P}<0.001$), and vascular leakage was reduced (Figs. 8A, 8C, 8E). The ratio of the nonperfused area to the entire retina in the WT normal exposure mouse group was 2.92%±0.18%; in the CRYAA(-/-) normal exposure group, the ratio was 2.54%±0.16%; in the WT OIR group, the ratio was 21.03%±0.49%; and in the CRYAA(-/-) OIR group, the ratio was 32.37%±1.81% (Fig. 8A).
Retinal neovascularization, as represented by endothelial cell nuclei protruding into the vitreous cavity (neovascular nuclei), was decreased in the CRYAA(−/−) mice (Fig. 9). As shown in Figure 9, the average numbers of neovascular nuclei were 23.13 ± 1.52 in the WT mouse eyes exposed to hyperoxia (n = 14) and 10.19 ± 0.57 in CRYAA(−/−) eyes (n = 16) (Fig. 9A). These data indicated a significant reduction in NV when CRYAA was depleted (P < 0.001; Figs. 9B, 9C).

**Choroidal Neovascularization Suppression in the CRYAA(−/−) Murine CNV Model**

Histologic CNV sections from 10 WT and 10 CRYAA-deficient mice demonstrated the attenuation of NV. Representative light micrographs of HE-stained sections of the posterior segment showed evidence of NV and proliferative membranes in the middle of the laser lesion. As shown in Figure 10, invasion decreased in the retina surrounding the laser scars in CRYAA(−/−) mice (B/C ratio of 1.49 ± 0.04) compared to the WT group (B/C ratio of 2.67 ± 0.04) (Figs. 10C, 10D). There were significant differences in the B/C ratio between the CRYAA(−/−) group and the controls (P < 0.001).

Choroidal neovascularization leakage measurements in murine retinas were performed by using FA to determine whether CRYAA knockout inhibited CNV. At 14 days after laser-induced CNV, cataracts had developed in the CRYAA(−/−) group, as shown by FA (Supplementary Fig. 1D). Fundus could not be seen, although leakage could be observed at the lesion site in WT mice (Supplementary Fig. 1). However, this difference could not be evaluated quantitatively.

**CRYAA Knockout Inhibited VEGF Secretion by HUVECs and in the Murine OIR and CNV Models**

Vascular endothelial growth factor is the most important growth factor in the pathology of retinal diseases, and its secretion was found to be decreased by CRYAA siRNA in vitro. To determine whether CRYAA siRNA modulated the expression of VEGF, Western blot analysis was performed. The results showed that at 48 and 72 hours, VEGF secretion was attenuated by CRYAA knockout (Fig. 11 shows the results at 48 hours). In the OIR and CNV murine models, VEGF...
p38 MAPK were not significantly different. In vivo, p-VEGFR2, the levels of total VEGFR2, AKT, PLC(48 hours (Fig. 12) and 72 hours (P < 0.001), without significant changes in the levels of total VEGFR2, AKT, PLCγ1, FAK, Src, p44/42, or p38 MAPK (Fig. 12).

**The Mechanism of CRYAA Action on NV via the VEGF Signaling Pathway**

CRYAA knockout could downregulate the expression of VEGF and downstream of VEGF, the phosphorylation of VEGFR2, AKT, PLCγ1, FAK, Src, p44/42 MAPK, and p38 MAPK was found to be decreased after CRYAA knockout (Fig. 13).

**DISCUSSION**

αA-crystallin is a major structural protein in the lens; it accounts for nearly 40% of the total protein in the lens and is required for the maintenance of lens transparency.45 Studies of αA-crystallin have mainly focused on lens diseases, such as age-related cataracts and congenital cataracts.46,47 In 2003, Xi et al.27 demonstrated the expression of 20 different lens proteins, using gene chips and quantitative PCR in the mouse retina. The study found for the first time that the expression of crystallins could be observed in retinal nuclear layers and retinal ganglion cells in the absence of any external stress. Changes in the expression profiles of crystallins during development and aging strongly argue in favor of their significance in maintaining homeostasis in the retina.48 An increasing number of studies have demonstrated that αA-crystallin is associated with a variety of retinal diseases, including AMD,49 autoimmune uveitis,50 optic nerve injury,51 and diabetic retinal disease.52 It has also been demonstrated that αA-crystallin might influence corneal NV.51,52 The specific action of αA-crystallin on retinal NV has remained unclear. Our research attempted to use CRYAA siRNA and CRYAA-knockout mice to determine its effects both in vitro and in vivo. The results have six direct implications. First, the loss of αA-crystallin could significantly inhibit the proliferation, migration, and tube formation of HUVECs. Second, knockdown of αA-crystallin could increase the apoptosis rate of HUVECs. Third, the pathologic NV of CRYAA(−/−) mice was inhibited in both the OIR and CNV models. Fourth, VEGF expression was downregulated in both HUVECs and the two murine models after the loss of αA-crystallin expression. Fifth, the reduction of αA-crystallin

