

KLF6 Induces Apoptosis in Human Lens Epithelial Cells Through the ATF4-ATF3-CHOP Axis

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Background: Many studies have confirmed that high myopia is related to the high prevalence of cataracts, which results from apoptosis of lens epithelial cells (LECs) due to endoplasmic reticulum stress. Krüppel-like factor 6 (KLF6) is a tumor suppressor that is involved in the regulation of cell proliferation and apoptosis.

Purpose: In this study, our purpose was to find the relationship between KLF6-induced apoptosis in LECs and ATF4 (activating transcription factor 4)-ATF3 (activating transcription factor 3)-CHOP (C/EBP homologous protein) signaling pathway.

Methods: KLF6, ATF4, ATF3, and CHOP were ectopically expressed using cDNAs subcloned into the pCDNA3.1+ vector. ATF4, ATF3, and CHOP knockdown were performed by small interfering RNA (siRNA). Expression of relative gene was tested using QT-PCR and western-blot. Then, accompanied by UVB stimulation, cell viability was measured by CCK-8 assay; The cell damage was examined by live & dead staining; The apoptotic markers Bax and Bcl-2 were detected by immunoblotting; Quantitative apoptotic levels were measured with the Apoptosis Detection Kit; The expression level of reactive oxygen-free radical (ROS) was analyzed by DCFH-DA` probe.

Results: Ectopically expressed ATF4, ATF3, and CHOP-induced apoptosis in cells, whereas ATF4, ATF3, and CHOP knockdown by small interfering RNA (siRNA) blocked KLF6-induced apoptosis. In addition, we determined that ATF4 regulates ATF3 and CHOP expression and that ATF3 silencing reduces CHOP upregulation without changing ATF4 levels; however, ATF4 and ATF3 expression was unaffected by blockade of CHOP, suggesting that KLF6 triggers endoplasmic reticulum stress in LECs by mediating the ATF4-ATF3/CHOP axis. Besides, KLF6 overexpression significantly induced LEC apoptosis under UV radiation, as demonstrated by the elevated Bax/Bcl-2 ratio.

Conclusion: The ATF4-ATF3-CHOP pathway plays an important role in KLF6-induced apoptosis in HLECs. Our results increase our understanding of the mechanisms that regulate LEC apoptosis and contribute to the development of a new preventative strategy for cataract.

Keywords: KLF6, ATF4, apoptosis, HLECs, endoplasmic reticulum stress

Introduction

Cataract is a clouding of the lens in the eye that affects vision. The process of cataract initiation remains unclear, even though more and more related mechanisms have been revealed. It was demonstrated that all age-related cataract patients had a remarkable increase in apoptotic epithelial cells compared to healthy people, indicating apoptosis in lens may lead to cataract. It is generally accepted that endoplasmic reticulum stress (ERS) induces lens epithelial cell apoptosis.¹ Although several drugs that can block LEC apoptosis have been identified, none

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has shown a definitive effect on cataracts. Therefore, non-invasive means for preventing LEC apoptosis are being investigated.

Many studies have revealed a strong correlation between high myopia (myopia exceeding 6.00 diopter (D) or axial length longer than 26 mm) and cataracts.²⁻⁴ It is well documented that myopia is a substantial risk factor for several subtypes of cataract, such as cortical cataract and posterior subcapsular cataract.^{5,6} Currently, the only effective treatment for cataract is phacoemulsification. However, operating on highly myopic eyes is challenging because of zonular weakness or posterior capsular rupture; moreover, this procedure could increase the risk of retinal detachment and macular edema postoperatively. In addition, a retrospective study in Taiwan reviewed that high myopia might accelerate nuclear sclerotic cataract in younger patients. Our initial object was to understand the mechanism of high myopia cataracts and develop targeted treatment protocols. In previous work, we performed mRNA microarray to find the differentially expressed genes between high myopia patients versus non-myopia patients and perform microarray analysis. GO functional enrichment analysis suggested the functions of differentially expressed genes between the two groups can be mainly divided into three parts: biological behavior regulation, cell component formation and molecular function. Differentially expressed genes participate in molecular biological processes and affect cell energy metabolism and protein synthesis (paper has not been published yet). We also identified two differentially expressed genes: Krüppel-like factor 6 (KLF6) and ATF4. According to this information, we designed a series of experiments to verified specialized function and interactions between KLF6/ATF4.

Our previous results have confirmed that KLF6 suppresses the IGF-1-induced proliferation of human LECs,^{7,8} a finding that is consistent with the inhibition of rat LEC proliferation by KLF6 overexpression.⁹ We also found that high KLF6 expression can induce apoptosis, suggesting that KLF6 might be a potential factor in the regulation of apoptosis.¹⁰ To further characterize the mechanism by which KLF6 induce apoptosis in LECs, we explored the molecular mechanism of KLF6 on the induction of ER stress and demonstrate a novel mechanism of ATF4 as a key mediator in KLF6-induced apoptosis.¹¹

KLF6 is a tumor suppressor gene that is involved in regulating cell proliferation and apoptosis.¹² The overexpression of KLF6 upregulates the proapoptotic genes FAS, TNF,

TNFSF12, and PYCARD and inhibits the expression of the antiapoptotic gene IL10. FAS protein expression is positively correlated with KLF6 expression in Schwann cells.¹³ ERS is caused by the dysfunction of ER homeostasis and correlates with apoptosis in LECs.¹⁴ ATF4 is an activating transcription factor and is a key molecule in the induction of apoptosis during ER stress.^{15,16} ATF4, an upstream regulator of CHOP and a proapoptotic factor in cellular stress,¹⁷ mediates ER stress-induced cell apoptosis in cataract lens epithelium.^{18,19} To study this further, we put insight in several ER-related genes, such as ATF3 and CHOP.

CHOP is a downstream component of endoplasmic reticulum (ER) stress pathways, which can be triggered by pancreatic ER kinase-like ER kinase-, activating downstream target and cause ER-related apoptosis. By now, CHOP-induced apoptosis in ER stress was related to various human diseases, such as neurodegenerative diseases, diabetes and tumor.²⁰ ATF3, like ATF4, is also a stress responsive protein, which can be triggered by ER stress. It was previously reported that ATF3 played an integral part in the PERK/eIF2 signaling branch of the UPR.²¹ PERK/eIF2 cascade has been proved to be a remarkable pathway that could influence apoptosis.²²

For the past few years, multiple researchers have reported a potential correlation in ATF4/ATF3/CHOP. Liu et al reported that ATF4-ATF3-CHOP could induce human pulmonary cancer cells apoptosis by mediating DR5.²³ Joo et al demonstrate ATF4-ATF3-CHOP cascade act as an intrinsic pathway of the ER induced apoptosis.²⁴ Those studies implying that there might be a connection between the three genes.

UV exposure is inevitable in daily life and is closely linked to eye diseases. There is growing evidence that oxidative stress under UV exposure plays a critical role in the HLECs apoptosis in cataract pathogenesis. UV radiation stimulate intracellular ROS production, a complicated event involving several potential pathways, such as p53 pathway or Nrf2/ARE pathway.²⁵⁻²⁷ Therefore, studying UV-induced apoptosis in human LECs may provide clues for the exploration of the causes of cataract formation and lead to the development of novel treatments. In our investigation, we used UV radiation to simulate the daily sunlight in natural environment, followed by comparison of cellular apoptosis level between KLF6 up-regulated HLECS and normal cells. Our data showed that KLF6 up-regulation was highly susceptible to UV-induced cell apoptosis cascade.

In our study, we examine the interaction between ATF4/ATF3/CHOP under HLECs apoptosis induced by

KLF6. Our results represent a novel therapeutic target for ER stress-based cataract therapeutic approaches.

