Effects of ELL-associated factor 2 on ultraviolet radiation-induced cataract formation in mice

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Abstract. ELL-associated factor 2 (Eaf2) has an important role in crystalline lens development and maturation; however, its role in ultraviolet radiation (UV)-induced cataract formation has remained elusive. The present study compared UV-induced cell apoptosis, activation of caspase-3 and caspase-9 and changes in protein expression levels of B-cell lymphoma 2 (bcl-2), bcl-2-associated X protein (bax) and phosphorylated extracellular signal-regulated kinase in wild-type and Eaf2-knockout mice. The results showed that Eaf2 knockout can reduce UV-induced apoptosis in crystalline lenses and mitigate the formation of cataracts. Further functional studies indicated that Eaf2 can induce the activation of caspase-3 and caspase-9, increase the protein expression of the pro-apoptotic protein bax and inhibit the expression of the anti-apoptotic protein bcl-2; thereby, Eaf2 promotes cell apoptosis and is implicated in the formation and development of cataracts. The present study laid a theoretical foundation for the development of drugs for cataract treatment.

Introduction

Cataracts are a type of visual impairment due to reduction of lens transparency and changes of crystalline lens color (1). They are the premier cause of blindness worldwide (2), accounting for 47.8% of all causes of blindness (3). Although high-quality surgical treatments of cataracts have had certain positive effects, the number of patients with cataract-induced blindness is increasing (4). Ultraviolet radiation (UV), oxidative stress and other factors lead to the formation of cataracts (5,6). UV can induce DNA damage and result in cell apoptosis, thereby disrupting the physiological functions of the crystalline lenses and causing a series of physiological changes. Loss of the crystalline lens microstructure interferes with the transmission of light to the retina, leading to visual impairment. UV exposure is inevitable in daily life and is closely linked to eye diseases. Therefore, studying UV-induced apoptosis in crystalline lenses may provide clues for the exploration of the causes of cataract formation and lead to the development of novel treatments.

ELL-associated factor 2 (Eaf2) is a potential tumor suppressor. It was discovered as a protein binding to ELL to form a complex. Eaf2, as a potential regulator of transcription, interacts with ELL and increases the extension activity of RNA polymerase II (7). Eaf2 is involved in multiple physiological processes, including regulation of transcription activation, cell apoptosis and embryonic development (8). Compared to normal cells, prostate cancer cell lines have significantly lower Eaf2 expression levels. The loss of the Eaf2 gene promotes tumorigenesis of prostate cancer (9), whereas over-expression of Eaf2 in prostate cancer cells can induce apoptosis (10). *In vivo* studies showed that Eaf2 can inhibit the growth and induce apoptosis of xenografted prostate tumors (10). Eaf2 knockout causes the formation of tumors, including lung adenocarcinoma, B-cell lymphoma and hepatocellular carcinoma (11). In addition, Eaf2 has important roles in embryonic development, in particular during the development of the eyes (12,13). Eaf2 expression is undetectable in proliferating epithelial cells of the anterior lenses, but can be detected in terminally differentiated and non-proliferating lens fibroblasts. These studies indicated that Eaf2 has important roles in the regulation of crystalline lens development and maturation (13).

Eaf2 has an important role in the regulation of crystalline lens development. However, its roles in UV-induced cataract formation have yet to be fully elucidated. In the present study, the roles of Eaf2 in UV-induced apoptosis were investigated in crystalline lenses of mice; furthermore, the activity of caspase-3 and caspase-9 and the expression levels of B-cell lymphoma 2 (bcl-2), bcl-2-associated X protein (bax) and phosphorylated extracellular signal-regulated kinase (p-ERK) were assessed in wild-type (WT) and Eaf2 knockout (Eaf2 KO) mice. The present study laid a theoretical foundation for the development of drugs for cataract treatment.