![Figure 10](image-url1)

**Figure 10.** Hematoxylin-eosin staining of histologic CNV sections. (A) Statistical analysis of the neovascular proliferative membrane invading into the RPE layer. CRYAA(−/−) murine model of CNV exhibited significantly reduced levels of invasion. (B) Wild-type normal eyes without any invasion or proliferative membrane. (C) CRYAA(−/−) normal eyes without any invasion or proliferative membrane. (D) Wild-type CNV mice showed neovascularization and proliferative membranes in the middle of the laser lesion, as indicated by the white circle. (E) CRYAA(−/−) CNV mice exhibited decreased invasion, as indicated by the white circle. **P < 0.001.

![Figure 11](image-url2)

**Figure 11.** Vascular endothelial growth factor secretion by the CRYAA-knockdown HUVECs and CRYAA(−/−) murine OIR and CNV models. Top: Statistical analyses of VEGF. Bottom: Immunoblot images of decreased VEGF expression in the CRYAA siRNA-transfected HUVECs and in the CRYAA(−/−) OIR and CNV models. Western blot analyses were performed three times, and qualitatively similar results were obtained for each replicate. The data are presented as above. *P < 0.01 and **P < 0.001.
resulted in higher levels of cleaved caspase-9 and caspase-3 both in vitro and in vivo. Finally, the phosphorylation of VEGFR2 and its downstream signaling pathway, including AKT, PLC$_c$1, FAK, Src, p44/p42 MAPK, and p38 MAPK, decreased both in vitro and in vivo after CRYAA knockout.

A previous study has demonstrated that aB-crystallin affects neither upstream Hif-1α signals nor downstream VEGFR2 signals, and only VEGF-A expression is altered. As a result, researchers have attempted to explore the direct association between aB-crystallin and VEGF-A by means of coimmunoprecipitation. However, in our study, knockout of aA-crystallin decreased not only the expression of VEGF but also the phosphorylation of VEGFR2 and its downstream effectors. In other words, aA-crystallin could upregulate the expression of VEGF; downstream of VEGF, the phosphorylation levels of VEGFR2, AKT, PLC$_c$1, FAK, Src, and p44/p42 MAPK were increased by the synergistic effect of aA-crystallin, as shown in Figure 13.

The process of angiogenesis is complex and includes endothelial cell proliferation, migration, invasion, basement membrane degeneration, and new tube formation from existing blood vessels. Vascular endothelial growth factor is one of the most important proangiogenic molecules owing to its regulation of the angiogenic process. Signals mediated by VEGF are mainly mobilized by its interaction with VEGFR2. Then, its downstream intermediaries are activated, including Src kinase, FAK, and its substrate paxillin. The binding of VEGF to VEGFR2 also results in tyrosine phosphorylation and the activation of PI3K, followed by the activation of AKT, the intracellular Ca$^{2+}$ chelator PLC$\gamma_1$, and two members of the MAPK family (p42/p44 MAPK [Erk1/2], p38 MAPK), which regulate endothelial cell survival, proliferation, and migration. To define the effects of CRYAA on VEGF-signaling cascades, we tested the effects of CRYAA on the VEGFR2, AKT, p38 MAPK, PLC$\gamma_1$, Src, and p44/p42 MAPK pathways, which have been linked to vascular cell survival, proliferation, migration, and vascularization. Our results demonstrated that knockout of CRYAA significantly reduced

![Figure 12](https://iovs.arvojournals.org/downloads/assets/figure12.jpg)

*Figure 12.* The phosphorylation of VEGFR2, AKT, PLC$\gamma_1$, p38 MAPK, FAK, Src, and p44/p42 MAPK is reduced in CRYAA siRNA-transfected HUVECs and in the OR and CNV models of CRYAA(-/-) mice. Left: Statistical analyses of (A–G) p-VEGFR2, p-AKT, p-PLC$\gamma_1$, p-p38 MAPK, FAK, Src, and p44/p42 MAPK. Right: (A–G) Immunoblot images of decreased p-VEGFR2, p-AKT, p-PLC$\gamma_1$, and p-p38 levels in vitro and in vivo after CRYAA knockout. Western blot analyses were repeated three times, and qualitatively similar results were obtained for each replicate. The data are presented as above. **P < 0.01 and ***P < 0.001.

