Eaf2 protects human lens epithelial cells against oxidative stress-induced apoptosis by Wnt signaling

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Abstract. The tumor suppressor protein ELL-associated factor 2 (Eaf2) serves an important role in lens development and maturation; however, its role in oxidative stress-induced cataract formation remains unclear. In the present study, an in vitro apoptosis model was constructed by treating HLE-B3 cells with 50 μM hydrogen peroxide (H₂O₂), and was confirmed by flow cytometry. Subsequently, overexpression of Eaf2 was induced in H₂O₂-induced HLE-B3 cells by ligating Eaf2 cDNA to a pcDNA3.0 plasmid and the role of Wnt3a in the function of Eaf2 was also assessed by inhibiting the expression of the gene in Eaf2-overexpression cells. The expression levels of glycogen synthase kinase 3β, β-catenin, Eaf2, caspase 3, Wnt3a, B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein were examined using reverse transcription-quantitative polymerase chain reaction and western blot analysis. Immunocytochemistry was used to locate Eaf2 and Wnt3 protein expression in the H₂O₂-induced HLE-B3 cells. The results indicated that Eaf2 was able to effectively suppress H₂O₂-induced apoptosis of HLE cells via inhibition of caspase 3 production and activation of Wnt3a signaling. In addition, knockdown of Wnt3a in Eaf2-overexpression cells evidently counteracted the effect of Eaf2 in antagonizing H₂O₂-induced apoptosis. Taken together, these findings suggested that Eaf2 may suppress oxidative stress-induced apoptosis of HLE-B3 cells exerted through the activation of Wnt3a signaling.

Introduction

The reduction in lens transparency caused by cataracts induces severe visual impairment (1), which can seriously effect patients' quality of life. Currently, cataract-induced blindness accounts for ~50% of blindness cases worldwide (2). It is well-recognized that damage resulting from oxidative stress in the ocular lens is a major cause of cataracts (3). The lens is a transparent organ that consists of a single layer of epithelial cells, which are the main target of oxidative stress (4). Oxidative stress caused by exposure to hydrogen peroxide (H₂O₂) results in DNA damage, and impairs the function of cells and tissues (5). H₂O₂, which is a non-free radical member of the active oxygen family, generates hydroxyl radicals that irreversibly damage the lens epithelium, resulting in cell death and cataract formation (6). Some patients with cataracts have markedly increased levels of H₂O₂ within their lenses (7), indicating the possible involvement of H₂O₂ in the genesis of nuclear cataracts in humans. Therefore, a better understanding of the mechanisms underlying H₂O₂-induced apoptosis of lens cells may provide information regarding the cause of cataract formation.

ELL-associated factor 2 (Eaf2) is a protein that was discovered based on its ability to bind with a second protein (eleven-nineteen lysine-rich leukemia protein; ELL), which is independently coded by a gene that is upregulated by androgen in the rat prostate gland and is also named human U19 (8). As a regulator of transcription, Eaf2 interacts with ELL to efficiently stimulate the extension activity of RNA polymerase II (9). It has previously been suggested that Eaf2 serves as a tumor suppressor in prostate cancer (10). Furthermore, knockdown of Eaf2 has been reported to promote tumorigenesis in mouse models of adenocarcinoma and hepatocellular carcinoma (11). In addition to its role in cancer, Eaf2 serves important roles in embryonic development via its involvement in non-canonical Wnt signaling (12,13), which is thought to be essential for convergence and extension movements, as well as the midline convergence of organ precursors (14,15). Liu et al (16) demonstrated that Eaf2 acts as an upstream modulator of non-canonical Wnt signaling to mediate convergence and extension movements. In addition, the Wnt family of secreted signaling proteins has important roles in organogenesis, tissue homeostasis and tumor formation (17). Overexpression of Wnt3a has been reported to promote the proliferation of human lens epithelial (HLE) cells (18). Furthermore, Eaf2 is required for normal eye development and the regulation of crystalline lens development and maturation in Xenopus laevis (12,13).

Key words: apoptosis, ELL-associated factor 2, H₂O₂, lens epithelial cell, Wnt3a
Recently, a related study identified an important role for Eaf2 in ultraviolet-induced cataract formation (19). However, the mechanism underlying the effects of Eaf2 on lenses undergoing oxidative stress remains unknown.

The present study aimed to investigate the role of Eaf2 in HLE cells undergoing H$_2$O$_2$-induced apoptosis, and to determine the underlying molecular mechanism. The results indicated that in HLE cells, Eaf2 protects against H$_2$O$_2$-induced cell death by inhibiting caspase 3 enzymatic activity and activating the Wnt3 signaling pathway.