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Materials and methods

Animals. A total of 40 14-week-old WT or Eaf2 KO mice (14) were divided into four groups (n=10 in each): i) WT-nonUV, ii) WT-UV, iii) Eaf2 KO-nonUV and iv) Eaf2 KO-UV. The right eyes of the WT-UV mice and Eaf2 KO-UV mice were exposed to UV radiation, while the left eyes received no radiation. WT-nonUV mice and Eaf2 KO-nonUV mice were not exposed to UV. The Eaf2 KO mice were obtained from Professor Yi Sin Liu (University of Southern California, Los Angeles, CA, USA). The mice were maintained in an environment with a constant temperature of 23 ± 2 ˚C, a relative humidity of 50±5 % and a 12 h light-dark cycle. Chow and water were provided *ad libitum*. The mice were sacrificed by decapitation. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals. The present met the standards of, and was approved by, the ethics committee of China Medical University (Shenyang, China).

Construction of the Eaf2 overexpression plasmid (Eaf2 OE). The Eaf2 coding sequence (CDS) was amplified by polymerase chain reaction (PCR) using murine cDNA, which was reverse transcribed from total RNA extracted from the rat cells. This was maintained in our laboratory as a template. The primers used were as follows: Eaf2-CDS forward, 5'-GTATGAAAGCTTAAGGCCAAAAGCGG-3' and reverse, 5'-GCCCGAATTCATCTCACAAATGTTTTCTCTGT-3'. The primers were synthesized by Sangon Biotech (Shanghai, China). The PCR was performed using a Life Express PCR Instrument (Bioer, Hangzhou, China) and the following cycling conditions were used: 95 ˚C for 5 min; 95˚C for 30 sec, 55˚C for 30 sec, 72˚C for 60 sec, 30 cycles; 72˚C for 5 min and 4˚C for 2 min. The products were purified using a multifunctional DNA purification kit (BioTeke, Beijing, China). The sequencing was performed by Sangon Biotech). The coding sequence was inserted into the p enhanced green fluorescence protein-N1 vector (Clontech, Mountain View, CA, USA) by the restriction enzymes FastDigest *Hin*dIII and FastDigest *Eco*RI (Fermentas, Burlington, CA, USA). The vector was sequenced for verification and was named as Eaf2 OE.

Cell culture and transfection. The rat crystalline lens epithelial cell line α-TN4 was cultured in Dulbecco's modified Eagle's medium (Gibco Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, USA). The cells were incubated in an incubator at 37˚C in an atmosphere containing 5% (v/v) CO_2. Cells were seeded into six-well plates and transfected at 70-80% confluence with the empty vector (Vector) or the Eaf2 overexpression plasmid (Eaf2 OE) using Lipofectamine 2000 reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. 48 h after transfection, the transfection efficiency was analyzed by quantitative real-time PCR and western blotting.

Mouse model of UV-induced cataracts. UV at a wavelength of 302 nm and intensity of 200 mW/cm^2 was generated by a UV transilluminator (Spectroline XX-15N/F; Spectronics, Westbury, NY, USA). The UV transilluminator was covered with aluminum foil, leaving only a small hole with a diameter of 5 mm for irradiating the eyes of the mice. The mice were not anesthetized, and only the right eyes were irradiated with UV. The left eyes were not irradiated and used as a control. 5 min prior to UV irradiation, the eyes of the mice were injected with 1% (w/v) tropicamide (cat. no. T9778; Sigma-Aldrich, St. Louis, MO, USA) and 0.1% (w/v) atropine sulfate to induce mydriasis. Prior to UV irradiation, the mice were checked with a slit lamp to exclude pre-existing cataracts. One eye of each mouse was exposed to UV for 100 sec twice per week for three weeks. 48 h after the last UV irradiation, the mouse lens opacity was observed by slit lamp examination.