Materials and Methods

Cell Culture and Transient Transfection

Human lens epithelial cell line was kindly provided by Yang Chunbo (Tianjin Medical University, Eye Institute). The cells were grown in Eagle's minimum essential medium that contained 10% fetal bovine serum. For transient transfection, plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.^{8,28,29}

Plasmid Construction: Human cDNAs encoding the full-length KLF6 gene, ATF4, ATF3, and CHOP were obtained by PCR. The resulting cDNAs were then subcloned into the pCDNA3.1+ vector to overexpress each protein, respectively.

siRNA design: To suppress KLF6, ATF3, CHOP and ATF4 expression, we used the following siRNAs: KLF6 sense (5'-GCCGAGCUUUUGUACAAtt-3') and antisense (5'-UUGUAACAAAAGCUCGGGctg-3'); ATF3 sense (5'-UCACAAAAGCCGAGGUAGctt-3') and antisense (5'-GCUA CCUCGGCUUUUGUGAtg-3'); ATF4 sense (5'-ggagcgc auugauacuagad-3') and antisense (5'-ucuaguaucaaugcgcucc d-3), and CHOP sense (5'-gccuaggucucuagaugad-3') and antisense (5'-ucaucuaagagaccuaggcd-3'), respectively. Non silencing siRNA (5'-uucuccgaacgugucacgudTdT-3' and 5'-acgugacacguucggagaadTdT-3') was used as a negative control. HLECs were transfected with KLF6, ATF3, ATF4, and CHOP using Lipofectamine RNAiMAX Transfection Reagent (Cat. # 13778150) in accordance with the manufacturer's instructions. Protein samples were collected for Western blot analysis 48 h after transfection.

UVB Irradiation

At 80% to 90% confluence, HLECs were washed twice with PBS, and UVB irradiation was performed under a thin layer of PBS. After 24 hrs transfection, cells were exposed to UVB radiation (20 mJ/cm²) for 200 seconds, incubated for another 48 hrs, and collected for further use.³⁰

Cell Viability Assay

Cell viability was measured using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Briefly, HLEC cells (5×10⁴ cells/well) were seeded into 96-well plates and left to adhere overnight. After the specific treatments, the cells were incubated with plain DMEM basic (100 μL) that contained 10%

CCK-8 solution (10μL) for 2 h at 37°C. The absorbance of the cell solution was measured at 450 nm on an Infinite 200 PRO Multimode Microplate Reader (Tecan Group Ltd., Switzerland). This experiment was repeated 3 times.

Hoechst Staining

Cell apoptosis was observed using Hoechst staining. HLECs were fixed in 4% formaldehyde for 15 min at room temperature. After being washed with PBS, the cells were stained with ready-to-use Hoechst staining buffer (Yeasan, China) at room temperature for 5 min. After absorbing the dye solution, the cells were washed with PBS twice and subsequently observed under a fluorescence microscope (Olympus) using a 350-nm excitation filter. Apoptotic cells were counted under 10 views in each condition and the average was calculated. This experiment was repeated 3 times.³¹

Live/Dead Staining Assay

Cell apoptosis was detected using LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells (L3224, Thermo Fisher). HLECs were seeded in a 12-well plate, and after 24 hrs of incubation, the cells were subjected to different treatments and washed with ice-cold PBS twice. Then, the cells were incubated in 500 μL tissue culture-grade D-PBS containing 1 μM calcein AM (Component A) and 2 μM ethidium homodimer-1 (EthD-1) (Component B) for 30 min at room temperature in the dark. Following the incubation, the live cells were observed using a 490-nm excitation filter, whereas the dead cells were observed using a 545-nm excitation filter under a fluorescence microscope (Olympus). Dead cells were counted under 10 views in each condition and the average was calculated. This experiment was repeated 3 times.

ROS Assay

DCFH-DA assay was employed to determine intracellular ROS (reactive oxygen species) levels. HLECs were seeded in a 24-well plate, and after 24 hrs of incubation, the cells were subjected to different treatments and washed with cold PBS twice. Then, the cells were treated with 30 μL PBS containing 10 μM DCFH-DA (Sigma-Aldrich, St. Louis, MO, USA) and 4 μM ER-tracker Red (Yeasan, China) for 30 mins at 37°C in the dark. ER-tracker Red staining was used to mark the endoplasmic reticulum. After the incubation, the cells were rinsed with PBS three times to remove excess DCFH-DA. Intracellular ROS levels were measured under a fluorescence microscope (Olympus). This experiment was repeated 3 times.²⁹

TUNEL Assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed to confirm the existence of apoptotic cells. A total of 2×10^4 cells were seeded onto coverslips. After overnight incubation, the cells were treated or left untreated as indicated, fixed with 4% paraformaldehyde for 30 min, and permeabilized with 0.1% Triton X-100. Then, the cells were incubated with TUNEL reaction solution (Yeasan, China) for 1 h in the dark at 37°C. After the incubation, the cells were treated with a drop of 5 µg/mL DAPI (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min and washed with PBS three times. Apoptotic cells were visualized, based on green fluorescence, under a fluorescence microscope. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole). The positive control for apoptosis was pretreated with DNase I to enzymatically induce DNA strand breaks. This experiment was repeated 3 times.

Apoptosis Assays

Apoptosis was quantified using the Cell Death Detection ELISA PLUS kit (Roche Applied Science, Catalog No. 11774425001, Mannheim, Germany) following the manufacturer's protocol. This assay evaluates apoptosis by measuring the contents of mono- and oligonucleosomes in the lysates of apoptotic cells. The cell lysates were placed in a streptavidin-coated microplate and incubated with a mixture of anti-histone /biotin and anti-DNA/oxidase. The amount of oxidase that was retained in the immunocomplex was determined photometrically with 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as the substrate. Absorbance was measured at 405 nm. This experiment was repeated 3 times.

RNA Extraction, Reverse Transcription, and Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from LECs using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, Beijing, China) as previously described.^{32,33} RNA concentration and purity were

determined on a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (6 µg) was digested with DNase I to eliminate residual DNA, and then, 1 µg of total RNA was subjected to reverse transcription in a 20-µL reaction mixture using the RevertAid cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Real-time PCR was performed on an HT7900 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction mixture contained SYBR Green FastStart 2X Master Mix (Roche, Beijing, China), cDNA template, and gene-specific primers. The primers are listed in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal control. The cDNA content of related genes in each LEC sample was normalized to that of GAPDH. The relative expression levels of genes were quantified using the comparative threshold cycle ($2^{-\Delta\Delta C_t}$) method. This experiment was repeated 3 times.^{34,35,36}

Western Blot Analysis

Lysates of LECs were prepared in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS] in PBS) following previously described protocols.¹⁴ A complete protease inhibitor mixture (catalog no. 04693159001, Roche Applied Science, Mannheim, Germany) was added to the lysis buffer prior to analysis. Protein concentration was determined by BCA protein assay (Catalog No. 23228, Pierce, Thermo Scientific, Rockford, IL, USA). Equal amounts of proteins were separated on 10% SDS polyacrylamide gels (100 V for 120 min) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA) on a semi-dry transfer apparatus (Bio-Rad) for 40 min at 20 V. Binding of nonspecific proteins to the membrane was blocked with blocking buffer (5% nonfat milk, 200 mM NaCl, 50 mM Tris, 0.05% Tween 20) at room temperature (RT) for 1 h. The blocked membrane was then incubated with the following primary antibodies: anti-KLF6, C-terminal (Abcam, ab135783); anti-ATF4 [EPR18111] (Abcam, ab184909); anti-ATF3 [44C3a] (Abcam, ab58668), anti-DDIT3 [9C8] (Abcam, ab11419), anti-Bax [E63] (Abcam,