Materials and methods

Cell culture. HLE-B3 cells were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). HLE-B3 cells were cultured in minimum essential medium (MEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere containing 5% CO$_2$ at 37°C.

Induction of apoptosis in HLE-B3 cells. H$_2$O$_2$-induced HLE-B3 cells used in subsequent experiments were gradually deprived of serum via an overnight culture in MEM containing 2% FBS, prior to being treated with 50 µM H$_2$O$_2$ for various time periods (4, 8 and 12 h) at 37°C.

Vector construction and cell transfection. To induce Eaf2 overexpression, the full-length cDNA (NC_000003.12) for the human Eaf2 gene was obtained from the total RNA of human HLE-B3 cells isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized from the RNA using SuperScript II RT (200 U/ml; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. β-actin served as an endogenous control in the qPCR analysis. The following primers were used: β-actin, forward 5'-GCC ACAAGATTACAAGAAGCG-3′, reverse 5'-TGGGCCACCA ATATCAAATCTCC-3′; caspase 3, forward 5'-TGGTTCTAC CAGTCCCTTG-3′, reverse 5'-AATTCTGTTGCCCACC TTTCG-3′; Eaf2, forward 5'-AGGTGACCATTAACCTGCA AAAT-3′, reverse 5'-AGGCCAATCTCCCTGATC-3′; Wnt3a, forward 5'-GTCACCCGATTTGCTCAG-3′, reverse 5'-GACACCATCCCACCAAACTC-3′; β-catenin, forward 5'-GGTGTCTCAAGGC-3′, reverse 5'-GCACCTCCGGCCA CAAGA-3′; and Bcl-2, forward 5'-TGTGGCCTCTTCT TTAGTTCG-3′ and reverse 5'-ATCCACGCTCTCGGT ATCC-3′: β-actin, forward: 5'-CTTAGTGGCTGTAACC TTTCCTG-3′, reverse: 5'-CTGTCACCTCCTACCTCAG TTT-3′. The RT-qPCR analyses were performed in triplicate on a Bio-Rad Connect Real-Time PCR platform (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a 20 µl reaction mixture containing 10 µl 2X SYBR Premix Ex Taq, 0.5 µl each primer (2.5 µM), 5 µl cDNA and 4.0 µl ddH$_2$O. The RT-qPCR procedure was conducted as follows: Initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing extension at 60°C for 20 sec, followed by 72°C for 20 sec. Human β-actin was used as an internal loading control for RT-qPCR. The 2$^{-ΔΔCq}$ (20) method was used to determine the relative mRNA expression levels of human β-catenin, caspase 3, Eaf2, Wnt3a, Bcl-2 and Bax. These levels were normalized to those of β-actin.

Western blot analysis. Treated HLE-B3 cells were harvested via centrifugation (14,000 x g 4 min) at 4°C. Subsequently, the pellets were lysed in a 2X SDS lysis buffer containing 100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4% SDS and 10% glycerine. The lysed cells were then centrifuged at 15,000 x g for 15 min at 4°C, the supernatant fractions were collected and protein concentrations were assessed using the bicinchoninic acid protein assay. Supernatant aliquots containing ~30 µg protein were separated by 7.5% SDS-PAGE. The proteins were then transferred onto polyvinylidene fluoride membranes by electrophoresis at 300 mA for 2 h. The membranes were then blocked with 5% non-fat milk, and were washed with TBST and probed overnight at 4°C with 1:1,000 dilutions of primary antibodies, all from Cell Signaling Technology.
Inc., Danvers, MA, USA; β-catenin (8480), GSK3e (12456), p-GSK3β (5558), Bcl-2 (15071), Bax (5023), caspase 3 (9662), Eaf2 (14159), Wnt3a (2721), GAPDH (5174). Subsequently, the blots were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.) for 45 min at 37˚C. Immunodetection was performed using the Super Enhanced Chemiluminescence Detection reagent according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.).

Immunocytochemistry. Then, 24 h post-transfection, immunostaining was performed as previously described (3). Briefly, the treated HLE-B3 cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, washed with PBS, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Subsequently, the cells were blocked with 1 h with PBS containing 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature and were incubated with primary antibodies (Eaf2; ab28357; Abcam and Wnt3a; ab28472; Abcam) for 1 h at room temperature. The cells were then incubated with the corresponding secondary antibodies (BM3894; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 1 h at room temperature. DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) was used for counterstaining. Images of the immunostained cells were captured using a fluorescence microscope (VANOX-S; Olympus Corporation, Tokyo, Japan).