Preparation of lens tissue. 48 h after UV irradiation, the eyeballs of all the mice were surgically removed, together with a ~2-mm optic nerve. The eyeballs were incubated in fixing buffer, containing acetic acid, formaldehyde solution (Kemiou Chemical Reagent Co., Lt., Tianjing China), physiological saline (Cisen Pharmaceutical Co., Ltd., Shandong, China) and 75% ethanol at a ratio of 1:2:7:10. After fixing, the crystalline lenses were removed under a microscope OLYMPUS DP73 (Olympus Corporation, Tokyo, Japan) for subsequent experiments.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis analysis. Apoptosis was analyzed using the In Situ Cell Death Detection kit (cat. no. 11684817910; Roche, Basel, Switzerland). Fixed eyes were paraffin-embedded and cut into tissue sections of 5 μm. Cultured cells were seeded and fixed with 4% (w/v) paraformaldehyde. Prior to the TUNEL assay, the samples were permeabilized with 0.1% (v/v) TritonX-100 (Beyotime Institute of Biotechonlogy, Shanghai, China) and blocked with 3% (v/v) H_2O_2. The TUNEL reagent was mixed with Enzyme Solution and Label Solution and then added to the sample surfaces dropwise. The samples were incubated at 37˚C in dark in a humid environment for 60 min. The Converter-POD was added to the sample surfaces dropwise and incubated at 37˚C for 30 min followed by diaminobenzidine substrate (Solarbio, Beijing, China). The samples were counterstained with hematoxylin (Solarbio), mounted with neutral balsam and subjected to microscopic imaging.

Western blot analysis. Proteins in tissues were extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) containing 1% phenylmethanesulfonylfluoride (Beyotime Institute of Biotechnology). Proteins in cultured cells were extracted using NP-40 lysis buffer (Beyotime Institute of Biotechnology). Proteins extracted were quantified using a Bicinchoninic Acid Protein Assay kit (cat. no. P0012; Beyotime Institute of Biotechnology). Equal amounts of each protein sample were subjected to SDS-PAGE. The separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). PVDF membranes were blocked with 5% (w/v) skimmed milk or 5% (w/v) bovine serum albumin (Biosharp, Hefei, China) and incubated at 4˚C overnight with the corresponding primary rabbit antibodies against bax (rabbit anti-mouse polyclonal antibody; 1:500; cat. no. WL0101; Wanleibio, Shenyang, China), bcl-2 (rabbit anti-mouse polyclonal antibody; 1:500; cat. no. WL0104; Wanleibio), ERK (rabbit anti-mouse polyclonal antibody; 1:1,000; cat. no. WL0323; Wanleibio), p-ERK (rabbit anti-mouse polyclonal antibody;

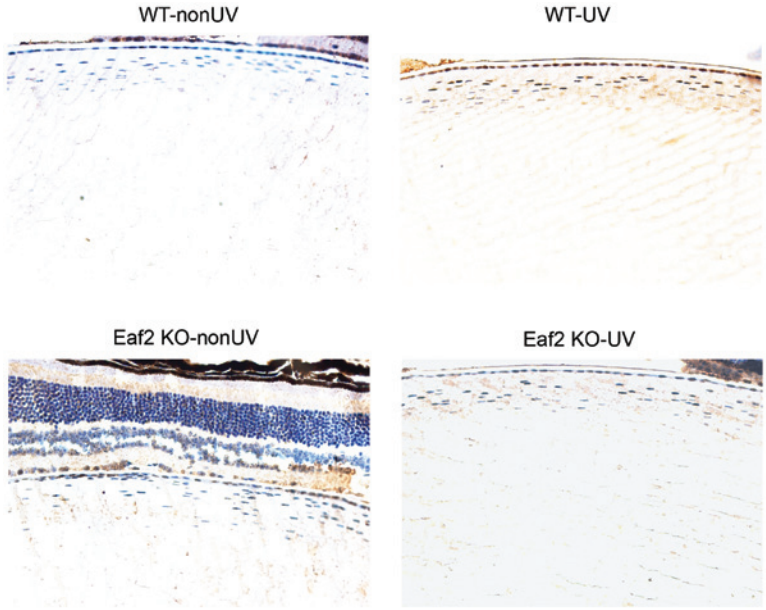


Figure 1. UV radiation induces apoptosis in crystalline lenses. After UV irradiation, apoptosis in the crystalline lenses of mice in each group was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling assay (magnification, x400). WT-nonUV, wild-type mice without UV radiation; WT-UV, wild-type mice with UV radiation; Eaf2 KO-nonUV, Eaf2-KO mice without UV irradiation; Eaf2 KO-UV, Eaf2-KO mice with UV radiation. UV, ultraviolet; Eaf2, ELL associated factor 2; WT, wild-type; KO, knockout.

