Long-term and potent IOP-lowering effect of \( \text{IkB}\alpha \)-siRNA in a nonhuman primate model of chronic ocular hypertension

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Highlights
Knocking down \( \text{IkB}\alpha \) could upregulate the expression of MMP2 and MMP9 in MCM and MTM.
LP could induce COHT model in rhesus monkeys successfully.
\( \text{IkB}\alpha \)-siRNA has a long-term and potent IOP-lowering effect in LP-induced monkeys with COHT.
Long-term and potent IOP-lowering effect of IκBα-siRNA in a nonhuman primate model of chronic ocular hypertension

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SUMMARY
Glaucoma is one of the most common causes of irreversible blindness. It is acknowledged that lowering intraocular pressure (IOP) is the effective treatment to slow glaucoma disease progression. The main obstacle of existing drugs is that the effect of reducing IOP does not last long. Degradation of IκB stimulates the transcription of NF-κB, which could upregulate the expression of matrix metalloproteinases (MMPs). Whether an IκB-targeted gene therapy works in glaucoma is unclear. Here, we established a chronic ocular hypertension (COHT) model in rhesus monkey by laser photocoagulation and verified that intracameral delivery of IκBα-siRNA showed long-lasting and potent effects of reducing IOP without obvious inflammation in monkeys with COHT. We also verified that IκBα-siRNA could increase the expressions of MMP2 and MMP9 by knocking down IκB in vitro and in vivo. Our results in nonhuman primates indicated that IκBα-siRNA may become a promising therapeutic approach for the treatment of glaucoma.

INTRODUCTION
Glaucoma is a multifactorial progressive neurodegenerative disease characterized by irreversible loss of visual function, which affected almost 76.5 million people worldwide in 2020 and is estimated to reach 111.8 million by 2040 (Baudouin et al., 2021). Although there are many causes of glaucoma progression, intraocular pressure (IOP) remains the major risk factor, so as to reduce IOP is the main therapeutic mechanism for slowing disease progression in patients with glaucoma (Garway-Heath et al., 2013; Varma et al., 2011). However, existing drugs are limited due to several reasons, such as the inability of monotherapy to achieve the therapeutic target IOP, drug-related side effects, and the low compliance of patients to take multiple eye drops every day. Thus, in recent years, scientific and medical circles have paid more attention to the development of potent and long-acting new drugs to reduce IOP. However, until now, no FDA-approved drugs targeting the long-acting efficiency in reducing IOP of glaucoma have been developed.

Nonhuman primates, which are homologous with humans, constitute an indispensable laboratory model for various fields of biomedical research (Evans and Silvestri, 2013; Roelfsema and Treue, 2014; Tomalka et al., 2021; Wegener et al., 2021), and have become ideal glaucoma experimental animals because of their highly consistent anatomical structure and physiological characteristics with humans eyeballs (Pasquale et al., 2021; Rasmussen and Kaufman, 2005; Stewart et al., 2011). There are several kinds of chronic ocular hypertension (COHT) model in nonhuman primates, including topical dexamethasone treatment (steroid-induced glaucoma), intracameral injection of microbeads made of different materials, and laser photocoagulation (LP) of the trabecular meshwork (TM) (Fingert et al., 2001; Pelzel et al., 2006; Quigley and Hohman, 1983; Tu et al., 2019; Weber and Harman, 2005). Dexamethasone (Dex)-induced ocular hypertension is one of the most commonly used and developed murine ocular hypertension model (Kasetti et al., 2018; Zode et al., 2014), but the success rate of this model in nonhuman primates is only 45% with a short maintenance time (approximately 2 weeks) of high IOP after stopping medication (Fingert et al., 2001). Injection of microbeads with different materials in the anterior chamber could induce a model of chronic glaucoma, but invasive and repeated intracameral injections to maintain stable IOP are needed (Chen et al., 2011; Rodrigo et al., 2021; Samsel et al., 2011; Weber and Harman, 2005). Compared with Dex-induced and microbeads-induced COHT, LP-induced COHT is more advanced, due to the advantages of being noninvasive, having a mild inflammatory response, keeping the refractive stroma clear, and maintaining consistently high IOP for several weeks to 11 months (Pelzel et al., 2006; Quigley and
Eyes, which have a clear anatomy, relative immune privilege, and relatively isolated compartment that limits systemic exposure, are a good target for RNAi therapy. RNAi-based mechanisms, especially the use of small interfering RNAs (siRNAs), have been used in research to treat several chronic diseases and have shown promising results for ocular diseases, including glaucoma (Cassidy et al., 2021; Diaz-Canestro et al., 2021; Feng et al., 2021; Nguyen et al., 2012; Ray et al., 2017). Due to their high susceptibility to enzyme hydrolysis, rapid removal from circulatory system siRNA, virus vectors or nanoparticle carriers are needed. Notably, the silenced gene has to be re-synthesized in order to recover its biological activity after siRNA treatment, so the siRNA compound strategies have much more prolonged effect compared with commercial pharmaceutical products (Behlke 2006; Liu et al., 2009; Lu et al., 2005). Matrix metalloproteinases (MMPs)-mediated extracellular matrix (ECM) remodeling in the TM and ciliary muscle (CM) reduces outflow resistance in the conventional (trabecular) and unconventional (uveoscleral) outflow pathways, and helps maintain IOP homeostasis (Acott et al., 2021; Bradley et al., 1998; De Groef et al., 2014; De Groef et al., 2013; O’Callaghan et al., 2017; Toris et al., 2008; Weinreb et al., 2020).

The secretion of MMPs depends on the activation of NF-κB in vascular smooth muscle and macrophages (Bond et al., 2001; Chase et al., 2002). In TM and human ciliary muscle (HCM) cells, upregulation of MMPs has been also shown to be mediated by NF-κB (Lan et al., 2009; Porter et al., 2012). NF-κB remains inactive when bound to IκB in the cytoplasm. Phosphorylation of IκB results in its ubiquitination and degradation, which releases NF-κB for nuclear translocation and stimulates the transcription of genes (Ramkumar et al., 2011). In addition, downregulation of IκBα plays a crucial role in reducing IOP in rats (Zeng et al., 2020). However, whether and how IκBα-siRNA working in MTM and/or MCM of COHT nonhuman primate, and whether it further achieves the goal of long-lasting IOP-lowering are unknown.

