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Original Article

αB crystalline upregulates the expression of matrix metalloproteinases in trabecular meshwork cells through TLR1/2

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Abstract: Purpose: Our previous study identified the TLR1/2 complex as the receptor for αB crystalline (CRYAB). Here, we aimed to determine whether CRYAB could influence the expression of matrix metalloproteinases (MMPs) in trabecular meshwork (TM) cells through TLR1/2. Methods: The expression of P65, P38, ERK and JNK, which are downstream of TLR1/2, was identified by western blot (WB) with the interference of a TLR1/2 inhibitor or siRNA-TLR1 (siRNA-TLR2). MMP2 and MMP9 were tested by WB analyses using the same interference conditions in TM cells. C57BL/6N mice were used to study the effects *in vivo* by anterior chamber injection of CRYAB. Finally, immunohistochemistry was performed to evaluate the alterations in MMP2 and MMP9 between the CRYAB injection group and the normal control group. Results: For the NF-κB pathway, the expression of P65 was increased in TM cells with the addition of exogenous CRYAB (*P<0.01*), which was dramatically reduced with inhibition of TLR1/2 by its inhibitor (CU-CPT22) or knockdown of TLR1 (or TLR2) with siRNA-TLRs (*P<0.01*). For the MAPK pathway, P38, ERK and JNK showed no significant difference when CRYAB or CU-CPT22 was added. Subsequently, MMP2 and MMP9 were upregulated, which was consistent with the increased level of p65 (*P<0.001*). Finally, elevated expression of MMP2 and MMP9 in mice was demonstrated in trabecular meshwork tissue compared to that of the normal control. Conclusion: CRYAB triggers the activation of MMPs in TM cells *in vitro* and *in vivo,* possibly following the TLR1/2-mediated proinflammatory effect on TM cells.

Keywords: αB crystalline (CRYAB), trabecular meshwork cells (TM cells), TLR1/2 complex, NF-κB, extracellular matrix

Introduction

CRYAB is a member of the small heat shock protein family that functions as a molecular chaperone, responding to prevent aggregation of abnormal proteins [1-3]. This protein was initially found in the lens of the eye, but later, it was also detected at high levels in heart and skeletal muscle tissues [4-6]. Studies have shown that elevated expression of CRYAB in the nervous system protects tissue and cells from damage under extreme stress, inflammation, and oxidation [7, 8]. Because of the high expression of CRYAB in multiple cells in the eye, we explored the possible function of CRYAB on TM cells, which play an important role in the regulation of aqueous outflow.

Recently, we identified that CRYAB binds to TLR1/2 as a signaling molecule, and we also reported that CRYAB promotes the proliferation and migration of TM cells, which may play a role in the progression of secondary glaucoma [9]. TLR1/2 downstream pathways are related to immune and inflammation regulation [10, 11]. P65 is the main molecule of NF-κB that participates in inflammation, immune reactions and apoptosis regulation. P38, JNK and ERK are the main factors of the MAPK pathway, which is involved in multiple functions, including cell cycle, proliferation, morphologic maintenance of cells and apoptosis. The possible relationship between the activation of these pathways and the function of TM cells interests us.

Anatomically, the TM tissue can be divided into three layers [12, 13]. The outermost is the juxtacanalicular tissue (JCT), which forms the main resistance of the aqueous humor. The JCT layer is composed of extracellular matrix (ECM) and the discontinuous basement membrane of Schlemm's canal (SC) endothelial cells, the main components of which are types I and IV collagens. MMP2 and MMP9 play a key role in the remodeling of collagens. Based on previous knowledge, elevated inflammation induces the activity of matrix metalloproteinases (MMPs), which help remodel TM tissue and increase the outflow rates.

Therefore, whether CRYAB has the same effect on TM tissue through TLR1/2 remains unknown. Here, we tested the changes in TLR1/2 downstream molecules, including P65, P38, ERK and JNK, with CRYAB and CU-CPT22 (or siRNA-TLRs) as interference [14]. MMP2 and MMP9 were selected as the target function proteins. The expression was detected *in vitro* and *in vivo*. This may explain the possible effect of CRYAB on TM tissue.