Table 1 Primers Used for qRT-PCR

Gene	Forward Primer	Reverse Primer
KLF6	GGTCAGCTCGGGAAAATTGA	CCTGCTCAGTTCGGGAGAAG
ATF4	AATTGGCCATCTCCCAGAAA	GGGAAGAGGTTGTAAGAAGGTGAA
ATF3	CTCTGCGCTGGAATCAGTCA	CCTCGGCTTTTGTGATGGA
CHOP	GGGAGCTGGAAGCCTGGTAT	CCCCCATTTTCATCTGAAGACA
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGAA

ab32503); and anti-Bcl-2 [E17] (Abcam, ab32124), with GAPDH as a reference at 4°C for overnight. After the membrane was washed three times with TBST (20 mM Tris, 500 mM NaCl, 0.1% Tween 20) for 10 min each time at RT, the membrane was incubated with Goat Anti-Rabbit IgG H&L (HRP) (ab6721) in a dark place for 1 h at RT. Then, the membrane was washed three times with TBST for 10 min each time at RT in the dark. Detection was performed using an ECL chemiluminescence kit (GE Healthcare, Freiburg, USA), and the WB film was scanned on a MultiSpectral Imaging System (EC3 410, UVP, Upland, CA, USA) and analyzed with Image J software to quantify protein band intensities. This experiment was repeated 3 times.^{37,38,39}

Statistics

All data were expressed as mean±SD. All experiments were performed at least three times. Statistical analyses were performed using two-tailed Student *t* test and one-way ANOVA followed by Tukey post-test. A *p*-value of less than 0.05 was considered to be significant.⁴⁰

Results

KLF6 Upregulates ATF4/ATF3/CHOP Expression

To confirm the relationship between KLF6 and ATF4/ATF3/CHOP, pCDNA3.1+-KLF6 plasmid was employed to increase KLF6 levels in HLECs, and the effects were measured by Western blot and qRT-PCR. As shown in Figure 1A–C, p 3.1+-KLF6 transfection upregulated KLF6 mRNA and protein compared with the empty vector (Vec) group. Upregulation of ATF4/ATF3/CHOP by pCDNA3.1+-KLF6 transfection was detected by Western blot and qRT-PCR. pCDNA3.1+-KLF6 transfection can significantly increase ATF4/ATF3/CHOP expression in HLECs (Figure 1A and D–I).

ATF4, ATF3, and CHOP are Involved in the Regulation of HLEC Apoptosis

To determine the role of ATF4 in the apoptosis of HLECs, ATF4 was overexpressed in HLECs by transient transfection of pCDNA3.1+-ATF4, and the upregulation of ATF4 was verified by real-time PCR and Western blot. As shown in Figure 2A and B, overexpression of ATF4 by transient transfection significantly increased ATF4 mRNA and protein, compared with cells that were transfected with empty vector. Furthermore, ATF4 expression significantly induced the apoptosis of HLECs (Figure 2C). Based on these results, we also determine the effects of ATF3 and

CHOP overexpression on HLECs apoptosis. Figure 2D and E show that pCDNA3.1+-ATF3/CHOP upregulated ATF3/CHOP mRNA and protein in HLECs (Figure 2D, E and G, H). Moreover, the increase in ATF3/CHOP was positively related to HLECs apoptosis (Figure 2F and I). These data indicate that ATF4, ATF3, and CHOP play a role in the regulation of cell apoptosis.

ATF4, ATF3, and CHOP Mediates KLF6-Induced Apoptosis

To determine the importance of ATF4 in KLF6-induced apoptosis in HLECs, we employed a siRNA that targeted ATF4 (Figure 3A) to specifically knock down the expression of ATF4 in HLECs. As shown in Figure 3A, transfection with ATF4 siRNA significantly downregulated KLF6-induced ATF4 expression. Although KLF6-induced apoptosis in HLECs that were transfected with the negative control siRNA, it failed to do so in cells in which ATF4 was knocked down by siRNA (Figure 3B). Furthermore, similar results were observed with ATF3 (Figure 3C) and CHOP (Figure 3E) siRNA. Knockdown of ATF4, ATF3, or CHOP reduced that ratio of apoptotic cells that were induced by KLF6 (Figure 3D and F). These data indicate that in addition to ATF4, ATF3 and CHOP regulate KLF6-induced apoptosis.

The Relationship Between ATF4, ATF3, and CHOP Under KLF6 Induction

Knocking down each protein in turn reduced the level of apoptosis (Figure 3). Following this experiment, we validated the relationship between ATF4, ATF3, and CHOP in KLF6-dependent cell apoptosis. Cells were transfected with ATF4, ATF3, or CHOP siRNA, respectively. Transfection with ATF4 siRNA decreased the expression of ATF3 and CHOP (Figure 4A). Furthermore, by inhibiting ATF3 expression using siRNA, we found that the induction of ATF4 was unaffected, whereas the expression of CHOP declined (Figure 4B). In cells that were transfected with CHOP siRNA, KLF6-induced expression including ATF4 and ATF3 was still unchanged (Figure 4C). These results suggest that KLF6 mediate induction of the ATF4-ATF3/CHOP axis, which leads to KLF6-dependent apoptosis.

KLF6 Aggravates Apoptosis in LECs Under UV Treatment

UV radiation was used as a classic oxidative stressor to induce cell apoptosis. To determine the effect of KLF6 on the survival of HLECs under UV radiation, pCDNA3.1+-KLF6 plasmid