In this study, we investigated whether IκBα-siRNA could inhibit the synthesis of IκBα in vitro and in vivo, and then increase the secretion of MMPs by activating NF-κB to play a long-term and potent role in reducing IOP in nonhuman primates COHT models.

**RESULTS**

**Characterization of MCM and MTM cells**

Primary MCM and MCM cells were successively harvested at least three times to produce three cell lines that were used in the following experiments. The primary MCM cells were validated in immunofluorescence using two markers: α-smooth muscle actin (α-SMA) (a variant of actin expressed specifically in smooth muscle cells) and desmin (a muscle-specific intermediate filament protein) (Hutchinson et al., 2010; Tamm et al., 1991; Zhao et al., 2003). Our cultured MCM cells showed both α-SMA and desmin immunoreactivity in all cells (Figure S1A). No fluorescence was detected in the negative control (NC) group (secondary antibodies only) both in MCM and MTM cells (Figure S1B). Besides, a number of criteria, including the expression of RSPO4 (spreading throughout the TM but not surrounding tissues (Patel et al., 2020)), neuron-specific enolase (NSE), fibronectin (FN), and collagen type IV (Col IV) (Fan et al., 2019; Guo et al., 2012; Mao et al., 2013; Patel et al., 2020), as well as Dex-induced increased expression of myocilin (MYOC) (Clark et al., 2001; Fan et al., 2019; Fingert et al., 2001), were used to characterize isolated MTM cells. We first studied the expression RSPO4, NSE, FN, and Col IV in MTM cells by immunofluorescence. All four proteins were expressed in our MTM cells (Figure S1C). Furthermore, the MTM cells showed increased expression of MYOC following induction by Dex (p = 0.0026) (Figure S1D).

**IκBα-siRNA colocalizes with lysosomes in vitro**

As shown in the merged images in Figure 1A, the Pearson’s correlation coefficient (PCC) value of the fluorescent signals of IκBα-siRNA-Cy5 and lysosomes was up to 0.79 and 0.84 in the primary MCM and
MTM cells separately after incubation for 24 h, providing additional quantitative confirmation of the obviously strong colocalization of IκBα-siRNA and lysosomes. In comparison, the PCC value decreased to 0.39 and 0.37 in MCM cells and MTM cells after 48 h, respectively. These results demonstrated great consistency with that observed by confocal imaging experiments, the IκBα-siRNA located primarily in lysosomes after 24 h incubation, while it was gradually released from lysosomes after 48 h incubation (Figures 1A and 1H).

IκBα-siRNA transfection knockdowns IκBα and activates NF-κB P65 in vitro
As described previously, we identified primary MCM cells and MTM cells, and screened the optimal sequence and concentration of IκBα-siRNA to inhibit the expression of IκBα (Ou et al., 2020). IκBα mRNA expression was significantly decreased in the experimental group compared with the blank and negative control groups after transfection of IκBα-siRNA for 48 h in the primary MCM cells (p = 0.0006) (Figure 1B) and in the MTM cells (p = 0.0004) (Figure 1I). Likewise, the decrease in IκBα protein expression was statistically significant in the experimental group compared with the blank and negative control groups in primary MCM cells by immunofluorescence (IF) (p = 0.0124) and western blotting (WB) (p = 0.0178) analysis (Figures 1C and 1D), while similar trend obtained by IF (p < 0.0001) and WB (p = 0.0002) analysis in primary MTM cells (Figures 1J and 1K). In addition, we found that although the mRNA and protein expression of NF-κBp65 did not change significantly both in primary MCM and MTM cells by qPCR (p = 0.3765, p = 0.7751) and WB (p = 0.5078, p = 0.9190) analysis (Figures 1E and 1F and 1L and 1M), the protein expression of phospho-NF-κBp65 increased after transfection of IκBα-siRNA both in the primary MCM and MTM cells by WB analysis (p = 0.0125, p = 0.0119) (Figures 1G and 1N).

Knockdown of IκBα upregulates the expression of MMPs in vitro
To further investigate the effects of IκBα on the expression of MMPs in the primary MCM and MTM cells, we verified it at the mRNA and protein levels.

qPCR analysis revealed the upregulated mRNA expression of MMP2 (p = 0.0013) and MMP9 (p = 0.0189) compared with the blank and negative control groups after knockdown of IκBα for 48 h in the primary MCM cells (Figure 2A). We found similar regulatory effects in the primary MTM cells (MMP2, p = 0.0049; MMP9, p = 0.0046) (Figure 2E).

WB analysis showed the upregulated protein expression of MMP2 (p = 0.0092) compared with the blank and negative control groups after knockdown of IκBα for 48 h in the primary MCM cells (Figure 2B). We also found similar regulatory effects in the primary MTM cells (p = 0.0122) (Figure 2F).

IF analysis supported the upregulated protein expression of MMP2 (p = 0.0158) and MMP9 (p = 0.0017) compared with the blank and negative control groups after knockdown of IκBα for 48 h in the primary MCM cells (Figures 2C and 2D), the similar regulatory effects was found in the primary MTM cells (MMP2, p = 0.0020; MMP9, p = 0.0047) (Figures 2G and 2H).