Materials and methods

Cell culture and treatment

Human TM cells were purchased from ScienCell (6590), cultured in DMEM (Gibco 11965-092, USA) supplemented with 10% fetal bovine serum (Gibco 10099141, Australia), 100 U/ml penicillin and 100 ug/ml streptomycin (Solarbio, Beijing, China). All cells were kept in a humidified incubator (5% CO_2) at 37°C. TM cells were divided into four groups: 1: normal control group; 2: TM cells stimulated with CRYAB (10 ng/ml) as an activator for 24 hr; 3: TM cells treated with the TLR1/2 inhibitor CU-CPT22 (5 μmol/L, RD4884) for 24 hr; and 4: TM cells pretreated with CU-CPT22 (5 μmol/L, RD4884) 12 hr before incubation with CRYAB (10 ng/ml) for another 24 hr. Total protein samples were collected for western blot (WB) analysis.

siRNA transfection

TLR1-siRNA, TLR2-siRNA and scrambled siRNA were obtained from Santa Cruz Biotechnology (sc-40254, sc-40256, sc-37007). TM cells were cultured in 6-well plates (Corning Life Sciences, Acton, MA, USA) for 36 hr to ensure 70%-80% confluence. The cells were then transfected with a mixture containing 4 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and 8 μl of siRNA in 800 μl of Opti-MEM (Gibco 31985070, USA). After transfection, the cells were cultured for another 5 hr, and the medium was then replaced with 1× normal growth medium. An additional 24 hr incubation was needed for the following experiments. The normal group, negative group (NC), TLR1-siRNA group, and TLR2-siRNA group were treated with CRYAB in the presence of interference. Knockdown efficiency was detected by WB analysis. Total protein samples were used for WB analysis.

Western blot analysis

After treatments, TM cells were collected and homogenized in RIPA lysis buffer (Thermo 89-900, USA) containing PMSF (Beyotime ST506) and protease and phosphatase inhibitors (Beyotime P1045) and incubated on ice for 30 min. The whole-cell lysates were then centrifuged at 14,000 g and 4°C for 15 min. The precipitate was discarded, and the protein concentrations were determined using a BCA kit (Beyotime P0010). Equal amounts of total protein (20 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat skim milk in Tris-buffered saline solution with the detergent Tween-20 (TBST) at room temperature for 1 hr and subsequently incubated with specific primary antibodies against P65 (ab32536, 1:50000), p38 (CST 8690s, 1:1000), JNK (CST 9252, 1:1000), ERK (CST 4695s, 1:1000), MMP2 (ab110186), MMP9 (ab137867), and β-actin (CST 4970s, 1:1000) at 4°C overnight. The membranes were washed three times with TBST for 10 min and then incubated with an HRP-conjugated secondary antibody (CST 7074, 1:2000) at 37°C for 1 hr. After three washes with TBST, the protein bands were visualized by chemiluminescence detection and quantified by ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). β-actin was used as the loading control.

Animals

Female C57BL/6N mice (n=9; age: 8 weeks) purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and were housed