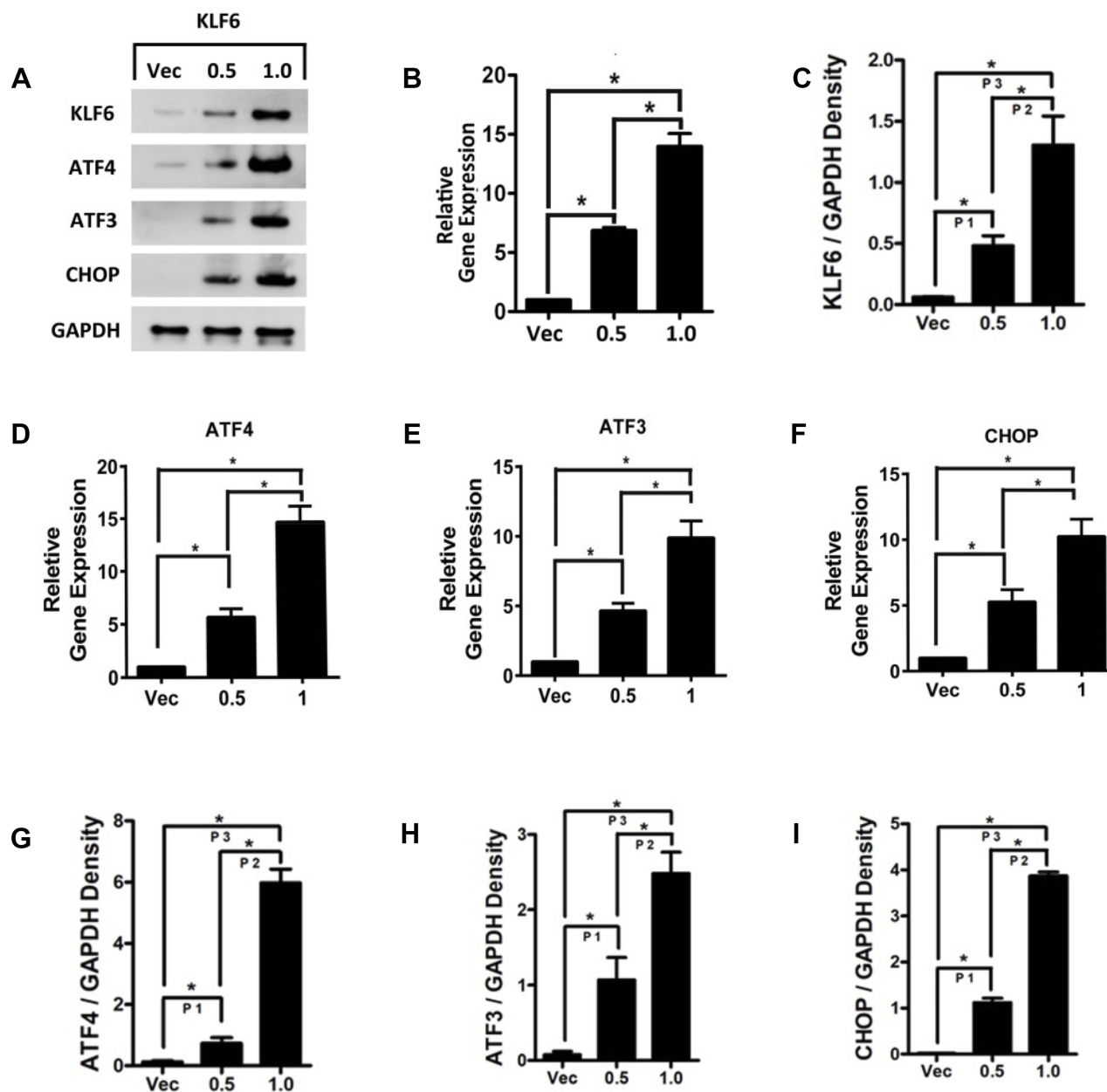


Figure 1 KLF6 upregulates ATF4/ATF3/CHOP Expression. HLECs were transfected with empty p or various amounts of pCDNA3.1+-KLF6 plasmids in 6-well plates. (A) Overexpression of KLF6 by transient transfection. Western blot was performed using anti-CDNA3.1+ with cell lysates collected at 48 hrs after transfection. KLF6 upregulates ATF4/ATF3/CHOP protein expression, detected by Western blot. (B) KLF6 gene expression levels were elevated with increasing amounts of pCDNA3.1+-KLF6 plasmid. (C) Grey level analysis suggested KLF6 protein expressions were elevated with increasing amounts of pCDNA3.1+-KLF6 plasmid. (D–F) Overexpression of ATF4/ATF3/CHOP in HLECs. HLECs were transfected with empty vector (Vec) or various amounts of pCDNA3.1+-KLF6. ATF4/ATF3/CHOP and GAPDH mRNA levels were detected by qRT-PCR. (G–I) HLECs were transfected with empty vector (Vec) or various amounts of pCDNA3.1+-KLF6. ATF4/ATF3/CHOP and GAPDH protein levels were detected by Western-blot. * $p < 0.05$.

was employed to increase KLF6 levels in HLECs. Viability and cytotoxicity were measured to assess viability under UV treatment with or without KLF6. As shown in Figure 5A and B, living cells showed green signals, whereas dead cells generated red signals. There were only a few red cells in the normal group by fluorescence microscopy; however, the

decrease in green signals was accompanied by a dramatic increase in red signals and KLF6 levels.

We performed Hoechst staining to determine the effect of KLF6 on UV-induced HLEC apoptosis. UV treatment significantly increased the number of apoptotic cells, as shown by the increase in apoptotic nuclei (condensed,

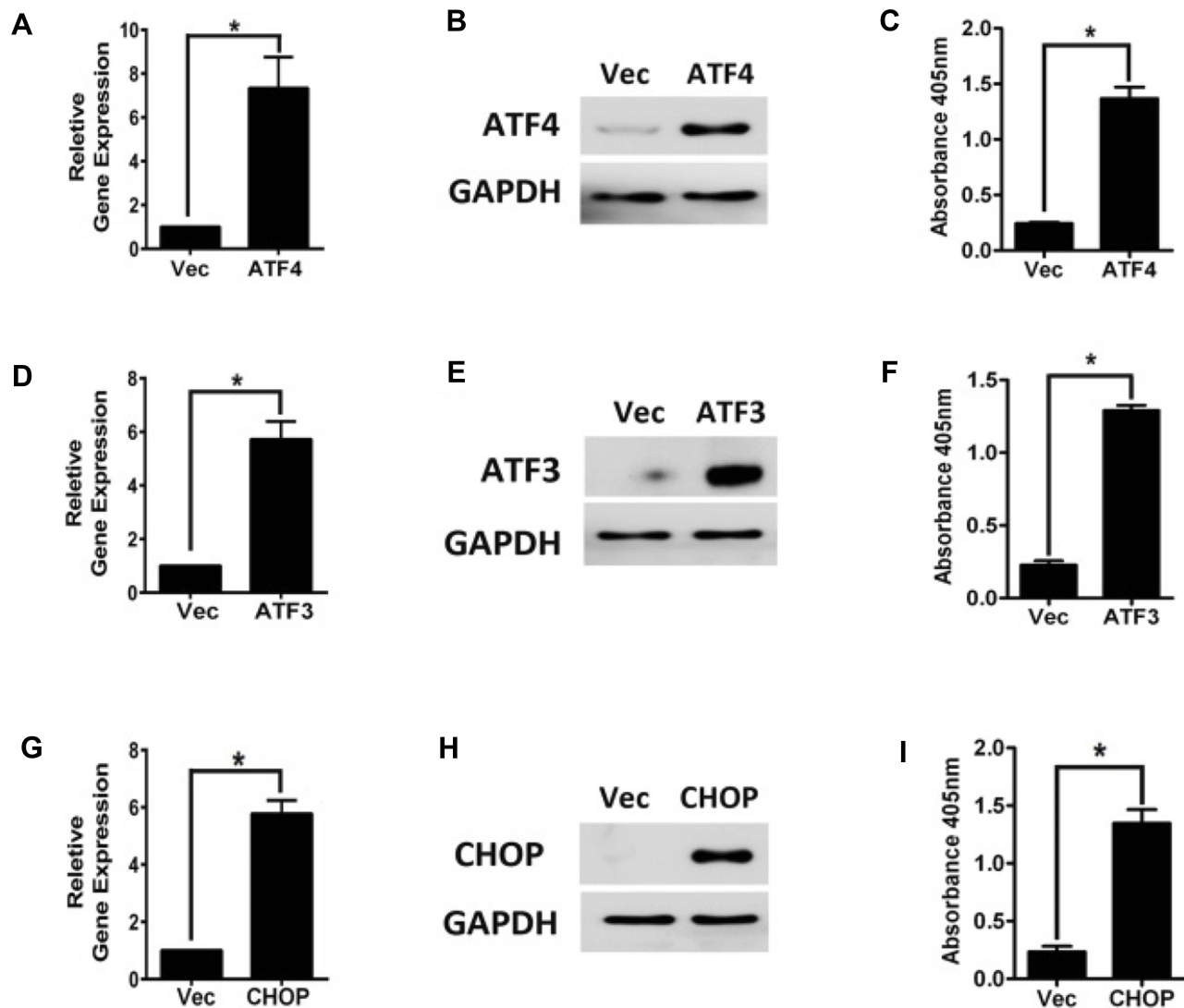


Figure 2 ATF4, ATF3, and CHOP are involved in the regulation of HLEC apoptosis. **(A, B)** Overexpression of ATF4 in HLECs. HLECs were transfected with empty vector (Vec) or pCDNA3.1+ATF4. ATF4 and GAPDH expression levels were detected by qRT-PCR and Western blot. **(C)** ATF4 induces apoptosis in HLECs. HLECs were transfected with empty vector or pCDNA3.1+ATF4. Apoptosis was analyzed using the Cell Death Detection ELISA kit 48 h after transfection. **(D, E and G, H)** HLECs were transfected with empty vector (Vec) or pCDNA3.1+ATF3/CHOP. Compared with the Vec group, ATF3/CHOP mRNA and protein expression was elevated in the pCDNA3.1+ATF3/CHOP group. **(F, I)** ATF3/CHOP-induced apoptosis was analyzed using the Cell Death Detection ELISA kit 48 hrs after transfection. * $p < 0.05$.

crescent-shaped, and fragmented nuclei), which KLF6 exacerbated. Taken together, these results suggest that KLF6 aggravates HLECs death during UV-induced injury *in vitro* (Figure 5C and D).