LP induces stable and last-lasting COHT in rhesus monkeys
Six eyes from three monkey models successfully established COHT (Figure 3A), and two eyes from one monkey were set as blank controls. The baseline IOP was 13.50 ± 2.51 mmHg, which was similar to those of previous studies (Lin et al., 2021; Pasquale et al., 2021; Quigley and Hohman, 1983; Tu et al., 2019). There was no elevated IOP in any eye after the first LP, and fluctuating IOP appeared after the second LP. Inflammation of the anterior chamber occurred in the early stage after LP. Two eyes maintained stable high IOP during the observation period of 2 months after the second LP, and the mean IOP reached 45.90 ± 8.25 mmHg. After the inflammation subsided, fluctuating IOP decreased to normal levels in four eyes, stable COHT was obtained in the remaining four eyes after the third LP, and the mean IOP reached 49.35 ± 7.15 mmHg during the observation period of 1 month (Figure 3B).
Figure 3. Establishment of COHT in rhesus monkeys

(A) A representative operation photo of LP in a rhesus monkey.
(B) Time course of IOP changes in six eyes after LP compared to two blank eyes.
(C) The thickness of the peripapillary RNFL at baseline and IOP remained high for 2, 6, and 10 weeks in the infratemporal (IT), infranasal (IN), nasal (N), supranasal (SN), supratemporal (ST), and temporal (T) regions.
(D) SD-OCT scanning of the macula was performed before LP and measured again after 10 weeks of elevated IOP in the same eye. Scale bar 200 μm.
(E–G) The GCL/GCIP/L retina thickness at distances of 1, 2, and 3 mm from the macular fovea at baseline and the IOP remained high for 2, 6, and 10 weeks in the inferior (I), superior (S), nasal (N), and temporal (T) regions. See also Tables S1A–S1C.
(H and I) H&E staining of paraffin sections showed that the RNFL and GCL around the optic disc in the COHT group were reduced compared with those in the control group (Figure 3H). The same changes were found in the peripapillary and peripheral retina. Scale bar 100 μm.
(J) Representative OCT scanned images at the anterior chamber corner before and after three LPs.
(K) Masson staining of paraffin sections of blank control eyes and COHT eyes in the TM. Scale bar 100 μm. See also Figure S2.

The thickness analysis of the peripapillary retinal nerve fiber layer (RNFL) via SD-OCT scanning showed that the thickness of the infranasal (IN) (reduced by 29.02%), infratemporal (IT) (reduced by 20.0%), nasal (N) (reduced by 19.99%), and supratemporal (ST) (reduced by 19.41%) became obvious thinner two weeks after IOP increased. The follow-up two-month observation period, when the IOP remained high and stable, the RNFL thickness in all quadrants gradually became thinner to varying degrees, especially in the IN (reduced by 59.62%), IT (reduced by 51.48%), supranasal (SN) (reduced by 63.61%), ST (reduced by 54.43%), and N (reduced by 46.23%) (Figure 3C).

According to the statistics of retinal thickness around the macular fovea scanned by SD-OCT, it was found that the thickness of ganglion cell layer (GCL) and ganglion cell inner plexiform layer (GCIP) was obvious thinner than baseline after 10 weeks of elevated IOP in a long-term observed monkey (Figure 3D). There was no significant change in macular retinal thickness after 10 weeks of elevated IOP (Figure 3E and Table S1A). The scan of the macular fovea showed that the thickness of GCL and GCIP decreased in four quadrants (inferior, superior, nasal, and temporal) after IOP increased. IOP began to rise over 2 weeks, the thickness of GCL and GCIP in the nasal quadrant which was 3 mm away from the macular fovea, that was, the area close to the optic disc, became thinner. During the next two months, IOP remained high, and the thickness of the GCL and GCIP in the four quadrants around the macular fovea became thinner and gradually reduced with time. Except for similar temporal changes, the GCL was more sensitive to the increase in IOP than the GCIP (Figures 3F, 3G, Tables S1B and S1C). The changes in the GCL and GCIP in the macula and peripapillary RNFL were consistent with the changes in glaucoma observed by SD-OCT.

H&E staining of paraffin sections showed that the RNFL and GCL around the optic disc in the COHT group were reduced compared with those in the control group (Figure 3H). The same changes were found in the peripheral retina (Figure 3I).

In vivo, anterior OCT scan showed the patent Schlemm’s Canal (SC) and smooth surface of the TM in the anterior chamber angle before LP, while collapsed SC and compact TM could be observed after LP (Figure 3J). Also, Masson staining of paraffin sections showed detailed damage to the TM structure after LP. The destroyed collagen structure of TM and collapsed SC were observed (Figures 3K and S2).

Intracameral injection of IκBα-siRNA has a potent and long-lasting efficacy

The effects of intracameral injection of 90–100 μg IκBα-siRNA on the IOP of one COHT eye compared with one COHT eye without any drug intervention are shown in Figure S3A. IκBα-siRNA lowered the IOP in the monkey eye from Day 2 to Day 28 after injection compared with the baseline and blank control eye, especially from Day 4 to Day 16. However, mild corneal edema, anterior chamber exudation, and cells were observed in the eye injected with 90–100 μg IκBα-siRNA on the first day after injection, which were improved on the Day 4 after the operation (Figure S3B).

The effects of the intracameral injection of 5–10 μg IκBα-siRNA or 5–10 μg NC-siRNA on the IOP of COHT monkeys are shown in Figure 4B. IκBα-siRNA was injected in the left eye of two monkeys with COHT as the experimental group, while NC-siRNA was injected in the right eye as the negative control group. The pretreatment IOP of the four eyes from two monkeys involved was 44 ± 9.17 mmHg (5–10 μg NC-siRNA) and 42.55 ± 6.56 mmHg (5–10 μg IκBα-siRNA). Impressively, the dose of 5–10 μg showed similar IOP-lowering effect to 90–100μg. IκBα-siRNA (5–10 μg) lowered the IOP in the eyes of monkey with COHT from Day 2 to Day 28 after injection compared with the baseline and negative control group. The effect of IOP lowering was very significant, especially from Day 4 to Day 16. The IOP decreased to 14.36 ± 5.15 mmHg, and the
Figure 4. Efficacy of 5–10 μg IkBa-siRNA intracameral injection

(A) Flow chart of rhesus monkey in vivo.

(B) Time course of IOP changes in COHT eyes after 5–10 μg IkBa-siRNA, or 5–10 μg NC-siRNA intracameral injection separately (n = 2).

(C) Time course of IOP changes in COHT eyes after 5–10 μg IkBa-siRNA or 5–10 μg NC-siRNA intracameral injection again (n = 3).