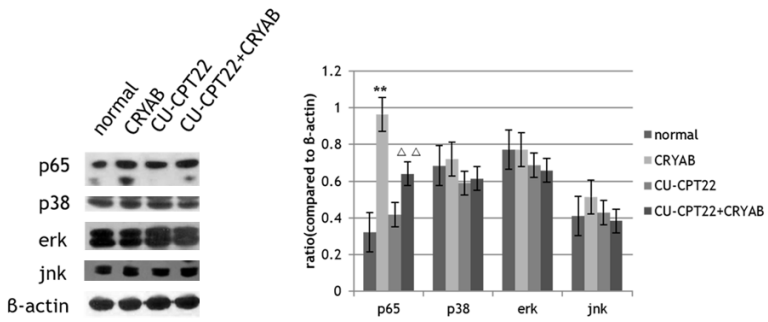

Figure 1. CRYAB demonstrated a proinflammatory effect on TM cells through TLR1/2 with CU-CPT22 treatment. Alterations in P65, P38, ERK, and JNK were tested by WB analyses. CRYAB acted as an activator of TLR1/2, while CU-CPT22 functioned as an inhibitor of TLR1/2. (n=3 for each test; mean ± SEM; **P<0.05, **P<0.01* vs. normal at the same time point; *ΔP<0.05, ΔΔP<0.01* vs. CRYAB at the same time point).

with a 12 hr: 12 hr light: dark cycle in a temperature- and humidity-controlled environment. Standard lab chow and water were provided ad libitum. The animals were divided into three groups. The left eyes were selected for testing. The experimental group was treated by anterior chamber injection with 2 μl CRYAB (10 ng/μl). The negative control group was treated by anterior chamber injection with 2 μl PBS. The normal group was not treated. The eyeballs were obtained 24 hr after surgery for subsequent experiments. The research was performed according to the NIH Guidelines. All animal procedures were approved by the Peking University People's Hospital MEC/IACUC (Approval No. 2018PHC088).

Immunohistochemistry

The fixed eyeballs were embedded in paraffin wax. Tissue slices were stained with hematoxylin-eosin (H&E). Immunohistochemistry analysis of MMP2 and MMP9 expression was performed with primary antibodies (ab110186, ab76003), and the secondary antibodies were purchased from Zsbio (PV-9001, Beijing). The optical density was quantified by ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All results were analyzed using SPSS19.0 software (Chicago, USA). All data are expressed as the mean ± standard error of mean (SEM). Statistical analysis was performed by t-test. A *P*-value of less than 0.05 was considered significant (**P< 0.05, **P<0.01,* and ****P< 0.001*).

Results

CRYAB showed an effect on the NF-κB and MAPK pathways in the presence of CU-CPT22 or siRNA-TLRs

Our previous study demonstrated that CRYAB could bind to TLR1/2 on TM cells. The main pathways downstream of TLR1/2 are NF-κB, MAPK (P38, ERK, JNK), and caspase-8-related pathways. Activation of these pathways is involved in cell proliferation, immune regulation, inflammation and survival. To determine the effect of CRYAB on these pathways, we tested the expression levels of proteins such as P65, P38, ERK, and JNK in TM cells treated with CRYAB at a concentration of 10 ng/ml. The results demonstrated that CRYAB increased the expression of p65, while the MAPK-related molecules showed no significant difference in expression. Then, we used CU-CPT22 to block TLR1/2, and the expression of P65 dropped markedly even in the presence of CRYAB (**Figure 1**).

Subsequently, the function of TLR1 or TLR2 was tested by knockdown technology. The knockdown efficiency of TLRs was determined by WB (**Figure 2**). Because only P65 demonstrated significant changes in the presence of CU-CPT22, we focused on NF-κB in the knockdown assay with siRNAs. CRYAB markedly increased the expression of p65, which dramatically declined with siRNA treatment (**Figure 3**).

The expression of MMPs changed after incubation with CRYAB

MMP2 and MMP9 play a key role in the remodeling of juxtacanalicular and Schlemm's canal inner wall basement membrane, which are composed of type I and type IV collagens. Because of the significant CRYAB-mediated changes in P65 during inflammation activation, we explored the relationship between CRYAB