To confirm the role of KLF6 in UV-induced HLEC apoptosis, cells were pretreated with or without KLF6 overexpression and subjected to TUNEL assay. As shown in Figure 6A, the increase in TUNEL-positive cells was accompanied by a rise in KLF6.

On this basis, we also checked the expression of Bax and Bcl-2 in HLECs. Consistent with the increased apoptosis in

HLECs, by Western blot, KLF6 upregulated the pro-apoptosis protein Bax, whereas the active form of the pro-viability protein Bcl-2 decreased (Figure 6B). Considering that the ratio of Bax to Bcl-2 protein is a decisive factor in transmitting apoptosis signals, we compared the gray values of the two corresponding bands and make clear the ratio of Bax/Bcl-2 to be augmented with KLF6 upregulation (Figure 6C–E). HLECs died through apoptosis, as confirmed by ELISA of mono- and oligonucleosome content in the lysates of apoptotic cells (Figure 6F). These data confirm that KLF6 increased the susceptibility of UV-induced apoptosis in HLECs.

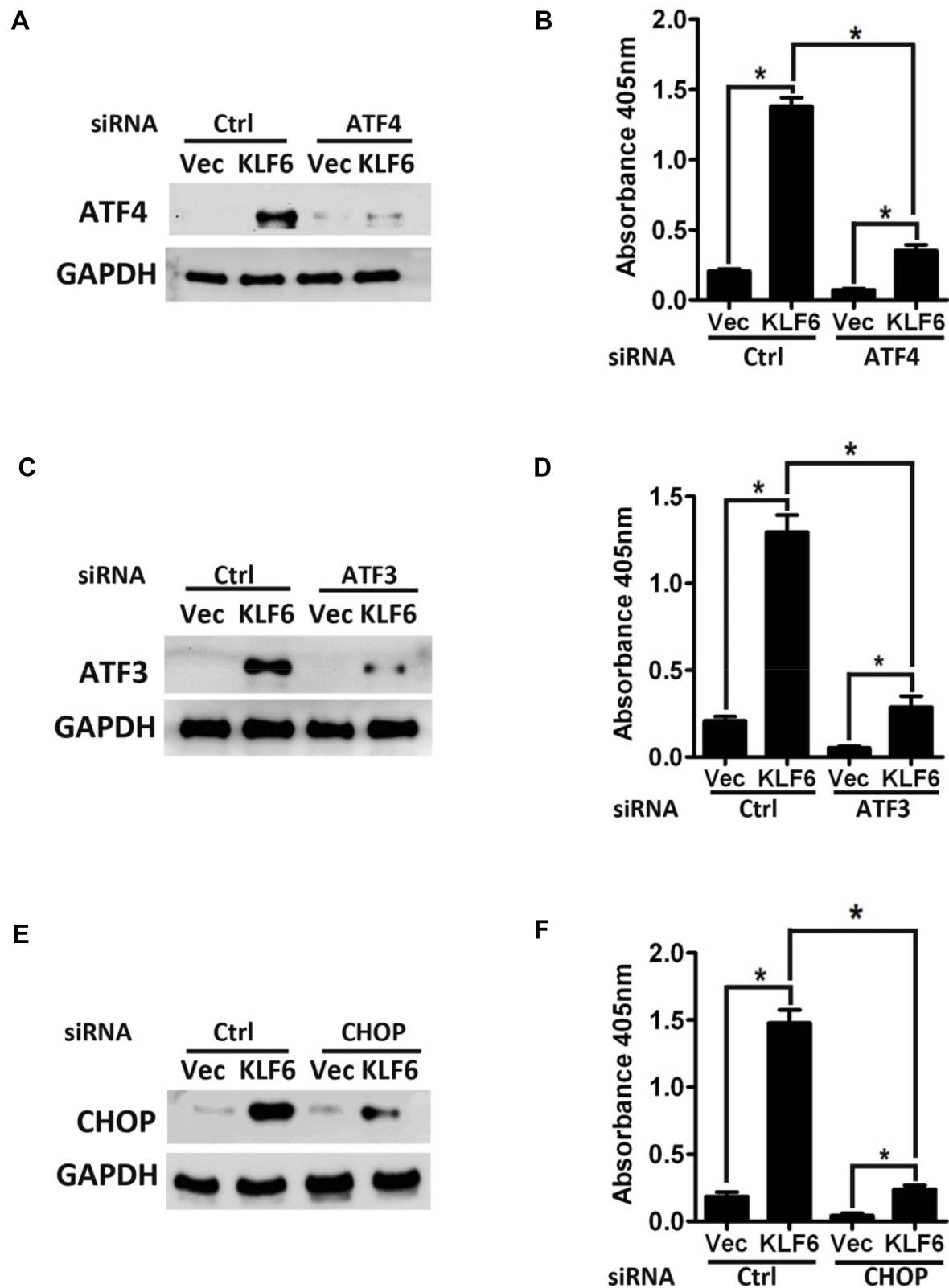


Figure 3 ATF4, ATF3, and CHOP Mediates KLF6-Induced Apoptosis. (**A–C**) ATF4-, ATF3-, and CHOP-targeting siRNAs or negative control siRNA were transfected into HLECs. At 24 hrs post-siRNA transfection, empty pCDNA3.1+ or pCDNA3.1+-KLF6 was transfected into cells. Downregulation of ATF4, ATF3, and CHOP was confirmed by Western blot of protein samples collected at 48 hrs after siRNA transfection. (**D–F**) HLECs were transfected with ATF4, ATF3, and CHOP siRNA or negative control siRNA, followed by empty pCDNA3.1+ or pCDNA3.1+-KLF6, as described in **A–C**, and apoptosis was analyzed 48 h after siRNA transfection using the Cell Death Detection ELISA kit. * $p < 0.05$.