(D and E) SD-OCT images showing the progression of peripapillary RNFL in the negative control group (5–10 μg NC-siRNA) and in the experimental group (5–10 μg IkBa-siRNA) from baseline to COHT and finally to 1 month after injection in one rhesus monkey. Peripapillary RNFL in different time is distinguished by green, yellow, and red respectively.

mean IOP decreased by 66.25% compared to the baseline. In the following 12 days, the effect of IkBa-siRNA still persisted, the IOP was 28.67 ± 5.42 mmHg, and the mean IOP decreased by 32.62% compared to the baseline. Overall, the mean IOP from Day 2 to Day 28 after injection was 21.86 ± 9.42 mmHg, which decreased by 48.63% compared to the baseline. However, the mean IOP from Day 2 to Day 28 after NC-siRNA injection was 47.25 ± 4.63 mmHg. And the IOP in the NC-siRNA-treated eyes did not show obvious changes post treatment compared to the baseline.

Three monkeys with COHT eyes were injected with 5–10 μg IkBa-siRNA in the left eye and 5–10 μg NC-siRNA in the right eye again (Figure 4A). A slit-lamp biomicroscope and the IOP were monitored every day after the second injection. The effect of reducing IOP began on the second day as before. The maximum effect of reducing IOP was observed on the fourth and fifth days (7.83 ± 2.14 mmHg), which
was 84.13% (41.50 mmHg) lower than the baseline (49.33 mmHg) (Figure 4C). The effect and trend of reducing IOP were similar to the previous treatment. We showed the progression of peripapillary RNFL in the negative control group (5–10 μg NC-siRNA) and in the experimental group (5–10 μg IkBa-siRNA) from one monkey with COHT (Figures 4D and 4E). Before any intervention, the peripapillary RNFL thickness of both eyes was in the normal range. The RNFL thickness became thinner to some extent after 6 weeks of COHT. One month after intracameral injection, the IOP in the negative control group was maintained at a high level (50.21 ± 2.46 mmHg), and the thickness of the peripapillary RNFL became obvious thinner, while the IOP in the experimental group was maintained at 22.43 ± 11.77 mmHg. And the peripapillary RNFL thickness continued to thin, but it was not as severe as that in the negative control group.

**Downregulation of IkBa upregulates MMPs of the MCM and MTM in vivo**

We investigated the changes in IkBa, MMP2, and MMP9 proteins and mRNA of the eyes in the negative control group and in the experimental group by IF and qPCR. As shown in Figure 5, mRNA expression of IkBa was significantly decreased in the MCM and MTM after IkBa-siRNA intracameral injection compared to that in the NC-siRNA group (MCM, p = 0.0050; MTM, p = 0.0116) (Figure 5A), and the protein expression of IkBa showed similar trend both in the MCM and MTM (MCM, p = 0.0003; MTM, p = 0.0106) (Figures 5B and 5C). As expected, the mRNA expression increasing trends of MMP2, MMP9 in the MCM, and MTM were consistent with the expression in vitro (MMP2 in the MCM, p = 0.0222; MMP9 in the MCM, p = 0.0407; MMP2 in the MTM, p = 0.0051; MMP9 in the MTM, p = 0.0254) (Figures 5D and 5G). In addition, the protein expression of MMP2 and MMP9 in the MCM and MTM also showed an increasing
trend after I\textsubscript{k}B\textsubscript{a}-siRNA intracameral injection by IF (MMP2 in the MCM, \(p = 0.0011\); MMP9 in the MCM, \(p = 0.0013\); MMP2 in the MTM, \(p = 0.0040\); MMP9 in the MTM, \(p = 0.0106\)) (Figures 5E, 5F, 5H and 5I). An increased protein expression of MMP2 and MMP9 in the MCM and MTM might promote ECM turnover in vivo, which lowered the IOP by reducing aqueous humor outflow resistance.

Obvious eyes’ adverse reactions do not occur after I\textsubscript{k}B\textsubscript{a}-siRNA intracameral injection

There was no corneal edema or serious inflammatory reaction in the negative control eye (5–10 \(\mu\)g NC-siRNA) during the observation period of one month after intracameral injection. Mild aqueous cells occurred in eyes injected with 5–10 \(\mu\)g I\textsubscript{k}B\textsubscript{a}-siRNA, which were absorbed in following 2 days. Slit-lamp examination recorded the general conditions of the eyes on the first day and 1 month after intracameral injection (Figure 6A). There was no significant change in the anterior segment of the eyes both in the negative control group (5–10 \(\mu\)g NC-siRNA) and the experimental group (5–10 \(\mu\)g I\textsubscript{k}B\textsubscript{a}-siRNA) one month after intracameral injection.

Anterior segment OCT scan showed that there was no obvious difference in central corneal thickness (CCT) between the two groups 1 month after intracameral injection (Figure 6B). And there was no significant difference in CCT before and 1 month after intracameral injection both in the negative control group (\(p = 0.1302\)) and in the experimental group (\(p = 0.5790\)) (Figure 6D). H&E staining of paraffin sections showed corneal structure changes between the two groups’ eyes (Figure 6C). There was no edema or thickening of the corneal stroma and no obvious abnormality of the corneal structure.

The ECD of both eyes was counted by specular microscopy before and 1 month after intracameral injection. There was no obvious abnormality in the morphology of the corneal endothelium before treatment of
either eye, and there was no significant change one month after injection in the two groups (p = 0.4661, p = 0.8759) (Figures 6E, 6F and Table S2).

**DISCUSSION**

Our previous studies showed that the IκB/NF-κB signaling pathway plays an important role in the expression of MMP2 in HCM cells and in the CM of rats (Lan et al., 2009; Zeng et al., 2020). Inhibition of IκBα expression by IκBα-siRNA led to translocation of NF-κBp65 from the cytoplasm to the nucleus, increased secretion of MMP2 in HCM cells (Lan et al., 2009). In addition to the above changes, IOP of rats decreased after IκBα-siRNA intracameral injection (Zeng et al., 2020). In the present study, we found that with the knockdown of IκBα after RNAi, NF-κBp65 was activated, and the secretion of MMP2 and MMP9 increased in the primary MCM and MTM cells. Moreover, IκBα-siRNA intracameral injection showed a significant and long-lasting IOP-lowering effect in vivo with a well-characterized LP-induced nonhuman primate model of COHT, and the expression of the above associated miRNAs and proteins was also confirmed in the MCM and MTM tissues of rhesus monkeys.