Statistical analysis. All statistical data were analyzed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA), and results are expressed as the mean ± standard deviation (n=3). One-way analysis of variance was performed followed by post hoc least significant difference test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Construction of an H$_2$O$_2$-induced apoptotic HLE cell model. HLE-B3 cells were induced to enter apoptosis by exposure to H$_2$O$_2$ for various time periods (4, 8 and 12 h). The apoptotic cells were detected by flow cytometry (Fig. 1A). Determination of the proportion of Annexin V- and PI-stained gated cells revealed that the population contained cells in both early stage (Annexin V$^+$/PI$^-$) and late stage (Annexin V$^+$/PI$^+$) apoptosis. The statistical analysis indicated that, when compared with the control cells, cell populations exposed to 50 µM H$_2$O$_2$ for 4 (P<0.01), 8 (P<0.001) and 12 h (P<0.001) exhibited increased apoptosis (Fig. 1B). Similar patterns were also recorded in an LDH release assay (Fig. 1C). To further investigate the mechanism underlying H$_2$O$_2$-induced apoptosis, the expression levels of caspase 3 and Eaf2 were monitored in HLE-B3 cells. The results indicated that the mRNA (Fig. 1D; P<0.01) and protein expression levels (Fig. 1E) of caspase 3 were markedly increased in HLE-B3 cells exposed to H$_2$O$_2$. Conversely, in HLE-B3 cells exposed to H$_2$O$_2$, caspase 3 and Eaf2 were reduced.
the mRNA (Fig. 1D; \( P<0.05 \)) and protein expression levels (Fig. 1E) of Eaf2 were markedly downregulated, as determined by RT-qPCR and western blot analysis. Therefore, it may be hypothesized that Eaf2 protects HLE cells from entering apoptosis. \( \text{H}_2\text{O}_2 \) treatment for 12 h increased the number of early and late stage apoptotic cells by 6- and 12-fold, respectively, making those cells lose the potential to recover viability during subsequent analysis. Consequently, HLE-B3 cells that underwent \( \text{H}_2\text{O}_2 \) treatment for 8 h were used in subsequent experiments.

**Overexpression of Eaf2 alleviates \( \text{H}_2\text{O}_2 \)-induced apoptosis of HLE cells.** To further investigate the effects of Eaf2 on \( \text{H}_2\text{O}_2 \)-induced HLE-B3 cells, cells were induced to overexpress Eaf2 by transfection with a pcDNA-Eaf2 plasmid, after which various experiments were conducted (Figs. 2 and 3). Apoptosis was analyzed by Annexin V/PI double staining (Fig. 2A); the results indicated that a significantly lower percentage of cells overexpressing Eaf2 had entered apoptosis (Fig. 2B). Overexpression of Eaf2 also decreased LDH activity (Fig. 2C), suggesting that the apoptotic process was suppressed. To investigate whether Eaf2 improves HLE-B3 cell survival rates by inhibiting apoptosis, the cellular levels of caspase 3 (a protease marker for apoptotic cell death) were analyzed by RT-qPCR and western blotting. The results demonstrated that,
when compared with the control cells, the mRNA (Fig. 2D; P<0.01) and protein expression levels (Fig. 2G) of caspase 3 were markedly decreased in cells overexpressing Eaf2. Since it has been reported that a hyperactive Wnt pathway serves a role in early eye development (21), the present study examined the expression levels of β-catenin, phosphorylated (p)-GSK3β and Wnt3a expression in HLE-B3 cells overexpressing Eaf2. As presented in Fig. 2D-G, cells overexpressing Eaf2 exhibited elevated expression levels of β-catenin, p-GSK3β, Wnt3a and Bcl-2 while decreased the expression of Bax, thus suggesting a close association between Eaf2 and Wnt3a signaling. An immunofluorescence assay further confirmed the upregulation of Wnt3a in Eaf2-overexpressing cells compared with the control cells (Fig. 3).

**Eaf2 inhibits H₂O₂-induced apoptosis in HLE cells by regulating Wnt3a.** To further investigate whether Eaf2 inhibits apoptosis of HLE-B3 cells by affecting Wnt3a signaling, Wnt3a expression...
was specifically knocked down in cells overexpressing Eaf2, and the cells were then examined by Annexin V/PI double staining (Fig. 4A). A statistical analysis indicated that the knockdown of Wnt3a significantly increased the overall percentage of Eaf2-overexpressing HLE-B3 cells undergoing apoptosis (Fig. 4B; *P<0.05). In addition, knockdown of Wnt3a reversed the Eaf2 overexpression-induced suppression of LDH release, thus suggesting a key role for Wnt3a in the function of Eaf2. That the role of Eaf2 in HLE-B3 cells depended on the activation of Wnt3a (Fig. 4C). Furthermore, the results suggested that Wnt3a knockdown promoted cell apoptosis by increasing the expression levels of caspase 3 and Bax while decreasing the expressions of β-catenin, p-GSK3β, GSK3β, Bcl-2 and Wnt3a (Fig. 4D and G). As presented in Fig. 5, an immunofluorescence analysis of Wnt3a expression was conducted in the various cell groups and the results showed that suppressed expression and distribution of Wnt3a was achieved in HLE-B3 cells.