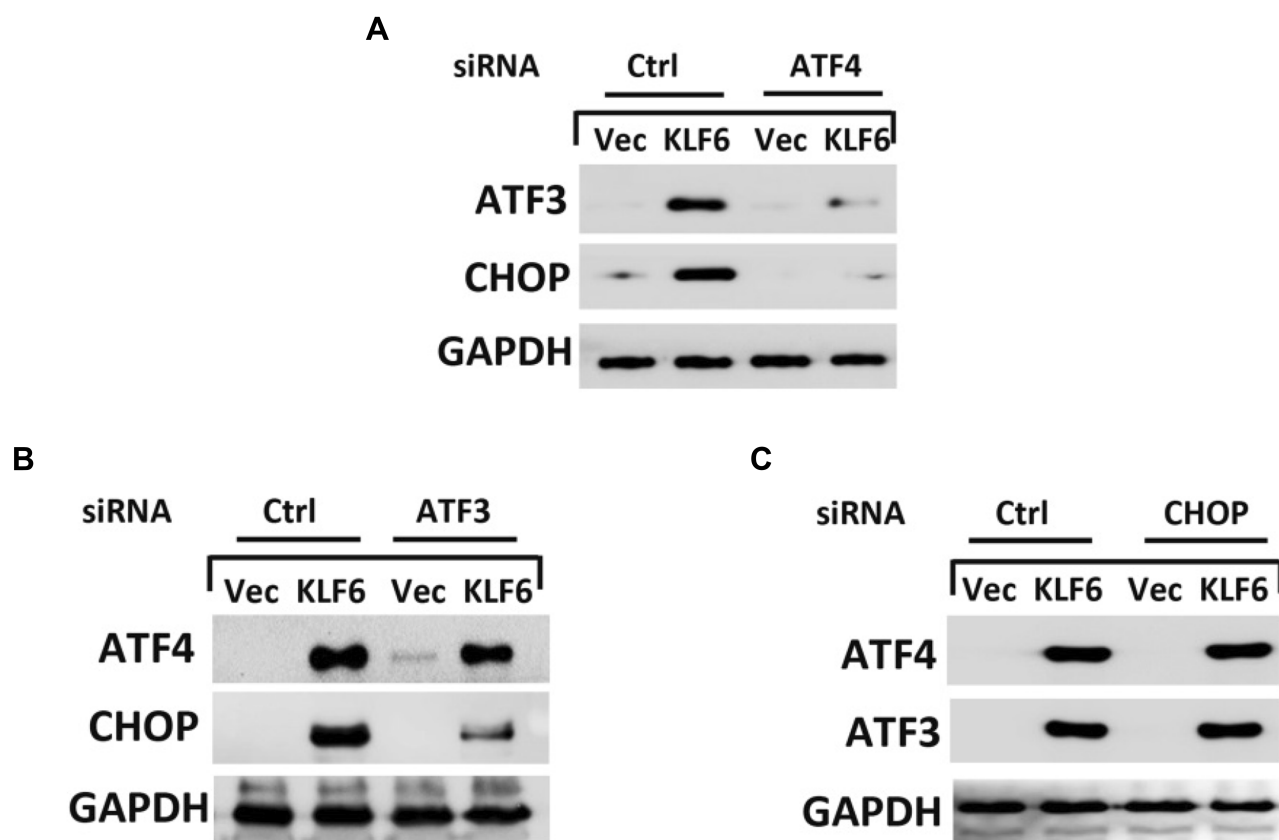


Figure 4 The relationship between ATF4, ATF3, and CHOP under KLF6 induction. Cells were transfected with (A) ATF4, (B) ATF3, or (C) CHOP siRNA. At 24 hrs post-siRNA transfection, cells were treated with pCDNA3.1+-KLF6 for 24 hrs and harvested, and Western blot was used to detect the expression of related proteins.

Discussion

Cataract is the leading cause of blindness worldwide, and its prevalence rises in high myopia patients with much younger age. As the relationship between LEC apoptosis and cataract is studied in greater detail, scientists are inclined to believe that the apoptosis of lens epithelial cells is the common pathogenesis of all types of cataract, except congenital cataract. UV radiation, aging, hypoxia, and other stimuli could cause excess apoptosis of lens epithelial cells, thus causing cytoskeletal degradation, accumulation of crystal protein, and ultimately cataract.³⁷ It has been approved that the apoptosis of lens epithelial cells is much more different in high myopia cataract and ordinary age-related cataract patients. We analyzed lens capsule gene microarray data in high myopia patients and identified two differentially expressed genes: Krüppel-like factor 6 (KLF6) and ATF4. We supposed that the two difference may play a pivotal role in the apoptosis of lens epithelial cells so that the stimuli above can cause more damage and protein degradation followed by subsequent cataract formation in lens of high myopia. Therefore, first

of all, understanding the mechanisms by which KLF6 induces apoptosis in the lens is important for developing the novel pathogenic hypothesis of cataracts.

KLF6 is a member of the specificity protein 1/Krüppel-like transcription factor family (Sp1/KLF) and was initially cloned from leukocytes.⁴¹ KLF6 is a tumor suppressor protein that is downregulated or mutated in several types of cancers, including prostate cancer.^{42–44} KLF6 suppresses tumor growth by activating p21WAF1/Cip1, an inhibitor of cyclin-dependent kinases, in cultured cells and a transgenic mouse model.^{45,46} KLF6 also interacts directly with cyclin D1 to suppress cyclin-dependent kinase 4 and causes cell-cycle arrest.⁴⁷ In addition, the overexpression of KLF6 increases the susceptibility of hepatic stellate cells to apoptotic stress, as evidenced by PARP cleavage in the liver.⁴⁸ Moreover, KLF6 overexpression induces apoptosis in several human cancers, including prostate cancer, non-small-cell lung cancer, and osteosarcoma.^{49–52} However, the mechanism of KLF6-induced apoptosis remains unknown. In previous study, we found that KLF6 induces apoptosis via ATF4 activation

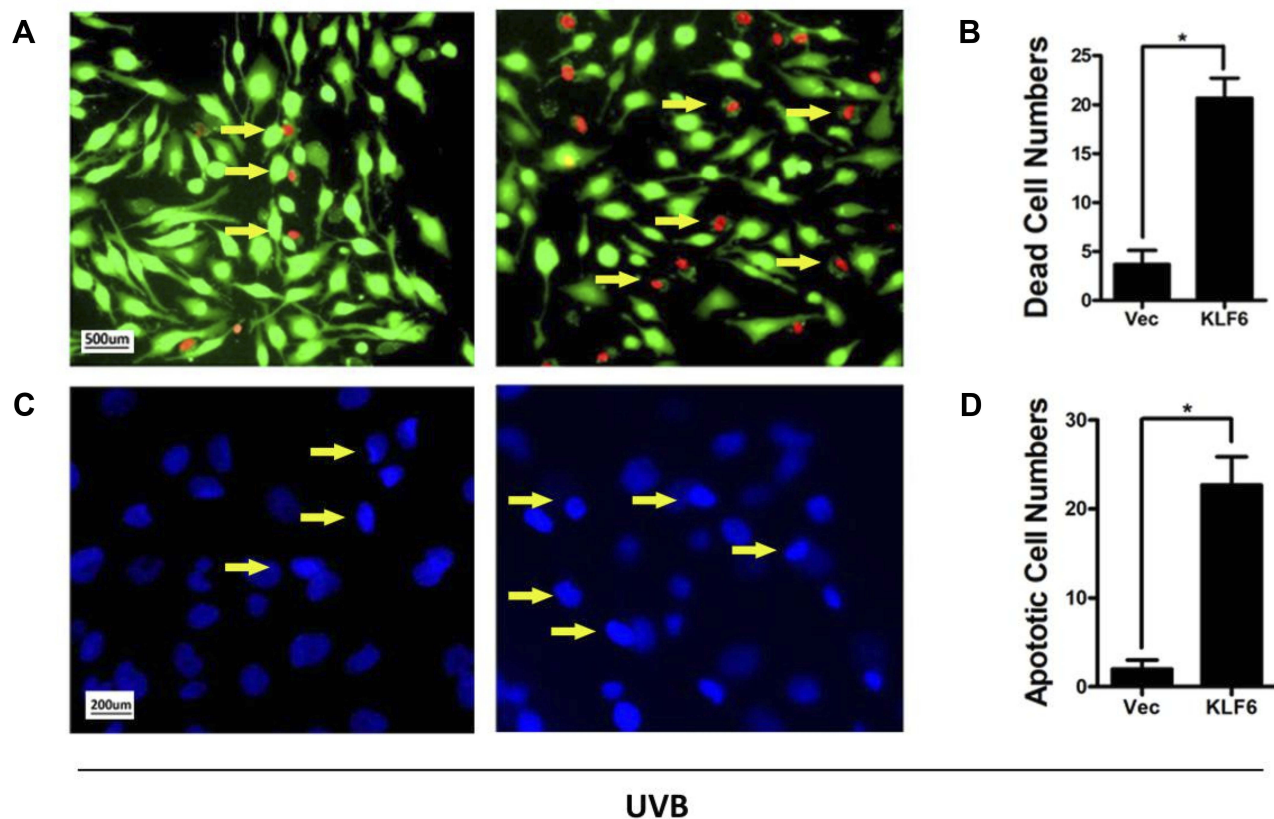


Figure 5 KLF6 affects survival in LECs under UV radiation. HLECs were transfected with pCDNA3.1+KLF6 plasmids in 6-well plates. **(A)** LIVE/DEAD staining was used to identify live (green) and dead (red) cells. **(B)** The number of dead cells was significantly increased in KLF6-transfected cells compared with the normal group under UV radiation. **(C)** Hoechst staining showed more condensed and crescent-shaped nuclei in the KLF6 versus Vec group, as indicated by the arrows. **(D)** The number of apoptotic cells was significantly increased in KLF6-transfected cells compared with the normal group under UV radiation. **p* < 0.05.