![Figure 13](https://iovs.arvojournals.org/downloads/assets/figure13.jpg)

*Figure 13.* The mechanism of CRYAA action on neovascularization through the VEGF signaling pathway.
the phosphorylation levels of VEGFR2, Akt, PLC\(\gamma\)1, p38 MAPK, Src, FAK, and p44/p42 MAPK in HUVECs and in the OIR and CNV models. However, the expression of VEGFR2, Akt, PLC\(\gamma\)1, p38 MAPK, Src, FAK, and p44/p42 MAPK showed no significant alterations. Thus, CryAA is likely to synergize not only with VEGF but also with the downstream signaling pathway of VEGFR2, thus altering the phosphorylation levels of VEGFR2, Akt, PLC\(\gamma\)1, p38 MAPK, Src, FAK, and p44/p42 MAPK.

We performed experiments to determine how adult-crystallin altered NV in vivo by using CryAA(\(-/-\)) murine OIR and CNV models. Angiography revealed a prominent decrease in neo-vessels, and histopathology revealed markedly reduced neovascular tufts in CryAA(\(-/-\)) murine retinas. In the CNV model, analysis of histologic CNV sections showed reduced CNV volumes. It was striking that NV was significantly attenuated in CryAA(\(-/-\)) mice in two distinct models. To our knowledge, the finding that NV was significantly attenuated in CryAA(\(-/-\)) mice is novel.

In addition, we discovered that the CryAA(\(-/-\)) murine OIR model exhibited a larger nonperfusion area. We believe that depletion of CryAA had large suppressive effects on vascularization; thus, normal vascular growth was inhibited to some extent in CryAA(\(-/-\)) mice. For potential clinical applications, the dose required to specifically inhibit pathologic NV instead of normal vascularization should be evaluated more precisely.

In vitro, HUVEC functions that play crucial roles in NV were inhibited to some extent, including VEGF-dependent proliferation, migration, and tube formation. Additionally, the level of apoptosis was increased, which corresponded with the previously mentioned protective effects of CryAA.\(^5\) The intrinsic apoptosis pathway is mainly regulated by Bcl-2 family members,\(^8\) which regulate mitochondrial outer membrane permeabilization to promote the release of cytochrome C (cytC) and other apoptotic molecules. In the cytosol, cytC, Apaf1, and procaspase-9 are assembled into the apoptosome to activate downstream effector caspases 3, 6, and 7, leading to cellular component degradation and cell death.\(^9\) Caspase-9 and caspase-3 are the two major apoptosis regulators.\(^50\) Our study assumed that the high apoptosis rate of the CryAA(\(-/-\)) group might be due to the activation of caspase-9 and caspase-3. However, HUVECs are derived from umbilical veins, which are large vessels. Therefore, they cannot completely represent intraocular NV, which principally affects the capillaries. This was a limitation of our research. Further experiments on retinal vascular endothelial cells should be performed in the future.

In conclusion, the main finding of these studies is 2-fold: (1) Knockout of CryAA could inhibit survival and angiogenesis signals both in vitro and in vivo, suggesting that CryAA might be a potential target for the treatment of angiogenesis-related diseases. (2) CryAA knockout strongly inhibited VEGF-induced vascularization by downregulating VEGF secretion and blocking VEGFR2, Akt, PLC\(\gamma\)1, p38 MAPK, Src, FAK, and p44/p42 MAPK phosphorylation both in vitro and in vivo. In addition, CryAA knockout could induce the cleavage of caspase-9 and caspase-3, leading to apoptosis. Further studies are needed to determine whether a CryAA antagonist could be safely used in human newborns and to clarify the timing and dose required to provide the safest and most efficacious treatment for human infant ROP and CNV.

Acknowledgments

Supported by the National Basic Research Program of China (81470651), the National Basic Research Program of China (973 Program, 2011CB510200), and the National Basic Research Program of China (800965) to ZMW. The authors alone are responsible for the content and writing of the paper.

Disclosure: Q. Xu, None; Y. Bai, None; L. Huang, None; P. Zhou, None; W. Yu, None; M. Zhao, None

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