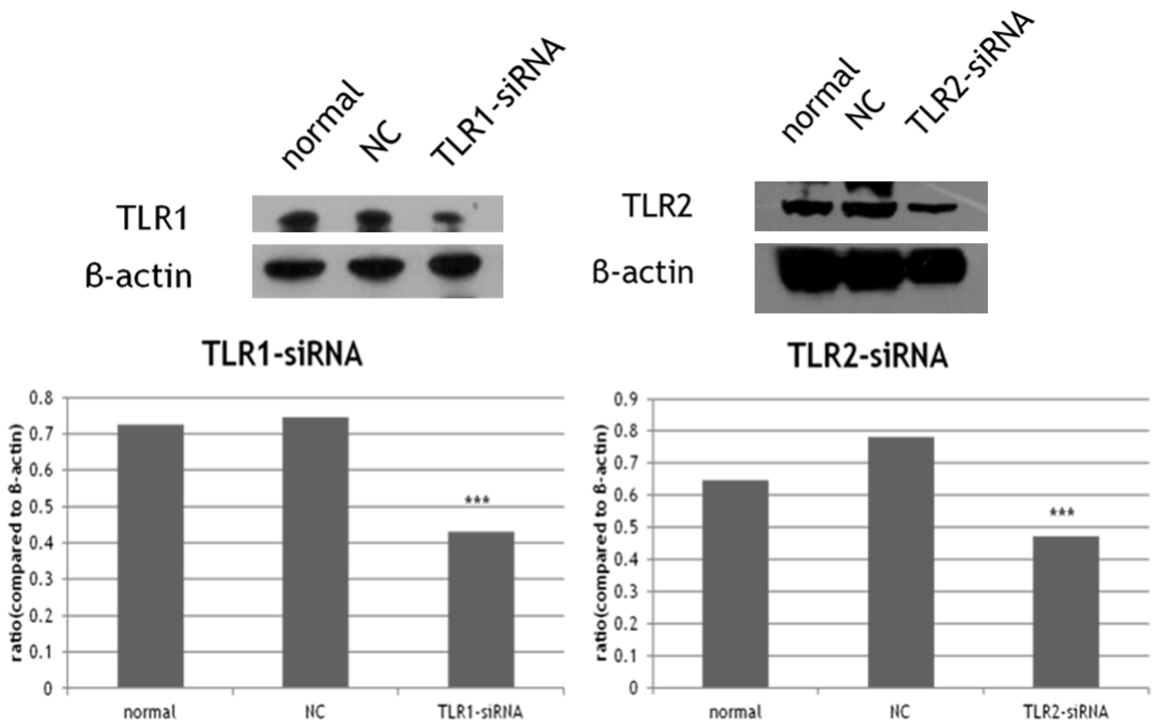

Figure 2. Knockdown efficiency of siRNA. (n=3 for each test; mean ± SEM; **P<0.05, **P<0.01, ***P<0.001* vs. negative control group (NC) at the same time point).

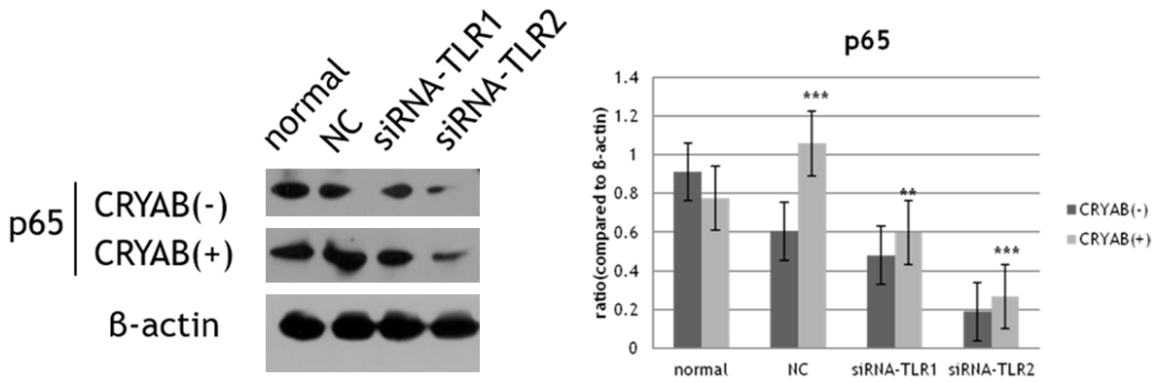

Figure 3. CRYAB promoted the expression of P65 in TM cells through TLR1/2 with siRNA. The functional differences of TLR1 and TLR2 in the activation of NF-κB were identified by WB analysis. siRNAs were used to knock down TLR1 and TLR2. (n=3 for each test; mean ± SEM; **P<0.05, **P<0.01, ***P<0.001* vs. CRYAB (-) at the same time point).

and MMPs using MMP2 and MMP9 as markers of aqueous outflow.