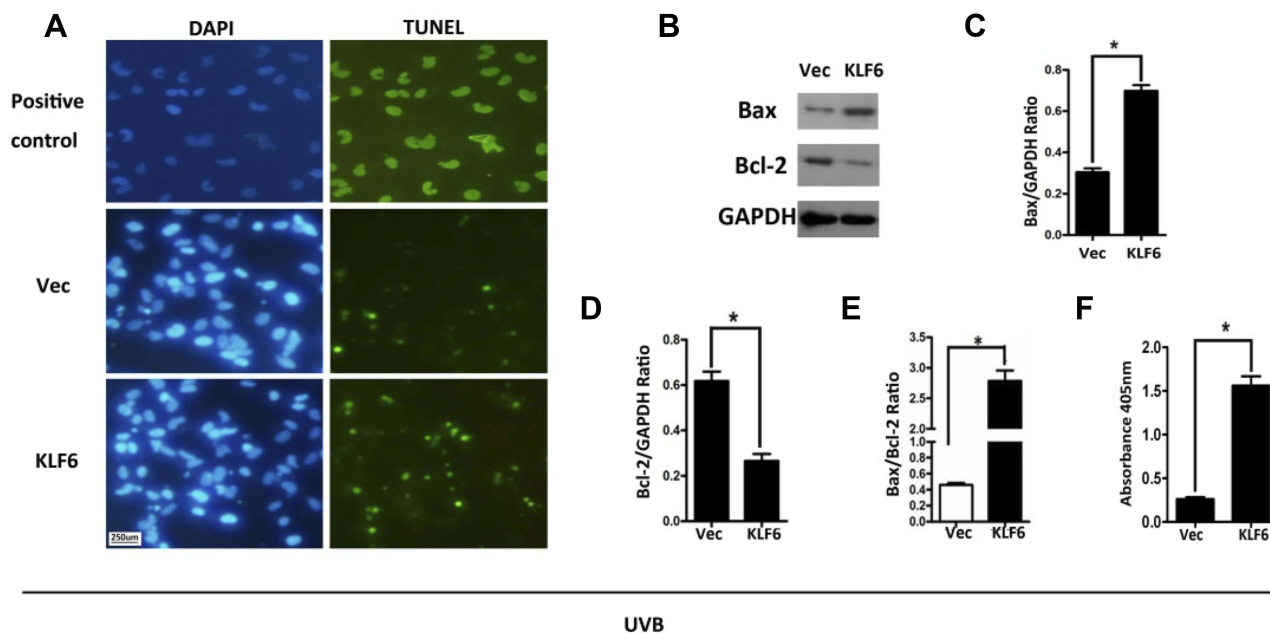


Figure 6 KLF6 induces apoptosis in LECs under UV radiation. HLECs were transfected with pCDNA3.1+KLF6 plasmids in 6-well plates. **(A)** The normal group showed regular round and normal nuclei, with almost no sign of staining by TUNEL; however, the KLF6 group showed typical characteristics of apoptosis. **(B)** Expression of Bax and Bcl-2, with GAPDH as a control. **(C, D)** Densitometric quantification of Bax and Bcl-2 normalized to GAPDH. **(E)** Relative Bax/Bcl-2 ratio was determined via Western blot. **(F)** KLF6-induced apoptosis in HLECs. Apoptosis was analyzed using the Cell Death Detection ELISA kit at 48 h after transfection. **p* < 0.05.

and that the induction of apoptosis likely has a key role in cataract formation.

In the presence of disequilibrium in the endoplasmic reticulum, such as hypoxia and oxidative stress, endoplasmic reticulum stress that is induced by the accumulation of misfolded endoplasmic reticulum proteins is called the unfolded protein response (UPR).^{53,54} Three pathways are known to mediate the UPR according to different transmembrane receptors: inositol-requiring enzyme 1 (IRE1), protein kinase R-like ER kinase (PERK), and activating transcription factor 6 (ATF6), the foremost of which is the PERK-mediated PERK-eIF2 α -ATF4 pathway.⁵⁵ PERK phosphorylation is activated during endoplasmic reticulum stress, leading to eIF2 α phosphorylation, which subsequently inhibits protein translation and reduces protein synthesis, thereby reducing false protein folding and maintaining endoplasmic reticulum homeostasis. This procedure can improve ATF4 mRNA translation, and ATF4 combined with amino acid regulatory element, AARE, increased the expression of CHOP, and further induces oxidative damage through the endoplasmic reticulum stress pathway. Initially, activation of PERK is a protective measure under mild stress. However, activation of PERK also leads to the induction of CHOP, which is an important factor that switches the survival signal to a pro-death signal.^{54,56–58} ATF4 plays an important role in anti-tumor responses and anti-oxidation and promotes renal interstitial fibrosis through the endoplasmic reticulum stress pathway (Figure 7).^{59–61} Previously, our study has revealed that the direct activation of ATF4 mRNA expression can elevate apoptosis in HLECs. In addition, ATF4 knockdown by siRNA blocks KLF6-induced apoptosis, indicating that ATF4 is a key component in HLECs apoptosis.

KLF6 could induce endoplasmic reticulum stress under UV radiation, large amount of unfolded protein can bind to BIP (immunoglobulin binding protein) and inhibit the bound of BIP to PERK, thereby activate the PERK pathway. PERK activation leads to phosphorylation of the subunit of eukaryotic initiation factor 2 (eIF2 α), which inhibits protein synthesis and subsequently inhibits protein translation, thereby reducing false protein folding and maintaining endoplasmic reticulum homeostasis. This procedure can improve ATF4 mRNA translation, and ATF4 is known to activate the ATF3 gene by binding to a ATF/cAMP response element binding (ATF/CREB). ATF3 controls CHOP expression and CHOP leads to decrease in

Bcl-2 but increase in DOCs, TRB3, directly induce cell apoptosis.

ATF3 and ATF4 belong to the ATF/cAMP response element binding (ATF/CREB) family of transcription factors.^{21,62} Members of the ATF/CREB family sense environmental stimulation signals and participate in cellular processes. Liu reported that the UPR increases ATF3 expression via p-eIF2-ATF4 signaling.²¹ Studies have shown that ATF3 is an adaptive response gene that has dual functions in cell apoptosis and the cell cycle.⁶³ ATF3 can induce glomerular mesangial cell apoptosis under stress conditions via KLF6 upregulation.⁶⁴ It also triggers HDAC1-induced proapoptotic activity through the ATF4/ATF3/CHOP pathway.⁶⁵ Conversely, ATF3 might have anti-apoptotic effects. Nakagomi found that ATF3 protects PC12 cells and superior ganglion neurons from JNK-induced apoptosis.⁶⁶ Several genes have been found to be its targets, including p53, CHOP, DR5, and Nrf2.^{23,67,68} CHOP is a target gene of ATF3. ATF3 can promote the transcription of CHOP and induce apoptosis of tumor cells.⁶⁹ Liu has indicated that salernide promotes the expression of CHOP by upregulating ATF3, which contributes to overexpression of DR5 and promotes salernide-induced apoptosis.²³ The mainly accepted mechanism of how CHOP lead to cell apoptosis is CHOP worked as a transcriptional factor mediating multiple apoptotic/anti-apoptotic genes such as DOCs (for down-stream of CHOP), Bcl-2 (B-cell lymphoma-2), TRB3 (tribbles-related protein3).²⁰ DOCs TRB3 is an intracellular pseudokinase that modulates the activity of several signal transduction cascades. During ER stress, TRB3 is up-regulated by an ER stress-inducible transcriptional factor, over-expression of TRB3 would work as feedback to down-regulates its own expression via the repression of ATF4/CHOP transcriptional activity;⁷⁰ Bcl-2 family contains both pro-apoptotic (BAX, Bak, Bok) and antiapoptotic (Bcl-2, Bcl-X, Bcl-W). Under ER stress, CHOP down-regulates the expression of Bcl-2 but not Bcl-X, leading cells to apoptosis.⁷¹ DOCs are a family composed of three members: DOC1, DOC4, and DOC6. However, the mechanism of DOCs effect either survival or death fell far behind the pharmacological and biochemical research.⁴⁰