1:1,000; cat. no. WLP002; Wanleibio), Eaf2 (rabbit anti-mouse polyclonal antibody; 1:1,000; cat. no. WL0333; Wanleibio), cleaved-caspase-3 (rabbit anti-mouse polyclonal antibody; 1:1,000; cat. no. ab2575; Abcam, Cambridge, MA, USA) and cleaved-caspase-9 (rabbit anti-mouse polyclonal antibody; 1:1,000; cat. no. WL0001; Wanleibio). The PVDF membranes were then incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:5,000; cat. no. A0208; Beyotime Institute of Biotechnology) at 37˚C for 45 min. Using β-actin as an internal reference, the objective proteins were detected by an enhanced chemiluminescence detection system (cat. no. E002-5; 7Sea Biotech, Shanghai, China). The signal intensities of the protein bands were analyzed using Gel-Pro-Analyzer 4.5 software.

Caspase-3 and caspase-9 activity assay. The crystalline lens tissues were mixed with nine volumes of phosphate-buffered saline, homogenized using a High speed Homogenizer (Scientz, Ningbo, China) and then frozen and thawed three times in liquid nitrogen. The samples were centrifuged at 10010 x g at 4˚C for 10 min, and the supernatants were retained for the experiments. The protein concentrations were measured using the Bradford Protein Assay kit (Beyotime Institute of Biotechnology) and diluted to equal concentrations. Caspase-3 and caspase-9 activities were analyzed using Caspase-3 Activity Assay kit (cat. no. C1116) and Caspase-9 Activity Assay kit (cat. no. C1158; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The absorbance at 409 nm was measured using a microplate reader (ELX-800; BioTek, Winooski, VT, USA).

Reverse transcription quantitative PCR (RT-qPCR). Total RNA was extracted using the RNA simple Total RNA kit (cat. no. DP419; Tiangen, Beijing, China), according to the manufacturer's instructions. The total RNA was reverse transcribed into cDNA by Moloney mouse leukemia virus reverse transcriptase (BioTeke) and oligo $(dT)_{15}$ (Sangon Biotech). Eaf2 mRNA levels were determined by RT-qPCR using the SYBR GREEN method with cDNA as template. SYBR GREEN reagent was purchased from Solarbio. The primers used are as follows: Eaf2 forward, 5'-CTTGCATACCTGGACCGT-3' and reverse, 5'-GTTCACCTTTGCCAACCTCA-3'; β-actin forward, 5'-CTGTGCCCATCTACGAGGGCTAT-3' and reverse, 5'-TTTGATGTCACGCACGATTTCC-3'. The primers were synthesized by Sangon Biotech. The RT-qPCR reaction volume (20 μl), contained cDNA (1 μl), forward primer (10 μM; 0.5 μl), reverse primer (10 μM; 0.5 μl), SYBR GREEN mastermix (10 μl) and water. An Exicycler™ 96, BIONEER Real-Time PCR system was used (Bioneer Corporation, Daejeon, Korea) and the reaction conditions were as follows: 95˚C for 10 min followed by 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 30 sec, and final incubation at 4˚C 5 min. The relative mRNA expression levels for each sample were calculated using the $2^{-\Delta\Delta Ct}$ method with β-actin as an internal reference (15).

Statistical analysis. Values are expressed as the mean ± standard deviation. All experiments were repeated three times. Differences between groups were analyzed using one-way analysis of variance and Bonferroni's Multiple Comparison. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

UV induces apoptosis in mouse cataract lenses. In order to study the role of Eaf2 in UV-induced formation of cataracts in