CU-CPT22 and siRNA were also used to inhibit TLR1/2. The expression of MMP2 and MMP9 increased after activation of TLR1/2 with CRYAB (*P<0.001*). After inhibiting the TLR1/2 complex, CRYAB failed to increase the expression of MMP2 and MMP9 (*P<0.01*) (**Figure 4A**). In the siRNA interference group, MMP2 and MMP9 increased with CRYAB treatment; the effects of siRNA were ambiguous and require further study (**Figure 4B**). The expression of MMPs were consistent with the expression of inflammatory factors, which confirmed our previous knowledge [15, 16].

CRYAB induced the expression of MMP2 and MMP9 in TM tissue in vivo

We demonstrated that *in vitro* CRYAB activates TM cells, leading to the increased expression of MMP2 and MMP9. We then examined this effect in an animal experiment. CRYAB was

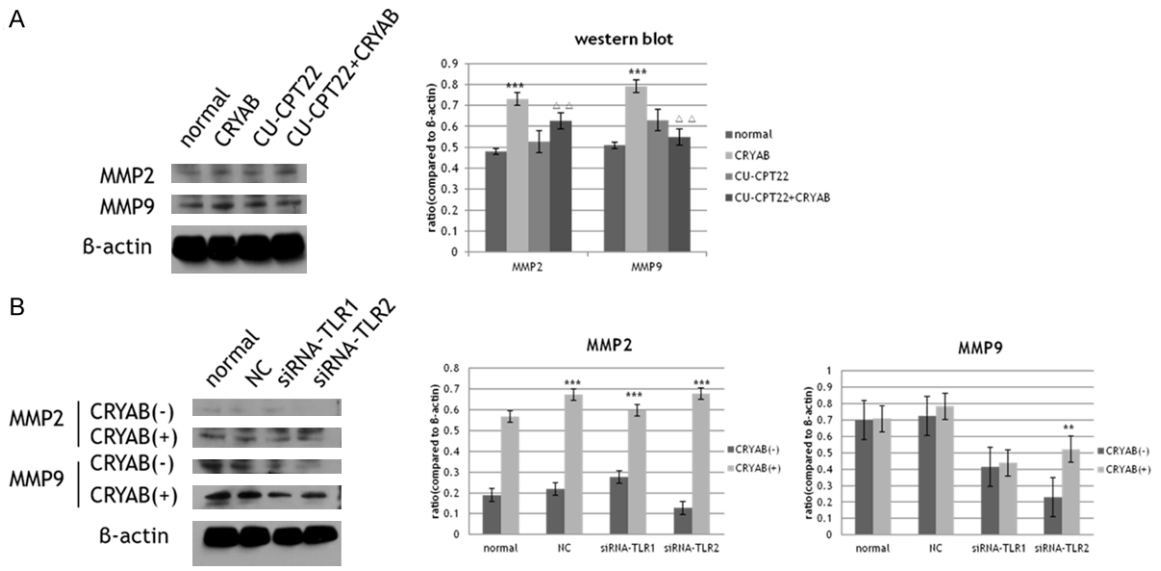

Figure 4. CRYAB elevated the expression of MMP2 and MMP9. A. The effect of CRYAB on the expression of MMP2 and MMP9 in TM cells was detected by WB analyses, in which CU-CPT22, an inhibitor of TLR1/2, was used as an interference agent. (n=3 for each test; mean ± SEM; **P<0.05, **P<0.01, ***P<0.001* vs. normal at the same time point; *ΔP<0.05, ΔΔP<0.01* vs. CRYAB at the same time point). B. siRNAs were also used to identify the functional differences of TLR1 and TLR2 in the process. (n=3 for each test; mean ± SEM; **P<0.05, **P<0.01, ***P<0.001* vs. CRYAB (-) at the same time point).