To simulate human glaucoma, we chose LP-induced COHT in rhesus monkeys as the disease model to verify the effect of reducing the IOP of IκBα-siRNA. Previous studies have found that the COHT model achieved IOP >30 mmHg after LP, and the maintenance time varies from weeks to months due to individual differences (Pelzel et al., 2006; Quigley and Hohman, 1983; Tu et al., 2019). In our study, the IOP was stable at 47.63 ± 7.81 mmHg after two to three LPs within 3 weeks to 2 months of the observation period, and there was no significant decrease in the negative treatment control group during the follow-up period. As expected, in our results, histopathology showed that the TM structure was damaged, and most SC collapsed after LP. In addition, the loss of ZO-1 and tricellulin siRNA-targeting SC endothelial tight junctions enhancing outflow facility showed an average IOP decrease of approximately 2 mmHg in rabbits after administration over a 4-day period by decreasing the production of aqueous humor (Martinez et al., 2014). Topical instillation of β2 adrenergic receptor siRNA induced a reduction in IOP of 30% ± 5% for 78–120 h in rabbits (Loma et al., 2018). Besides, ZO-1 and tricellulin siRNA-targeting SC endothelial tight junctions enhancing outflow facility showed an average IOP decrease of approximately 2 mmHg in DEX-treated mice eyes after 48 h intracameral injection, representing twice that measured in normotensive controls (approximately 1 mmHg) (Cassidy et al., 2021). Intracameral injection of adenovirus vector encoding wild-type human metalloproteaseinase 1 (AdhGRE. MMP1) quickly induced a maximum hypotensive effect both in normal and steroid-induced ocular hypertension sheep eyes within 48 h, and the IOP-lowering effect lasted for at least 15 days (Gerometta et al., 2010). Viral vectors are the preferred system for gene delivery because of their higher in vivo transfection efficiency. It shows a longer-lasting effect due to being more persistent with being self-complementarity. However, viral gene delivery systems have disadvantages related to limited payload capacity, potential immunogenicity, and no known pathogenicity (Dang et al., 2021).
Activation of the NF-κB signaling pathway is often contributed to the occurrence of inflammation and glaucoma (Vernazza et al., 2020). Under chronic stress conditions, the ER accumulates reactive oxygen species and promotes oxidative stress-induced TM damage (Anholt and Carbone, 2013; Cullinan and Diehl, 2006). Damaged ER activates inflammatory processes via NF-κB, mitochondrial changes, and enhanced TM cell apoptosis, which lead to elevated IOP (Rao et al., 2004). Hernandez et al. showed that NF-κB was necessary for TGFβ2-induced ECM production and ocular hypertension (Hernandez et al., 2020), whereas the level of MMPs was not detected and the molecular mechanisms by which NF-κB upregulates the ECM is not fully understood. However, several studies had indicated the opposite conclusion. NF-κB mediated ECM degradation in osteoarthritis cartilage and skin (Hussain et al., 2018; Kaur et al., 2015). In addition, the DEX-induced reduction in MMP-2 and MMP-9 secretion was prevented by NF-κB activation in HTM cells (Mohd Nasir et al., 2020). Besides, it had been demonstrated that the mechanism of IOP lowering by prostaglandin analogs (PGAs, i.e., the first-line treatment for open-angle glaucoma) involves the increasement of MMPs in TM and CM, and tissue remodeling that enhances conventional and unconventional outflow (Toris et al., 2008; Weinreb et al., 2020). Just as PGAs were initially found to have IOP-lowering effects, in 1977, Camras et al. found that low doses of topical PGAs decreased IOP (Camras et al., 1977; Eakins 1977). In contrast, large quantities of PGAs infused into animal eyes led to ocular inflammation with breakdown of the blood-aqueous barrier. However, appropriate amount of PGAs have emerged as the most potent IOP-lowering topical medication and are rarely accompanied by uveitis. A statistical study showed that the incidences of uveitis and cystoid macular edema among PGAs users were very rare at 62/28232 (0.22%) and 25/28232 (0.09%), respectively (Hu et al., 2020). In the current study, corneal edema and inflammatory reactions only occurred in the anterior segment of the eye 1–4 days after intracameral injection of 90–100 μg IκBα-siRNA without an obvious increase in IOP, and corneal edema and inflammatory reactions completely disappeared during our observation. What’s more, IκBα-siRNA (5–10 μg) showed only a slight inflammatory reaction (mild aqueous cells) 1–3 days after intracameral injection, and there was no significantly increased IOP compared with the negative control group. Interestingly, it showed a similar effect of reducing IOP in the following 1 month. Therefore, the effect of IκBα-siRNA on reducing IOP is worth affirming, and we speculate that the observation of inflammation may be related to the dose, and the optimal dose needs to be further studied.

In conclusion, IκBα-siRNA increased the secretion of MMP2 and MMP9 by activating the NF-κB signaling pathway in vitro and in vivo. And IκBα-siRNA with nonviral vectors showed long-term and potent effects of reducing IOP in monkeys with COHT. Therefore, IκBα-siRNA may become a new and promising therapeutic approach for the treatment of glaucoma.

Limitations of the study
In this study, we verified the definite IOP-lowering effect of IκBα-siRNA in monkeys with COHT by promoting the expressions of MMP2 and MMP9 in MCM and MTM. However, the specific effect of IκBα-siRNA on the aqueous humor outflow resistance in the trabecular meshwork and uveoscleral pathways needs further investigation.
• **METHOD DETAILS**
  - Identification of primary MCM and MTM cells
  - IkBα-siRNA transfection
  - Colocalization assays
  - Real-time PCR (qPCR) analysis
  - Western blotting (WB)
  - Immunofluorescence (IF)
  - Anesthesia
  - Establishment of a COHT monkey model and IOP measures
  - OCT measurements
  - Drug delivery to the anterior segment
  - Corneal endothelial cells
  - Tissue preparation and histology
  - Histological immunofluorescence

• **QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104149.