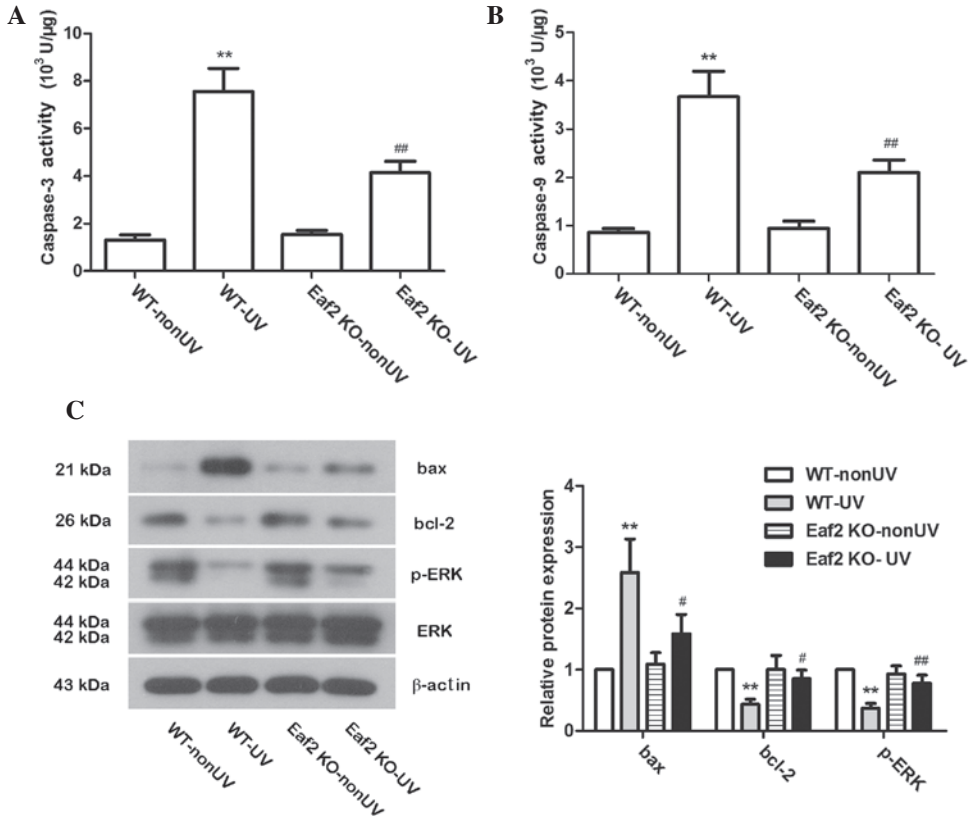


Figure 2. Eaf2 knockout reduces UV-induced apoptosis in the crystalline lenses. (A and B) Caspase-3 and caspase-9 activity in the crystalline lenses of mice in each group were detected after UV irradiation. (C) Protein expression levels of bax, bcl-2, p-ERK and ERK were detected by western blotting. β-actin was used as reference when the relative expression levels of proteins were calculated. Values are expressed as the mean ± standard deviation of three experiments. **P<0.01 compared with WT-nonUV; $^{\#\#}$P<0.01 compared with WT-UV. UV, ultraviolet; Eaf2, ELL associated factor 2; WT, wild-type; KO, knockout; bcl-2, B-call lymphoma 2; bax, bcl-2-associated X protein; p-ERK, phosphorylated extracellular signal-regulated kinase.

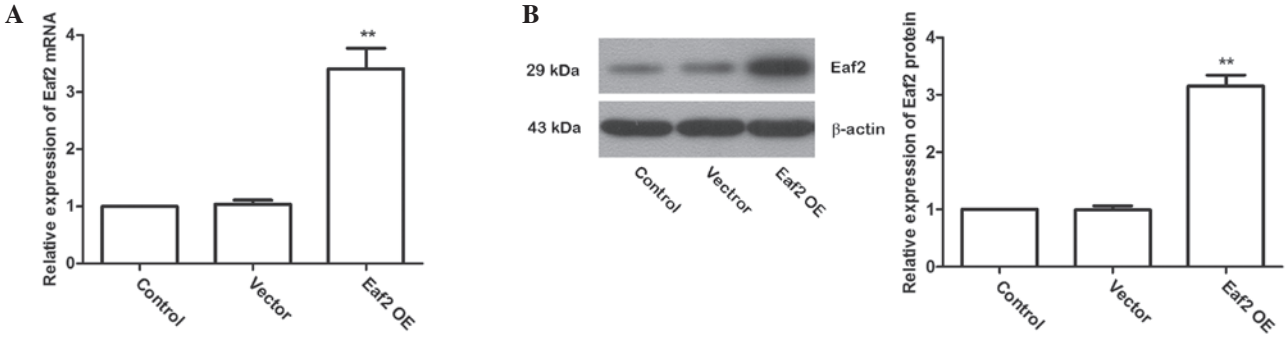


Figure 3. Eaf2 overexpression. (A) mRNA expression levels of Eaf2 were detected by reverse transcription quantitative polymerase chain reaction. Relative mRNA expression levels were calculated and analyzed using the $2^{-\Delta\Delta Ct}$ method. (B) The Eaf2 protein expression levels were detected by western blotting using β-actin as a reference. Values are expressed as the mean ± standard deviation of three experiments. **P<0.01 compared with the vector. OE, overexpression; Eaf2, ELL associated factor 2.