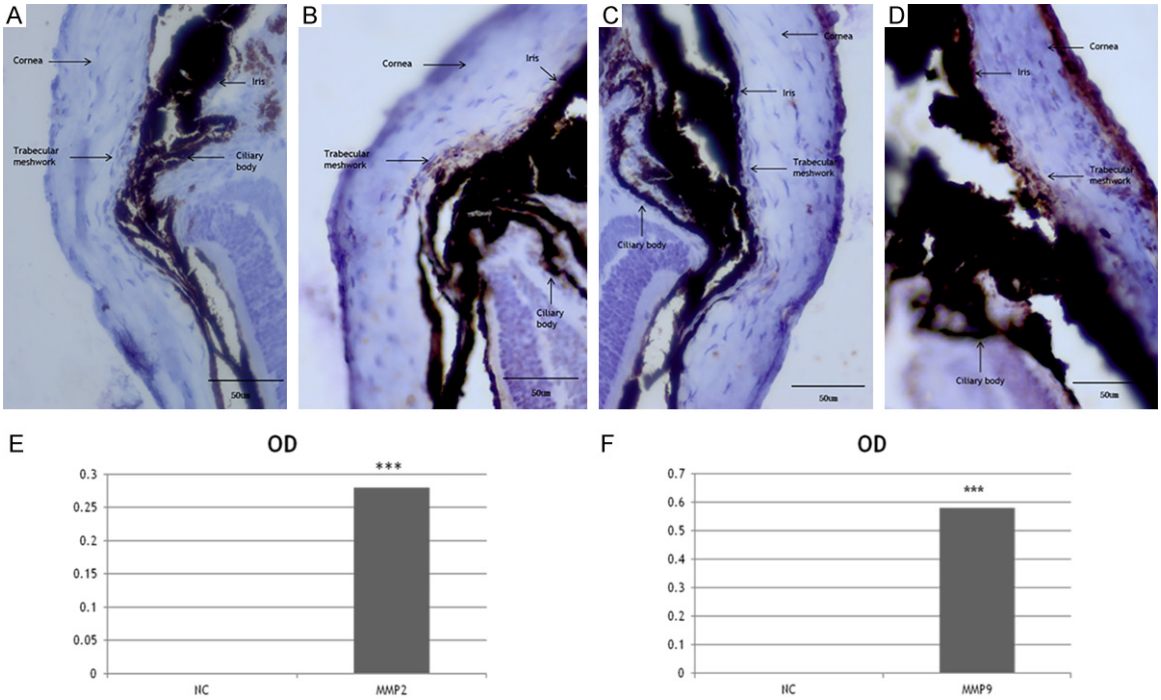

Figure 5. Immunohistochemistry analysis of TM tissues of mice. A and B. Expression of MMP2. MMP2 was stained brown. C and D. Expression of MMP9. MMP9 was stained brown. A and C. Negative control group (NC): anterior chamber injection with 2 μl PBS. B and D. Experimental group: anterior chamber injection with 2 μl CRYAB (10 ng/ul). E and F. Statistical analysis of the optical density. (**P<0.05, **P<0.01, ***P<0.001* vs. NC).

injected into the anterior chamber, imitating the release of the protein from residual LEC after cataract surgery. Immunohistochemistry was used to evaluate the expression of MMPs. We found that *in vivo*, CRYAB also acted on TM cells and increased the expression of MMP2

and MMP9 (brown staining in **Figure 5B** and **5D**), which might have a protective effect on the aqueous humor outflow rate.