Discussion
Oxidative stress is a major cause of cancer and cell death, and is thought to serve a major role in cataract formation (22).
Previous studies have demonstrated that \( \text{H}_2\text{O}_2 \)-induced oxidative stress can stimulate apoptosis of HLE cells, which form a single layer in the ocular lens (3,5,23). Furthermore, this type of cellular stress may be considered the initiating factor for non-congenital cataract formation (24). In the present study, the role of the tumor suppressor Eaf2 was determined in \( \text{H}_2\text{O}_2 \)-induced apoptosis of lens cells. The results of the in vitro study suggested that Eaf2 protects HLE cells from \( \text{H}_2\text{O}_2 \)-induced apoptosis by regulating the expression levels of apoptosis-associated proteins. This conclusion was based on an analysis of data obtained from loss and gain-of-function experiments. Cellular apoptosis is an important physiological and pathological process, which results in the destruction of cell membranes and condensation of chromosomes (25). Furthermore, the activation of caspase proteases is regarded as the central mechanism of apoptosis. The levels of caspase 3 can be used to accurately reflect the levels of apoptosis in a cell population, particularly the levels of early stage apoptosis (26). Meanwhile, Bax and Bcl-2 expression serve as key roles in cell apoptosis. The present study observed that HLE cells began undergoing apoptosis in a time-dependent manner following exposure to \( \text{H}_2\text{O}_2 \). Furthermore, their entry into apoptosis was accompanied by the activation of caspase 3 and Bax, and suppression of Bcl-2. In HLE cells, oxidative stress leads to apoptosis, which is a common cellular mechanism underlying cataract formation (27).

It has previously been reported that Eaf2 is spatially regulated in the lenses of embryonic mice (12). Another study demonstrated that Eaf2 is highly enriched in the developing eye, and is essential for normal eye development (13). In the present study, gradually decreasing levels of Eaf2 expression were detected over time in HLE cells undergoing \( \text{H}_2\text{O}_2 \)-induced apoptosis. A further analysis indicated that in HLE cells overexpressing Eaf2, the rate of \( \text{H}_2\text{O}_2 \)-induced apoptosis was decreased, thus suggesting that it protects HLE cells against oxidative stress-induced apoptosis.

Overexpression of Eaf2 resulted in a marked increase in the expression levels of Wnt3a in \( \text{H}_2\text{O}_2 \)-induced cells. Conversely, knockdown of Wnt3a decreased Eaf2 expression, and thereby promoted apoptosis of HLE cells. Wnt3a signaling is reportedly involved in the regulation of ocular cell proliferation and eye development (21,28). A previous study demonstrated that Wnt3a overexpression promotes the proliferation of HLE-B3 cells by increasing the percentage of cells in S phase (18). Furthermore, the loss of Eaf2 function has been reported to result in a loss of eye function, and the loss of Wnt-4 function can be reversed by Eaf2 (13). Based on these findings, it may be concluded that Eaf2 suppresses \( \text{H}_2\text{O}_2 \)-induced apoptosis via its effects on Wnt3a signaling. In addition, immunocytochemistry studies also revealed that Eaf2 can influence the expression of Wnt3a, and affect Wnt3a expression levels. This finding may further explain how Eaf2 regulates the percentage of lens cells undergoing \( \text{H}_2\text{O}_2 \)-induced apoptosis.

In conclusion, to the best of our knowledge, these data are the first to demonstrate that Eaf2 gene transcription products can protect HLE cells from oxidative damage caused by exposure to \( \text{H}_2\text{O}_2 \). In addition, the results of the present study demonstrated that when overexpressed, Eaf2 can reduce \( \text{H}_2\text{O}_2 \)-induced cell death by decreasing the expression levels of caspase 3 and Bax, and increasing Wnt3a and Bcl-2 expression. These results provide a theoretical foundation for the development of novel drugs for the treatment of cataracts. The present study focused on cell apoptosis and the suppressive effects of Eaf2 on apoptosis; however, further studies are required to explain how Eaf2 affects apoptosis.
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