mice, UV was used to irradiate the crystalline lenses of WT mice and Eaf2 KO mice, and a TUNEL assay was used to quantify the apoptosis in the crystalline lenses. For Eaf2 KO and WT mice, the apoptosis rates of the crystalline lenses after receiving UV irradiation were higher than those in animals that were not exposed to UV (Fig. 1). These results indicated that UV induced apoptosis in the crystalline lenses. In addition, the apoptotic rates in crystalline lenses in the Eaf2 KO-UV group were lower than those in the WT-UV group (Fig. 1). These results implied that Eaf2 knockout reduces UV-induced apoptosis in the crystalline lenses, thereby mitigating the formation of UV-induced cataracts.

Eaf2 knockout reduces UV-induced apoptosis. To further study the role of Eaf2 in UV-induced cataract formation in mice, the activities of caspase-3 and caspase-9 were examined. In WT-UV and Eaf2 KO-UV mice, after UV irradiation, the activities of caspase-3 and caspase-9 were significantly increased. Compared with WT-UV mice, Eaf2 KO-UV mice had significantly lower caspase-3 and caspase-9 activities (Fig. 2A and

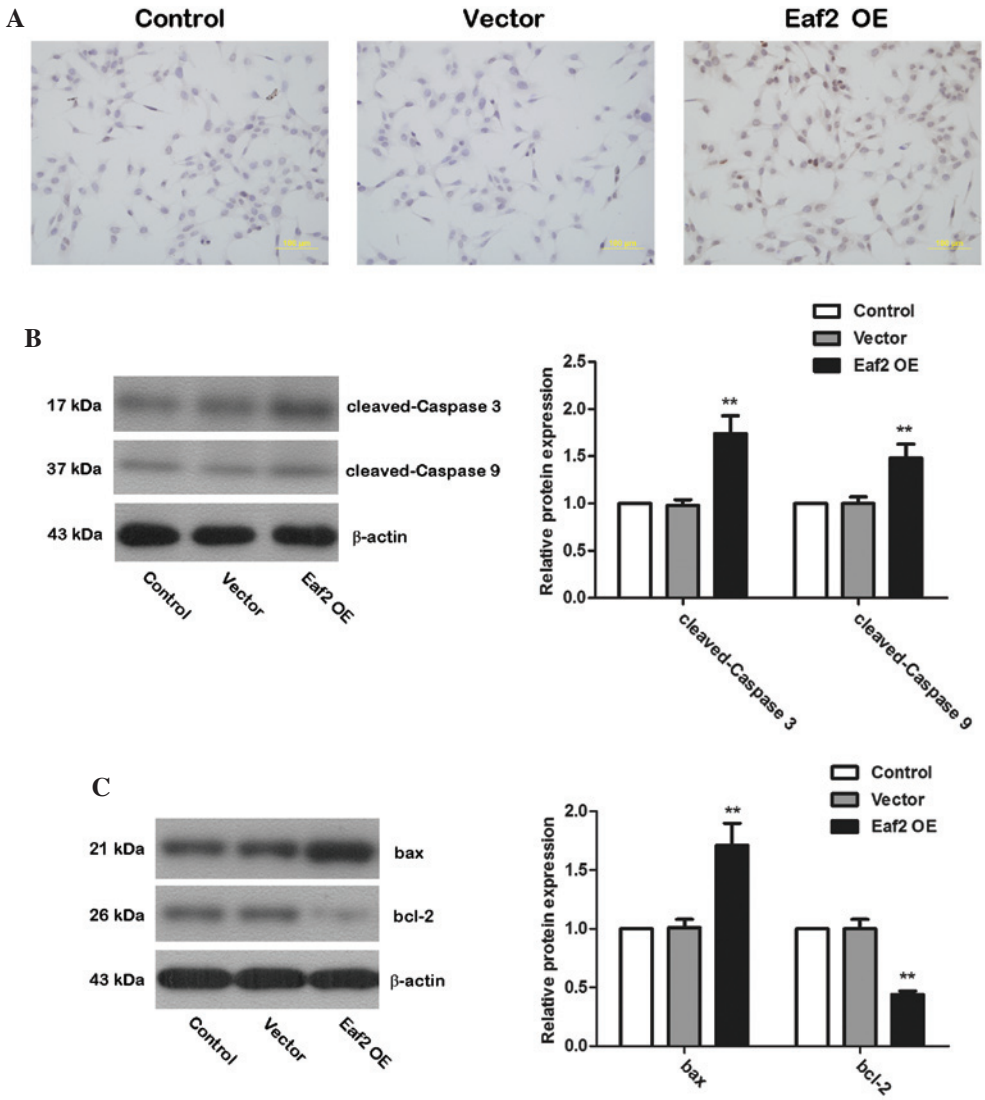


Figure 4. OE of Eaf2 induces apoptosis. (A) Cell apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling assay after transfection with Eaf2 OE vector (scale bar, 100 μm). (B) Western blot analysis revealed the effects of Eaf2 OE on cleaved caspase-3 and cleaved caspase-9 protein levels. (C) The protein levels of bax and bcl-2 were detected by western blotting after transfection with Eaf2 using β-actin as reference. Values are expressed as the mean ± standard deviation of three experiments.**P<0.01 compared with the vector. bcl-2, B-call lymphoma 2; bax, bcl-2-associated X protein; OE, overexpression; Eaf2, ELL associated factor 2.