In this study, we assumed that KLF6 induces HLECs apoptosis through ATF4 and ATF3. We employed siRNA to block ATF4, ATF3, and CHOP expression separately. The results showed that ATF4 regulates ATF3 and CHOP expression and that ATF3 regulates CHOP expression; however, when blocking CHOP, ATF4 and ATF3 expression remained unchanged. Our further work would be focusing on how the

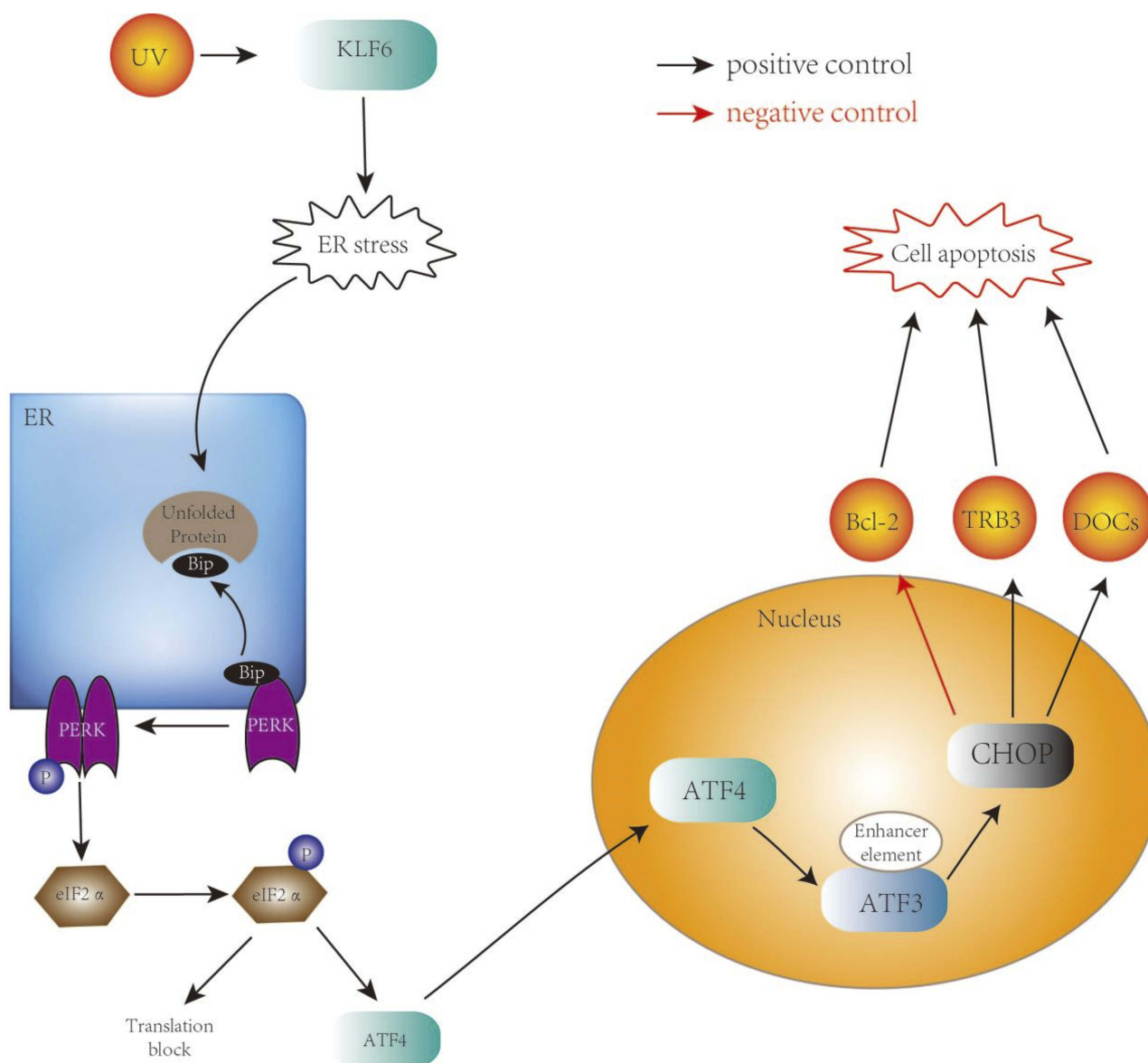


Figure 7 A model of KLF6-induced cell apoptosis via ATF4-ATF3/CHOP pathway under UV.

three molecules interact with each other. Wang speculates that ATF4 promotes ATF3 expression; thus, ATF4 and ATF3 could form a complex and subsequently enhance the transcription of CHOP.⁷² Some earlier studies have found that ATF3 can suppress CHOP gene transcription, whereas CHOP inhibits ATF3 protein function.⁷³ However, our data show that ATF3 can promote CHOP expression. Anyway, how CHOP is regulated by ATF3 and ATF4 remains unknown and needs to be studied further.

In our research, we stressed HLECs with UV, which is a physiological stressor in vivo and relevant for study of oxidative stress-induced ocular disorders. KLF6 overexpression significantly accelerated UV-induced HLECs apoptosis, as

verified by cell death detection and cell staining, such as Hoechst, and live and dead staining. We proved that KLF6 upregulation can activate the proapoptotic protein Bax, inactivate the anti-apoptotic protein Bcl-2, and simultaneously elevate the Bax/Bcl-2 ratio in HLECs that are transfected with KLF6. Bax/Bcl-2 ratio is a crucial “molecular switch” in cell apoptosis reflecting the apoptotic state of HLECs.^{74,75}

In summary, KLF6 is a novel transcriptional activator that induces apoptosis in HLECs via the ATF4-ATF3-CHOP pathway. Moreover, our data provide evidence that KLF6 could make HLECs prone to ER stress and elevated apoptosis level in HLECs under UV radiation. Our results increase our understanding of the regulatory mechanisms of HLECs

apoptosis for the development of novel approaches to prevent and treat high myopia cataract.

Abbreviations

AARE, amino acid regulatory element; ATF3, activating transcription factor 4; ATF4, activating transcription factor 3; ATF6, activating transcription factor 6; ATF/CREB, ATF/cAMP response element binding; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; CHOP, C/EBP homologous protein; DAPI, 4',6-diamidino-2-phenylindole; DR5, death receptor 5; ERS, endoplasmic reticulum stress; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IRE1, inositol-requiring enzyme 1; JNK, c-Jun N-terminal kinase; KLF6, Krüppel-like factor 6; LECs, lens epithelial cells; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PERK, protein kinase R-like ER kinase; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; UPR, unfolded protein response; UVB, ultraviolet radiation B.

Data Sharing Statement

The data used to support the findings of this study are included within the article.

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The use of gifted HLECs was approved by Tianjin Medical University Eye Hospital ethics committee.

Disclosure

The authors declare that they have no conflicts of interest.

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