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**AUTHOR CONTRIBUTIONS**
Conception and design: YL, YS, DS, ZZ, and RZ. Performed the research and data collection: DS, XL, ZZ, FY, SH, YL, and ZY. Data analysis and interpretation: DS, BW, and ZZ. Funding: YL. Writing–original draft: DS. Writing–review & editing: YS, ZZ, and YL.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

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### STAR★METHODS

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| rabbit monoclonal anti-IkBz | Cell Signaling Technology | Cat# 4812S; RRID: AB_10694416 |
| rabbit monoclonal anti-NF-κB P65 | Cell Signaling Technology | Cat# 3033S; RRID: AB_331284 |
| rabbit monoclonal anti-phospho-NF-κB P65 | Cell Signaling Technology | Cat# 8242S; RRID: AB_10859369 |
| mouse monoclonal anti-MMP2 | Invitrogen | Cat# 436000; RRID: AB_1501404 |
| rabbit monoclonal anti-β-actin | Cell Signaling Technology | Cat# 8457S; RRID: AB_10950489 |
| rabbit monoclonal anti-s-smooth muscle actin (α-SMA) | Bioss | Cat# bsm-33187M; RRID: AB_2910163 |
| goat polyclonal anti-desmin | R&D Systems | Cat# AF3844; RRID: AB_2092419 |
| rabbit anti-RSPO4 | Bioss | Cat# bs-18878R; RRID: AB_2910164 |
| rabbit polyclonal anti-NSE | Bioss | Cat# bs-1027R; RRID: AB_10855053 |
| rabbit anti-FN | Bioss | Cat# bs-4859R; RRID: AB_2910165 |
| goat polyclonal anti-Col IV | Millipore | Cat# A8769; RRID: AB_92262 |
| mouse monoclonal anti-MYOC | Santa Cruz Biotechnology | Cat# sc-137233; RRID: AB_2148737 |
| mouse monoclonal anti-IkBz | Cell Signaling Technology | Cat# 4812T RRID: AB_2910165 |
| mouse monoclonal anti-MMP9 | R&D Systems | Cat# MAB936, RRID: AB_2282047 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Lipofectamine® RNAiMAX | Invitrogen | Cat# 13778150 |
| LysoTracker® Green DND-26 | Invitrogen | Cat# L7526 |
| Fetal bovine serum | Gibco | Cat# 10099-141 |
| Mounting medium with DAPI - Aqueous, Fluoroshield | Abcam | Cat# ab104139 |
| **Experimental models: Cell lines** | | |
| Rhesus monkey ciliary muscle cells and trabecular meshwork cells | This study | N/A |
| **Experimental models: Organisms/strains** | | |
| Rhesus monkey | Guangdong Laboratory Animals Monitoring Institute | N/A |
| **Oligonucleotides** | | |
| ACTB forward primer:5’-AGATCAAGATCATTTGGCTTCCTCCTG-3’ | This study | N/A |
| ACTB reverse primer:5’-TCACATGGCCCGCTAGAAAGCA-3’ | This study | N/A |
| IkBz forward primer:5’-CTGGTGTGCTGCTCCCTGGAATG-3’ | This study | N/A |
| IkBz reverse primer:5’-GTGTCTAGCTCTCTCCTCATCCTCCTG-3’ | This study | N/A |
| NF-κB forward primer:5’-TCACGCGATCCAGAGACAAAC-3’ | This study | N/A |
| NF-κB reverse primer:5’-AACAGCAGGCGCACACGCATC-3’ | This study | N/A |
| MMP2 forward primer:5’-CACCTACACCAAAGAATCGGCTG-3’ | This study | N/A |
| MMP2 reverse primer:5’-GTGCCAAGTCAATGTCAGGAGAG-3’ | This study | N/A |
| MMP9 forward primer:5’-TCTGCCCGAGCTGCTCCTGCTCAG-3’ | This study | N/A |
| MMP9 reverse primer:5’-GCAGGATGTCATAAGGTGACGCAG-3’ | This study | N/A |
| **Software and algorithms** | | |
| GraphPad Prism 8.0 | GraphPad Software | https://www.graphpad.com/ |
| ImageJ | National Institute of Health | https://imagej.nih.gov/ij/ |
| ZEN | Zeiss | https://www.zeiss.com/microscopy/us/products/microscope-software/zen.html |
RESOURCES AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by
the lead contact, Yuqing Lan (lanyq@mail.sysu.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability
All data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead
contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal
All of the experiments were performed in accordance with the ARVO Statement for the Use of Animals in
Ophthalmic and Vision Research. The study protocol was approved by the Ethics Committee of Guangdong
Laboratory Animals Monitoring Institute (IACUC2020141). All efforts were made to limit the number of an-
imals and to minimize animal suffering. The number of animals was in line with the principles of the 3Rs.

Four healthy male rhesus monkeys from the Guangdong Laboratory Animals Monitoring Institute, which
were 3 to 4 years of age, were used in this study. All monkeys were housed in a room at 16–26°C and
40–70% humidity with a 12-h light-dark cycle. The monkeys' health was monitored daily by animal care staff
and veterinary personnel.

Isolation and culture of primary MCM and MTM cells
Rhesus monkey (3 to 5 years old, sex balance) eyes were presented by Guangdong Laboratory Animals
Monitoring Institute. Primary MCM cells and MTM cells were harvested at least three times. As described
previously, we identified primary MCM cells and MTM cells, screened the optimal sequence and concen-
tration of IκBa-siRNA to inhibit the expression of IκBa (Ou et al., 2020). The medium was composed of
Dulbecco’s modified Eagle’s medium and Ham’s F12 nutrient mixture (DMEM/F12, Gibco, USA) supple-
mented with 20% fetal bovine serum (FBS, Gibco, Australia), 1% penicillin-streptomycin (HyClone, USA),
and 1 ng/mL recombinant human basic fibroblast growth factor (bFGF) (Gibco, USA). The cultures were
incubated in a 37°C humidified incubator with 5% CO2. The experiments were taken in the well-grown cells
of 3-5 generations.