B; P<0.01). These results suggested that Eaf2 knockout can reduce UV-induced apoptosis, which was consistent with the results of the TUNEL assays. Protein levels of bax, bcl-2, ERK and p-ERK were also detected by western blotting. In WT-UV mice, bax protein levels were increased, while bcl-2 and p-ERK protein expression levels were decreased as compared with those in the WT-nonUVmice. Of note, compared with WT-UV mice, Eaf2 KO-UV mice had decreased bax levels and elevated bcl-2 and p-ERK levels (Fig. 2C). These results, on the molecular level, suggested that Eaf2 knockout can reduce UV-induced apoptosis in crystalline lenses.

Eaf2 overexpression. To investigate the role of Eaf2 in UV-induced apoptosis in crystalline lenses, a plasmid named Eaf2 OE to over-express Eaf2 in murine crystalline lens cells was constructed and the over-expression efficiency was detected by RT-qPCR and western blotting. The RT-qPCR results showed that after transfection with Eaf2 OE, the Eaf2 mRNA levels were significantly increased (Fig. 3A; P<0.01). The western blotting results also showed that after transfection with Eaf2 OE, Eaf2 protein levels were significantly increased (Fig. 3B; P<0.01). These results indicated that transfection of Eaf2 OE effectively increased the levels of Eaf2 in murine crystalline lens cells.

Eaf2 overexpression induces apoptosis in murine crystalline lens cells. The TUNEL assay was used to detect cell apoptosis after transfection with Eaf2 OE. Compared with non-transfected (Control) cells and cells transfected with empty vector (Vector), cells transfected with Eaf2 OE exhibited a significantly increased level of apoptosis (Fig. 4A). Western blotting was then used to examine protein levels of cleaved caspase-3 and cleaved caspase-9. After transfection with Eaf2 OE, the levels of cleaved caspase-3 and cleaved caspase-9 were significantly increased (Fig. 4B; P<0.01), indicating an increase in the activation of caspase-3 and caspase-9. Western blotting was also used to detect the protein

levels of the pro-apoptotic protein bax and the anti-apoptotic protein bcl-2. In cells over-expressing Eaf2, bax expression was significantly elevated, whereas bcl-2 expression levels were significantly reduced (Fig. 4C; $P<0.01$). These results illustrated that Eaf2 promoted the activation of caspase-3 and caspase-9 and affected the expression of apoptosis-associated proteins, thereby inducing apoptosis.