Discussion

To our knowledge, this study described for the first time the influence of CRYAB on the extracellular matrix of TM tissues. In our previous study, we demonstrated that TM cells express TLR1/2 and that CRYAB could bind to TLR1/2, acting as a signaling molecule to trigger the downstream pathway and lead to changes in cell function. Here, we report for the first time that CRYAB primarily activates NF-κB through TLR1/2, leading to the elevated expression of MMPs in TM cells *in vitro* and *in vivo*.

The TLR1/2 complex is a conservative protein complex involved in inflammation regulation and immunity in macrophage-like cells [17-19]. NF-κB and MAPK signaling pathways are the main downstream pathways of TLR1/2. Here, we identified that CRYAB activates the pathways and induces high expression of inflammation-related molecules, especially NF-κB. CU-CPT22, an inhibitor of TLR1/2, was used to suppress the whole receptor complex [14], and we found that the expression of P65 declined dramatically even with the addition of CRYAB. However, the factors representing MAPK demonstrated no significant difference, which indicated that the NF-κB pathway is the main pathway by which CRYAB acts on TM cells. Then, we further explored the function of TLR1 or TLR2 in the NF-κB pathway independently with siRNA knockdown of specific parts of the complex. The results also suggested that CRYAB plays a proinflammatory function through TLR1/2 by increasing the expression of P65. Knocking down TLR1 or TLR2 depressed the proinflammatory process activated by CRYAB. Activated NF-κB translocates into the nucleus, promoting the expression of TNF-α and IL-1 [20, 21].

The TM tissue includes layers of sieved tissue composed of ECM and TM cells. The influences of inflammation on the quality and quantity of ECM are not well understood. Most of the outflow resistance of the aqueous humor is due to the ECM of the JCT and the basement lamina of the SC inner wall endothelium [22-25]. The remodeling of ECM activated by MMPs is the main factor in maintaining the IOP balance [26]. Given that type I and type IV collagens are the main components of the aqueous outflow tract, we chose type IV collagenases, such as MMP2 and MMP9, as our markers representing the ECM turnover levels. In the current study, we found that CRYAB induced high expression of MMP2 and MMP9 in accordance with the activation of the NF-κB pathway. This result followed the findings that activation of NF-κB led to increased production of TNF-α, IL-1α, IL-1β and IL-8, which subsequently caused the elevation of MMPs regulating the outflow rates to maintain a relatively normal IOP [27, 28]. Increased MMPs indicate a more active replacement of ECM, which might decrease the outflow resistance. Additionally, it was presumed that the modified ECM would be somewhat different in composition, organization and amount, which helped to maintain the rebalanced outflow resistance. The same results were also found *in vivo*. We compared the *in situ* expression of MMP2 and MMP9 before and after anterior chamber injection with CRYAB, and the increased staining of the MMPs strongly supported our research at the cellular level. Although we could not find a reliable way to directly measure the changes in outflow rate, all these findings partially explain the phenomenon of the reduced IOP after cataract surgery.

Thus, until now, there have been two viewpoints about the function of CRYAB in TM cells in our series of studies. One study showed that CRYAB plays an anti-apoptotic function and promotes the proliferation and migration of TM cells, which might exacerbate the density and stiffness of TM tissue [9]. Another study demonstrated a possible protective effect of CRYAB by elevating the expression of MMPs, which play a role in decreasing the resistance of the outflow pathway. However, the two viewpoints might not oppose each other. In normal TM tissue, MMPs are relatively highly expressed during nonstress conditions and function to maintain open outflow pathways [29]. Once the appropriate conditions change, such as high perfusion pressure, stretching of TM structures or inflammation, the TM tissue responds to offset the influences to restore the normal balance within suitable limits [30, 31]. In our current study, the highly expressed MMP2 and MMP9 might be the remedy of TM cells to the unwelcomed inflammation triggered by CRYAB. The final balance between the two sides determines the ultimate function of TM tissue.

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Disclosure of conflict of interest

None.

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