METHOD DETAILS

Identification of primary MCM and MTM cells
Primary cells were identified as MCM and MTM cells separately by immunofluorescence. In detail, the pri-
mary MCM cell cultures were validated by detecting the presence of α-SMA and desmin, while MTM cell
cultures were certificated by detecting the expression of RSPO4, NSE, FN, and Col IV. Besides, cultured
MTM cells stimulated with 100 nM Dex (Sigma-Aldrich Corp., USA) were added fresh every 3 days to the
media for 10 days. The level of MYOC was analysed by immunofluorescence.

IκBa-siRNA transfection
Three pairs of siRNA against the IκBa gene and a pair of nonspecific control siRNA (NC-siRNA) were pur-
chased from Guangzhou Ruibo Biotech Co., Ltd, China. Then, we screened the optimal one (5’-GCAC
UUAGCCUUACAUCAU-3’) and selected 10 nM as the optimal transfection concentration through the
detection of transfection efficiency and cytotoxicity (Ou et al., 2020). Primary MCM cells and MTM cells
were transfected with IκBa-siRNA or NC-siRNA and Lipofectamine® RNAiMAX Reagent (Invitrogen,
USA) complex following the manufacturer’s instructions. Both primary cells were divided into 3 groups:
blank control (PBS), negative control group transfected with NC-siRNA and experimental group
transfected with IkBα-siRNA. Forty-eight hours after transfection, cells were collected for subsequent experiments.

**Colocalization assays**

To monitor whether IkBα-siRNA could be absorbed and released in MTM and MCM cells, colocalization with lysosome were examined after incubation for 24 hours and 48 hours, respectively. After transfection for 24 hours and 48 hours, LysoTracker Green DND-26 (ThermoFisher, USA) was added for 1 hour following the manufacturer’s protocol. The images were taken under a laser confocal microscope (Zeiss LSM 880 with Airyscan, Germany). Pearson’s correlation coefficient (PCC) was applied to describe colocation analysis by ImageJ software.

**Real-time PCR (qPCR) analysis**

Total RNA was extracted from each group in vitro and in vivo using a RNA Quick Purification kit (ESscience, China). RNA was reverse transcribed into cDNA using PrimeScript™ RT Master Mix (Perfect Real Time) (Takara, Japan), and RT–PCR was performed with Green Premix Ex Taq II (Tli RNase H Plus) (Takara, Japan). The related primers were designed and synthesized by Shanghai Biotech Co., Ltd. and were listed in the key resources table. The relative expression levels were calculated and analyzed using the \(2^{-\Delta \Delta Ct} \) method. Among them, the experiment in vitro was repeated three times independently, and the results were used for subsequent statistical analysis. Besides, MTM and MCM tissues were collected from 1 eye of the negative control group and 1 eye of the experimental group, the experiment was repeated three times for subsequent statistical analysis.

**Western blotting (WB)**

Total protein was extracted from MCM and MTM cells with RIPA lysates (Epizyme, China) containing protease inhibitors (1:100; Epizyme, China) and nucleases (1:100; Hai-gene, China). The protein concentration of each sample was measured with a BCA Protein Assay Kit (Cwbio, China). After electrophoresis and transfer, the proteins were transferred to a 0.45-μm polyvinylidene fluoride (PVDF) membrane (Millipore, USA). Then, the membranes were blocked at room temperature (RT) for 15 minutes with a fast blocking solution (1:5; Epizyme, China) and incubated with selected primary antibodies at 4°C overnight. The membranes were washed in Tris-buffered saline containing 0.1% Tween 20 (1X TBST) and incubated with secondary antibodies for 1 hour at RT. Finally, the membranes were washed and imaged using the ChemiDoc Touch Imaging System (Bio–Rad, USA). The following primary antibodies were used: rabbit anti-IkBα (1:5000; CST, USA), rabbit anti-NF-κB P65 (1:1000; CST, USA), rabbit anti-phospho-NF-κB P65 (1:1000; CST, USA), mouse anti-MMP2 (1:500; Invitrogen, USA), and rabbit anti-β-actin (1:10000; CST, USA). The secondary antibodies were HRP-labeled goat anti-rabbit IgG (1:5000; CST, USA) and HRP-labeled goat anti-mouse IgG (1:5000; CST, USA). The experiment was repeated three times independently. The gray value of each band was calculated and analyzed using ImageJ software.

**Immunofluorescence (IF)**

MCM and MTM cells were seeded on coverslips and transfected for 48 hours. They were fixed in a 4% paraformaldehyde solution (Bestbio, China) for 15 minutes and then permeabilized in 0.3% Triton X-100 (Solarbio, China) for 10 minutes at RT. Then, they were blocked with normal goat serum (Bioss, China) or 5% BSA for 1 hour at RT and incubated with selected primary antibodies at 4°C overnight. The coverslips were washed in TBST and incubated with secondary antibodies for 40 minutes at RT under dark conditions. After washing with TBST again, mounting medium with DAPI (Abcam, UK) was used, and images were taken by a Zeiss laser confocal microscope. The following primary antibodies were used: rabbit anti-α-SMA (1:100; Bioss, China), goat anti-desmin (1:100; R&D Systems, USA), rabbit anti-RSPO4 (1:100; Bioss, China), rabbit anti-NS5 (1:100; Bioss, China), rabbit anti-FN (1:100; Bioss, China), goat anti-Col IV (1:200; Millipore, Germany), mouse anti-MYOC (1:100; Santa Cruz Biotechnology, USA), mouse anti-IkBα (1:100; CST, USA), mouse anti-MMP2 (1:100; Invitrogen, USA), mouse anti-MMP9 (1:100; R&D Systems, USA). The secondary antibodies were anti-mouse IgG (H + L), F(ab’)2 Fragment (Alexa Fluor® 555 Conjugate) (1:500; CST, USA) anti-rabbit IgG (H + L), F(ab’)2 Fragment (Alexa Fluor® 555 Conjugate) (1:500; CST, USA), and anti-mouse IgG (H + L), F(ab’)2 Fragment (Alexa Fluor® 488 Conjugate) (1:500; CST, USA), donkey anti-goat IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 (1:500; Invitrogen, USA). The experiment was repeated three times independently, and the mean fluorescence intensities were calculated and analyzed by using ImageJ software.
Anesthesia
Rhesus monkeys were anesthetized with an intramuscular injection of ketamine hydrochloride (5 mg/kg) plus medetomidine (0.05 mg/kg). Topical corneal anesthesia was presented with oxybuprocaine hydrochloride eye drops (Santen Pharmaceutical Co., Ltd, Japan).