Discussion

UV (5), smoking (16,17), vitamin deficiency (18), oxidative stress (19) and other factors may cause cataracts. Among these factors, UV is an inevitable risk factor encountered in daily life. In the present study, the role of Eaf2 in UV-induced cataract formation in mice was investigated. The *In vivo* experiment suggested that Eaf2 knockout was able to mitigate UV-induced cataract formation in mice. Furthermore, the results of the *in vitro* study suggested that Eaf2 activates caspases, regulates the expression levels of apoptosis-associated proteins and promotes apoptosis of crystalline lens cells. These results provided a theoretical basis for the development of novel cataract treatments.

UV-induced apoptosis in the crystalline lenses is an important cause of UV-induced cataracts. The present study found that UV irradiation can induce apoptosis of crystalline lens cells in WT and Eaf2-KO mice. It has been reported that UV-induced cataracts are associated with the activation of P53 and caspase-3 (20). UV activates P53 and caspase-3 and ultimately induces apoptosis in crystalline lens cells, resulting in cataracts. In addition, UV alters the translocation of nuclear factor (NF)-κB. Blocking the NF-κB pathway with an NF-κB inhibitor decreased the degree of UV-induced cell death (21). UV can also cause DNA damage (22), interfering with DNA replication and transcription. When a large number of cells undergo apoptosis, the physiological functions of the crystalline lens are disturbed, causing cataract lesions.

Eaf2 has an extensive role in the development of crystalline lenses. During mouse embryo development, Eaf2 has a spatial regulation mode in the developing crystalline lens (13). A study showed that in *Xenopus laevis*, Eaf2 deficiency leads to the loss of the eyes (12). These studies provided important evidence for the importance of Eaf2 in the developing eye. The present study found that the extent of apoptosis in Eaf2 KO-UV mice was significantly lower than that in WT-UV mice. These results implied that Eaf2 knockout, to a certain degree, has a mitigating role in UV-induced cataracts. Furthermore, the present study examined the UV-induced apoptosis in Eaf2-KO murine crystalline lenses. UV irradiation activated caspase-3 and caspase-9, promoted the expression of bax, and inhibited the expression of bcl-2 as well as the activation of ERK. These results are consistent with the results of a previous study (20). Eaf2-KO reduced the UV-induced activation of caspase-3 and caspase-9, changes in bax and bcl-2 expression levels, and ERK pathway activation. These results suggested that Eaf2 may modulate UV-induced apoptosis through regulating caspase activation, the expression of apoptosis-associated proteins and the activation of the ERK pathway. Further study of gene functions showed that Eaf2 promoted caspase-3 and caspase-9 activation, altered bax and bcl-2 expression and thus affected apoptosis of crystalline lens cells. Similar to the present study, Xiao *et al* (14) showed that Eaf2 can affect the activation of caspase-3, as well as the expression levels of bax, BH3 interacting-domain death agonist and P53, thus affecting UV-induced apoptosis in crystalline lenses. In the process of UV-induced cataract formation, UV can lead to DNA damage, which affects the distribution of Eaf2. It has been indicated that UV irradiation may promote Eaf2 translocation to the nucleolus (8). Studies have shown that Eaf2 can also act as a tumor suppressor gene either by regulating ERK phosphorylation (23) or by binding to the retinoblastoma protein to inhibit the Ras pathway (24). However, how Eaf2 induces apoptosis during UV-induced cataract formation is worth pursuing in in-depth studies.

In the present study, the effects of Eaf2 in UV-induced apoptosis of murine crystalline lens cells were investigated. The results showed that Eaf2 promoted the activation of caspase-3 and caspase-9, increased the expression of the pro-apoptotic protein bax and inhibited the expression of the anti-apoptotic protein bcl-2, thereby promoting apoptosis of crystalline lens cells. Eaf2-KO inhibited UV-induced activation of caspase-3 and caspase-9 as well as changes in the expression levels of apoptosis-associated proteins, and Eaf2 KO was shown to mitigate UV-induced cataracts. These results laid a theoretical foundation for developing novel drugs for cataract treatment.

Acknowledgements

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