Establishment of a COHT monkey model and IOP measures
In this study, a COHT monkey model was established by destroying the TM with LP. Six eyes of 3 rhesus monkeys were treated with a TX532 laser photocoagulation instrument (Oculight TX; IRIDEX, USA). Pupils were sufficiently contracted with pilocarpine nitrate eye drops (Zhenrui®, Bausch & Lomb, China) before LP. The laser parameters were as follows: 50 μm spot size, 0.5s duration, 800–1000 mW laser power and 150–250 spots. Photocoagulation of the middle trabecular meshwork in the entire circumference was completed, and each effective light spot was based on the generation of a bubble (Quigley and Hohman, 1983; Tu et al., 2019). Damage to the ciliary body band would be avoided as much as possible. Tobramycin and dexamethasone eye ointment (Tobradex®, Alcon, USA) and Compound tropicamide eye drops (Zhuobian®, Sinqi, China) were used for alleviating the noninfectious inflammation for 3-7 days.

All rhesus monkeys underwent a regular ophthalmological examination before and after LP, including IOP measurements, slit-lamp biomicroscopy, direct ophthalmoscopy, and OCT measurements. IOP was measured using the rebound tonometer (Tono Vet; Icare, Finland) according to the manufacturer’s instructions. IOP measurements in this study were completed between 10 and 12 a.m. Each measurement of IOP was completed within 5 minutes after anesthesia took effect. The IOP of both eyes was measured three times, and the mean was calculated. The anterior segments of animals were regularly examined with a slit-lamp biomicroscope (KJSDII, Suzhou Kangjie Medical Co., Ltd, China) and recorded, such as corneal clarity, anterior chamber cells and flares.

The baseline mean IOP of the rhesus monkeys was similar to that of humans (Lin et al., 2021; Pasquale et al., 2021). If the IOP was not consistently higher than 21 mmHg, additional LP was performed again at intervals of three weeks until stable IOP was obtained.

OCT measurements
Spectral-domain optical coherence tomography (SD-OCT; Heidelberg Engineering, Heidelberg, Germany) was applied to measure changes in the cornea and anterior chamber angle, especially central corneal thickness (CCT) and Schlemm’s canal (SC).

SD-OCT was also applied to measure changes in the optic nerve head (ONH) and macula. The retinal nerve fiber layer (RNFL) thickness of the ONH and the retina, ganglion cell layer (GCL) and ganglion cell inner plexiform layer (GCIPL) thickness of the macula were measured. To minimize the risk of corneal dryness, artificial tears were applied during examination. Peripapillary RNFL thickness data were automatically obtained and recorded by the software. The retina, GCL and GCIPL thickness of the macula and CCT were manually measured by two researchers (D.F. Sun and Z.Y. Zhan), and the average value was taken.

Drug delivery to the anterior segment
There were 5–10 μg/90–100 μL lBa-siRNA (2OMe+5Chol) (Guangzhou Ruibo Biotech Co., Ltd, China) or 5–10 μg NC-siRNA dissolved in 20 μL of sterile RNase free water; then, we added 3 μL of Lipofectamine® RNAiMAX Reagent(LIPO) and incubated at RT for 10 minutes. Intracameral injection was performed in six eyes of 3 monkeys with stable high IOP. The appropriate dose was selected according to the treatment results. Tobramycin eye ointment (Tobrex®, Alcon, USA) was topically administered to prevent ocular infection.

Corneal endothelial cells
The corneal endothelial cell density (ECD), average area, standard deviation, coefficient of variation, and hexagonality measurements were performed by specular microscopy (SP-300P; TOPCON, Japan),and ECD was counted to evaluate the corneal endothelial damage before and after drug intervention.

Tissue preparation and histology
Animals were anesthetized and euthanized 6 days after intracameral injection. Two blank control eyes, 2 negative control eyes (5–10 μg of NC-siRNA complexed with 3 μL of LIPO), and 2 experimental eyes.
(5–10 μg of IxBα-siRNA complexed with 3 μL of LIPO) were immediately enucleated, washed in cold saline, fixed in FAS eye fixative (Wuhan Servicebio, Wuhan, China) for at least 24 h, embedded in paraffin, and cut into 4-μm sections. Some sections were stained with hematoxylin and eosin (H&E) and modified Masson trichrome stain (Bioss, Beijing, China), and others were used for later IF. The H&E– and Masson-stained slides were examined to detect pathological changes in the cornea, TM and retina using a light microscope (Axio Planz imaging; Zeiss, Germany). Another 1 negative control eye and 1 experimental eye were immediately enucleated and washed in cold saline. Then, the MTM and MCM were dissected quickly on ice and preserved at −80°C for follow-up qPCR.

**Histological immunofluorescence**

The paraffin sections from 2 negative control eyes and 2 experimental eyes were sequentially dewaxed and hydrated, and antigen was retrieved with 10 mM sodium citrate. The sections were then incubated in 3% hydrogen peroxide for 20 minutes and permeabilized in 0.3% Triton X-100 for 20 minutes. Each section was blocked with 10% normal goat serum for 1 hour at RT and then incubated with primary antibody overnight at 4°C. After three rinses in TBST, the slices were incubated with the corresponding secondary antibodies at RT for 40 minutes in the dark. After three washes, mounting medium with DAPI (Abcam, Britain) and coverslips were applied, and images were taken under a laser confocal microscope. The experiment was repeated 3 times for each eye for subsequent statistical analysis.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The statistical results of all experimental data in vitro were from independent three repeated experiments. Statistical analysis was performed using Prism 8.0 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± standard deviation (SD). Differences between two groups were compared using Student’s t test. Multiple comparisons were calculated using one-way ANOVA. Data were considered statistically significant at p < 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001).